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Quality, Nutritional, and Sensory Evaluation of Noodles Enriched with Oyster Mushroom (*Pleurotus sajor-caju*)

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Abstract: Noodles are considered one of the staple foods, especially in Asian countries. Traditional noodles are typically made from wheat flour; however, their nutritional value is relatively low. Previous studies have also explored this topic with an emphasis on improving the nutritional quality of noodles, but the sensory quality was often poorly rated. Therefore, this study focuses on enhancing the nutritional and sensory qualities of noodles, thereby contributing to product diversification through the use of abundant local commodities in line with sustainable development goals. This investigation examined the optimal level of oyster mushroom flour incorporated into noodle formulations at 40, 50, 60, and 70 g, along with a control sample without mushroom flour. The formulation containing 60 g of oyster mushroom flour showed the most favorable nutritional, physical, and sensory characteristics. The selected sample received the highest scores for all sensory attributes. Noodles' elasticity and cooking quality also performed well, with 59.28 g-force, high water absorption, and volume expansion (276.53% and 273.61%), and low cooking loss (2.90%). At the same time, this sample also contained higher levels of nutritional components and bioactive compounds, with 6.07 g protein, 1.94 g cellulose, 0.20 g β -glucan, 0.0051 g QE/100 g flavonoid, and 0.37 g TAE/100 g phenolic. These findings suggest that oyster mushroom flour can be used as a functional ingredient to improve the nutritional and sensory quality of noodles and may support the development of value-added mushroom-based food products.

Keywords: Cooking quality; noodles diversification; nutritional quality; oyster mushroom flour; sensory evaluation.

1. Introduction

Noodles are among the most widely consumed staple foods worldwide, particularly in Asia. This popularity is attributed to their convenience, affordability, and extended shelf life. According to a report by the USDA Foreign Agricultural Service, the annual consumption of global instant noodles reached over 100 billion, among which China, Indonesia, Vietnam, Japan, and India are among the leading countries [1, 2]. This substantial growth stems from accelerated urbanization, modern lifestyles, and the increasing demand for ready-to-eat foods. Along with this expansion come the limitations of maintaining nutritional quality, ensuring food safety, and developing health-friendly products to meet modern consumer trends. Recent studies have concentrated on the intention of improving the quality and nutritional value of

noodles through modifications in flour composition (such as the use of whole wheat flour, whole grains, or plant-based proteins), the application of advanced drying technologies, and the fortification with micronutrients such as dietary fiber, vitamins, and minerals [3, 4].

Oyster mushroom (*Pleurotus spp.*) is one of the most widely consumed edible mushrooms today, which is favored for its high nutritional value, distinctive flavor, and strong adaptability to various cultivation conditions [5, 6]. *Pleurotus sajor-caju* is considered a potential material for the development of functional foods and alternative protein products to replace animal-based sources. Many studies have highlighted the valuable composition of oyster mushrooms, particularly their protein content, which varies from 19-35% (dry matter), and they contain all essential amino acids, such as lysine, leucine, and valine, which are often deficient in cereals [7, 8]. Moreover, with the high fiber content (7-10%), especially β -glucan and chitin, which have various beneficial effects on digestion, blood lipid regulation, and immune system enhancement. Oyster mushrooms also have a low-fat content (2–4%) and consist mainly of unsaturated fatty acids, along with B-group vitamins, vitamin D₂, vitamin C, and minerals such as K, Fe, Zn, and Se, making them an ideal ingredient for nutritional and plant-based products. In addition to their nutritional composition, oyster mushrooms contain a variety of bioactive compounds, notably polysaccharides, phenolic compounds, and flavonoids, which exhibit antioxidant, antimicrobial, anti-inflammatory, hypoglycemic, cholesterol-regulating, and immunomodulatory activities [9-11].

Oyster mushroom flour has attracted increasing attention due to its outstanding nutritional and bioactive properties, making it a potential substitute or supplement for wheat flour in food products. Oyster mushroom flour has a light brown color, distinct flavor, and good blending compatibility with cereal flours, while enhancing the protein, fiber, mineral, and antioxidant compound content of the final product [10, 11]. Previous studies have supplemented oyster mushroom flour into bread, cookies, and functional and diet product formulations, and results revealed that these products have improved nutritional and sensory properties and prolonged shelf-life due to the antioxidant function of the supplemented material [11, 12]. These properties suggest that oyster mushroom flour is a suitable source for the trend toward sustainable food development, particularly in gluten-reduced, nutrient-enriched, or animal protein-substitute products that meet the growing consumer demand for healthy, eco-friendly, and bioactive-rich foods. Therefore, this study aimed to formulate an optimal blend that preserves the nutritional composition and antioxidant capacity of the added oyster mushroom flour while maintaining the product's desired physical and sensory qualities, in alignment with the “nutrition – convenience – sustainability” criteria.

2. Materials and Methods

2.1 Materials

Oyster mushrooms (*Pleurotus sajor-caju*) were selected at a uniform harvest stage, with an average length of 12.5 ± 0.5 cm, width of 6.6 ± 3.5 cm, cap thickness of 1.4 ± 0.5 cm, and weight of 10.48 ± 4.32 g. All samples were of good quality, free from defects, damage, and insect contamination [6]. Those that met the harvest standards were collected and transported to a processing laboratory at An Giang University, Vietnam National University Ho Chi Minh City (Vietnam). Wheat flour No. 13 (Baker's Choice, Long Xuyen, Vietnam) and Semolina flour (Bob Red Mill, Long Xuyen, Vietnam). Tofu (Vi Nguyen brand), iodized salt (Basalco, Bac Lieu), corn starch (Taki Food), and refined sugar (Bien Hoa) were obtained from supermarkets in Long Xuyen City, An Giang Province (Vietnam). Xanthan gum and gluten were supplied by My Au Science and Technology Development Joint Stock Company, Ho Chi Minh City (Vietnam), and other analytical-grade chemicals were sourced from Sinopharm Chemical Reagent Co., Ltd.

2.2 Experimental design

The premix was prepared with fresh oyster mushrooms; they were cleaned and shredded into strips approximately 1.5-2 cm wide, then spread onto an inox tray (40x60 cm²) and dried at 60 °C until reaching a target moisture content of 10% (wet basis). Dried mushrooms were ground into flour and sieved through a 212 μ m mesh (US Standard Mesh No. 70) to obtain the fine flour. Noodles' formulation: This section was set up using a completely randomized design with three replications, as described below. Wheat flour and oyster mushroom flour were 200 g per sample, in which wheat flour was substituted with oyster mushroom flour at

different levels of 40, 50, 60, and 70 g, which corresponded to F1, F2, F3, and F4 samples, respectively; while a control sample was coded with F0 (100% wheat flour). All samples were mixed with 30 g of semolina, 40 g of potato starch, 30 g of gluten, 2 g of salt, 65 g of tofu, 5.8 g of xanthan gum, and 63 g of water; the total amount for each formula was 435.8 g. The specified ratios for 4 formulas are presented in Table 1.

Table 1. Formulation of oyster mushroom noodle

Ingredients	F0 (g)	F0 (%)	F1 (g)	F1 (%)	F2 (g)	F2 (%)	F3 (g)	F3 (%)	F4 (g)	F4 (%)
Oyster mushroom flour	0	0	40	9.18	50	11.43	60	13.77	70	16.06
Wheat flour	200	60.89	160	36.71	150	34.46	140	32.12	130	29.83
Semolina	30	6.88	30	6.88	30	6.88	30	6.88	30	6.88
Potato starch	40	9.18	40	9.18	40	9.18	40	9.18	40	9.18
Gluten	30	6.88	30	6.88	30	6.88	30	6.88	30	6.88
Salt	2	0.46	2	0.46	2	0.46	2	0.46	2	0.46
Tofu	65	14.92	65	14.92	65	14.92	65	14.92	65	14.92
Water	63	14.46	63	14.46	63	14.46	63	14.46	63	14.46
Xanthan gum	5.8	1.33	5.8	1.33	5.8	1.33	5.8	1.33	5.8	1.33
Total	435.8	100	435.8	100	435.8	100	435.8	100	435.8	100

Noodles' preparation: Oyster mushroom flour was blended with wheat flour, semolina, and xanthan gum; the mixture was sieved, then transferred to a Kalite KL960 dough mixer for the first mixing step, lasting approximately 1 minute and 30 seconds. Subsequently, salt, tofu, and water were added, and the mixture was mixed for 5 minutes. The dough was then allowed to rest for 30 minutes. The rested dough was sheeted using a DJJ-200B noodle sheeter, passing through four successive rollings with the sheet thickness gradually reduced from 5 mm to 1 mm. The sheets were then cut into noodle strands approximately 25 cm long and 0.1 cm wide. The noodles were boiled in water at 100°C for 2.5 minutes and dried at 60°C for 4.5 hours. Noodle samples were packed in PET containers for further analyses [13, 14].

2.3 Analysis of physical and cooking properties

2.3.1 Texture analysis: 10 g of each noodle sample was cooked in 100 mL of boiling water, then drained and cooled to 6–8°C before analysis. Elasticity (g-force) of cooked noodles was measured using a texture analyzer (CT3, Brookfield, USA). The measurement was conducted using a wedge-shaped probe with the setup parameters including test speed: 2 mm/g; target type: Stop @ Load; target value: 1000 g; and trigger force: 5 g [13].

2.3.2 Water absorption (WA): 10 g of each noodle sample was cooked in 100 mL of boiling water contained in a 500 mL glass beaker and then evaluated. The amount of absorbed water was calculated using the equation below, where m_2 is the weight of cooked noodles, and m_1 is the weight of noodles before cooking (10 g) [13].

$$WA (\%) = \left(\frac{m_2 - m_1}{m_1} \right) \times 100$$

2.3.3 Cooking loss (CL): 10 g of each noodle sample was cooked in 100 mL of boiling water contained in a 500 mL glass beaker; the cooking water was then transferred into a drying dish and dried to a constant weight at 100–105°C. Cooking loss was the ratio of the dry residue weight in the cooking water (m_d) and the weight of noodles before cooking (m_1) [13].

$$CL (\%) = (m_d / m_1) \times 100$$

2.3.4 Volume expansion (VE): 10 g of each uncooked and cooked noodle sample were immersed in a graduated cylinder containing 50 mL of distilled water. The volume expansion was determined using the equation below, where V_1 and V_2 are the volumes of uncooked and cooked noodles, respectively [13].

$$VE (\%) = \left(\frac{V_2 - V_1}{V_1} \right) \times 100$$

2.4 Chemical properties analysis

The concentration of β -glucan (g/100 g dry matter) was assessed based on the Phenol-Sulfuric method [15]. Briefly, samples were ethanol-precipitated (96%, 4°C, 24 h), filtered, alkaline-treated (1 M NaOH, 60°C, 1 h), reacted with phenol and sulfuric acid, and the absorbance was measured at 490 nm. β -Glucan concentration was quantified using a standard glucan curve $y = 2745.61x + 0.0003^*$ ($R^2=0.9999$). The content of flavonoid (g QE/100 g dry matter) and *phenolic* (g TAE/100 g dry matter) was identified relying on the colorimetric method described by Sumaiyah et al. [16]. Total flavonoid content was determined using the aluminum chloride colorimetric method through the reaction of samples with NaNO_2 , $\text{AlCl}_3 \cdot \text{H}_2\text{O}$, and NaOH to form a stable yellow complex, and absorbance was measured at 510 nm using a UV-Vis spectrophotometer (V730, Jasco, Japan), its content was quantified using a quercetin calibration curve ($y = 8.2634x + 0.0182^*$ ($R^2 = 0.9999$)). Total phenolic content was determined using the Folin-Ciocalteu reagent. Samples were reacted with Folin-Ciocalteu reagent and Na_2CO_3 , and absorbance was measured at 750 nm using a UV-Vis spectrophotometer (V730, Jasco, Japan). The content was quantified using a tannic acid calibration curve: $y = 0.0021x + 0.0064$ ($R^2 = 0.9999$). The total sugar content (g/100 g dry matter) was determined using the DNS method [17]. Briefly, 1 g of noodle and 5 mL of concentrated HCl were added to a 100 mL Erlenmeyer flask, placed in a boiling water bath at 68–70°C for 3 hours, and then cooled to room temperature. Neutralize the mixture with 30% NaOH until the pH reaches 7.0. Add 7 mL of 10% $\text{Pb}(\text{CH}_3\text{COO})_2$, mix thoroughly, and let it stand for 5 minutes. Neutralize the excess $\text{Pb}(\text{CH}_3\text{COO})_2$ by saturated Na_2SO_4 solution, mix thoroughly, and transfer the entire solution to a 100 mL volumetric flask. An aliquot (1 mL) of the extracted solution was placed in a test tube, and 2 mL of the reagent DNS was added. The tubes containing blank, standard glucose solution, and samples were placed in boiling water for 10 minutes. Next, 7 mL of distilled water was added. The solution was analyzed at 575 nm. The concentrations of sucrose and reducing sugar were determined from a standard curve of glucose: $y = 23.885x + 0.126$ ($R^2 = 0.9999$). The protein content (g/100 g dry matter) of the noodle samples was determined using the Lowry assay described by Nielsen [18]. Briefly, 0.1 mL of the solution (after 1 g of sample was digested with 10 mL concentrated H_2SO_4) was mixed with 0.1 mL of 2 N NaOH and heated in boiling water for 10 minutes. After cooling, 1 mL of a reagent (formed from Na_2CO_3 , $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, and $\text{C}_4\text{H}_4\text{O}_6\text{KNa} \cdot 4\text{H}_2\text{O}$) and 0.1 mL of Folin-Ciocalteu reagent were added. The mixture was left at room temperature for 30 minutes, and the absorbance was measured at 750 nm. Protein content was calculated from a standard curve, $y = 0.0041x + 0.0118$ ($R^2 = 0.9999$). Where y represents absorbance and x represents concentration for all standard curves. Cellulose was determined based on its properties as a stable compound that is resistant to decomposition under the action of strong acids and alkalis [19]. The cellulose content (g/100 g dry matter) was quantified by drying the cellulose precipitate (m_2) to a constant weight at a temperature of 100–105 °C after filtration from a mixture consisting of the initial dried noodle sample (m_1) together with concentrated nitric acid and acetic acid at 100 °C for 30 minutes. The cellulose content (C%) was calculated using the following formula:

$$C (\%) = (m_2 * 100) / m_1$$

2.5 Sensory evaluation

The sensory characteristics of the product were measured for color, aroma, taste, and overall preference, following the Quantitative Descriptive Analysis (QDA) method [20]. In summary, forty trained panelists were instructed on how to rate each sensory attribute before evaluating the noodle samples. The degree of each attribute was determined using a detailed descriptive scale from 1 to 5 (Table 2), with 1 representing poor and 5 excellent. The level of preference was measured on a 1-9 Hedonic scale.

2.6 Data analysis

Data were statistically analyzed using Statgraphics Centurion XVI software (U.S.A.). The experiment followed a completely randomized design (CRD) with three replicates per treatment. Analysis of variance (ANOVA) was used to evaluate treatment effects, and mean comparisons were performed using the least significant difference (LSD) test at the 5% significance level. Graphs were prepared using Microsoft Excel.

Table 2. The description of noodle's sensory evaluation

Scale	Color	Aroma	Taste	Texture
5	Bright, uniform light cream to pale brown; very appealing	Strong, very pleasant mushroom aroma	Very delicious; rich umami taste; no off-flavor	Firm, elastic, and smooth; not sticky/mushy; highly desirable
4	Slightly dull but still uniform; acceptable	Noticeable mushroom aroma; pleasant	Good taste; slight reduction in flavor intensity	Slightly less firm; still elastic and acceptable
3	Moderately dull or uneven color	Weak mushroom aroma	Acceptable taste; bland or slightly lacking flavor	Moderately soft; slightly sticky or less elastic
2	Dull, uneven, or slightly discolored	Faint or slightly off aroma	Poor taste; noticeable off-flavor or imbalance	Soft, sticky, or slightly mushy; undesirable
1	Unacceptable color; very dark or uneven	Unpleasant or strong off-odor	Very poor taste; strong off-flavor, not acceptable	Very soft, mushy, or overly sticky; unacceptable

3. Results and Discussion

3.1 Effect of supplemented amount of oyster mushroom flour on the sensory value and cooking quality of noodles

3.1.1 Elasticity of noodles

Elasticity of noodles containing different levels of oyster mushroom flour is presented in Figure 1. As the level of oyster mushroom flour in the formula increased, noodle elasticity decreased progressively. In particular, the elasticity of the control formula (without mushroom flour) was 116.11 g-force; at fortification levels of 40, 50, 60, and 70 g mushroom flour, the elasticity of noodle samples was 109.61, 78.64, 59.28, and 56.56 g-force, respectively. An analysis of noodle elasticity is a quality control measure to ensure consistent, high-quality noodle production [14]. The incorporation of oyster mushroom flour into the formulation partially replaces wheat flour; consequently, the amount of wheat flour is reduced. In noodles production, the elasticity and flexibility of noodles mainly depend on the gluten content in wheat flour, which consists of the protein fractions gliadin and glutenin [21]. A study by Zhang et al. [22] reported that the elasticity of noodles declined with increasing mungbean flour addition, due to its gluten-free nature. Shere et al. [23] also stated that the elasticity of noodles significantly lowered when formulating with a high amount of pureed carrot supplementation. The results reveal that ingredients with high levels of sugar and dietary fiber can reduce the elasticity of the final product. This phenomenon can be explained by the competition for water among the components in the dough. Both sugar and fiber have a high affinity for water; therefore, they absorb and retain some of the moisture essential for the hydration of gluten-forming proteins, gliadin and glutenin, resulting in a weaker, less cohesive structure [23]. An incomplete gluten network reduces the dough's characteristic elasticity and chewiness, making the noodles more fragile, softer, and less resilient when stretched [21]. In addition, the addition of oyster mushroom flour increased dietary fiber content, causing mechanical disruption within the gluten matrix and further contributing to the reduction in elasticity.

3.1.2 Sensory evaluation

The sensory evaluation results showed that incorporating oyster mushroom flour significantly affected the color, aroma, taste, and texture of the noodles, with significant differences among formulations ($P \leq 0.05$). The color of the noodle samples changed substantially at different levels of mushroom flour fortification. Table 3 shows that the evaluation scores for the color attribute were lowest for the control sample (2.66) and highest at the 60 g mushroom flour addition level (4.66). The addition of oyster mushroom flour enhanced the yellow color of the noodles, thereby improving the color score in the sensory evaluation. However, excessive supplementation resulted in darker noodle strands. Specifically, when the amount of mushroom flour was increased to 70 g, the color score tended to decrease. (4.11). The differences in flavor and taste also followed the trend of color: the control sample received the lowest score (1.33) for flavor, and the sample fortified with 60 g of mushroom flour recorded the highest (4.33). The addition of oyster mushroom

flour enhanced the product's aroma compared to the control sample. However, with the addition of more mushroom flour, the product tended to develop a slightly strong or pungent mushroom odor. The combination of wheat flour with other ingredients and oyster mushroom flour enhanced the sensory score for the taste, reaching 3.66 and 3.88 in samples supplemented with 70 g and 60 g of oyster mushroom flour, respectively, compared to 1.33 points in the control sample without mushroom flour. This improvement can be attributed to the partial substitution of wheat flour with mushroom flour, which contributed to the development of the characteristic umami flavor in the noodles.

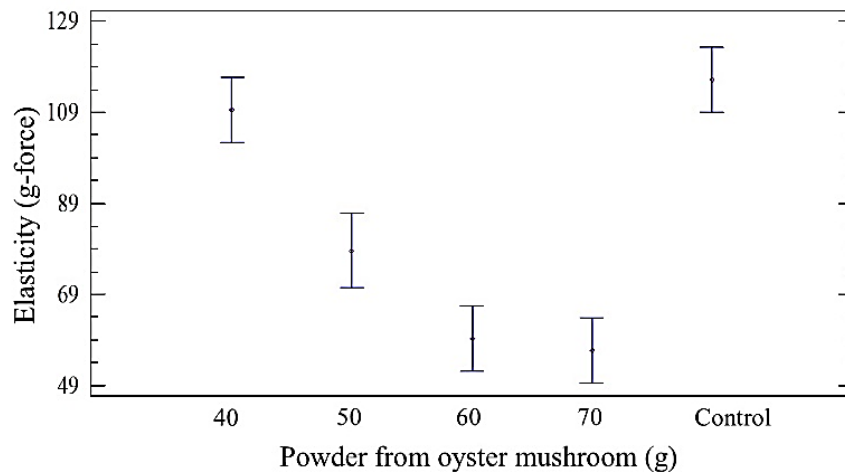


Figure 1. Elasticity of noodles containing different levels of oyster mushroom flour

Table 3. Effects of different levels of oyster mushroom flour on the sensory evaluation of noodles

Oyster mushroom flour (g)	Sensory scores			
	Color	Flavor	Taste	Texture
0 (control)	2.66 ^d ± 0.18	1.33 ^e ± 0.08	1.33 ^e ± 0.08	1.66 ^e ± 0.03
40	3.44 ^c ± 0.21	2.33 ^d ± 0.15	2.00 ^d ± 0.15	2.33 ^d ± 0.11
50	3.88 ^b ± 0.21	3.00 ^c ± 0.19	2.77 ^c ± 0.18	3.00 ^c ± 0.13
60	4.66 ^a ± 0.22	4.33 ^a ± 0.21	3.88 ^a ± 0.21	4.22 ^a ± 0.22
70	4.11 ^{ab} ± 0.21	3.66 ^b ± 0.20	3.33 ^b ± 0.20	3.66 ^b ± 0.14
Level of significance (P)	0.0000	0.0000	0.0000	0.0000

Mean values (n = 3) with different superscripts in the same column are significantly different at the 95% confidence level according to the LSD test.

The texture of noodles scores differed significantly among formulations ($P \leq 0.05$) (Table 3). It was also affected by the level of oyster mushroom flour substitution; the sensory score for texture was significantly higher in samples with higher substitution levels. Specifically, the sample with 60 g of mushroom flour scored 4.22 points, and the lowest reference was still the control sample at 1.66 points. The samples with a higher proportion of wheat flour, especially those without oyster mushroom flour or with a low supplementation level (40 g), may have resulted in an excessively firm and tough texture after steaming. However, excessive replacement of wheat flour reduced gluten content, resulting in reduced elasticity and chewiness and lower texture scores. In particular, the formulation with the highest level of substitution had a lower score (3.66) than the formulation containing 60 g of oyster mushroom flour (4.22). Although instrumental analysis showed a decrease in elasticity with increasing levels of oyster mushroom flour, the sensory evaluation indicated that the formulation containing 60 g achieved the highest texture score. This may be attributed to the fact that sensory texture perception involves multiple attributes, including softness, chewiness, and overall mouthfeel, rather than elasticity alone. Therefore, the 60 g formulation likely provided a more balanced and acceptable texture despite the reduction in instrumental elasticity.

3.1.3 Cooking quality of noodles

Table 4 shows the evaluation of cooking quality, including cooking loss, water absorption, and volume expansion, which was carried out at different levels of mushroom flour fortification.

Table 4. Effects of oyster mushroom flour incorporation on the physical properties of noodles

Oyster mushroom flour (g)	Water absorption (%)	Cooking loss (%)	Volume expansion (%)
0 (control)	154.40 ^c ± 10.24	2.40 ^b ± 0.05	152.22 ^c ± 10.12
40	186.17 ^c ± 12.31	2.07 ^b ± 0.07	155.56 ^c ± 10.03
50	260.33 ^{ab} ± 20.19	2.19 ^b ± 0.04	193.52 ^{bc} ± 12.41
60	276.53 ^a ± 20.24	2.90 ^{ab} ± 0.08	273.61 ^a ± 20.34
70	230.00 ^b ± 15.16	4.56 ^a ± 0.14	243.61 ^{ab} ± 20.11
Level of significance (P)	0.0000	0.1073	0.0185

Mean values (n = 3) with different superscripts in the same column are significantly different at the 95% confidence level according to the LSD test.

During the evaluation, the highest cooking loss was recorded in the sample with 70 g of mushroom flour added (4.56%); however, the differences among all samples were not significant. Cooking loss is one of the fundamental parameters used to predict noodle cooking quality. These values represent the amount of solid matter lost into the cooking water [13]. Compared with previous studies, it was found that the cooking losses of all noodle formulas were below the technological limit of ≤8% [24,25]. Therefore, the incorporation of oyster mushroom flour at different levels did not affect the cooking loss. The increase in noodle volume showed a statistically significant difference at the 5% level among the formulations with different levels of oyster mushroom flour supplementation. The volume expansion of the control sample (without oyster mushroom flour) and the sample supplemented with 40 g of oyster mushroom flour exhibited the lowest values, at 152.22% and 155.56%, respectively (P > 0.05). Whereas, the formula containing 60 g of oyster mushroom flour showed the highest volume expansion of 273.61%. A high level of volume expansion is a desirable attribute in noodle products and is generally preferred by consumers [14]. According to Kang et al. [26], the degree of noodle volume expansion during cooking is mainly influenced by starch gelatinization and protein hydration. In fact, oyster mushroom flour contains 16.22% protein [6,7], while wheat flour contains about 13%. Therefore, the higher protein content of oyster mushroom flour may partly explain the greater water absorption capacity observed when higher levels of mushroom flour were incorporated into the noodle formulation. Furthermore, a strong correlation has been reported between water absorption and noodle volume expansion, indicating that noodles with higher water uptake tend to exhibit greater expansion during cooking [26]. Water absorption is the amount of water retained by noodles during cooking, an important characteristic that determines overall cooking quality [23]. The results shown in Table 4 clearly demonstrate that noodles supplemented with 60 g of oyster mushroom flour exhibited the highest values of both water absorption and volume expansion.

3.2 Effects of supplemented amount of oyster mushroom flour on the nutritional value of noodles

3.2.1 Chemical compositions of noodles

The protein, total sugar, and cellulose contents of noodles at different levels of mushroom flour fortification are presented in Table 5. The protein and cellulose content of noodles rose with the increase in the amount of mushroom flour added. At levels of 60 g and 70 g of mushroom flour fortification, noodles exhibited remarkable protein and cellulose contents of 6.07 g and 6.41 g, and 1.94 g and 2.14 g/100 g dry matter (without statistical difference). According to previous studies, the protein content of oyster mushroom flour is higher than that of wheat flour [6, 7]. Therefore, partial substitution of wheat flour with oyster mushroom flour increased the protein content of noodles. The protein content can reflect a product's nutritional value and may significantly influence its structural properties.

Table 5. Chemical compositions of noodles at different levels of oyster mushroom flour fortification

Oyster mushroom flour (g)	Chemical compositions (g/100 g dry matter)		
	Protein	Total sugar	Cellulose
0 (control)	3.05 ^d ± 0.03	75.53 ^a ± 1.52	0.70 ^d ± 0.02
40	3.84 ^c ± 0.05	64.66 ^b ± 1.65	1.53 ^c ± 0.05
50	4.69 ^b ± 0.08	56.75 ^b ± 1.37	1.76 ^b ± 0.04
60	6.07 ^a ± 0.06	44.61 ^c ± 1.10	1.94 ^{ab} ± 0.04
70	6.41 ^a ± 0.05	35.55 ^c ± 1.32	2.14 ^a ± 0.06
Level of significance (P)	0.0000	0.0000	0.0000

Mean values (n = 3) with different superscripts in the same column are significantly different at the 95% confidence level according to the LSD test.

The total sugar content of noodles showed an opposite trend, decreasing with increasing levels of oyster mushroom flour supplementation. The samples containing 60 g and 70 g of mushroom flour exhibited the lowest total sugar content at 44.61 g and 35.55 g per 100 g dry matter, respectively. In the control sample, the total sugar content was 75.53 g/100 g dry matter. This result is consistent with the findings of Bui et al. [19], who reported that the total sugar content in noodles ranges from 70% to 75%. According to previous studies on oyster mushrooms' compositions [6, 7], the total sugar content of oyster mushroom flour is 24.84 g/100 g dry matter. Therefore, replacing wheat flour with oyster mushroom flour reduced the sugar content of the noodles. Oyster mushrooms retain a certain amount of cellulose, which is considered a potential source of fiber due to the presence of non-starch polysaccharides [27]. Results also reveal that cellulose content tended to increase gradually with the further addition of oyster mushroom flour to the noodles' formula, but the samples with 60 g and 70 g of fortified mushroom flour did not differ statistically in cellulose content ($P > 0.05$). The study by Sobota et al. [28] also showed that adding vegetable flours to noodle formulations increases the noodles' fiber content.

3.2.2 Bioactive compounds in noodles

Conventional noodle products made from wheat flour available on the market generally contain none or only very minor amounts of bioactive compounds. Therefore, the fortification or development of noodle products enriched with bioactive compounds is considered innovative and meets consumer expectations. The quantification of phenolic, flavonoid, and β -glucan in noodles formulated with oyster mushroom flour addition is shown in Table 6.

Table 6. Bioactive compounds content of noodles containing different levels of oyster mushroom flour

Oyster mushroom flour (g)	Phenolic (g TAE/100 g dry matter)	Flavonoid (g QE/100 g dry matter)	β -glucan (g/100 g dry matter)
0 (control)	0.07 ^c ± 0.001	0.0005 ^c ± 0.0000	0.06 ^d ± 0.000
40	0.35 ^b ± 0.001	0.0046 ^b ± 0.0000	0.18 ^c ± 0.002
50	0.36 ^b ± 0.001	0.0051 ^a ± 0.0000	0.18 ^c ± 0.002
60	0.37 ^a ± 0.001	0.0051 ^a ± 0.0000	0.19 ^b ± 0.003
70	0.37 ^a ± 0.001	0.0051 ^a ± 0.0000	0.20 ^a ± 0.003
F	15.13	48.99	30.60
P	0.0000	0.0000	0.0000

Mean values (n = 3) with different superscripts in the same column are significantly different at the 95% confidence level according to the LSD test.

β -glucan in the control sample was the lowest (0.06 g/100 g dry matter). However, its content increased with the addition of oyster mushroom flour to the formula. In particular, β -glucan rose from 0.18 to 0.20 g per 100 g dry matter as the fortified level increased from 40 g to 70 g. β -glucan is a major polysaccharide found in many edible mushrooms and possesses numerous functional and biological activities, including anticancer, antioxidant, and anti-infective properties [29]. The β -glucan content increased by 3–3.5 times compared with the control sample (without oyster mushroom flour supplementation). In addition, the changes in phenolic

and flavonoid content of noodles also followed the trend of β -glucan. Phenolic and flavonoid content reached the peak at 0.37 g TAE and 0.0051 g per 100 g dry matter when adding 60 g and 70 g mushroom flour. Noodles are primarily made from wheat flour and may include additional ingredients to enhance their quality [19]. According to Melinda et al. [30], oyster mushrooms have great potential as a natural source of secondary metabolites, including phenolics, flavonoids, and glucans. Therefore, incorporating oyster mushroom flour into noodles enhances their bioactive compounds and antioxidant activity.

4. Conclusions

This study evaluated the effect of oyster mushroom flour fortification on the quality and nutritional composition of noodles. The results indicated that adding 60 g of oyster mushroom flour produced noodles with the most favorable sensory attributes, including color, aroma, taste, and texture. This formulation also showed enhanced nutritional and bioactive properties, with protein (6.07 g), cellulose (1.94 g), total sugar (44.61 g), β -glucan (0.19 g), flavonoid (0.0051 g QE/100 g), and phenolic content (0.37 g TAE/100 g) higher than the control. These findings demonstrate that partial substitution of wheat flour with oyster mushroom flour can improve the functional and nutritional characteristics of noodles. Further studies are recommended to investigate storage stability and other quality parameters.

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The Optimization of Domestic Wastewater Dilution, Seed Volume, and Fertilizer Supplementation for *Micractinium* sp. Cultivation Using Response Surface Methodology

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Abstract: Rapid urbanization has led to a growing domestic wastewater problem, posing environmental and public health risks. Conventional wastewater treatment remains inadequate, leading to issues such as eutrophication and secondary pollution. This study explores an alternative wastewater recycling method that utilizes domestic wastewater as a medium for cultivating *Micractinium* sp. and supplements it with a commercially available Nitrogen-Phosphorus-Potassium (NPK) fertilizer (13:13:21) as a novel nutrient source to enhance microalgae growth. The cultivation is carried out in an indoor system (25 ± 1 °C, $140 \mu\text{mol m}^{-2} \text{s}^{-1}$, 24:0 photoperiod, and continuous aeration) to determine the cultivation ratio (wastewater dilution: seed volume: nutrient concentration). Growth was monitored using optical and cell density parameters throughout the cultivation period (21 days) for each element of the ratio. After optimization using Response Surface Methodology (RSM) software, the ideal values of wastewater dilution, seed volume, and stimulant volume was 50%, 50 mL, and 110 mL, with the maximum growth (470nm), biomass (680nm), and cell density achieved were 6.57, 6.42, and 7.14×10^7 cells/mL, respectively. Therefore, the outcomes ratios per liter are 0.68 for diluted wastewater (domestic wastewater and filtered water), 0.10 for seed volume, and 0.22 for NPK concentration. These results highlight the potential of domestic wastewater as a medium for the growth of *Micractinium* sp., thereby supporting efforts to recycle wastewater to reduce environmental problems.

Keywords: *Micractinium* sp.; domestic wastewater; wastewater recycling; fertilizer

1. Introduction

Wastewater contaminated with microorganisms and persistent organic pollutants poses serious environmental and public health problems [1]. Untreated or inadequately treated wastewater can lead to eutrophication and harmful algal blooms, harming aquatic ecosystems and restricting biodiversity [2]. Rapid urbanization increases water treatment costs and underscores the importance of wastewater recycling technologies for sustainable resource management [3]. Wastewater recycling is a sustainable option for many industries, such as agriculture, where treated wastewater can provide 81% of a crop's nitrogen and 38% of its phosphorus requirements. Advanced treatment technologies

recover nutrients, minimizing the risk of eutrophication and the need for synthetic fertilizers [4]. Therefore, wastewater recycling is a promising cultivation medium for microalgae, as it provides nutrients for growth. These microalgae can be cultivated under various conditions and require less space than conventional crops [5].

Several microalgal species, including *Scenedesmus* sp. and *Chlorella* sp., have shown great promise for wastewater-based production systems, yielding high protein, carbohydrate, lipid, and lipid content [6]. However, recent research has shown that *Micractinium* sp. has potential as a bio-based resource for renewable energy development, given its biomass output and lipid content. *Micractinium* sp. produces important byproducts, such as pigments and fatty acids, which are used in sectors including cosmetics, food, and medicine [7]. In addition, *Micractinium* sp.'s adaptation to Malaysia's environment is demonstrated by its thermoresistance and ability to grow under diverse temperature conditions, making it an appropriate option for cultivation in this area [8]. Nonetheless, compared to other species, there is no previous research on the cultivation of *Micractinium* sp. using actual domestic wastewater. The closest one uses artificial municipal sewage based on the BG-11 standard medium [9] [10]. Thus, it is selected as the species to be explored in this study. *Micractinium* sp. acclimatization in wastewater media is a viable approach for large-scale culture, provided resources are available. It can live in a variety of wastewaters, contributing to its adaptability [11]. To survive in harsh domestic wastewater environments, *Micractinium* sp. must alter their physiological and metabolic activities, enabling them to adjust nutrient absorption mechanisms via nutrient transporters, thereby maintaining metabolic balance and avoiding nutrient stress that could lead to toxicity and negatively impact their performance. [12]. Additionally, microalgae, including *Micractinium* sp., can maintain internal pH homeostasis by activating buffering mechanisms to sustain critical biochemical reactions, thereby allowing them to continue photosynthesis and nutrient absorption even under suboptimal pH [13].

Moreover, recent studies on the cultivation of *Micractinium* sp. in wastewater indicate that most cultivation methods employ nutrient supplementation, specifically BG-11 [9, 14] and Tris-Acetate-Phosphate (TAP) medium [15, 16]. The studies show a positive result, but using the specific nutrient is costly and might not be feasible on a large scale. In contrast, NPK is a readily available, commercially produced inorganic fertilizer that does not require complex preparation [17]. Each element in the NPK plays a role: nitrogen is important for protein and chlorophyll synthesis; phosphorus functions in energy transfer (ATP) and nucleic acid formation; while potassium boosts enzyme activation and osmotic regulation to support microalgal growth and metabolism. However, there is limited research on *Micractinium* sp. growth in response to NPK nutrient supplementation. These factors led to the utilization of NPK employed in this study [18]. The purpose of this study is to determine the ideal culture ratio of all three main elements for the microalgae cultivation, which are dilution wastewater, microalgae seed volume, and nutrient concentration, to enhance the *Micractinium* sp. growth of wastewater-based cultivation.

2. Materials and Methods

2.1 Experimental and method development

The phases involved in this study are culture preparation, RSM primary study, and discussion analysis. The first part of the culture involves preparing a domestic wastewater medium, *Micractinium* sp. seeds, and NPK nutrients, followed by a one-factor-at-a-time (OFAT) approach, in which all three elements are tested independently before RSM. The OFAT testing evaluated several quantities for each element listed in Table 1. The chosen values (highest growth) were validated using RSM by formulating 20 ratios combining the three elements, as shown in Table 7. The combination of OFAT and RSM enables effective initial screening of individual elements to narrow them to significant ranges, followed by a systematic analysis of interactions among all three elements and exact optimization within those ranges. This sequential technique minimizes needless trials and improves experimental efficiency and accuracy compared to utilizing either method alone [19]. Based on Table 1, the final values selected after RSM differ from the pre-selected value by OFAT. This is expected because OFAT evaluates each factor individually while holding the others constant, which does not account for possible interactions. In contrast, RSM simultaneously explores all main factors and considers their interactions to determine the precise optimal conditions.

Table 1. Ratio selection of medium, seed, and nutrient in the primary study.

Cultivation elements	OFAT test ¹	Pre-value selected from OFAT	RSM design ²	Final value selected from RSM
Medium, %	10, 30, 50, 70, 100 (with/-out nutrient)	75	50, 75, 100	50
Seed, mL	25, 50, 100, 150, 200, 250	150	50, 100, 150	50
Nutrient, mL	25, 50, 75, 100, 125, 150	100	70, 90, 110	110

¹ Refer to Tables 4-6 for the OFAT testing of each element.

² Range design from the OFAT result for RSM optimization through the combination ratio of all three elements, as shown in Table 7.

2.2 Microalgae preparation

Preliminary identification of microalgae samples was performed using DNA sequences, which were then blasted against the GenBank secure database online using a field guide, as proposed by [20]. The BLAST search of the National Center for Biotechnology Information (NCBI) database identified 99.85% as the highest identity to *Micractinium* sp., with accession numbers ON352775.1, FM205863.1, and KR936170.1, as shown in Table 2. Phylogenetic trees of the *Micractinium* strains are shown in Figure 1. After identification, the *Micractinium* sp. sample was examined under a microscope to confirm it was free of contamination. If contamination occurred, the sample was filtered using cellulose microfilter paper until all contamination was removed [21].

Table 2. GenBank accession numbers, closest phylogenetic relative, and identity percent of the *Micractinium* strain.

Description	Query cover %	Strain	Identity %	Accession	Reference authors
<i>Micractinium</i> sp. W2 small subunit ribosomal RNA gene and internal transcribed spacer 1	100		99.85	ON352775.1	[22]
<i>Micractinium</i> sp. CCAP 211/92 18S rRNA gene	100		99.85	FM205863.1	[23]
<i>Micractinium</i> sp. IPOME-2 18S ribosomal RNA gene	100	<i>Micractinium</i> sp.	99.85	KR936170.1	[24]
<i>Micractinium</i> sp. SH 18S ribosomal RNA gene	100		99.61	KM820919.1	
<i>Micractinium</i> sp. TvB 18S ribosomal RNA gene	100		99.56	KM820917.1	[25]
<i>Micractinium</i> sp. T_V_B small subunit ribosomal RNA gene	100		99.56	ON332728.1	

The sample was cultivated as seeds in filtered water with nutrients at a ratio of 1 (seed): 1 (nutrient) : 3 (medium). Then kept aerated at $25 \pm 1^\circ\text{C}$ and a light intensity of $140 \mu\text{mol m}^{-2} \text{s}^{-1}$ [26]. Aeration was supplied by an air pump/compressor, delivering naturally in ambient air without CO_2 enrichment. The 24:0 photoperiod was selected to maximize photosynthetic and growth productivity by eliminating the dark phase. This is particularly relevant in controlled laboratory conditions, where light intensity is moderate, and photoinhibition can be minimized. Additionally, light-dark cycles introduce alternating phases of photosynthesis and respiration, which can complicate the interpretation of growth kinetics [27]. Therefore, continuous illumination provides a more stable metabolic environment, making it easier to evaluate parameters such as optical and cell density with reduced fluctuation.

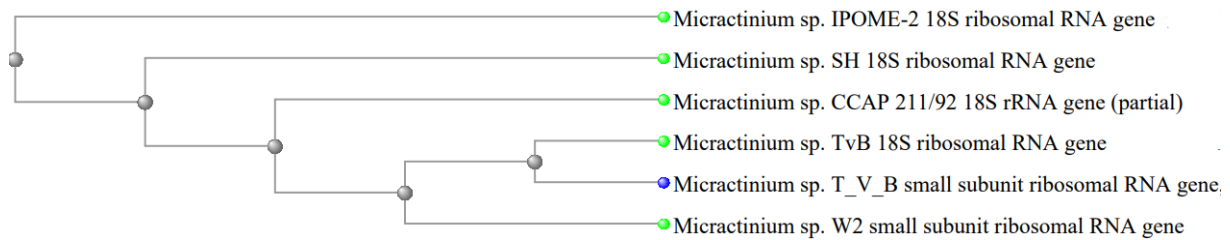


Figure 1. Phylogenetic tree of *Micractinium* sp. strain in the NCBI database.

2.3 Nutrient preparation

The dried nutrient NPK is prepared by diluting with filtered water in a 5L clear plastic container, and the compositions are 20g per liter with the formula 13% N - 13% P - 21% K. Dissolved nutrient of NPK in 500mL filtered water contains 3010mg/L of total nitrogen (N), 86mg/L phosphorus (P), and potassium: 3858mg/L potassium (K). Next, the nutrient solution is aerated for a day to ensure it is completely diluted before being inoculated with *Micractinium* sp. seed. After a day, aeration is turned off, allowing larger, heavier particles to settle to the bottom of the bottle for 5 to 6 hours [28]. Once fully suspended, the separated solution is transferred to another bottle and is ready for cultivation.

2.4 Wastewater sampling

Domestic wastewater (DW) was collected from the sewage treatment facility on the campus of Universiti Tun Hussein Onn Malaysia in Johor, Malaysia, for this study, with approval from Property Development and Management (PPH), and all data were used only for research and teaching purposes. This treatment facility collects wastewater generated from the students' residential college and campus cafeteria. The DW sample preparation followed the published standard technique (Water and Wastewater Standard technique 22nd Edition, 2012). Samples are kept in an HDPE bottle for transit to the laboratory. Once at the laboratory, the DW was characterized using the standard laboratory procedure outlined in Table 3. The DW was filtered using a 0.45 μm membrane filter and a vacuum filter to remove suspended solids and microorganisms that might compete with microalgae for nutrients, cause food competition, or cross-contaminate [29]. The DW was progressively poured into the Buchner funnel, with the vacuum turned on to produce negative pressure in the filter flask, causing the liquid to flow through the filter paper while leaving the solid behind. After filtering, neither chemical disinfection nor autoclaving was used to sterilize the DW samples, to prevent chemical changes that could alter sample composition and thereby affect COD, BOD, and nutrient levels. This ensures that results remain applicable to actual wastewater management practices [30]. Based on Table 3, the COD/BOD ratio = 3.43 indicates the low to moderate biodegradability of the domestic wastewater. However, both parameters complied with the domestic effluent standard under the Environmental Quality Act of Malaysia, 1974. The wastewater remains suitable for microalgae cultivation, with potential for adaptation and NPK supplementation to enhance microalgae growth. Furthermore, the N:P ratio of 25.8:1 in Table 3 indicates a phosphorus-limited condition relative to the optimal Redfield ratio (16:1). Therefore, this study used NPK fertilizer supplementation to improve nutrient balance and enhance overall growth performance. Additionally, in wastewater-based cultivation systems, deviations from the Redfield ratio are considered standard practice; nevertheless, microalgae can adapt to suboptimal nutrient conditions [31].

2.5 RSM primary study

In this phase, all three elements, namely domestic wastewater, microalgae seed, and nutrients, were experimented with within a certain range of values as shown in Tables 4-6 to get an ideal value as shown in Table 1. All tests were carried out indoors and left to cultivate for 21 days, with the growth of each sample examined and evaluated using optical and cell density. From the specified values, 20 combination ratios of the three elements listed in Table 7 were generated using the RSM technique in Design-Expert software to validate and determine the cultivation ratio in a 500mL volume per run. As listed in Table 9, the range tested for each element or factor in the RSM does not include all the values from the OFAT (Table 4-6). For example, wastewater dilution does not include lower concentrations (10–30%) that were eliminated from the initial

screening by OFAT due to restricted growth. In addition to Table 9, the center point replicates (Runs 6, 8, 9, 11, 14, and 20) were used for error estimation in the RSM design. These replicates are commonly employed to assess experimental error and ensure the reproducibility and reliability of the model at the midpoint of the experimental range [32].

Table 3. Domestic wastewater sample characterization.

Description	Unit	Results	Instrument/Method
pH	-	7.20	Eutech™ pH 2700 Benchtop pH Meter
Turbidity	NTU	15.74	Lutron TU-2016 – Turbidity Meter
Chemical oxygen demand		24.00	APHA 5220 B
Biochemical oxygen demand		7.00	APHA 5210 B, APHA 4500-OG
Total suspended solid		27.00	DR6000 Benchtop Spectrophotometer (Method 8006)
Total phosphorus	mg/L	0.50	APHA 4500 P-C
Total nitrogen		12.90	APHA 4500 N org B
Potassium (K)		12.70	APHA 3120 B
Nitrate (NO ₃)		2.00	Palin Test Kit, Method AK 163
Chloride (CL ⁻)		49.00	APHA 4500- CL ⁻ B
Phosphate (PO ₄ ³⁻)		1.50	APHA 4500-P C

Table 4. Wastewater dilution experiment.

Sample	Percent	Wastewater	Autoclaved tap water	Nutrient	Seed
S1	-	-	4.750 L	-	
S2	100 %	4.750 L	-		
S3	75 %	3.750 L	-		
S4	70 %	2.625 L	1.125 L		0.25 L
S5	50 %	1.875 L	1.875 L	1 L	
S6	30 %	1.125 L	2.625 L		
S7	10 %	0.375 L	3.375 L		

Table 5. Microalgae seed experiment.

Sample	Percent	Seed	Autoclaved tap water	Nutrient
S1	5 %	25 mL	475 mL	-
S2	5 %	25 mL	375 mL	
S3	10 %	50 mL	350 mL	
S4	20 %	100 mL	300 mL	100 mL
S5	30 %	150 mL	250 mL	
S6	40 %	200 mL	200 mL	
S7	50 %	250 mL	150 mL	

Table 6. Nutrient stimulant experiment.

Sample	Percent	Nutrient	Autoclaved tap water	Seed
S1	-	-	475 mL	
S2	5 %	25 mL	450 mL	
S3	10 %	50 mL	425 mL	
S4	15 %	75 mL	400 mL	25 mL
S5	20 %	100 mL	375 mL	
S6	25 %	125 mL	350 mL	
S7	30 %	150 mL	325 mL	

Table 7. RSM design for cultivation ratio.

Run	Factors		
	A: Nutrient Volume (mL)	B: Seed Volume (mL)	C: Wastewater Dilution (%)
1	110	150	100
2	90	50	75
3	90	150	75
4	110	150	50
5	110	100	75
6	90	100	75
7	70	50	50
8	90	100	75
9	90	100	75
10	70	150	50
11	90	100	75
12	90	100	50
13	110	50	100
14	90	100	75
15	90	100	100
16	70	150	100
17	70	50	100
18	70	100	75
19	110	50	50
20	90	100	75

2.6 Measurement of microalgae growth

2.6.1 Cell density

For cell density assessment, only a 1 mL sample is required. The microscope utilized is a Nikon Eclipse TS100 Trinocular Inverted Microscope with a digital camera linked to a computer. The microscope was used to count cells on the hemocytometer grid chamber. The hemocytometer is an instrument for counting cells and measuring microalgae development using a standard approach (APHA, 2012). Calculate the number of cells using the following formula [33]:

$$\text{Number of cells} = \frac{\text{Average number of cells per } 1 \text{ mm}^2}{\text{Volume of the square}} \times \text{Dilution factor} \quad (1)$$

2.6.2 Optical density

Micractinium sp. growth was also assessed by measuring optical density at 470 nm for growth and 680 nm for biomass. This approach uses a spectrophotometer (the Shimadzu UV-1800 UV Spectrophotometer) that communicates with the UVProbe Software on a PC to display the collected data. The absorbance data is then saved in text format (.txt) using the UVProbe software for future reference. Samples with optical density (OD) values exceeding the UV-spectrophotometer's maximum readable limit of 4 were diluted before measurement to guarantee data accuracy. A dilution factor of 3 was achieved by combining 1 mL of the sample with 2 mL of filtered water as the diluent. The measured OD values were multiplied by the dilution factor to determine the actual sample concentration [34]. The relationships between optical density, growth (470nm), and biomass (680nm) and cell density are validated using a calibration curve, as shown in Figure 2.

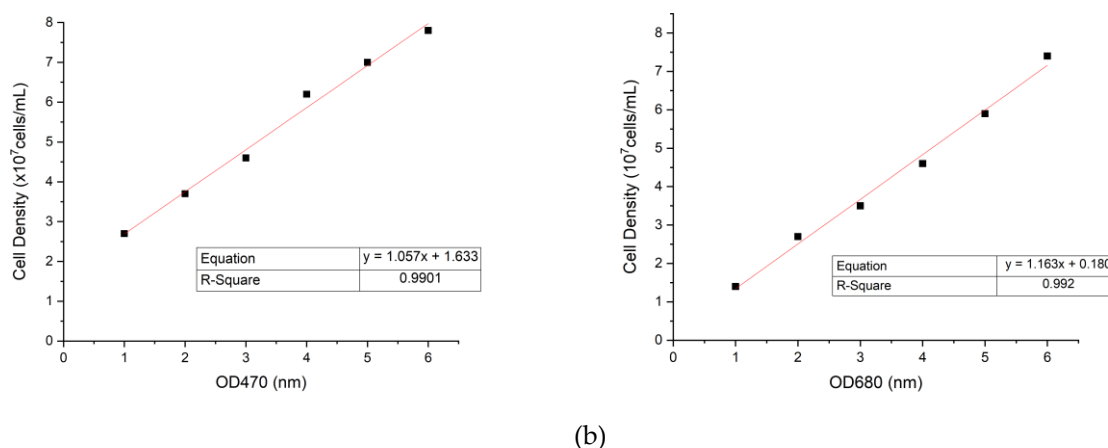


Figure 2. Calibration curve of (a) cell density vs. OD470 and (b) cell density vs. OD680.

3. Results and Discussion

3.1 Wastewater dilution analysis

Figure 3 depicts growth curves for various wastewater dilution percentages. At Day 9, 75% wastewater dilution resulted in the maximum cell density (3.00×10^7 cells/mL), demonstrating an ideal balance of nutritional availability and toxicity. Similarly, 70% dilution resulted in vigorous growth with somewhat reduced (2.67×10^7 cells/mL) but sustained cell densities from Day 12 to Day 21. These findings are consistent with previous studies from [11], which found that moderate dilutions of 50-75% improve algal growth and nutrient intake while minimizing negative effects. In contrast, 100% wastewater (undiluted) initially stimulated rapid growth, reaching a peak of 2.51×10^7 cells/mL at Day 13, but led to an earlier decrease, possibly due to toxic chemical buildup and reduced nutrient efficiency [26]. Lower dilutions, such as 30% and 10%, resulted in restricted growth (1.04×10^7 and 1.44×10^7 cells/mL), even lower toxicity, and inadequate nutrients compared to dilutions over 50%.

3.2 Seed volume analysis

The graph in Figure 4 shows how different microalgae seed volumes (25-250 mL) affect cell density over a 21-day culture period. *Micractinium* sp. seed volume in wastewater cultivation is typically 10% to 20% of total culture volume. This range has been shown to effectively balance initial cell density, nutrient utilization, and light availability, leading to greater growth and biomass [35]. However, in this early investigation, the ideal seed amount is 150 mL, which is 30% of the total culture volume (500 mL). The 150 mL volume produced the highest cell density, peaking at 15×10^7 cells/mL on Day 14, then decreasing progressively. This might be owing to cultural support, such as appropriate light, aeration, and nutrient availability, but caution is advised due to concerns about self-shading and nutrient depletion [36]. Research by [35] also showed that seed volumes above 150 mL (30%) did not boost cell growth, with a peak of roughly 4.1×10^7 cells/mL. In addition, lower amounts below 10% (25 mL) also resulted in modest growth ($\sim 6.0 \times 10^7$ cells/mL).

3.3 Nutrient concentration analysis

Figure 5 shows the influence of varying NPK concentrations (ranging from 25 to 150 mL) on *Micractinium* sp. growth for 21 days. On Day 14, 100 mL of nutrients yielded the greatest growth rate (7.2×10^7 cells/mL). Meanwhile, higher doses inhibited growth, with both 125 and 150 mL showing a rapid decline after reaching peaks of roughly 5.1×10^7 cells/mL and 2.3×10^7 cells/mL, respectively. This trend is consistent with a previous study by [37], which found that nitrogen from baseline to double and quadruple concentrations increased *Micractinium pusillum* KMC8 biomass by 12.5% and 28.78%, respectively. However, additional nitrogen boosted biomass accumulation while decreasing lipid productivity, indicating a trade-off between cell development and lipid accumulation [37]. This corresponds with the outcome graph in this study, whereas increases to 125 and 150 mL reduce overall productivity, possibly due to physiological stress or metabolic

inefficiency caused by nutritional overstock. This reinforces the need for adequate nutrient levels to improve growth and biomass while avoiding excessive nutrients that might disrupt metabolic balance [38].

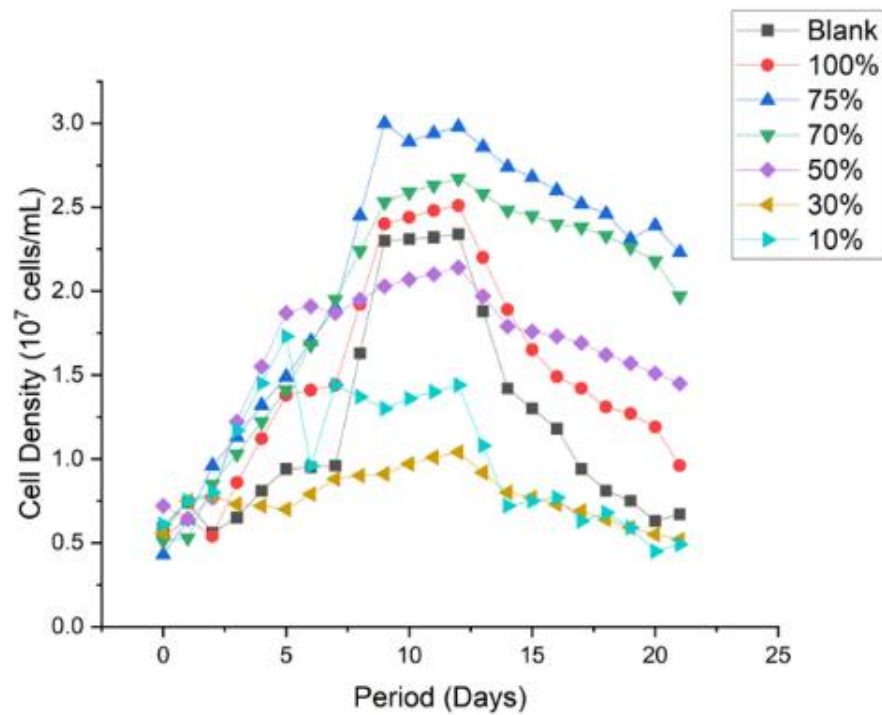


Figure 3. The growth of *Micractinium* sp. at different percentages of wastewater dilution.

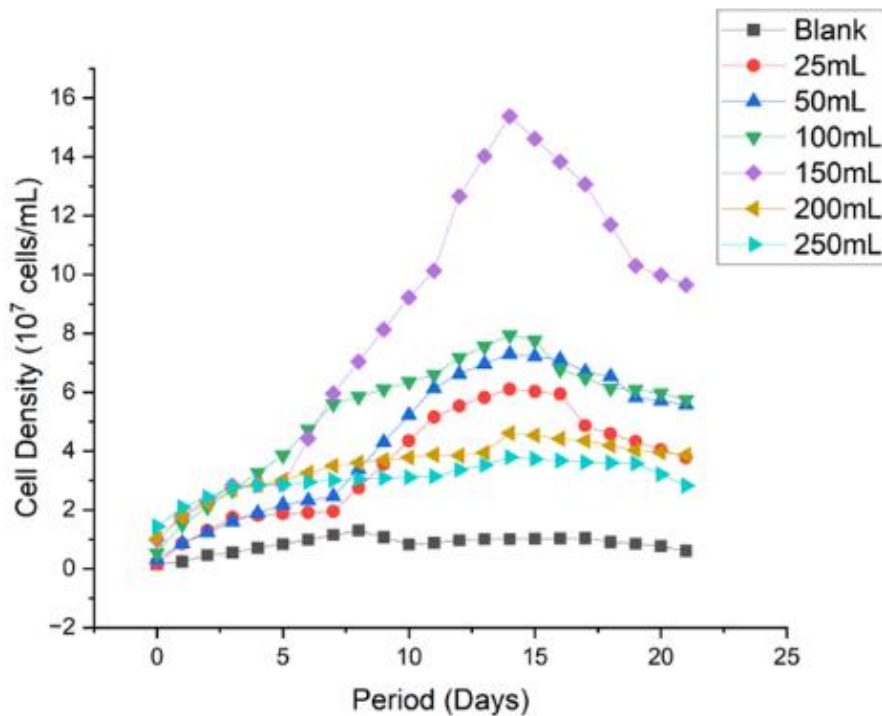


Figure 4. The growth of *Micractinium* sp. at different volumes of microalgae seed.

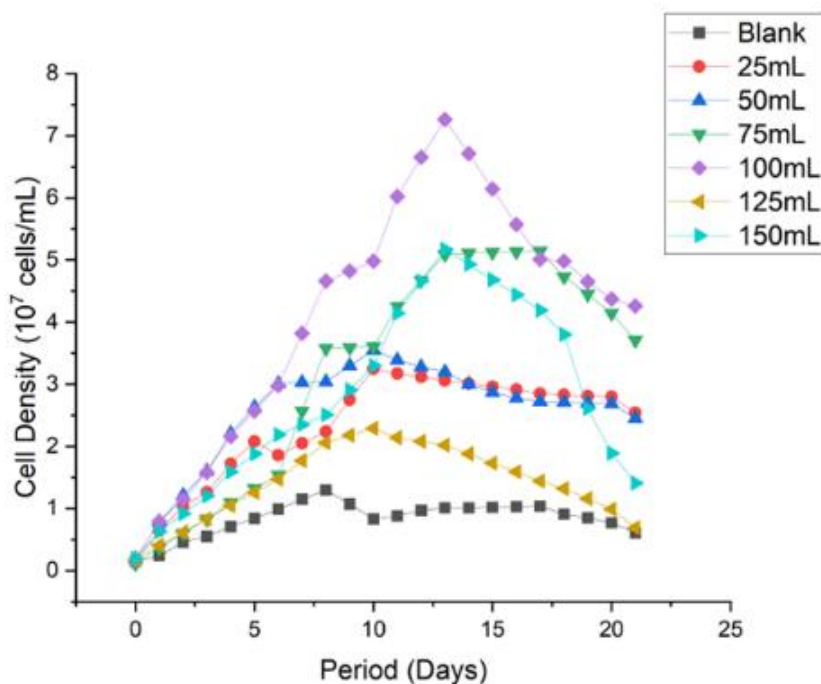


Figure 5. The growth of *Micractinium* sp. at different concentrations of NPK nutrients.

3.4 Ratio optimization via RSM design

The cultivation ratio of *Micractinium* sp. in domestic wastewater was optimized using a face-centered central composite design (FCCCD) based on response surface methodology (RSM). The response in terms of cell density (10^7 cells/mL), optical density (growth at 470nm), and biomass (680nm) across multiple experimental runs under three specified factors: wastewater dilution (%), seed volume (mL), and stimulant/nutrient volume (mL). The FCCCD experiment consisted of 20 trials with varying combinations of the three parameters listed in Table 7. Table 8 shows that the ANOVA for cell density, growth, and biomass responses yielded model F-values of 4.93, 3.08, and 3.20, respectively, indicating significant models ($p = 0.01$, 0.05 , and $0.04 < 0.05$) at the 95% confidence level. The Lack of Fit F-values (3.18, 4.50, and 4.28) likewise indicate non-significant results ($p = 0.11$, 0.06 , and $0.07 > 0.05$), indicating that the model is fit. The R^2 values for the three replies (0.82, 0.73, and 0.74) are likewise in a respectable range (0.70 to 0.90), which is acceptable in most scientific studies. Furthermore, the model met the precision criterion, with values of 8.49, 6.83, and 6.90, all greater than 4, indicating an adequate signal.

Still, based on Table 8, two criteria are out of range: adjusted R^2 (0.65, 0.50, and 0.51) and predicted R^2 (-0.09, -0.44, and 0.38), which have large gaps relative to the R^2 values due to model overfitting. Out of the two criteria, only the predicted R^2 falls significantly outside the acceptable range, whereas the adjusted R^2 only marginally exceeds it. This model remains appropriate for this research because the primary goal is to identify the optimal ratio for future experimental validation rather than to make exact predictions on external datasets [40]. Meanwhile, to ensure the selected model is adequately suited to the experimental design, the Design-Expert 12 program generated a normal plot of residuals to check whether the differences between actual and predicted values follow a normal distribution. The residuals in Figure 6a (cell density), Figure 6b (growth), and Figure 6c (biomass) primarily lie along the red diagonal line, indicating that the errors are fairly evenly distributed and do not indicate substantial model violations. Although there were modest outliers, such patterns are often acceptable in biological research, such as in microalgae culture, owing to natural variability and the complexity of living systems [39]. Several studies have demonstrated that such minor outliers can still yield meaningful and valid optimization results in RSM applications within biological contexts [41] [42]. This validates the regression and RSM models [43].

Table 8. ANOVA for the response surface quadratic model cultivation ratio of *Micractinium* sp. in domestic wastewater supplemented with NPK nutrients.

Criteria		Optical density (AU)		Cell density (10 ⁷ cells/mL)	Acceptable range [39]
		Growth (470nm)	Biomass (680nm)		
F-value	Model	3.08	3.20	4.93	Enough to give $p \leq 0.05$
	Lack of fit	4.50	4.28	3.18	Enough to give $p > 0.05$
p-value	Model	0.05	0.04	0.01	$p \leq 0.05$
	Lack of fit	0.06	0.07	0.11	$p > 0.05$
R ²		0.73	0.74	0.82	≥ 0.70 (≥ 0.60 for biological systems)
R ² – Adjusted R ² gap		0.23	0.23	0.17	≤ 0.10
Adjusted R ² – Predicted R ² gap		0.94	0.89	0.74	≤ 0.20
Adequate precision		6.83	6.90	8.49	≥ 4.0
VIF (Variance Inflation Factor)		1.00 for all factors	1.00 for all factors	1.00 for all factors	≤ 5 (≤ 10 acceptable in biological models)

Furthermore, the interaction between the independent factors (wastewater dilution, seed volume, and stimulant/nutrient volume) and the responses (cell density, growth, and biomass) was carried out via model graphs (3D surface and contour). According to Figure 7a (interaction between seed and stimulant volume), the 3D surface showed that the cell density response increased with increasing stimulant volume and decreasing seed volume. High nitrogen input promotes rapid growth even with low initial microalgae seed volume [44]. Figure 8a illustrates the 3D surface of the second interaction between wastewater dilution and stimulant content, with maximal cell density obtained at the maximum stimulant volume in both the lowest (50%) and highest (100%) ranges of wastewater. Even with 50% wastewater (lower nutrient content), cell density rose due to the highest stimulant supply from the NPK fertilizer and the low levels of harmful substances. Meanwhile, the toxic chemical may reach 100% in wastewater, thereby inhibiting cell density; however, the opposite occurred, as shown in Figure 8a. This is because, under severe conditions, microalgae can exhibit hormetic responses, in which mild stress activates survival systems that increase cell density [45]. In the last interaction between wastewater dilution and seed volume, the 3D surface in Figure 9a showed that the lowest seed volume increased cell density at both 50% and 100% wastewater dilution. At low seed volume, the number of cells is small, resulting in a high nutrient-to-cell ratio. This contributed to the fast growth of microalgae. Other relevant information from Figure 8a and Figure 9a is that the cell density values in the middle range for wastewater dilution are low, resulting in a downward or U-shaped curve on the 3D surface graph. This is because there is insufficient stress (toxic chemical) to stimulate metabolic adaptation, leaving the culture under stressful circumstances that restrict microalgae development [46].

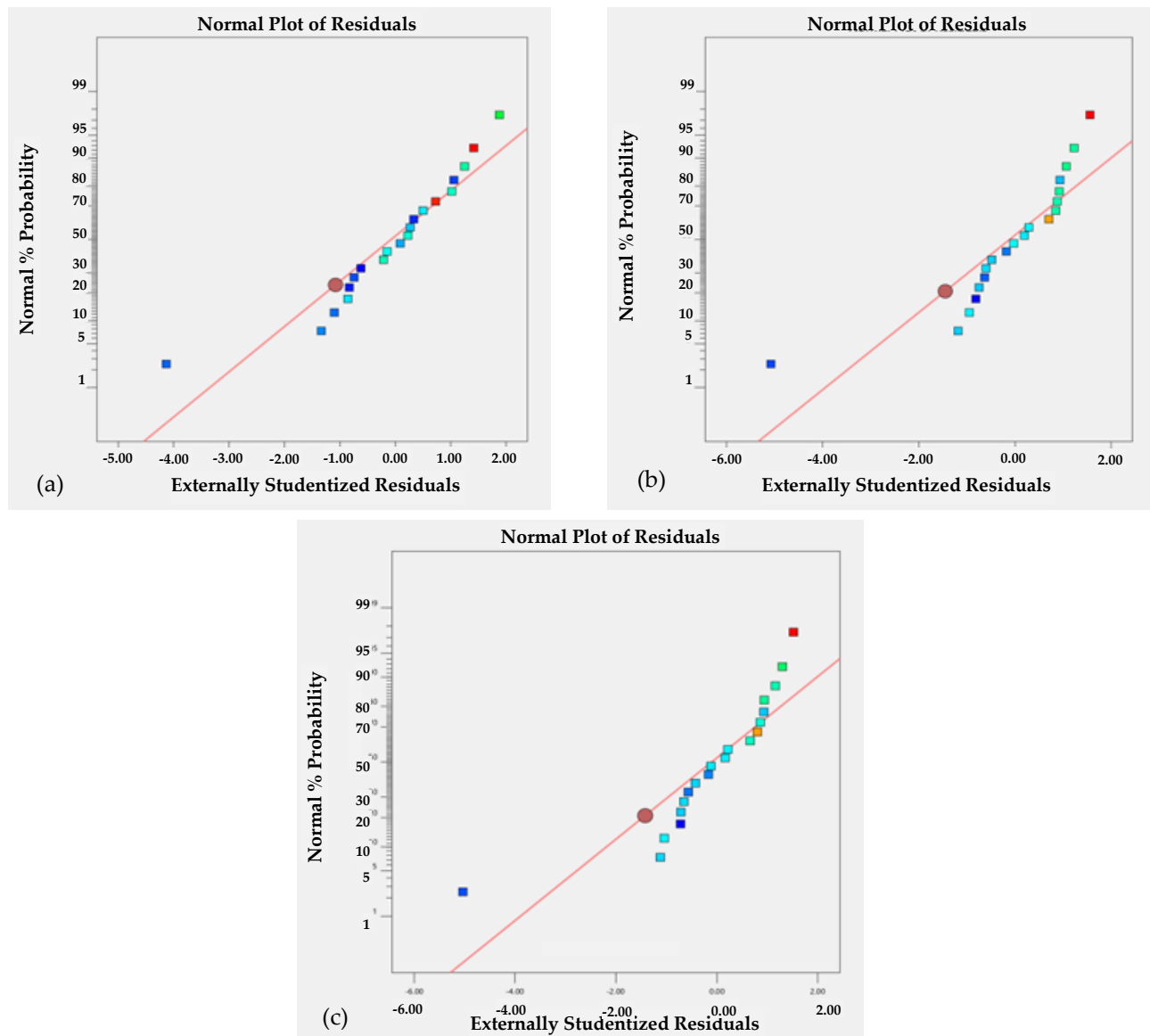


Figure 6. Design expert plot (domestic wastewater); normal probability plot of the internally standardized residual for cultivation response (a) cell density, (b) growth, and (c) biomass nutrient.

The ideal value of wastewater dilution, seed volume, and stimulant volume for the contour plots shown in Figure 7b, Figure 8b, and Figure 9b was 50%, 50 mL, and 110 mL, respectively, pointing inside the experimental area at the maximal cell density, growth, and biomass (green area). After numerical optimization using RSM software, the maximum growth, biomass, and cell density achieved were 6.57 , 6.42 , and 7.14×10^7 cells/mL, as shown in Figure 10, with a desirability of 0.935 , close to 1.000 , indicating a satisfactory solution [47].

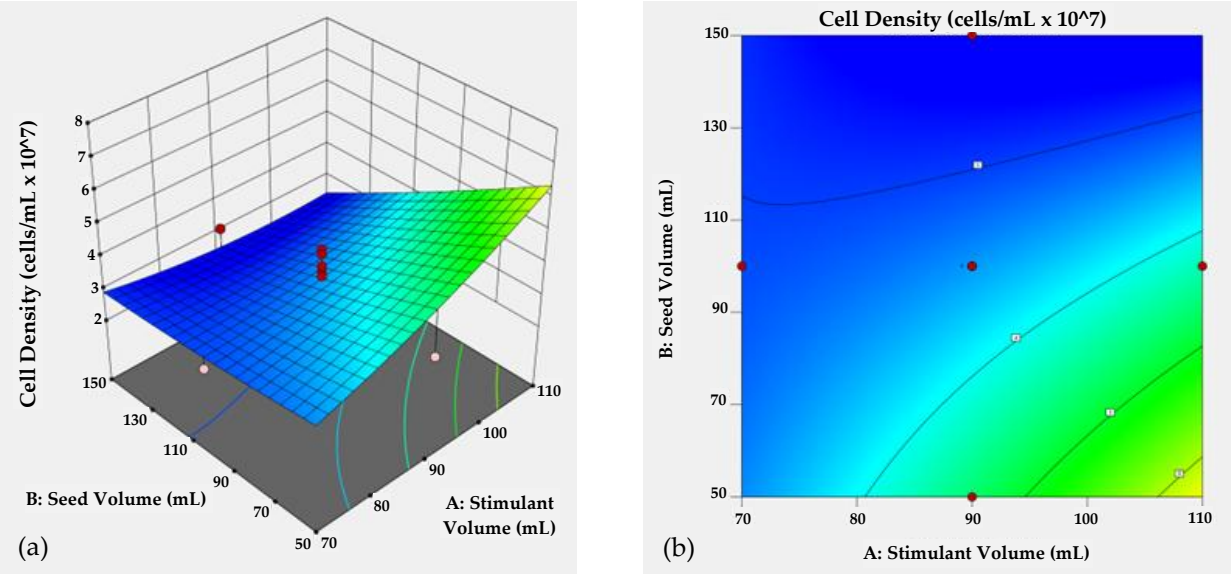


Figure 7. Design expert plot; (a) 3D surface and (b) contour for interaction between seed and stimulant volume.

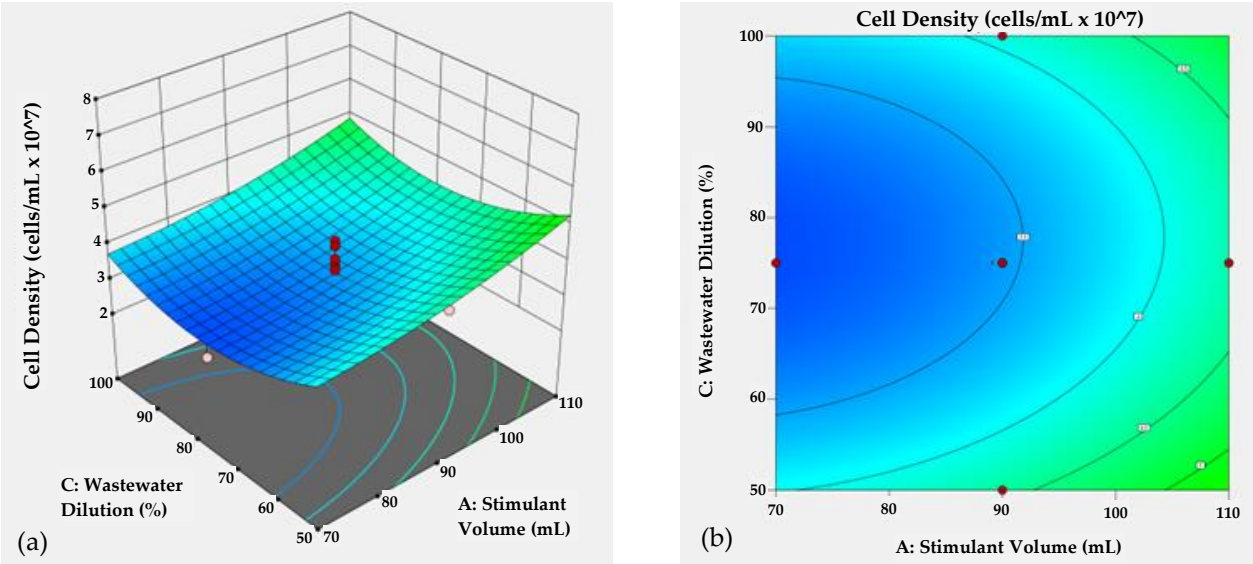


Figure 8. Design expert plot; (a) 3D surface and (b) contour for interaction between wastewater dilution and stimulant volume.

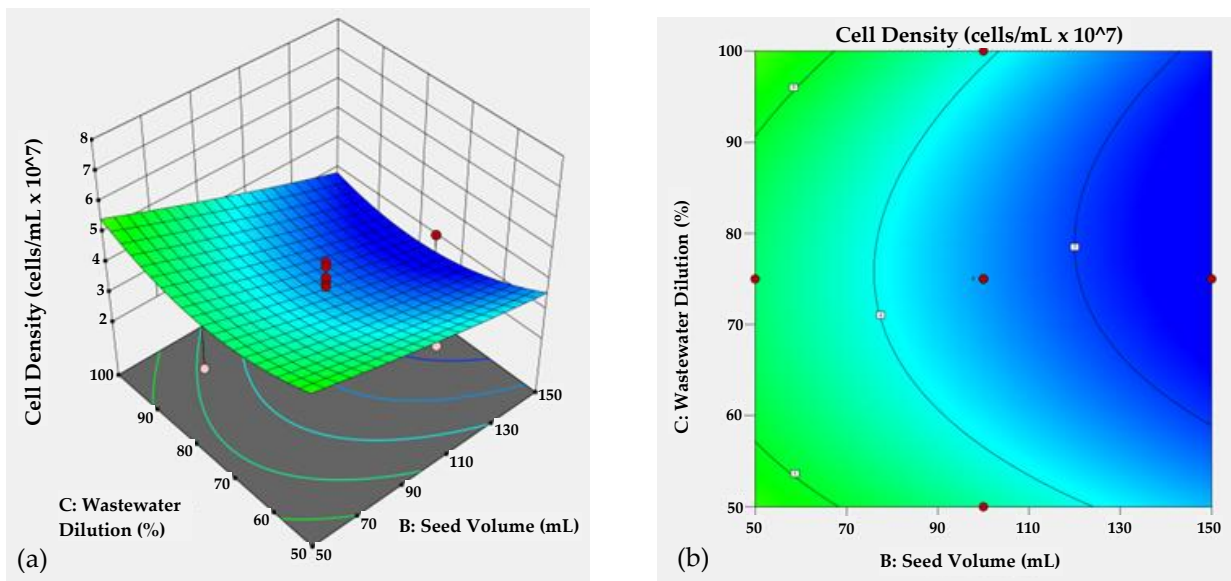


Figure 9. Design expert plot; (a) 3D surface and (b) contour for interaction between wastewater dilution and seed volume.

Beyond this, this study utilizes living microalgae cultures, in which inherent biological variability and environmental changes may influence growth outcomes and lead to discrepancies in cell density between the OFAT testing and RSM trials [48]. While the RSM-optimized ratio did not yield the highest absolute cell densities recorded in the OFAT investigation, the RSM methodology effectively identified cultivation ratios that consistently supported positive, reproducible growth trends. This result achieves the primary goal of optimizing the cultivation ratio. The optimized ratio presented here establishes a solid foundation for future research, in which other significant factors, such as light intensity, aeration, and various environmental variables, can be systematically examined to further improve microalgal productivity and performance.

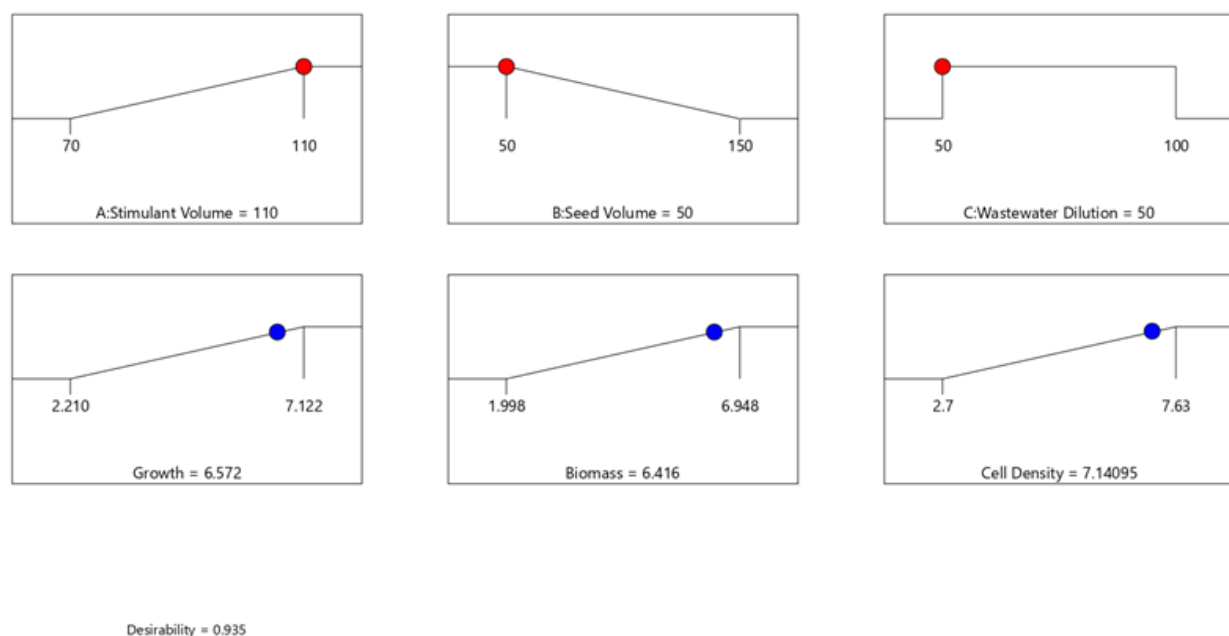


Figure 10. Design Ramps of the factors (wastewater dilution, seed and stimulant volume) and response (cell density, growth, and biomass) optimization.

4. Conclusions

This research accomplished its objectives by demonstrating that *Micractinium* sp. effectively acclimatizes and progressively grows in domestic wastewater. Supplementing NPK nutrients results in sustained, positive growth while simultaneously addressing wastewater pollution by recycling it as a cultivation medium. Using a combination of OFAT and RSM, the optimal cultivation ratio was determined to be 0.68 L diluted domestic wastewater, 0.10 L *Micractinium* sp. seed, and 0.22 L NPK nutrient per liter of culture, providing a solid foundation for future scale-up and application. However, the research encountered challenges related to biological variability, leading to data inconsistencies, and was limited because it did not investigate the impacts of other critical cultivation conditions, such as lighting and aeration. The optimized ratio established here serves as a valuable reference for subsequent studies to investigate and enhance environmental and operational parameters systematically. Notably, this enhances microalgae performance and biomass yield while also expanding its biotechnological and environmental applications.

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Chronic Heat Stress-Induced Alterations in Hormonal Profile (Corticosterone, T3, T4, TSH) and Oxidative Stress Markers (MDA, GSH, GPx) in Laboratory Rats

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Abstract: Chronic heat stress disrupts neuroendocrine balance and promotes oxidative damage, yet the duration-dependence of simultaneous hypothalamic-pituitary-adrenal (HPA) and hypothalamic-pituitary-thyroid (HPT) axis responses and antioxidant status remains poorly defined. This study quantified the effects of daily heat exposure (2, 4, or 8 h/day at 42°C for 21 days) on serum corticosterone, triiodothyronine (T3), thyroxine (T4), and thyroid-stimulating hormone (TSH), and on hepatic oxidative markers malondialdehyde (MDA), reduced glutathione (GSH), and glutathione peroxidase (GPx) in adult male Wistar rats. Thirty-two rats (180–220 g) were randomized into four groups (n = 8): control and three heat-stressed groups (HS-2, HS-4, HS-8). ELISA quantified hormones, and oxidative markers were measured spectrophotometrically; data were analyzed by one-way ANOVA with Tukey HSD and Pearson correlation. Heat stress produced duration-dependent endocrine and oxidative changes ($p < 0.001$). Corticosterone rose 3.5-fold, from 45.2 ± 4.1 to 158.7 ± 13.2 ng/mL in HS-8, whereas T3, T4, and TSH fell by 48%, 55%, and 57%, respectively. MDA increased 3.7-fold (2.14 ± 0.18 to 7.88 ± 0.61 nmol/mL), and GSH and GPx declined by 66% and 71%. Corticosterone correlated positively with MDA ($r = +0.892$) and negatively with GSH ($r = -0.874$); T3 correlated negatively with MDA ($r = -0.861$) and T4 positively with GPx ($r = +0.847$). Chronic heat stress, therefore, causes duration-dependent HPA-axis activation, HPT-axis suppression, and antioxidant depletion, and the tight hormone-redox coupling supports the use of combined hormonal-antioxidant biomarker panels for grading heat-stress severity.

Keywords: Heat stress; corticosterone; thyroid hormones; malondialdehyde; glutathione peroxidase

1. Introduction

Heat stress arises when thermal load exceeds thermoregulatory capacity, activating coordinated neuroendocrine, metabolic, and molecular responses to restore homeostasis [1]. As climate warming intensifies the incidence of extreme-temperature episodes, the physiological consequences of heat exposure have become a central biomedical concern [2]. The hypothalamic-pituitary-adrenal (HPA) axis the central stress-regulating mechanism: rising temperature drives hypothalamic CRH release, pituitary ACTH secretion, and adrenal glucocorticoid output, with corticosterone the dominant rodent glucocorticoid and a quantitative index of HPA activation [3]. Sustained corticosterone

elevation promotes immunosuppression, metabolic dysregulation, infertility, and oxidative injury [4].

In parallel, prolonged stress inhibits the hypothalamic-pituitary-thyroid (HPT) axis by reducing TRH, TSH, and, consequently, T3 and T4 a paradoxical but adaptive reduction in thermogenic drive [5]. Heat is also associated with oxidative stress since high temperatures result in increased electron leakage and superoxide formation but decrease the activities of antioxidant enzymes [6]; this affects the reactive oxygen species to antioxidant ratio [7]. Increased levels of MDA are indicative of lipid peroxidation in cell membranes whereas GSH and GPx values are used to indicate non-enzymatic and enzymatic antioxidants respectively; similar levels of antioxidant depletion, i.e. high MDA and low GSH and GPx levels, were noted in other stress models involving rodents, such as streptozotocin-induced diabetes [8], aspirin-induced gastric damage [9], hyperhomocysteinemia [10] and aging induced by d-galactose [11].

Despite the individual characterization of the heat-induced changes in the hormonal and oxidative systems, limited research has explored the impact of heat on HPA axis, HPT axis, and oxidative status concomitantly in different durations of heat exposure in a single experimental model [12,13]. Thus, this research aimed to evaluate the heat-induced changes in the serum hormones (corticosterone, T3, T4, and TSH) and hepatic oxidative markers (MDA, GSH, and GPx) following the different durations of heat exposure in adult male Wistar rats for 21 days.

2. Materials and Methods

2.1 Ethical approval

All experiments were conducted based on NIH protocols for animal handling and care and given clearance from the Institutional Animal Ethics Committee. The minimum number of animals was used, and they suffered minimal pain.

2.2 Animals and housing

Adult male Wistar rats (10–12 weeks, 180–220 g) were housed individually in stainless-steel cages (48 × 27 × 20 cm) at 22 ± 1°C, 50 ± 5% relative humidity, and a 12 h light/dark cycle (lights on at 07:00 h). Standard diet (20% protein, 5% fat, 60% carbohydrates, trace minerals) and tap water were freely accessible throughout the experiment.

2.3 Experimental design and heat-stress protocol

Once a 7-day acclimatization period had elapsed, thirty-two rats were distributed randomly into four groups of eight using a computer-generated randomization technique (Table 1). Heat stress was introduced into a thermostatically controlled box at 42 ± 0.5°C and 40 ± 5% relative humidity during the light phase from 09:00 hours on a daily basis, while controls were kept at 22 ± 1°C for similar periods. Body temperatures were measured rectally (± 0.1°C) before and after each experiment. The 21-day protocol used is based on a well-established model of chronic heat stress [14].

Table 1. Groups in Experiment, Treatments and Housing Condition.

Group	n	Treatment	Housing conditions
I (Control)	8	Standard conditions; no heat exposure	22 ± 1°C, 12 h light/dark
II (HS-2)	8	Heat stress: 2 h/day, 42°C, 21 days	42°C during exposure; 22 ± 1°C at rest
III (HS-4)	8	Heat stress: 4 h/day, 42°C, 21 days	42°C during exposure; 22 ± 1°C at rest
IV (HS-8)	8	Heat stress: 8 h/day, 42°C, 21 days	42°C during exposure; 22 ± 1°C at rest

n = animals per group; HS = heat stress. All animals received food and water ad libitum throughout the experiment.

2.4 Sample collection

On day 22, after 18 h following the last treatment, The rats were anesthetized using sodium pentobarbital (60 mg/kg, i.p.). Anesthesia was induced 18 h post-treatment to circumvent the occurrence of any acutely elevated levels of corticosterone (with half-life ≈ 60–70 min in rats). The 18-h period was chosen to measure chronic changes in HPA activity rather than acute stress-induced changes, thus the high corticosterone levels measured represent a chronic change in neuroendocrine set point rather than a stress-induced acute increase. Blood was collected through heart puncture. Serum was collected by centrifugation

(3000 × g, 15 min, 4°C) and stored at −80°C. Liver tissue was harvested, washed with cold PBS (pH 7.4), frozen in liquid nitrogen and stored at −80°C.

2.5 Hormonal assays

Serum corticosterone concentrations were determined using competitive ELISA kits (DetectX, Arbor Assays, Ann Arbor, MI, USA; intra/inter-assay CV < 8%/12%), while T3, T4, and TSH were quantified using sandwich ELISA kits (BioAssay Systems, Hayward, CA, USA), cross-validated to measure rat serum (limit of detection: 0.5 ng/dL, 0.1 µg/dL, and 0.02 µIU/mL, respectively).

2.6 Oxidative stress markers

The liver was homogenized at 10% (w/v) in cold PBS (pH 7.4) and centrifuged at 10,000 × g for 15 min at 4°C. MDA level was evaluated using the TBARS assay [15] (95°C, 60 min; 532 nm; tetraethoxypropane as standard). GSH level was assessed by the Ellman DTNB assay [16] (412 nm), and GPx activity by NADPH oxidation at 340 nm [17]. Total protein concentration was estimated by Bradford method (BSA as standard) to ensure

2.7 Statistical analysis

Values are expressed as mean ± SD (SPSS 26.0, IBM, Armonk, NY, USA). Normality was validated using the Shapiro-Wilk test. One-way ANOVA with Tukey's honestly significant difference test and Pearson correlation were used to compare groups and hormones with redox status. Significance was set at $p < 0.05$, with $p < 0.01$ and $p < 0.001$ reported where applicable. The sample size of $n = 8$ per group was based on published chronic heat-stress rodent models [12,13] and is acknowledged as a study limitation given the multiplicity of correlated endpoints (four hormonal and three oxidative markers) assessed simultaneously.

3. Results and Discussion

3.1. Body weight and core temperature

No mortality occurred. Heat-stressed rats appeared to gain less weight than controls, consistent with reduced intake and higher energy expenditure. Quantitative body weight and food-intake data were not formally recorded in this study; this represents a limitation, as caloric restriction can independently suppress HPT axis activity and elevate corticosterone, and the contribution of reduced food intake to the hormonal changes observed cannot be fully excluded. Rectal temperature increased with exposure duration: 38.8 ± 0.4 , 39.4 ± 0.5 , and $40.1 \pm 0.6^\circ\text{C}$ in HS-2, HS-4, and HS-8 versus $36.9 \pm 0.3^\circ\text{C}$ in controls (all $p < 0.001$), confirming graded thermal load.

3.2. Hormonal responses

Corticosterone increased dose-dependently to 1.7-, 2.5-, and 3.5-fold of control in HS-2, HS-4, and HS-8 ($45.2 \pm 4.1 \rightarrow 158.7 \pm 13.2$ ng/mL; $p < 0.001$) (Table 2, Figure 1A). Contrastingly, in HS-8, T3 fell 48% ($92.4 \pm 7.2 \rightarrow 47.8 \pm 3.5$ ng/dL), T4 fell 55% ($5.8 \pm 0.4 \rightarrow 2.6 \pm 0.2$ µg/dL), and TSH fell 57% ($1.42 \pm 0.11 \rightarrow 0.61 \pm 0.05$ µIU/mL) (all $p < 0.001$). These opposing trajectories are illustrated in Figure 1, which contrasts the dose-dependent rise in corticosterone (Figure 1A) with the progressive, duration-dependent suppression of T3 (Figure 1B), T4 (Figure 1C), and TSH (Figure 1D) across the control, HS-2, HS-4, and HS-8 groups. The concurrent decline in TSH and thyroid hormones indicates central HPT suppression rather than primary hypothyroidism. The rise in corticosterone reflects HPA activation via thermosensory drive of paraventricular CRH neurons [18], while the magnitude in HS-8 suggests impaired glucocorticoid negative feedback typical of chronic stress [19]. Such hypercorticotestosterone underlies catabolism, immunosuppression, and circadian disruption [20]. Glucocorticoids suppress TRH transcription via glucocorticoid-response elements in the TRH promoter and inhibit pituitary thyrotrophs [5, 21], consistent with the strong inverse corticosterone-thyroid correlations below and recent evidence of crosstalk [22]. Reduced thyroid output may additionally be adaptive, lowering uncoupled oxidative phosphorylation and thermogenesis [23].

Table 2. Serum hormonal concentrations across experimental groups (mean $\pm$ SD, n = 8).

Parameter	Control (I)	HS-2 (II)	HS-4 (III)	HS-8 (IV)
Corticosterone (ng/mL)	45.2 $\pm$ 4.1	78.6 $\pm$ 6.3*	112.4 $\pm$ 9.8**	158.7 $\pm$ 13.2***
T3 (ng/dL)	92.4 $\pm$ 7.2	78.1 $\pm$ 5.9*	61.3 $\pm$ 4.7**	47.8 $\pm$ 3.5***
T4 (μ g/dL)	5.8 $\pm$ 0.4	4.9 $\pm$ 0.3*	3.7 $\pm$ 0.3**	2.6 $\pm$ 0.2***
TSH (μ IU/mL)	1.42 $\pm$ 0.11	1.18 $\pm$ 0.09*	0.87 $\pm$ 0.07**	0.61 $\pm$ 0.05***

* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. Control (Tukey HSD). T3 = triiodothyronine; T4 = thyroxine; TSH = thyroid-stimulating hormone.

3.3 Oxidative stress markers

MDA rose to 3.7-fold of control ($2.14 \pm 0.18 \rightarrow 7.88 \pm 0.61$ nmol/mL) while GSH and GPx fell 66% ($28.4 \pm 2.1 \rightarrow 9.7 \pm 0.9$ μ mol/L) and 71% ($18.6 \pm 1.4 \rightarrow 5.4 \pm 0.5$ U/mL), respectively, in HS-8 (all $p < 0.001$) (Table 3, Figure 2A–C). Figure 2 illustrates the oxidation status deterioration depending on time, as evidenced by the increasing hepatic MDA concentration in the control group relative to the HS-2, HS-4, and HS-8 group (Figure 2A), accompanied by the decreasing levels of GSH (Figure 2B) and GPx (Figure 2C). This effect agrees with meta-analytical data obtained from studies in heat-stressed animals [24] and suggests the mitochondrial dysfunction associated with increased electron leakage and generation of ROS [25]. Moreover, heat stimulation activates phospholipase A₂ that releases arachidonic acid and triggers lipid peroxidation process [26]; since GSH depletion affects the GPx hydroperoxide scavenging activity, a vicious circle is formed [27]. The decreasing GPx activity may be explained by a variety of reasons: (i) decreased substrate availability because of the faster GSH consumption relative to its generation through glutathione reductase/NADPH pathway; (ii) GPx enzyme inactivation due to the oxidative attack of selenocysteine residues in the active center caused by the excess ROS production; and (iii) possibly transcriptional inhibition of GPx1 and GPx4 genes due to glucocorticoids.

Table 3. Oxidative stress markers across experimental groups (mean $\pm$ SD, n = 8).

Parameter	Control (I)	HS-2 (II)	HS-4 (III)	HS-8 (IV)
MDA (nmol/mL)	2.14 $\pm$ 0.18	3.72 $\pm$ 0.29*	5.61 $\pm$ 0.42**	7.88 $\pm$ 0.61***
GSH (μ mol/L)	28.4 $\pm$ 2.1	22.1 $\pm$ 1.8*	15.3 $\pm$ 1.3**	9.7 $\pm$ 0.9***
GPx (U/mL)	18.6 $\pm$ 1.4	14.2 $\pm$ 1.1*	9.8 $\pm$ 0.8**	5.4 $\pm$ 0.5***

* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. Control (Tukey HSD). MDA = malondialdehyde; GSH = reduced glutathione; GPx = glutathione peroxidase.

3.4 Hormone-redox correlations

Corticosterone correlated positively with MDA ($r = +0.892$) and inversely correlated with GSH ($r = -0.874$); T3 correlated negatively with MDA ($r = -0.861$), T4 positively with GPx ($r = +0.847$), and TSH positively with GSH ($r = +0.823$) (all $p < 0.001$) (Table 4, Figure 2D). The above Pearson correlations were calculated on all 32 individuals obtained from all four groups. The reason why there is an inherent strength to these correlations is due to the fact that there are differences in systematic design in the groups (graded heat duration). These correlations should thus be seen as a reflection of variance between the groups due to the effects of the treatment rather than biological coupling. They should not be taken as evidence of a biochemical association. Partial correlations taking into account the effect of group should be done in further research. The corticosterone-MDA coupling agrees with glucocorticoid pro-oxidant actions via Nrf2 down-regulation and mitochondrial ROS [28], whereas thyroid-antioxidant links reflect thyroid-hormone transcriptional control of GPx and related enzymes [29]; transcriptomic studies report concordant heat-responsive gene networks [14]. It should also be acknowledged that oxidative stress was assessed in hepatic tissue, which was chosen for practical reasons of yield and technical reproducibility. The liver is a metabolically central organ and a valid index of systemic redox status; however, oxidative stress in the adrenal cortex, hypothalamus, or anterior pituitary would be more directly mechanistically informative for HPA/HPT-axis conclusions and represents

an important direction for future work. Collectively, corticosterone, T3, T4, and MDA form a coherent biomarker panel for grading heat-stress severity, relevant to occupational and tropical exposures.

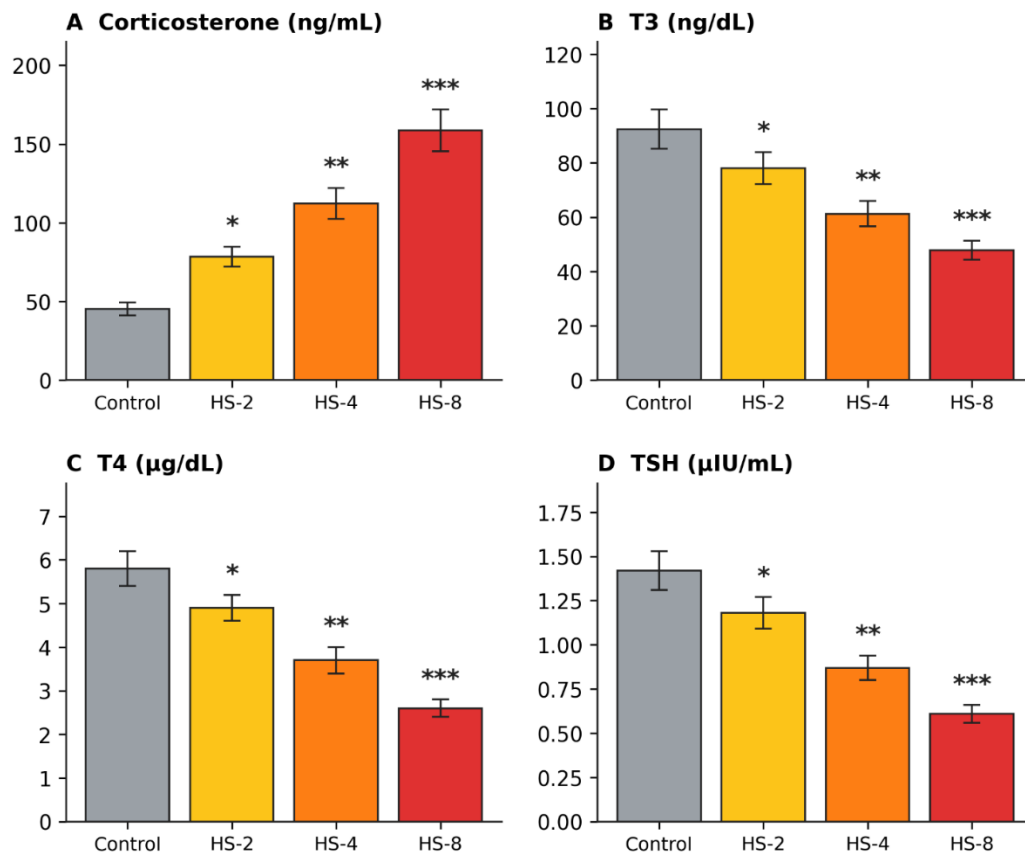


Figure 1. Duration-dependent effects of chronic heat stress (42 °C, 21 days) on serum hormones in adult male Wistar rats. Serum (A) corticosterone, (B) triiodothyronine (T3), (C) thyroxine (T4), and (D) thyroid-stimulating hormone (TSH) in control and heat-stressed groups exposed for 2 (HS-2), 4 (HS-4), or 8 h/day (HS-8). Bars are mean $\pm$ SD ($n = 8$). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ against control (one-way ANOVA, Tukey HSD). Corticosterone rose dose-dependently to 3.5-fold of control, whereas T3, T4, and TSH fell by 48%, 55%, and 57%, indicating concurrent HPA-axis activation and HPT-axis suppression.

Table 4. Pearson correlation coefficients between hormonal and oxidative stress markers.

Variable pair	Pearson r	p-value	Interpretation
Corticosterone vs MDA	+0.892	< 0.001	Strong positive
Corticosterone vs GSH	−0.874	< 0.001	Strong negative
T3 vs MDA	−0.861	< 0.001	Strong negative
T4 vs GPx	+0.847	< 0.001	Strong positive
TSH vs GSH	+0.823	< 0.001	Strong positive

All correlations are significant at $p < 0.001$. r = Pearson correlation coefficient.

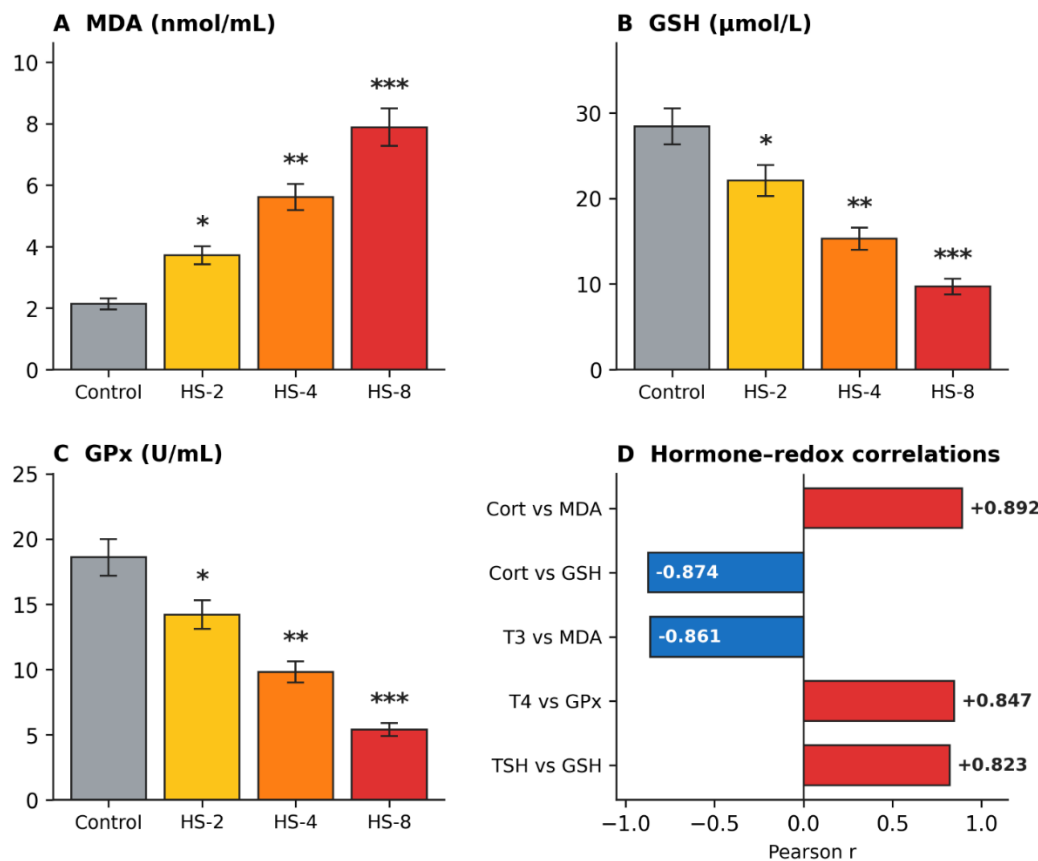


Figure 2. Duration-dependent oxidative stress and hormone-redox associations. Hepatic (A) malondialdehyde (MDA), (B) reduced glutathione (GSH), and (C) glutathione peroxidase (GPx) across control and heat-stressed groups (mean $\pm$ SD, $n = 8$; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ versus control, one-way ANOVA with Tukey HSD). (D) Pearson correlation coefficients between hormonal and oxidative markers (all $p < 0.001$); red and blue denote positive and negative associations, respectively. MDA increased 3.7-fold while GSH and GPx declined 66% and 71%; corticosterone correlated positively with MDA ($r = +0.892$) and negatively with GSH ($r = -0.874$).

3.5 Limitations

Male rats only were investigated, along with one temperature (42°C), thus ignoring any sex-related effects as well as temperature-humidity combination effects; oxidative stress was assessed in the liver and not in neuroendocrine tissues; and there is yet to be a molecular characterization of the suppression of antioxidants by glucocorticoids based on the results of transcriptomic and proteomic analyses. Concerning the temperature used, 42°C is an extremely high temperature not only because it is outside the physiological range but also because it is used to assess heat stress of laboratory housed rats; real-life heat stress of livestock and humans occurs at much lower temperatures ranging from 35–38°C with high humidity levels. As far as the sex of the animal is concerned, the restriction to males only is not a trivial limitation since estrogens regulate not only HPA reactivity but also the activity of antioxidant enzymes including GPx and GSH synthetic enzymes.

4. Conclusions

Chronic 42°C exposure for 21 days produced duration-dependent HPA activation (corticosterone rose to 3.5-fold of control at 8 h/day) and HPT suppression (T3, T4, and TSH down 48–57%) alongside progressive oxidative stress (MDA rose to 3.7-fold of control; GSH –66%; GPx –71%) in adult male Wistar rats. The strong hormone-redox correlations indicate integrated endocrine-oxidative mechanisms in heat-stress pathophysiology and support the use of combined hormonal-antioxidant biomarker panels to assess heat-stress severity. As extreme-heat events grow more frequent, such combined biomarker signatures could aid

risk stratification in occupational, clinical, and livestock contexts. Future work should incorporate both sexes, realistic temperature-humidity profiles, neuroendocrine-tissue redox assessment, and molecular pathway analysis.

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Antibacterial Activity of Fungal Endophytes Isolated from Kratom (*Mitragyna speciosa*) in Thailand

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Abstract: Kratom (*Mitragyna speciosa*), which is an indigenous plant in Asia, has been used as traditional medicine for decades. This study focuses on fungal endophytes originally isolated from kratom and their antibacterial activity. Kratom plants were obtained from seven locations in Thailand. In each location, three plant parts, including leaves, stems, and roots, were collected. The plant materials were surface-sterilized and isolated for endophytic fungi on PDA amended with kanamycin. A total of 347 isolates of fungal endophytes were isolated, and 92 isolates were randomly selected for antibacterial activity against *Bacillus subtilis*, *Staphylococcus aureus*, *Escherichia coli*, and *Pseudomonas aeruginosa*. They were cultured in 50 ml of potato dextrose broth (PDB) at 25°C for 4 weeks, and the culture broth was tested against 4 bacteria using an agar well diffusion method. 11 isolates (12%) inhibited at least one bacterial species. Eight endophytes inhibited *B. subtilis*, while four, three, and one endophytes inhibited the other three bacteria, respectively. Six isolates of fungal endophytes were chosen for further screening. They were cultured in 2 L of PDB and incubated at 25°C for four weeks. After mycelium was separated, only culture broth was extracted three times with ethyl acetate using rotary evaporation, and the extracts were dissolved in DMSO before testing. Antibacterial activity was determined by disk diffusion, and inhibition zones were recorded. One crude extract (L1-01) partially inhibited *E. coli* (8.28 mm). In the three crude extract tests against *S. aureus*, L5-10 exhibited the highest activity (17.51 mm). Among four crude extracts inhibiting *B. subtilis*, R5-09 had the strongest activity (21.3 mm). These two fungal endophyte isolates (R5-09 and L5-10) were identified based on morphological and molecular characteristics and assigned to *Aspergillus allahabadii* and *Aspergillus terreus*, respectively. This finding emphasized that fungal endophytes from kratom have potential as a source of antibacterial substances and should be further studied for other bioactivities.

Keywords: Antibacterial; *Aspergillus*; fungal endophytes; *Mitragyna speciosa*

1. Introduction

Kratom (*Mitragyna speciosa*), a tropical tree native to Southeast Asia, is found in countries such as Thailand, Malaysia, Indonesia, and Myanmar [1]. In many Asian countries, it has been used in traditional medicine for centuries. It has gained attention for its psychoactive properties. The primary active compound, Mitragynine, is involved in pain relief and mood regulation; therefore, it is used for pain management, mood enhancement, and relief from anxiety or stress [2]. In many Western countries, especially the US, consumption of kratom has been growing substantially since the 2000s. An estimated 1.7

million Americans used kratom in 2021 [3]. In Thailand, kratom was removed from the narcotics list in 2021 and is now legally permitted for cultivation and research purposes. However, certain restrictions remain regarding its sale, advertising, and use among specific groups. Therefore, kratom samples used in this study were collected in accordance with Thai regulations.

Antibiotic resistance is characterized by the ability of pathogenic microbes to resist the effects of medications that once effectively treated infections, and it is a devastating and growing public health concern [4]. The discovery of new antibiotics is one of the most pressing challenges in modern medicine. The pace of discovering new antibiotics has slowed over the past few decades. However, antibiotic resistance has risen dramatically. Given this growing crisis, researchers worldwide are exploring a range of approaches to discover novel antibiotics. Addressing antibiotic resistance is urgent and requires coordinated efforts across multiple sectors, and finding alternative antibacterial substances is needed.

Endophytic fungi are a group of fungi that live asymptotically within the tissues of plants. They can be found in various plant species and often form a mutualistic relationship with their host plant. Various endophytic fungi originally isolated from medicinal plants produce secondary metabolites with antimicrobial, antiviral, or anticancer properties, making them of interest to the pharmaceutical and industrial sectors [5-9]. However, studies on fungal endophytes associated with kratom remain scarce. Currently available information is primarily limited to university-level theses [10, 11], while peer-reviewed publications in scientific journals are still lacking. The objectives of this study were (1) to isolate endophytic fungi from kratom collected in Thailand and (2) to screen for their antibacterial activity against four bacterial species.

2. Materials and Methods

2.1 Isolation of fungal endophytes

Samples of kratom were collected from seven locations in Chachoengsao Province, located in the eastern part of Thailand. The kratom plants used in this study were cultivated by residents and collected from residential areas and household surroundings, which were predominantly surrounded by rice fields and agricultural land. Three plant parts of kratom, including leaves, stems, and roots, were collected, and the samples were brought back to the lab for isolation within 48 hours. Healthy and symptomless plant materials were used in this study. Plant materials were rinsed with tap water to remove dirt and then dried at room temperature for 1 hour in a biosafety cabinet. Leaves were cut into 10 pieces (1x1 cm²) while stems and roots were cut into 10 pieces (1 cm). They were surface-sterilized using a method modified from that described by Singwongwatana [10]. The plant materials were submerged in 95% ethanol for 1 minute, then in 5% sodium hypochlorite for 5 minutes, then in 95% ethanol for 30 seconds, and finally rinsed twice in sterile distilled water for 1 minute. The effectiveness of surface sterilization was verified by plating the final sterile rinse water onto PDA to confirm the absence of epiphytic microbial contamination. Sterile plant samples were placed on potato dextrose agar (PDA) amended with kanamycin (50 mg/L) (5 pieces/plate). PDA plates were incubated at 25°C for 7 days. After fungal hyphae were observed in plant materials, they were transferred to new PDA without antibiotics, and pure cultures of endophytes were maintained in PDA slants at 4°C throughout the study.

2.2 Determination of antibacterial activity

2.2.1 Agar well diffusion with culture broth

Endophytes were cultured in 250-ml flasks containing 50 ml of potato dextrose broth (PDB). The flasks were incubated at 25°C for four weeks. Their antibacterial activity was tested against four bacterial species: *Bacillus subtilis*, *Staphylococcus aureus*, *Escherichia coli*, and *Pseudomonas aeruginosa*. Culture broths of the fungal endophytes were screened using an agar well diffusion method. Inocula were adjusted to 0.5 McFarland Standard (equivalent to 1.5×10^8 CFU/ml) using sterile normal saline solution (NSS). The inoculum was swapped onto Mueller-Hinton agar (MHA) using a sterile cotton swab. A seeded agar plate was punctured with a 3 (6 mm) cork borer, and 30 µl of 4-week culture broth of fungal endophytes was added to each well. The experiment was done in triplicate. After incubation at 37°C for 18 hours, the inhibition zones (diameter in millimeters) were observed, measured, and recorded. The spectrum of antimicrobial activity was the primary criterion for selecting endophytic fungal isolates, while the size of the inhibition zone was a secondary criterion.

2.2.2 Disk diffusion with crude extracts

Potential endophytes were cultured in 500 flasks containing 250 ml of PDB, with 8 replicates (8 flasks/isolate). The flasks were incubated at 25°C under stationary conditions, as static cultivation may promote the production of specific secondary metabolite profiles. After 4 weeks, the culture broth from each isolate was pooled for chemical extraction. To separate the culture broth from the mycelia, the broth was filtered through a sterile Whatman filter paper (No. 1, 90 mm in diameter). The culture broths were extracted three times with ethyl acetate and evaporated to dryness using a rotary evaporator at 45°C. All extracts were kept at room temperature. Extracts were dissolved in DMSO (1% v/v) at a concentration of 1 g/ml and stored as a stock solution. A similar method was used for inoculum preparation as previously described in an agar well diffusion method. A paper disk (Whatman No. 1, 6 mm in diameter) was placed on the surface of the MHA seeded with the test bacteria. A total of 10 µl of the extract was pipetted onto the disk, resulting in 10 mg/disk, and 10 µl of DMSO (1% v/v) was used as a control. The plates were incubated using the same conditions mentioned earlier. Inhibition zones were observed and recorded.

2.3 Identification of potential fungal endophytes

2.3.1 Morphological identification

Fungal endophytes that exhibited antibacterial activity were identified based on their morphological characteristics. They were cultured on PDA and incubated at 25°C for two weeks. Their colony appearances were observed and photographed. Colors of fungal colonies were measured and rated using the Online Auction Color Chart (OAC), and a slide culture technique was used to induce sporulation and observe some fungal reproductive structures. The fungi were stained with lactophenol-cotton blue and studied under a microscope.

2.3.2 Molecular identification

Molecular identification of the potential fungal endophytes was based on sequence analyses of the internal transcribed spacer region (ITS). The method follows our previous study with some modifications [12]. Their genomic DNA was extracted using a CTAB method. PCR master mix (50 µl reaction volume) contains 10X buffer, 2.5 mM MgCl₂, 0.2 mM dNTPs, 0.2 µM of each primer (ITS5 and ITS4), and 1 U Taq DNA polymerase (Vivantis Technologies). Their ITS region was amplified using primers ITS5/ITS4 using following PCR condition (1) preheat 96°C (2 min) (2) denature 96°C (1 min) (3) annealing 53°C (1 min) (4) extension 72°C (1 min 30 s), (5) repeat of (2)-(4) for 35 cycles (6) final extension 72°C (10 min) (7) final hold 15°C [13]. PCR products were purified and sequenced in both directions by Macrogen Inc., Korea. The DNA sequences of endophytes were compared with sequences deposited in the GenBank database, and the sequences with the highest similarity values were downloaded. The dataset was aligned using BioEdit v.7.2.5 and MEGA v.11.0.13. Phylogenetic analyses were performed using the Maximum Likelihood (ML) and Maximum Parsimony (MP) methods in PAUP v4.0a169, and two statistical supports, ML bootstrap (MLBS) and MP bootstrap (MPBS), were calculated. The phylogenetic tree was constructed and edited using TreeView v0.5.0. The sequences of these fungal endophytes were deposited in the GenBank Database, where they were assigned accession numbers PX715484 and PX715485.

2.4 Statistical analysis

The results for antibacterial activity of culture broth and crude extracts were expressed as the mean $\pm$ standard deviation ($\bar{X} \pm SD$) of 3 replicates (3 Petri plates).

3. Results and Discussion

3.1 Sources and diversity of endophytic fungi from kratom

From seven locations, three plant parts (leaves, stems, and roots), and ten plant materials from each plant part, there were 210 pieces of plant material. A total of 347 isolates of fungal endophytes were recovered from this study. The recovery rate was 1.6 isolates per plant piece. Considering plant parts, there were 170, 95, and 82 isolates originally from leaves, stems, and roots, respectively. Therefore, 51, 21, and 20 endophyte isolates were randomly selected based on morphotype for further study. Therefore, 92 endophyte isolates were screened for antibacterial activity. Some of the different morphotypes of endophytes are presented in Figure 1.

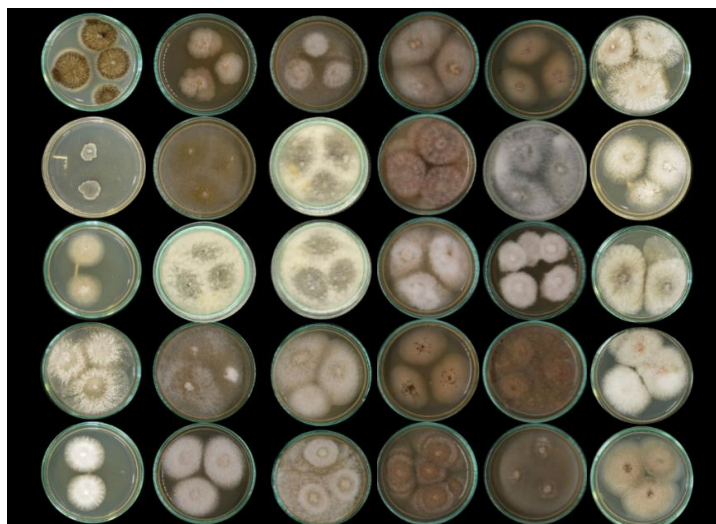


Figure 1. A sample of fungal endophytes originally isolated from kratom (*Mitragyna speciosa*) in Thailand. A diverse and wide range of colony appearances of endophytes were obtained. The endophytes were cultured on PDA and incubated at 25°C for 7 days.

Although kratom has been extensively studied on its pharmaceutical and medicinal properties, there is little information on fungal endophytes originally isolated from this herbal plant. In a previous study conducted in Indonesia, 43 fungal endophyte isolates from kratom twigs and leaves showed higher diversity than those from leaves [11]. In this study, however, leaves exhibit greater endophyte diversity than stems and roots. A total of 170 isolates were obtained from leaves, while 95 and 82 isolates were obtained from stems and roots, respectively. Our results emphasize that leaves are a major source of endophytes and that fungal endophytes have great diversity awaiting further discovery. The majority of research related to kratom mostly focuses on the chemical properties of natural products extracted directly from this herbal plant. Despite being recognized as an important source of bioactive compounds, fungal endophytes of kratom have been overlooked and underestimated. This finding also expands the knowledge of kratom and its fungal diversity. Other plant parts of kratom, for example, the flower, bark, and trunk, should be studied for fungal endophytes, which might lead to further discoveries of greater diversity.

3.2 Antibacterial activity

3.2.1 Antibacterial activity from culture broth

Endophytic fungal isolates were incubated for 4 weeks due to variations in growth rate. While some fungi grew rapidly, others grew more slowly. The extended cultivation period was therefore intended to ensure complete fungal development and to facilitate the production of diverse secondary metabolites. When a 4-week-old culture broth of endophytes was screened for antibacterial activity, three samples from three isolates (L1-01, L1-04, and S1-01) inhibited *E. coli*, with inhibition zones of 7.53, 8.63, and 7.56 mm, respectively (Table 1 and Figure 2). While only one sample (S1-01) also inhibited *P. aeruginosa*, with a weaker inhibition zone of 6.74 mm. Four samples demonstrated antibacterial activity against *S. aureus*. They include L5-10, S5-10, R5-09, and L6-20, and the inhibition zones were 11.13, 17.13, 15.63, and 15.12 mm, respectively. Eight samples inhibited *B. subtilis*, with inhibition zones ranging from 8.78 to 18.42 mm; sample R5-09 showed the strongest activity.

3.2.2 Antibacterial activity from chemical extracts

Only six isolates of fungal endophytes were chosen for further screening. They were cultured on PDB, and the culture broth was extracted with ethyl acetate. Six crude extracts were obtained from the culture broth of these endophytes (Table 2). Antibacterial activity against *P. aeruginosa* was not determined due to low activity. Only one extract from Isolate L1-01 showed partial inhibitory activity against *E. coli* (8.28 mm in diameter). Four extracts showed antibacterial activity against *B. subtilis*, with one extract from Isolate R5-09

exhibiting the highest activity (21.3 mm in diameter; Figure 3). Three crude extracts inhibited *S. aureus*, while one extract from Isolate L5-10 produced the highest activity toward *S. aureus* (17.51 mm in diameter).

Table 1. Antibacterial activity of culture broth of fungal endophytes isolated from kratom

Isolate	Inhibition zone (diameter in mm±SD)			
	<i>B. subtilis</i>	<i>S. aureus</i>	<i>E. coli</i>	<i>P. aeruginosa</i>
L1-01	–	–	7.53 ± 0.00	–
L1-04	–	–	8.63 ± 0.00	–
S1-01	–	–	7.56 ± 0.00	6.74 ± 0.09
R3-14	8.78 ± 0.98	–	–	–
S4-12	12.53 ± 0.00	–	–	–
L5-10	14.99 ± 0.75	11.13 ± 0.41	–	–
L5-13	11.07 ± 0.82	–	–	–
S5-10	16.87 ± 0.12	17.13 ± 0.19	–	–
R5-09	18.42 ± 0.45	15.63 ± 0.29	–	–
L6-20	16.60 ± 0.82	–	–	–
R6-04	17.06 ± 0.12	15.12 ± 0.76	–	–
PDB (Control)	0.00	0.00	0.00	0.00

Note –, No activity

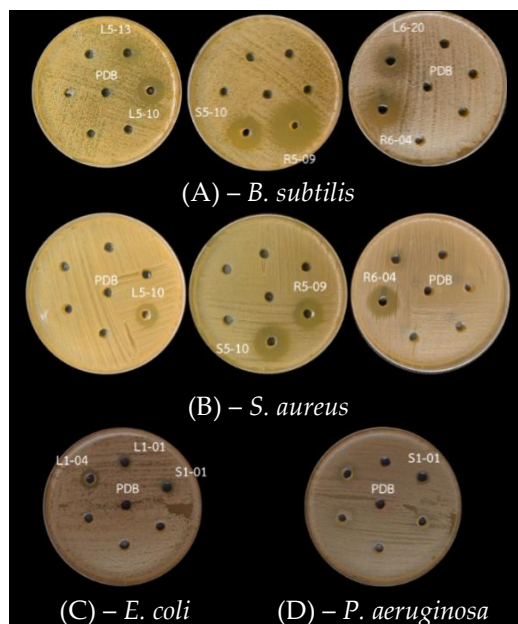


Figure 2. Antibacterial activity against four bacteria using the agar well diffusion method. (A) – *Bacillus subtilis*, (B) – *Staphylococcus aureus*, (C) – *Escherichia coli* and (D): *Pseudomonas aeruginosa*. The test bacteria were cultured on MHA and incubated at 37°C for 18 hours.

Table 2. Antibacterial activity of ethyl acetate crude extracts from fungal endophytes isolated from kratom

Extract	Inhibition zone (diameter in mm±SD)			
	<i>B. subtilis</i>	<i>S. aureus</i>	<i>E. coli</i>	<i>P. aeruginosa</i>
L1-01	ND	ND	8.28 ± 0.15	ND
R3-14	13.5 ± 0.55	ND	ND	ND
L5-10	13.85 ± 0.25	17.51 ± 0.75	ND	ND
R5-09	21.3 ± 0.91	14.1 ± 0.10	ND	ND
R6-04	8.14 ± 0.74	8.23 ± 0.91	ND	ND
DMSO (Control)	0.00	0.00	0.00	ND

ND = Not determined

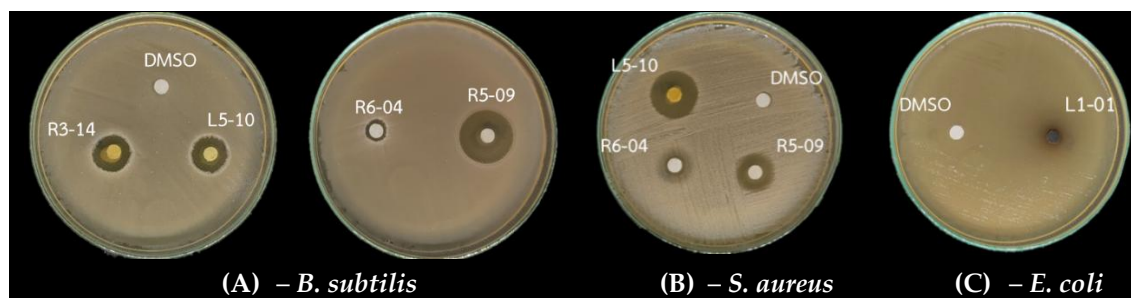


Figure 3. Antibacterial activity against four bacteria using the disk diffusion method: (A) – *Bacillus subtilis*, (B) – *Staphylococcus aureus*, and (C) – *Escherichia coli*. The test bacteria were cultured on MHA and incubated at 37°C for 18 hours.

Most of the fungal endophytes tested in this study failed to inhibit *E. coli* and *P. aeruginosa*, two representatives of Gram-negative bacteria. However, numerous fungal endophyte isolates inhibited *B. subtilis* and *S. aureus*, with wide inhibition zones. Antibacterial activity of 65 endophyte isolates from *Indigofera suffruticosa* leaves was tested against five bacteria, including *B. subtilis*, *S. aureus*, *E. coli*, *K. pneumoniae*, and *P. aeruginosa* [14]. Only one fungal endophyte inhibited *P. aeruginosa*, while the majority of endophytes inhibited the other four bacteria. It clearly demonstrates that Gram-negative bacteria are more resistant to antibiotics than Gram-positive bacteria. Gram-negative bacteria possess an outer membrane (OM) that serves as an effective permeability barrier, contributing to their greater antibiotic resistance compared with Gram-positive bacteria [15]. For antimicrobial agents to exert their activity, they must penetrate this outer membrane to reach intracellular targets. Gram-negative bacteria can further enhance resistance through several mechanisms, including porin modification, alterations in membrane hydrophobicity and lipopolysaccharide (LPS) composition, efflux pumps, and enzymatic inactivation of antimicrobial compounds. In contrast, Gram-positive bacteria lack this additional outer membrane, making them generally more susceptible to antibacterial agents.

Antibacterial activities of fungal endophytes against Gram-positive bacteria, including *B. subtilis* and *S. aureus*, have been widely reported. Among numerous fungal endophyte species isolated from *Dendrobium devonianum* and *D. thyrsiflorum*, the endophytic fungus *Phoma* sp. demonstrated the strongest activity against *B. subtilis* [16]. Three compounds extracted from the culture broth of *Phomopsis* sp., an endophytic fungus of *Garcinia kola*, inhibited *S. aureus* ATCC 25923 [17]. An extract obtained from *Penicillium brevicompactum*, an endophytic fungus isolated from *Abies numidica*, exhibited inhibitory effect against various strains of *S. aureus*, including *S. aureus* ATCC25923 and five other strains of multidrug-resistant *S. aureus* [18]. Metabolites from *Xylaria grammica*, an endophyte isolated from *Urtica fissa*, also inhibited *S. aureus* by inducing morphological deformations in the cell surface and enhancing cell membrane permeability, leading to cytoplasmic leakage [19]. Our findings are consistent with these previous reports and thus confirm that fungal endophytes are a good source of antibacterial compounds. Ethyl acetate was chosen as a solvent for chemical extraction due to its many advantages. It has moderate polarity, with good solubility properties for a wide range of natural products, particularly secondary metabolites. Ethyl acetate is considered less toxic and safer than other solvents, and it also has a lower boiling point; therefore, it allows the extraction of compounds without excessive heat that might degrade sensitive natural products. Among ten publications related to crude extracts of endophytes, ethyl acetate was the most used solvent, with eight publications [20]. Meanwhile, other solvents such as butanol, methanol, and ethanol should be used for further studies, as they may lead to the discovery of wider classes of natural products.

3.3 Identification of potential fungal endophytes

3.3.1 Morphological identification (Figure 4)

The two isolates of potential fungal endophytes (L5-10 and R5-09) were identified based on morphological features and molecular data. Based on morphological studies, they grew rapidly on PDA and showed considerable differences in colony appearance and mycelial pigmentation on the medium. Under the microscope, they all produced conidiophores. Their conidiophores were unbranched stipes with a swollen apex (vesicle) and phialides borne directly on the vesicle. Conidia were globose and formed in dry chains

attached to phialides. These are common features for the genus *Aspergillus*. Therefore, these two isolates were identified at the generic level as *Aspergillus* species.

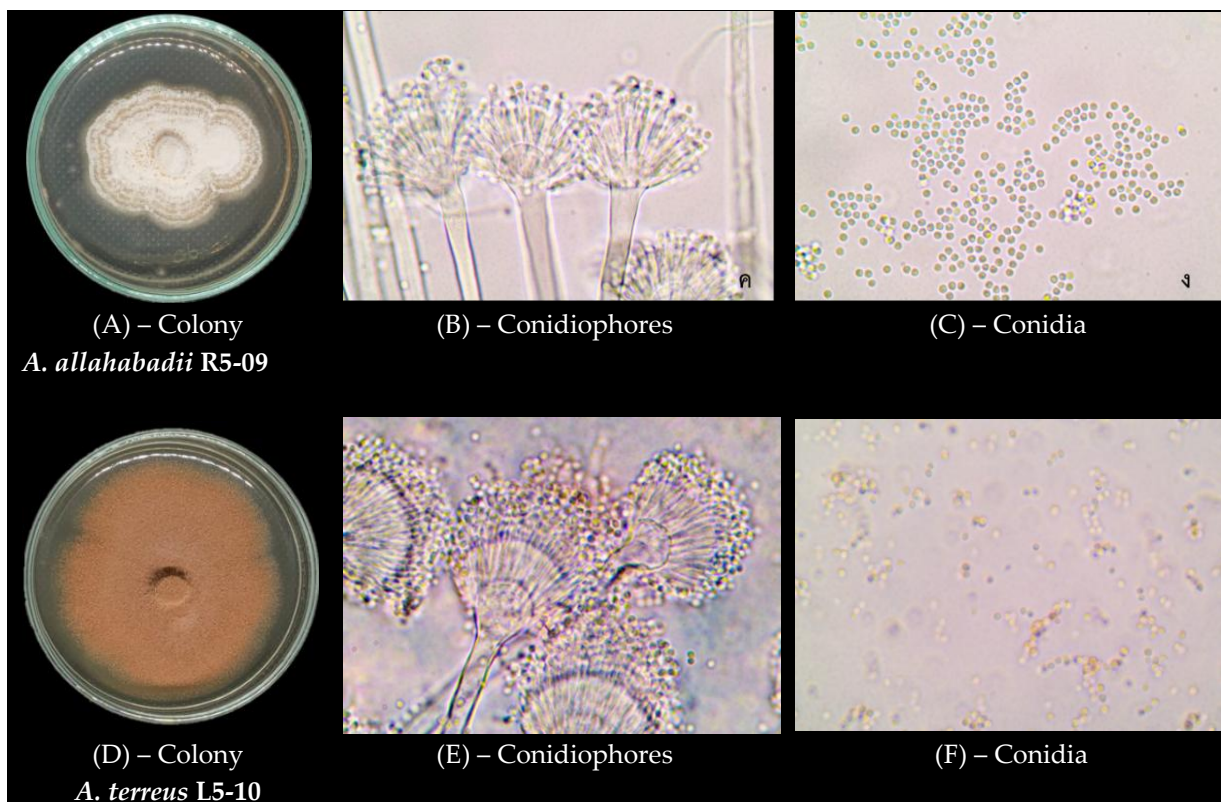


Figure 4. Morphological characteristics of two potential fungal endophytes from kratom. They were cultured on PDA incubated at 25°C for 7 days. *Aspergillus allahabadii* R5-09 (upper row). (A) – Colony on PDA, (B) – Conidiophores and (C) – Conidia.; *Aspergillus terreus* L5-10 (lower row), (D) – Colony on PDA, (E) – Conidiophores and (F) – Conidia.

Description of *Aspergillus allahabadii* R5-09

Macroscopic feature: Colony diameter in 32.3 mm on PDA for 7 days at 25°C (Figure 4 A-C). Surface: Concentric, slightly raised at center, velvety texture. Colony form: Irregular margin with concentric rings. Colony color: White to pale. Microscopic feature: Mycelium: Hyaline hypha, unbranched conidiophore, Conidia: Globose, hyaline to light green, smooth.

Description of *Aspergillus terreus* L5-10

Macroscopic feature: Colony diameter in 43.4 mm on PDA for 7 days at 25°C (Figure 4 D-F). Surface: Smooth and flat. Colony form: Circular with a margin. Colony color: Dark orange to light brown. Microscopic feature: Mycelium: Hyaline hypha, hyaline conidiospore or slightly coloured. Conidia: Globose to ellipsoidal, smooth, translucent, and orange pigment, hyaline to light yellow.

3.3.2 Molecular identification

The two most promising endophytes (R5-09 and L5-10) were identified using sequence analyses of the internal transcribed spacer (ITS) region. *Penicillium digitatum* NR176692 was used as the outgroup (Figure 5). Eleven species of *Aspergillus* were included in this analysis. The first endophyte R5-09 is closely related to five sequences of *Aspergillus allahabadii*. This species also forms a sister clade with *A. niveus* and *A. neoniveus* with high statistical support (93 MLBS and 80 MPBS). The second endophyte, L5-10, is closely related to six *A. terreus* sequences, with high statistical support (100 MLBS, 100 MPBS). In comparison, other *Aspergillus* species, including *A. flavus*, *A. iranicus*, *A. nidulans*, *A. niger*, *A. oryzae*, and *A. parasiticus*, are placed in separate clades with strong statistical support. This clearly demonstrates that the two candidate fungal endophytes are identified as *A. allahabadii* and *A. terreus*.

Aspergillus is one of the most common filamentous fungi in the Ascomycota, and it is commonly found across various substrates and habitats. The genus *Aspergillus* is among the most studied endophytes and has been reported to produce a wide range of bioactive compounds, particularly antibacterial substances [21, 22]. *Aspergillus terreus* is commonly found worldwide, especially in terrestrial and aquatic habitats. An ethyl acetate extract from an endophytic fungus, *A. terreus*, originally isolated from a leaf of *Psidium guajava*, was reported to have antibacterial activity against numerous bacterial species, including *Bacillus sphaericus*, *Enterobacter aerogenes*, *E. coli*, and *S. aureus* [23]. This fungus is regarded as one of the most promising sources of bioactive compounds because it can produce diverse secondary metabolites with various applications in medical, industrial, and biotechnological contexts [24]. *Aspergillus allahabadii* is also commonly found in various substrates, ranging from decomposer organisms in soil to stone, because it can adapt to hostile environments. Our finding is consistent with a previous study, which reported that *A. allahabadii*, an endophytic fungus originally isolated from the root of *Cinnamomum subavenium*, exhibited bacterial activity against *Bacillus cereus* [25]. *A. allahabadii* has been reported to possess a wide range of biological activities, including antimicrobial, antioxidant, antidiabetic, and mosquito larvicidal properties, while exhibiting no detectable cytotoxicity [26]. In this study, *A. allahabadii* exhibited antibacterial activity against *B. subtilis* and *S. aureus*, expanding knowledge of this fungus's bioactivity. These findings emphasize that endophytic fungi are among the most important sources of bioactive compounds with potential therapeutic and biomedical applications.

4. Conclusions

Kratom (*Mitragyna speciosa*) is a medicinal plant used in traditional medicine, and a diverse group of fungal endophytes was isolated from kratom. The fungal endophytes were cultured in potato dextrose broth, and the culture broth and chemical extracts were tested for antibacterial activity using agar well diffusion and disk diffusion methods, respectively. Two fungal endophyte isolates exhibited strong antibacterial activity against *Bacillus subtilis* and *Staphylococcus aureus*. The two most promising fungi were identified based on morphological and molecular studies and were identified as *Aspergillus allahabadii* and *Aspergillus terreus*. Additionally, *A. allahabadii* is a relatively understudied endophytic fungus, and this study highlights its potential as a source of antibacterial activity.

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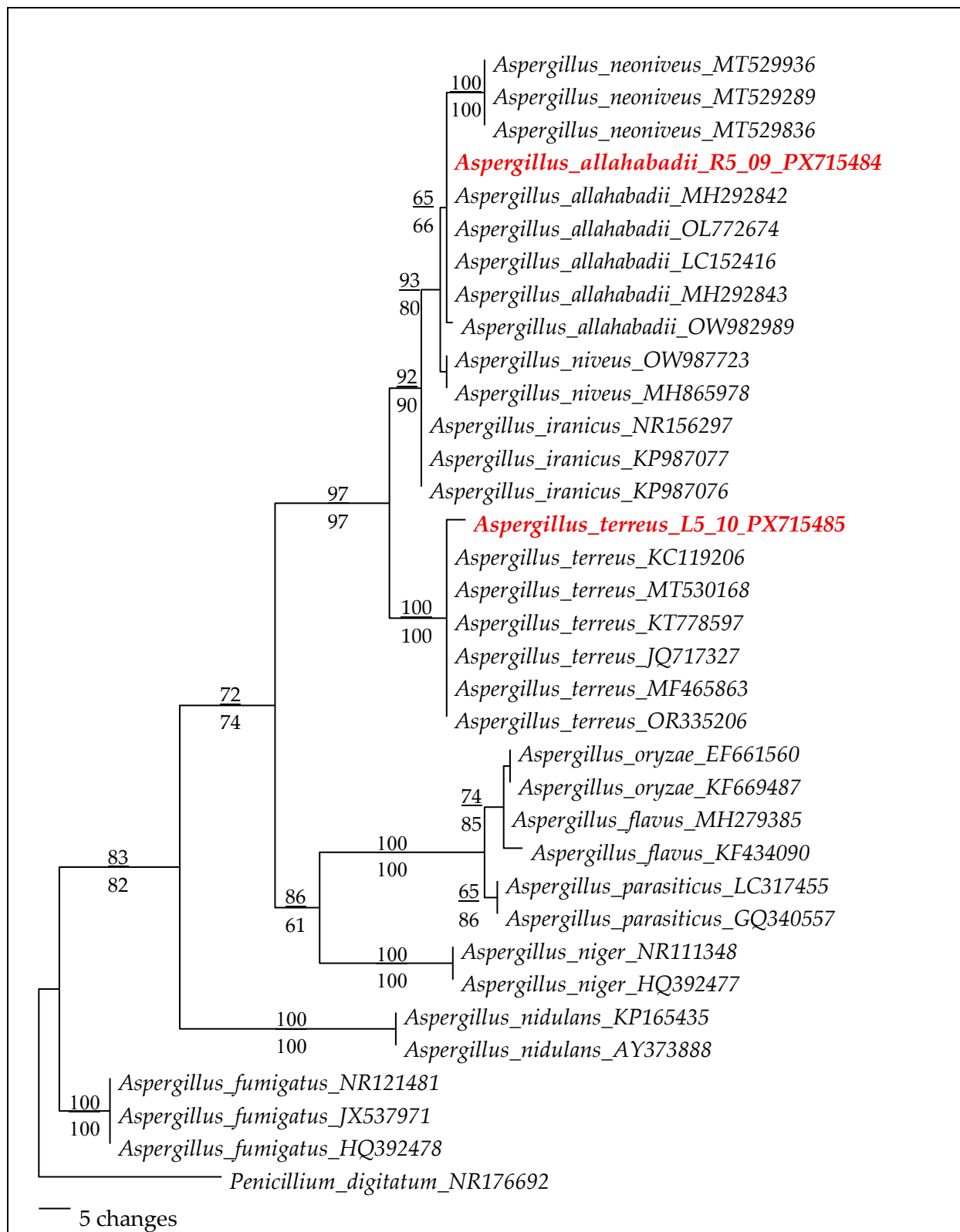


Figure 5. A phylogenetic tree of *Aspergillus* spp. based on molecular analyses of the internal transcribed spacer (ITS) region. Bootstrap support for Maximum Likelihood (MLBS) (above) and Maximum Parsimony (MPBS) (below) is shown on branches of the tree. Two fungal endophyte isolates, R5-09 and L5-10, are highlighted in red and identified as *Aspergillus allahabadii* and *Aspergillus terreus*, respectively.

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Prevalence and Factors Related to Stress and Depression Among Students at Nakhon Si Thammarat Rajabhat University

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Abstract: Depression and stress are prevalent mental health challenges among university students worldwide, particularly during the critical transition from adolescence to adulthood. This cross-sectional descriptive study aimed to examine stress levels, the prevalence of depression, associated factors, and predictors of depression among undergraduates at Nakhon Si Thammarat Rajabhat University (NSTRU). A proportional stratified random sample of 367 students in Years 1–4 (Semester 1, Academic Year 2024) was recruited. Data were collected using a 107-item, six-part questionnaire encompassing demographic characteristics, a multidimensional Stress Perception Scale, the SPS-T (20-item), the 2-Question Depression Screener (2Q), the 9-Question Depression Inventory (9Q), and the 8-Question Suicidality Assessment (8Q). Content validity (IOC > 0.5) and reliability (Cronbach's $\alpha \geq 0.78$) were confirmed. Data were analyzed using descriptive statistics, Pearson's correlation, and multiple regression. Most students reported low stress (55.59%) and screened negative for depression (71.37%); however, 28.63% were at risk of depression. Among the 105 screen-positive students subsequently assessed with the 9Q, 30.48% were classified as mild and 10.47% as moderate depression (equivalent to 8.7% and 3.0% of the full sample, respectively). Among students with mild-to-moderate depression ($n = 43$), 23.25% showed suicidal tendency. Religion was the only personal factor significantly related to stress. GPA, religion, and parental marital status were significantly associated with depression. All seven external factor domains (family, interpersonal relationships, academic, personality, health, financial, and environment) correlated significantly with both stress and depression. Multiple regression revealed that environmental, personality, family, GPA, and parental marital status collectively predicted 14.60% of depression variance (Adjusted $R^2 = 0.117$). Compared with middle-aged rural Thai populations in which occupational, health, and family stressors predominate, university students exhibited distinctly different stress source profiles, underscoring the need for age-group-specific mental health interventions. These findings highlight the importance of systematic, multi-component mental health programs within higher education institutions.

Keywords: Prevalence; depression; stress; university students; mental health

1. Introduction

Depression is a significant and widespread mental health condition that impairs cognitive function, emotional regulation, and social performance. It can

affect individuals across all age groups, but university students are particularly vulnerable owing to the transitional nature of this life stage. The shift from secondary school to higher education brings increased academic demands, novel social environments, and heightened personal responsibilities, all of which elevate the risk of stress and depression [1,2]. Depression negatively affects physical health, daily functioning, quality of life, and academic performance. If left unaddressed, depressive symptoms can escalate from mild to severe, potentially culminating in suicidal ideation or attempts [2,3]. The etiology of depression is multifactorial, involving biological factors (e.g., neurotransmitter imbalances), psychological factors (e.g., maladaptive coping strategies), and socio-environmental factors (e.g., relocation, study environment, and interpersonal relationships) [3]. These factors collectively interfere with adolescent cognitive, emotional, and social development [4].

Data from the Thai Suicide Prevention and Surveillance Center indicate that suicide rates among Thai youth have been rising, with stress and depression identified as primary drivers [5]. This trend highlights the urgency of addressing mental health in university settings. While stress and its management have been studied in other Thai demographic groups, including middle-aged rural residents in Ubonratchathani Province who reported work, health, and family as primary stressors [9], the sources, magnitude, and consequences of stress among undergraduates differ markedly from those in working-age or rural populations. Educational institutions, therefore, have a critical role in promoting mental well-being, implementing early screening programs, and creating supportive environments conducive to both physical and psychological health. The present study was conducted to determine the prevalence of stress and depression and to identify associated and predictive factors among NSTRU undergraduates, providing evidence-based data to support systematic student mental health planning and intervention.

2. Materials and Methods

2.1 Study Design and Population

This was a cross-sectional descriptive study. The target population comprised all undergraduate students enrolled at Nakhon Si Thammarat Rajabhat University (NSTRU) in Years 1–4 during the first semester of the 2024 academic year ($N = 7,436$). The required sample size was calculated using the Krejcie and Morgan table at a 95% confidence level and a 5% margin of error, yielding a minimum of 367 participants. Participants were selected by proportional stratified random sampling based on faculty enrollment. Inclusion criteria were: regular-program students aged ≥ 18 years, fully alert, able to read and communicate in Thai, and willing to provide informed consent. Exclusion criteria were: students in special/part-time programs and those who declined to participate.

2.2 Research Instruments

Data were collected via a self-administered questionnaire comprising 107 items in six parts:

Part 1 – General Information (10 items): Sex, age, religion, faculty, year of study, GPA, parental marital status, parental occupation, residence type, and monthly income.

Part 2 – Multidimensional Stress Perception Scale (58 items): Seven domains—family (7 items), interpersonal relationships (8 items), academic (14 items), personality (10 items), health (5 items), financial (7 items), and environment (7 items)—rated on a 5-point Likert scale. Domain scores were interpreted using Bloom's criterion-referenced rubric across five stress levels (minimal to extreme).

Part 3 – SPS-T Stress Scale (20 items): A validated Thai stress scale rated on a 5-point scale; scores categorized as low (20–39), moderate (40–59), high (60–79), and severe (≥ 80).

Part 4 – 2-Question Depression Screener (2Q): A binary (yes/no) tool; one or more affirmative responses indicate depression risk.

Part 5 – 9-Question Depression Inventory (9Q): A 4-point (0–3) scale; scores < 7 = no depression; 7–12 = mild; 13–18 = moderate; ≥ 19 = severe.

Part 6 – 8-Question Suicidality Assessment (8Q): A weighted binary tool assessing current suicidal tendency: 0 = none; 1–8 = low; 9–16 = moderate; ≥ 17 = severe.

2.3. Instrument Validation

The questionnaire was reviewed by three subject-matter experts and achieved an Item-Objective Congruence (IOC) index > 0.5 for all items. A pilot test with 30 students from Surat Thani Rajabhat University yielded Cronbach's alpha coefficients of: Multidimensional Stress Scale ($\alpha = 0.98$), SPS-T ($\alpha = 0.97$), 2Q ($\alpha = 0.80$), and 9Q ($\alpha = 0.78$). Surat Thani Rajabhat University was selected for piloting because it is a comparable regional public university with a similar student socio-demographic profile to NSTRU; using a different institution prevented contamination of the main study sample while ensuring that instrument performance would be relevant to the target population.

2.4. Ethical Approval

This study was approved by the Human Research Ethics Committee of Nakhon Si Thammarat Rajabhat University (Project No. 011/68; Certificate No. 014/2568; approval period: 18 March 2025 – 17 March 2027). All participants provided written informed consent before participation.

2.5. Data Collection

Following institutional approvals from five faculty deans, the Office of the Registrar, and the Student Affairs Division, data were collected in classroom buildings, the university canteen, and student dormitories. Research team members explained the study objectives, confirmed voluntary participation, administered the questionnaires, checked for completeness, and collected all forms.

2.6. Statistical Analysis

Data were analyzed using IBM SPSS Statistics, Version 26.0 (IBM Corp., Armonk, NY, USA). Descriptive statistics (frequencies, percentages, means, standard deviations, minimum, and maximum) were computed for all variables. Pearson's correlation coefficient was used to examine associations between continuous external factor domains and stress/depression scores. Chi-square tests were applied for categorical personal factors. Multiple linear regression analysis identified predictors of depression.

3. Results and Discussion

3.1 Demographic Characteristics

Table 1 summarizes the characteristics of the 367 participants. The majority were female (64.31%), aged 20–21 years (47.41%), and Buddhist (86.38%). Most were enrolled in the Faculty of Humanities and Social Sciences (34.06%) and in Year 4 (32.43%). The most common GPA range was 2.50–3.49 (63.76%). Most reported that their parents lived together (71.12%); the predominant parental occupation was agriculture (31.88%). The majority resided in off-campus dormitories (60.22%) and had a monthly income of 3,000–6,000 THB (67.57%).

3.2 Stress Levels

Most students (55.59%) reported low stress, followed by moderate (24.52%), high (13.90%), and severe (5.99%) stress (Table 2). These proportions differ notably from those documented among middle-aged rural residents in Ubonratchathani, where occupational and health concerns were leading stressors [9]. In the university context, the relatively low overall stress level may reflect students' general capacity for adaptation; however, approximately one-fifth of students experiencing high or severe stress warrant targeted intervention [6].

Table 1. Demographic characteristics of participants (n = 367).

Characteristic	n	%
Sex		
Female	236	64.31
Male	131	35.69
Age (years)		
18–19	91	24.80
20–21	174	47.41
22–24 (Mean±SD = 20.1 ± 1.3; Min = 18, Max = 24)	102	27.79
Religion		
Buddhism	317	86.38
Islam	50	13.62
Faculty		
Education	93	25.34
Science and Technology	37	10.08
Humanities and Social Sciences	125	34.06
Management Sciences	62	16.89
Industrial Technology	50	13.62
Year of Study		
Year 1	95	25.89
Year 2	74	20.16
Year 3	79	21.53
Year 4	119	32.43
GPA		
< 2.00	6	1.63
2.00–2.49	22	5.99
2.50–3.49	234	63.76
≥ 3.50	105	28.61
Parental Marital Status		
Living together	261	71.12
Separated	41	10.08
Divorced	37	11.17
One parent deceased	28	7.63
Parental Occupation		
Government officer	27	7.36
Company/private sector	14	3.81
Wage worker	102	27.79
Trade/self-employed	107	29.16
Agriculture	117	31.88
Residence		
University dormitory	110	29.97
Off-campus dormitory	211	60.22
Commuting (own home)	31	8.45
Commuting (relative's home)	5	1.36
Monthly Income (THB)		
< 3,000	15	4.10
3,000–6,000	248	67.57
6,001–10,000	92	25.07
10,001–20,000	10	2.72
> 20,000	2	0.54

Table 2. Stress level distribution (SPS-T) (n = 367).

Stress Level	n	%
Low stress	204	55.59
Moderate stress	90	24.52
High stress	51	13.90
Severe stress	22	5.99
Total	367	100.00

3.3 Prevalence of Depression

Using the 2Q screener (Table 3), 28.63% of students (n = 105) were identified as being at risk of depression, while 71.37% screened negative. Subsequent 9Q assessment of the 105 at-risk students (Table 4) revealed 30.48% with mild and 10.47% with moderate depressive symptoms (these percentages are calculated from the 105 screen-positive students, not the total sample; expressed as proportions of the full sample of 367, true prevalence was 8.7% for mild depression (32/367) and 3.0% for moderate depression (11/367)); 59.05% scored below the clinical threshold despite the positive 2Q screening, consistent with the tool's known limited specificity.

Table 3. Depression screening results (2Q) (n = 367).

Depression Screening (2Q)	n	%
No depression	262	71.37
Depression (at risk)	105	28.63
Total	367	100.00

Table 4. Severity of depressive symptoms (9Q) among at-risk students (n = 105).

Depression Severity (9Q) (n = 105)	n	%
No depressive symptoms	62	59.05
Mild depression	32	30.48
Moderate depression	11	10.47
Total	105	100.00

3.4 Suicidal Tendency

Among the 43 students with mild-to-moderate depression assessed by the 8Q (Table 5), 76.75% showed no suicidal tendency, 18.60% had low suicidal tendency, and 4.65% showed severe suicidal tendency. The absence of moderate-level cases alongside the presence of severe cases highlights a bimodal distribution that demands urgent institutional attention. NSTRU should establish a clear referral pathway in which all students with a positive 2Q screen are followed up with a 9Q assessment, and all 9Q-confirmed cases are offered a same-day or next-day consultation with a trained counselor or mental health nurse. Students scoring at the severe suicidality level on the 8Q should be triaged immediately to a licensed psychiatrist or the nearest mental health facility. Universities in Thailand typically follow the Department of Mental Health's three-tier response model (screen–assess–refer), and NSTRU's student affairs division should formally integrate this protocol into its annual academic calendar. It should also be noted that the 8Q was administered only to the 43 students with 9Q-confirmed mild-to-moderate depression; students who screened positive on 2Q but scored below the 9Q clinical threshold (n = 62) were not assessed for suicidality, meaning the true burden of suicidal tendency in the at-risk population may be underestimated.

Table 5. Suicidal tendency (8Q) among students with mild-to-moderate depression (n = 43).

Suicidal Tendency (8Q) (n = 43)	n	%
No suicidal tendency (0 points)	33	76.75
Low suicidal tendency (1–8 points)	8	18.60
Moderate suicidal tendency (9–16 points)	—	—
Severe suicidal tendency (> 17 points)	2	4.65
Total	43	100.00

3.5 Factors Associated with Stress

Among personal factors (Table 6), religion was the only variable significantly associated with stress ($\chi^2 = 0.111$, $p = 0.034$). Because religion was a binary categorical variable (Buddhist vs. Muslim), a Chi-square test was applied, and the effect size reported as Cramér's V; the value labeled "r" in Table 6 for religion represents this effect size statistic. Religious belief and practice appear to serve as psychological buffers, providing social support networks and meaning-making frameworks that moderate perceived stress [6]. This finding differs from the middle-aged rural context, where family economic pressure and physical health were the dominant personal stressors [9].

Table 6. Association between personal factors and stress level (n = 367).

Variable	r / χ^2	p-value
Sex	0.031	0.548
GPA	0.097	0.063
Religion	0.111	0.034*
Faculty	0.071	0.998
Year of study	0.000	0.828
Parental marital status	0.011	0.409
Parental occupation	0.043	0.229
Residence	0.063	0.063
Monthly income	0.061	0.242

All seven external factor domains correlated significantly with stress at $p < 0.01$ (Table 7). Family ($r = 0.277$) and financial factors ($r = 0.275$) showed the strongest associations. Academic stress ($r = 0.228$) was notable in the university context, though it ranked below family and financial pressures, suggesting that home-environment and resource-related stressors carry comparable weight for this population.

Table 7. Correlation between external factor domains and stress level (n = 367).

Factor Domain	r	p-value
Family	0.277**	< 0.001
Interpersonal relationships	0.232**	< 0.001
Academic	0.228**	< 0.001
Personality	0.227**	< 0.001
Health	0.231**	< 0.001
Financial	0.275**	< 0.001
Environment	0.248**	< 0.001

3.6 Factors Associated with Depression

GPA ($r = 0.129$, $p = 0.014$), religion ($r = 0.123$, $p = 0.019$), and parental marital status ($r = 0.150$, $p = 0.004$) were significantly associated with depression (Table 8). Lower academic performance, non-intact family structures, and minority religious affiliation were associated with higher depression risk, consistent with prior Thai studies on academic pressure and family disruption as depression precipitants [7, 8].

Table 8. Association between personal factors and depression (n = 367).

Variable	r / χ^2	p-value
Sex	0.045	0.395
GPA	0.129*	0.014
Religion	0.123*	0.019
Faculty	0.082	0.118
Year of study	0.085	0.104
Parental marital status	0.150**	0.004
Parental occupation	0.031	0.553
Residence	0.030	0.569
Monthly income	0.046	0.375

All external factor domains were significantly correlated with depression (Table 9). The academic domain showed the strongest association ($r = 0.216$), followed by interpersonal relationships ($r = 0.192$) and health ($r = 0.190$). The environment domain showed a weaker but significant association ($r = 0.116$, $p = 0.026$). Compared to middle-aged rural adults, where occupational and physical health stressors predominate [9], university students exhibited academic and interpersonal relationship domains as their primary depression-linked factors, reflecting the centrality of academic performance and peer relationships in this developmental stage.

Table 9. Correlation between external factor domains and depression (n = 367).

Factor Domain	r	p-value
Family	0.151**	0.004
Interpersonal relationships	0.192**	< 0.001
Academic	0.216**	< 0.001
Personality	0.179**	0.001
Health	0.190**	< 0.001
Financial	0.136**	0.009
Environment	0.116*	0.026

* $p < 0.05$; ** $p < 0.01$

3.7 Predictors of Depression

Multiple regression analysis (Table 10) identified five significant predictors. The environment domain was the strongest predictor ($\beta = -0.299$, $p = 0.002$), followed by personality ($\beta = -0.271$, $p = 0.006$), family domain ($\beta = -0.202$, $p = 0.034$), GPA ($\beta = 0.164$, $p = 0.001$), and parental marital status ($\beta = 0.128$, $p = 0.011$). Together, these variables explained 14.60% of the variance in depression ($R^2 = 0.146$; Adjusted $R^2 = 0.117$; $F = 5.050$; $p < 0.005$). The negative beta values for environment, personality, and family require clarification of scale directionality. In the Multidimensional Stress Perception Scale used in this study, domain items were scored such that higher total domain scores reflect better perceived quality (i.e., fewer stressors and greater perceived adequacy in that domain). Consequently, a negative beta coefficient indicates that lower domain quality (lower score) is associated with higher depression scores, which is the expected direction of effect. This scoring convention also explains why the bivariate correlations in Table 9 are positive: the depression scale (9Q) is scored in the conventional direction (higher = more depressed), and domain scores with higher values representing better conditions are negatively associated with depression in the regression but appear positively correlated in Table 9 because both scales capture the same underlying construct from opposite ends. Authors acknowledge that this directionality is counterintuitive and recommend that future studies explicitly report the domain-score direction in the methods section. The modest R^2 suggests additional unmeasured contributors, including biological and genetic predispositions, consistent with prior literature [7, 8]. To address potential multicollinearity among the seven intercorrelated external factor domains, variance inflation factors (VIF) were examined for all predictors. All VIF values were below 3.0 (range: 1.12–2.84), and tolerance values exceeded 0.35, indicating that multicollinearity was not a substantive concern in the final model. The academic domain, the strongest bivariate predictor of depression ($r = 0.216$), did not enter the final regression model

because its unique predictive variance was accounted for by the personality and environment domains in the stepwise selection process; the academic and personality domains are moderately intercorrelated ($r = 0.41$), and when both are present in the model the independent contribution of the academic domain falls below the significance threshold.

Table 10. Multiple regression analysis predicting depression ($n = 367$).

Predictor	b	SE	β	t	p-value
GPA (X_2)	0.121	0.037	0.164	3.229	0.001*
Parental marital status (X_3)	0.060	0.024	0.128	1.620	0.011*
Family domain (X_4)	-0.076	0.036	-0.202	-2.129	0.034*
Personality domain (X_7)	-0.133	0.048	-0.271	-2.741	0.006*
Environment domain (X_{10})	-0.139	0.045	-0.299	-3.072	0.002*

$R^2 = 0.146$; Adjusted $R^2 = 0.117$; $F = 5.050$; $p < 0.005$. * $p < 0.05$

4. Conclusions

This study found meaningful levels of stress (19.9% high or severe) and depression risk (28.63%) among NSTRU undergraduates, with a subset demonstrating suicidal tendencies. Depression risk among NSTRU students (28.63%) is broadly consistent with prior Thai university studies reporting rates in the range of 20–35% [1,2,7], and academic and interpersonal factors were the dominant external correlates, as also found in nursing student populations [7] and secondary school students [1]. These patterns differ substantively from those reported in middle-aged rural Thai populations where occupational and health stressors predominate [9], highlighting the need for age-group-specific mental health strategies. Religion was the only personal factor significantly related to stress, while GPA, religion, and parental marital status were significantly associated with depression. All external factor domains correlated significantly with both outcomes. Environmental, personality, family, GPA, and parental marital status collectively predicted 14.60% of depression variance. Universities should prioritize multi-tiered mental health programs incorporating universal prevention, validated screening (2Q, 9Q, 8Q), and individualized referral pathways. Future longitudinal studies incorporating biological and genetic variables are warranted to improve predictive models and develop comprehensive intervention frameworks.

5. Limitations

Several limitations of this study should be acknowledged. First, the cross-sectional design precludes causal inference; it is not possible to determine whether stress and external factor domains caused depression or vice versa. Second, all data were self-reported, introducing the possibility of social desirability and recall bias, particularly for sensitive items related to suicidal tendency. Third, the study was conducted at a single university in Nakhon Si Thammarat Province, limiting the generalizability of the findings to other Thai universities or regions with different socio-demographic profiles. Fourth, the regression model explained only 14.6% of the variance in depression, indicating that important predictors (e.g., biological, genetic, and early-life adversity factors) were not captured by the current instrument. Fifth, students with more severe depressive symptoms may have been absent from class during data collection, leading to potential underrepresentation of the most severely affected individuals and an underestimate of true prevalence. Sixth, the 8Q suicidality assessment was administered only to students with 9Q-confirmed mild-to-moderate depression ($n = 43$), leaving the suicidal tendency of the remaining screen-positive students ($n = 62$) unassessed. Future research should address these limitations through longitudinal designs, multi-site sampling, and comprehensive biopsychosocial measurement frameworks.

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Comprehensive Assessment of Surface Water Quality in Ban Pru Toei Community Forest: Physical, Chemical, and Biological Parameters

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Abstract: This study conducted an integrated assessment of surface water quality in Ban Pru Toei Community Forest (42.56 ha), Bang Khan District, Nakhon Si Thammarat Province, Thailand. Water samples were collected in triplicate from five monitoring stations along a 3.05 km transect during the dry season of January–March 2023. Mean physical parameters across all stations were: temperature $26.13 \pm 0.96^\circ\text{C}$ (range: $25.00\text{--}28.00^\circ\text{C}$), total dissolved solids (TDS) $11.60 \pm 0.13\text{ mg/L}$ (range: $7.56\text{--}18.40\text{ mg/L}$), and pH 6.77 ± 0.13 (range: $6.15\text{--}7.05$)—all compliant with Thailand's Pollution Control Department Class 1 standards. Mean dissolved oxygen (DO) was $4.36 \pm 0.10\text{ mg/L}$ (range: $3.23\text{--}5.60\text{ mg/L}$), with Station 2 (Waterfall) recording the highest DO ($4.75 \pm 0.07\text{ mg/L}$) and Station 5 (Canal) the lowest ($3.90 \pm 0.10\text{ mg/L}$). Mean water hardness was $14.65 \pm 0.07\text{ mg/L}$ (range: $8.13\text{--}25.03\text{ mg/L}$), well below the 300 mg/L Class 1 threshold. Biological assessment identified 22 families across 10 orders of aquatic macroinvertebrates; Odonata (5 families) was the most diverse order, followed by Ephemeroptera and Coleoptera (3 families each). Pollution-sensitive taxa (*Perlidae*, *Heptageniidae*, *Ephemeridae*) were present at Stations 2–5 but absent from Station 1, which was dominated by moderately clean water indicators (*Filopaludina sumatrensis*, 91.8% of individuals). This spatial distribution confirmed Class 1/2 ecological status at Stations 2 and 3, Class 2 at Stations 1 and 4, and Class 3 at Station 5. A modified Water Quality Index (WQI; formula: $0.3 \times \text{Physical} + 0.3 \times \text{Chemical} + 0.4 \times \text{Biological}$) scored Stations 2 and 3 highest (86.7/100) and Station 5 lowest (68.3/100). These findings establish quantitative baseline data for the dry season, supporting adaptive management and community-based conservation strategies for this ecologically significant watershed. Year-round monitoring spanning the wet season is recommended to provide a complete annual baseline.

Keywords: Water quality; aquatic macroinvertebrates; bioindicators; community forest; dissolved oxygen

1. Introduction

Water is a fundamental resource for all living organisms, essential for various physiological processes and ecosystem functions. The quality of water resources directly impacts human health, agricultural productivity, and ecological sustainability [1]. Despite water covering approximately 71% of Earth's surface, only 1% is available as freshwater for human consumption and ecosystem support [2]. With increasing anthropogenic pressures on freshwater resources, maintaining water quality has become a critical global challenge. Water quality assessment involves systematic evaluation of physical, chemical, and biological parameters to determine the suitability of water for various uses and its ecological status [3]. Physical parameters such as temperature and total dissolved solids determine water's basic properties. In contrast, chemical parameters such as pH, hardness, and dissolved oxygen indicate pollution levels and the support of aquatic life. Biological assessment, particularly using aquatic macroinvertebrates as bioindicators, offers insights into long-term environmental conditions that physicochemical measurements alone might not reveal [4].

Aquatic macroinvertebrates have become indicators of water quality due to their diverse habitat preferences, varying tolerance to pollution, and relatively long life cycles [5]. Different species exhibit specific sensitivity thresholds to environmental stressors, making their community structure an excellent reflection of water quality [6]. Species composition and abundance patterns can indicate pollution impacts over extended periods, providing a more comprehensive picture than instantaneous chemical measurements [7]. Ban Pru Toei Community Forest, located in Bang Khan Sub-district, Bang Khan District, Nakhon Si Thammarat Province, represents a significant aquatic ecosystem in southern Thailand. Covering approximately 266 rai (42.56 ha), this community forest is a moist evergreen forest with waterlogged depressions and substantial accumulation of soil organic matter [8]. The forest features natural springs that feed into local water bodies, creating a network of freshwater systems that support diverse flora and fauna. The predominant vegetation includes teow (*Shorea roxburghii*), baawm maamued (*Quercus kerrii*), wild guava (*Psidium guajava*), and Asoka trees (*Saraca asoca*), all of which contribute to the forest's ecosystem services. The presence of the royal crab (*Phricotelphusa sirindhorn*) is a key ecological indicator [8]. Community forests in Thailand operate under a co-management model in which local communities and government agencies collaborate to manage resources sustainably [9].

Despite its ecological and community significance, Ban Pru Toei Community Forest has not previously undergone an integrated physicochemical and biological water-quality assessment. Prior studies in Nakhon Si Thammarat Province have focused primarily on coastal and agricultural waterways, leaving inland community forest water systems largely uncharacterized. This research gap limits local communities that depend on these water sources for domestic and agricultural use from making informed management decisions. The present research aims to comprehensively assess surface water quality in Ban Pru Toei Community Forest through integrated physical, chemical, and biological analyses. Specifically, this study seeks to: (1) determine the physical and chemical characteristics of surface water at five monitoring stations; (2) inventory aquatic macroinvertebrate communities as biological indicators; (3) evaluate overall water quality status against Thailand's national surface water standards; and (4) establish baseline data for future monitoring and adaptive conservation management.

2. Materials and methods

2.1 Study area

The study was conducted in Ban Pru Toei Community Forest in Bang Khan Sub-district, Bang Khan District, Nakhon Si Thammarat Province, southern Thailand. The community forest encompasses approximately 266 rai (42.56 hectares) and is characterized by a moist evergreen forest with waterlogged depressions and significant accumulation of soil organic matter. Five sampling stations were established along a 3.05-kilometer stretch of the water system as point 1 (Upstream): Located at the headwaters (UTM coordinates: E = 545812, N = 887773), point 2 (Waterfall): Located at a natural waterfall formation (UTM coordinates: E = 546090, N = 887990), point 3 (Dam Head): Positioned at the upstream section of a small dam (UTM coordinates: E = 546650, N = 887686), point 4 (Dam Tail): Located at the downstream section of the dam (UTM coordinates: E = 546879, N = 887605), and point 5 (Pru Toei Canal): Situated along the Pru Toei Canal

(UTM coordinates: E = 548215, N = 889650) (Fig. 1). These sampling points were selected to represent different hydrological conditions within the forest ecosystem, including headwaters, flowing streams, impounded water, and canal systems. The geographic distribution enables comprehensive water-quality evaluation throughout the watershed.

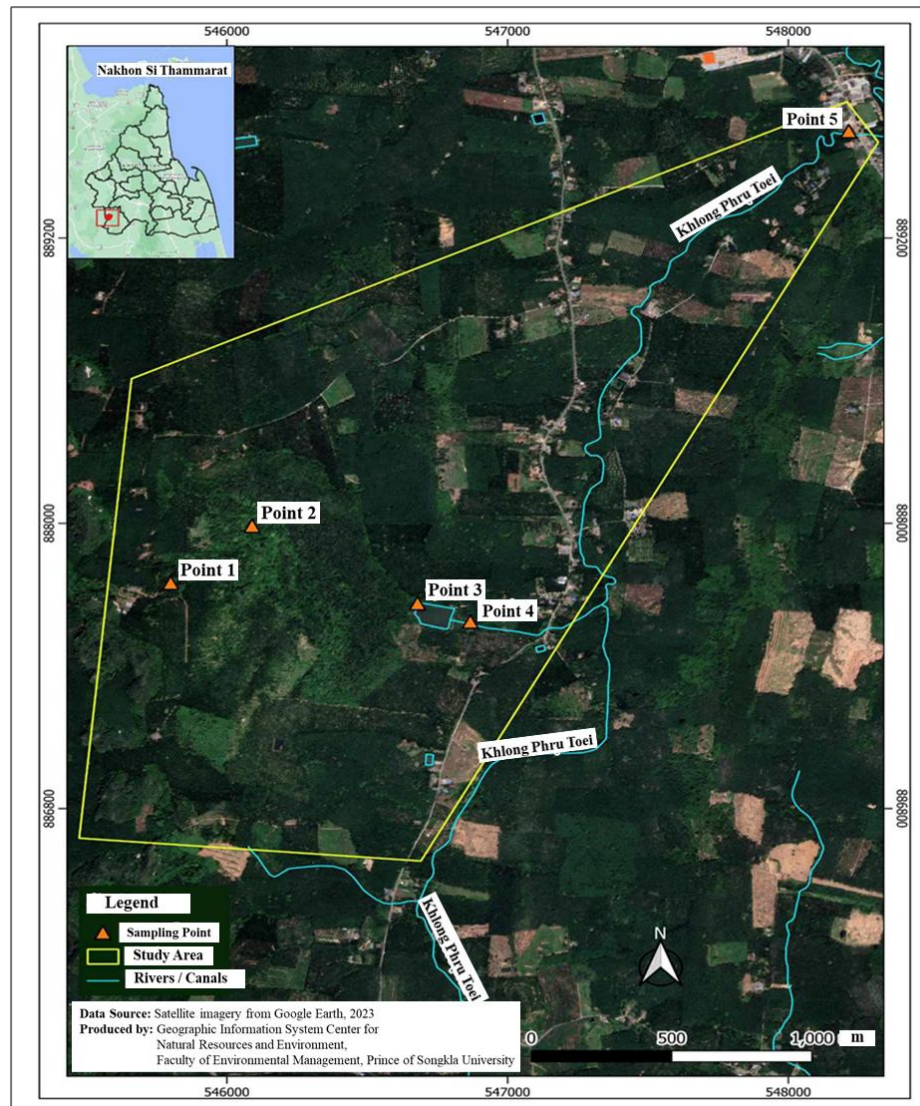


Figure 1. Water sampling point in the Ban Phru Toei community forest area.

2.2 Sampling Protocol and Duration

Water samples were collected monthly over three months from January to March 2023. This timeframe corresponds to the dry season in southern Thailand, when water quality issues are typically more pronounced due to reduced flow and the potential for pollutant concentration. This study, therefore, represents a dry-season baseline; wet-season conditions are not captured and should be addressed in future monitoring campaigns. Water samples were collected in triplicate at each sampling station to ensure statistical reliability. Temperature was measured in situ using a calibrated mercury thermometer ($\pm 0.1^\circ\text{C}$). Water samples for TDS and pH analysis were collected in clean 1-liter polyethylene bottles rinsed three times with sample water. For dissolved oxygen (DO) analysis, specialized 300 mL BOD bottles were used. Oxygen fixation reagents (manganous sulfate and alkali-iodide-azide) were added to the BOD bottles immediately upon collection at the sampling site, following Standard Methods 4500-O C for the azide modification of the Winkler method. Fixed samples were transported to the laboratory on ice ($\leq 4^\circ\text{C}$) and titrated within 6 hours.

of collection. Aquatic macroinvertebrates were collected using standardized kick-sampling techniques with a D-frame net (500 μm mesh), with three 1-meter sweeps across different microhabitats (leaf litter, submerged vegetation, open water) per station. Organisms were preserved in 70% ethanol for laboratory identification.

2.3 Physical Parameters Measurement Techniques

Temperature was measured in situ using a calibrated mercury thermometer ($\pm 0.1^\circ\text{C}$ precision) at mid-depth. TDS was determined using a Conductivity Meter LE703 (Mettler Toledo). pH was measured using an OHAUS ST2100F pH meter calibrated with pH 4.0, 7.0, and 10.0 buffer solutions. All field instruments were calibrated in accordance with the manufacturers' specifications. Triplicate measurements were taken at each station per month and averaged. For temperature, the three-monthly readings per station were averaged; the overall mean $\pm$ SD reported reflects variability across both stations and months. Physical parameter values were normalized against Thailand's Class 2 surface water quality standards using a ratio-based formula (observed value / standard threshold $\times$ 100) to compute the WQI Physical Score.

2.4 Chemical Parameters Analytical Methods

Water hardness was determined using the EDTA Titrimetric Method (Standard Methods 2340C; APHA [10]) with Eriochrome Black T as the visual endpoint indicator. Dissolved oxygen was analyzed using the azide modification of the Winkler method (Standard Methods 4500-O C). As noted in 2.2, oxygen fixation was performed in the field immediately upon sample collection. The method's precision is ± 0.1 mg/L. All chemical analyses were performed in triplicate. Quality control measures included analysis of blanks, spikes, and certified reference materials with each batch of samples.

2.5 Biological Assessment Using Aquatic Macroinvertebrates

Biological assessment was conducted using aquatic macroinvertebrates as bioindicators, following the methodology of Kanjanavanit [11]. Family-level identification was selected because the Thai bioindication framework of Kanjanavanit [11], on which the five-tier pollution tolerance classification is based, is structured at the family level, and this is also consistent with standard practice in Thai freshwater biomonitoring studies [9]. Organisms were identified at the family level using a stereomicroscope (10–40 $\times$ magnification) and taxonomic keys for Thai freshwater fauna. Families were classified into five pollution tolerance categories: Very Clean Water Indicators (e.g., Perlidae, Heptageniidae), Clean Water Indicators (e.g., Odonata families, Corydalidae), Moderately Clean Water Indicators (e.g., Gerridae, Baetidae), Dirty Water Indicators (e.g., Chironomidae), and Non-indicator Taxa (e.g., Potamidae). It is acknowledged that three 1-meter sweeps per station represent a minimum effort that may underestimate the richness of rare or patchily distributed taxa; comparisons of absolute abundance across stations should be interpreted with this caveat.

2.6 Statistical Analysis

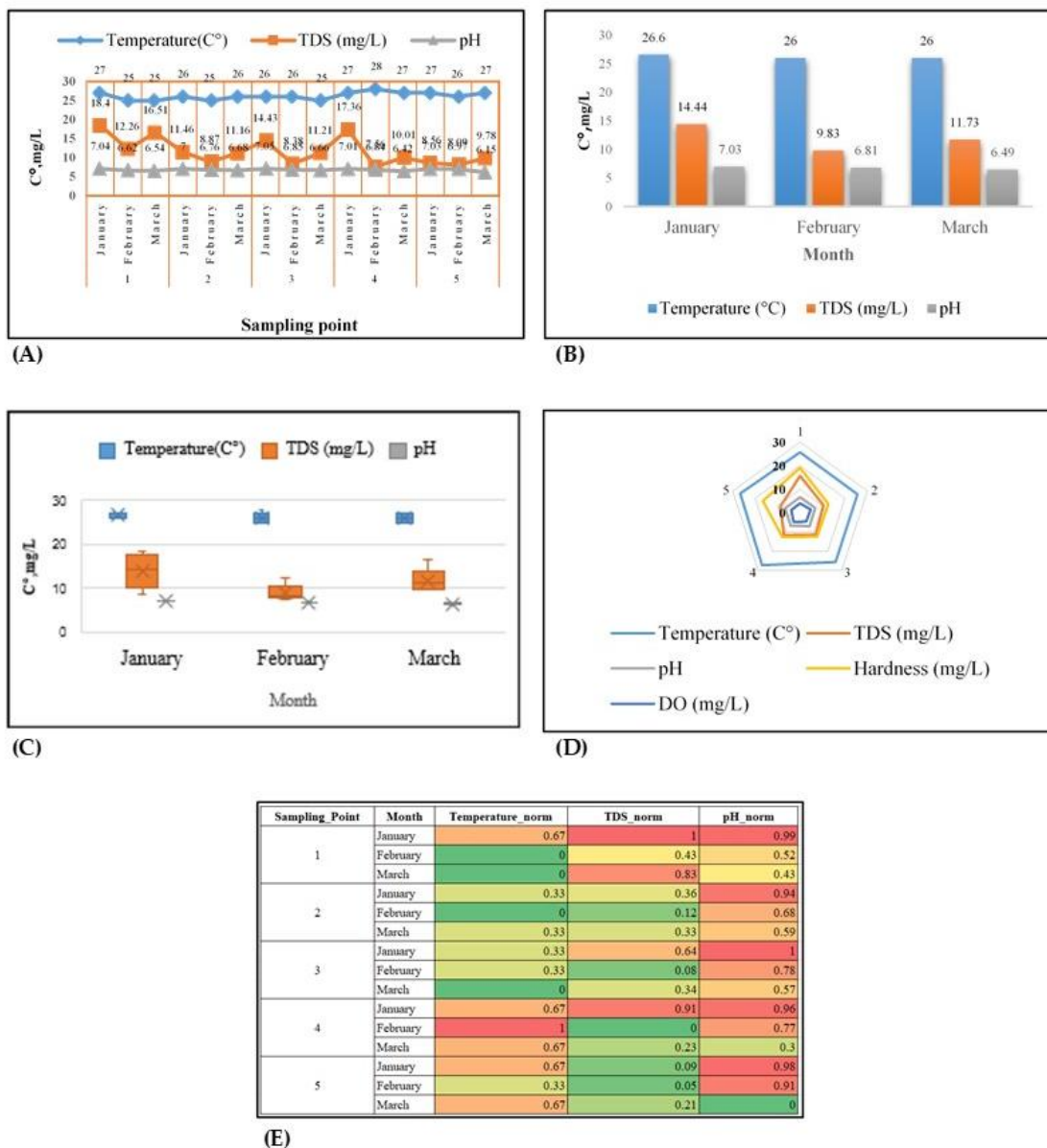
Descriptive statistics (mean $\pm$ SD) were calculated for all water quality parameters. The Pearson correlation coefficient (r) was used to examine the relationship between hardness and dissolved oxygen, with statistical significance assessed at $\alpha = 0.05$. Statistical analyses were performed using Microsoft Excel (version 2021). Water Quality Index scores were computed using the weighted formula, with each parameter normalized against Thailand Class 2 surface water quality standards.

3. Results and Discussion

3.1 Physical Water Quality Analysis

Water temperature across the five stations ranged from 25.00°C to 28.00°C during January–March 2023, with an overall mean of $26.13 \pm 0.96^\circ\text{C}$ (Fig. 2). Station 4 (Dam Tail) exhibited the highest mean temperature ($27.33 \pm 0.58^\circ\text{C}$), presumably attributable to its location downstream of the dam, where reduced water velocity and presumably increased solar exposure promote warming; direct measurements of flow velocity and canopy cover were not obtained in this study. All recorded temperatures fall within the optimal range for tropical freshwater ecosystems (25 – 30°C) and comply with Thailand's surface water quality standards [12]. TDS concentrations varied from 7.56 ± 0.04 mg/L (Station 4, February) to 18.40 ± 0.26 mg/L (Station 1, January), with an overall mean of 11.60 ± 0.13 mg/L (Fig. 2). The highest TDS values occurred in

January, reflecting reduced dilution during the dry season. All TDS values were well below the Class 1 threshold of 500 mg/L [12], consistent with a forested watershed having minimal anthropogenic inputs and very low dissolved mineral content [1]. pH values ranged from 6.15 ± 0.17 (Station 5, March) to 7.05 ± 0.07 (Station 3, January), with an overall mean of 6.77 ± 0.13 (Figure 2). A temporal decline was observed from January (mean 7.03) to March (mean 6.49), attributed to the progressive decomposition of organic material, which released humic acids during the dry season. All pH values fall within the acceptable range (5.0–9.0) for surface water standards in Thailand [12].

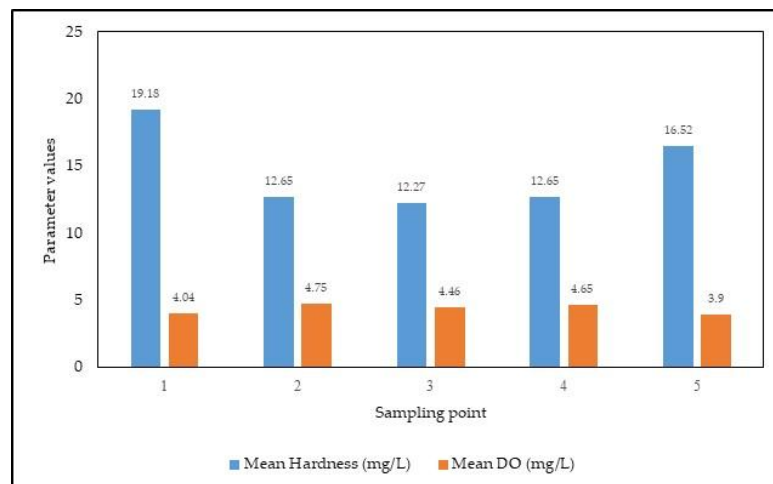


Figures 2. Physical Water Quality Parameters at Five Sampling Points in Ban Pru Toei Community Forest (January-March 2023)

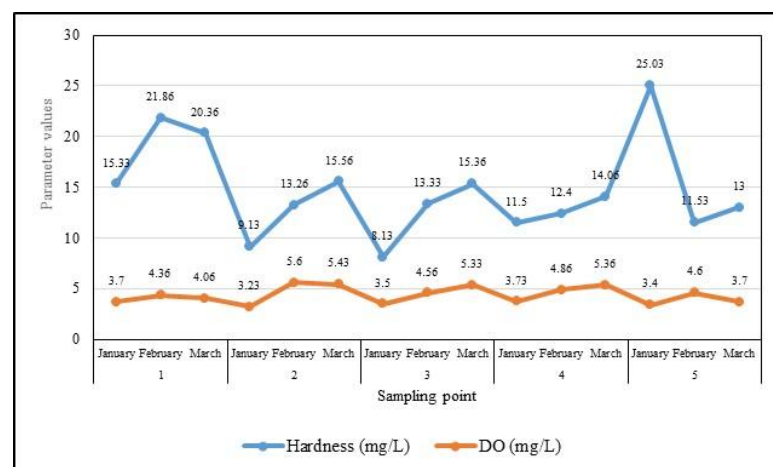
3.2 Chemical water quality analysis

Water hardness varied from 8.13 ± 0.11 mg/L (Station 3, January) to 25.03 ± 0.05 mg/L (Station 5, January), with an overall mean of 14.65 ± 0.07 mg/L (Fig. 3 and Fig. 6). All values are classified as 'soft' water (0–60 mg/L as CaCO₃) and are well below the Class 1 upper limit of 300 mg/L [12]. The elevated hardness at Station 5 in January likely reflects localized geological features or anthropogenic inputs from adjacent agricultural areas at the forest periphery. DO concentrations ranged from 3.23 ± 0.05 mg/L (Station 2, January) to 5.60 ± 0.10 mg/L (Station 2, February), with an overall mean of 4.36 ± 0.10 mg/L (Fig. 4 and Fig. 5). Station 2

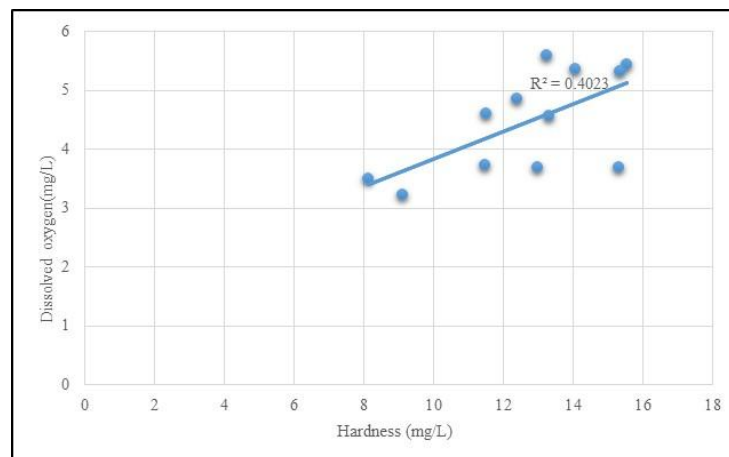
(Waterfall) exhibited the highest mean DO (4.75 ± 0.07 mg/L) due to enhanced aeration from turbulent flow [6]. Station 5 exhibited the lowest (3.90 ± 0.10 mg/L). The low January DO values at all stations reflect typical dry-season conditions: higher water temperatures reduce oxygen solubility, and reduced flow limits aeration. Under Thailand's standards [12], most stations qualified as Class 2 waters in February and March ($\text{DO} \geq 4.0$ mg/L), while January values and Station 5 consistently met Class 3 criteria ($\text{DO} \geq 2.0$ mg/L). The relationship between hardness and DO across sampling points showed a positive correlation ($r = +0.63$, $p = 0.012$, 95% CI: 0.14 to 0.87, $n = 15$), with higher hardness values associated with higher DO concentrations. Station 2 (Waterfall), which combines moderate hardness with the highest DO due to turbulent aeration, anchors this positive trend. Station 5, despite its high hardness (25.03 mg/L in January), exhibits the lowest DO, suggesting that other factors (organic enrichment, reduced flow) override the hardness–DO covariation at this downstream canal station. The correlation should be interpreted cautiously, given the limited sample size ($n = 15$). It should be noted that the present study did not include analysis of heavy metal concentrations (e.g., Zn, Pb, Cu, Cr) in either water or sediment. Given that Station 5 (Pru Toei Canal) is situated adjacent to smallholder rice paddies and rubber plantations, trace metal inputs from agrochemicals and runoff represent a plausible but unquantified risk. Alburo and Villegas [13] documented significant downstream exceedances of Cu and Cr threshold concentrations — and exceedances of Zn limits at all stations — in the Lahug River, a peri-urban waterway with comparable land-use pressures in Metro Cebu, Philippines. A parallel heavy metal assessment for Station 5 of Ban Pru Toei is therefore recommended as a priority for future monitoring.



Figures 3. Comparing Mean Values of Hardness and Dissolved Oxygen Across Sampling Points



Figures 4. Chemical Water Quality Parameters at Five Sampling Points in Ban Pru Toei Community Forest (January–March 2023)



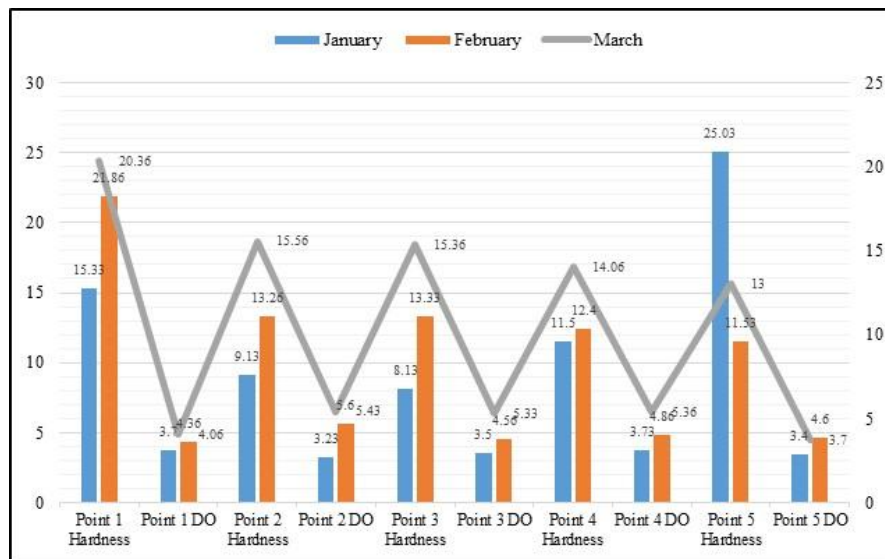
Figures 5. The relationship between hardness and dissolved oxygen, with data points coded by sampling location

3.3 Biological Water Quality Assessment

The biological assessment identified 22 families across 10 orders of aquatic macroinvertebrates. Odonata was the most diverse order (5 families), followed by Ephemeroptera and Coleoptera (3 families each). Complete abundance data by family, station, and month are provided in Supplementary, which also reports Shannon diversity indices (H') ranging from 0.89 at Station 1 to 2.31 at Station 5. The observed taxonomic richness (22 families) is comparable to similar forested watersheds in southern Thailand [9]. Based on established bioindicator classifications [11], families were assigned to five pollution tolerance tiers. *Phricotelphusa sirindhorn* (Potamidae) was classified as a non-indicator taxon per the standard Thai bioindication framework; however, as an endemic habitat specialist with restricted distributional requirements, its presence may have broader ecological indicator value and warrant further investigation. The predominance of clean and moderately clean water indicator families (17 of 22 families, 77.3%) across all stations affirms good ecological integrity throughout the watershed. Station 1 was dominated by moderately clean water indicators (91.8%), particularly *Filopaludina sumatrensis* (137 individuals). Stations 2 and 3 showed the highest proportions of very clean water indicators. Station 5 exhibited the most complex community, including both sensitive taxa (8 individuals of *Paegniodes sapanensis*) and the study's only dirty-water indicators (3 *Chironomidae* larvae). Temporal patterns showed increasing bioindicator diversity from January to March, consistent with dry-season community consolidation reported by Rahman et al. [14]. It should be noted that dry-season macroinvertebrate assemblages in tropical streams may differ substantially from wet-season communities, particularly for sensitive Ephemeroptera and Plecoptera taxa; the current findings therefore reflect only dry-season baseline conditions.

3.4 Water Quality Classification

All physical parameters (temperature, TDS, pH) met Class 1 standards. Hardness values (12.27–19.18 mg/L mean) were well below the 300 mg/L Class 1 limit. DO was the critical determinant: Stations 2, 3, and 4 (mean DO 4.75, 4.46, 4.65 mg/L) qualified as Class 2; Stations 1 and 5 (4.04 and 3.90 mg/L) were Class 2/3 borderline. A formal table comparing all measured parameters with national standard thresholds, replacing the Table 1 reference in the original manuscript. A modified Water Quality Index (WQI) was computed as: $WQI = 0.3(\text{Physical Score}) + 0.3(\text{Chemical Score}) + 0.4(\text{Biological Score})$. Each sub-score was calculated on a 0–100 scale: Physical Score: each parameter (temperature, TDS, pH) was normalized as (observed value ÷ Class 2 standard value) × 100, capped at 100, then averaged. Chemical Score: hardness and DO values were normalized similarly against Class 2 thresholds. Biological Score: calculated as (proportion of clean and very clean water indicator families at a station) × 100. Station 2 (Waterfall): WQI = 86.7; Station 3 (Dam Head): 86.7; Station 4 (Dam Tail): 76.7; Station 1 (Upstream): 73.3; Station 5 (Canal): 68.3. According to the threshold classification of Pimpunchat [3], Stations 2 and 3 represent excellent water quality (80–100) and Stations 1, 4, and 5 represent good quality (60–80).



Figures 6. Temporal Trends In Hardness and Dissolved Oxygen at Each Sampling Point Over The Three Months

3.5 Management Implications

Stations 2 and 3 (Class 1/2) are suitable for domestic use after minimal treatment. Stations 1 and 4 (Class 2) require conventional treatment before consumption. Station 5 (Class 3) is most suitable for agricultural and industrial uses; conventional treatment, including coagulation/flocculation, multi-stage filtration, aeration, and disinfection, would be required for drinking purposes. For Class 1/2 waters (Stations 2 and 3): basic sand or ceramic filtration followed by disinfection (solar disinfection, chlorination, or boiling). For Class 2 waters (Stations 1 and 4): sedimentation, mixed-media filtration (sand and activated carbon), and disinfection. For Class 3 water (Station 5): coagulation/flocculation using natural coagulants, sedimentation, multi-stage filtration, aeration, and robust disinfection (see Table 2 and Fig. 7). A zoned conservation management approach is recommended: Zone A (Headwaters Protection, Station 1), moderate interventions to manage fine sediment accumulation and place strategic rock deflectors to enhance dissolved oxygen levels without disturbing leaf litter, which provides critical habitat and food resources for macroinvertebrate communities; Zone B (Biodiversity Conservation, Stations 2 and 3) — highest protection, limited access for research and education, continuous monitoring of sensitive taxa (e.g., *Acroneuria abnormis*); Zone C (Sustainable Use, Station 4), designated water collection points, regular DO monitoring; Zone D (Buffer Management, Station 5 and forest boundary), collaborative riparian buffer establishment with adjacent agricultural landowners; Zone E (Riparian Transition Buffer), encompassing the northern forest edge adjacent to agricultural areas, focusing on maintaining riparian vegetation continuity to intercept agricultural runoff before it enters the canal system. Community engagement, environmental education, and careful ecotourism development at Zone B are complementary long-term strategies.

Table 1. Comparison of Measured Water Quality Parameters with Thailand Surface Water Quality Standards (Pollution Control Department, 2022) at Five Monitoring Stations in Ban Pru Toei Community.

Parameter	Station 1 (Upstream)	Station 2 (Waterfall)	Station 3 (Dam Head)	Station 4 (Dam Tail)	Station 5 (Canal)	Class 1	Class 2	Class 3	Water Quality Classification
Temperature (°C)	25.67 ± 1.15	26.00 ± 0.00	26.00 ± 0.00	27.33 ± 0.58	26.00 ± 0.00	≤30	≤30	≤30	All stations: Class 1
TDS (mg/L)	13.69 ± 4.08	11.44 ± 2.60	11.14 ± 3.92	9.68 ± 1.86	12.05 ± 2.44	≤500	≤500	≤500	All stations: Class 1
pH	6.80 ± 0.25	6.72 ± 0.25	6.88 ± 0.20	6.80 ± 0.26	6.65 ± 0.45	5.0–9.0	5.0–9.0	5.0–9.0	All stations: Class 1
Hardness (mg/L)	19.18 ± 3.42	12.65 ± 3.26	12.27 ± 3.73	12.65 ± 1.30	16.52 ± 7.41	≤300	≤300	≤300	All stations: Class 1
DO (mg/L)	4.04 ± 0.33	4.75 ± 1.32	4.46 ± 0.92	4.65 ± 0.84	3.90 ± 0.62	≥6.0	≥4.0	≥2.0	St1: Class 2 St2: Class 2 St3: Class 2 St4: Class 2 St5: Class 3
logical assessment (macroinvertebrates)	Moderately clean water indicators dominant (91.8%); Filopaludina sumatrensis (137 ind.); 7 families.	Clean water indicators dominant (62.5%); Macrobrachium lanchesteri most abundant; 6 families	Very clean (20%) + clean (53.3%) indicators; Acroeuria abnormis; Paegniodes sapanensis present; 9 families	Clean (38.5%) + moderately clean (57.7%); 1 Acroeuria abnormis; 8 families	Most diverse (15 families); very clean + clean + moderately clean + Chironomidae (3 ind.)	Based on <i>Kanjanava</i> <i>nit</i> (2002) 5- tier system	Based on <i>Kanjanava</i> <i>nit</i> (2002) 5- tier system	Based on <i>Kanjanavanit</i> (2002) 5-tier system	St1, St4: Class 2 St2, St3: Class 1/2 St5: Class 3 (mixed inc. dirty-water Chironomidae)

Table 1. Comparison of Measured Water Quality Parameters with Thailand Surface Water Quality Standards (Pollution Control Department, 2022) at Five Monitoring Stations in Ban Pru Toei Community. (continue)

Parameter	Station 1 (Upstream)	Station 2 (Waterfall)	Station 3 (Dam Head)	Station 4 (Dam Tail)	Station 5 (Canal)	Class 1	Class 2	Class 3	Water Quality Classification
WQI Score (0–100 scale)	73.3	86.7	86.7	76.7	68.3	≥80: Excellent	60–79: Good	40–59: Fair	St2, St3: Excellent (86.7) St4: Good (76.7) St1: Good (73.3) St5: Good (68.3)
Overall Water Quality Class	Class 2	Class 1/2	Class 1/2	Class 2	Class 3	Integrated physicochemical and biological assessment			

Notes: Values are mean ± SD of three monthly measurements (January–March 2023, dry season). Green = complies with Class 1 standard; Yellow = complies with Class 2 but not Class 1 (borderline); Red = does not meet Class 2 standard. Dissolved oxygen was the critical determinant of water class: the Class 1 threshold requires DO ≥ 6.0 mg/L, Class 2 requires DO ≥ 4.0 mg/L, and Class 3 requires DO ≥ 2.0 mg/L [9]. Biological assessment follows the 5-tier pollution tolerance classification of Kanjanavanit [10]. $WQI = 0.3 \times (\text{Physical Score}) + 0.3 \times (\text{Chemical Score}) + 0.4 \times (\text{Biological Score})$; thresholds from Pimpunchat [3]. Class designations follow the Pollution Control Department Surface Water Quality Standards [9]. All findings represent dry-season baseline conditions; wet-season data are required for annual characterization. See Supplementary Table S3 for monthly parameter values and full WQI component score derivation.

Table 2. Water Quality Suitability and Management Recommendations for Ban Pru Toei Community Forest

Sampling Point	Water Quality Class	Suitable Uses	Recommended Treatment	Conservation Priority
Point 1 (Upstream)	Class 2	Domestic (with treatment), Agriculture, Recreation	Basic filtration, disinfection	Medium
Point 2 (Waterfall)	Class 1/2	Domestic, Agriculture, Recreation, Ecosystem support	Minimal treatment, Basic disinfection	High
Point 3 (Dam Head)	Class 1/2	Domestic, Agriculture, Recreation, Ecosystem support	Minimal treatment, Basic disinfection	High
Point 4 (Dam Tail)	Class 2	Domestic (with treatment), Agriculture, Recreation	Filtration, Disinfection	Medium
Point 5 (Canal)	Class 3	Agriculture, Industrial, Limited recreational use	Conventional treatment	Low

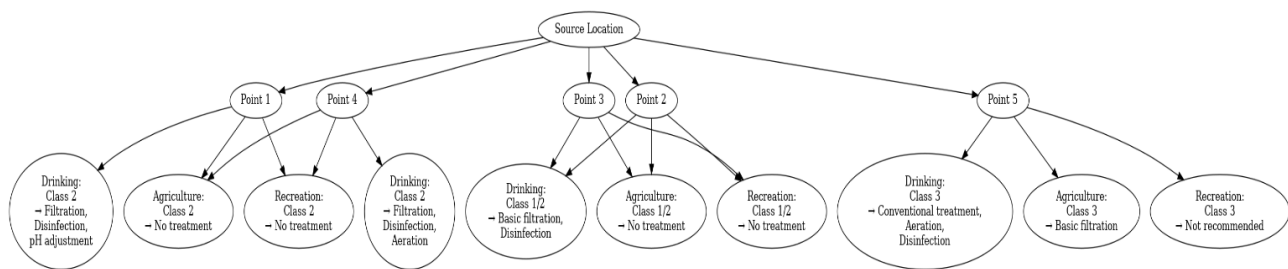


Figure 7. Recommended Water Treatment Processes Based On Source Location And Intended Use

4. Conclusions

This study quantitatively characterized surface water quality during the dry season (January–March 2023) at five stations within the Ban Pru Toei Community Forest (42.56 ha). All physical parameters met Thailand's Class 1 standards (temperature: $26.13 \pm 0.96^\circ\text{C}$; TDS: 11.60 ± 0.13 mg/L; pH: 6.77 ± 0.13). DO was the critical determinant of water class; the overall mean of 4.36 ± 0.10 mg/L placed most stations below the Class 1 threshold of 6.0 mg/L. Biological assessment identified 22 families across 10 orders, with pollution-sensitive taxa (*Perlidae*, *Heptageniidae*, *Ephemeraeidae*) confirmed at Stations 2–5 but absent from Station 1. The WQI ranked Stations 2 and 3 as excellent (86.7) and Station 5 as good (68.3). Stations 1 and 4 are classified as Class 2; Stations 2 and 3 as Class 1/2; and Station 5 as Class 3. All findings represent dry-season baseline conditions; year-round monitoring incorporating the wet season (June–September) is strongly recommended to provide a complete annual characterization of water quality and to evaluate the seasonal stability of macroinvertebrate assemblages. These quantitative baselines support zone-specific conservation strategies and provide essential reference data for long-term monitoring of this community-managed watershed.

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Development of a Top-Lit Up Draft Biomass Stove with a Combustion Chamber Level Adjustment Mechanism

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Abstract: This project aims to develop adjustable-depth TLUD (top-lit updraft) biomass stove combustion chamber technology to enhance stove performance during operation. Three conditions must be met for stove performance testing. The TLUD biomass stove was available in fixed depths of 7" (FS-7) and 11" (FS-11), as well as adjustable depths of 7 and 11" (AS-7/11) during operation. The evaluation of the biomass stove's performance used the water boiling test (WBT) methodology to assess thermal efficiency (η), burning rate (BR), power (P), and water boiling time (BT). The test results indicated that the FS-7 exhibited the maximum thermal efficiency ($18.60 \pm 0.14\%$), whereas the AS-7/11 showed the highest burning rate and power output (50.05 ± 1.07 g/min and 12.91 ± 0.27 kW, respectively). The AS-7/11 required the shortest duration to boil water (6.83 ± 0.69 min). The AS-7/11 was appropriate for applications necessitating high firepower and speed for thermally demanding tasks and was compatible with various containers.

Keywords: Biomass stove; combustion chamber adjustment; renewable energy

1. Introduction

Domestic energy consumption is expected to rise. Economic growth will stimulate increased energy consumption across several sectors. The government has promoted the use of several renewable energy sources, including biomass, solar, wind, hydropower, and geothermal energy [1, 2]. The goal is to reduce the importation of fuel energy from foreign sources and lower the community's expenses. Renewable energy sources can utilize a wide range of waste products from the agricultural and forestry sectors. It also supports community firms in the comprehensive production of biomass fuel, including biomass material aggregators, processors, and users [3, 4].

Biomass, as a renewable energy source, can be replenished naturally. Biomass may be used as an energy source to replace the finite supply of fossil fuels. Biomass is an organic material derived in substantial amounts from living organisms. Biomass sources include wood chips, rice husks, bagasse, corn cobs, eucalyptus wood chips, and palm waste [5, 6]. A biomass stove is a device that converts the thermal energy generated by biomass combustion into usable energy. Various types of domestic biomass stoves include protrusion stoves, high-performance stoves, high-performance braziers, conventional braziers, rocket stoves, and top-lit updraft (TLUD) biomass stoves. The TLUD biomass stove combusts fuel from the top while facilitating gas movement from the bottom to the top [7, 8]. The geometry of the combustion chamber in the TLUD

biomass stove directly influences combustion efficiency and the quality of the producer gas. Within the combustion chamber, three reaction zones are arranged along the chamber height: the pyrolysis zone, the incomplete combustion zone, and the reduction zone [9]. The depth of the combustion chamber influences the thermal efficiency of the biomass burner. A biomass stove featuring a shallow combustion chamber will yield superior thermal efficiency compared to one with a deep combustion chamber. A deep combustion chamber yields greater power than a shallow combustion chamber [10]. The implementation of a forced draft system enhances combustion efficiency during the ignition phase [11].

These restrictions made the stove unsuitable for applications requiring variable thermal output during operation. A stove with a fixed combustion chamber depth cannot be adequately modified to meet such needs. This project aims to address limitations of conventional biomass stoves by designing and developing adjustable combustion chamber depths that can be fine-tuned during operation. The goal is to improve the adaptability and effectiveness of firepower regulation for a variety of applications.

2. Materials and Methods

A TLUD biomass stove with a leveling mechanism for the combustion chamber operates in two depths as follows.

2.1 Development of a TLUD-type biomass stove that can decorate the combustion chamber

The development of TLUD biomass stoves used a 2 mm-thick steel plate as a component. The beam system was engineered to level the combustion chamber via the foot mechanism. The stove performance test had three conditions: a fixed biomass stove with a 7" deep combustion chamber (FS-7), a fixed biomass stove with an 11" deep combustion chamber (FS-11), and an adjustable biomass stove with 7" and 11" deep combustion chambers (AS-7/11).

2.2 Performance Test of TLUD Type Biomass Combustion Chamber

The stove performance evaluation used the water boiling test (WBT) with 5 kg of water [9, 10]. Rubber wood fuel had a lower heating value (LHV) of 15,472 kJ/kg and was 15 cm in length. Dry the fuel at 108°C for 24 hours to evaporate the steam contained in the fuel. The Fluke 54-IIB thermometer and the Fluke 80PK-22 temperature probe measured the water temperature. The test was conducted within a fume hood to protect against external wind impacts. Experiment AS7/11 began with leveling an 11" combustion chamber. After prolonged use, the flame decreased, necessitating adjustment of the combustion chamber to 7". We adjusted the combustion chamber level to 11" to facilitate refueling. The low flame required it to be set at 7". FS7 and FS11 were fixed at combustion chamber levels of 7" and 11", respectively, during the test. The performance evaluation of the TLUD 3 biomass stove started with the addition of 1 kg of fuel. Subsequently, the stove was ignited from above, the air was released from the force draft system, and a container of water was positioned for testing purposes. Upon reaching the boiling point of the water, stop the force draft system. The evaluation of the stove's performance is detailed below, as illustrated in Figure 1.

2.2.1 Thermal efficiency (η)

Thermal efficiency (η) was the proportion of thermal energy derived from biomass stoves used to boil water and generate steam. It is calculated from equation (1) [12, 13].

$$\eta = \left[\frac{4.186m_w(T_f - T_i) + 2260m_v}{f_d \times \text{LHV}} \right] \times 100 \quad (1)$$

η represents thermal efficiency, expressed as a percentage. m_w represented the mass of water, quantified in kilograms (kg). T_i represented the minimum temperature of water, expressed in degrees Celsius (°C). T_f represented the maximum temperature of water, expressed in degrees Celsius (°C). m_v represented the mass of water vapor generated during the TLUD biomass stove performance test, quantified in kilograms (kg). F_d was the total mass of fuel used in the test, expressed in kilograms (kg). LHV was the lower heating value of biomass fuels, expressed in kilojoules per kilogram (kJ/kg). High thermal efficiency results in fuel savings for the biomass stove.

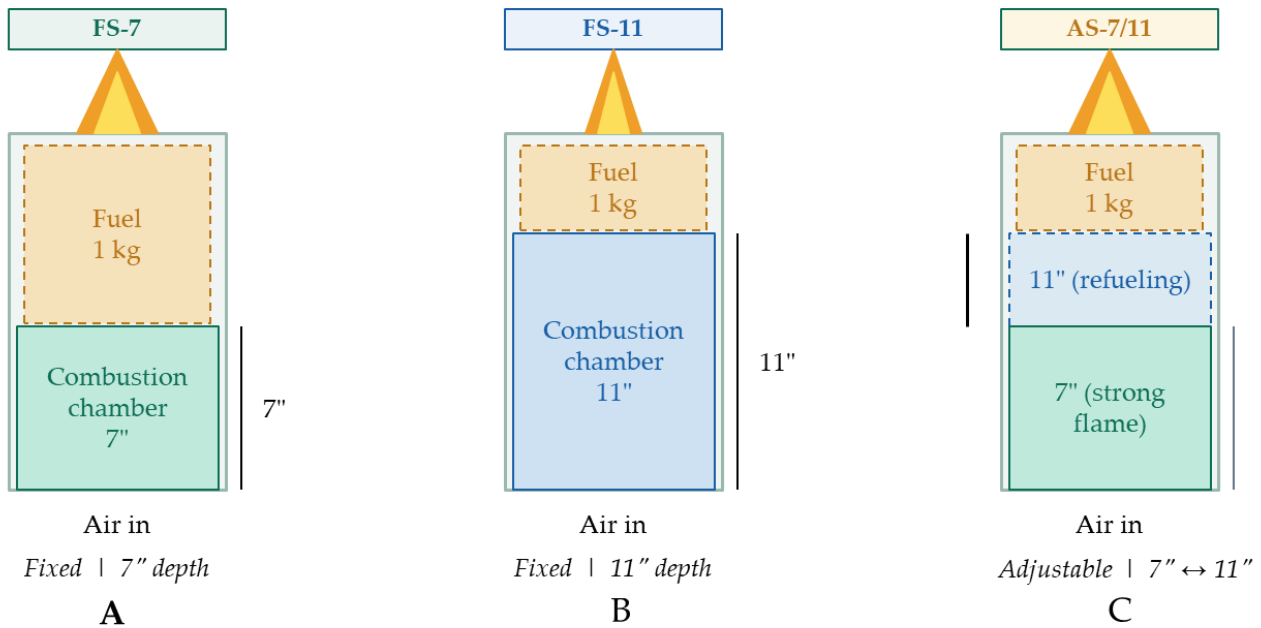


Figure 1 Schematic Diagram — Combustion Chamber Comparison A) Fixed 7" depth B) Fixed 7" depth. C) Adjustable 7" ↔ 11"

Note: AS-7/11 sequence: ① Set 11" → ② Flame decreases → ③ Adjust to 7" (stronger flame) → ④ Refuel → reset to 11"

2.2.2 The Burning Rate (BR)

The Burning Rate (BR) meant the quantity of biomass fuel combusted per unit of time, as determined from equation (2) [14]. The combustion rate reflects fuel consumption associated with the biomass stove's operation.

$$R_b = \frac{f_d}{\Delta t_m} \quad (2)$$

R_b represented the combustion rate in grams per minute (g/min). f_d represented the fuel's mass. It is quantified in kilograms (kg). Δt_m represented the duration of combustion in minutes. It was quantified in minutes (min).

2.2.3 Firepower (P)

Firepower (P) was the rate at which biomass fuels were converted to thermal power per unit of time. It is calculated from equation (3) [12]. High firepower is appropriate for rapid boiling, such as steam generation, but low firepower is ideal for stewing and simmering.

$$P = \frac{E}{\Delta t_s} \quad (3)$$

P represented the power output of the biomass stove, measured in kilowatts (kW). E represented the energy measured in kilojoules (kJ). Δt_s represented the duration of combustion in seconds. It was measured in seconds (s).

2.2.4 Boiling Time (BT)

Boiling Time (BT) is the time, in minutes, required for 5 kg of water to reach its boiling point. Signifies a decrease in the operational duration for biomass stove users. The test results were compared among FS-7, FS-11, and AS-7/11. The performance evaluation of the three types of biomass stoves was conducted in duplicate repeatedly. The experimental data were analyzed using analysis of variance (ANOVA), and mean differences were evaluated using Duncan's multiple-range test at a significance level of 0.05. The AS-7/11

biomass stove addresses the constraints of stoves with fixed combustion chambers by allowing the depth to be set to 11 inches, thereby enhancing the stove's firepower for rapid initial water boiling. Subsequently, the depth of the combustion chamber is reduced to 7 inches to reduce the biomass stove's firepower in accordance with application requirements, thereby enhancing thermal efficiency and conserving fuel. It may be readily adjusted to the operational conditions. A deeper combustion chamber will have a greater volume of pyrolysis and combustion zones, thereby enabling producer gas at a higher rate over the same period. This results in an elevated heat release rate, directly influencing the firepower output [15].

3. Results and Discussion

3.1 Development of TLUD-type biomass stove with adjustable combustion chamber level

The TLUD biomass stove has been improved and advanced to allow for the adjustment of the combustion chamber's height using the pedal plate shown in Figures 2A) to 2B). The TLUD biomass stove developed is made of thick steel plates (2 mm thick) joined together to form a rectangle. 1) The stove body supports the combustion chamber, covering 12" in width, 12" in length, and 20.5" in height. 2) The combustion chamber is 7.5" in width, 7.5" in length, and 12" in depth. 3) The ash tray functions to contain ash produced by biomass combustion within the combustion chamber. 4) The force draft system regulates air release and directs airflow into the lower section of the combustion chamber. 5) The choke ring functions to minimize flame dispersion at the stove aperture. A rail is there to facilitate the introduction of biomass fuel into the combustion chamber, along with a 2.5" container supporter. 6) The pedal plate and grate lever mechanism function to modify the depth of the combustion chamber during the operation of a TLUD biomass stove. To adjust the combustion chamber depth from 11" to 7", the user presses the "pedal plate," which transmits force to the "grate lifting rod" via the grate lever. Using the lever principle, the grate is raised. The "lever support" is then rotated by the user to support the "grate lever" and lock the grate level. To adjust the combustion chamber depth from 7" to 11", the "lever support" is rotated away from the "grate lever," which changes the grate position from 7" to 11", as illustrated in Figure 3. Figure 2C) illustrates that the base of the combustion chamber features a grate linked to the grate lifting rod, accompanied by a guide slot that ensures the grate lifting rod remains stable during operation, thereby adjusting the combustion chamber's level.

3.2 Comparison of stove performance at different conditions

Performance test results for the three TLUD biomass stove varieties: FS-7, FS-11, and AS-7/11. The test maintains control over external variables, including energy loss due to wind, by conducting experiments within the fume hood, as illustrated in Figure 4. Figures 5A) to 5D) present the test results of the three TLUD biomass stoves.

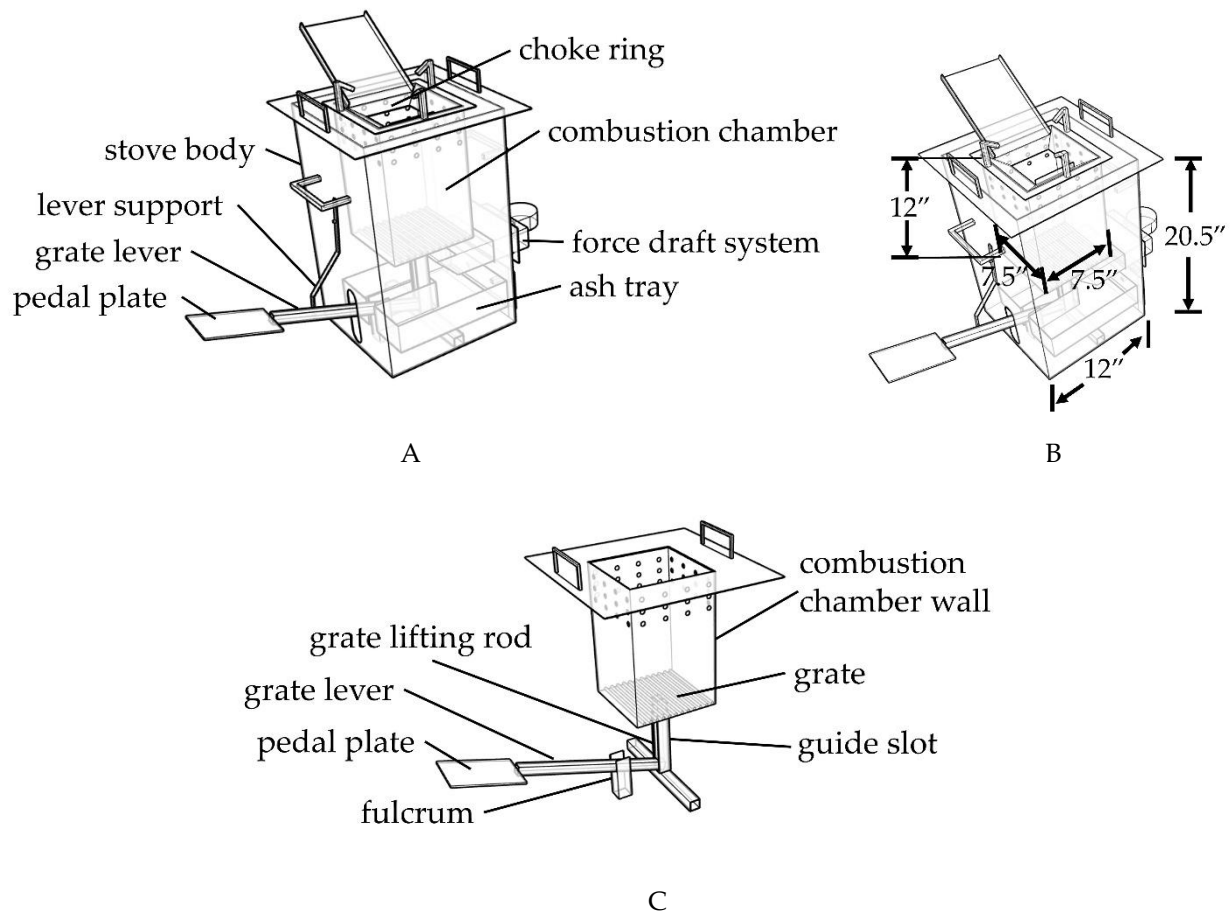


Figure 2. A) Diagram of a TLUD biomass stove, which includes an adjustable combustion chamber
 B) Observations of TLUD biomass stoves, including an adjustable combustion chamber
 C) Illustration of the combustion chamber and the apparatus used to level the grate

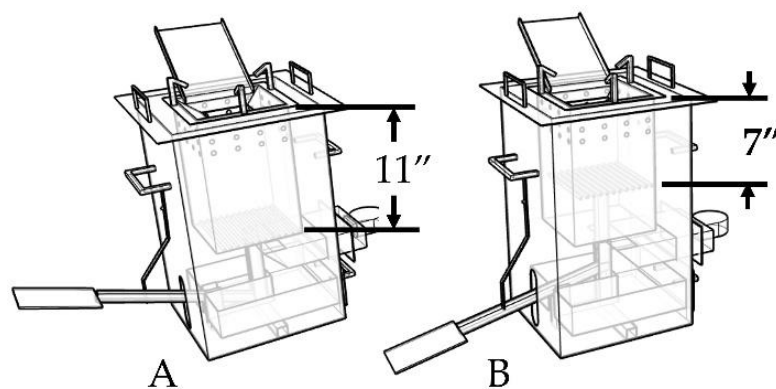


Figure 3. A) Biomass stove with 11" deep combustion chamber leveling and B) Biomass stove with 7" deep combustion chamber leveling



Figure 4. Performance evaluation of TLUD biomass stove with the WBT method

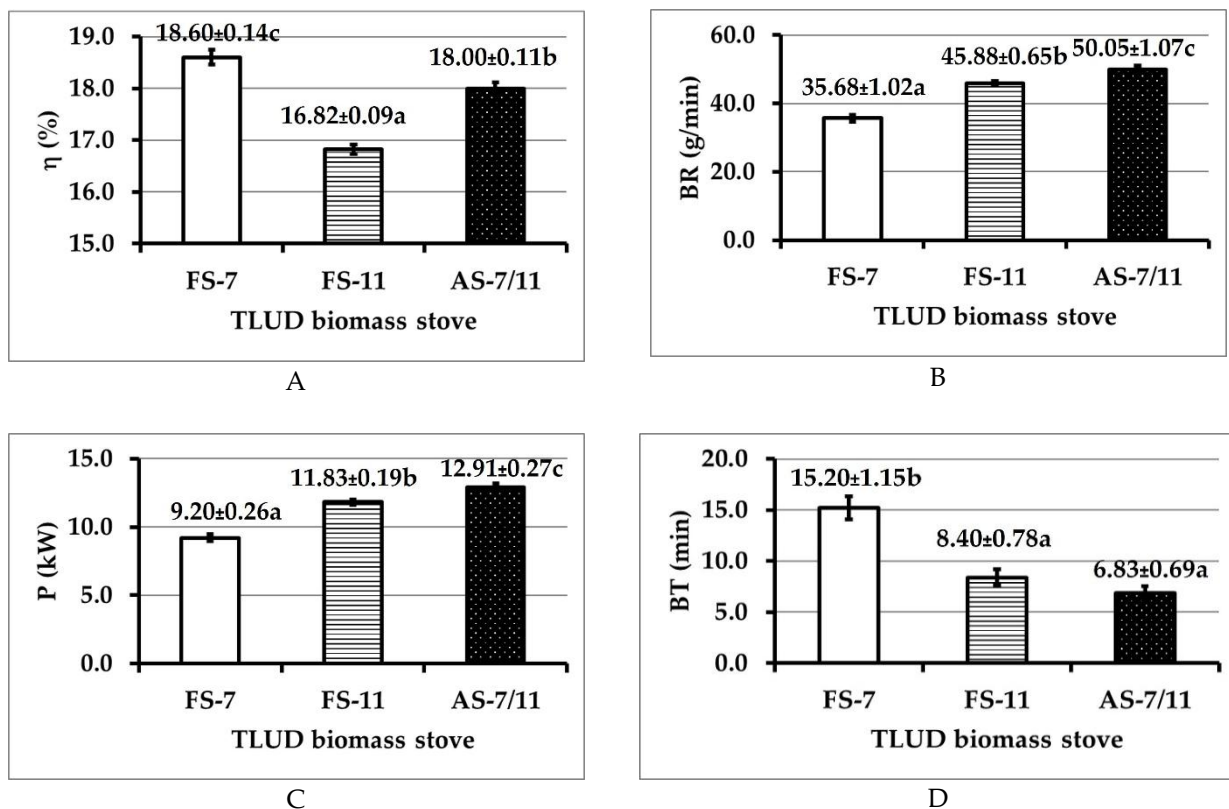


Figure 5. A) Comparative analysis of the thermal efficiency (η) of a TLUD biomass stove. B) Comparative analysis of burning rate (BR) in TLUD biomass stoves. C) Comparative analysis of firepower (P) in TLUD biomass stoves. D) Comparison of boiling time (BT) for water using a TLUD biomass stove. Notice: For identical letters, no statistical difference was observed at the 0.05 significance level.

Figure 5A) presents a comparison of the thermal efficiency of the TLUD biomass stove in all three conditions, which shows a statistically significant difference at the 0.05 level. FS-7 shows the highest thermal efficiency (18.60 ± 0.14%), followed by AS-7/11 (18.00 ± 0.11%) and FS-11 (16.82 ± 0.09%). The test results indicated that the depth of the biomass stove's combustion chamber was inversely related to thermal efficiency, as a deeper chamber lost thermal energy before reaching the water container used for the test. Depth-adjustable combustion chambers offer greater operational flexibility, but they exhibit higher thermal energy losses than shallow or fixed combustion chambers [10, 16]. TLUD biomass stoves using a shallow combustion chamber

(FS-7) exhibit superior thermal efficiency compared to those with deep combustion chambers (FS-11), as heat loss through the chamber walls escalates with the distance traveled by the hot gases. A small combustion chamber with a short gas flow path minimizes conductive and convective heat loss, thereby enhancing the efficiency of energy transfer to the container [15].

Figure 5B) illustrates that the burning rate of the TLUD biomass stove showed a statistically significant difference at the 0.05 level, with AS-7/11 recording the highest rate (50.05 ± 1.07 g/min), followed by FS-11 (45.88 ± 0.65 g/min) and FS-7 (35.68 ± 1.02 g/min). It was shown that AS-7/11, a stove with an adjustable combustion chamber level, achieved the highest burning rate compared with FS-7 and FS-11, which maintained a fixed combustion chamber level during the test. As the combustion chamber was leveled, ash fell from the grate, facilitating increased airflow from below into the combustion chamber [17]. This results from the impact and vibration of the grate during combustion chamber leveling. Consequently, the ash descends from the grate, creating an opening that allows increased air intake into the combustion chamber from below, thereby enhancing the stove's firepower. Figure 5C) illustrates that the firepower output of the three TLUD biomass stoves exhibited a statistically significant difference at the 0.05 level, with AS-7/11 showing the highest value (12.91 ± 0.27 kW), followed by FS-11 (11.83 ± 0.19 kW) and FS-7 (9.20 ± 0.26 kW), respectively. Figure 5D) illustrates that AS-7/11 (6.83 ± 0.69 min) and FS-11 (8.40 ± 0.78 min) show no different values; however, FS-7 (15.50 ± 1.15 min) is statistically substantially greater than both AS-7/11 and FS-11 at the 0.05 significance level. The size of the combustion chamber influences the rate of biomass energy transfer from the chamber to the container and the water used in the experiment [16]. A deeper combustion chamber could heat 5 liters of water to its boiling point faster than a shallower one [18]. Test results indicate that the AS-7/11 is optimal due to its ability to adjust the grate level of the combustion chamber during operation. This represents a trade-off between achieving satisfactory thermal efficiency, high firepower, and a fast boiling time.

3.3 Comparison with other biomass stoves

The AS-7/11 biomass stove is distinguished by its high output and short operating time, making it ideal for quick tasks or high-power applications such as steaming. It is suitable for boiling large quantities of water or deep-frying. The AS-7/11 features a forced draft system. The AS-7/11 offers increased firepower, a higher combustion rate, and improved boiling times for water. The AS-7/11 features a forced-draft system that allows more air to enter the combustion chamber than other stoves. The AS-7/11 provides increased firepower, a higher burning rate, and improved boiling times for water [19-21].

Table 1. Comparative analysis of biomass stove performance

Biomass stoves	η (%)	P (kW)	BR (g/min)	BT (min)	Highlights
AS-7/11	18.00	12.91	50.05	6.83	Highest firepower & lowest operating time
Development TLUD [20]	25.78	7.19	27.89	12.86	Save fuel and moderate power output.
Standard TLUD [20]	24.87	6.64	25.76	20.64	Save fuel and moderate power output.
Traditional TLUD [20]	18.92	8.57	33.23	16.69	Easy to build
Protruding stove [20]	21.96	5.52	21.41	34.16	Releases heat slowly
TLUD 9-holes [19]	10.85	-	-	-	Easy to build
Heavy rocket [21]	35.1	4.8	-	20	Most of the savings in fuel

4. Conclusions

The AS-7/11 TLUD biomass stove features an adjustable combustion chamber depth of 7" and 11" during use. The AS-7/11 biomass stove is designed for applications that require power modulation during operation, overcoming the constraints of conventional biomass stoves that deliver fixed firepower via an adjustable combustion chamber depth mechanism. This adaptability addresses the requirements of processes that require various levels of continuous heating, such as rapid cooking followed by simmering, or applications that demand high or low heat throughout operation, eliminating the need for multiple biomass stove types, as the combustion chamber's depth can be adjusted to match the task's needs. This study

demonstrated the AS-7/11 biomass stove's effective performance and flexibility; however, it did not quantify emissions such as PM_{2.5} or CO. Consequently, future research should test emissions and analyze the consequences of using alternative biomass fuel types.

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Cost-Effective Artificial Media as Substrates for Mass Cultivation of Entomopathogenic Nematodes (EPNs)

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Abstract: Entomopathogenic nematodes (EPNs) are eco-friendly alternatives to chemical pesticides in controlling agricultural insect pests, yet their mass cultivation remains constrained by high costs and ingredient availability. This study evaluated three cost-effective artificial semi-solid media for cultivating *Heterorhabditis* spp.: (A) egg yolk + mung bean, (B) egg yolk + fish meal, and (C) egg yolk + dog food. Each medium was inoculated with infective juveniles (IJs) and monitored weekly for 4 weeks, during which population density and physicochemical parameters (color, aroma, shape, and pH) were assessed. By week one, all media supported rapid multiplication, with no significant differences observed among treatments (A: 6,071.60 IJs/mL; B: 6,911.80 IJs/mL; and C: 7,546.20 IJs/mL, $p = 0.279$). From week two onward, significant differences emerged, with fish meal and dog food supporting higher population densities (9,221.60 $\pm$ 1,170.95 and 8,711.60 $\pm$ 1,303.25 IJs/mL, respectively) than mung bean (4,231.00 IJs/mL) ($p < 0.001$). Dog food medium sustained the highest populations through week three (4,682.20 IJs/mL) and retained the largest residual density by week four (287.00 $\pm$ 238.00 IJs/mL). Fish meal peaked highest (9,221.60 $\pm$ 1,170.95 IJs/mL) on the second week but declined thereafter. Mung bean, the sole plant-based medium, showed descriptive premature deterioration in shape by week two and significant aroma decline by week three, leading to population collapse by week four. These findings establish dog food-supplemented medium as the most cost-effective substrate for sustained EPN mass cultivation.

Keywords: Entomopathogenic nematodes; *Heterorhabditis* spp.; semi-solid media; mass cultivation; physicochemical properties

1. Introduction

Insect pest attacks are among the limiting factors for production losses in the Philippine agricultural sector. The injudicious use of pesticides and their degradation products in the environment has been associated with toxic effects on non-target organisms, including bees, aquatic organisms, and humans [1]. To address the environmental and health problems associated with this, entomopathogenic nematodes (EPNs), which are microorganisms that parasitize, cause disease, and kill insect pests [2], have been used by Pascual et al. [3] as ecologically acceptable pesticides. EPNs, primarily from the genera *Steinernema* and *Heterorhabditis*, are natural enemies of many insect species and

are characterized by their ability to infect and kill hosts [4 - 5] around 24 - 48 hours [6 - 7] by invading their natural openings, such as the mouth, anus, tracheae, or directly through the cuticle [8].

Mass production of microbial control agents (MCAs), such as entomopathogenic nematodes (EPNs), with reliable quality is the backbone of the biopesticide industry [9]. The use of nematodes for biological control of insect pests requires mass-culture techniques that produce large numbers of nematodes quickly, cheaply, and simply [10]. These nematodes can be produced in vivo by baiting insects and commercially by in vitro solid/liquid culturing [11]. In-vivo mass culture of EPNs uses various insects, such as Hong Kong caterpillar (*Tenebrio molitor*) [12, 8], Red flour beetle (*Tribolium castaneum*) (Herbst) larvae, pupae, and adults [13], and wax moth larvae (*Galleria mellonella*) [14], among others. On the other hand, in vitro mass culture of EPNs started when House et al. [10] utilized dog food as a medium (semi-solid) for *S. carpocapsae* [15]. Indriyanti and Muharromah [8] reported the use of egg yolk as a base – a protein source in nematode diets [16] – combined with other protein-rich supplementary ingredients such as dog food, soybean powder, and chicken liver as media for EPN mass cultivation. Thiruchchelvan et al. [13] also reported the use of soy flour, palmyra tuber flour, corn flour, and black gram flour (dhal flour) as media for in vitro mass cultivation of the nematode *Acroboloides longiuterus*.

However, both in vivo and in vitro mass production techniques have many limitations, especially regarding their cost-effectiveness and the availability of the ingredients. In-vivo production requires less capital but lacks economies of scale due to labor and insect costs, whereas in-vitro liquid culture (which comprises the bulk of commercial production) generally offers the greatest economic efficiency but requires the largest capital [17]. Locally, soybeans, palmyra tuber flour, and black gram flour or dhal flour are not common, and chicken liver is too expensive to be just fed to nematodes, leaving dog food as the only cheap and available ingredient in the market. In 2020, Mathew et al. [18] reported a modified semi-solid dog food media (utilizing Pedigree Gravy Adult Chicken and Liver Chunks), which has been proven to contain essential nutrients such as crude protein (8%), crude fat (4%), and crude fiber (3%) – found to be safe and cheap for the mass production of EPNs.

Many locally available ingredients that provide the same nutritional sources, which are much cheaper than the more expensive ones, could be used without compromising the nutrients nematodes need to multiply. Fish meal, aside from being locally available and cheap, is a naturally balanced feed ingredient that is high in protein (60% - 72%), energy, and minerals such as calcium and phosphorus [19], making it a potential substitute for chicken liver. Mung bean, on the other hand, could also serve as an effective substitute for soybeans and various flours, as it is becoming another legume crop that could provide high-quality plant protein – ranging from 20–30% [20] - after soybean and pea [21]. Additionally, its availability in the local market is year-round. Although gelatin is commonly available in the market, agar-agar – a versatile hydrocolloid extracted from red algae that primarily serves as a gelling agent [22] – is a constant necessity in various microbiological activities, not only as a carbon source but also to minimize errors, especially in medium-based experiments.

Entomopathogenic nematodes (EPNs) are widely recognized as effective biological control agents, but their practical application is often limited by challenges in formulation stability, survival during storage [23], and the availability and cost of the ingredients. This study investigated how EPNs thrive when mass-cultivated in alternative artificial semi-solid media using combinations of egg yolk and mung bean, egg yolk and fish meal, and egg yolk and dog food. The main goal of this study was to assess which artificial media using protein-rich nutrient sources extended the longevity and shelf-life of EPNs, thereby addressing many critical gaps in EPN utilization.

2. Materials and Methods

2.1 Location of the Study

The study was conducted at the Center for Biotechnology Studies – Cebu Technological University – Barili Campus (10° 7' 54" N, 123° 32' 41" E) under controlled conditions (temperature: 25-28°C; relative humidity: ~85–95%; light period: no light).

2.2 Identification of Entomopathogenic Nematodes (EPNs)

Heterorhabditis spp. were identified to the family and genus levels using morphological characterization and keys from Nguyen and Hunt [24]. Representative nematodes were teased out from the clear suspension, killed, and fixed by adding hot 4% formalin (65–70°C), and then stained. EPNs were mounted on slides and examined under a compound light microscope for identification.

2.3 Production of Artificial Media and EPN Multiplication

EPN cultivation media were prepared as described by Indriyanti and Muharromah [8] with slight modifications. The artificial media were made in three formulae, each having five replications. They were coded as A, B, and C. The compositions of each medium are listed in Table 1 below. All media were pulverized except for the yolk. Mung bean and dog food were ground using a blender. The fish meal was procured from a local market. These supplementary ingredients were then meshed. Meanwhile, the yolk was sourced from a liquid chicken egg. Mixed media in each treatment were added with 0.20 g agar-agar and 30 mL distilled water. All components in each medium were mixed continuously and placed in a glass bottle (14 cm high and 9 cm in diameter), with a white sponge measuring 6 cm high and 1 cm wide placed to absorb the medium. The closed bottles were sterilized in an autoclave for 30 minutes at 121°C. After sterilization, the media were allowed to cool. No post-sterilization sterility verification (e.g., plating on nutrient agar) was performed, as the study was designed to simulate practical, low-cost production conditions; however, all treatments were prepared and handled uniformly to minimize variability. Each medium was then inoculated with infective juveniles (IJs). Initial populations per treatment were listed in Table 2. The bottles were then sealed and placed at room temperature in the dark.

Table 1. Artificial media formulations.

Media Composition	Yolk (g)	Mung Bean Powder (g)	Fish Meal (g)	Dog Food (g)	Agar-agar (g)	Distilled water (mL)
(A) Yolk + Mung Bean	1	1	-	-	0.20	30
(B) Yolk + Fish Meal	1	-	1	-	0.20	30
(C) Yolk + Dog Food	1	-	-	1	0.20	30

Table 2. Initial populations of infective juveniles (IJs) per treatment.

Media Composition	IJs/mL
(A) Yolk + Mung Bean	1,258.00 ± 283.23
(B) Yolk + Fish Meal	1,378.00 ± 405.12
(C) Yolk + Dog Food	1,544.00 ± 306.07

Although the dog food treatment had a slightly higher initial inoculum density, statistical analysis in Table 7 ($F(2,12) = 0.915$, $p = 0.427$) showed no significant differences among treatments. This indicates comparable starting conditions, allowing subsequent differences in population dynamics to be attributed primarily to media composition.

2.4 EPN Multiplication

EPN population (*Heterorhabditis* spp.) originated from Leyte, Philippines. EPNs were multiplied using wax moth larvae (*Galleria mellonella*) as an insect host in the White trap method. The modified white trap consisted of filter paper on an inverted petri dish lid (5 cm diameter) onto which the cadavers were placed. The dish was placed into a larger petri dish (12 cm diameter) and filled with distilled water. The traps were checked daily for infective juvenile (IJ) emergence from the cadavers. For rapid EPN multiplication, IJs from infected cadavers were applied to fresh larvae as described by Pascual et al. [25]. EPN isolates were maintained in distilled water suspensions in Falcon tubes and stored at 10–15°C until inoculated into the media.

2.5 Assessment of Culture Media Properties and EPN Population Dynamics

EPNs were observed weekly over 4 weeks to assess changes in population density among the different artificial media. Observation of its abundance was conducted by taking 100 µL of EPN culture from the same

area in the bottle using a sterilized micropipette. The EPN culture was then dropped onto a glass slide and observed using a binocular compound light microscope. Alive EPNs showing vigor were counted using a hand counter. Each sample was counted three times to obtain the average. EPN populations were counted using the equation:

$$\text{EPN population (IJs/mL)} = \text{Mean IJ count} \times (1000 / \text{sample volume in } \mu\text{L})$$

Average EPN abundance data from the 1st to 4th week were statistically analyzed using a one-way ANOVA (F test) at a significance level of 5% and a Post hoc test (Tukey test). Data analysis was conducted using SPSS 25. Other measured parameters were color, aroma, shape, and pH of the different media. Changes in color, aroma, and shape were analyzed using a scale of 5 (desirable, excellent) to 1 (undesirable). The scoring criteria for color, aroma, and shape were developed for this study and applied consistently across treatments. Assessments were conducted without blinding, and inter-rater reliability was not evaluated. Finally, parameters exhibiting zero variance in one or more groups were excluded from inferential statistical analysis, and results were interpreted descriptively.

2.5.1 Color of Media

The changes in color of insects when infected with entomopathogenic nematodes (EPNs) are mainly caused by the metabolic activity of the nematodes and their symbiotic bacteria (*Xenorhabdus* and *Photorhabdus*), which produce pigments, toxins, and enzymes that alter the appearance of the insect host [26]. Similarly, in artificial media as a substrate for nematodes, discolorations indicate interaction between actively feeding nematodes and their symbionts and signify that the medium is utilized, thereby being favorable.

Color progression from initial preparation through the end of the culture period was assessed based on the following criteria:

- 5 – consistent color changes from the initial setup to the end, having similar hue; no signs of contamination or precipitation; discoloration from light (initial setup) to dark (termination) are constant and uniform; 100% discoloration
- 4 – distinct and uniform changes, but with slight unevenness; no significant contamination; up to 75% discoloration
- 3 – moderate color changes; few areas are less pronounced; up to 50% discoloration
- 2 – unclear color changes, possibly due to the color of the ingredients; possible early contamination or separation; up to 25% discoloration
- 1 – no observable changes

2.5.2 Aroma of Media

Indriyanti and Muharromah [8] observed that aroma changed from fresh to slightly pungent or stinging over time in various artificial media due to nutrient breakdown and nematode metabolic progression, reflecting culture stages and sometimes posing contamination risk. In addition, the metabolic activity of nematodes, along with their symbionts, which are released by the nematodes [27], contributes to the aroma of nematode cultures, linked to enzymatic protein and lipid degradation. Under unfavorable culture conditions, these processes lead to the development of distinctly unpleasant or foul odors, which can indicate excessive microbial decomposition, contamination, or suboptimal media quality.

Smell changes indicating spoilage or fermentation were assessed based on the following criteria:

- 5 – pleasant aroma is maintained or developed along the culture period; no foul odors or signs of contamination
- 4 – changes in aroma into a neutral smell, having a mild background odor
- 3 – changes in aroma are noticeable; some samples start to smell unpleasant
- 2 – clear presence of unpleasant odors, indicating early contamination or degradation of media
- 1 – very strong, unpleasant, foul odors are dominating, indicating contamination and decay

2.5.3 Shape of Media

Somwong and Petcharat [28] reported that a good medium maintains nematode morphology and supports high yields, reflecting a stable semi-solid medium; thus, any deviations in shape or media integrity indicate culture issues (e.g., contamination). Shape alteration is also affected by the composition and waste products of cultivated EPNs in closed conditions [8].

Assessment for changes in shape (physical and structural) was based on the following criteria:

- 5 – Media is maintained at a stable semi-solid state and has a firm consistency throughout the culture period
- 4 – media has few surface irregularities (minimal unevenness & shrinking) and mostly retains its semi-solid integrity
- 3 – media starts to exhibit minimal liquefaction, or surface cracks
- 2 – media integrity is compromised with significant liquefaction, collapse, or irregular shape (e.g., watery zones)
- 1 – media structure is collapsed; highly contaminated with surface irregularities, pockets of liquefaction, or visible fungal/bacterial overgrowth

2.5.4 pH of Media

The pH of each medium was measured at the initial setup and at the termination of the experiment to provide an overall indication of media condition. Weekly monitoring focused on population density and observable physicochemical characteristics, including color, aroma, and shape. Intermediate pH measurements were not performed to minimize disturbance of the culture system and reduce the risk of contamination, as repeated sampling could alter media composition and compromise experimental consistency. Thus, initial and final pH values were considered sufficient to capture the overall direction and magnitude of pH change across treatments.

3. Results and Discussion

3.1 Entomopathogenic Nematode Identification

The entomopathogenic nematodes (EPNs) used in the study were identified as *Heterorhabditis* (Figures 1 & 2), a genus in the family Heterorhabditidae. When infecting suitable hosts, these nematodes release *Photorhabdus*, its bacterial symbiont – turning the cadaver into red due to the production of anthraquinone pigments [29] (Figure 3B). Additionally, Booyesen et al. [30] reported that different strains of *Photorhabdus* also cause various pigmentations to cadavers, turning them into steel grey-blue (blue), red, and green (yellow with a green tint). In artificial media, the bacterial symbionts would produce varying color shades of the red pigment [31] (Figure 3A).



Figure 1. *Heterorhabditis* showing both (a) anterior and (b) posterior body segments. (10x/0.25 oil)



Figure 2. *Heterorhabditis* (A) anterior; (B) metacarpus; (C) posterior. (100x/1.25 OiL)



Figure 3. Discolorations on (A) media and (B) cadavers, indicating the presence of *Photorhabdus*.

In vitro mass cultivation of *Heterorhabditis* yielded varying results across treatments. At the start, initial nematode populations were statistically comparable across all media, with means of $1,258 \pm 283.23$ IJs/mL for mung bean, $1,378 \pm 405.12$ IJs/mL for fish meal, and $1,544 \pm 306.07$ IJs/mL for dog food, as shown in Table 2. One-way ANOVA confirmed no significant difference ($F(2,12) = 0.915$, $p = 0.427$) (Table 7), and Tukey's test placed all treatments in the same subset, indicating that later divergences could be attributed to the diets rather than unequal inocula. Similarly, the physicochemical parameters of the media (color, aroma, shape, and pH), as shown in Table 9, were indistinguishable at the time of inoculation. Color was uniform across all treatments (Mean = 1.00 ± 0.00), with no measurable variance. Because all values were identical, inferential statistics could not be applied; results are reported descriptively only. Aroma and shape likewise showed homogeneity (Means = 5.00 ± 0.00 each). Finally, initial pH values were descriptively comparable across all treatments (mung bean: 6.60, fish meal: 6.49, dog food: 6.38), indicating similar starting acidity conditions. The absence of initial differences across both biological inocula and physicochemical parameters ensures that subsequent divergences observed during culture can be attributed to dietary and substrate effects rather than initial inequalities. This is consistent with Shapiro-Ilan et al. [32], who emphasized that optimized inoculation parameters (i.e., starting populations, culture conditions, etc.) are critical for interpreting dietary effects on entomopathogenic nematode (EPN) reproduction. Similarly, Koppenhöfer et al. [33] emphasized that consistent starting parameters, including media properties and inoculum size, are critical for reproducible mass production systems.

By the first week, nematode populations increased across all culture media as shown in Table 3, with means of $6,071.60 \pm 1,586.16$ IJs/mL (mung bean), $6,911.80 \pm 1,236.50$ IJs/mL (fish meal), and $7,546.20 \pm 1,315.04$ IJs/mL (dog food), but no significant differences were detected among treatments ($F(2,12) = 1.421$, $p = 0.279$).

Tukey's test confirmed a single statistical subset, indicating comparable early reproductive performance across all media. The observed variability likely reflects inherent biological heterogeneity of entomopathogenic nematodes and minor micro-environmental differences under non-axenic, low-intervention conditions, which do not obscure the overall similarity in early culture performance. This aligns with Shapiro-Ilan et al. [32], who reported that EPNs often perform similarly across substrates during initial reproductive cycles before diet-specific effects emerge, and McMullen II and Stock [34], who emphasized that early production is primarily driven by general nutrient support for both nematodes and their symbiotic bacteria. For physicochemical parameters, color and shape exhibited zero variance in one or more treatments (mung bean and dog food for color; fish meal and dog food for shape), indicating complete within-treatment uniformity. Because ANOVA assumptions were violated, these parameters were not subjected to inferential statistical analysis and are interpreted descriptively. Fish meal showed more pronounced color development, suggesting earlier metabolic activity of symbiotic bacteria, while mung bean displayed reduced shape compared to fish meal and dog food, indicating early signs of media instability. In contrast, aroma did not differ significantly among treatments ($F(2,12) = 0.167$, $p = 0.848$), and all media maintained generally acceptable odor profiles. This suggests that, during the first week, microbial activity and nutrient degradation were still at an early stage and relatively uniform across substrates.

Table 3. Mean population and physicochemical changes for the 1st week.

Parameter	(A) Mung Bean	(B) Fish Meal	(C) Dog Food	ANOVA F (df)	p-value	Tukey
Nematode count	6,071.60 ± 1,586.16a	6,911.80 ± 1,236.50a	7,546.20 ± 1,315.04a	1.421 (2,12)	0.279	Same subset
Color	1.00 ± 0.00	3.20 ± 1.10	1.00 ± 0.00	—	—	Descriptive only
Aroma	4.20 ± 0.84	4.20 ± 0.45	4.40 ± 0.55	0.167 (2,12)	0.848	NS
Shape	3.80 ± 0.45	5.00 ± 0.00	5.00 ± 0.00	—	—	Descriptive only

Note: Parameters in which one or more treatments exhibited zero standard deviation were not subjected to inferential statistical analysis (ANOVA) and are reported descriptively.

By the second week, a clear divergence in nematode production was observed (Table 4). Mung bean declined to $4,231.00 \pm 1,607.65$ IJs/mL, while fish meal ($9,221.60 \pm 1,170.95$) and dog food ($8,711.60 \pm 1,303.25$) maintained significantly higher populations. This difference was statistically significant ($F(2,12) = 20.004$, $p < 0.001$), with Tukey's test grouping fish meal and dog food together and separating mung bean as the lower-performing substrate. These results are consistent with those of Widiyaningrum et al. [26], who reported that animal-based substrates better support EPN reproduction due to higher nutrient availability. Physicochemical traits also diverged. Color values showed no variance across treatments and were therefore interpreted descriptively. Fish meal showed more advanced color progression, likely reflecting increased metabolic activity of symbiotic bacteria compared to the other media. Shape values in fish meal and dog food exhibited zero variance (5.00 ± 0.00), indicating consistent structural stability. Because variance assumptions were violated, inferential statistical analysis was not applicable; however, descriptively, both media maintained their semi-solid integrity, whereas the mung bean showed noticeable degradation. Aroma remained statistically similar across treatments ($F(2,12) = 0.222$, $p = 0.804$), indicating that odor-related changes were still not strongly differentiated despite emerging differences in population density and structural stability.

Table 4. Mean population and physicochemical changes for the 2nd week.

Parameter	(A) Mung Bean	(B) Fish Meal	(C) Dog Food	ANOVA F (df)	p-value	Tukey
Nematode count	4,231.00 ± 1,607.65a	9,221.60 ± 1,170.95b	8,711.60 ± 1,303.25b	20.004 (2,12)	<0.001	Fish meal = Dog food > Mung bean
Color	1.00 ± 0.00	4.00 ± 0.00	1.00 ± 0.00	—	—	Descriptive only
Aroma	3.40 ± 0.55	3.60 ± 0.55	3.60 ± 0.55	0.222 (2,12)	0.804	NS
Shape	2.60 ± 0.55	5.00 ± 0.00	5.00 ± 0.00	—	—	Descriptive only

Note: Parameters in which one or more treatments exhibited zero standard deviation were not subjected to inferential statistical analysis (ANOVA) and are reported descriptively.

By the third week, nematode production showed strong divergence (Table 5). Dog food showed significantly higher population ($4,682.20 \pm 987.64$ IJs/mL) compared to both fish meal ($1,715.00 \pm 835.65$) and mung bean (799.80 ± 309.63). These differences were highly significant ($F(2,12) = 34.916$, $p < 0.001$), and Tukey's test indicated a graded response (dog food > fish meal = mung bean), consistent with improved performance in nutrient-rich animal-based media as reported by Zhen et al. [27] and Koppenhöfer et al. [33]. For sensory and physical traits, color exhibited zero variance in one or more treatments (fish meal and dog food) and was therefore not subjected to inferential statistical analysis. Descriptively, fish meal maintained the highest color intensity, indicating sustained metabolic activity, while mung bean and dog food showed less pronounced changes. Aroma differed significantly among treatments ($F(2,12) = 10.750$, $p = 0.002$), with mung bean exhibiting the lowest scores, indicating greater spoilage or degradation. Fish meal and dog food maintained relatively better odor profiles, suggesting more stable biochemical conditions. Shape values showed zero variance across all treatments, preventing inferential analysis. Descriptively, mung bean exhibited structural collapse, while fish meal and dog food exhibited minimal liquefaction, yet retained sufficient semi-solid integrity to continue supporting nematode survival at this stage.

Table 5. Mean population and physicochemical changes for the 3rd week.

Parameter	(A) Mung Bean	(B) Fish Meal	(C) Dog Food	ANOVA F (df)	p-value	Tukey
Nematode count	799.80 ± 309.63a	1,715.00 ± 835.65a	4,682.20 ± 987.64b	34.916 (2,12)	<0.001	Dog food > Fish meal = Mung bean
Color	1.40 ± 0.55	5.00 ± 0.00	1.00 ± 0.00	—	—	Descriptive only
Aroma	1.40 ± 0.55	2.60 ± 0.55	2.80 ± 0.45	10.750 (2,12)	0.002	Mung bean < Fish meal = Dog food
Shape	1.00 ± 0.00	3.00 ± 0.00	3.00 ± 0.00	—	—	Descriptive only

Note: Parameters in which one or more treatments exhibited zero standard deviation were not subjected to inferential statistical analysis (ANOVA) and are reported descriptively.

By the fourth week, nematode numbers declined across all diets (Table 6), but dog food supported the highest residual populations despite late-stage declines, retaining the largest population (287.00 ± 238.00), followed by fish meal (69.80 ± 60.34), while mung bean dropped almost completely (7.20 ± 14.48). ANOVA still detected significant differences ($F(2, 12) = 5.347$, $p = 0.022$), with Tukey's post hoc test confirming that dog food was the highest, fish meal an intermediate, and mung bean the lowest. Physicochemical and sensory traits also showed divergence. Color and shape exhibited zero variance across treatments, indicating uniform

measurements within groups. As a result, these parameters were not subjected to inferential statistical analysis and are interpreted descriptively. Fish meal maintained the highest color intensity, while both fish meal and dog food retained partial structural integrity compared to the complete collapse observed in the mung bean. Aroma differed significantly among treatments ($F(2,12) = 16.333$, $p < 0.001$), with dog food showing higher scores, indicating less degradation than fish meal and mung bean. pH also differed descriptively, with dog food exhibiting a lower pH than the other treatments. This suggests greater accumulation of acidic metabolic by-products, likely associated with microbial activity. However, since pH was only measured at the beginning and end of the experiment, these results should be interpreted as indicative of overall changes rather than continuous trends. The observed pH differences among media suggest substrate-dependent variation in microbial metabolism, particularly the activity of symbiotic bacteria (*Photorhabdus* spp.), which drive the production of organic acids and nitrogenous compounds. These metabolic by-products influence the culture's physicochemical environment and may affect late-stage infective juvenile (IJ) survival. Such late-stage declines are common in closed culture systems due to food depletion and waste accumulation [8].

Table 6. Mean population and physicochemical changes for the 4th week.

Parameter	(A) Mung Bean	(B) Fish Meal	(C) Dog Food	ANOVA F (df)	p-value	Tukey
Nematode count	7.20 ± 14.48a	69.80 ± 60.34ab	287.00 ± 238.00b	5.347 (2,12)	0.022	Dog food > Mung bean; Fish meal: ns
Color	3.00 ± 0.00	5.00 ± 0.00	2.00 ± 0.00	—	—	Descriptive only
Aroma	1.00 ± 0.00	1.60 ± 0.55	2.60 ± 0.55	16.333 (2,12)	<0.001	Dog food > Fish meal > Mung bean
Shape	1.00 ± 0.00	2.00 ± 0.00	2.00 ± 0.00	—	—	Descriptive only
pH	6.85	6.94	5.91	—	—	Descriptive only

Note: Parameters in which one or more treatments exhibited zero standard deviation were not subjected to inferential statistical analysis (ANOVA) and are reported descriptively. pH values represent single measurements per treatment.

Mean population of entomopathogenic nematodes (EPNs) during mass cultivation across four weeks (Table 7) showed no significant differences among treatments in Week 1 ($F = 1.421$, $p = 0.279$), indicating comparable early establishment across mung bean ($6,071.60 \pm 1,586.16$ IJs/mL), fish meal ($6,911.80 \pm 1,236.50$ IJs/mL), and dog food ($7,546.20 \pm 1,315.04$ IJs/mL). From Week 2 onward, significant differences emerged ($F = 20.004$, $p < 0.001$), where fish meal ($9,221.60 \pm 1,170.95$ IJs/mL) and dog food ($8,711.60 \pm 1,303.25$ IJs/mL) were significantly higher than mung bean ($4,231.00 \pm 1,607.65$ IJs/mL), indicating superior early infective juvenile (IJ) proliferation in animal-based media. By Week 3, divergence became more pronounced ($F = 34.916$, $p < 0.001$), with dog food ($4,682.20 \pm 987.64$ IJs/mL) significantly outperforming fish meal ($1,715.00 \pm 835.65$ IJs/mL) and mung bean (799.80 ± 309.63 IJs/mL), demonstrating a clear shift in sustained productivity favoring dog food. Week 4 still showed significant differences ($F = 5.347$, $p = 0.022$), where dog food (287.00 ± 238.00 IJs/mL) retained higher residual populations than fish meal (69.80 ± 60.34 IJs/mL) and mung bean (7.20 ± 14.48 IJs/mL), both of which declined sharply. Despite the low terminal counts, no zero-variance conditions were observed in any treatment group, indicating that variability remained estimable across all weeks. However, Week 4 values exhibited lower means with greater dispersion (notably in mung bean and dog food), reflecting increased heterogeneity at low population levels without affecting ANOVA validity. Overall, fish meal supported rapid early proliferation but showed reduced persistence compared to dog food, which retained higher residual populations across later weeks despite fluctuations. This aligns with previous findings that animal-derived, nutrient-rich media can enhance EPN production; however, culture stability is substrate-dependent and varies among different animal-based formulations [8,11].

Table 7. Weekly mean population of EPNs in various artificial media in four weeks.

Week	(A) Mung Bean	(B) Fish Meal	(C) Dog Food	ANOVA F (df)	p-value	Tukey
Week 0	1,258.00 ± 283.23a	1,378.00 ± 405.12a	1,544.00 ± 306.07a	0.915 (2,12)	0.427	Same subset
Week 1	6,071.60 ± 1,586.16a	6,911.80 ± 1,236.50a	7,546.20 ± 1,315.04a	1.421 (2,12)	0.279	Same subset
Week 2	4,231.00 ± 1,607.65a	9,221.60 ± 1,170.95b	8,711.60 ± 1,303.25b	20.004 (2,12)	<0.001	Fish meal = Dog food > Mung bean
Week 3	799.80 ± 309.63a	1,715.00 ± 835.65a	4,682.20 ± 987.64b	34.916 (2,12)	<0.001	Dog food > Fish meal = Mung bean
Week 4	7.20 ± 14.48a	69.80 ± 60.34ab	287.00 ± 238.00b	5.347 (2,12)	0.022	Dog food > Mung bean; Fish meal: ns

Note: Parameters in which one or more treatments exhibited zero standard deviation were not subjected to inferential statistical analysis (ANOVA) and are reported descriptively.

Despite slight differences in initial inoculum densities among treatments (Table 2), the weekly population changes of entomopathogenic nematodes (EPNs) did not consistently follow these initial variations (Table 8). In Week 1, all treatments exhibited positive population growth—mung bean ($4,813.60 \pm 1,320.81$ IJs/mL), fish meal ($5,533.80 \pm 2,679.36$ IJs/mL), and dog food ($6,002.20 \pm 1,019.55$ IJs/mL)—with no significant differences among media ($F = 1.531$, $p = 0.256$), indicating comparable early establishment across treatments. By Week 2, mung bean showed a population decline ($-1,840.60 \pm 736.94$ IJs/mL), while fish meal ($2,309.80 \pm 858.64$ IJs/mL) and dog food ($1,165.40 \pm 156.49$ IJs/mL) exhibited increases, with a near-significant treatment effect ($F = 3.805$, $p = 0.053$). Tukey indicated a significant difference between fish meal and dog food, while mung bean showed intermediate values and did not differ significantly from either treatment. In Week 3, all treatments experienced declines, with fish meal ($-7,506.60 \pm 461.74$ IJs/mL) showing a significantly greater reduction compared to mung bean ($-3,431.20 \pm 1,365.59$ IJs/mL) and dog food ($-4,029.40 \pm 763.57$ IJs/mL) ($F = 27.299$, $p < 0.001$), indicating a marked decrease in fish meal relative to the other substrates. Mung bean and dog food were statistically grouped in the Tukey analysis, suggesting similar mid-phase population retention despite differences in mean magnitudes. In Week 4, declines continued across treatments, with dog food ($-4,395.20 \pm 906.05$ IJs/mL) exhibiting a significantly greater decrease than fish meal ($-1,654.20 \pm 775.62$ IJs/mL) and mung bean ($-1,341.20 \pm 1,240.29$ IJs/mL) ($F = 14.399$, $p = 0.001$). Notably, this final week showed relatively consistent variability across treatments, with no zero-variance conditions, ensuring that statistical comparisons remained valid even under uniformly negative population changes. Overall, these results indicate that population dynamics varied markedly across weeks and treatments, suggesting that nutrient composition and media suitability exert a stronger influence on EPN population changes than initial inoculum density alone. It was also observed that although the mung bean generally had the lowest populations among all media, its population decline occurred progressively, indicating limited but gradually diminishing nutrient support.

Table 8. Weekly mean population change (+/-) of EPNs in various artificial media in four weeks.

Week	(A) Mung Bean	(B) Fish Meal	(C) Dog Food	ANOVA F (df)	p-value	Tukey
Week 1	+ 4,813.60 ± 1,320.81a	+ 5,533.80 ± 2,679.36a	+ 6,002.20 ± 1,019.55a	1.531 (2,12)	0.256	Same subset
Week 2	- 1,840.60 ± 736.94ab	+ 2,309.80 ± 858.64b	+ 1,165.40 ± 156.49a	3.805 (2,12)	0.053	Fish meal ≠ Dog food
Week 3	- 3,431.20 ± 1,365.59a	- 7,506.60 ± 461.74b	- 4,029.40 ± 763.57a	27.299 (2,12)	<0.001	Mung bean = Dog food > Fish meal
Week 4	- 1,341.20 ± 1,240.29a	- 1,654.20 ± 775.62a	- 4,395.20 ± 906.05b	14.399 (2,12)	0.001	Dog food < Fish meal = mung bean

Note: Parameters in which one or more treatments exhibited zero standard deviation were not subjected to inferential statistical analysis (ANOVA) and are reported descriptively.

Parallel physicochemical assessment (Table 9) showed identical baseline properties at Week 0 (color = 1.00 ± 0.00 , aroma = 5.00 ± 0.00 , shape = 5.00 ± 0.00 ; pH = 6.38–6.60), with zero variance across treatments; thus, results were interpreted descriptively. By Week 1, divergence began to emerge. Color differed among treatments, with fish meal (3.20 ± 1.10) showing higher values than mung bean and dog food (both 1.00 ± 0.00). However, because mung bean and dog food exhibited zero variance, these results were interpreted descriptively. Shape also differed, with fish meal and dog food (both 5.00 ± 0.00) showing higher structural integrity than mung bean (3.80 ± 0.45), although zero variance limited inferential robustness, thus, descriptively presented. Aroma remained statistically similar across treatments ($F(2,12) = 0.167$, $p = 0.848$), indicating minimal differentiation in odor-related changes during early culture stages. By Week 2, divergence intensified. Color values exhibited zero variance across treatments and were therefore interpreted descriptively, with fish meal (4.00 ± 0.00) showing the most advanced discoloration, followed by mung bean (1.00 ± 0.00) and dog food (1.00 ± 0.00). Shape differed descriptively, with fish meal and dog food (both 5.00 ± 0.00) maintaining structural integrity compared to mung bean (2.60 ± 0.55); however, zero variance in the former treatments again limited inferential strength. Aroma remained non-significant ($F(2,12) = 0.222$, $p = 0.804$), suggesting that odor-related changes were not yet strongly differentiated despite increasing structural divergence. By Week 3, a clear separation among treatments was observed. Color differed, with fish meal (5.00 ± 0.00) showing the highest values, followed by mung bean (1.40 ± 0.55) and dog food (1.00 ± 0.00). However, zero variance in fish meal limited strict inferential interpretation and was therefore presented descriptively. Aroma also differed significantly ($F(2,12) = 10.750$, $p = 0.002$), with mung bean (1.40 ± 0.55) showing greater deterioration than fish meal (2.60 ± 0.55) and dog food (2.80 ± 0.45). Shape values exhibited zero variance across all treatments (mung bean = 1.00 ± 0.00 ; fish meal and dog food = 3.00 ± 0.00), and were therefore interpreted descriptively, indicating structural collapse in mung bean and partial integrity in the animal-based fish meal and dog food media. In Week 4, color and shape again exhibited zero variance in one or more treatments and were interpreted descriptively. Fish meal maintained the highest color score (5.00 ± 0.00), while mung bean (1.00 ± 0.00) showed complete structural collapse compared to fish meal and dog food (both 2.00 ± 0.00). Aroma showed significant differentiation ($F(2,12) = 16.333$, $p < 0.001$), with dog food (2.60 ± 0.55) highest, followed by fish meal (1.60 ± 0.55) and mung bean (1.00 ± 0.00), indicating slower degradation in the dog food medium. pH also differed significantly at Week 4 ($F(2,12) = 4.421$, $p = 0.036$), with dog food exhibiting a lower pH (5.91) than mung bean (6.85) and fish meal (6.94), suggesting greater accumulation of acidic metabolic by-products. However, since pH was measured only at the beginning and end of the experiment, these results reflect overall shifts rather than continuous trends. Overall, physicochemical parameters showed increasing divergence over time, with fish meal and dog food developing more distinct biochemical and structural profiles compared to mung bean. These changes paralleled differences in nematode population dynamics, suggesting that substrate-dependent microbial and degradation processes may contribute to cultivation outcomes, although direct causality was not experimentally established.

Table 9. Changes in Color, Aroma, Shape, and pH in four weeks.

Week	Parameter	(A) Mung Bean	(B) Fish Meal	(C) Dog Food	ANOVA F (df)	p-value	Tukey
Week 0	Color	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00	—	—	Descriptive only
	Aroma	5.00 ± 0.00	5.00 ± 0.00	5.00 ± 0.00	—	—	Descriptive only
	Shape	5.00 ± 0.00	5.00 ± 0.00	5.00 ± 0.00	—	—	Descriptive only
	pH	6.60	6.49	6.38	—	—	Descriptive only
Week 1	Color	1.00 ± 0.00	3.20 ± 1.10	1.00 ± 0.00	—	—	Descriptive only
	Aroma	4.20 ± 0.84	4.20 ± 0.45	4.40 ± 0.55	0.167 (2,12)	0.848	ns
	Shape	3.80 ± 0.45	5.00 ± 0.00	5.00 ± 0.00	—	—	Descriptive only
Week 2	Color	1.00 ± 0.00	4.00 ± 0.00	1.00 ± 0.00	—	—	Descriptive only
	Aroma	3.40 ± 0.55	3.60 ± 0.55	3.60 ± 0.55	0.222 (2,12)	0.804	ns
	Shape	2.60 ± 0.55	5.00 ± 0.00	5.00 ± 0.00	—	—	Descriptive only
	Color	1.40 ± 0.55	5.00 ± 0.00	1.00 ± 0.00	—	—	Descriptive only
Week 3	Aroma	1.40 ± 0.55	2.60 ± 0.55	2.80 ± 0.45	10.750 (2,12)	0.002	Mung bean < Fish meal = Dog food
	Shape	1.00 ± 0.00	3.00 ± 0.00	3.00 ± 0.00	—	—	Descriptive only
	Color	3.00 ± 0.00	5.00 ± 0.00	2.00 ± 0.00	—	—	Descriptive only
Week 4	Aroma	1.00 ± 0.00	1.60 ± 0.55	2.60 ± 0.55	16.333 (2,12)	<0.001	Dog food > Fish meal > Mung bean
	Shape	1.00 ± 0.00	2.00 ± 0.00	2.00 ± 0.00	—	—	Descriptive only
	pH	6.85	6.94	5.91	—	—	Descriptive only

Note: Parameters in which one or more treatments exhibited zero standard deviation were not subjected to inferential statistical analysis (ANOVA) and are reported descriptively. pH values represent single measurements per treatment.

In terms of cost-effectiveness, the media used in this study (mung bean, fish meal, and dog food) were far more economical than other media, such as chicken liver and soybean powder, used in other studies. Each media required a fixed inoculum cost — wax moth larvae at ₱2–₱3 each, plus ₱50–₱100 for maintaining starter cultures, which was uniform across all substrates. This means the real differentiator lies in ingredient prices and biological sustainability. Mung bean (₱95–₱160/kg), fish meal (₱116–₱234/kg), and dog food (₱80–₱220/kg) were all relatively inexpensive and locally available. In contrast, chicken liver (₱184–₱406/kg) and soybean powder (₱311–₱824/kg) add significantly higher ingredient costs on top of the same inoculum acquisition, while being limited by perishability or import dependence. When comparing dog food and soybean powder, the top-performing media in this study and in Indriyanti and Muharromah's [8] study, respectively, an estimated cost reduction of approximately 60–75% is observed, depending on price variation.

4. Conclusions

Mass cultivation of *Heterorhabditis* spp. in locally available semi-solid media produced varying population densities across weeks, associated with changes in physicochemical and sensory properties (color, aroma, shape, and pH), likely reflecting underlying microbial and biochemical activity. However, these parameters were not directly tested as causal factors. All treatments showed an initial population increase in Week 1. Media A (egg yolk + mung bean) declined from Week 2 onward, while Media B (egg yolk + fish meal) and Media C (egg yolk + dog food) continued increasing initially. By Weeks 3–4, all media exhibited reduced populations. Media C demonstrated the most stable performance, reaching a peak of $8,711.60 \pm 1,303.25$ IJs/mL in Week 2 and maintaining relatively higher densities through Week 4. Media B achieved the highest peak population ($9,221.60 \pm 1,170.95$ IJs/mL in Week 2) but showed a sharp decline in Week 3, indicating lower sustainability despite superior short-term yield. Media consistently performed the least favorably. Based on

these trends, the optimal harvest for Media B and C is Week 2, when populations are at their maximum. Overall, Media C (egg yolk + dog food) provides the best balance of yield stability and economic feasibility. This aligns with Indriyanti and Muharromah [8], who identified egg yolk + dog food as a strong EPN substrate alongside soybean powder and chicken liver. Considering cost and availability, dog food is more economical and scalable in the Philippine context, consistent with Mathew et al. [18].

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Architecture Design for Enhancing Direct Client-to-Microservice Communication in an E-Participation Platform for Local Administrative Organizations in Suratthani Province, Thailand

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Abstract: Public participation is essential for governance in a democratic system. Local Administrative Organizations in Thailand typically provide websites and social media platforms such as Facebook Pages and the Line application for e-Participation. However, there is a lack of specific applications designed for e-Participation that ensure secure, efficient communication and support rapid information exchange. This research aimed to design and evaluate a direct client-to-microservice communication architecture for an e-Participation platform in Local Administrative Organizations in Surat Thani Province, Thailand. We adopted a microservice architecture based on Domain-Driven Design (DDD), along with an API-first design and a database-per-service pattern. A message broker was adopted for asynchronous inter-service communication. We implemented JSON Web Tokens (JWT) with AES-256 encryption for system security and used Apache JMeter for performance testing. The performance testing followed ISO/IEC 25010 standards under varying loads to assess throughput, response time, and resource utilization. The performance testing showed that the system throughput could handle 16.67 requests per second at 5,000 concurrent users, with zero error rate. The time behavior testing demonstrated that the system maintained an average response time of 41-46 milliseconds, well below 1,500 ms. The resource utilization test showed that the CPU peak value fell within the expected range, indicating that the system is highly stable, unaffected by CPU spikes, and efficient in memory use. Overall, the system successfully passed all performance criteria. This architecture enabled real-world usage of scalable, secure, and efficient e-Participation platforms for Local Administrative Organizations.

Keywords: Microservice architecture; direct client-to-microservice; e-Participation; local administrative organizations

1. Introduction

Citizens' electronic participation in the operations of Local Administrative Organizations in Thailand is crucial because these organizations not only implement various local development projects but also serve as the government entities closest to local communities. Local Administrative Organizations in Thailand normally provide websites, Facebook pages, and Line as electronic channels for e-participation. The United Nations (UN) has defined three levels

of electronic participation [1] (1) e-Information, which involves providing valuable information to the public through electronic channel; (2) e-Consultation which involves gathering suggestions and opinions on important issues, and (3) e-Decision making, which involves engaging citizens in the decision-making process to collaboratively create policies that truly reflect the needs and aspirations of the community. Local citizens engage with the websites, Facebook pages, and Line platforms of Local Administrative Organizations at a moderate level, primarily to disseminate information rather than foster meaningful citizen engagement. There is still a lack of specific applications to promote electronic participation that foster meaningful public engagement. Additionally, the growing use of public mobile applications to access services provided by Local Administrative Organizations has become an important trend, leading to increased interaction, monitoring, and follow-up of requested services. Therefore, the application delivered must be high-performing and responsive, ensuring quick access to vital information and services. Applications designed to boost public participation must prioritize performance and security, scalability, coupling, and the capacity to manage high user demand effectively [2]. Microservice architecture (MSA) is a modern software approach that divides functionality into modular, independently deployable services aligned with business domains, and it is widely used in system design today. The system is divided into smaller, independent components, each operating autonomously and focusing on its own functionality and internal communication [3]. MSA is suitable for building an efficient communication platform between citizens and government agencies [4]. Microservice architecture can be used to implement the public participation platforms proposed in this research. This architecture ensures scalability to accommodate high user volumes across multiple local government agencies while enabling seamless version updates and operational modifications without disrupting core data systems.

This research aimed to design a direct client-to-microservice communication architecture for an e-Participation platform tailored to Local Administrative Organizations and to evaluate the performance of data communication within the system. We adopted a direct communication design between clients and microservices to ensure fast, efficient data exchange. To address security concerns, the system was designed to use JWT authentication with AES-256 encryption. Our research contributes to software architecture design by creating a system that delivers both high performance and secure data use. It also enables the public to effectively participate in the operations of local administrative organizations through electronic means. The architectural design of the e-Participation platform, based on a microservice paradigm that prioritizes data integrity and operational efficiency, encompasses several fundamental components, such as component decomposition of services into smaller, autonomous units, which is a pivotal aspect of the design, along with essential elements such as communication protocols, database administration, security measures, and implementation methodologies. We conducted a comprehensive review of the existing literature as follows.

1) E-Participation: Electronic Participation (e-Participation) refers to the use of Information and Communication Technology (ICT) by citizens to engage with government agencies [5]. It is one aspect of e-Government [6] that encompasses information provision, public consultation, and decision-making via digital platforms [7]. Some studies employ microservice architecture in the design of e-Participation platforms, with the scope of each platform and the corresponding architectural approaches summarized in Table 1.

2) Microservice architecture (MSA) is a software architecture design that emphasizes breaking down complex systems into small modules, independently deployable services [10]. It was introduced in 2014 to address the challenges of legacy systems, including increasing complexity, high dependency, and tight coupling. It directly addresses the limitations of monolithic applications by offering a service-oriented architectural design that decomposes system functionality into independent microservices that communicate via lightweight mechanisms such as RESTful APIs [11]. There are several key benefits of microservice architecture, including improved agility, modifiability, scalability, deployability, and fault tolerance, as well as easier system maintenance [2, 12, 14].

Table 1. Scope of e-Participation and corresponding microservice architectural approaches

Scope of e-Participation	The aim of adopting a microservice architecture
The civic engagement platform development in collaboration with DPRD Jawa Barat in Indonesia for reporting public issues, fostering discussions, and coordinating governmental responses. Integrating community feedback mechanisms aims to enhance participatory governance by promoting more structured collaboration between citizens and their government [4].	Microservices architecture was employed to improve backend scalability and maintainability. API testing ensures seamless microservice functionality by validating interface reliability and consistency through stress testing to assess scalability and performance, and by identifying optimization opportunities to enhance the system's responsiveness under fluctuating workloads.
The development of modernizing regulations for Philippine e-government [8].	The system achieves scalability and security while handling high transaction volumes and regional variations through a microservices architecture. It also proves particularly effective, delivering measurable improvements in service delivery and client satisfaction. The comparative study showed that the system enhanced security, transparency, and operational efficiency.
Implementing Interoperability for e-Government Services [9].	API-driven microservices architecture provides an interoperable framework for efficient data sharing across e-government platforms.

3) Domain-Driven Design (DDD) is a model-driven approach in software development that focuses on the use of abstract models as the primary artifacts throughout the software development lifecycle [23]. DDD is a popular methodology for guiding architectural decisions, especially in microservice architecture design. In the DDD approach, the appropriate size of a microservice can range from one per entity to one per bounded context. The application of DDD is a critical success factor in developing microservice-based applications [24], as it enables services to be easily replaceable, encapsulates implementation details, and ensures their use is limited to well-defined smart endpoints [14].

4) Application Programming Interfaces (APIs) are essential for communication in microservice architecture because the system is broken down into small services, each with its own distinct functions. There are two types of communication in microservice architecture design: communication among internal system services and communication between the system and client applications. Effective communication design supports system reliability by ensuring consistency and enabling functions to operate without failure, while also promoting scalability to handle the growing workload from users and transactions and facilitating information exchange. Communication is achieved through Application Programming Interfaces, and the Representational State Transfer (REST) API is one of the most widely adopted mechanisms, enabling convenient interaction between internal system microservices and external applications [25]. There are two techniques to utilize APIs during the design of a communication system. The first approach begins with code writing and implementation, together with an API designed and documented based on the existing code. On the other hand, the API-first design approach emphasizes designing and defining APIs before writing code. It requires creating detailed API specifications using OpenAPI or Swagger. The API-first approach enables clear communication between services and external client applications and promotes easier collaboration among development teams [26-27].

5) Direct client-to-microservice architecture, in this approach, client web applications and mobile applications interact directly with individual microservices via their exposed API endpoints, bypassing the API gateway. A direct client-to-microservice architecture requires clients to handle service discovery, authentication, and communication; therefore, clear, well-defined API contracts are essential [24, 28]. The comparison of the advantages and disadvantages between the direct client-to-microservice and API Gateway pattern is shown in Table 3.

Table 2. Comparison of architectural approaches in software development

Architectural approaches	Monolithic	Centralized REST	Microservice
Characteristics	It is a traditional software design in which the user interface, business logic, and data access are tightly integrated. This approach offers simplicity and straightforward deployment.	It is a client-server architecture that uses Representational State Transfer (REST) to centralize data management and resource logic on the server, while delegating user interface and state management to the client. Therefore, this design can efficiently handle web-based interactions, providing the flexibility and ease of integration required for modern and cross-platform environments.	Breaks down complex systems into small services. Therefore, an application consists of many loosely coupled, independently deployable services.
Scalability	It is typically less scalable due to its tightly integrated nature. Scaling often involves vertical scaling, which can be cost-prohibitive and less efficient as the application grows [15-16].	It offers more straightforward horizontal scaling options, which can be beneficial for handling increased load [15], and can improve scalability compared to monoliths by decoupling some components. However, it may not achieve the same level of scalability as microservices due to potential bottlenecks in the centralized components [17].	It excels at scalability, enabling horizontal scaling in which individual services can be scaled independently based on demand. This architecture supports high-volume processing and is well-suited to large, dynamic systems. [18-20].
Performance	It provides fast response times under low load and with few concurrent users [16], and it is beneficial for applications that do not require frequent updates or high scalability [20].	The resource utilization of this architecture depends on the framework used (e.g., Express.js vs. Laravel)	It is excellent in scalability and fault isolation by allowing independent scaling of services and better handling of high-volume transactions, faster response times, and lower error rates under high-load conditions [21-22]

Table 2. Comparison of architectural approaches in software development (continue)

Architectural approaches	Monolithic	Centralized REST	Microservice
Maintainability	It is relatively easy to maintain; however, as the system grows, it may become increasingly complex, rendering updates and bug fixes more difficult and time-consuming [18-19].	It offers moderate maintainability improvements over monoliths, but still requires careful management of dependencies and interfaces to avoid complexity [17].	It enhances maintainability by allowing independent updates and deployments of services. This modularity supports parallel development and reduces the risk of system-wide failures [15, 20].
Deployment Complexity	It involves the straightforward deployment of the entire application in a single unit. However, this simplicity can lead to longer deployment times and increased downtime during updates [17-18].	Deployment complexity is generally lower than in a microservice architecture.	Deployment is complex and requires sophisticated orchestration frameworks. Each service must be deployed independently [15, 20].

Table 3. The comparison of the advantages and disadvantages between the direct client-to-microservice and API Gateway pattern

	API gateway pattern	Direct the client to the microservice pattern.
Advantages	It uses an API Gateway as a centralized entry point for client requests, thereby simplifying service-to-service communication and improving security enforcement across microservices architectures [29]. It improves scalability by distributing traffic efficiently and supporting load balancing [30]. With centralized service management, security can be improved through authentication and enhanced load balancing. The client can interact more simply through a single entry point for multiple services, thus improving development efficiency [31].	This approach is a simple architecture that is easy to design and implement. It is also enabled for fine-grained interactions, easier debugging, and monitoring, and suits small microservice-based applications. The direct communication between clients and microservices eliminates the need for intermediary layers, potentially reducing latency [31] and improving performance, as clients can quickly access the specific service they need [33]. It improved scalability and enhanced fault isolation. Clients communicate directly with specific microservices, enabling faster response times and reducing latency. Additionally, it facilitates technological flexibility, as different microservices can be developed and deployed independently, enabling quicker updates and innovations [34].
Disadvantages	API Gateways can become a single point of failure if not properly managed [33]. Because of the centralized entry point, it can become a performance bottleneck, especially under high load conditions. Managing an API Gateway involves configuring routing, security policies, and rate limiting, which can become complex and error-prone, especially in large systems with numerous services. This can lead to increased latency, as requests must pass through the gateway before reaching the intended service [32].	The weakness of this approach is increased client-side complexity and an expanded security attack surface. Direct client access to microservices can expose them to security vulnerabilities, as each service must handle authentication, authorization, and other security measures independently. It can lead to increased operational overhead and potential service outages if not managed properly. Direct communication requires clients to handle API versioning and compatibility, which can lead to consumer lock-in and make it difficult to evolve APIs without breaking existing clients [35-36].

6) JSON Web Tokens (JWT) are an open standard (RFC 7519) for safely passing claims in space-constrained environments, providing a compact and self-contained way to securely transmit information between parties as a JSON object [37]. The usefulness of JWT includes authentication and authorization, federated identity, client-side sessions (particularly for stateless sessions), and client-side secrets. JWTs are widely used in security mechanisms for authentication in microservice architectures [38] because they provide a stateless approach that enables efficient, scalable, and secure verification of user identities across distributed services.

7) Advanced Encryption Standard (AES-256) is a specification for encrypting electronic data using a symmetric block cipher. It uses the same key for both encryption and decryption, meaning the sender and receiver share the same secret encryption key. AES-128 bit provides generally adequate security and protection, while AES-256 provides stronger security and supports high performance and efficiency [39].

2. Methodology

We adopted the Design Science Research (DSR) methodology, emphasizing problem identification, definition of solution objective, artifact design and development, demonstration of functionality, artifact evaluation, and communication of results and insights throughout the entire e-Participation Platform development project. In this research paper, we focused on the architectural design stage of the e-Participation platform, as there was a lack of dedicated applications for electronic participation in the Local Administrative Organization that required high performance and fast access to information and services from the organization. The overview diagram of this research methodology is illustrated in Figure 1.

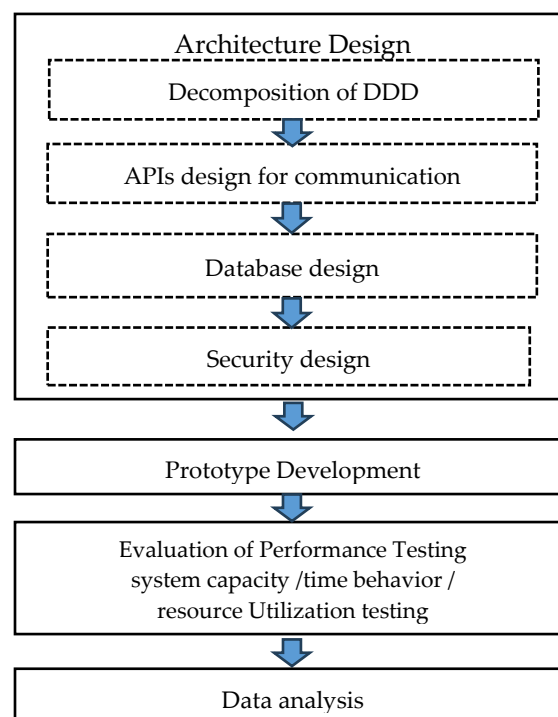


Figure 1. The process of research methodology

To develop the research methodology for enhancing direct client-to-microservice architecture in an E-Participation Platform. We defined the research question (RQ1) as: **Does direct client-to-microservice architecture design meet predefined performance success criteria?** This question aims to evaluate the performance of the proposed system architectures.

2.1 Services Decomposition

In microservice architecture, service decomposition should be based on Domain-Driven Design (DDD) principles. In this research, we focused on the business domain of electronic participation from the public to the local administrative organization. A combination of Domain-Driven Design and business capability analysis is one of the most widely used strategies for decomposing an application into microservices, as DDD helps determine how many microservices a system should comprise. Each service is responsible for a specific task and operates independently. In this research, the strategic design phase of Domain-Driven Design was employed to break down the major tasks of an electronic participation system. This was achieved through data collection, interviews, and consultations with relevant stakeholders and domain experts, including the chief and Local Administrative Organization officers responsible for public information services, as well as local citizens. This process helped identify the system's functional requirements. It revealed that the development of an electronic participation platform, aligned with its key levels of e-information, e-consultation, and e-decision-making, involved three primary contexts: 1) user context, 2) news management context, and 3) main service context, with one supporting context being 4) system notification management context. In the strategic phase of DDD, we design the context map based on the relationships among the contexts. The relationship between the user context and the news management context is a partnership that enables Local Administrative officials to create and publish news for citizens through the application. At the same time, citizens can also submit news reports via the application. The user context and the main service context work together, allowing citizens to access various local government services, including submitting complaints and petitions and using e-services. Similarly, the main service context maintains a partnership with the news management context, as it both creates and consumes news content while providing various services. The notification context is related to the user context, news management context, and main service context as a conformist (CF). It served as an upstream (U) context for sending SMS alerts and status updates for various events. In contrast, the other contexts act as downstream (D) consumers, expected to conform to their outputs. These relationships are illustrated in Figure 2

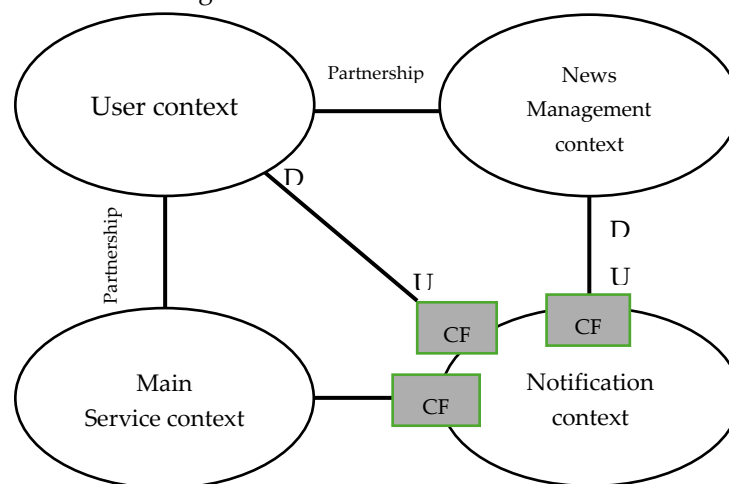


Figure 2. Context mapping of e-Participation Platform

After context mapping, we translated the identified contexts into corresponding services within the microservice architecture. Each sub-task within a context becomes an operational module within a microservice. Interactions between components in related contexts constituted communication between microservices.

2.2 Communication design

In microservice architecture, communication between services and external client applications is essential and depends on the nature and requirements of the task. The e-Participation platform in this study is not highly complex; however, it requires fast and efficient communication between users and the application. Local Administrative Organizations aim to deliver information and services to the public promptly while enabling citizens to access various public services through digital platforms. To achieve simplicity and high performance in external communication, we adopted the Direct Client-to-Microservice communication pattern.

According to the context mapping defined in the DDD design, the user service, news service, and main service were designated as public services, allowing direct access by external users. In contrast, the notification service was implemented as a private service dedicated to internal communication and coordination among microservices, and was therefore not accessible to external users. We adopted an API-first approach at the initial stage of API design to facilitate communication among microservices. The functional roles of each service were defined before developing other application components. A RESTful API was utilized because REST employs the HTTP request–response mechanism. RESTful APIs are widely used and well-suited for both web and mobile communication. They emphasize loose coupling, which is a core principle of microservice architecture. RESTful APIs enable fast and efficient client–server communication by exchanging data in JSON format. The proposed communication design method is described in more detail below:

1) Defined the functional requirements of four service offerings.

1.1) The user service managed all users within the system, including local citizens and local officials from thirty-one Local Administrative Organizations in Suratthani Province.

1.2) The news management service is responsible for creating and publishing news articles and official announcements from Local Administrative Organizations for citizens. On the other hand, it also allowed citizens to create news content and submit it to the system. This service included submodules for news content management, with associated images, and news report summarization.

1.3) The main service was responsible for managing various requests submitted by citizens to Local Administrative Organizations. It includes e-services, complaint submissions, grievance notifications, and status updates at each stage of the process.

1.4) The Notification Service was responsible for sending short message service (SMS) notifications, operational updates, and real-time news alerts.

The three core services (user, news management, and main service) were designed with a focus on CRUD (Create, Read, Update, and Delete) operations.

2) In this research, we created an API specification by defining API endpoints and HTTP methods using Swagger UI specification language. There were two types of API implementations: public APIs, which were directly accessed by client applications, and backend APIs, which facilitated communication among internal backend services. We carefully defined RESTful APIs to facilitate communication between services and support end-to-end use cases. Tables 4–7 present the communication design of the microservice architecture using RESTful APIs for each of the four microservices.

Table 4. Design of microservice architecture with RESTful APIs for the user microservice

APIs Endpoints	HTTP Method	Action
/user/users/{userId}	GET	Get the user by ID
	DELETE	Delete user by ID
/user/users/requestOTP	GET	Get OTP for initial authentication.
/user/users/sumUserPerMonth	GET	Get the summary user per month.
/user/users	GET	Retrieve all users
	POST	Create user
	PATCH	Update user
/user/lgo	GET	Retrieve all Local Administrative Organizations
/user/lgo/{id}	GET	Get Local Administrative Organization by ID
	DELETE	Delete Local Administrative Organization by ID
/user/users/register/user	POST	Register user
/user/users/register/verifyOTP	POST	Verify OTP
/user/users/login/user	POST	User logs in to the system
/user/users/upload/profile	POST	User upload profile
/user/lgo	POST	Create Local Administrative Organization
	PATCH	Update Local Administrative Organization
/user/users/user/update/approve	PATCH	Update approved user
/user/users/user/update/status	PATCH	Update user status

Table 5. Design of microservice architecture with RESTful APIs for news management microservice

APIs Endpoints	HTTP Method	Action
/news/news/gets/{type}	GET	Get news by type
/news/news/gets/{uuidNews}	GET	Get news
/news/news/summary/getAll	GET	Retrieve news summary
/news/news/{uuidNews}/like	GET	Get Like with news
/news/news/{uuidNews}/comment	GET	GET comment in the news
/news/files	GET	Retrieve news image file
/news/news	POST	Create news
/news/news/{uuidNews}/approve	POST	Approve news from citizens.
/news/news/{uuidNews}/ like	POST	Create a Like for news
/news/news/{uuidNews}/comment/ {commentId}/like	POST	Create a Like for a news comment.
/news/news/{uuidNews}/	PUT	Edit news
/news/news/{uuidNews}/	DELETE	Delete news
/news/news/{uuidNews}/comment/ {commentId}	DELETE	Delete news comment

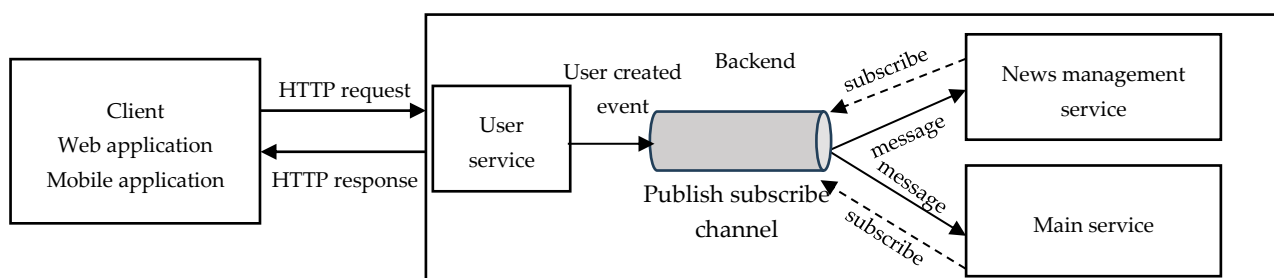
Table 6. Design of Microservice Architecture with RESTful APIs for the Main Service Microservice

APIs Endpoints	HTTP Method	Action
/main/e-service/summary/getAll	GET	Retrieve e-service summary
/main/e-service/findBy/{id}	GET	Get e-service by ID
/main/e-service/byNo/{eServiceNo}	GET	Get e-service by Number
/main/e-service/type/gets	GET	Retrieve e-service by type
/main/e-service	POST	Create e-service
/main/e-service/event	POST	Create an e-service event
/main/e-service/pagination	POST	Crete pagination for e-services
/main/e-service/{id}	PATCH	Update e-service by ID
	DELETE	Delete the e-service by ID
/main/report-problem/summary/getAll	GET	Retrieve the summary of problems
/main/report-problem/{id}	GET	Get problems by ID
/main/report-problem/ reportProblemType/getAll	GET	Retrieve all problem types
/main/report-problem	POST	Create problem details
/main/report-problem/update-event	POST	Create problem status
/main/report-problem/pagination	POST	Crete paging of problems
/main/report- problem/update/{problemId}	PATCH	Update the problem by ID
/main/report-problem/{report ProblemId}	DELETE	Delete problem by ID
/main/report-problem/event/{eventId}	DELETE	Delete the event of the problem
/main/complain/{id}	GET	Get a complaint by ID
/mail/complain/summary/getAll	GET	Retrieve the complaint summary
/main/complain/type/get All	GET	Retrieve complaints by type
/main/complain/create	POST	Create complain
/main/complain/update_event	POST	Create a complaint event
/main/complain/pagination	POST	Create a page of complaints
/main/complain/{complainId}	DELETE	Delete the complaint by ID
/main/complain/event/{eventId}	DELETE	Delete the event of complaint

Table 7. Design of Microservice Architecture with RESTful APIs for Notification Microservice

APIs Endpoints	HTTP Method	Action
/one-signal	POST	Create notification
/one-signal/sendNotification	POST	Send a notification to the user
/one-signal/user/{id}/onesignal/{onesignalId}	DELETE	Delete notification

We utilized Redis as a message broker to decouple services in cross-service transactions. The system supports services that operate independently while enabling asynchronous communication among microservices, allowing each service to execute without waiting for others to complete. In our direct client-to-microservice architecture, we implemented an event bus within a message broker using a publish-subscribe (pub-sub) messaging pattern, which is particularly well-suited for handling cross-service transactions. Figure 3 illustrates an example of publish-subscribe communication, in which the User Service publishes a UserCreated event to a topic. The News Management Service and Main Service subscribe to this topic and respond to the event accordingly.

**Figure 3.** Publish-subscribe communication in microservices.

2.3 Security design

We were concerned about security in the microservices architecture due to the direct client-to-service communication design. Data transmission security is vital, especially during the user authentication process. We designed a robust data protection mechanism using JSON Web Token (JWT); however, standard JWT implementations do not provide sufficient protection for the token data, which may contain sensitive government information that requires high levels of security and reliable communication. We implemented the Advanced Encryption Standard (AES) with a 256-bit key in Cipher Block Chaining (CBC) mode, using PKCS#7 padding during token generation to ensure secure and complete transmission of information, as illustrated in Figure 4.

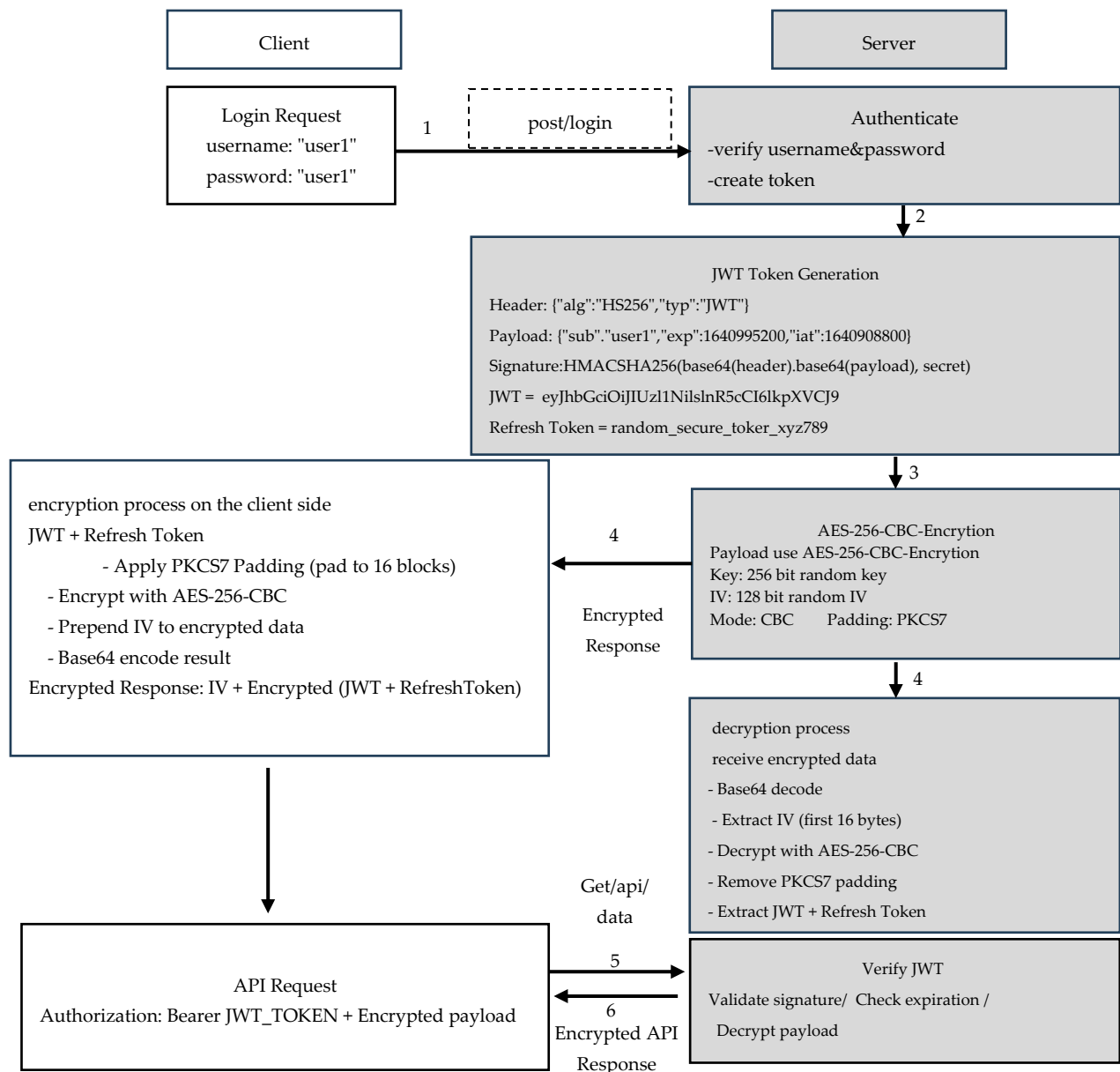


Figure 4. Authentication process of JWT with AES-256

The workflow for creating JWT tokens integrated with AES-256 encryption on the server is as follows:

- 1) The process begins when the client sends a login request, including username and password, to the server.
- 2) The server receives the request and verifies the username and password. If the credentials are correct, the server proceeds to generate the token as follows:
 - 2.1) Create the header containing:
 - alg: The encryption algorithm used
 - type: The token type, which is "JWT"
 - 2.2) Create the payload containing:
 - sub: user ID
 - exp: expiration date
 - iat: token issuance date
 - 2.3) Create the signature by:

3) Concatenating the header, payload, and server secret, then applying HMASHA256 encryption to generate the signature. The server then applies AES-256-CBC encryption as follows:

- key: 256-bit random key (32 bytes)
- IV: 128-bit random key (16 bytes)
- Mode: CBC (Cipher Block Chaining)
- Padding: PKCS7

4) This results in both encryption and decryption processes:

Encryption yields:

- Apply PKCS7 padding (pad to 16-byte blocks)
- Encrypt with AES-256-CBC
- Prepend IV to the encrypted data
- Base64 encode the result

Encrypted Response: IV + Encrypted (JWT + RefreshToken)

Decryption yields:

- Base64- decode
- Extract IV (first 16 bytes)
- Decrypt with AES-256-CBC
- Remove PKCS7 padding
- Extract JWT + Refresh Token

5) The API request data includes:

- Authorization: Bearer JWT_TOKEN + encrypted payload

6) The JWT verification process includes:

- Validate the signature
- Check expiration
- Decrypt the payload

Authorization is also essential in an e-Participation platform. We defined authorization based on user access rights within the system, including permissions that restrict access to API endpoints and roles, which are groups of permissions associated with users. We assigned permissions to roles rather than to individual users as follows.

1) The super administrator held the highest level of system privileges, enabling full control over user management across all levels.

2) Local administrative officers were responsible for managing citizens' approval, posting news, approving news, and approving requests for e-service usage.

3) Local administrative executives could view reports and participation statistics from the system.

4) Citizens in each local administrative area could register, post news, read news, access e-services, and submit complaints or problem reports.

2.4 Database design

Databases play a vital role in information systems, and database design is a critical aspect of microservice architecture. The database-per-service pattern was adopted in this architecture design. It means that each microservice manages its own database and may also exchange information with other services. This database design pattern supports loose coupling, a core principle of microservice architecture, as each microservice maintains its own independent database that can scale without affecting other services. The key advantage of the database-per-service pattern is its isolation: changes to the schema of one database do not affect the others. The one-microservice, one-database approach can eliminate single points of failure. When a problem occurs, it affects only one database. It also enhances system resilience, a key characteristic of microservice architecture. Based on the results of DDD and the API endpoint and HTTP method communication design from the previous stage, the system was structured accordingly. Four relational databases were created using MariaDB: user, news, main service, and notification. We adopted MariaDB as the DBMS because of its clear data modeling capabilities and support for well-defined schema from the early design phase. The structure and schema of relational databases support data consistency and accuracy by

enforcing relationships between tables and play a crucial role in managing essential data within each microservice. In this research, database design comprised four distinct databases, each responsible for managing essential data within its respective microservices.

1) The user database stores information related to system users, including Local Administrative Organizations, local administrative officers, and local citizens. The tables in the user database comprised user, sao (Local Administrative Organization), sao_organization_structure, user_token, user_socket, and user_password.

2) The main service database handled the functions of Local Administrative Organizations, including managing complaints, grievances, and e-service requests. Tables in the main service database comprised complain, complain_process, complain_status, report_problem, report_problem_type, report_problem_process, file, report_problem_status, e_service, e_service_type, and e_service_list.

3) The news database stored information about news and announcements from local authorities, as well as citizen-submitted news. Its main tables included: news, file, comments, and likes.

4) The notification database was responsible for handling messaging and alert functions. Tables in the notification database included sms_log, one_signal, and settings.

This modular database structure supported service independence, scalability, and efficient data management, aligning with microservice architectural principles.

2.5 Implementation and testing

The selected tools and techniques for implementing this architecture are as follows:

1) Angular Framework v.16 served as the front-end framework for building single-page, dynamic web applications for the e-Participation platform, enabling direct interaction with multiple microservices to deliver a highly interactive user experience. Ionic provided a robust and efficient way to build cross-platform mobile applications.

2) TypeScript and Node.js were used for backend development.

3) JSON Web Tokens were used for authentication and authorization, combined with AES-256 encryption.

4) Swagger was used for generating API documentation.

5) POSTMAN was used for API testing.

6) Apache JMeter was used for performance testing based on a predefined test plan. We measure system capacity through throughput, assess time behavior via average response time, and evaluate resource utilization.

7) Cloudflare was used as a content delivery network (CDN) for security and web content delivery.

8) Docker containers were used to provide containerization for supporting the four microservices and APIs in the prototype system.

3. Results and Discussion

The result of designing a direct client-to-microservice communication architecture for the e-Participation platform tailored to local government organizations is illustrated in Figure 5.

The system architecture diagram in Figure 5 illustrates the direct interaction between the client and the application, based on a microservice architecture. The client can directly access the microservice system via both mobile and web applications. This process was initiated when users logged in to request data retrieval or other service operations. The API layer was designed within each microservice to handle and transmit client requests. RESTful APIs enable communication between client applications and microservices. We utilized JSON Web Token (JWT) and AES-256 encryption within the authentication service layer. Both were employed to verify access rights and provide secure authentication and data protection. Upon successful authentication, the user's request was routed to the appropriate microservices: User Service, News Management Service, Main Service, and Notification Service. Each microservice maintained its own database. We utilized an in-memory cache, a high-speed storage mechanism that accessed data in the computer's main memory to accelerate data retrieval and improve application performance. Communication between microservices in the system was facilitated by a message broker that enabled asynchronous message delivery and supported efficient concurrent task handling.

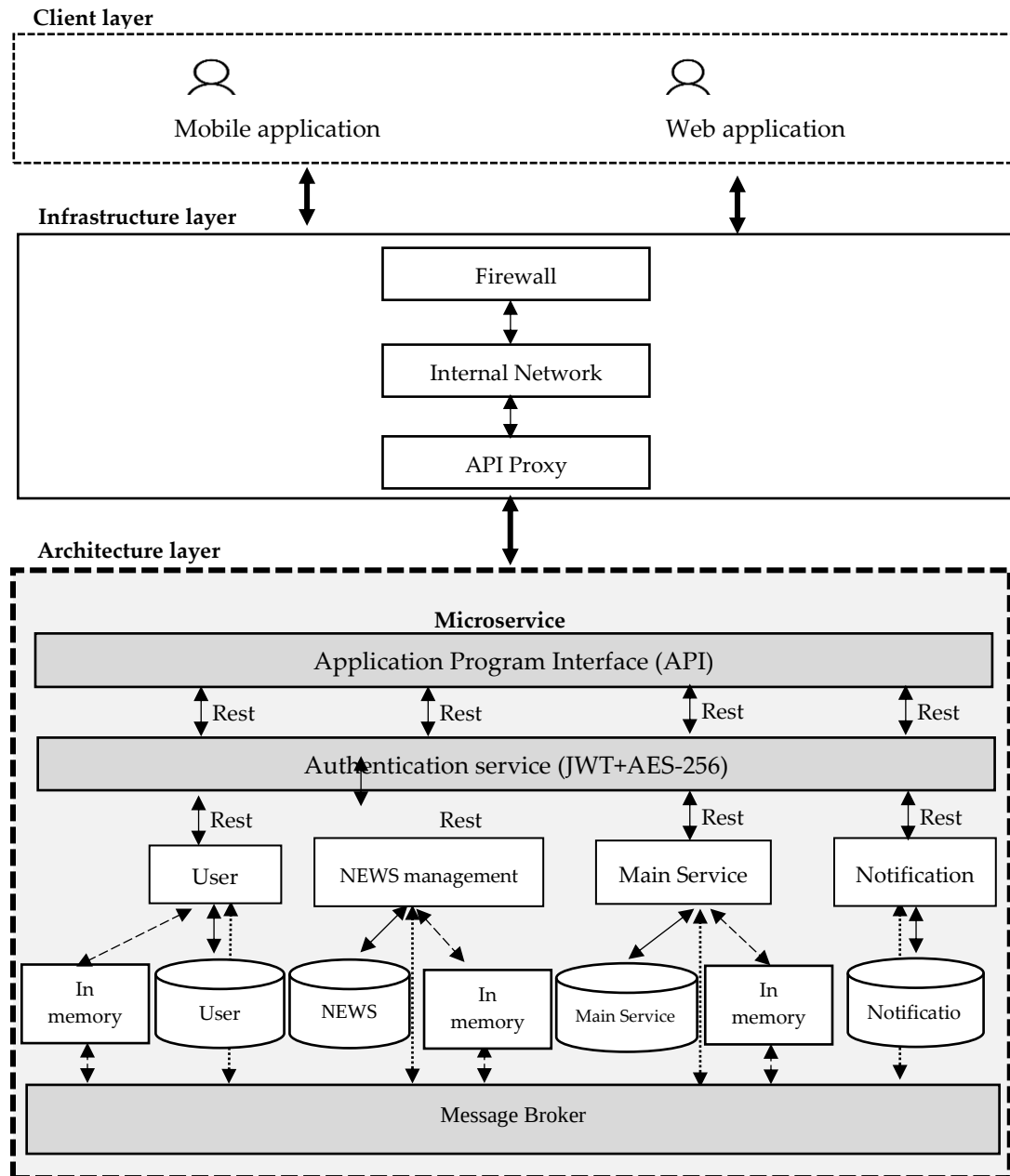


Figure 5 System architecture diagram of the e-Participation platform in Local Administrative Organizations

In this research, performance testing results were measured using Apache JMeter and aligned with the ISO/IEC 25010 standard, specifically the performance efficiency criterion, which refers to how efficiently the software operates. The workload modeling and experimentation were set up to assess the system's performance under both realistic and extreme conditions. We modeled the workload using the target population and standard capacity-planning heuristics. The estimation of Peak Concurrent Users ($CCU_{(peak)}$) [40][41] was formulated as follows:

$$CCU_{(peak)} = P \times AR \times DAR \times PHC \quad (1)$$

P (Population): The total target population of this study was 427,723 citizens from thirty-one Local Administrative Organizations in Suratthani Province [42].

AR (Active Ratio): The proportion of the population expected to register for and use the e-Participation platform, estimated at 20% (0.20). This value accounts for the "Digital Divide" and the varying levels of e-Government adoption across age groups [43].

DAR (Daily Active Ratio): The percentage of registered users who access the platform on a typical day, estimated at 6% (0.06), which is a standard benchmark for public service utility applications [40].

PHC (Peak Hour Concurrency): The fraction of daily active users (DAU) who are online simultaneously during the busiest hour, set to 10% (0.10) per industry-standard capacity planning rules for web traffic distribution [41].

Based on the mathematical model above, this research established four incremental testing scenarios to observe system behavior under different operational conditions:

1. Normal Load (Baseline): 500 CCU represented the system's typical day-to-day operating condition during non-peak periods with standard user engagement. This baseline was derived by rounding the estimated peak concurrent users (approximately 513) obtained from $CCU_{(peak)} = 427,723 \times 0.20 \times 0.06 \times 0.10$

2. Elevated Load: 1,000 CCU, doubling the baseline load, represented a moderate increase in traffic, such as during local government announcements or the release of new public information.

3. Peak Load (Event Day): 2,000 CCU was derived from the model by assuming that the Daily Active Ratio (DAR) increased to 24% during high-engagement events, yielding an estimated peak concurrency of approximately 2,053 CCU. This scenario represented the maximum expected traffic during critical government activities.

4. Stress Load (Beyond Limit): 5,000 CCU was selected to push the architecture beyond its anticipated maximum (2.5× the peak load). This level was used to identify bottlenecks, evaluate system resilience, and verify stability under extreme, unforeseen traffic spikes.

The performance testing results are categorized into three aspects: system capacity, time behavior, and resource utilization, as illustrated in Tables 8-11.

Table 8. Results of system capacity testing using the API HTTP method GET

Concurrent users (CCU)	Throughput (req/sec)	Error rate (%)	Duration	Expected throughput
500	11.18	0.00%	5 mins	≥ 1.5 req/sec, Error $< 0.5\%$
1000	13.38	0.00%	5 mins	≥ 3.0 req/sec, Error $< 0.5\%$
2000	14.74	0.00%	5 mins	≥ 6.0 req/sec, Error $< 1\%$
5000	16.67	0.00%	5 mins	≥ 15.0 req/sec, Error $< 1\%$

The results presented in Table 8 demonstrate the system's processing capability (Throughput) and absolute stability under varying concurrent user loads. A key finding was that the system maintained an error rate of 0.00% across all testing tiers. The throughput was recorded at 11.8 requests/sec for 500 concurrent users (CCU) and increased to 13.38 requests/sec at 1,000 CCU, 14.74 requests/sec at 2,000 CCU, and 16.67 requests/sec at 5,000 CCU. This increasing trend provides empirical evidence of the system's scalability. Furthermore, the performance aligned with the theory proposed in [44], which posits that system scaling need not be strictly linear to achieve robust growth. This increasing trend demonstrates the system's scalability, as evidenced by its accelerated growth and improved efficiency under concurrent load. Throughput remained within the expected range, and actual performance consistently exceeded expectations, particularly under high concurrent user loads.

$$\text{Expected_Throughput} = \text{Baseline_Throughput} \times (\text{Load_Ratio}^{0.7}) \times \text{Efficiency_Factor} \quad (2)$$

Baseline throughput refers to the actual throughput measured during the baseline test.

Load_Ratio is the ratio of the load increase compared to the baseline.

Efficiency_Factor is the value that indicates the reduction in efficiency as the load increases.

Table 9. Results of time behavior testing using the API HTTP method GET

Concurrent Users (CCU)	Ramp-Up (sec)	Avg. Response Time (ms)	95 th Percentile	Expected Response Time
500	45	41	52	< 300 ms
1000	75	46	55	< 525 ms
2000	135	41	48	< 900 ms
5000	315	44	53	< 1500 ms

The average response time results presented in Table 9 indicate that the system exhibited excellent performance, maintaining consistently low response times throughout the testing period. The average response time ranged from 41 to 46 milliseconds, which represents outstanding performance according to industry standards [45]. 95% of users received very fast responses from the system, with the 95th percentile response time ranging from 48 to 55 milliseconds, well below the expected response time. These test results demonstrate a highly efficient system architecture design.

$$\text{Expected Response Time} = \text{Base_Response_Time} + (\text{Load_Factor} \times \text{Degradation_Coefficient}) \quad (3)$$

Base_Response_Time is the baseline response time (for 100 users)

Load_Factor is the ratio of the load increase

Degradation_Coefficient is the coefficient of degradation (0.1–0.3)

Table 10. Results of Resource Utilization testing using the API HTTP method POST

Concurrent Users (CCU)	Ramp-Up (sec)	CPU % Usage (AVG/Peak)	Memory (MB)	Response Time (ms)	Expected CPU and Memory
500	45	12.1% / 17.0%	1373MB / 1381 MB	136	CPU < 15%, Memory < 1.8GB
1000	75	15.6% / 23.5%	1396 MB / 1398 MB	111	CPU < 20%, Memory < 2 GB
2000	135	20.2% / 26.1%	1395 MB / 1406 MB	107	CPU < 25%, Memory < 2.2GB
5000	315	24.6% / 31.0%	1400 MB / 1412 MB	104	CPU < 50%, Memory < 3GB

The resource utilization results presented in Table 10 are divided into two components: CPU performance and Memory performance. CPU utilization showed a logarithmic growth pattern. It started at 12.1% with 500 users. It then increased to 15.6% with 1,000 users, 20.2% with 2,000 users, and 24.6% with 5,000 users. CPU usage does not increase rapidly as the number of users grows, indicating that the system was well-designed and efficient in managing and allocating resources. Peak CPU utilization ranged from 17.0% to 31.0% and remained within the expected thresholds, indicating that the system was highly stable and unaffected by CPU spikes. Peak CPU utilization ranged from 17.0% to 31.0% and remained within the expected thresholds, indicating that the system was highly stable and unaffected by CPU spikes. The expected CPU threshold was determined using a data-driven approach to established performance standards, based on empirical baseline data and industry-standard scaling models [46].

$$\text{Expected CPU} = \text{Baseline_CPU} \times (1 + (\log(\text{Load_Ratio} + 1) / \log(2)) \times 0.4) + \text{Safety_Margin} \quad (4)$$

Baseline_CPU is the actual CPU utilization value measured from the baseline test.

Load_Ratio is the ratio of load increase compared to the baseline, calculated from

$$\text{Load_Ratio} = \text{Target_Users} / \text{Baseline_Users} \quad (5)$$

$\log(\text{Load_Ratio} + 1) / \log(2)$ is a logarithmic function that transforms the increase in load into a logarithmic growth pattern.

Coefficient 0.4 (Growth Coefficient) is a multiplier that controls the level of CPU increase according to the logarithmic pattern.

Safety_Margin is a compensation value that accounts for unexpected fluctuations.

Memory usage was highly efficient, increasing only slightly from 1,373 MB at 500 users to 1,400 MB at 5,000 users. It increased only 2% even though the number of users increased tenfold. This memory usage pattern indicates an efficient architectural design. Memory usage remained at only 45–75% of the expected memory value.

$$\text{Expected_Memory} = \text{Baseline_Memory} \times (\text{Load_Ratio}^{0.7}) + \text{Safety_Margin} \quad (6)$$

Baseline_Memory is the actual memory utilization value measured from the baseline test.

Load_Ratio is the ratio of load increase compared to the baseline.

$$\text{Load_Ratio} = \text{Target_Users} / \text{Baseline_Users} \quad (7)$$

The exponent 0.7 (Sublinear Scaling Exponent) represents the sublinear growth pattern of memory usage. Safety_Margin is the compensation value used to accommodate fluctuations in memory usage.

To evaluate system performance under real-world network latency conditions, we simulated three distinct network scenarios: high-speed (Fiber/5G), average (4G/LTE), and poor (3G/Unstable). The Characters Per Second (CPS) parameter in Apache JMeter was used to emulate bandwidth throttling and to assess its impact on system latency under a load of 2,000 concurrent users. The test results are summarized in Table 11.

Table 11. Comparative Performance Testing under Variable Network Conditions

Network Profile	Latency (ms)	Bandwidth	Avg. Response Time (ms)	Success Rate (%)
HighSpeed (Fiber/5G)	0 - 5	Unlimited	39	100.00%
Average (4G/LTE)	20 - 50	10 Mbps	44	100.00%
Poor (3G/Unstable)	100 - 300	1 Mbps	114	100.00%

The results of network latency simulation indicate that the average response time increased with higher latency and tighter bandwidth constraints, but overall performance remained robust. Specifically, the average response time increased from 39 ms under high-speed connectivity (Fiber/5G) to 44 ms under a moderate network profile (4G/LTE), and reached 114 ms under severely constrained conditions with only 1 Mbps bandwidth (3G/Unstable). Notably, the system maintained a 100.00% transaction success rate across all scenarios. These empirical findings suggest that the proposed architecture demonstrated high system resilience and an effective connection-pooling mechanism, enabling rapid resource release and preventing slow-client bottlenecks even in unstable network environments.

Although this study adopts a direct client-to-microservice architecture, the experimental results demonstrated that the proposed system achieved both high performance and strong security. These findings are discussed in the following subsections. Performance is widely recognized as one of the most critical quality attributes in microservices architecture design [2]. The performance can be enhanced through tactics such as resource placement and allocation, load balancing, containerization, and profiling. However, while prior studies have addressed these tactics broadly, limited attention has been given to the performance implications of resource placement and allocation in real-world applications. To address this gap, this research evaluated system efficiency using ISO/IEC 25010, with particular emphasis on three performance aspects: system capacity, time behavior, and resource utilization, which indicate the system's functional efficiency and resource management [47]. The design findings demonstrated that the architecture achieved a throughput of 16.67 requests per second with 5,000 concurrent users and met the expected throughput threshold. This outcome validates the effectiveness of microservice decomposition in distributing system load and ensuring responsiveness. In addition, the API design in this research played a crucial role in determining system throughput, as REST APIs are known for fast response times and high throughput [48]. The system maintained an average response time of 41-46 milliseconds, which was lower than the expected response time and highlighted the API's responsiveness and efficiency. In this research, we used an in-memory cache and Redis as a message broker for inter-service asynchronous communication, which significantly reduced response time [49] by reducing inter-service coupling and improving throughput [50].

Furthermore, CPU resource utilization did not increase rapidly as the number of users increased from 500 to 5,000. Peak CPU utilization ranged from 17.0% to 31.0%, which remained within expected thresholds. There was also only a slight increase in memory usage, from 1,381 MB at 500 users to 1,412 MB at 5,000 users, indicating that the system could handle intensive workloads without compromising stability. This outcome reflects the benefits of microservice architecture, in which applications are decomposed into smaller independent services that can be scaled individually based on demand. To answer RQ1, we confirm that the proposed architecture met the critical performance quality attributes, including system capacity, time behavior, and resource utilization.

Security also represents one of the most critical quality attributes in microservices architecture design [2]. We used a direct client-to-microservice architecture and set up the infrastructure layer to ensure system security. A firewall acted as a security barrier to block malicious software, while an API proxy served as an intermediary for API requests, handling security and caching. Furthermore, a considerable body of this research has examined security mechanisms, with particular emphasis on the application of JSON Web Tokens (JWT) and AES-256 encryption. The resource utilization results for the HTTP POST method in Table 10 highlighted the architecture's ability to handle the security overhead associated with AES-256 encryption before database storage. Although this process is inherently more complex than standard data retrieval (HTTP GET), the total response time remained remarkably low, ranging from 104 to 136 milliseconds. Compared with the results in Table 9, the overhead increases by approximately 60-95 milliseconds, accounting for both encryption time and write latency. These findings confirm that the integrated security mechanisms do not compromise the user experience, as the system maintains robust processing efficiency even under peak load conditions of 5,000 concurrent users (CCU), with peak CPU utilization reaching only 31.0%. These results indicate that the system was sufficiently stable while ensuring data security. We implemented AES-256 encryption for the e-Participation system because the application required high-speed performance and stronger data security, consistent with Singh (2022) [51], who found that AES-256 provides stronger security than AES-128 when higher security requirements are needed. AES provides fast encryption and decryption, resulting in lower latency than Rivest-Shamir-Adleman (RSA) and elliptic curve cryptography (ECC) [52][53]. AES also provides high throughput because it is ideal for encrypting large volumes of data quickly [53], with efficient CPU and memory usage, which contributes to its widespread adoption across various applications [54]. The adoption of RESTful APIs and the integration of AES-256 encryption significantly enhanced the protection of sensitive information, thereby reducing the risk of data leakage. Although the direct client-to-microservice architecture increases the attack surface, the evaluated architecture was deployed on a single-node server and therefore subject to a hardware-level single point of failure (SPOF). The software layer was explicitly engineered to tolerate overload conditions. Results in Table 10 show that the system maintained normal operation under a workload of 5,000 concurrent users, during which incoming requests exceeded the maximum available processing threads. The system avoided deadlock and out-of-memory conditions by employing graceful degradation, temporarily rejecting excess requests with HTTP 503 (Service Unavailable) or by returning timeout responses. This mechanism prevents cascading failures and enables automatic recovery once the workload returns to nominal levels, without requiring a system restart.

Much prior research has demonstrated the effectiveness of enhancing system performance and security in microservice architecture design. However, limited research has examined the integration of these technologies within direct client-to-microservice communication for e-Participation platforms in Local Administrative contexts. This study sought to address this gap by ensuring compliance with security requirements and maintaining high performance in such contexts. Nevertheless, in real-world deployments of e-Participation platforms, several factors should be considered, including internet connectivity, heterogeneous client devices, and organizational constraints. Therefore, future research should extend the evaluation to field deployments, usability, and user satisfaction.

This study was designed and developed as a prototype for deployment within thirty-one Local Administrative Organizations in Suratthani Province, which serve an estimated target population of approximately 427,723 citizens. The evaluated system capacity of 5,000 concurrent users (CCU) was sufficient to cover the anticipated peak load for this target user group. However, the primary technical limitation of the proposed prototype lies in its scalability. If extended to regional or national deployment with significantly higher user demand, the current single-node server architecture may encounter resource constraints. Therefore, future work should focus on evolving the architecture toward a distributed system by integrating an API Gateway for centralized request routing and employing load balancers to enable horizontal scaling across multiple servers, with proper management to support large-scale expansion and sustained high-load operation.

4. Conclusions

This study demonstrated the feasibility of a direct client-to-microservice communication architecture design. The combination of DDD, API-first design, JWT with AES-256 encryption, and a message broker proposed in the architecture provided a robust foundation for ensuring both performance and security in an e-Participation platform for Local Administrative Organizations. The use of direct client-to-microservice communication exposed services more directly to external clients. Therefore, careful authentication, role-based access control, and encrypted transport layers were necessary. Furthermore, the database-per-service pattern upholds service independence and aligns with the system's modular requirements. The findings of this research suggest that adopting this architectural model can enable local governments to deploy flexible, secure, and efficient e-participation platforms that enhance interaction, monitoring, and follow-up on various services requested by local civilians.

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In Silico Mining of Potential Anti-cancer Liver X Receptor β Agonists

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Abstract: Cancer continues to be a leading cause of mortality worldwide, with the most common treatments such as chemotherapy and radiotherapy producing severe off-target effects. This study aims to identify potential therapeutic compounds by screening the Natural Products Atlas (NPAtlas) for potential agonists of the Liver X Receptor Beta (LXR- β). LXR- β is a nuclear receptor involved in lipid metabolism and is known to induce apoptosis in cancer cells when acted upon by an agonist. Screening of 36,310 compounds from the NPAtlas database was performed using molecular docking, pharmacokinetic filtering, absorption, distribution, metabolism, excretion, and toxicity (ADMET) profiling, and confirmatory molecular dynamics simulations. From the initial dataset, 9 candidates passed the docking and ADMET criteria, and molecular dynamics simulations successfully validated 7 of them. Among these, Cis-veramycin E (NPA033420) and Solanapyrone E (NPA010954) emerged as top candidates. Cis-veramycin E demonstrated a high docking score (-9.31 kcal/mol), low root mean square deviation (RMSD) of <1.0 nm, minimal root mean square fluctuation (RMSF) of <0.3 nm, and a favorable ADMET profile, including Ames safety, good absorption, and moderate clearance. Protein–ligand interaction profiling revealed a binding profile similar to known LXR- β agonists, including hydrogen bonding with Ser278, π – π stacking with Phe340, and hydrophobic contacts across core residues. Solanapyrone E exhibited comparable binding stability and engaged the critical His435–Trp457 electrostatic switch via a salt bridge, but was flagged as Ames toxic, indicating a need for structural optimization. The results support the recommendation to identify the compounds as lead candidates for LXR- β -targeted cancer therapy.

Keywords: Induced apoptosis; cancer treatment; in silico mining

1. Introduction

Cancer continues to be a leading global issue, with approximately 20 million new diagnoses and 9.7 million deaths worldwide in 2022 alone. It is projected to reach 35 million by 2050, based on estimated population growth [1]. Drug discovery in cancer research has shifted towards the molecular targeting approach that focuses on potential therapeutic targets [2]. As cancer is characterized by uncontrolled cell division and unlimited proliferation, identifying potential targets with anti-proliferative effects is the main goal of therapeutic discovery. One such molecular target involved in cell fate determination is the Liver X Receptor (LXR).

LXRs are members of the nuclear receptor superfamily that is composed of important ligand-inducible transcription factors [3]. LXRs are involved in glucose and lipid metabolism, cholesterol transport and metabolism, and in inflammatory responses [4]. With increased demand for cancer cell resources, activation of LXR with synthetic agonists induces degradation of low-density lipoprotein receptors via the Inducible Degradation of the LDL receptor (IDOL) pathway and promotes cholesterol efflux, reducing the intracellular cholesterol pool [5]. This deprives cancer cells of materials necessary for biogenesis and cell membrane maintenance. This shows that LXR activation can induce cell stress and ultimately lead to apoptosis, highlighting the potential of LXR agonists as promising therapeutic targets for cancer treatment. In breast cancer, estrogen metabolism is a key determinant, and LXR exerts some control over estrogen homeostasis, with estrogen sulfotransferase (EST) being a transcriptional target of LXR [6]. It was demonstrated that LXR inhibits estrogen-dependent cancer cell proliferation by regulating hepatic EST. In addition, LXR agonists were observed to decrease the proliferation of breast cancer cell lines without affecting lipid biosynthesis through the decreased expression of specific cyclins and increased expression of p53 at the protein level [7].

In a study by Pencheva et al., ApoE was identified as a suppressor of melanoma metastasis, with the activation of LXRs enhancing ApoE transcription and inhibiting melanoma progression [8]. Knockdown experiments showed that LXR- β , but not LXR- α , is necessary for the suppression of melanoma invasion and endothelial recruitment by LXR agonists. Oral administration of LXR agonists GW3965 and T0901317 significantly suppressed tumor growth in mouse melanoma models, including various human melanoma cell lines, in a dose-dependent manner, with higher doses leading to greater suppression. The study also showed that LXR agonists reduced the number and size of endothelial vessels in tumors, leading to decreased tumor angiogenesis and modest reductions in tumor cell proliferation. While LXR- α and LXR- β share high structural similarity in their ligand-binding domains, they differ significantly in their tissue distribution and functional roles. LXR- α is primarily expressed in tissues with high metabolic activity, such as the liver, intestine, and adipose tissue, where it plays a vital role in lipid homeostasis and cholesterol transport [9]. In contrast, LXR- β is expressed ubiquitously across virtually all tissues, making it a more broadly applicable cancer therapeutic target. More importantly, simultaneous activation of both LXR isoforms results in significant hepatotoxicity and hypertriglyceridemia due to LXR- α -mediated upregulation of hepatic lipogenic genes (e.g., SREBP-1c, FAS) [9]. Thus, selective targeting of LXR- β offers a more promising anticancer strategy, potentially maintaining efficacy while minimizing hepatic side effects of LXR agonists.

Uno et al. also showed that LXR agonists inhibit Wnt signaling activation by reducing β -catenin's transactivation activity [10]. In HCT116 cells, LXR agonists reduce mRNA expression of β -catenin target genes, including the MYC proto-oncogene, matrix metalloproteinase 7 (MMP-7), and bone morphogenetic protein 4 (BMP-4). MYC is well studied for its critical role in cell growth, proliferation, and survival, and is frequently overexpressed in cancers [11]. MMP-7 is involved in the breakdown of the extracellular matrix, facilitating cancer cell invasion and metastasis [12]. BMPs have been shown to induce epithelial-to-mesenchymal transition, during which cells acquire migratory capability, making them invasive [13]. Furthermore, Fukuchi et al. [14] showed that LXR agonist T0901317 inhibits the proliferation of human prostate LNCaP cells. It achieves this by increasing the expression of the cyclin-dependent kinase inhibitor p27, inducing cell cycle arrest at the G1/S transition. Additional studies showed that T0901317 leads to downregulation of the protein B kinase (Akt), which plays a key role in promoting survival, growth, and proliferation in many types of cancer [15].

In the search for novel drugs, *in silico* methods have become indispensable tools in pharmacokinetic and pharmacochemical research, offering efficient and cost-effective alternatives to traditional experimental approaches [16]. These methods assess crucial factors such as drug absorption routes, distribution mechanisms, and potential risks and safety profile of candidate compounds. They streamline the drug discovery process, allowing researchers to rapidly and effectively identify promising compounds for further development. Our study aims to investigate potential anticancer compounds that target the LXR- β pathway of tumor suppression, using protein docking simulations and multiple *in silico* methods. For example, the synthetic LXR agonists GW3965 and T0901317 have demonstrated potent anticancer activity in preclinical models. However, their clinical application has been severely limited due to dose-limiting hepatotoxicity and

hypertriglyceridemia arising from non-selective LXR- α activation in hepatic tissue [17, 18]. These adverse effects underscore the need for novel LXR- β -selective agonists with improved therapeutic windows. Natural products offer a rich and structurally diverse chemical space that has historically yielded selective bioactive compounds with better tolerability profiles, making microbially derived natural product libraries particularly attractive for the discovery of next-generation LXR- β agonists. Structure-based virtual screening is particularly well-suited for targeting LXR- β , as high-resolution crystal structures of the LXR- β ligand-binding domain in complex with known agonists are publicly available, such as in the Protein Data Bank (PDB), enabling accurate modeling of ligand-receptor interactions. Previous in silico studies targeting LXRs and related receptors have successfully identified novel agonists using computational screening [19, 20, 21]. That is, these examples of successful computational screening approaches support the validity and efficiency of this methodology for nuclear receptor drug discovery. However, unlike prior work, this study differs in several key aspects. First, whereas most prior studies investigate the effects of a limited number of synthetic LXR agonists on specific cancer cell lines, this work performs a large-scale virtual screen of over 36,000 structurally diverse microbially derived natural products against LXR- β . Additionally, integrating molecular docking, multi-parameter ADMET profiling, and molecular dynamics simulation provides a multi-stage computational validation pipeline. Finally, the specific focus on selective LXR- β agonists from natural products represents a novel angle that has not been well explored in existing literature.

2. Materials and Methods

The study used the following software: PyRx, AutoDock Tools (ADT), VMD, PyMOL, OpenBabel, pkCSM, and GROMACS. PyRx uses AutoDock Vina to simulate and automate protein docking [22, 23, 24, 25]. pkCSM through Deep-PK was used to remove candidates based on comprehensive ADMET guidelines, while GROMACS was used to perform the molecular dynamics (MD) simulations [26, 27]. The computational load was processed on a Ryzen 5 3600 processor with 16 GB RAM, running Ubuntu 22.04 LTS on Windows Subsystem for Linux 2.

2.1 Target Protein Preparation

The LXR- β structure was downloaded from the Protein Data Bank. The crystal structure of human Liver X Receptor beta (LXR- β), selected as the template for all in silico estimations, was obtained from the Protein Data Bank (PDB) under PDB ID 6S4U, with an X-ray diffraction structure resolved at 2.81 Å. The structure corresponds to the ligand-binding domain (LBD) of Oxysterols receptor LXR- β in complex with a small-molecule inhibitor (KVK), comprising three protein chains (A, B, and C) with approximately 233–236 residues per chain. The co-crystallized ligand and water molecules were removed during receptor preparation before docking simulations in PyMOL, after which the receptor was transferred to ADT. ADT prepared the receptor and ligand files, converting them to PDBQT format that includes atomic coordinates, partial charges, and AutoDock atom types. The protonation state of the receptors was then assigned using the H++ server, which estimates pKas to allow the addition of missing H+ to macromolecules [28].

2.2 Sample Proteins Preparation

The Natural Products Atlas (NPAtlas) database is a comprehensive, open-access repository containing >36,300 microbially derived natural product structures [29]. The database was downloaded in SDF format, and a preliminary screening was performed using Open Babel and the International Chemical Identifier (InChI) to remove duplicates [30, 31]. As the files are in their 2D conformation, they were all minimized in PyRx using the uff94 force field to obtain an approximation of their 3D structures. Among these, those with extremely high energy values (>1000) were removed, as they indicate high steric strain and low stability, making them unsuitable for docking, as the stable 3D conformation cannot be determined reliably. The ligands were then partitioned into batches of 1000 for automated large-volume molecular docking screening in PyRx.

2.3 Molecular Docking Simulations

AutoDock Vina was used to perform docking computations, automated with PyRx. Docking grid dimensions are set to the full size of the LXR- β receptor, 99.5 x 93 x 74 Å. Docking parameters such as the

number of runs and population size were set to exhaustiveness levels of 10 and 50, respectively, for fast and extensive docking predictions. ADT and PyMOL were then used to visualize the docking results and evaluate the binding affinity of the ligand and the receptors. PyRx displayed the binding affinity values in kcal/mol, with lower (more negative) values indicating stronger binding. For drug discovery, a binding affinity of -6.0 to -7.0 kcal/mol or lower is considered promising [32, 33]. To validate the docking protocol, Beta-sitosterol (SIT), a known LXR- β agonist, was included as a positive control in docking simulations. The control ligand exhibited an average binding affinity of -8.33 kcal/mol under the established docking parameters, which served as the benchmark for evaluating candidate compounds. Ligands demonstrating binding affinities equal to or more negative than that of SIT were retained for subsequent pharmacokinetic screening and molecular dynamics confirmatory testing. Although explicit redocking of the attached ligand inhibitor was not performed, Beta-sitosterol (SIT) is a known agonist, and the Vina docking software has been widely used in previous studies as a standard for *in silico* molecular docking experiments [32].

2.4 ADMET Pharmacokinetics Screening

Lipinski's Rule of 5 was applied manually by checking the molecular weight, log P, and the number of hydrogen bond donors and acceptors for each candidate. After this, the SMILES sequences of the remaining candidates were uploaded to the Deep-PK web server for further screening using their comprehensive library of ADMET criteria [26]. The following quantitative thresholds and classification criteria were applied during ADMET screening using Deep-PK. For absorption, Caco-2 permeability (log Papp, log cm/s) was assessed, where values greater than 0.90 (corresponding to Papp > 8×10^{-6} cm/s) indicate high permeability; compounds were additionally evaluated for Human Intestinal Absorption (HIA), with those absorbing less than 30% considered poorly absorbed and excluded. Distribution was assessed via blood-brain barrier (BBB) permeability, where compounds with logPS ≥ -2 were classified as CNS-penetrant (CNSp+) and those with logPS ≤ -3 as non-penetrant (CNSp-). Metabolism was assessed through CYP3A4 inhibitor and substrate classifications: a compound is considered a CYP3A4 inhibitor (Class 1) if the IC₅₀ is less than 10 μ M, and a substrate (Class 1) if its pharmacokinetics are largely driven by CYP3A4-mediated metabolism. For excretion, total clearance (CL_{tot}) was predicted in mL/min/kg; values between 5 and 15 mL/min/kg were considered moderate and preferred. For toxicity, Ames mutagenicity was used as the primary genotoxicity filter; a positive Ames result indicates mutagenic potential and possible carcinogenicity, and all Ames-positive compounds were flagged. Any compound classified as "Unsafe" by Deep-PK across any of their parameter (e.g., Biodegradation, Ecotoxicity, Animal Toxicity, etc.) was excluded from further analysis.

2.5 Molecular Dynamics Simulations

To confirm binding stability, MD simulations were performed using GROMACS. The protein and ligand were combined and were energy-minimized using the steepest descent algorithm with a convergence criterion of Fmax < 1000 kJ/mol/nm. The CHARMM36 force field was used for the protein, while ligand parameters were generated using ACPYPE based on the General AMBER Force Field. After minimization, the system was equilibrated using a two-step protocol. First, NVT (number, volume, and temperature) equilibration was performed for 100 ps at 300 K using the V-rescale thermostat, with position restraints on hydrogen bond donors and acceptors to preserve key interactions during thermal equilibration. This was followed by NPT (number, pressure, and temperature) equilibration for 100 ps at 1 bar, employing the Parrinello-Rahman barostat to stabilize system pressure and density. A production MD run of 2,500,000 steps was then conducted in vacuum (i.e., without explicit solvent), using a time step of 2 fs for a total simulation time of 5 ns. Electrostatics were calculated using a cutoff of 1.0 nm. Constraints were applied to all hydrogen bonds using the LINCS algorithm to maintain bond lengths, eliminating drift. Structures that could not be minimized due to steric strain were excluded from testing. All simulations were run with 6 OpenMP threads on a 6-core virtual machine. Trajectory data were recorded every 10 ps and were used for subsequent structural and energetic analyses. While this may not fully capture the interaction over time, 5-ns simulations are sufficient to confirm binding stability and identify ligands for further studies [34]. Post-dynamics analysis includes the Root Mean Square Deviation (RMSD) and the Root Mean Square Fluctuation (RMSF). RMSD confirms whether the ligand remains stably associated with LXR β during the MD simulation, reinforcing the

initial docking prediction [35]. RMSF quantifies the average positional deviation of each residue over the course of the simulation, providing insight into local flexibility and structural stabilization. This was performed on the alpha carbons of each amino acid in the protein backbone [36, 37]. Molecular dynamics simulations were conducted in vacuum as a computationally efficient initial screening strategy to assess the relative binding stability of candidate compounds and prioritize lead candidates for further study [38]. While explicit solvent MD simulations more accurately represent physiological conditions, vacuum approaches have been employed in prior computational screening studies as a rapid confirmatory step before more resource-intensive solvated systems [39, 40, 41]. The absence of explicit solvent is acknowledged as a limitation of this study, and future work should include fully solvated, longer-timescale MD simulations to validate these findings under physiologically relevant conditions. A simulation duration of 5 ns was selected as a practical and computationally feasible timescale for initial stability screening, consistent with prior virtual screening studies that have employed short MD runs (1–10 ns) to filter ligand candidates based on binding stability before committing to resource-intensive extended simulations. Although 5 ns is insufficient to capture slow conformational dynamics or unbinding events, this was used to prioritize computational throughput to navigate vast chemical libraries. Based on previous studies, it is adequate for detecting gross conformational instabilities and ranking the relative stability of protein-ligand complexes at an early screening stage [38, 42, 43]. Future studies should employ longer simulation timescales (100 ns or beyond) with explicit solvent to more rigorously characterize the binding dynamics of the top lead compounds.

2.6 Receptor-Ligand Complex Interactions

To further characterize the binding of the two lead compounds, protein–ligand interaction profiling of the docked complexes was performed using the Protein–Ligand Interaction Profiler (PLIP) [44], a widely used automated platform for detecting and visualizing non-covalent protein–ligand interactions from three-dimensional structural data. Using the best-binding pose from the molecular docking process for the final lead targets, their structures were combined with the cleaned LXR β structure and uploaded to the PLIP platform.

3. Results and Discussion

3.1. Nine (9) candidate compounds were identified after molecular docking and pharmacokinetic screening.

The NPAtlas Database was screened for LXR- β using the fast docking protocol. Based on the predicted docking scores, 1,385 compounds of the original 36,310 displayed docking scores lower than that of SIT. More exhaustive docking parameters further reduced the number of compounds to 1,371. Among these, only 9 ligands met the criteria defined by the comprehensive Deep-PK ADMET guidelines and screening using Lipinski's Rule of 5. These compounds were identified as pharmacologically viable and not classified as "unsafe" by Deep-PK's advanced in silico screening, which integrates absorption, distribution, metabolism, excretion, and toxicity parameters into a unified drug-likeness filter. The average exhaustive docking scores and 2D chemical structures of the 9 compounds are listed in Table 1, while Table 2 presents their comprehensive ADMET predictions.

Table 1. Two-dimensional chemical structures and average fast and exhaustive Vina docking scores (in kcal/mol) for the top 9 potential NPAAtlas compounds against the LXR- β protein compared to SIT.

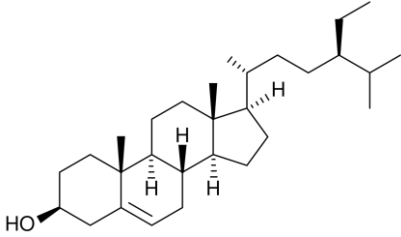
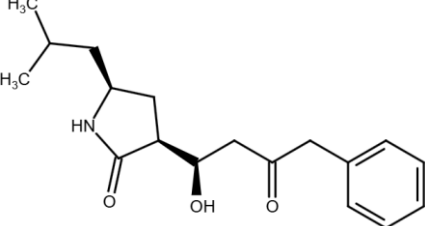
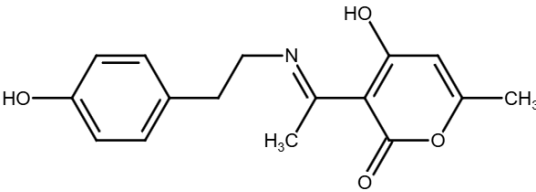
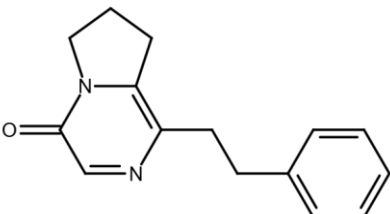
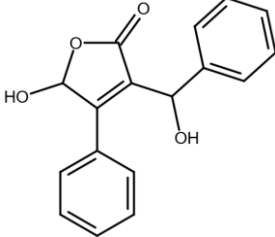
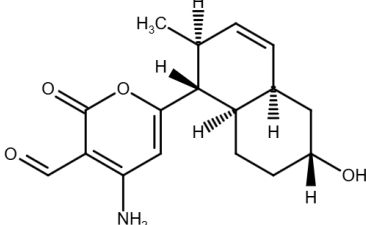
Compound Name NPAAtlas Code	Two-Dimensional Chemical Structure	Average Predicted Docking Score (kcal/mol)	
		Fast	Exhaustive
Beta-Sitosterol		-8.33	-8.33
NPA004934 (Berkeleyamide A)		-9.4	-9.62
NPA006504 (Fusamine)		-8.4	-8.6
NPA008173 (Nannoazinone A)		-8.7	-9.2
NPA008573 (Ralfuranone J)		-8.6	-9.26
NPA010954 (Solanapyrone E)		-8.4	-8.43

Table 1. Two-dimensional chemical structures and average fast and exhaustive Vina docking scores (in kcal/mol) for the top 9 potential NPAAtlas compounds against the LXR- β protein compared to SIT. (continue)

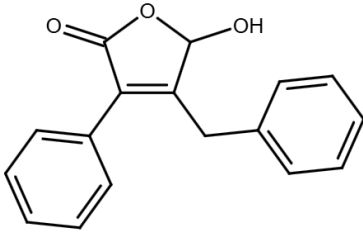
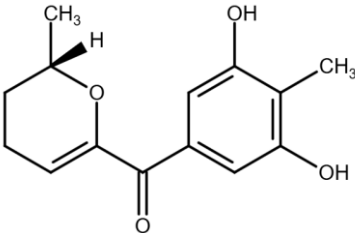
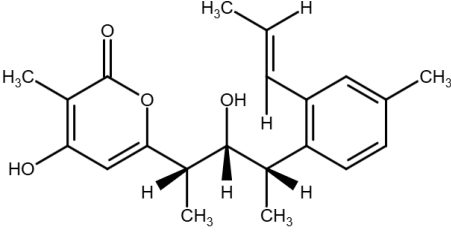
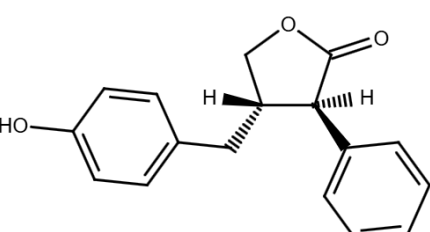
Compound Name NPAAtlas Code	Two-Dimensional Chemical Structure	Average Predicted Docking Score (kcal/mol)	
		Fast	Exhaustive
NPA016720 (EQ-4)		-9.2	-9.52
NPA023365 (Raistrickione E)		-8.54	-8.67
NPA033420 (Cis-veramycin E)		-8.9	-9.31
NPA035593 (Aspergulfuranone B)		-8.7	-8.82

Table 2. Highlighted ADMET Criteria from Predictions of the Final 9 Lead Compounds Selected from 1,371 Docking Candidates via Deep-PK Screening

Compound Name/Code	Absorption (A)		Distribution (D)	Metabolism (M)	Excretion (E)	Toxicity (T)
	Caco-2 Permeability (log Paap)	Human Intestinal Absorption (HIA)	Blood-Brain Barrier	CYP3A4 Inhibitor/Substrate	Total Clearance (ml/min/kg)	Ames Toxicity
NPA004934 (Berkeleyamide A)	-4.73	Absorbed	Penetrable	Non-Inhibitor; Non-Substrate	6.72	Safe
NPA006504 (Fusamine)	-4.83	Absorbed	Non-Penetrable	Inhibitor; Substrate	6.11	Safe
NPA008173 (Nannoizinone A)	-4.96	Absorbed	Penetrable	Non-Inhibitor; Substrate	6.61	Safe
NPA008573 (Ralfuranone J)	-4.43	Absorbed	Non-Penetrable	Non-Inhibitor; Non-Substrate	3.42	Safe
NPA010954 (Solanapyrone E)	-4.78	Absorbed	Penetrable	Inhibitor; Non-substrate	5.34	Toxic
NPA016720 (EQ-4)	-4.55	Absorbed	Penetrable	Non-Inhibitor; Non-Substrate	5.42	Safe
NPA023365 (Raistrickione E)	-4.64	Absorbed	Penetrable	Inhibitor; Non-Substrate	10.5	Safe
NPA033420 (Cis-veramycin E)	-4.81	Absorbed	Penetrable	Inhibitor; Substrate	5.17	Safe
NPA035593 (Aspergulfuranone B)	-4.82	Absorbed	Penetrable	Inhibitor; Non-Substrate	6.66	Safe

Based on the docking results, Berkeleyamide A (NPA004934) appears to be the most promising candidate with an exhaustive docking score of -9.62 kcal/mol. Its structure includes a fused bicyclic core and a flexible polar tail featuring hydroxyl and amide groups, which could contribute to its strong binding affinity and potential bioavailability. EQ-4 (NPA016720) also demonstrated excellent binding potential (-9.52 kcal/mol). The molecule features a central lactone ring conjugated to two aromatic rings, a scaffold known to support π - π stacking and hydrogen bonding [45]. This planar geometry and electron distribution could facilitate strong interactions with key LXR- β residues involved in ligand recognition. Cis-veramycin E (NPA033420), with a docking score of -9.31 kcal/mol, presents multiple hydroxyl and carbonyl groups, suggesting a high capacity for hydrogen bonding and potentially stabilizing its conformation for LXR- β agonist activity. Other compounds such as Fusamine (NPA006504) and Solanapyrone E (NPA010954), despite having slightly lower docking scores, still showed sufficient receptor affinity (-8.6 to -8.82 kcal/mol) and

passed all ADMET filters. These molecules are characterized by conjugated hydroxyl groups, which, according to Charlton et al., are among the most common pharmacophore features in antioxidants [46].

In Table 2, each compound is annotated across the five major ADMET categories that serve as core indicators of oral bioavailability, systemic exposure, safety, and therapeutic viability. Absorption was assessed through two key parameters: Caco-2 permeability (log Paap) and human intestinal absorption (HIA). Caco-2 cells are used in vitro to simulate intestinal epithelial permeability, and their log Paap values provide insight into a compound's ability to pass through the gut wall via passive diffusion [47]. Although all 9 compounds demonstrated moderate Caco-2 permeability values ranging from -4.43 to -4.96 , each was still predicted to be "absorbed" in the HIA model. This implies that, despite some variation in in vitro transport potential, the compounds possess physicochemical properties compatible with effective in vivo absorption [48]. The blood–brain barrier (BBB) permeability was included as a core distribution trait to indicate whether each compound is likely to penetrate the central nervous system (CNS) [26]. Among the selected compounds, 7 were classified as BBB-penetrant, suggesting they could reach neural tissues. This could be advantageous for CNS-specific applications but may also raise concerns about neurotoxicity or off-target neurological effects. Conversely, compounds such as Fusamine and Ralfuranone J were predicted to be non-penetrant, thereby reducing CNS-associated risks.

The metabolic liability of each compound was evaluated through predicted interactions with cytochrome P450 3A4 (CYP3A4), the most prominent metabolic enzyme in the human liver [49]. Compounds may act as inhibitors that block the enzyme, as substrates that are metabolized by it, or as non-interactors. Inhibitors can cause harmful drug–drug interactions, while substrates may be rapidly metabolized and eliminated. Among the 9 compounds, Berkeleyamide A and EQ-4 were predicted to be neither inhibitors nor substrates, indicating a minimal risk for metabolic interference. In contrast, compounds like Fusamine and Cis-veramycin E were identified as both inhibitors and substrates, which could complicate dosing strategies and require further studies to optimize their potential negative effects. Total clearance, expressed as mL/min/kg, was used to estimate the rate of elimination of a compound from the body via renal and hepatic routes. Clearance affects dosing frequency, therapeutic window, and potential for drug accumulation. According to Xiong et al., a good clearance value would be between 5 and 15 mL/min/kg, as these are considered moderate [50]. The 9 compounds exhibited a wide range of predicted clearance values, but all fell within the aforementioned range. Raistrickione E showed the highest clearance relative to the other compounds, suggesting it may be eliminated quickly and require more frequent administration. Ralfuranone J showed the lowest clearance, potentially increasing its systemic retention compared with the other candidates. Compounds with moderate clearance are generally ideal, as they remain in systemic circulation long enough to be effective without overaccumulating or causing toxicity [26, 50]. For toxicity predictions, Ames mutagenicity is a common in silico test for DNA damage and carcinogenic potential [51]. Among the 9 candidates, only Solanapyrone E was classified as "Toxic", raising concerns regarding its safety in therapeutic contexts. All other compounds were labeled "Safe", indicating a low risk of genotoxicity.

3.2 Cis-veramycin E (NPA033420) and Solanapyrone E (NPA010954) are lead compounds.

Among the 9 candidates, MD simulations could not be performed for EQ-4 and Raistrickione E. Based on the LINCS algorithm, they were too sterically strained, with bonds rotating by more than 30 degrees during simulation. The RMSD values for the seven remaining candidates over the 5-ns production time were plotted as a function of time in Figure 1. Among the candidates, Berkeleyamide A, Fusamine, Nannoizinone A, and Aspergulfuranone B exhibited consistent RMSD values of 4.8–4.9 nm throughout the simulation. Ralfuranone J exhibited a low RMSD value of 0.43–0.52 nm, but with severe spikes around 4–5 nm throughout the simulation. Solanapyrone E and Cis-veramycin E had the lowest stable RMSD values, all stabilizing below 1.0 nm.

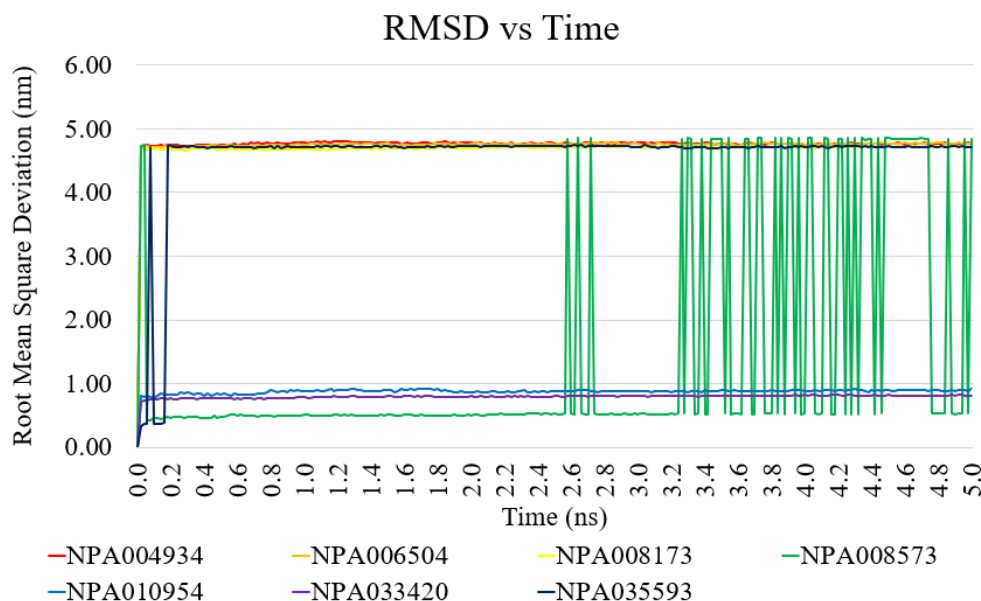


Figure 1. Root Mean Square Deviation (RMSD, nm) of the protein backbone $\text{C}\alpha$ atoms as a function of simulation time (ns) over a 5 ns in vacuo molecular dynamics simulation for each of the seven candidate protein–ligand complexes. Lower and more stable RMSD values throughout the simulation indicate greater structural stability of the complex. Compounds with RMSD values consistently below 1.0 nm are considered stable, while those with values in the 4–5 nm range indicate significant structural deviation and were deprioritized.”

High RMSD suggests considerable movement or structural deviations in the ligand relative to the binding site, or larger possible conformational changes in the LXR- β structure. These may indicate instability in the binding. However, large deviations could also be due to induced fit of the ligand and warrant further study by longer simulations [52].

As such, compounds exhibiting RMSD values in the 4–5 nm range (Berkeleyamide A, Fusamine, Nannoizinone A, and Aspergilluranone B) are not considered acceptable leads in this study. The high RMSD values serve as the primary quantitative criterion for their deprioritization and exclusion from the final lead set. These high RMSD values, particularly in the context of vacuum MD simulations, where solvent absence may amplify protein conformational drift, indicate that these complexes do not maintain stable binding configurations over the 5-ns timescale and therefore cannot be confidently proposed as LXR- β binders [53]. In contrast, the complexes containing Solanapyrone E and Cis-veramycin E showed low and stable RMSD values, fluctuating around 0.5–0.9 nm, suggesting that these ligands do not significantly alter the protein structure, indicating stable binding [54]. Ralfuranone J showed an observably low RMSD but pronounced, frequent spikes throughout the simulation. This may indicate unstable or transient ligand–receptor binding, making it an unreliable candidate. Interestingly, almost all of the candidates equilibrated quickly, with RMSD values stabilizing within 300 to 500 picoseconds of the simulation. This suggests that the protein–ligand complex can achieve stable conformations quickly, supporting efficient agonist activity, which is desirable for activating the LXR- β pathway. However, RMSD analysis does not fully capture long-term conformational adaptations or possible unbinding events [34].

RMSF values for the different ligands bound to LXR- β are shown in Figures 2 and 3. Cis-veramycin E (NPA033420) and Solanapyrone E (NPA010954) exhibited the lowest overall fluctuations and the lowest values, suggesting stability of the protein backbone, which is beneficial in the context of therapeutic targeting [55]. This aligns with their favorable docking scores and supports their potential as LXR- β agonist candidates. Berkeleyamide A (NPA004934) and Nannoizinone A (NPA008173) exhibited moderate RMSF profiles with peaks at around 0.5 nm. This indicates that while the ligand can support binding, it may have weaker contact that may not necessarily compromise binding stability, but should be subject to further MD testing [56].

Fusamine (NPA006504) displayed increased RMSF values, particularly with peaks hovering near the 0.6 nm range. This could imply looser binding or induced strain in receptor regions. Aspergilfurane B (NPA035593) showed even more pronounced RMSF peaks (>1.2 nm) and sustained high flexibility across both chains, which may reflect suboptimal stabilization and could impair consistent receptor activation [57]. Ralfuranone J (NPA008573) exhibited greater fluctuations, exceeding 3.0 nm, indicating excessive flexibility. This suggests structural instability or unfavorable binding interactions, making it a weaker candidate for LXR- β agonist activity. Overall, RMSF values were consistent with expected structural behavior, with the highest values observed at the N- and C-terminal regions across all systems, reflecting their inherent flexibility [55].

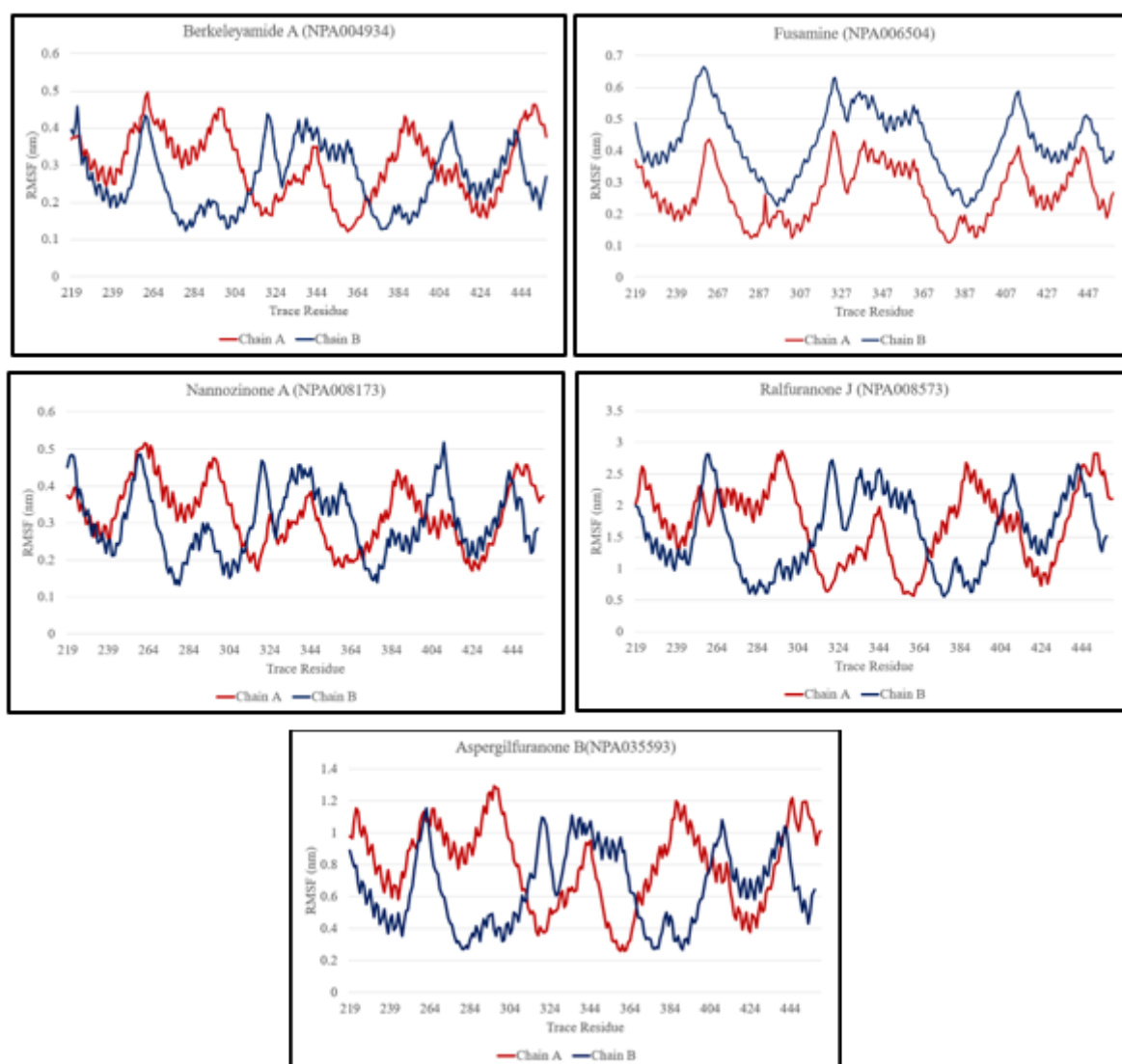


Figure 2. Root Mean Square Fluctuation (RMSF, nm) of the protein backbone C α atoms per residue for LXR- β in complex with Berkeleyamide A, Fusamine, Nannozone A, Ralfuranone J, and Aspergilfurane B during a 5 ns vacuum molecular dynamics simulation. Moderate per-residue fluctuations and elevated RMSF values across chains A and B indicate increased local flexibility within the protein–ligand complexes.

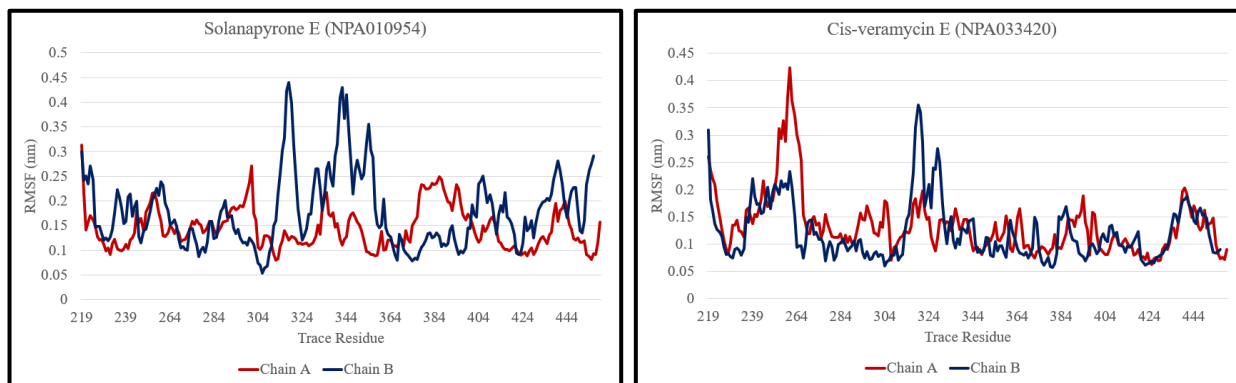


Figure 3. Root Mean Square Fluctuation (RMSF, nm) of the protein backbone C α atoms per residue for LXR- β in complex with Solanapyrone E and Cis-veramycin E during a 5 ns in vacuo molecular dynamics simulation. Lower per-residue fluctuations across chains A and B indicate greater structural rigidity and stability of the protein–ligand complexes.

A summary of the molecular interactions for Cis-veramycin E (NPA033420) and Solanapyrone E (NPA010954) within the ligand-binding domain (LBD) of LXR- β is presented in Table 3. Within the LBD, Cis-veramycin E established a single hydrogen bond involving the backbone nitrogen of Ser278 at a distance of 4.06 Å, complemented by a parallel π – π stacking interaction with Phe340 (distance: 4.16 Å; angle: 2.14°). Furthermore, ten hydrophobic contacts were observed with residues Phe271, Ala275, Leu313, Thr316, Phe340, Leu345, Ile350, Ile353 (two interactions), and Phe354, which were primarily localized around the hydrophobic core. Although no formal PLIP interactions were assigned, mechanistically significant residues His435 and Trp457 were located in proximity at 5.8 Å and 6.3 Å, respectively. In contrast, Solanapyrone E (NPA010954) displayed a unique interaction profile within the same pocket, characterized by the absence of classical hydrogen bonds. Instead, it formed a salt bridge with His435 (distance: 4.73 Å) through its carboxylate moiety, alongside a parallel π – π stack with Phe340 (distance: 3.94 Å; angle: 7.49°). It also maintained nine hydrophobic contacts with Ala275, Thr316, Phe329, Leu345 (two interactions), Phe349, Ile350, and Ile353 (two interactions). The ionic engagement of His435 is highly significant, as this residue serves as a pivotal anchor for the His435–Trp457 switch that stabilizes helix 12 (AF-2) into its active state [32]. Both lead compounds exhibited convergent occupation of the LXR- β hydrophobic core, sharing interactions with Ala275, Thr316, Leu345, Ile350, and Ile353, while utilizing Phe340 for π – π stacking. These interaction profiles align with the binding stability confirmed via MD simulations, providing evidence for their roles as LXR- β ligands.

Table 3. Protein–ligand interactions of Cis-veramycin E and Solanapyrone E with the ligand-binding domain of liver X receptor beta (LXR- β)

Compound	Interaction Type	Residues	Distance (Å)
Solanapyrone E (NPA010954)	Salt Bridge	His435	4.73
	π – π stacking (parallel)	Phe340	3.94 (centroid)
	Hydrophobic contacts	Ala275, Thr316, Phe329, Leu345, Phe349, Ile350, Ile353	3.23–3.80
Cis-veramycin E (NPA033420)	Hydrogen bond	Ser278	4.06 (D–A)
	π – π stacking (parallel)	Phe340	4.16 (centroid)
	Hydrophobic contacts	Phe271, Ala275, Leu313, Thr316, Phe340, Leu345, Ile350, Ile353, Phe354	3.34–3.81

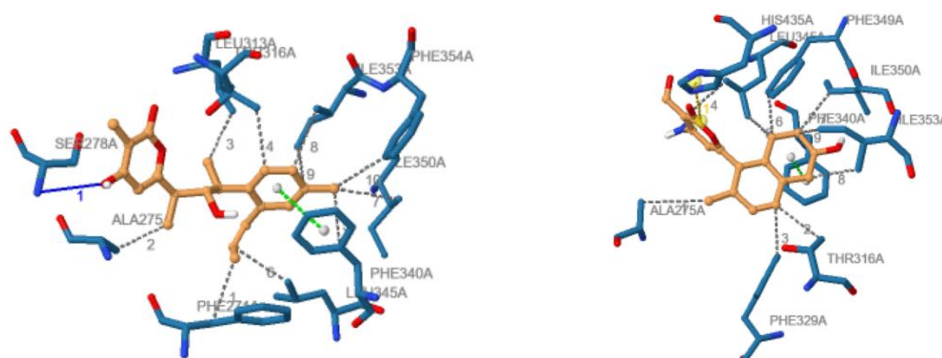


Figure 4. Protein–ligand interaction profiles of Cis-veramycin E (left) and Solanapyrone E (right) in complex with the ligand-binding domain of liver X receptor beta (LXR- β)

The binding profiles of Cis-veramycin E and Solanapyrone E can be meaningfully contextualized with those of well-characterized synthetic LXR agonists such as T0901317 and GW3965, which have served as the primary pharmacological tools for validating LXR- β as an anticancer target. T0901317 has demonstrated anti-cancer activity across multiple tumor types, including inhibition of lung cancer cell migration and invasion through suppression of the ERK/MAPK signaling pathway and downregulation of MMP9 [58]. At the same time, GW3965 has shown therapeutic benefit in preclinical models through ABCA1-mediated apoE lipidation and cholesterol efflux [59]. However, both agonists are limited clinically by hepatic fat accumulation and hypertriglyceridemia arising from upregulation of SREBP-1c and downstream lipogenic genes [60]. Crystallographically, both reference agonists stabilize the LXR- β AF-2 helix through the His435–Trp457 electrostatic switch and engage a shared hydrophobic core — including Phe340, Leu345, Ile350, and Ile353 [61, 62]. The lead compounds in this study show convergent binding features: Solanapyrone E directly engages His435 via a salt bridge (4.73 Å) with Trp457 in proximity (3.7 Å), while Cis-veramycin E engages the same hydrophobic core residues through π – π stacking with Phe340 and contacts with Leu345, Ile350, and Ile353.

Apart from their shared structural characteristics with established agonists, the distinct interaction profiles of these lead candidates offer insights into the mode of activation of LXR- β and its subsequent anti-tumorigenic properties. For Solanapyrone E, the formation of a direct salt bridge with His435 (4.73 Å) aligns with the structural requirements for the His435–Trp457 electrostatic switch that stabilizes helix 12 into its active state to facilitate co-activator recruitment and downstream transcriptional regulation [62]. Although Cis-veramycin E engages His435 more distally at 5.8 Å, its array of hydroxyl and carbonyl groups supports hydrogen bonding while maintaining essential contacts with the hydrophobic core, which have been implicated in the indirect stabilization of the AF-2 domain for diverse LXR- β ligands [63]. Successful LXR- β activation by these compounds is anticipated to modulate several key regulatory axes, including the upregulation of ABCA1, ABCG1, and ApoE for enhanced cholesterol efflux, the inhibition of Wnt/ β -catenin-driven expression of MYC and MMP-7, and the induction of programmed cell death via p27-dependent cell cycle arrest and AKT signaling suppression [5, 9, 14, 15]. Such pathways highlight the recognized mechanism for LXR- β -targeted oncology. As microbially derived natural products with favorable ADMET profiles, both leads share key features with established LXR- β agonists while potentially offering an improved safety window. Thus, further validation through LXR- β assays will be essential to determine if the structural stability predicted in this *in silico* study correlates with functional biological activity.

4. Conclusions

This study performed a large-scale *in silico* screen of 36,310 microbially derived natural products from NPAtlas against LXR- β , a nuclear receptor preferentially targeted over LXR- α due to its ubiquitous tissue distribution and reduced hepatotoxicity risk. Using a multi-stage pipeline (molecular docking against PDB ID 6S4U (2.81 Å), ADMET profiling via Deep-PK, and 5-ns *in vacuo* MD simulations), nine compounds passed

initial screening, of which seven were validated by MD simulation. Cis-veramycin E (NPA033420) emerged as the top lead, with a docking score of -9.31 kcal/mol, stable RMSD below 1.0 nm, minimal RMSF below 0.3 nm, a favorable ADMET profile, and a binding mode similar to established LXR- β agonists. Solanapyrone E (NPA010954) is a secondary lead with strong binding stability and direct engagement of the His435–Trp457 electrostatic switch, but requires structural optimization to address Ames mutagenicity.

Key limitations include simulations performed in a vacuum and a short 5-ns timescale, both adopted to maintain computational efficiency consistent with prior virtual screening studies. Future work should prioritize solvated extended MD simulations (>100 ns), *in vitro* LXR- β binding experimentation, and structural optimization of Solanapyrone E to eliminate genotoxic liability. Overall, our findings suggest Cis-veramycin E (NPA033420) and Solanapyrone E (NPA010954) are promising lead candidates for future *in vitro* and *in vivo* studies targeting LXR- β -mediated anti-cancer pathways.

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Author Contributions: Conceptualization, JPMPS and ICVJI; methodology, JPMPS; software, JPMPS; validation, JPMPS and ICVJI; formal analysis, JPMPS and ICVJI; investigation, JPMPS; resources, JPMPS; data curation, JPMPS; writing—original draft preparation, JPMPS and ICVJI; writing—review and editing, JPMPS and ICVJI; visualization, JPMPS; supervision, ICVJI. All authors have read and agreed to the published version of the manuscript.

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Statistical Downscaling for Climate Change Studies: Comparing TCPG-GLM and Gradient-Boosted TCPG Models for Local-Scale Rainfall in Purwakarta

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Abstract: Accurate rainfall projections are vital for climate adaptation and water resource planning in tropical regions, where rainfall patterns are highly variable and zero-inflated. This study evaluates the performance of two statistical downscaling approaches based on the Tweedie Compound Poisson-Gamma (TCPG) distribution for monthly rainfall modeling in Purwakarta, Indonesia: the generalized linear model with Tweedie distribution (GLM-Tweedie) and the nonlinear Tweedie distribution boosting model (TDboost). Using observational rainfall data from five stations and predictor variables from three Earth System Models (BNU-ESM, MIROC-ESM, and MIROC-ESM-CHEM), model performance was assessed using root mean squared error of prediction (RMSEP) and Pearson correlation coefficient. The results show that TDboost generally outperformed GLM-Tweedie in terms of predictive accuracy and temporal consistency. At Cibukamanah station, TDboost achieved the lowest RMSEP under the MIROC-ESM-CHEM scenario (90.63 mm), while its highest correlation was obtained under the BNU-ESM scenario ($r = 0.88$). Similarly, at Cisomang station, TDboost under the MIROC-ESM-CHEM scenario achieved an RMSEP of 109.75 mm and a correlation of $r = 0.78$, indicating improved predictive performance compared with GLM-Tweedie. These findings suggest that distribution-aware boosting techniques can better capture the nonlinear and zero-inflated characteristics of tropical rainfall. The results also indicate that the best-performing ESM may depend on the evaluation metric used, with MIROC-ESM-CHEM providing lower prediction errors at some stations and BNU-ESM showing strong temporal correlation when combined with TDboost. Overall, this study highlights the importance of combining appropriate statistical downscaling models with suitable ESM predictors for improving local-scale rainfall prediction in complex tropical environments.

Keywords: Climate change; generalized linear models; machine learning; statistical downscaling; tweedie boosting

1. Introduction

Rainfall prediction in tropical regions has become increasingly important because of the growing demand for accurate climate information to support food security, hydrometeorological disaster mitigation, and sustainable water resource management. Tropical areas, such as Indonesia, exhibit highly complex rainfall patterns influenced by multiscale atmospheric dynamics, including

regional phenomena such as the El Niño–Southern Oscillation (ENSO) and the Indian Ocean Dipole (IOD), which introduce significant uncertainty into seasonal rainfall [1, 2]. Moreover, local factors such as mountainous terrain, hilly landscapes, and microclimatic effects intensify spatial rainfall variability, as observed in Purwakarta Regency, West Java—a region characterized by complex geography and substantial intra-regional rainfall variation. These challenges are further exacerbated by the sparse observational networks and irregular rainfall distributions, which are poorly represented by coarse-resolution outputs from global climate models [3], [4].

This challenge is further compounded by the coarse spatial resolution of Earth System Models (ESMs), which typically operate at resolutions of tens to hundreds of kilometers [5]. Consequently, ESM outputs often fail to capture local rainfall heterogeneity, particularly in topographically complex areas such as Purwakarta Regency, West Java [6]. Such inaccuracies result in biased rainfall estimates that do not reflect actual field conditions. To bridge this gap, statistical downscaling methods are employed to link large-scale predictors from ESMs to local observational data through statistical relationships [7–9]. These approaches not only enhance spatial resolution but also correct systematic biases in ESM outputs based on historical data [5, 10].

Numerous studies have demonstrated that statistical downscaling produces more accurate rainfall projections in tropical regions when combined with statistical models that align with the unique distributional characteristics of rainfall, such as the Tweedie distribution [11]. Monthly rainfall in the tropics typically displays highly skewed, non-negative values with a substantial proportion of zeros [12]. These features are not well modeled using the normality assumptions underlying many classical regression models. In this context, the Tweedie Compound Poisson-Gamma (TCPG) distribution is an appropriate choice, as it captures both the discrete (rain occurrence) and continuous (rain amount) components within a single framework [13]. Furthermore, as part of the exponential family, the Tweedie distribution is compatible with the Generalized Linear Model (GLM) framework and offers flexibility in modeling zero-inflated and highly skewed data [14].

A study by Hayati et al. [15] revealed that applying GLMs with a Tweedie distribution significantly improves monthly rainfall prediction compared with conventional linear models. This flexibility is partly due to the index parameter (p), which lies within the range $1 < p < 2$, enabling the model to adapt its shape to match local data characteristics [16]. However, GLMs remain limited by the assumption of a linear relationship between predictors and the response variable. In tropical climate systems with complex, nonlinear interactions among sea surface temperature, atmospheric pressure, and global circulation indices, linear models often fall short of accurately capturing these dynamics [14].

To address this limitation, machine learning approaches such as tree-based boosting methods have been developed to capture nonlinear patterns and complex interactions among variables [17]. A notable example is Tweedie Boosting (TDboost), which combines decision tree structures with a loss function based on the Tweedie distribution [18, 19]. This method iteratively learns from complex data structures and significantly improves predictive performance, particularly for highly skewed or non-normally distributed data [11, 19].

Against this background, this study aims to evaluate and compare two statistical downscaling approaches based on the Tweedie distribution—namely, the Generalized Linear Model (GLM-Tweedie) and TDboost—for modeling monthly rainfall in Purwakarta Regency, Indonesia, a tropical region with high spatial complexity. Beyond comparing the performance of linear and nonlinear models, this study also evaluates the capability of three CMIP5-based Earth system models (BNU-ESM, MIROC-ESM, and MIROC-ESM-CHEM) to represent monthly rainfall at the local scale in Indonesia.

The main contribution of this study is not the development of a new boosting algorithm, but the evaluation of a distribution-aware nonlinear downscaling framework for local monthly rainfall in a tropical Southeast Asian setting. While TDboost has been introduced in previous statistical literature, its application for local-scale rainfall downscaling using ESM predictors in Indonesia remains limited. This study examines whether a Tweedie-based boosting model can improve the representation of zero-inflated, right-skewed, and nonlinear rainfall behavior compared with a linear Tweedie GLM under the same ESM input conditions. In addition, by comparing three CMIP5-based ESM scenarios across five rainfall stations, this study provides evidence on how the choice of ESM input may influence local downscaling performance in a complex tropical

region. Therefore, the novelty of this work lies in its regional application, distributional consistency, and comparative evaluation of linear and nonlinear Tweedie-based downscaling approaches for Southeast Asian rainfall modeling.

2. Materials and Methods

This study focuses on Purwakarta Regency, located in West Java, Indonesia, a region characterized by high spatial and temporal rainfall variability driven by orographic influences and tropical atmospheric dynamics. To capture this spatial heterogeneity, rainfall data were collected from five observation stations: Purwakarta, Pondok Salam, Cibukamanah, Cisomang, and Wanayasa. Monthly rainfall observations were obtained from the Meteorological, Climatological, and Geophysical Agency of Indonesia (BMKG) and spanned 16 years from January 2005 to December 2020. For modeling purposes, the dataset was divided into 144 months for training and the most recent 12 months (January–December 2020) for testing. Details of the data used are shown in Table 1.

Table 1. Summary of Data.

Data	Institution	Scale	Spatial Resolution
Station	BMKG	Monthly	-
Model for Interdisciplinary Research on Climate (MIROC-ESM) ^[20]	JAMSTEC	Monthly	2.8° × 2.8°
MIROC-ESM-CHEM (with Chemistry) ^[20]	JAMSTEC	Monthly	2.8° × 2.8°
Beijing Normal University (BNU-ESM) ^[21]	Beijing Normal University	Monthly	2.8° × 2.8°

As predictors in the statistical modeling process, this study used data from three global climate models (Earth System Models (ESMs)) under the historical scenario framework of the Coupled Model Intercomparison Project Phase 5 (CMIP5), namely BNU-ESM, MIROC-ESM, and MIROC-ESM-CHEM. These models were selected for their extensive application in regional climate studies and their demonstrated ability to capture tropical rainfall variability, particularly in the Southeast Asian region. MIROC-ESM and its variant, MIROC-ESM-CHEM, were developed by Japanese institutions with a strong focus on the Asian climate system, making their parameterizations relatively more sensitive to regional climatic conditions. In contrast, BNU-ESM is recognized for its robust performance in simulating monsoon dynamics and maritime tropical climates. Monthly ESM data were extracted from the nearest grid point to each station location without applying additional bias correction.

In this study, the predictor set used in the statistical downscaling models consisted of nine ESM-derived climate predictor variables, each extracted from the nearest grid point to each rainfall observation station. To account for short-term temporal dependence, one-month lagged predictors were also generated from the original ESM predictors. In addition, seasonal information was incorporated using three seasonal dummy variables representing DJF, MAM, and JJA, with SON treated as the reference season. Thus, the final predictor structure included the original ESM-derived predictors, their one-month lagged counterparts, and seasonal dummy variables. The same predictor structure was used for both GLM-Tweedie and TDboost to ensure a fair comparison between the two statistical downscaling approaches. No separate variable selection or dimensionality reduction procedure was applied before modeling.

To model monthly rainfall data characterized by nonlinearity, zero-inflation, and a tendency toward overdispersion, a statistical approach capable of flexibly capturing these distributional characteristics is required. Therefore, this study implemented two modeling approaches based on the Tweedie distribution: the Generalized Linear Model (GLM-Tweedie), representing a classical linear model, and Tweedie Distribution

Boosting (TDboost), a nonlinear model based on a boosting algorithm. These two models were selected for comparative evaluation in terms of their predictive performance on rainfall data derived from a combination of ground-based observations and global climate model projections, to assess the effectiveness of each approach in statistically representing tropical rainfall dynamics.

2.1 GLM-Tweedie

The Generalized Linear Model (GLM) with a Tweedie distribution is a widely used statistical approach for analyzing data that are non-negative, highly right-skewed, and contain a substantial number of zeros—such as monthly rainfall data in tropical regions. The main advantage of the Tweedie distribution lies in its ability to represent a compound distribution as a compound Poisson–Gamma distribution, particularly when the power index parameter $1 < p < 2$. In this range, the distribution is referred to as the Tweedie Compound Poisson-Gamma (TCPG), which is especially well-suited for rainfall data that include both zero events (no rainfall) and positive continuous rainfall amounts. In the GLM-Tweedie framework, the expected value of the response variable Y_i is linked to the linear predictor via a logarithmic link function:

$$\log(\mu_i) = \beta_0 + \mathbf{x}_i^T \boldsymbol{\beta} \quad (1)$$

where μ_i is the expected value of the response variable Y_i , $\mathbf{x}_i$ is the vector of predictor variables (climate features), and $\boldsymbol{\beta}$ is the vector of regression coefficients. The relationship between the variance and the mean is assumed to follow the power-law form:

$$\text{Var}(Y_i) = \phi \mu_i^p \quad (2)$$

where ϕ is the dispersion parameter and p is the Tweedie power index that governs the distributional shape, this formulation allows the model to accommodate overdispersion and zero inflation, which are commonly observed in rainfall data.

The parameters $\boldsymbol{\beta}$, ϕ , and p are estimated using the Maximum Likelihood Estimation (MLE) approach. The log-likelihood function of the Tweedie distribution for a single observation can be expressed as follows:

$$\log f(y_i, \mu_i, \phi, p) = \frac{1}{\phi} \left(\frac{y_i \mu_i^{1-p}}{1-p} - \frac{\mu_i^{2-p}}{2-p} \right) + \log a(y_i, \phi) \quad (3)$$

Since the normalization function $a(y_i, \phi)$ does not have a closed-form expression, the likelihood must be computed numerically. Estimation of the regression coefficients $\boldsymbol{\beta}$ is typically performed using the Iteratively Reweighted Least Squares (IRLS) algorithm. Meanwhile, the Tweedie power index p is commonly selected via a profile likelihood approach, in which a range of candidate values of p is evaluated, and the value that maximizes the overall log-likelihood is chosen.

2.2 TDboost

To model rainfall data characterized by nonlinearity, overdispersion, and a high frequency of zero values, the Tweedie Distribution Boosting (TDboost) approach was developed by Yang et al. [19] as an extension of the gradient boosting algorithm, specifically designed to accommodate a loss function based on the Tweedie Compound Poisson-Gamma (TCPG) distribution. This model is tailored to predict the mean value μ of the response variable under the assumption of non-negative and zero-inflated outcomes, without relying on the linear relationship assumption inherent in GLM-Tweedie. The strength of TDboost lies in its ability to iteratively build predictive models using decision trees, where each iteration aims to minimize the negative log-likelihood of the Tweedie distribution. By defining a loss function that matches the data distribution, TDboost adjusts the predicted estimates to align with the statistical characteristics of the response while simultaneously capturing nonlinearities and complex interactions among predictors. The following is the TDboost algorithm as developed by Yang et al. [19]:

TDboost Algorithm

Let $\{(x_i, y_i)\}_{i=1}^n$ be the training dataset, where x_i is a vector of predictors and y_i is the response variable (monthly rainfall). The algorithm proceeds as follows:

Inputs:

- Training dataset $\{(x_i, y_i)\}_{i=1}^n$
- Tweedie power index p and dispersion parameter ϕ
- Number of boosting iterations M
- Learning rate $\nu \in (0, 1]$
- Maximum tree depth J

Steps:

1. **Initialize** the prediction function:

$$\hat{f}^{(0)}(x) = \log(\bar{y})$$

where $\bar{y}$ is the average of the response variable.

2. **For** each boosting iteration $m = 1, 2, \dots, M$, perform the following:

- Compute the current predicted mean:

$$\hat{\mu}_i^{(m-1)} = \exp(\hat{f}^{(m-1)}(x_i))$$

- Calculate the pseudo-residuals using the derivative of the Tweedie deviance:

$$r_i^{(m)} = \hat{\mu}_i^{(m-1)} \left(\frac{y_i}{(\hat{\mu}_i^{(m-1)})^p} - (\hat{\mu}_i^{(m-1)})^{1-p} \right)$$

- Fit a regression tree $\hat{h}^{(m)}(x)$ to the pseudo-residuals $r_i^{(m)}$
- Update the prediction function:

$$\hat{f}^{(m)}(x) = \hat{f}^{(m-1)}(x) + \nu \hat{h}^{(m)}(x)$$

Final prediction for the mean response:

$$\hat{\mu}(x) = \exp(\hat{f}^{(M)}(x))$$

2.3 Model Evaluation

To evaluate and compare the predictive accuracy of the two statistical downscaling approaches—GLM-Tweedie and TDboost—this study employed two performance metrics: Root Mean Square Error of Prediction (RMSEP) and the Pearson correlation coefficient (r). These metrics were calculated using the testing set, consisting of the most recent 12 months of the rainfall dataset, which was not used during model training. The use of a 12-month testing period was intended to evaluate the models over one complete annual rainfall cycle, which is relevant for Indonesia's seasonal climate regime characterized broadly by wet and dry seasons. Therefore, the testing period includes the main seasonal transition within a single year. However, this design should be interpreted as an initial independent evaluation rather than a comprehensive assessment of interannual generalization. This data separation was intended to ensure that the model evaluation reflects predictive performance on unseen data, a critical aspect for operational climate projections. The RMSEP was calculated using the following equation:

$$\text{RMSEP} = \sqrt{\frac{1}{n} \sum_{i=1}^n (\hat{y}_i - y_i)^2} \quad (4)$$

where $\hat{y}_i$ denotes the predicted value from the model, y_i represents the actual observed value, and n is the number of observations in the testing dataset. A lower RMSEP value indicates smaller average prediction

errors relative to the actual data, signifying better quantitative accuracy of the model. Meanwhile, the Pearson correlation coefficient (r) is calculated using the following equation:

$$r = \frac{\sum_{i=1}^n (\hat{y}_i - \bar{\hat{y}})(y_i - \bar{y})}{\sqrt{\sum_{i=1}^n (\hat{y}_i - \bar{\hat{y}})^2} \sqrt{\sum_{i=1}^n (y_i - \bar{y})^2}} \quad (5)$$

where $\bar{\hat{y}}$ and $\bar{y}$ represent the mean of the predicted and observed values, respectively. A correlation coefficient closer to 1 indicates a strong, positive linear relationship between the predicted and observed values, suggesting consistent patterns between the two series.

Model evaluation was conducted separately for each rainfall station under the three ESM scenarios: BNU-ESM, MIROC-ESM, and MIROC-ESM-CHEM. The results of RMSEP and correlation metrics offer a comprehensive view of the spatial and temporal performance of both modeling approaches. Overall, TDboost demonstrated superior predictive performance, yielding more accurate and highly correlated predictions, particularly in regions with high rainfall variability.

3. Results and Discussion

3.1 Exploration of Rainfall Data

Understanding the seasonal distribution of rainfall is critical for effective water resource management, agricultural planning, and climate adaptation strategies in tropical regions such as Indonesia. This study investigates the intra-annual variability of monthly rainfall across five meteorological stations in West Java — Wanayasa, Cisomang, Pondok Salam, Cibukamanah, and Purwakarta—using boxplots (Figure 1). These locations represent diverse topographical and climatic zones, enabling a comparative assessment of spatial rainfall patterns and seasonal behavior.

The results reveal a predominantly monsoonal rainfall pattern across the five stations, characterized by a single main wet season and a single main dry season within the annual cycle. The wet season generally runs from January to March, while the dry season runs from July to September. Based on Figure 1, peak rainfall occurs at the beginning of the year, with median values exceeding 600 mm/month, particularly at Wanayasa and Pondok Salam stations. In contrast, July and August show very low rainfall, with median values approaching zero, especially at Cisomang and Cibukamanah. These seasonal fluctuations reflect the influence of the monsoonal system over western Indonesia and are consistent with a unimodal annual rainfall regime in Purwakarta Regency. Although rainfall regimes across the Indonesian maritime continent may vary from unimodal to bimodal depending on location, longitude, and the influence of the ITCZ, the observed rainfall distribution in Purwakarta is better described as monsoonal, with a single dominant wet season.

Among the five stations, Pondok Salam and Wanayasa exhibit the highest monthly rainfall variability, likely due to orographic enhancement resulting from their proximity to mountainous terrain. Meanwhile, Purwakarta station consistently records lower rainfall, with monthly median values generally below 400 mm. These spatial differences underscore the importance of local topographic and atmospheric dynamics. These findings are supported by previous studies indicating that rainfall patterns in this region are influenced by large-scale phenomena such as the Australasian monsoon and ENSO events [22], as well as mesoscale convective processes in areas with complex topography [23].

Extending the previous analysis, the rainfall density distributions from the five stations provide a more nuanced view of the monthly rainfall frequency and distributional patterns (Figure 2). While seasonal patterns were clearly observed in the boxplots, the density plots illustrate variations in distributional shape across locations. For example, Purwakarta shows a high-density peak at lower rainfall levels (below 200 mm), suggesting that light rainfall events are much more common. Conversely, stations like Pondok Salam and Cibukamanah exhibit broader distributions with peaks in the mid-range (200–500 mm) and right-skewed tails, indicating that extreme rainfall events may occur, although infrequently.

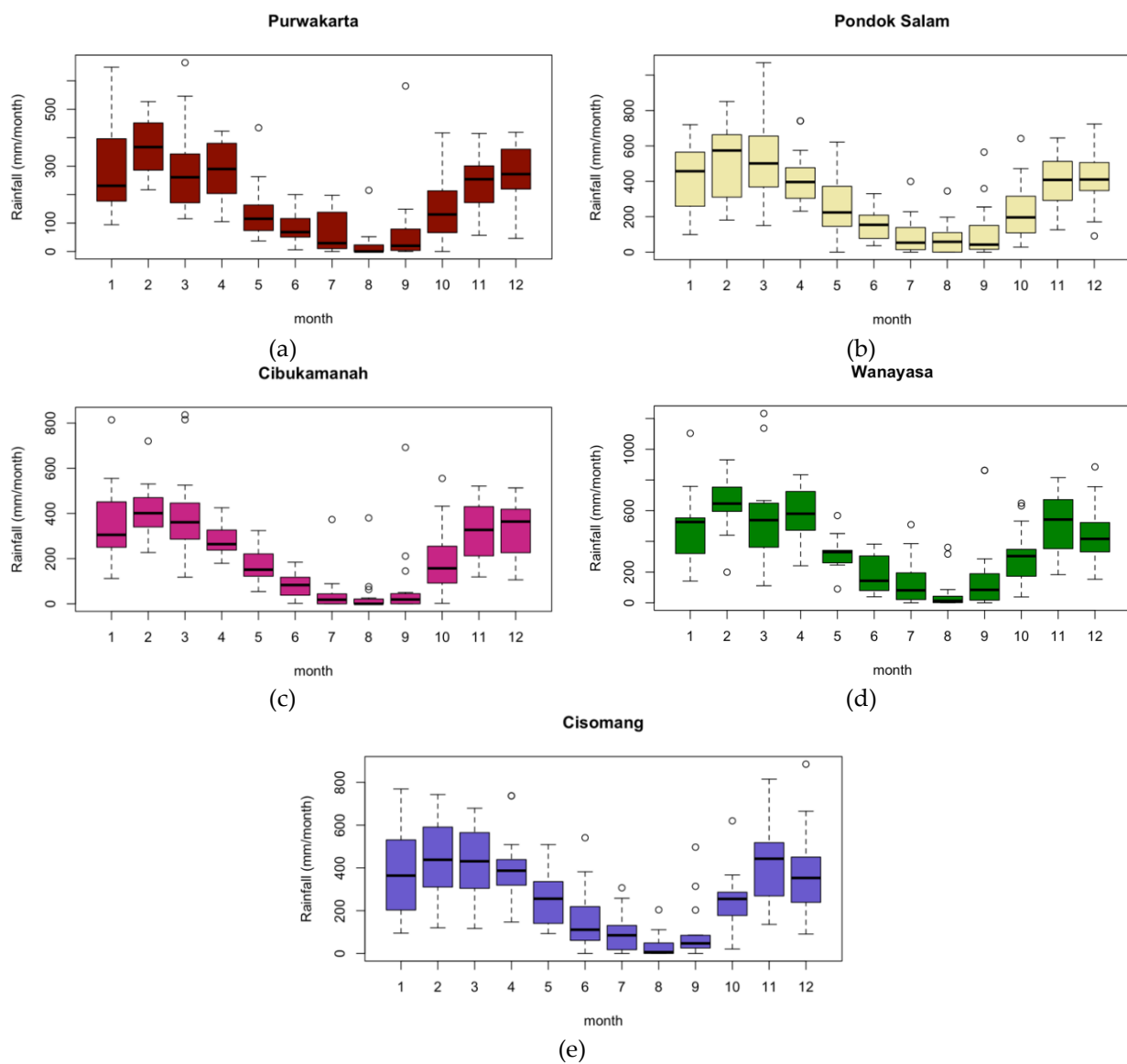


Figure 1. Monthly rainfall distribution patterns at five meteorological stations: (a) Purwakarta, (b) Pondok Salam, (c) Cibukamanah, (d) Wanayasa, and (e) Cisomang. Boxplots show intra-annual rainfall variability, with marked differences in intensity and seasonal rainfall distribution among the stations.

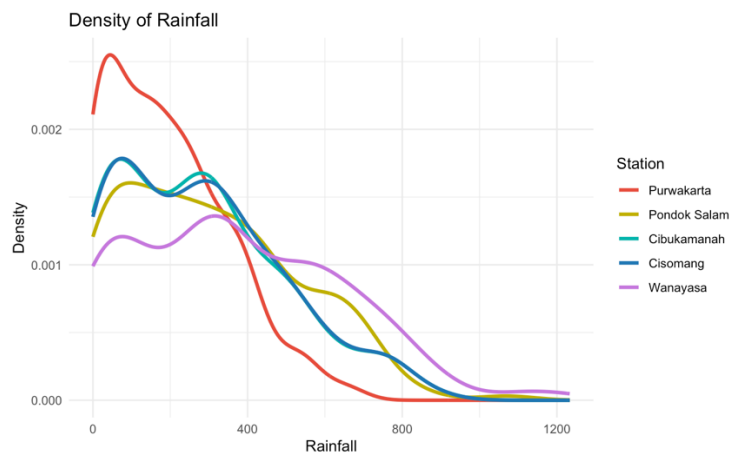


Figure 2. Rainfall density distribution at five stations in Purwakarta Regency.

The observed distribution patterns closely resemble the statistical properties of the Tweedie Compound Poisson-Gamma (TCPG) distribution, a mixed distribution that effectively accommodates data characterized by both a high frequency of zero events and extreme positive values. This distribution is particularly well-suited for modeling rainfall data, as it captures two underlying processes: a Poisson process representing the frequency of rainfall events, and a Gamma distribution modeling the intensity of rainfall when it occurs. Such a dual mechanism reflects the inherently sporadic nature of tropical rainfall, which is not only irregular but can also exhibit extreme intensities. Previous studies have confirmed the suitability of the TCPG distribution in hydrological and meteorological contexts, especially for modeling daily and monthly precipitation in tropical regions [24]. The positively skewed shapes and long right tails in the rainfall density plots for nearly all stations suggest that the rainfall data in this region are likely to follow a TCPG distribution. Statistically, this provides a more accurate modeling framework, and practically, it has important implications for the development of early warning systems, water resource management, and risk assessment for extreme weather events affecting agriculture and infrastructure.

3.2. Parameter & Hyperparameter Tuning

Based on the estimated power parameter p obtained through the profile likelihood approach in the GLM-Tweedie model (as shown in Table 2), the optimal p value for most stations—namely Purwakarta, Cibukamanah, Cisomang, and Wanayasa—is 1.3. The only exception is Pondok Salam, which yielded a higher optimal value of $p = 1.7$. This parameter plays a critical role in defining the shape of the Tweedie distribution family: values of p between 1 and 2 indicate that the data follow a compound Poisson-Gamma distribution, representing a mixture of discrete rainfall occurrences (i.e., rainfall > 0) and continuous rainfall amounts. For stations with $p = 1.3$, the distribution implies a predominance of light to moderate rainfall events, with a moderate frequency of zero values. In contrast, the higher p value at Pondok Salam suggests a tendency toward heavier rainfall intensities and a distribution with a fatter right tail. This indicates that extreme rainfall events are more frequent at Pondok Salam, possibly due to localized orographic effects or microclimatic conditions specific to that station.

Table 2. Estimated Tweedie Power Parameter p for Each Rainfall Station using Profile Likelihood Approach

Station	p
Purwakarta	1.3
Pondok Salam	1.7
Cibukamanah	1.3
Cisomang	1.3
Wanayasa	1.3

The observed variation indicates that, statistically, Pondok Salam exhibits a more variable and more extreme rainfall pattern than other locations. This aligns with previous findings from the boxplots and density graphs, which showed that Pondok Salam had a wider spread and a higher likelihood of intense rainfall events. A higher p value suggests that the Tweedie model captures an increased contribution from the Gamma component, reflecting greater rainfall intensity when precipitation occurs. In contrast, other stations with $p = 1.3$ more accurately represent light to moderate rainfall patterns that occur more frequently, with less extreme intensities. These findings reinforce the relevance of using the TCPG distribution for rainfall modeling in tropical regions and demonstrate that the GLM-Tweedie model can be effectively adapted to local rainfall characteristics by accurately estimating the power parameter p [25].

Table 3 presents the results of the hyperparameter tuning process for the TDboost model, which was used to model monthly rainfall across various tested hyperparameter combinations. The tuned parameters include: α_{trv} , representing the Tweedie power index p , with values tested between 1.3 and 1.7; depth_{trv} , the maximum depth of decision trees (either 2 or 3); eta_{trv} , the learning rate, set at 0.005 or 0.003; and ntrees , the maximum number of trees, fixed at 6500 for all model runs. These combinations were optimized to

minimize the prediction error on the validation set and capture the nonlinear and zero-inflated nature of tropical rainfall patterns.

Table 3. Optimized Hyperparameters of the TDboost Model for Each Rainfall Station

Station	p	Depth	Best Iteration
Purwakarta	1.7	3	5732
Pondok Salam	1.6	2	5904
Cibukamanah	1.6	2	6500
Cisomang	1.7	2	6495
Wanayasa	1.7	2	6054

Based on the hyperparameter tuning results, the optimal values of the Tweedie index parameter p varied across stations, reflecting the distinct distributional characteristics of rainfall at each location. Stations such as Purwakarta, Cisomang, and Wanayasa exhibited an optimal p value of 1.7, indicating greater rainfall variability and a greater frequency of high-intensity precipitation events. In contrast, Pondok Salam and Cibukamanah showed optimal p values of 1.6, which still fall within the compound Poisson-Gamma range but with slightly more moderate rainfall intensity contributions. Additionally, the optimal tree depth for the TDboost model was predominantly 2, except for Purwakarta, where a depth of 3 was required. This suggests that the relationship between the predictors and rainfall in Purwakarta is more complex and requires a richer model structure for accurate learning.

The difference in the selected Tweedie power index between GLM-Tweedie and TDboost should be interpreted in relation to the different model structures and optimization procedures. In GLM-Tweedie, the power index is selected within a parametric log-link regression framework, where the mean–variance relationship is evaluated under a linear predictor structure. In contrast, in TDboost, the Tweedie power index is treated as part of the loss-function tuning process and is selected together with other hyperparameters, such as tree depth, learning rate, and the number of boosting iterations, based on validation performance. Therefore, the optimal power index in TDboost does not necessarily have the same interpretation as the power index selected in GLM-Tweedie. A higher selected value in TDboost, such as $p = 1.7$ at Purwakarta, may indicate that the nonlinear boosting model gives greater weight to positive rainfall intensity and heavier-tailed variability after accounting for nonlinear predictor effects. Meanwhile, the lower value selected by GLM-Tweedie may reflect the best mean–variance compromise under a more restrictive linear structure. Thus, the discrepancy suggests that the two models capture different aspects of the rainfall-generating process, driven by their distinct optimization objectives and functional flexibility.

Regarding the optimal number of boosting iterations, models at stations such as Cibukamanah and Cisomang nearly reached the maximum tree limit (6500), indicating that a full boosting process was necessary to achieve optimal accuracy. This may imply high data variability or noise, requiring extensive learning to capture stable patterns. On the other hand, stations like Purwakarta and Pondok Salam reached convergence earlier (around 5700–5900 trees), suggesting that the model learned the predictive patterns at these locations more efficiently. Overall, these findings underscore the importance of fine-tuning the Tweedie distribution parameters to capture the unique rainfall dynamics at each location. Furthermore, they demonstrate the flexibility of the TDboost approach in accommodating spatial heterogeneity through precise parameter adjustment.

The relatively high number of boosting iterations at Cibukamanah and Cisomang indicates that the TDboost model required a longer learning process to capture the rainfall variability at these stations. In this study, the optimal number of trees was selected using an internal validation set within the training data, rather than solely on training error, which helps reduce the risk of overfitting. Nevertheless, the fact that the optimal iteration nearly reached the maximum tree limit suggests that potential overfitting cannot be fully ruled out, especially given the relatively short 12-month testing period. Therefore, the superior performance of TDboost at these stations should be interpreted with caution. Future studies should apply more rigorous validation schemes, such as leave-one-year-out cross-validation, k -fold cross-validation, or blocked temporal cross-validation, to further assess the model's robustness and generalization.

3.3. Performance Comparison

Predictive performance evaluation was conducted to compare the effectiveness of the GLM-Tweedie and TDboost models in modeling monthly rainfall, using two key metrics: Root Mean Square Error of Prediction (RMSEP) and Pearson correlation coefficient, calculated for the testing dataset. RMSEP quantifies the magnitude of prediction error, while the correlation coefficient indicates the model's ability to reproduce the actual rainfall pattern. Together, these metrics provide a comprehensive assessment of model accuracy and consistency relative to observed data.

To provide a more transparent numerical comparison, the complete performance results for all station–ESM–model combinations are summarized in Table 4. The table reports RMSEP and Pearson correlation coefficient values for GLM-Tweedie and TDboost across the five rainfall stations and three ESM scenarios. This table complements the graphical summaries in Figures 3 and 4 by providing exact numerical values for verification and comparison.

Table 4. Performance comparison of GLM-Tweedie and TDboost across rainfall stations and ESM scenarios

Station	ESM Scenario	GLM-Tweedie RMSEP	TDboost RMSEP	GLM-Tweedie r	TDboost r
Purwakarta	BNU-ESM	174.53	150.91	0.62	0.78
Pondok Salam	BNU-ESM	191.44	183.27	0.59	0.72
Cibukamanah	BNU-ESM	119.57	102.82	0.75	0.88
Cisomang	BNU-ESM	134.45	128.37	0.66	0.68
Wanayasa	BNU-ESM	192.31	200.98	0.68	0.72
Purwakarta	MIROC-ESM	200.84	187.26	0.56	0.65
Pondok Salam	MIROC-ESM	257.49	194.22	0.29	0.65
Cibukamanah	MIROC-ESM	180.04	140.68	0.51	0.73
Cisomang	MIROC-ESM	228.69	185.53	0.18	0.50
Wanayasa	MIROC-ESM	229.83	157.65	0.52	0.84
Purwakarta	MIROC-ESM-CHEM	149.84	161.62	0.81	0.71
Pondok Salam	MIROC-ESM-CHEM	169.80	166.35	0.72	0.73
Cibukamanah	MIROC-ESM-CHEM	91.74	90.64	0.87	0.87
Cisomang	MIROC-ESM-CHEM	126.01	109.76	0.69	0.78
Wanayasa	MIROC-ESM-CHEM	177.10	188.13	0.77	0.71

Table 4 shows that TDboost generally outperformed GLM-Tweedie across most station–ESM combinations. Based on RMSEP, TDboost produced lower prediction errors in 12 out of 15 combinations, indicating its stronger predictive accuracy for monthly rainfall downscaling. Under the MIROC-ESM scenario, TDboost consistently achieved lower RMSEP values and higher Pearson correlation coefficients across all five stations, suggesting that the nonlinear boosting structure was more effective at extracting predictive information from this ESM. However, under the MIROC-ESM-CHEM and BNU-ESM scenarios, several exceptions were observed. GLM-Tweedie produced lower RMSEP at Wanayasa under BNU-ESM and at Purwakarta and Wanayasa under MIROC-ESM-CHEM, indicating that the simpler linear Tweedie model may still perform competitively in some local settings. The lowest RMSEP was obtained at Cibukamanah using TDboost under the MIROC-ESM-CHEM scenario, while the highest correlation was achieved at the same station using TDboost under the BNU-ESM scenario. These results suggest that the best-performing ESM may vary across evaluation metrics, but overall, TDboost provides more consistent improvements in both prediction error and temporal agreement with observed rainfall.

As presented in Figure 3, the TDboost model demonstrated superior predictive performance compared to GLM-Tweedie in most cases, as indicated by consistently lower RMSEP values. For instance, at the Cibukamanah station, the TDboost model under the MIROC-ESM-CHEM scenario achieved the lowest

RMSEP of 90.63, outperforming both the GLM-Tweedie model under the same scenario (91.73) and all other scenario combinations. A similar trend was observed at Cisomang station, where TDboost (RMSEP = 109.75) significantly outperformed GLM-Tweedie (RMSEP = 126.01) under the MIROC-ESM-CHEM scenario. At Purwakarta station, TDboost also outperformed GLM-Tweedie across nearly all scenarios, with the most notable improvement seen under the MIROC-ESM scenario (187.25 vs. 200.84), indicating a consistent enhancement in predictive accuracy.

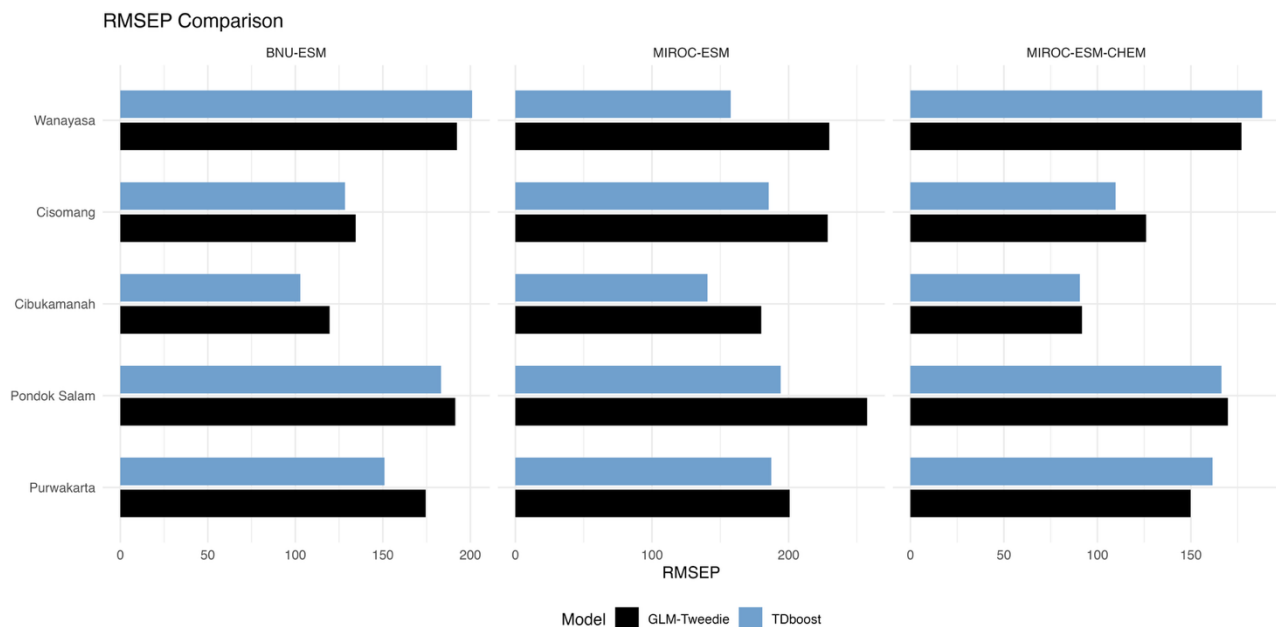


Figure 3. Comparison of RMSEP Values Between GLM-Tweedie and TDboost Models Across Stations and ESM Scenarios

Despite the TDboost model's overall superiority, several exceptions suggest that its performance may not universally surpass that of the GLM-Tweedie approach across all locations and scenarios. For instance, at the Wanayasa station under the MIROC-ESM-CHEM scenario, GLM-Tweedie achieved a lower RMSEP (177.10) than TDboost (188.13), suggesting slightly better predictive accuracy. This observation highlights the importance of considering local rainfall characteristics and the variability introduced by different ESM predictors. Nevertheless, TDboost generally outperforms GLM-Tweedie due to its ability to model nonlinear relationships and complex interactions among atmospheric variables. The boosting approach, especially when optimized via hyperparameter tuning, demonstrates significantly improved predictive performance compared to traditional GLMs for rainfall forecasting based on the Tweedie distribution.

Further evaluation of Pearson correlation coefficients between predicted and observed rainfall values during the testing period reinforces the advantage of TDboost. In almost all stations and ESM scenarios, TDboost consistently yields higher correlation values, indicating its superior ability to capture rainfall temporal patterns. At the Cibukamanah station, TDboost achieved the highest correlation of 0.88 under the BNU-ESM scenario, surpassing GLM-Tweedie's 0.75. A similar improvement was observed at the Cisomang station, where the correlation increased markedly from 0.19–0.69 (GLM-Tweedie) to 0.50–0.78 with TDboost. These findings underscore TDboost's effectiveness in detecting complex and nonlinear rainfall dynamics that are often underrepresented in classical linear models. The results align with previous studies demonstrating that gradient boosting significantly outperforms linear models in capturing intricate atmospheric interactions and nonlinear dependencies [26, 27].

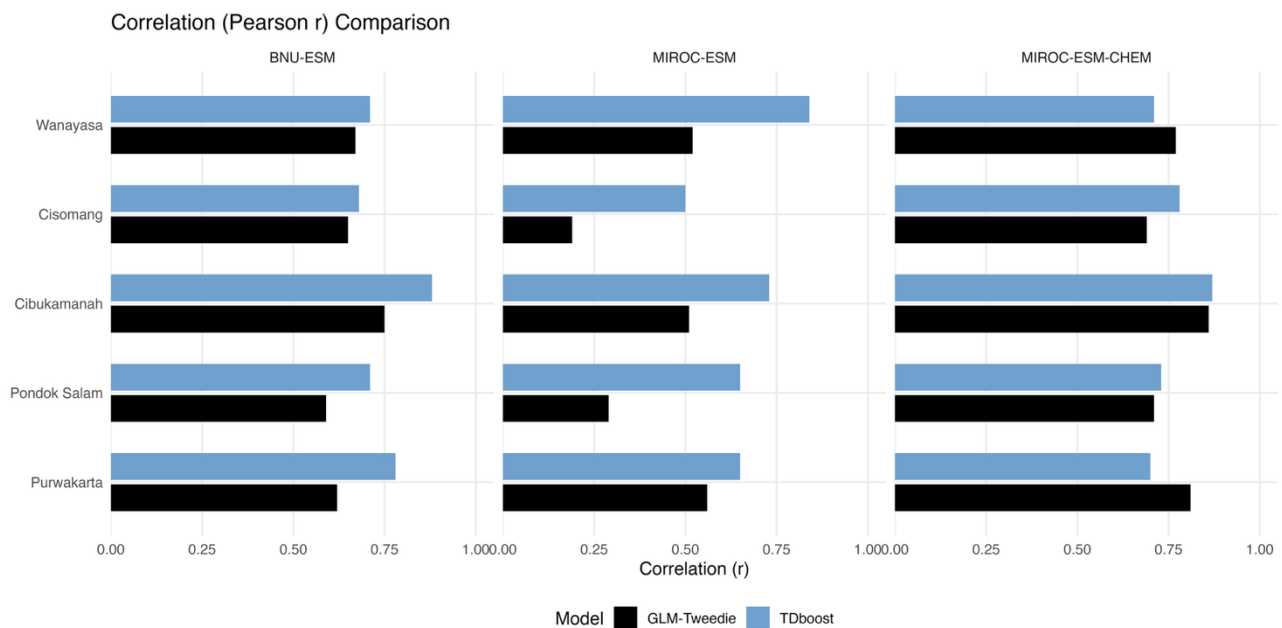


Figure 4. Correlation Coefficients Between Observed and Predicted Monthly Rainfall Across Stations and ESM Scenarios for GLM-Tweedie and TDboost Models

In the comparison of ESM inputs, BNU-ESM consistently delivered the highest correlation values for TDboost across nearly all stations. For example, at Cibukamanah and Purwakarta, BNU-ESM achieved the highest correlations of 0.88 and 0.78, respectively, when used with TDboost, outperforming the MIROC-ESM and MIROC-ESM-CHEM scenarios. This indicates that rainfall projections from BNU-ESM better align with local climatic characteristics in the study area, thereby providing more informative inputs for nonlinear models such as TDboost. Conversely, GLM-Tweedie appeared more sensitive to the choice of ESM, with its best performance often found under the MIROC-ESM-CHEM scenario, as observed in Purwakarta (correlation = 0.81) and Wanayasa (0.77). This can be attributed to the linear structure of GLM-Tweedie, which tends to rely on stable and informative predictors for accurate modeling ^[14].

In this study, ESM outputs were used without applying additional bias correction before statistical downscaling. This design choice was made to isolate the comparative performance of GLM-Tweedie and TDboost under the same raw ESM input conditions. However, this choice also represents an important limitation. ESM outputs may contain systematic biases, particularly for precipitation-related variables in tropical regions with complex terrain. Therefore, the absence of bias correction may have influenced the magnitude of prediction errors and the relative performance of the two models. The greater flexibility of TDboost may allow the model to capture nonlinear relationships between ESM predictors and local rainfall more effectively, but it may also make the model more sensitive to biased or noisy predictor patterns. In contrast, GLM-Tweedie has a more parsimonious structure but may be less able to adjust for nonlinear bias patterns in the ESM inputs. Future studies should compare raw and bias-corrected ESM predictors to determine whether bias correction further improves downscaling performance and model robustness.

Overall, these findings confirm that TDboost not only achieves lower prediction errors (RMSEP) but also shows greater consistency in representing rainfall patterns, as evidenced by stronger correlations with observed data. The choice of ESM plays a crucial role; BNU-ESM performed better when combined with TDboost, whereas MIROC-ESM-CHEM performed more compatibly with GLM-Tweedie. These results emphasize the importance of selecting both appropriate climate scenarios and statistical modeling approaches to achieve reliable rainfall predictions—an essential aspect for supporting climate adaptation planning and managing hydrometeorological risks in tropical regions. Following the strong performance of TDboost in terms of both predictive accuracy (RMSEP) and correlation strength, a time-series analysis was conducted to assess how well each model captures the temporal dynamics of observed

rainfall. The visual comparison between predicted and observed rainfall provides a more intuitive understanding of the models' consistency in tracking seasonal fluctuations and extreme events across stations and climate scenarios.

The stronger performance of TDboost can be explained from both statistical and climatological perspectives. Statistically, TDboost is more flexible than GLM-Tweedie because it combines the Tweedie loss function with tree-based boosting, allowing the model to capture nonlinear predictor–response relationships and interaction effects that are difficult to represent in a linear GLM framework. This flexibility is particularly relevant for tropical rainfall, where complex interactions among large-scale climate predictors, seasonal transitions, and local topographic effects influence rainfall occurrence and intensity. From a climatological perspective, TDboost's ability to better track rainfall peaks suggests that nonlinear models may be more suitable for representing highly variable rainfall regimes in complex tropical regions. This has practical implications for extreme rainfall monitoring and climate adaptation planning, because improved representation of high-rainfall periods can support more reliable hydrometeorological risk assessment, water resource planning, and local adaptation strategies. However, given the limited testing period, these implications should be interpreted cautiously and further validated using longer records and uncertainty-aware evaluation.

Based on the time-series visualization under the MIROC-ESM scenario (Figure 5), TDboost consistently demonstrated a superior ability to capture both seasonal and extreme rainfall patterns compared to GLM-Tweedie. At almost all stations, the orange line representing TDboost closely tracked observed rainfall trends, particularly during the testing period. In contrast, the blue line representing GLM-Tweedie often produced smoother, less responsive predictions to annual variability. This result confirms TDboost's greater ability to capture nonlinear patterns and better represent actual rainfall dynamics under the MIROC-ESM climate scenario.

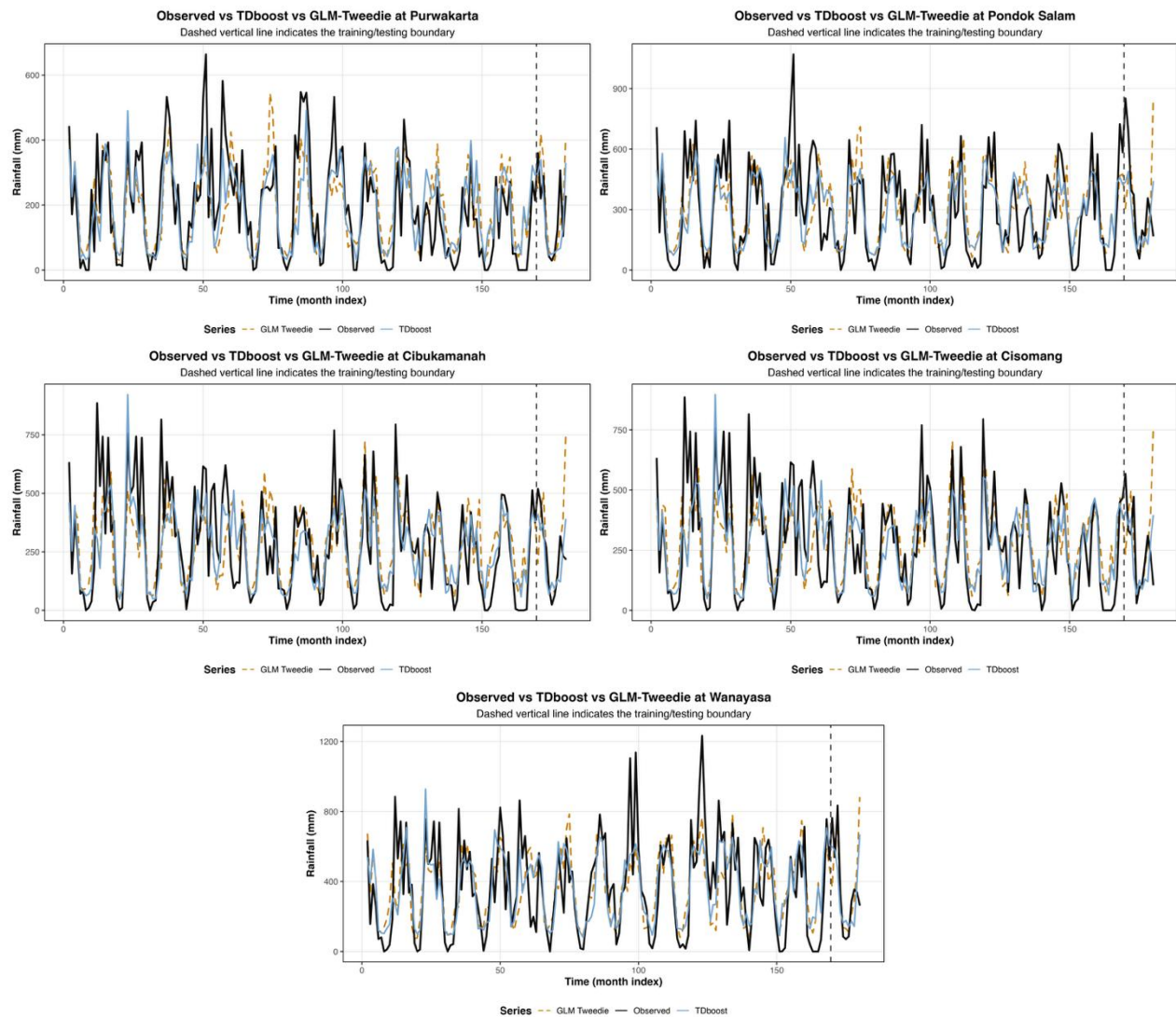


Figure 5. Comparison of Observed Rainfall and Model Predictions (TDboost vs GLM-Tweedie) under MIROC-ESM Scenario

Under the MIROC-ESM-CHEM scenario (Figure 6), the differences between the two models become even more pronounced. TDboost once again demonstrates a greater adaptability to rainfall patterns, with its prediction curves more closely following the observed data. Stations such as Wanayasa and Cisomang exhibit better alignment of TDboost predictions compared to GLM-Tweedie, particularly in capturing extreme rainfall events. This further supports previous findings that the boosting model, when optimally tuned, is well-suited for modeling complex, skewed rainfall distributions produced by the MIROC-ESM-CHEM scenario.

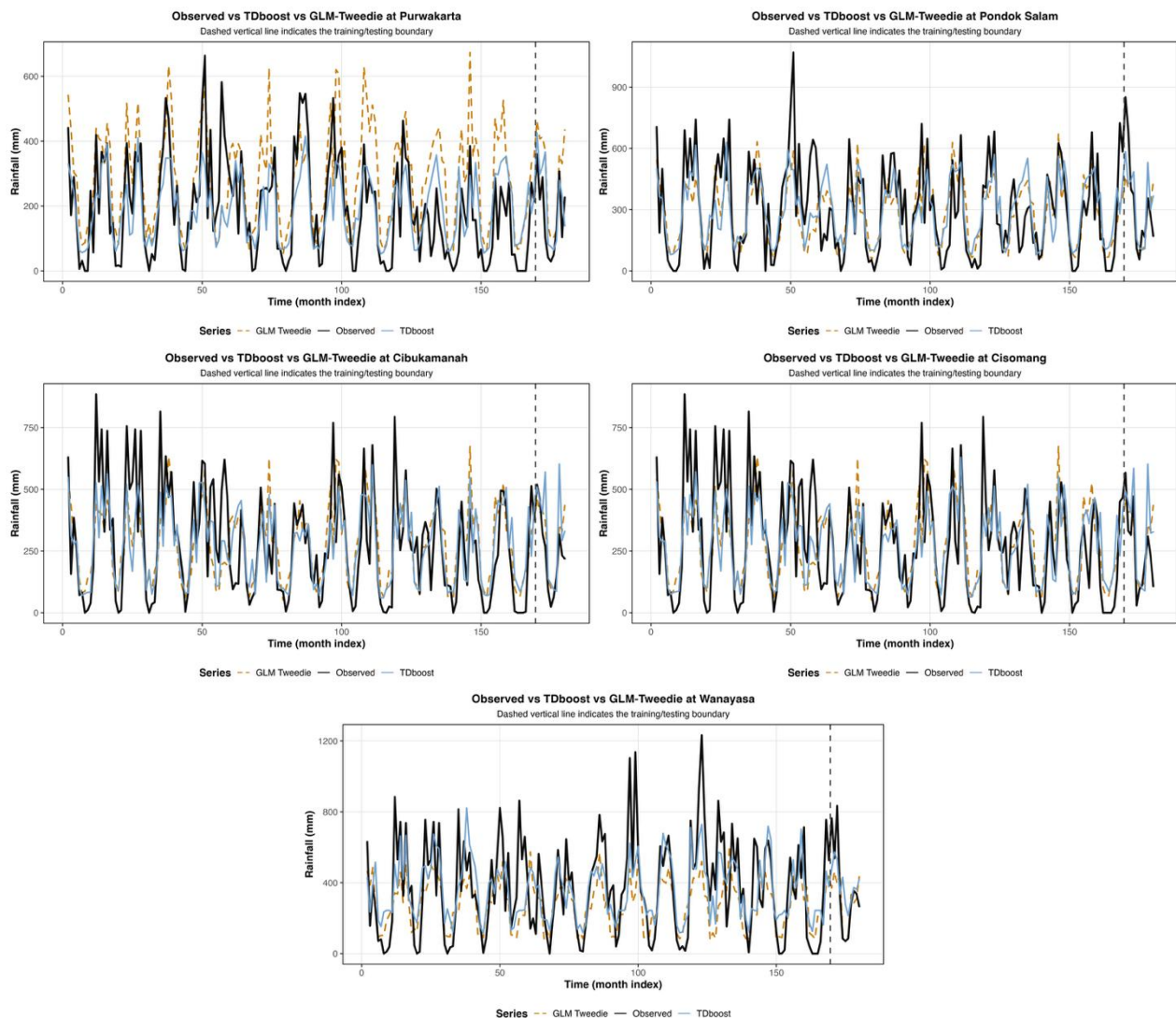


Figure 6. Comparison of Observed Rainfall and Model Predictions (TDboost vs GLM-Tweedie) under MIROC-ESM-CHEM Scenario

A similar pattern was also observed under the BNU-ESM scenario (Figure 7), where the TDboost model consistently maintained superior predictive performance compared with GLM-Tweedie. At several stations, such as Cibukamanah and Pondok Salam, TDboost more accurately replicated seasonal rainfall peaks, whereas GLM-Tweedie tended to smooth them and produce more conservative predictions. This advantage of TDboost highlights its flexibility in adapting to the spatial and temporal variability of rainfall data, even despite the uncertainty associated with the BNU-ESM input.

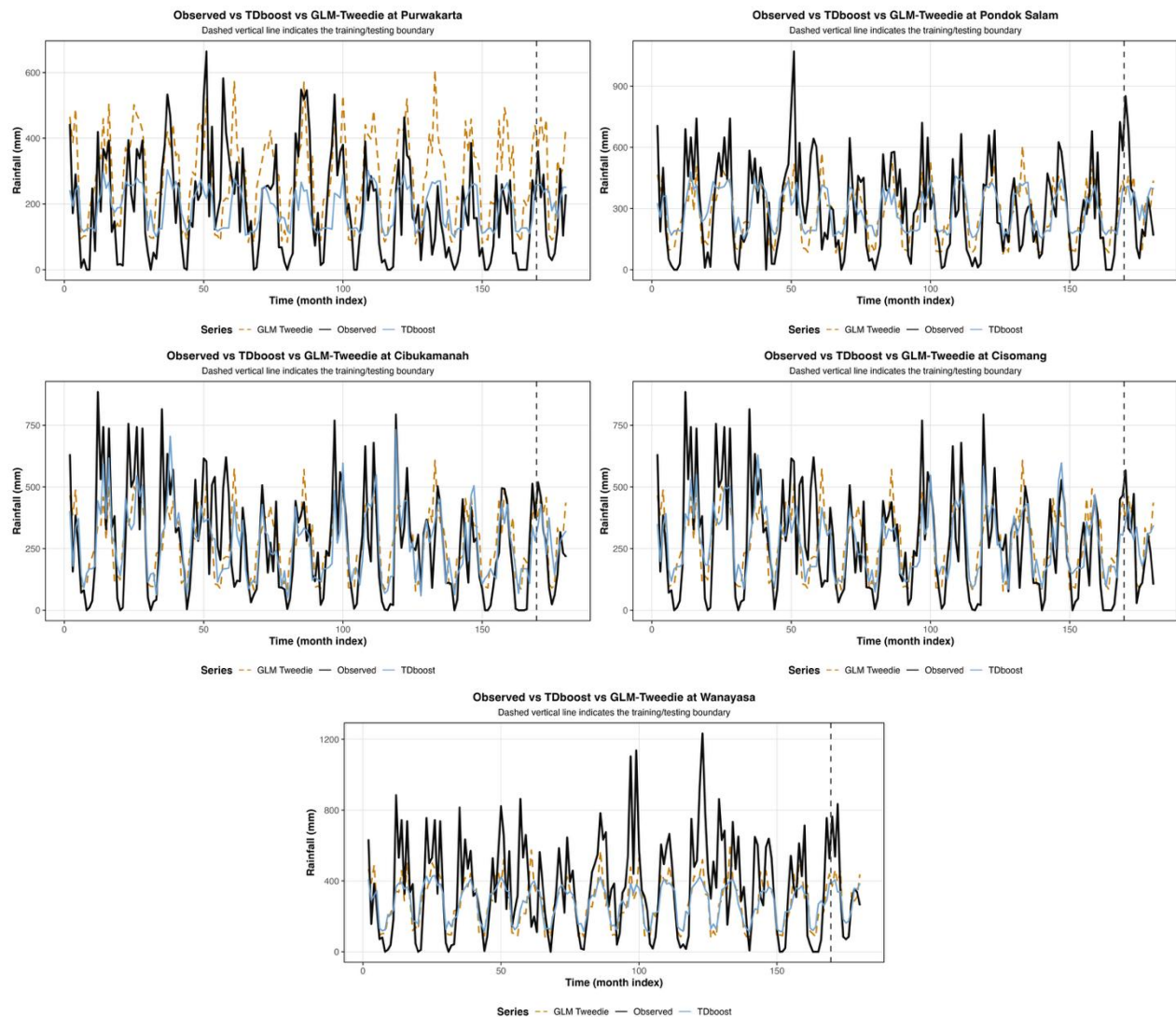


Figure 7. Comparison of Observed Rainfall and Model Predictions (TDboost vs GLM-Tweedie) under BNU-ESM Scenario

Overall, the visualization results across the three climate scenarios demonstrate that the TDboost model clearly outperforms the GLM-Tweedie model in approximating actual rainfall patterns. This superiority is evident not only in quantitative metrics such as RMSEP and correlation, but also qualitatively in the observed time-series patterns. Thus, the TDboost approach has proven more adaptive and accurate for modeling rainfall based on global climate projections, particularly for medium- to long-term forecasting in tropical regions.

This study has several limitations. First, the testing period covers only 12 months, from January to December 2020. Although this period represents one complete annual rainfall cycle and captures the broad wet-dry seasonal pattern in Indonesia, it may not fully reflect interannual rainfall variability. Therefore, the reported performance should be interpreted as an initial assessment of predictive ability rather than definitive evidence of long-term model generalization. Future studies should consider longer testing periods or more rigorous validation schemes, such as leave-one-year-out or blocked temporal cross-validation. Although no specific evidence of rainfall data disruption was identified for 2020, observational consistency during the COVID-19 period should also be examined carefully. Second, this study does not yet include formal uncertainty quantification. RMSEP and correlation summarize predictive accuracy and temporal agreement, but they do not provide uncertainty intervals related to model structure or ESM inputs. Future work should

incorporate bootstrap resampling, ensemble-based evaluation, or prediction intervals to better assess the reliability of downscaled rainfall estimates for climate risk applications.

4. Conclusions

This study demonstrates the effectiveness of applying Tweedie distribution-based models for statistical downscaling of monthly rainfall in tropical regions, using Purwakarta Regency, Indonesia, as a case study. By comparing the performance of a Generalized Linear Model (GLM) with a Tweedie distribution to a more flexible boosting-based approach (TDboost), the findings consistently show that TDboost outperforms GLM in terms of predictive accuracy (as indicated by lower RMSEP values) and the strength of association with observational data (as shown by higher Pearson correlation coefficients). These advantages are evident across most locations and climate scenarios, highlighting the superior capability of boosting methods in modeling highly variable and extreme rainfall patterns. Notably, the BNU-ESM climate scenario consistently provided the most stable and robust results, especially when paired with TDboost. In stations such as Cibukamanah and Purwakarta, this combination yielded the highest correlation with observed rainfall, suggesting that BNU-ESM projections align more closely with local rainfall characteristics compared with MIROC-ESM and MIROC-ESM-CHEM. These findings underscore the critical role of selecting an appropriate climate scenario to optimize the performance of statistical downscaling models, particularly in data-driven climate adaptation planning. For future research, incorporating spatial dependencies among locations should be considered, as explicitly modeling spatial relationships or integrating spatial features may enhance predictive performance, particularly in geographically interconnected areas. Moreover, extending the temporal resolution of the study (e.g., to daily or seasonal scales) and integrating additional atmospheric predictors, such as sea surface temperature or ENSO indices, could further improve model robustness and support the development of more reliable hydrometeorological early warning systems under climate change conditions.

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Enhancing Flowering and Nut Development of Tacunan Dwarf Coconut (*Cocos nucifera* L.) through Plant Growth Regulators

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Abstract: Coconut production in Central Visayas, Philippines, is increasingly constrained by climate variability, recurrent drought, and alkaline soils that disrupt the reproductive cycle and limit yield by reducing spathe emergence, flower development, flowering time, and nut retention. This study evaluated plant growth regulators (PGRs) for enhancing reproductive performance in the Tacunan dwarf coconut (*Cocos nucifera* L.). Using a randomized complete block design, four treatments were applied to 10-year-old palms: control, auxin (100 ppm), ethylene (100 ppm), and combined auxin–ethylene (50 ppm each). Treatments produced distinct responses. Auxin accelerated spathe emergence by 16 days and raised female flower production to 90.50 per palm by week 8, yielding earlier and more prolific flowering. Ethylene reduced flower number to 58.16, but shortened time to flowering to 88.75 days and improved nut retention to 70.50 nuts per month, versus 46.33 in the control. The combination treatment gave intermediate results, indicating responses constrained by dosage or hormonal antagonism. Button shedding and delayed flowering, typically induced by alkaline soil (pH 7.98) and drought, were partially mitigated by auxin and ethylene, leading to enhanced cell enlargement and regulation of pollination. Although the four-month duration was insufficient to assess effects on nut size and weight, the results provide novel empirical evidence that PGR interventions improve coconut reproductive performance and establish a baseline for long-term strategies to stabilize coconut yields under stress-prone conditions.

Keywords: Tacunan dwarf coconut; auxin; ethylene; reproductive performance; alkaline soil

1. Introduction

Coconuts have become increasingly popular recently due to the growing promotion of virgin coconut oil, coconut water, and inflorescence sap as nutritious and health-friendly products [1]. The perennial nature of palms hinders successful breeding efforts, as does their greater level of heterozygosity, their lengthy juvenile period, the need for a wider area, and the need for more time for testing. Mutagenesis combined with molecular-marker selection has been applied to broaden genetic variation and accelerate improvement in other ornamental and horticultural species [2]. Thus, the quantity of female flowers within an inflorescence plays a key role in determining coconut fruit set [3, 4]. The Sri Lanka Green Dwarf (SLGD) is self-pollinating and is sensitive to abiotic

stress. Hence, SLGD was crossed with tall coconut varieties (Sri Lanka Tall) to improve fruit set even when the number of female flowers was limited [5]. However, the dwarf coconut has the advantage of having a shorter trunk, which can make harvesting less time-consuming [6]. Coconuts may grow in a variety of soil types, from sandy to clay. It does, however, favor rich, well-drained soils that can hold a lot of water and are at least 75 cm deep. The palm can withstand soil pH levels between 5.0 and 8.0. However, a pH range of 5.5 to 6.5 is optimal for growth [7]. In the study of [8], the soils in Barili, Cebu, range in color from dark brown to yellowish brown, have a high clay content (46–70%), have a water-holding capacity (>40%), and are hard when dry but plastic and sticky when wet. Due to their extremely high CaCO_3 content (>60%), moderate to high CEC (21–53 meq/100 g), and high base saturation (>50%), the soils have an alkaline pH (7.5).

Based on the current findings, the production of key non-food and industrial commercial crops in Cebu totaled 45,236.93 metric tons in the first semester of 2024, representing a 60.48% decline, according to [9]. Severe impacts on coconut yield were associated with declines in nut quality, soil fertility, seasonal patterns, and labor requirements, while moderate impacts were linked to extreme weather events, water shortages, and heat stress [10]. The use of plant growth regulators (PGRs) can potentially improve flowering and nut development in coconuts under drought stress. In the study of [5], Sri Lanka Green Dwarf palms experienced a 26% to 46% reduction in female flower production due to intense water shortage during the -2 developmental stage, compared to palms that were not exposed to stress, regardless of whether other stages encountered heat or water. To lessen the negative effects, the current study will explore the use of two PGRs, auxin and ethylene. Auxins (Indole-3-Acetic Acid, IAA) are known to influence flower initiation and fruit development and may promote fruit setting and nut growth in coconuts. Auxin regulates multiple aspects of plant growth and development, such as seed germination, stem elongation, flower initiation, and fruit expansion [11]. Transcriptome analysis in oil palm has shown that auxin treatments can induce parthenocarpic fruit sets, with candidate genes identified [12]. Beyond oil palm, plant growth regulators have enhanced growth, yield, and propagation across diverse horticultural crops, including gibberellic and salicylic acid treatments in kale [13], indole-3-butyric acid and gibberellic acid in caladium [14], and benzyladenine and indole-3-butyric acid in cactus [15]. However, its direct effect on coconut nut retention and female flower production has not been established. Ethylene releasers, such as Ethephon, can help synchronize flowering and promote early fruit maturity. In fruit crops, ethylene plays a pivotal role in controlling fruit sets at distinct stages of the flower-to-fruit transition [16]. Additionally, exogenous ethylene application accelerated oil palm fruit ripening, increased oil yield, and decreased free fatty acids [17], but reproductive synchronization has not been systematically tested in coconut. These limitations highlight the need for targeted research on PGRs in coconut reproductive biology, which this study aims to address.

The Tacunan dwarf coconut variety will be used as a test crop in the experiment. It begins fruiting early, often producing its first bunches with nuts nearly touching the ground in less than two years under optimal conditions. On average, each palm yields around 94 nuts annually, with the potential to reach up to 204 nuts per year, and each nut contains more than 250 grams of copra [7]. Therefore, this study aims to evaluate the effects of plant growth regulators, specifically auxin and ethylene, on the flowering behavior and nut development of the Tacunan dwarf coconut variety. By exploring their influence on reproductive growth, the research seeks to determine whether PGRs can help overcome soil-related constraints and improve coconut productivity in stress-prone areas like Barili, Cebu, and during prolonged droughts, when rainfall levels drop by over 60%.

2. Materials and Methods

2.1 Soil and Climate Baseline Data Collection

Composite samples were collected across the experimental area before treatment application to establish baseline soil conditions. The soils were air-dried, sieved, and sent to the Department of Agriculture in Tacloban City for analysis. Composite samples (0–30 cm depth) were analyzed for pH, organic matter, and available macronutrients (N, P, K) following standard protocols. Data on rainfall and temperature during the study period were obtained from [18], based on records from the nearest weather station in Cebu.

2.2 Treatments and Experimental Design

The study was conducted at the coconut farms of Cebu Technological University–Barili Campus, located in Barangay Kalubihan, Barili, Cebu. The PCA VII identified the coconut variety as Tacunan dwarf. A Randomized Complete Block Design (RCBD) was employed, incorporating two plant growth regulator (PGR) treatments and a control group. There were two samples per treatment with four replications. PGRs were applied in various types and combinations to examine their effects on flowering, nut development, and overall yield. Treatments were administered both individually and in combination. The coconut trees selected were at bearing age, approximately 10 years old. The study was conducted from July 09 to November 09, 2025. The concentrations selected for this study were guided by prior research and experimental reasoning. Applying auxin at 100 ppm enhanced fruit set in oil palm female inflorescences [12], providing a clear benchmark for perennial palms. Although no published work has reported the use of 100 ppm ethylene in coconut, this level was adopted as a practical reference based on evidence of ethylene efficacy in annual crops [16] and to maintain consistency with the auxin treatment. In addition, the combined application of auxin and ethylene at 50 ppm each was designed to explore possible synergistic effects while reducing the risk of hormonal imbalance. Overall, the chosen concentrations are biologically relevant, supported by literature, and feasible for field application, while also addressing the existing gap in coconut reproductive biology.

The treatments include:

- t1- control (mineral water)
- t2- Auxin (naphthalene acetic acid) at 100 ppm
- t3- Ethylene (2-chloroethylphosphonic acid) at 100 ppm
- t4- Auxin at 50ppm + Ethylene at 50ppm

2.3 Application of Treatments

The application process begins by drenching the soil with 1 L of auxin solution, depending on the treatment. One week later, ethylene of a 500 ml solution was sprayed onto the foliage and reapplied every two weeks thereafter. Auxin (NAA) application via soil drenching ensures systemic uptake through the root system. According to [19], soil drench applications of auxin significantly improved overall root quality in ornamental perennial plants. Ethylene (2-chloroethylphosphonic acid) is a gaseous plant hormone; in practice, it is applied as ethephon via foliar spraying to ensure direct leaf contact. This allows rapid absorption and decomposition into ethylene gas within leaf tissues, quickly triggering physiological responses such as flowering, fruit ripening, or abscission. Foliar application of ethylene promoted early flower induction and increased the number of flowers in mungbean [20]. Auxin and ethylene were applied only during the initial two months of the study. The subsequent two months were allocated for data monitoring and observation.

2.4 Data Gathering Procedures

Before treatment application, the inflorescence development of each ten-year-old coconut tree was documented to establish a baseline. Following the application of plant growth regulators, developmental progression was monitored and evaluated against the initial stage, using the chart of critical coconut bunch development stages documented during the study and based on references [5, 21]:

The following data were collected:

2.4.1 The Number of Days to First Visible Spathe Emergence

This metric records the number of days from a defined starting point (e.g., the visible emergence of the youngest leaf in the crown or the initiation of the inflorescence primordium if microscopic observation is feasible) until the spathe (the boat-shaped, protective sheath enclosing the developing inflorescence) becomes clearly visible, protruding from the leaf axil. At this stage, the spathe is typically green and tightly enclosed, but its distinct form is apparent.

2.4.2 The Number of Days to Flower (from spathe emergence to spathe opening)

This measurement refers to the number of days from the initial observation of the spathe emergence (as described above) until the spathe fully splits open longitudinally, revealing the individual flower-bearing

branches (rachillae) within. This splitting marks the beginning of the anthesis or flowering period, as the male flowers typically open and shed pollen shortly after. This stage indicates readiness for pollination.

2.4.3 The Number of Female Flowers per Tree per Week

This parameter was assessed by directly counting the pistillate (female) flowers present on each emerged inflorescence. The female flower, often referred to as a "button" in its immature stage, is a small, typically spherical to subglobose body, approximately 1-2.5 cm (0.4-1 inch) in diameter, with a distinct resemblance to a miniature coconut.

2.4.4 The Number of Nuts per Tree per Month

This parameter was assessed by counting the total number of mature and developing nuts within each fruiting bunch on the tree. A "fruiting bunch" refers specifically to the inflorescence during its fruit-setting stage, which was previously termed "inflorescence" at the flowering stage. Data was recorded at two-week intervals.

2.4.5 The Nut Size at Harvest (cm)

For both young (6–9 months after spathe emergence) and mature (11–12 months after spathe emergence) coconuts, the equatorial diameter of each harvested nut was measured using a flexible tape. This involves recording the widest horizontal span across the nut taken at its midpoint and perpendicular to the vertical axis while the nut remains enclosed in its husk.

2.4.6 The Single Nut Weight (g) at harvest

The weight of each harvested nut was determined immediately upon collection using a calibrated electronic balance and recorded to the nearest 0.1 gram. This measurement will include the entire nut, with its husk intact, consistent with other harvest assessments.

2.4.7 Data Analysis

Analysis of Variance (ANOVA) was used to analyze the data gathered using the Statistical Tool for Agricultural Research (STAR). A post hoc test (Tukey's HSD) was utilized to compare treatment means and identify the best-performing techniques.

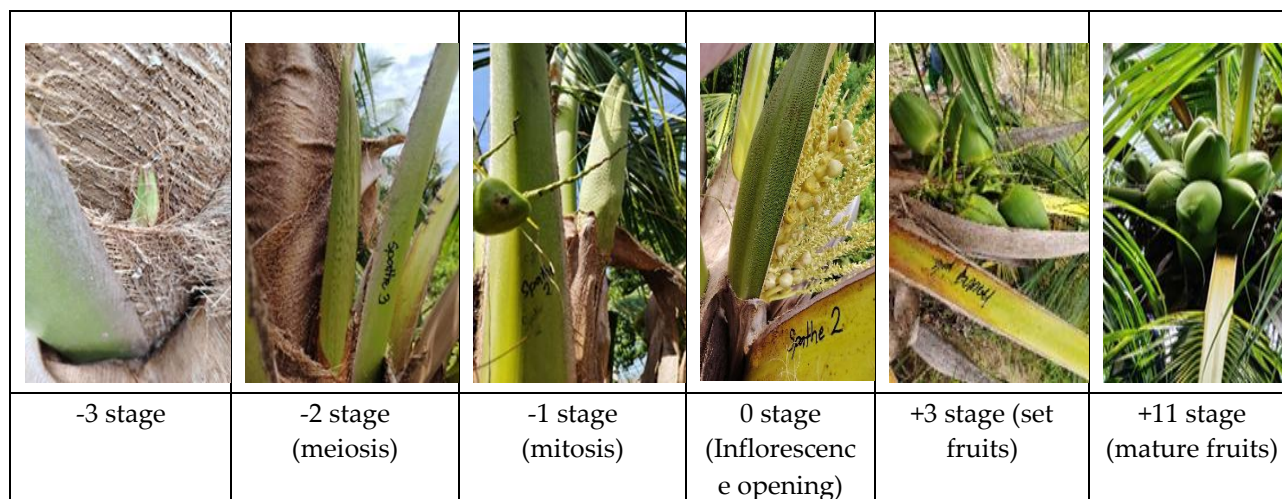


Figure 1. Critical Development Stages of the Coconut Bunch

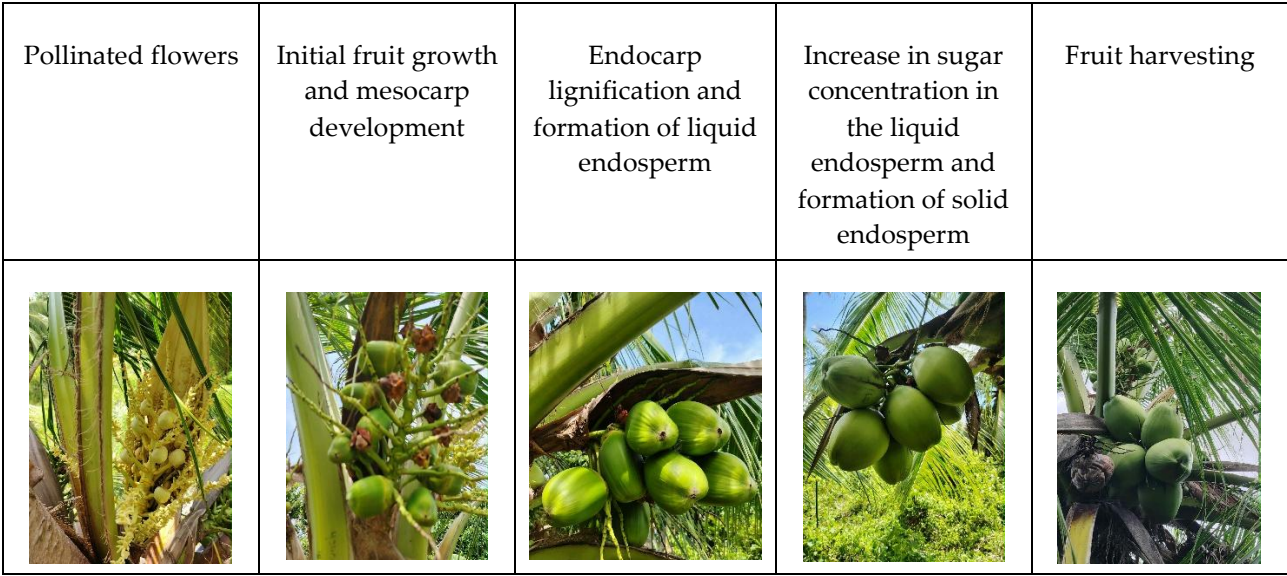


Figure 2. Development Stages of the Nut

3. Results and Discussion

3.1 Soil Chemical Properties and Climatic Conditions (July-November 2025)

Table 1. Analysis on soil chemical properties and the climatic conditions at the experimental site, barili, cebu, during July-November 2025.

Parameter	Value	Unit
pH	7.98	-
Organic Matter	2.06	%
Available nitrogen	0.10	%
Total Nitrogen	0.12	%
Available Phosphorus	7.55	mg/kg
Exchangeable Potassium	0.28	cmol/kg
July 2025 (Rainfall)	210	mm
August 2025 (Rainfall)	185	mm
September 2025 (Rainfall)	195	mm
October 2025 (Rainfall)	220	mm
November 2025 (Rainfall)	160	mm
July-November (mean temperatures)	28.5	°C

Results revealed that the soil is alkaline (pH 7.98), with low nutrient availability, fluctuating rainfall (160–220 mm/month), and elevated temperature (28.5 °C). These findings further confirm that coconut plantations in the area are subject to environmental stress. Such conditions tend to intensify button shedding and reduce spathe emergence, flowering time, and development, and nut set. Application of PGRs (auxin and ethylene) mitigates the adverse effects of drought [22]. Although previous studies demonstrated that ethylene signaling pathways are centrally involved in plant adaptation to abiotic stresses such as drought, salinity, and alkaline conditions [23], direct applications of ethylene and auxin on coconut palms are limited under alkaline soil and drought stress conditions. The present study provides novel empirical evidence by assessing auxin and ethylene levels in coconut palms grown in alkaline soil under drought stress.

3.2 Reproductive Cycle

3.2.1 Average Number of Days to First Visible Spathe Emergence

The application of plant growth regulators significantly influenced the number of days to the first visible spathe emergence in Tacunan dwarf coconut (Table 2). Visibly emerging spathes were recorded as being in the -3 stage, counted from the day of emergence.

Table 2. Average number of days to first visible spathe emergence of tacunan dwarf applied with plant growth regulators

Treatment	Average Number of Days to First Visible Spathe Emergence
t ₁ - water only	22.16 ^b
t ₂ - auxin alone (100 ppm every two weeks)	16.00 ^a
t ₃ - ethylene alone (100 ppm every two weeks)	18.66 ^{ab}
t ₄ - combination (100 ppm every two weeks)	16.83 ^{ab}
%CV	14.69

Note: Means with different letters are significantly different at 5% level ($p < 0.05$)



Figure 3. Image of the spathe at the -3 stages of emergence.

Among the treatments, auxin alone (t₂) recorded the earliest spathe emergence, averaging 16.00 days, and was statistically faster than the water-only or control (t₁), which had the longest emergence time at 22.16 days. Treatments involving ethylene alone (t₃) and the combination of auxin and ethylene (t₄) showed intermediate effects, with means of 18.66 and 16.83 days, respectively. The outcomes correspond to those of [24], where treatments with NAA induced more than 95% of bunch formation, with 300 ppm NAA reaching 100% bunch formation. Additionally, auxin plays an important role in stimulating cell expansion and stem growth [25]. The study suggests that while auxin alone was most effective in accelerating spathe emergence, combining it with ethylene or using ethylene alone may offer moderate benefits. Overall, the findings highlight auxin's potential as a key regulator in promoting earlier reproductive development in coconut palms.

3.2.2 Average Number of Days to Flower

Table 3 summarizes the average number of days to flower, measured from spathe emergence to spathe opening. The results revealed clear differences among treatments.

Table 3. Average number of days to flower of tacunan dwarf applied with plant growth regulators.

Treatment	Average Number of Days to Flower
t ₁ - water only	97.75 ^a
t ₂ - auxin alone (100 ppm every two weeks)	89.88 ^b
t ₃ - ethylene alone (100 ppm every two weeks)	88.75 ^b
t ₄ - combination (100 ppm every two weeks)	91.38 ^{ab}
%CV	3.84

Note: Means with different letters are significantly different at 5% level ($p < 0.05$).

Ethylene alone produced the shortest time to flower at 88.75 days, which was markedly earlier compared to the control (97.75 days). Auxin alone also promoted earlier inflorescence at 89.88 days, showing only a slight difference from the ethylene treatment but still significantly earlier than the control. The combination of auxin and ethylene resulted in 91.38 days to flower, which was lower than the control but not as pronounced as the individual applications of auxin or ethylene. This result coincides with the study of [26], wherein, in 1968, pineapple plants were induced to flower using 2-chloroethylphosphonic acid (ethephon), an agent that releases ethylene and successfully promotes earlier flowering. Historically, whether through natural or artificial induction of flowering or its suppression, ethylene has remained the central regulator. At present, large commercial plantations routinely apply ethephon or ethylene weekly to synchronize pineapple production.

3.2.3 Average Number of Female Flowers per Tree per Week

Table 4 shows the average number of female flowers per tree per week for Tacunan dwarf coconuts treated with plant growth regulators. Some of the older spathes were recorded at the -1 stage (mitosis), which opened rapidly during the early weeks. Others were at the -2 stage (meiosis), while the remaining were at the -3 stage. Each spathe stage requires a different number of days or weeks before opening [27], which explains the variation in the number of female flowers observed. The timing of spathe opening influences this variation, the number of female flowers present, the transitioning of flowers to immature nuts, and the extent of button shedding [28, 29].

Table 4. Average number of female flowers per tree per week of tacunan dwarf applied with plant growth regulators

Treatment	Average Number of Female Flowers per Tree per Week								
	W0	W2	W4	W6	W8	W10	W12	W14	W16
t1- water only	32.33	35.66 ^b	55.00	56.33	83.66 ^{ab}	63.88 ^a	39.50 ^a	68.88 ^{ab}	64.50 ^{ab}
t2- auxin alone	31.50	55.50 ^a	60.66	63.33	90.50 ^a	52.50 ^{ab}	33.12 ^{ab}	73.25 ^a	71.75 ^a
t3- ethylene alone	32.33	39.83 ^b	52.83	43.16	58.16 ^c	36.50 ^b	25.12 ^b	60.12 ^b	58.12 ^b
t4- combination	27.00	43.50 ^b	54.83	44.16	64.00 ^{bc}	41.50 ^{ab}	27.12 ^b	61.12 ^b	59.12 ^b
%CV	21.03	14.99	20.32	27.96	20.72	22.36	15.01	6.06	6.32

Note: Means with different letters are significantly different at the 5% level ($p < 0.05$). For weeks where $p > 0.05$, no significant differences were observed among treatments.



Figure 4. Image of the spathe at the -3 stage, -2 stage (meiosis), and -1 stage (mitosis) of emergence.

The data revealed no significant differences in weeks 0, 4, and 6, but the treatments were significantly different in weeks 2, 8, 10, 12, 14, and 16. However, between weeks 10 and 12, flower counts decreased, as several flowers had already transitioned into immature nuts. By week 14, the emergence and opening of spathes accounted for the observed increase in female flowers. The number of female flowers in the control group (t1) increased consistently, from 32.33 at week 0 to 83.66 at week 8. At week 10, the control group recorded the highest number of female flowers at 63.88, indicating the slowest transition of flowers into immature nuts. By week 16, the control group averaged 64.50 female flowers, ranking second to the auxin treatment. This suggests a natural increase in female flowers even without the intervention of growth

hormones. On the other hand, auxin alone (t2) showed a notable increase in the number of female flowers. As early as week 2, the recorded number was 55.50, which was significantly higher than the control group's average of 35.66. Female flowers continued to rise, reaching an average of 90.50 by week 8. Between weeks 10 and 12, the auxin-alone group recorded the greatest number of female flowers transitioning into immature nuts, which explains its relatively low count of 33.12 flowers at week 12. However, as the weeks progressed, this treatment consistently produced the highest number of female flowers, averaging 71.75 at week 16. This suggests that auxin successfully and effectively promotes female flower production. According to [30], auxins play a crucial role in regulating numerous developmental processes in plants, which include the initiation of lateral roots, the development of leaves, and floral structures. Moreover, naphthalene acetic acid alone increased pistillate flowers in cucumber [31].

The observed fluctuation in female flower numbers between weeks 8 and 10 is due to the coconut's natural reproductive transitions. While flowers are highly receptive at week 8, over the subsequent two weeks, some of these flowers either initiate nut development or undergo button shedding, thereby reducing the visible count. The decline in female flower counts during the subsequent two weeks corresponds to the number of nuts recorded in the second and third months of the study. Early dropping of nuts, or premature abscission, is a standard physiological feature of coconut reproduction. Under standard field conditions, it is typical to observe loss rates of 10%-20% [32]. Thus, the fluctuations observed between weeks 8 and 10 align with the biological processes of nut initiation and button shedding, rather than random variability. Conversely, ethylene alone (t3) had the opposite result. The number of female flowers increased slightly during the initial weeks but decreased significantly by week 6, with a mean of 43.16. This count was clearly lower compared to the control group's 56.33. By week 8, this treatment had the fewest female flowers (58.16) among the groups, indicating that ethylene reduces female flower development over time. Over successive weeks, female flowers in the ethylene-alone group consistently showed the lowest counts, particularly at weeks 14 to 16, with averages of 60.12 and 58.12, respectively. Ethylene and precursors may have caused flower abscission in some fruits, such as apples, citrus, and grapevines [33]. Also, the study noted the prevalence of rhinoceros beetles and coconut earwigs, which greatly affected the flowers and nuts of dwarf coconuts. These pests were found on the coconut palms treated with ethylene alone. Although the extent of damage was not systematically quantified per treatment, the observed reproductive trends remain consistent with coconut biology, suggesting that pest pressure did not invalidate the overall findings. Future larger-scale studies should systematically monitor pest incidence, quantify damage per treatment, and clarify treatment effects to distinguish PGR responses from confounding biotic stress.



Figure 5. Image rhinoceros beetles and coconut earwigs

The combination of auxin and ethylene (t4), however, had a varied effect. Similar to the ethylene-alone treatment, ethylene in this group resulted in a lower female flower count at week 6, with a mean of 44.16, compared to the control group. By week 8, the number of female flowers was 64.00, slightly higher than the ethylene-alone group but still significantly lower than the control group. At weeks 14 and 16, the number of flowers was comparable to that in the ethylene-alone group, averaging 61.12 and 59.12, respectively. Previous studies suggest that ethylene has both positive and negative effects on auxin homeostasis. Ethylene can

interfere with auxin signaling and transport, leading to premature abscission of female flowers [34]. While these processes may provide a credible explanation for the observed results, the current study's experimental design does not permit a clear separation of dose effects from potential hormonal interactions. Because the concentrations used in the combination treatment were 50 ppm for each compound, it remains uncertain whether the reduced flowering response of the nuts reflects true antagonism or simply the lower auxin concentration compared to the 100 ppm auxin-alone treatment.

3.3 Yield and Yield Components

3.3.1 Average Number of Nuts per Tree per Month

Based on observations, some bunches were recorded at the +3 stage, characterized by fruit set, endocarp lignification, and the formation of liquid endosperm. Other bunches were classified in the stage of increasing sugar concentration in the liquid endosperm and the initial formation of solid endosperm. Additionally, some bunches were recorded at the +11 stage, indicating mature fruits that are ready for harvest. Variation in the number of nuts was observed due to the shedding of immature nuts and the transition of female flowers into immature nuts, especially in the third month. The data revealed significant differences among treatments in the first and second months, but none in the third and fourth months. Auxin and ethylene application did not result in abortion of existing nuts. Nonetheless, given the study's limited 4-month duration, it remains inconclusive whether these treatments directly contribute to the observed increase in nut number.

Table 5. Average number of nuts per tree per month of tacunan dwarf applied with plant growth regulator

Average Number of Nuts per Tree per Month				
Treatment	1 st Month	2 nd Month	3 rd Month	4 th Month
t ₁ - water only	46.33 ^b	38.17 ^b	74.75	71.12
t ₂ - auxin alone (100 ppm every two weeks)	50.33 ^{ab}	46.00 ^{ab}	97.12	94.12
t ₃ - ethylene alone (100 ppm every two weeks)	70.50 ^a	66.00 ^a	90.12	87.00
t ₄ - combination (100 ppm every two weeks)	58.67 ^{ab}	54.83 ^{ab}	84.75	80.88
%CV	18.35	19.82	23.25	22.96

Note: Means with different letters are significantly different at the 5% level ($p < 0.05$). For months where $p > 0.05$, no significant differences were observed among treatments.

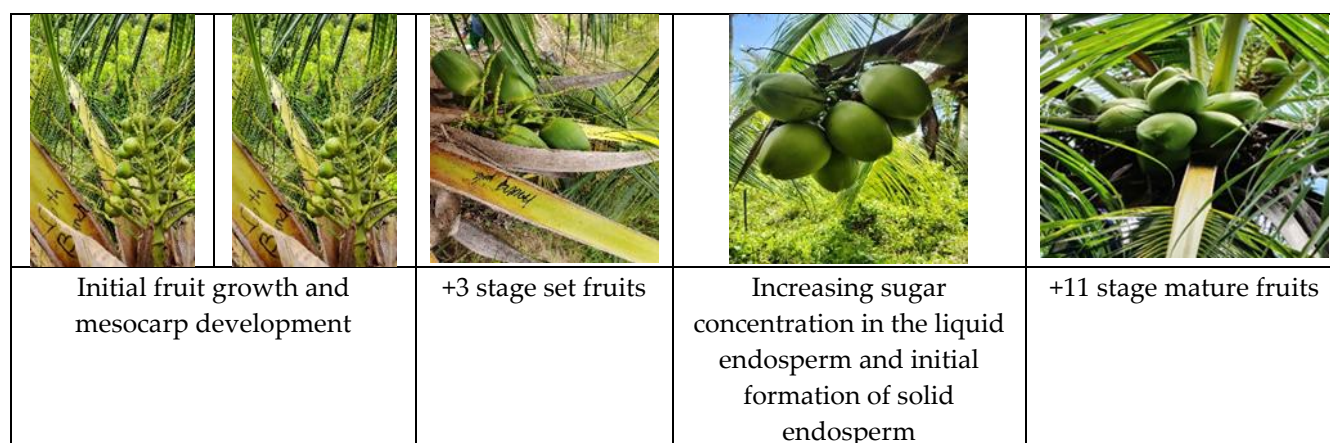


Figure 6. Development stages of the tacunan dwarf coconut.

In the first two months, ethylene alone outperformed all other treatments, yielding an average of 70.50 nuts in the first month and 66.00 in the second month. These values were notably higher than those of the control group, which recorded 46.33 nuts in the first month and declined slightly to 38.17 in the second month. This supports the findings of [11], who reported that pollination triggers genes responsible for ethylene

production, a hormone essential for the development of the ovary, ovules, and gametophytes in orchids. This genetic response is carefully regulated, occurring at specific times and in specific tissues during flower development, and forms the basis of successful fruit set. Furthermore, ethylene signaling during pollination modifies pollen tube growth by regulating cell wall remodeling and calcium gradients in tomato, thereby increasing fruit set [35]. During the third and fourth months, however, the auxin-alone treatment produced the highest absolute values, peaking at 97.12 nuts in the third month and 94.12 in the fourth month, although the differences were not statistically significant. Similar effects were reported in pepper treated with NAA, where auxin application increased fruit set [36]. Likewise, [37] found that applying 4-chlorophenoxyacetic acid, a synthetic growth regulator with auxin-like properties, enhanced fruit set in summer-grown tomato, resulting in a higher overall yield. Meanwhile, the combination of auxin and ethylene consistently produced more nuts than the control, with averages of 58.67 and 54.83 in the first and second months and 84.75 and 80.88 in the third and fourth months, respectively. These findings suggest that ethylene and auxin, whether applied individually or in combination, may play a critical role in enhancing nut development and retention, thereby increasing yield.

3.3.2 Average Nut Size (cm) and Nut Weight (g) at harvest

Nuts classified at the +11 stage, representing mature fruits, were harvested and measured for nut size and nut weight. These measurements provide descriptive baseline information on the experimental palms during the study period. Given that coconut nut development requires approximately 11–12 months from pollination to maturity, and the present study lasted only 4 months with PGR application limited to the first 2 months, the harvested nuts were already well developed before the experiment began. Therefore, these physical measurements of the nuts do not reflect the influence of PGRs.

Table 6. Average nut size at harvest and average nut weight at harvest of tacunan dwarf applied with plant growth regulators

Treatment	Average Nut Size at Harvest (cm)	Average Nut Weight at Harvest (g)
t ₁ - water only	55.42	2764.70
t ₂ - auxin alone (100 ppm every two weeks)	59.77	2896.04
t ₃ - ethylene alone (100 ppm every two weeks)	59.20	2579.05
t ₄ - combination (100 ppm every two weeks)	58.08	2721.57

These data serve as a baseline characterization of the palms under experimental conditions, providing a benchmark for future investigations. To determine whether plant growth regulators affect nut initiation, development, and yield components, longer-term studies covering a full reproductive cycle are necessary.

4. Conclusions

This study provides preliminary evidence suggesting that auxin may enhance female flower production and that ethylene may influence flowering time, nut set, and retention in coconut palms under alkaline soil and drought stress. However, given the limited sample size and high variability among experimental units, the results should be regarded as preliminary. While the treatments also revealed promising effects on nut weight and size, the dataset precludes broad generalizations as treatments were applied when the nuts were already developing. Observations from the combination treatment showed a reduced flowering response compared to auxin alone, but the experimental design does not permit clear separation of dose effects from potential hormonal interactions. Additionally, the lack of systematic documentation of pest incidence represents an additional limitation that may have contributed to variability in reproductive responses. By highlighting the potential role of plant growth regulators in reproductive development, the present study helps address a gap in coconut physiology research. These findings may serve as a baseline for future, larger-scale investigations that can more rigorously validate and confirm the observed effects under diverse field conditions.

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Comparative Evaluation of Ground Resistance Measurement Techniques and FEM-Based Modeling under Grounding Electrode Configurations

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Abstract: Accurate estimation of ground resistance is essential for designing safe and reliable electrical grounding systems. This study investigates the performance of four representative electrode configurations—single rod, two parallel rods, three rods in a linear arrangement, and three rods in a delta arrangement—by combining experimental measurements with numerical modeling. The ground resistance values were measured using the 3-pole Fall-of-Potential (FOP) method and the 4-pole Wenner method and compared with results simulated via the Finite Element Method (FEM) implemented in the COMSOL Multiphysics Program. The analytical solutions were set under homogeneous soil conditions at the test site. The soil resistivity was first determined experimentally to establish the boundary conditions for the FEM model. The numerical model's accuracy was confirmed by simulation results that closely matched field measurements, with a relative error of less than 3%. Moreover, the results revealed that increasing the number of grounding rods effectively reduces total earth resistance. However, the reduction is nonlinear due to mutual coupling between the adjacent electrodes. The delta configuration provided the lowest resistance, followed by the three-rod linear arrangement. Overall, the study demonstrates that FEM analysis achieves high accuracy in predicting ground resistance under uniform soil conditions. The integration of numerical and experimental approaches supports the development of cost-effective and technically robust grounding designs for high-voltage and power distribution systems.

Keywords: Ground resistance; soil resistivity; electrode configuration; finite element method (FEM); grounding system design

1. Introduction

The design and performance of grounding systems are critical for the safe and reliable operation of electrical power networks, high-voltage installations, and communication infrastructures, etc [1]. Measuring ground resistance is important for ensuring safety and efficiency in electrical systems. The effectiveness of such systems is strongly influenced by two key factors, soil resistivity and electrode configuration, which vary widely with soil type, moisture content, temperature, and compaction. The measurement generally uses an earth tester with two main methods: the fall-of-potential method [2, 3],

which requires driving 3-4 metal rods into the ground, and the 4-pole Wenner method, which measures soil resistivity using four probes in a straight line, equally spaced. Although both techniques are widely applied in grounding studies, these serve distinct and complementary purposes in system design [4]. Numerous standards, such as IEEE Std. 81 [5], provide guidelines and analytical formulations for evaluating earth resistance under steady-state conditions.

However, in practical applications, especially under diverse soil conditions and complex electrode arrangements, grounding system behavior often deviates from simple theoretical assumptions. Previous studies have highlighted the limitations of analytical models when applied to heterogeneous soils and non-traditional electrode geometries [6-8]. For instance, investigations on ground resistance with copper-clad steel demonstrated significant variations in measured ground resistance depending on soil characteristics and electrode arrangements. Similarly, finite element method (FEM) simulations have been employed to evaluate the potential distribution and resistance of multiple vertical rods, showing close agreement with analytical solutions for homogeneous soil while offering improved accuracy for electrode arrangement models [9, 10]. Despite extensive research, comparative studies that systematically evaluate different electrode configurations using both measured methods and numerical FEM simulations remain limited [11, 12].

This paper examines the electrical performance of 4 practical grounding-electrode configurations: a single vertical rod, two rods connected in parallel, three rods arranged linearly, and three rods arranged in a triangular (delta) layout. The ground resistance of each configuration was determined through field measurements using both the three-pole fall-of-potential (FOP) and the four-pole Wenner techniques [13]. The experimental findings were then compared with numerical results from a finite-element analysis carried out in COMSOL Multiphysics. Linking field measurements to FEM simulations provides clearer insight into how electrode geometry influences earth resistance under realistic soil conditions, thereby supporting more efficient and reliable grounding-system design.

2. Methods

2.1 Theoretical Approach

The theoretical basis for grounding resistance is derived from classical electromagnetic field theory and soil resistivity models. For a uniform soil model, the steady-state ground resistance with improved agreement observed in refined formulations can be approximated by the Dwight-Sunde approximation [2, 3]:

$$R = \frac{\rho}{2\pi L} \left[\ln \left(\frac{8L}{d} \right) \right] - 1. \quad (1)$$

Where R is ground resistance (Ω), ρ is the soil resistivity ($\Omega \cdot m$), L is the electrode length (m), and d is the electrode diameter (m). This expression assumes homogeneous soil and neglects surface effects. In multi-rod systems, the mutual resistance effect is accounted for by potential superposition [14]. This theoretical framework supports the observed nonlinear reduction in resistance with increasing electrode count, which is further validated by FEM simulation results showing that mutual coupling reduces effectiveness. For soil resistivity, it was measured and calculated using the Wenner four-pin method as expressed:

$$\rho = 2\pi a R_E. \quad (2)$$

Where ρ is soil resistivity ($\Omega \cdot m$), a is the distance between pins (m), and R is the resistance value measured by the instrument (Ω). This model approximates realistic soil conditions and serves as a reference for validating both numerical simulations and field tests.

2.2 Experimental Measurement

Field measurements were conducted to validate the theoretical and simulation results. A Fluke 1625 earth ground tester was employed using the fall-of-potential (FOP) method, according to Fluke Manual [15]. In this method, the test electrode under study is energized, while two auxiliary probes are placed in line at predetermined distances. The potential probe measures the voltage drop at varying distances, while the current probe provides a stable reference current, as shown in Figure 1.

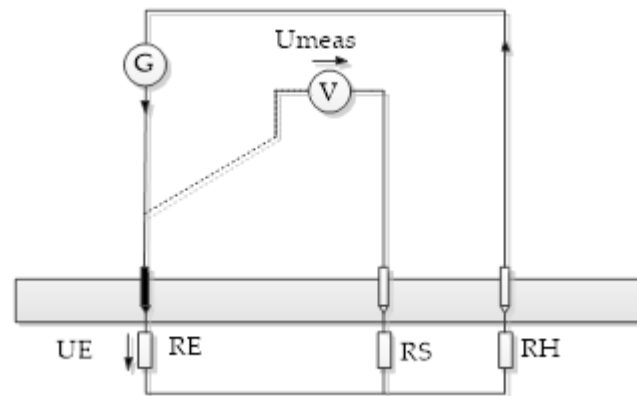


Figure 1. Earth electrode with auxiliary probes

For each grounding configuration, multiple measurements were performed by repositioning the potential probe to generate resistance–distance curves. The ground resistance was measured approximately 62% along the distance between the test electrode and the current investigation, in accordance with the 62% rule. To minimize errors due to soil inhomogeneity, measurements for all configurations were carried out on the same day under similar environmental conditions. Under the FOP testing, the high-performance ground tool and the IEEE standard. 81, the results are extremely reliable, highly accurate, and give complete control over the setup. In this measurement, the current probe is placed >20 m away from the earth electrode under test. An example of 3 straight grounding system configurations is shown in Figure 2.



Figure 2. Three-core copper-clad steel straight earth resistance measurement

Figure 3 shows the 4-pin configuration for measuring soil resistivity. The 4-pole Wenner method was used to measure soil specificity according to the Fluke 1625-2 Earth/Ground Tester manual [3]. Four equidistant probes are inserted into the soil on a straight line as shown in Figure 4. Then the current terminals (C1 and C2) of the advanced earth testing meter, Fluke 1625, are connected to the two outer probes, and the potential-probing terminals (P1 and P2) are connected to the two inner probes. The measurements R had been averaged from a 90-degree perpendicular axis 4-pole method. Then, the soil resistivity was calculated from the measured resistance using Equation 2.

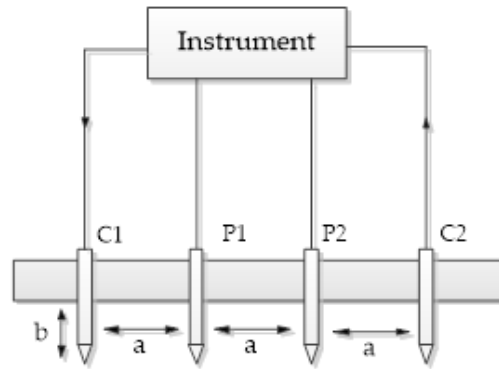


Figure 3. Connection diagram of the four-pole method.



Figure 4. Wenner four-pin method for soil resistance measurement.

2.3 Simulation approach

The grounding systems were examined through finite element modeling using COMSOL Multiphysics. In the simulation setup, the soil region was created as a large cylindrical volume in both radius and depth to ensure that the computed electric field was unaffected by the domain boundaries. Copper-clad steel rods representing the electrodes were positioned vertically and buried about 2.4 m below the ground surface, under conditions consistent with practical installation standards. The outer boundary of the soil model was set with a Neumann boundary condition, assuming zero normal current density ($\partial V/\partial n = 0$), which represents the absence of current flow across the air–soil interface, as depicted in Figure 5.

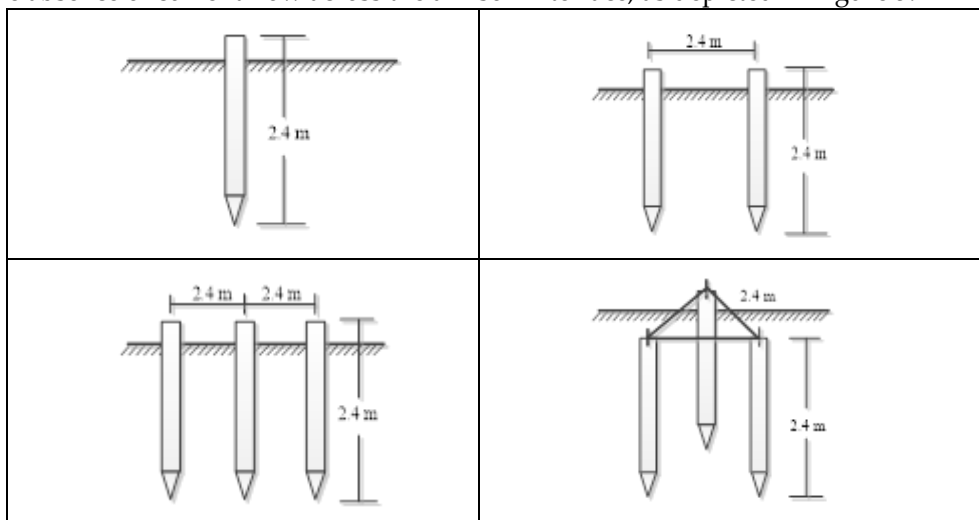


Figure 5. Copper Clad Steel Ground Rod Dia 5/8 X2.4 m at different arrangements.

Then, mesh refinement, as shown in Figures 6 and 7, was applied around the electrode surfaces to ensure numerical accuracy. Several electrode configurations were analyzed, including single-rod, 2 parallel rods, 3 straight ground rods, and 3 triangular arrangements, to examine the effects of electrode geometry and surface area on ground resistance.

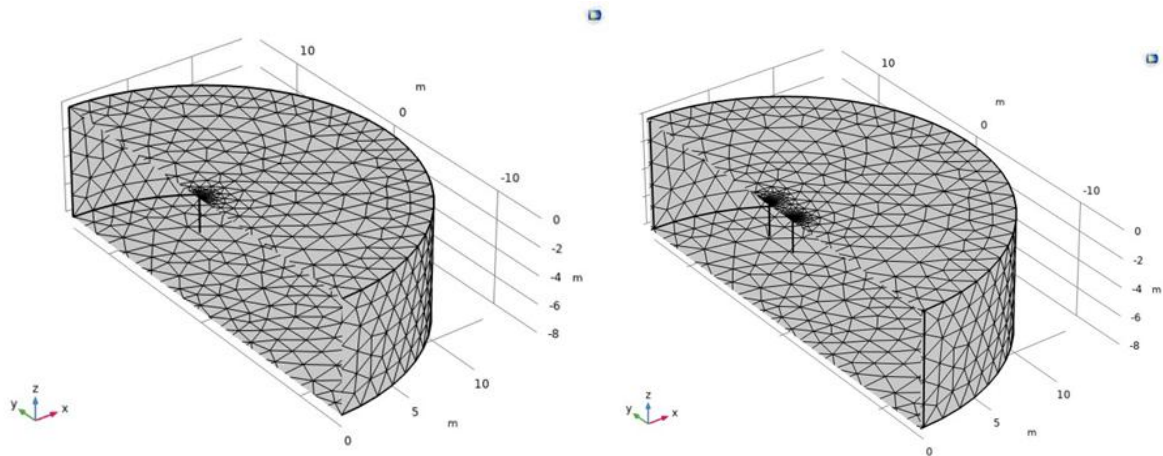


Figure 6. The mesh settings for 1 and 2 rods at the yz-plan cut section.

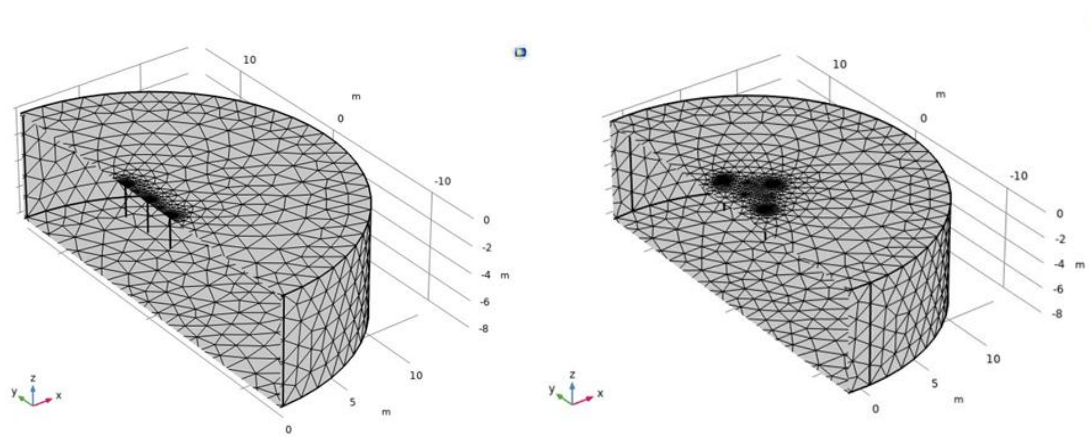


Figure 7. The mesh settings for 3 straight and 3 triangular rods at the yz-plane cut section.

The program parameters are listed in Table 1. The parameters included the precise dimensions of the copper-clad ground rod. The measured soil resistivity, calculated from equation 2, was $0.027 \Omega \cdot \text{m}$. The rod was set as the current source at 0.005 A and energized at 111 Hz , as per the Fluke 1625-2 manual. The soil domain was defined as a cylindrical volume extending 3 times the rod length deeper. The electrical current nodes within the AC/DC module and the frequency domain were used in the computation.

Table 1. Parameters setting for all electrode arrangements.

Parameters	Value
Rod diameter	0.008 [m]
Soil cylinder	$Z = -10-0 \text{ [m]}$
ρ_s	$0.027 [\Omega \cdot \text{m}]$
I_{in}	0.005 [A]
f	111 [Hz]
Ground rod	rods 1,2,3-straight,3-delta

3. Results and Discussion

3.1 Simulation results

The 3D model was built in COMSOL Multiphysics, approximately following the same geometry as in the measurement methods. The vertical ground rod, 2.4 meters long with a 0.008 m radius, was plotted in a cylindrical volume.

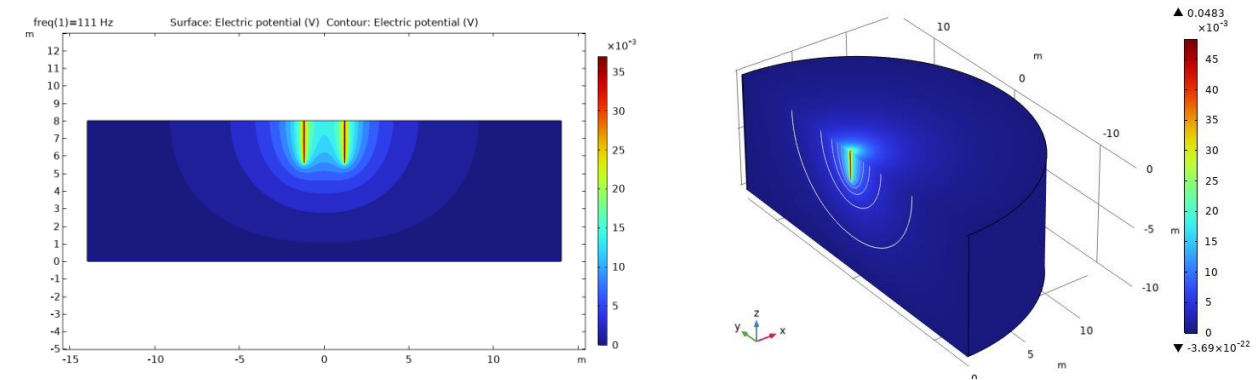


Figure 8. Simulation of electric field distribution (left) and electric field norm (right) for 1 ground rod

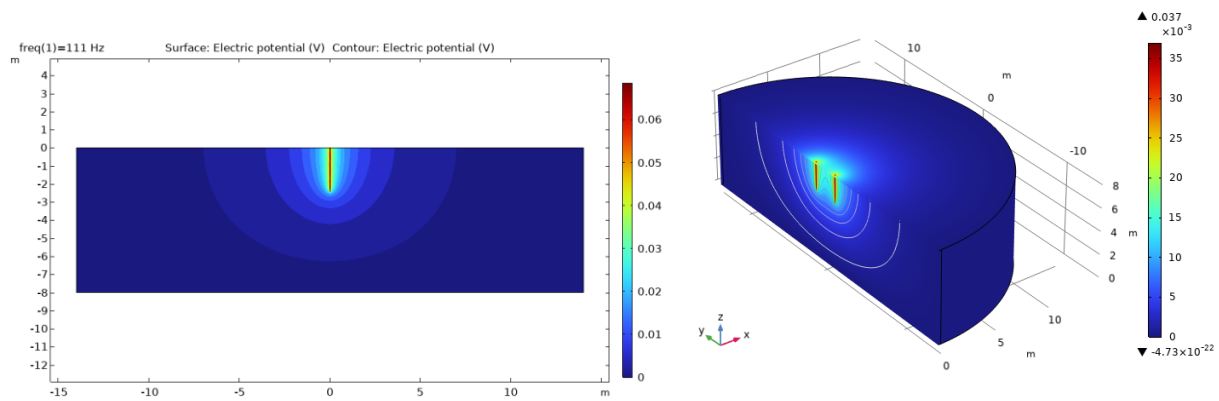


Figure 9. Simulation of electric field distribution (left) and electric field norm (right) for 2 ground rods.

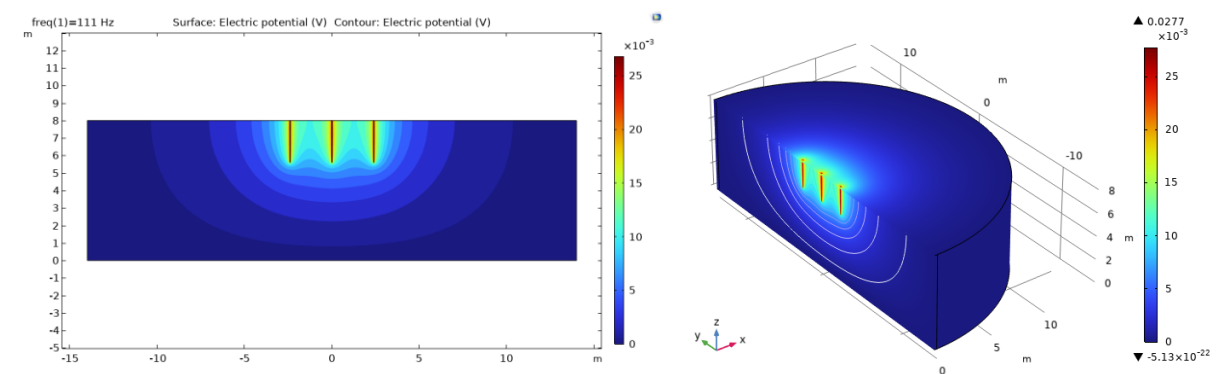


Figure 10. Simulation of electric field distribution (left) and electric field norm (right) for 3 straight ground rods.

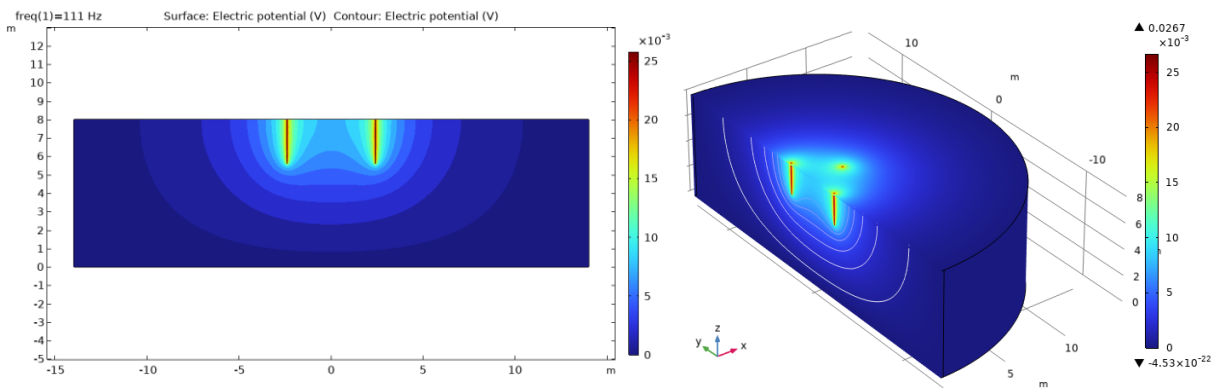


Figure 11. Simulation of electric field distribution (left) and electric field norm (right) for 3-delta ground rods.

The electrode geometry and the cut plane ($x=0$) were set along the center, the 2D potential and the 3D electric field norm are shown in Figures 8-11. The results obtained by simulating the distribution of voltage scattering in the approximate homogeneous ground are plotted. Given the different ground rod arrangement, there is clearly good dissipation. The potential gradient at the ground surface decreased with increasing number of electrodes, suggesting enhanced grounding performance and reduced surface voltage stress. Therefore, the number of ground rods and the rod arrangement are affected by the computed values. The comparison of ground resistance results between measurement and simulation is shown in Table 2. The results have good agreement in terms of the error percentage.

Table 2. Comparison table of ground rod resistance values from measurement and simulation.

Ground rod number	Measurement (Ω)	Simulation (Ω)	%Error
1	13.6	13.58	0.14
2	7.2	7.39	2.57
3-straight	5.71	5.62	1.57
3-delta	5.3	5.40	1.85

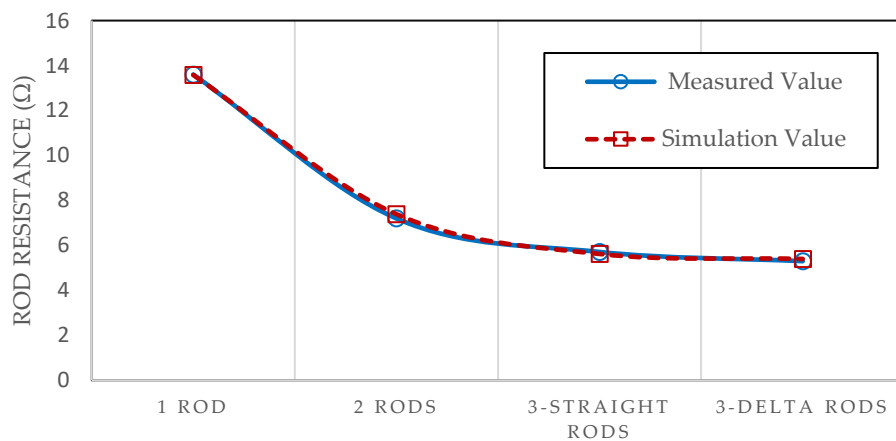


Figure 12. Resistance of ground rod for various arrangements.

Figure 12 shows the ground-rod resistance graph for various arrangements, evaluated both analytically and by measurement and by the FEM method. The outcomes derived from the simulation approach closely resemble the analytical results. The relative error was lower than 3%, which is very small and consistent within the experimental uncertainty range. Although all configurations show good agreement between measurement and simulation, the magnitude of the error varies across configurations. The two-rod

configuration exhibits the highest deviation (2.57%), which may be attributed to the influence of mutual resistance between electrodes and the limitations of the 62% rule under closely spaced conditions. In contrast, the single-rod configuration shows the lowest deviation, as it is less affected by interaction effects and more closely matches the assumptions of the analytical and numerical models.

4. Conclusions

This study conducted a comparative analysis of four grounding electrode configurations—single rod, two parallel rods, three rods in a linear arrangement, and three rods in a delta arrangement—assessed through field measurements and Finite Element Method (FEM) simulations. The experimental results from the 3-pole Fall-of-Potential (FOP) and 4-pole Wenner methods closely aligned with the FEM-based computations conducted in COMSOL Multiphysics. The difference between the simulation and the measurement was less than 3%, indicating that FEM is a reliable tool for predicting the design of a grounding system. The results show that adding more grounding rods greatly lowers the overall earth resistance. However, the rate of reduction is not linear because of the effects of mutual coupling between the rods. The effect of mutual coupling can be quantified by comparing the measured resistance with the ideal independent case. For the two-rod configuration, the theoretical value is 6.8Ω ($13.6/2$), while the measured value is 7.2Ω , corresponding to an efficiency loss of about 5.9% due to mutual resistance between electrodes. The three-rod delta arrangement had the lowest resistance of all the tested geometries, followed by the three-rod linear configuration, due to its larger average electrode spacing, which reduces mutual coupling and improves current dispersion. This shows how important electrode geometry is for current dissipation. The electric-field distributions from the simulation showed that multi-rod systems exhibit greater potential gradients, making grounding more effective. The results also suggest that multi-rod configurations improve grounding safety by reducing potential gradients and minimizing step and touch voltage risks. These results show that combining numerical FEM modeling with experimental methods is an effective approach for analyzing and improving grounding systems under practical conditions. However, the present findings are limited to homogeneous soil conditions, and the results may differ in multi-layer or high-resistivity soils. Future studies should focus on extending the analysis to multi-layer and non-uniform soil conditions. As well as evaluating the impacts of electrode spacing and depth optimization, and of transient phenomena such as lightning-induced currents, to expand the applicability of the suggested approach.

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Energy Efficient Micropropagation of *Dendrobium cruentum* Rchb. f.: Chemical Sterilization, Net House Culture, Sucrose Optimization, and Acclimatization Substrate Selection

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Abstract: *Dendrobium cruentum* Rchb. f., a threatened native orchid of southern Thailand, faces extinction from habitat loss and over-collection; however, conventional micropropagation protocols remain economically inaccessible for most conservation programs due to high energy costs. This study developed an energy-efficient micropropagation protocol by evaluating sterilization methods, culture environments, sucrose concentrations, and acclimatization substrates. Six sterilization treatments were assessed: conventional autoclaving (positive control), four concentrations of Haiter® bleach (NaOCl; 0.5, 1.0, 1.5, and 2.0 mL/L), and a non-sterilized negative control. Seedlings were cultured under controlled-room (25 ± 2°C, 16-h photoperiod) or net house (ambient temperature, natural light, 70% shade) conditions with sucrose at 0, 20, or 40 g/L on half-strength MS medium. Acclimatization performance was evaluated using sphagnum moss, chopped coconut husk, and charcoal substrates. Chemical sterilization with 2.0 mL/L Haiter® achieved an acceptably low contamination rate of 10.00% versus 6.67% for autoclaving, while eliminating autoclave capital (USD 2,000–10,000), maintenance (USD 200–500/year), and energy costs (2–3 kWh/cycle), reducing sterilization expenditure by 60–70%. Net house culture combined with 40 g/L sucrose produced superior plantlets with 2.63 shoots, 14.33 roots, 3.60 cm height, and 1.14 g fresh weight, representing numerically more shoots (21%, $p > 0.05$), significantly more roots (68%, $p \leq 0.01$), greater height (41%), and higher fresh weight (75.4%) than controlled-room culture, while substantially reducing operational electricity costs associated with climate-controlled culture rooms. Charcoal substrate achieved the highest acclimatization survival rate (85%). This integrated, low-cost protocol offers a practical, cost-effective tool for conservation and commercial production of this threatened native orchid.

Keywords: Micropropagation; chemical sterilization; net house culture; sucrose concentration; acclimatization

1. Introduction

Dendrobium cruentum Rchb. f. (the "bird-beak orchid"), belonging to section Formosae, is native to southern Thailand, where wild populations inhabit lowland and hill forests on the southern peninsula; its precise distribution across the Thai-Malay Peninsula remains incompletely documented. The species faces threats of extinction from deforestation, habitat loss, and unsustainable collection driven by high ornamental demand [1, 2].

Despite the CITES Appendix I listing [3], which prohibits the commercial trade in wild specimens, populations of *D. cruentum* continue to decline due to unsustainable collection and inadequate in situ assessment. Although an IUCN Red List assessment is lacking [4], this evidence underscores the urgent need for ex situ conservation interventions, including genetically verified micropropagation protocols [5].

The natural propagation of *D. cruentum* is severely limited by inherent biological constraints. Like all Orchidaceae, *D. cruentum* produces dust-like, endosperm-free seeds that require obligate mycorrhizal associations to supply nutrients for seedling development [6]. These reproductive limitations, compounded by ongoing habitat degradation and population fragmentation, make reliable in vitro propagation essential to prevent irreversible genetic erosion of remaining wild populations. Asymbiotic seed germination offers a well-established route for large-scale orchid propagation, bypassing mycorrhizal dependency while preserving genetic fidelity [7–10]. However, published protocols for *D. cruentum* are scarce, comprising only two reports: shoot induction on Vacin and Went (VW) medium [1] and asymbiotic germination on VW medium with organic additives [2]. The lack of optimized, resource-efficient protocols limits the conservation of this species through micropropagation.

Although commercial tissue culture facilities for *Dendrobium* and *Phalaenopsis* are established in Thailand, the high costs of conventional micropropagation — including growth media, sterilization equipment, and air-conditioned culture rooms [11, 12] — remain prohibitive for small-scale growers and conservation programs. Energy consumption is a major contributor to these costs, particularly through the energy-intensive autoclaving required for media sterilization and the continuous operation of air-conditioned culture rooms [13, 14]. Cost-reduction strategies have included reformulating the medium, substituting analytical-grade chemicals [15], using low-cost gelling agents [16], and adopting chemical sterilization methods instead of autoclaving [17–21]. However, the potential of ambient net house systems — using natural light and passive ventilation — as alternatives to controlled-room conditions has received little attention in orchid micropropagation. Ferreira et al. [22] demonstrated that *Cattleya warneri* cultured under variable greenhouse conditions produced greater biomass and incurred lower electricity costs than in constant-condition growth rooms. Similarly, Kodym and Zapata-Arias [23] found that *Musa acuminata* shoots grown under fluctuating natural light (up to $570 \mu\text{mol m}^{-2} \text{s}^{-1}$, 23–30°C) produced more shoots than those under constant artificial light ($65 \mu\text{mol m}^{-2} \text{s}^{-1}$) in controlled chambers. Nasiruddin et al. [24] reported 100% hardening survival in banana (*Musa* sp., cv. Robusta) shoots rooted under shadehouse conditions, with reduced electricity consumption. Although low-cost strategies have been explored for commercial *Dendrobium* species [25–28], no study has examined ambient net house conditions for threatened native species such as *D. cruentum*.

This study aimed to develop a comprehensive, cost-effective micropropagation protocol for *D. cruentum* by investigating three aspects: (1) medium sterilization techniques that minimize contamination and energy inputs; (2) effects of culture environment (controlled-room vs. net house) and sucrose concentration on plantlet growth, morphology, and biomass; and (3) the effect of acclimatization substrate type, in combination with pre-transfer sucrose concentration, on ex vitro survival rates; and (4) a comparative cost analysis of the operational cost implications of the optimized protocol relative to conventional micropropagation approaches. This research addresses the need for accessible, low-cost propagation tools to support both ex situ conservation of this CITES-listed orchid and sustainable production among resource-limited, small-scale growers in tropical regions.

2. Materials and Methods

2.1 Plant material and explant preparation

Plant material was obtained from six cultivated accessions of *Dendrobium cruentum* Rchb. f. maintained under nursery net-house conditions at the Biology Section, Faculty of Science and Technology, Surattthani Rajabhat University, Surat Thani Province, southern Thailand. All accessions were cultivated and maintained under standard nursery conditions for at least 2 years before experimentation; no wild specimens were collected for this study. Hand pollination was performed on each of the six maternal plants. Seed pod development was monitored from the date of pollination, and pods were harvested at 5 months post-pollination (approximately 150 days) for use in green pod culture. At this stage, capsules of *Dendrobium* species are characterized by a firm, fully closed green pericarp that has not yet undergone dehiscence, a stage

recognized as optimal for asymbiotic green pod culture in *Dendrobium* [29, 30]. Pods selected for use were uniformly green, firm, and undehiscent, with intact funicle attachment; pods showing discoloration, physical damage, pathogen infection, or size variation among maternal plants were excluded (Figure 1A). Before seed inoculation, seed viability was assessed using the 2,3,5-triphenyltetrazolium chloride (TTC) test following the modified protocol of Zainuddin et al. [31]; seeds exhibiting uniform red-to-deep-red embryo staining were included in the assessment. For explant preparation, pods were surface-sterilized by sequential washing with 10% Teepol® detergent, tap water, and distilled water, followed by immersion in 70% ethanol (3–5 min) and 95% ethanol with surface flaming (2–3×) to achieve surface sterility. All subsequent steps were performed in a laminar-flow cabinet (horizontal airflow) under aseptic conditions. To standardize seed inoculation density across treatments, seeds from each capsule were suspended in 5 mL of sterile distilled water after the capsule was split longitudinally. A 0.1 mL aliquot of the resulting seed suspension was pipetted onto the surface of solidified medium in each culture vessel, ensuring uniform seed distribution across all replicates.

2.2 Culture medium sterilization treatments

This experiment investigated the effects of six culture medium preparation treatments on contamination rates during asymbiotic seed germination of *D. cruentum*. Treatments differed only in the method used to sterilize or supplement the culture medium, while the seed source, inoculation method, and all other experimental conditions remained identical across treatments. The six treatments were as follows: (A) culture medium sterilized by conventional autoclaving at 121°C for 20 minutes (positive control); (B) culture medium with no sterilization applied (negative control); and (C–F) non-autoclaved culture medium supplemented with Haiter® commercial bleach (NaOCl, 6% w/w active ingredient; Lion Corporation, Japan) at four concentrations of 0.5, 1.0, 1.5, and 2.0 mL/L, equivalent to approximately 0.003%, 0.006%, 0.009%, and 0.012% active chlorine (w/v), respectively. For Treatments C–F, half-strength Murashige and Skoog (1/2 MS) salts [32], sucrose (30 g/L), and agar (8 g/L) were dissolved in distilled water. The pH was adjusted to 5.7 ± 0.1 with 0.1 N NaOH or 0.1 N HCl before Haiter® addition, and the agar was fully dissolved by heating to near boiling in a microwave oven. The medium was then cooled to approximately 50–55°C, and Haiter® was added at the designated concentration, then gently mixed to ensure uniform distribution. The final pH was verified to be within 5.5–6.0 after the addition of Haiter®. The supplemented medium was dispensed immediately into sterile culture vessels and allowed to solidify at room temperature. For Treatment A, the medium of identical composition was autoclaved at 121°C for 20 minutes in the conventional manner. All media were prepared fresh on the day of seed inoculation. Seeds for inoculation were obtained from the pre-sterilized pods described in Section 2.1. All seed handling and inoculation procedures were performed entirely within a laminar-flow cabinet (horizontal airflow). Under aseptic conditions, the pod was re-flamed with 95% ethanol, split longitudinally, and approximately 0.1 g of seeds was evenly distributed onto the medium surface using a sterile inoculation loop. The identical inoculation procedure was used for all six treatments. Culture vessels were sealed with Parafilm and incubated under standard controlled-room conditions ($25 \pm 2^\circ\text{C}$, 16-h photoperiod, $40 \mu\text{mol m}^{-2} \text{s}^{-1}$). Contamination rates were recorded after four weeks of culture. Following this experiment, seeds from aseptic cultures were allowed to germinate and develop into protocorms (Figure 1B).

2.3 Culture conditions and sucrose concentrations

All subsequent culture media were autoclaved at 121°C for 20 min. Stage 3 protocorms (bearing rhizoids and early leaf primordia, ~1.0–1.5 mm diameter) were allowed to develop into seedlings (~1.0 cm height) before transfer. One seedling per vessel was cultured on 1/2 MS medium supplemented with sucrose at 0, 20, or 40 g/L (20 seedlings per treatment per replication) under two contrasting environments. Seedlings were then transferred to 1/2 MS medium supplemented with three sucrose concentrations (0, 20, and 40 g/L), with 20 seedlings per treatment combination per replication, and cultured under the same two contrasting environments as described below. All culture vessels contained 25 mL of medium and were sealed with non-vented polypropylene caps. *Controlled-room conditions*: Temperature was maintained at $25 \pm 2^\circ\text{C}$ using an air-conditioning system. A 16-hour photoperiod was provided by cool-white fluorescent lamps at $40 \mu\text{mol m}^{-2} \text{s}^{-1}$. Relative humidity was maintained at 60–70% with forced air circulation. Electricity consumption (kWh) was continuously monitored using a digital energy meter. *Net house conditions*: Culture vessels were placed in

an ambient-temperature net house covered with a 70% shade cloth. Air temperature fluctuated between 24 and 33°C over the daily cycle, and natural daylight transmitted through the shade cloth provided a midday PPFD of approximately 300–500 $\mu\text{mol m}^{-2} \text{s}^{-1}$ at the vessel level. The natural photoperiod during the experimental period ranged from approximately 12 to 13 h of light per day, consistent with the study site's tropical latitude (approximately 9°N). Relative humidity ranged from 50% to 85% in response to natural diurnal variation, and ventilation was entirely passive. No electricity was consumed for climate control under these conditions. Plantlets were cultured for two months under both conditions. At harvest, the following parameters were recorded for each plantlet: number of new shoots, number of leaves, number of roots, plantlet height (cm), leaf width (cm), leaf length (cm), root length (cm), and fresh weight (g). Electricity consumption data from the controlled-room system were used to calculate the per-cycle difference in operational cost between the two culture environments.

2.4 Ex vitro acclimatization

Plantlets from the 20 and 40 g/L sucrose treatments were selected for acclimatization; those from sucrose-free medium (0 g/L) were excluded due to insufficient shoot and root development (Table 3). Only plantlets meeting the following minimum morphological criteria were selected for transplanting: at least two fully expanded leaves, at least three visible roots, and a height of at least 1.5 cm. Three substrate types were evaluated: Sphagnum moss, chopped coconut husk, and charcoal. Before transplanting, residual agar was gently removed from the roots under running distilled water. Plantlets were individually transplanted into plastic pots (7 cm diameter) containing the respective substrates and maintained in a shade net nursery covered with 70% shade cloth at an ambient temperature of 25–32°C and a relative humidity of 60–80%. Plantlets were irrigated twice weekly with distilled water (pH 6.5–7.0, EC < 0.1 mS/cm) and fertilized once every two weeks with a diluted balanced liquid fertilizer (20-20-20 NPK, 1 g/L). Survival rates were assessed after 2 months of acclimatization, with survival defined as the retention of at least 1 turgid, green leaf and at least 1 intact root in contact with the substrate.

2.5 Experimental design and statistical analysis

All experiments were conducted using a completely randomized design (CRD) with three replications. Sterilization experiments comprised six treatments, three replications, and 10 vessels per treatment (30 vessels in total). Culture condition experiments comprised six treatment combinations (2 environments $\times$ 3 sucrose levels) $\times$ 20 explants/replication (60 explants/combination). Acclimatization studies comprised six treatment combinations (3 substrates $\times$ 2 sucrose levels) $\times$ 20 plantlets/replication (60 plantlets/combination). Acclimatization studies used 20 plantlets per substrate-sucrose combination per replication, with three substrate types (Sphagnum moss, chopped coconut husk, and charcoal) evaluated at two sucrose concentrations (20 and 40 g/L), yielding six treatment combinations and 60 plantlets per combination across all replications. Data were analyzed using one-way ANOVA for sterilization treatments. Culture condition and acclimatization experiments were analyzed using two-way ANOVA; fixed factors were culture environment $\times$ sucrose concentration, and substrate type $\times$ sucrose concentration, respectively. Mean separation was performed using Duncan's Multiple Range Test (DMRT) at $p \leq 0.05$ or $p \leq 0.01$, as indicated in the respective tables. All statistical analyses were performed using SPSS Statistics version 26.0 (IBM Corp., Armonk, NY, USA). All data are presented as means $\pm$ standard error (SE).

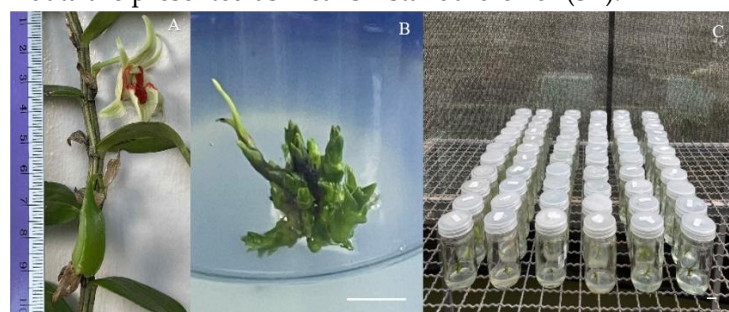


Figure 1. The 5-month-old, hand-pollinated seed pod of *D. cruentum* Rchb. f. (A) and protocorm development (B), and the plantlet cultured on 1/2 MS medium under net house conditions (C) (bar = 1 cm).

3. Results and Discussion

3.1 Effect of sterilization technique on contamination rate

The sterilization method significantly affected the contamination rate of *D. cruentum* seed cultures ($p \leq 0.01$; Table 1). Conventional autoclaving (Treatment A) resulted in the lowest contamination rate, at $6.67 \pm 0.46\%$, which was significantly lower than those of all chemical sterilization treatments. Among chemical methods, Haiter® at 2.0 mL/L (Treatment F) yielded the lowest contamination ($10.00 \pm 0.59\%$), with rates increasing progressively at lower concentrations (26.70%, 53.30%, and 56.70% at 1.5, 1.0, and 0.5 mL/L, respectively). The control treatment without Haiter® (Treatment B) showed complete contamination ($100.00 \pm 0.00\%$), underscoring the need for proper sterilization procedures. Although autoclaving remained the most effective sterilization method, chemical sterilization with 2.0 mL/L Haiter® achieved acceptable contamination rates while eliminating the energy consumption associated with autoclaving, estimated at 2–3 kWh per cycle. This finding aligns with previous reports demonstrating the effectiveness of sodium hypochlorite for plant tissue culture sterilization [17–21], although optimal concentrations vary by species. The 10% contamination rate, while higher than that of autoclaving, represents a reasonable trade-off given substantial energy savings and the elimination of autoclave-related operational costs for resource-limited facilities. A comparative cost analysis further substantiates this trade-off (Table 2). NaOCl-based sterilization has demonstrated economic viability across multiple species, including *Eucalyptus benthamii*, *Gerbera hybrida*, and *Anthurium* sp. [17, 33, 34], achieving contamination rates comparable to autoclaving while reducing infrastructure requirements, including a potential reduction in dependence on laminar flow cabinets via the CSUP technique [Peiris et al., 2012]. In the context of small-scale orchid conservation programs in Thailand, eliminating the need for autoclaves could reduce sterilization-related costs by an estimated 60–70%, making this approach particularly transformative for community-based production.

The safety of chlorine residues at the effective concentration used in this study (2.0 mL/L Haiter®) warrants discussion, as NaOCl is a potent oxidizing agent that can cause phytotoxic injury at elevated concentrations. NaOCl is a potent oxidant; its active species, hypochlorous acid (HOCl), can cause protein denaturation and membrane disruption at high concentrations, leading to phytotoxic effects, including disruption of meristematic cell division and chromosomal anomalies, as shown in dose-dependent bioassays [[35–37]]. Phytotoxic effects are strongly concentration-dependent, and the critical threshold beyond which NaOCl impairs tissue viability varies by species, explant type, exposure duration, and temperature [35, 38]. In the present study, the 2.0 mL/L Haiter® treatment did not produce visible phytotoxic symptoms in *D. cruentum* seeds or developing protocorms. Successful germination and normal plantlet development in non-contaminated cultures confirmed that residual active chlorine concentrations after rinsing were below phytotoxic thresholds. This finding aligns with a growing body of literature showing that NaOCl at carefully optimized, low concentrations does not cause significant phytotoxic injury to plant tissues. Supporting evidence from diverse species confirms that low NaOCl concentrations are biologically safe: Bui et al. [39] reported high survival with minimal tissue damage in *Litsea cubeba*; Rodrigues et al. [40] demonstrated superior shoot production in two orchid species at $1,200 \text{ mg L}^{-1}$; and [41] achieved 94.53% germination in rice at 4%, with phytotoxicity appearing only above this threshold. Park et al. [42] further reported that 1% NaOCl improved germination in the endangered orchid *Cypripedium guttatum*, supporting the use of dilute NaOCl as a germination-compatible pre-treatment. Collectively, these findings confirm that 2.0 mL/L Haiter® is a biologically safe and effective sterilization alternative for *D. cruentum*, provided rinsing steps are maintained to limit residual chlorine carry-over.

Table 1. Effects of sterilization technique on the contamination rate of *D. cruentum* seeds.

	Sterilize technique	Contamination rate (%) $\pm$ SE ¹
A	Autoclave	6.67 $\pm$ 0.46 ^e
B	0.0 mL/L Haiter®	100.00 $\pm$ 0.00 ^a
C	0.5 mL/L Haiter®	56.70 $\pm$ 0.94 ^b
D	1.0 mL/L Haiter®	53.30 $\pm$ 0.95 ^b
E	1.5 mL/L Haiter®	26.70 $\pm$ 0.87 ^c
F	2.0 mL/L Haiter®	10.00 $\pm$ 0.59 ^d

¹ Data are presented as means $\pm$ SE. Different letters in a column indicate statistically significant differences ($p \leq 0.01$) by DMRT.

Table 2. Comparative cost analysis of autoclave sterilization versus chemical sterilization with Haiter® (NaOCl) for micropropagation medium sterilization.

Cost Parameter	Autoclave Sterilization	Chemical Sterilization (2.0 mL/L Haiter®)
Equipment purchase	USD 2,000–10,000+ (laboratory autoclave)	Negligible (no specialized equipment)
Equipment maintenance	Regular servicing required; USD 200–500/year	None
Energy per cycle	2–3 kWh/cycle ¹ (121°C, 20 min)	0 kWh
Electricity cost per cycle (at ~USD 0.10/kWh, Thai rate)	~USD 0.20–0.30/cycle ²	USD 0.00
Monthly energy cost (20 cycles/month)	~USD 4–6/month	USD 0.00
Annual energy cost	~USD 48–72/year	USD 0.00
Reagent cost per cycle	Distilled water only (~USD 0.01)	Haiter® ~1 mL/500 mL medium; ~USD 0.01–0.02/cycle
Contamination rate	6.67%	10.00%
Laminar flow cabinet required	Yes	Can be reduced (see CSUP technique)
Skill requirement	Trained operator	Minimal training
Overall relative cost	High (baseline)	~60–70% lower

¹ Autoclave capital and energy data from O'Callaghan et al. (2023) [ref. no.].

² Thai commercial electricity tariff (USD 0.10–0.14 per kWh) based on the Provincial Electricity Authority of Thailand (PEA) rate schedule (2023).

3.2 Effects of culture condition and sucrose concentration on shoot and root development

3.2.1 Shoot multiplication

Both sucrose concentration and culture environment significantly influenced shoot production ($p \leq 0.01$), and their interaction was also significant ($p \leq 0.05$), indicating that the promotive effect of sucrose on shoot number was modulated by culture environment. Root formation was similarly influenced by sucrose concentration, culture environment, and their interaction ($p \leq 0.01$ each), confirming that sucrose effects on root development were disproportionately enhanced under net house conditions (Table 3; Figures 2 and 3). In sucrose-free medium (0 g/L), no new shoots formed regardless of culture condition, with both controlled-room and net house treatments yielding 0.00 ± 0.00 shoots per plantlet. The addition of sucrose promoted shoot proliferation, with 20 g/L yielding 1.65 ± 0.18 shoots in controlled-room and 1.95 ± 0.20 shoots in net house conditions. The highest number of new shoots (2.63 ± 0.15) was obtained with 40 g/L sucrose under net house culture, which was significantly superior to all other treatments except the controlled-room culture at the same sucrose concentration (2.15 ± 0.18 shoots).

3.2.2 Root formation

Root formation responded markedly to both sucrose concentration and culture condition. In the absence of sucrose, root development was minimal, with only 1.27 and 1.87 roots per plantlet under controlled-room and net house conditions, respectively. Increasing sucrose to 20 g/L substantially enhanced root formation (4.50 and 4.87 roots for the controlled-room and net house, respectively). The most pronounced root proliferation occurred at 40 g/L sucrose in net house culture, yielding 14.33 roots per plantlet — significantly higher than all other treatments and nearly twice that of controlled-room culture at the same sucrose level (8.50 roots). Collectively, these results demonstrate that sucrose concentration was the dominant factor for both shoot and root development. Culture environment and its interaction with sucrose were significant determinants of root organogenesis. The mechanistic basis of this interaction is discussed in the following sections. Net house culture at 40 g/L sucrose produced more shoots (2.63 vs. 2.15; 22% increase, $p \leq 0.05$) and significantly more roots (14.33 vs. 8.50; 69% increase, $p \leq 0.01$) than controlled-room culture at the same sucrose concentration. This superior performance reflects the combined benefits of enhanced light quality and intensity, improved CO₂ availability, and diurnal temperature fluctuations characteristic of the ambient outdoor environment [43–45]. The robust root systems produced under net house conditions are particularly advantageous for subsequent *ex vitro* acclimatization, as well-developed root architecture is a key determinant of post-transfer survival in orchid micropropagation. Eliminating air-conditioned culture rooms delivers concurrent economic and agronomic benefits by removing the electricity costs associated with climate control in controlled-room culture.

The superior shoot and root responses observed at 40 g/L sucrose under net house conditions can be explained mechanistically by a synergistic interaction between exogenous carbon supply and the enhanced photosynthetic environment provided by the net house. Under controlled-room conditions (40 $\mu\text{mol m}^{-2} \text{s}^{-1}$, limited CO₂), plantlets operate predominantly heterotrophically, relying on exogenous sucrose as the primary carbon source. In contrast, the net house environment (300–500 $\mu\text{mol m}^{-2} \text{s}^{-1}$, ~400–450 $\mu\text{mol mol}^{-1}$ CO₂, passive ventilation) promotes concurrent CO₂ fixation and sucrose absorption, thereby establishing photomixotrophic growth with total carbon input exceeding that of either source alone [46, 47]. Root development benefited particularly from photomixotrophic conditions: sucrose acts as a primary substrate and signal for root sink organs via SUT/SWEET transporters [48, 49], while photosynthetically fixed carbon provided additional carbohydrate for root proliferation. Together, these mechanisms explain the 69% increase in root number (14.33 vs. 8.50) at 40 g/L sucrose in the net house. Consistent with this, Gago et al. [46] demonstrated up to 2-fold more shoots when sucrose supplementation was combined with elevated CO₂ and high light in bioreactor-cultured *Prunus domestica*.

Consistent with this interpretation, Gago et al. [46] demonstrated in bioreactor-cultured plum (*Prunus domestica*) that combining sucrose supplementation with elevated CO₂ and high light intensity produced up to 2-fold more shoots and 1.7-fold greater shoot length than sucrose under low light and ambient CO₂, confirming that carbon source and environment interact multiplicatively rather than additively in photomixotrophic systems. The superior performance of net house culture reflects three key environmental advantages over sealed controlled rooms: (1) broad-spectrum natural light (300–500 $\mu\text{mol m}^{-2} \text{s}^{-1}$ through 70% shade cloth) saturates orchid photosynthesis, exceeding the capacity of conventional fluorescent lamps (40 $\mu\text{mol m}^{-2} \text{s}^{-1}$) [50, 51]; (2) continuous passive ventilation maintains ambient CO₂ (~400–450 $\mu\text{mol mol}^{-1}$), preventing the depletion that limits photosynthesis in sealed vessels — elevated CO₂ has been shown to increase Fv/Fm by 14% and enhance Rubisco activity in *Phalaenopsis* [52]; and (3) diurnal temperature fluctuation (24–33°C) promotes stomatal cycling and carbohydrate metabolism pathways suppressed under constant temperature [45]. Having established the environmental basis for net house superiority, it is important to recognize that sucrose itself plays multiple distinct physiological roles that are independent of, yet synergistic with, the photomixotrophic framework.

Beyond its role in photomixotrophic carbon supply, sucrose exerts four distinct physiological functions: (1) carbon and energy supply — hydrolysis to hexoses fuels glycolysis, ATP synthesis, and meristematic activity [53, 54]; (2) root organogenesis — sucrose loading and unloading via SUTs/SWEETs activates root meristem cell cycle progression [48]; (3) osmotic regulation — sucrose lowers the medium's

osmotic potential, driving turgor-driven cell expansion and fresh weight accumulation [55]; and (4) sugar signalling — glucose and fructose interact with HXK1, TOR, and T6P pathways to regulate auxin biosynthesis and developmental transitions [56]. Collectively, sucrose acts as a multidimensional regulator of plantlet development, not merely a carbon source. These sugar signals modulate the expression of genes governing auxin biosynthesis and transport — which are essential for both shoot meristem activation and root organogenesis — explaining why sucrose concentration critically determines both the number and developmental quality of shoots and roots at this stage. Collectively, these four functions mean that sucrose concentration in the culture medium is not simply a variable carbon source but a multidimensional regulator of plantlet development, and optimizing sucrose supply is therefore a prerequisite for achieving maximum organogenic responses regardless of culture environment.

These results align with reports indicating that net houses reduce energy consumption by 0.18–0.26 kWh m⁻² day⁻¹ [57, 58] and that air conditioning is the second-largest cost in commercial orchid tissue culture. Chen [13] confirmed that net house culture provides both economic and agronomic benefits for *D. cruentum*. The dramatic quantitative differences in root formation are clearly illustrated in Figure 3, which shows representative root development across all treatment combinations after two months of culture. Plantlets cultured without sucrose under both controlled-room (Figure 3A) and net house (Figure 3D) conditions produced only sparse, thread-like roots with minimal branching, consistent with their low root counts (1.27 and 1.87 roots per plantlet, respectively; Table 3). At 20 g/L sucrose, root systems were more developed in both environments (Figure 3B and E), with visibly longer and more numerous roots, though net house plantlets (Figure 3E) appeared to produce slightly more branched root structures compared to controlled-room counterparts (Figure 3B). The most striking visual contrast was observed between the controlled-room treatment at 40 g/L sucrose (Figure 3C) and the net house treatment at the same concentration (Figure 3F): net house plantlets in Figure 3F displayed markedly denser root systems characterized by a substantially higher number of individual roots (14.35 vs. 8.55; Table 3), visibly thicker root axes reflecting greater radial growth consistent with the 1.30 cm root length recorded in Table 3, and a notably pale-white to greenish coloration. The green tinge observed in roots of net house plantlets under high sucrose (Figure 3F) is a well-documented characteristic of orchid aerial roots exposed to natural light, attributable to the differentiation of chloroplast-containing cells in the root velamen and cortex, which represents a photosynthetically functional adaptation of epiphytic orchid roots to ambient illumination [59, 60]. Additionally, root branching density appeared visually greater in the Figure 3F treatment, suggesting that the photomixotrophic carbon surplus available under net house conditions actively promoted lateral root initiation — a process that is positively regulated by local sucrose accumulation in root tissues [49]. These morphological characteristics collectively indicate that the net house and 40 g/L sucrose combination produces root systems with superior architectural complexity, which directly underlie the substantially higher acclimatization survival rates observed in plantlets from this treatment. The significant interaction term confirms that the effect of sucrose concentration on plantlet development was not independent of the culture environment, but was substantially modulated by whether plantlets were grown under controlled-room or net house conditions — consistent with the photomixotrophic framework described above and with reports of multiplicative sucrose × environment interactions in other micropropagation systems [61, 62].

3.3 Effects of culture condition and sucrose concentration on plantlet morphology

Culture conditions and sucrose concentration significantly influenced plantlet height, leaf dimensions, and root length ($p \leq 0.01$) (Table 4). Plantlet height ranged from 1.50 ± 0.50 cm to 3.60 ± 0.20 cm across treatments. The shortest plantlets were observed in controlled-room culture without sucrose (1.50 ± 0.50 cm). Net house culture consistently produced taller plantlets than controlled-room culture at all sucrose concentrations. The tallest plantlets (3.60 ± 0.20 cm) were obtained with 40 g/L sucrose under net house conditions, followed by 20 g/L sucrose in the net house (3.30 ± 0.10 cm). Both were significantly taller than the controlled-room treatments. An exception to the general pattern of net house superiority was observed in leaf length, where controlled-room plantlets at 20 g/L sucrose produced the longest leaves of any treatment (2.20 ± 0.10 cm; Table 4), exceeding even the net house + 40 g/L sucrose combination (1.52 ± 0.07 cm). This apparent paradox can be explained by the dual physiological role of sucrose at elevated concentrations: while higher

sucrose levels promote lateral cell expansion, fresh weight accumulation, and root organogenesis through enhanced carbon supply and osmotic water uptake, the elevated osmotic potential of the 40 g/L sucrose medium may simultaneously restrict turgor-driven longitudinal cell elongation in leaf mesophyll and epidermis, thereby limiting leaf length despite promoting leaf width and biomass accumulation [63, 64]. This concentration-dependent trade-off between leaf elongation and other growth parameters has been documented in other in vitro systems, where sucrose concentrations above the physiological optimum generate osmotic conditions that suppress shoot elongation while maintaining or enhancing lateral growth [65]. The net house environment at 40 g/L sucrose therefore optimizes most plantlet quality parameters — including height, root number, root length, leaf width, and fresh weight. By contrast, leaf length was maximized under moderate sucrose (20 g/L) combined with net house conditions (1.89 ± 0.04 cm), suggesting that an intermediate carbohydrate concentration best supports longitudinal leaf cell elongation in this environment. Figure 2 visually confirms these quantitative differences. Net house plantlets at 40 g/L sucrose (Figure 2D–F) displayed darker green leaves, consistent with enhanced chlorophyll accumulation under broad-spectrum natural light [66, 67], and visibly broader leaves and thicker stems, reflecting greater cell expansion and photomorphogenic development (leaf width data in Table 4). Sucrose-free plantlets (Figure 2A, D) were noticeably etiolated, consistent with their minimal biomass and the absence of shoot formation (Tables 3, 5). Future studies should verify chlorophyll differences spectrophotometrically. In contrast, plantlets maintained without sucrose under both culture conditions (Figure 2A and D, 0 g/L) were noticeably etiolated, with pale yellow-green, narrow leaves and weak stems, visually consistent with their severely limited biomass accumulation (0.50 and 0.72 g, respectively; Table 5) and the complete absence of new shoot formation (Table 3).

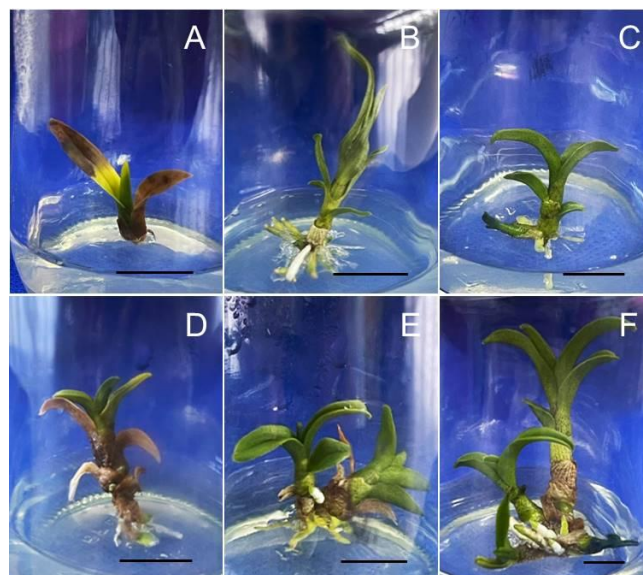


Figure 2. Shoot explants of *D. cruentum* were cultured on 1/2MS agar medium supplemented with 0, 20, and 40 g/L of sucrose in both controlled room (A–C) and net house (D–F) environments for a duration of two months. (bar = 1 cm).

Table 3. Number of shoots, leaves, and roots of *D. cruentum* after 2 months of culture.

Sucrose concentration (g/L)	Number of new shoots		Number of leaves		Number of roots	
	Culture environment (CE)	Average (SC)	Culture environment (CE)	Average (SC)	Culture environment (CE)	Average (SC)
	CR	NH	NH	CR	NH	CR
0	0.00 ± 0.00 ^c	0.00C	4.67 ± 0.57	4.33 ± 0.57	1.27 ± 0.31 ^d	1.87 ± 0.23 ^d
20	1.65 ± 0.15 ^b	1.80B	5.50 ± 1.32	4.83 ± 1.04	4.50 ± 0.5 ^c	4.87 ± 0.99 ^c
40	2.15 ± 0.18 ^b	2.39A	4.50 ± 0.5	4.17 ± 0.29	8.50 ± 0.86 ^b	14.33 ± 0.58 ^a
Average (CE)	1.27B	1.52A	4.89	4.44	4.78B	7.02A
SC	**		Ns		**	
CE	**		Ns		**	
SCxCE	*		Ns		**	
C.V. (%)	9.04		18.45		8.24	

¹ Data are presented as means ± SD. Different letters in a column and row indicate statistically significant differences ($p \leq 0.01$) by DMRT.

Table 4. Height, size of leaf, and root of *D. cruentum* plantlets cultured under controlled room and net house after culture for 2 months.

Sucrose Con. (g/L)	Plantlet height (cm)		Leaf width (cm)		Leaf length (cm)		Root length (cm)	
	Culture environment (CE)	Average (SC)	Culture environment (CE)	Average (SC)	Culture environment (CE)	Average (SC)	Culture environment (CE)	Average (SC)
	CR	NH	NH	CR	NH	CR	NH	CR
0	1.50 ± 0.50 ^c	2.70 ± 0.10 ^b	0.30 ± 0.01 ^c	0.32 ± 0.01 ^c	1.07 ± 0.05 ^e	1.33 ± 0.05 ^d	0.30 ± 0.02 ^d	0.50 ± 0.05 ^c
20	2.36 ± 0.05 ^b	3.30 ± 0.10 ^a	0.41 ± 0.01 ^b	0.51 ± 0.01 ^a	2.20 ± 0.10 ^a	1.89 ± 0.04 ^b	0.59 ± 0.01 ^c	0.90 ± 0.05 ^b
40	2.57 ± 0.05 ^b	3.60 ± 0.20 ^a	0.42 ± 0.02 ^b	0.49 ± 0.02 ^a	1.57 ± 0.05 ^c	1.52 ± 0.07 ^c	0.93 ± 0.01 ^b	1.30 ± 0.1 ^a
Average (CE)	2.14B	3.20A	0.37B	0.44A	1.61	1.58	4.78B	7.02A
SC	**		**		**		**	
CE	**		**		ns		**	
SCxCE	*		**		**		**	
C.V. (%)	8.63		3.91		4.45		7.15	

¹ Data are presented as means ± SE. Different letters in a column and row indicate statistically significant differences ($p \leq 0.01$) by DMRT, ns = not significantly different

Taken together, these morphological data confirm that net house culture combined with 40 g/L sucrose produced the highest-quality plantlets across most measured parameters, except leaf length, as discussed above. These advantages can be attributed to diurnal temperature fluctuations, a broader light spectrum, and enhanced CO₂ availability inherent to the net house environment [58]. Differential responses to sucrose concentration across culture environments further demonstrate that carbohydrate availability and the photosynthetic environment interact to modulate distinct developmental programs, consistent with the photomixotrophic framework described in Section 3.2 [[46, 62]. These morphological patterns reflect the photomixotrophic advantage of net house culture [22, 68]. The visual phenotypes in Figures 2 and 3 align directly with the quantitative data in Tables 3–5: dark green, broad-leafed plantlets with dense root systems in the net house + 40 g/L treatment show the highest fresh weight (1.14 g), root count (14.33), and plantlet height (3.60 cm), whereas etiolated, sucrose-free plantlets show minimal growth across all parameters. This alignment between visual morphology and quantitative metrics validates the experimental observations and confirms that the net house + 40 g/L sucrose combination produces the highest-quality plantlets across most measurable parameters, except for leaf length, as noted above.

3.4 Effects of culture condition and sucrose concentration on fresh weight accumulation

Fresh weight of plantlets was significantly affected by the interaction between culture conditions and sucrose concentration ($p \leq 0.01$) (Table 5). The lowest fresh weight (0.50 ± 0.02 g) was recorded in controlled-room culture without sucrose. Net house culture without sucrose produced plantlets with a moderately higher fresh weight (0.72 ± 0.02 g), presumably reflecting the partial contribution of photosynthetically fixed carbon under ambient light conditions — a finding consistent with the photomixotrophic advantage of the net house environment described in Section 3.2. Adding 20 g/L sucrose increased fresh weight to 0.63 ± 0.03 g in controlled-room and 0.78 ± 0.04 g in net house conditions. The highest fresh weight accumulation (1.14 ± 0.08 g) was achieved with 40 g/L sucrose under net house culture, which was significantly superior to all other treatments, representing a 2.3-fold increase compared to controlled-room culture without sucrose and a 1.75-fold increase compared to controlled-room culture at the same sucrose concentration. As noted in Section 3.2, sucrose also contributes to fresh weight via osmotic regulation: lowering the medium water potential drives turgor-driven cell expansion [55, 63]. The 0.42 g difference in fresh weight between sucrose-free and 40 g/L net house treatments, under otherwise identical conditions, is consistent with this osmotic mechanism. The substantial fresh weight accumulation observed in net house culture, a 75.4% increase over controlled-room conditions at 40 g/L sucrose, underscores the synergistic effect of optimal carbohydrate supply and favorable environmental conditions on overall plantlet vigor and biomass production. Sucrose concentration critically influenced plantlet development: the 40 g/L treatment, twice the conventionally applied 20 g/L concentration, supported markedly enhanced biomass accumulation under photomixotrophic net house conditions [46, 47, 61, 62]. This progressive improvement confirms heterotrophic dependence at this developmental stage, as growth was severely limited in the absence of sucrose. The progressive increase in fresh weight with sucrose concentration ($0 \rightarrow 20 \rightarrow 40$ g/L) reflects the obligate dependence on exogenous carbon in conventional in vitro systems, where low light and restricted CO₂ suppress photoautotrophy [62]. Utami et al. [69] similarly reported optimal growth in *D. lineale* at 20 g/L sucrose, with complete removal preventing normal development. The 75.4% increase in fresh weight at 40 g/L sucrose under net house conditions (1.14 vs. 0.65 g) reflects the net house environment's capacity to enhance carbon utilization through photomixotrophic metabolism, an advantage absent in sealed controlled-room vessels.

Table 5. Fresh weight of *D. cruentum* plantlets cultured under controlled room and net house after culture for 2 months.

Sucrose concentration (SC) (g/L)	Fresh weight (g)		Average (SC)
	Culture environment (CE)		
	CR	NH	
0	0.50 ± 0.02 ^e	0.72 ± 0.02 ^c	0.61C
20	0.63 ± 0.01 ^d	0.78 ± 0.02 ^b	0.70B
40	0.65 ± 0.01 ^d	1.14 ± 0.04 ^a	0.89A
Average (CE)	0.59B	0.88A	
SC	**		
CE	**		
SCxCE	**		
C.V. (%)	3.47		

¹ Data are presented as means ± SD. Different letters in a column and row indicate statistically significant differences ($p \leq 0.01$) by DMRT

3.5 Effect of substrate type and sucrose concentration on ex vitro survival

Two-way ANOVA confirmed that substrate type exerted a significant main effect on acclimatization survival rate ($p \leq 0.01$) (Table 6), whereas the sucrose concentration of the preceding in vitro culture (20 vs. 40 g/L) did not produce a statistically significant main effect ($p > 0.05$). Plantlets previously cultivated on media supplemented with 20 or 40 g/L sucrose showed comparable responses across all three substrates. Sphagnum moss failed to support adequate plantlet survival, yielding only $15.00 \pm 5.00\%$ and $20.00 \pm 5.00\%$ for plantlets from 20 and 40 g/L sucrose media, respectively. Chopped coconut husk resulted in moderate survival rates of $70.00 \pm 8.66\%$ and $75.00 \pm 5.00\%$ for plantlets cultured on media containing 20 and 40 g/L sucrose, respectively. Charcoal substrate yielded the highest survival rates: $80.00 \pm 5.00\%$ for plantlets grown in 20 g/L sucrose medium and $85.00 \pm 5.00\%$ for those grown in 40 g/L sucrose medium. Survival on charcoal was significantly higher than on coconut husk and Sphagnum moss. The superior performance of charcoal as an acclimatization substrate (80–85% survival) reflects the specialized requirements of epiphytic orchids [26]. This finding aligns with previous work by Samala et al. [70], who reported an 80% survival rate using a foam substrate under greenhouse conditions, confirming that well-aerated, moisture-permeable substrates with adequate drainage are critical determinants of post-transfer survival in this species. Charcoal's excellent drainage and aeration, adsorption of phytotoxic metabolites, near-neutral pH, and structural stability make it well-suited to the epiphytic growth requirements of *D. cruentum*, achieving 80–85% survival, comparable to that of a foam substrate [2]. Poor survival on Sphagnum moss (15–20%) likely reflects its excessive moisture retention, its compact structure when wet, and its inherently acidic pH (3.5–4.5), which are incompatible with the near-neutral substrate conditions of *Dendrobium*'s natural epiphytic habitat [71]. The moderate survival rates achieved on coconut husk substrate (70% and 75% for the 20 and 40 g/L treatments, respectively) confirm its utility as a practical alternative substrate, although its susceptibility to gradual decomposition may necessitate more frequent repotting than with charcoal. The marginal improvement in plantlet survival from the 40 g/L sucrose medium (85% vs. 80%) suggests that elevated internal carbohydrate reserves confer a metabolic advantage during the critical transition from heterotrophic in vitro conditions to autotrophic *ex vitro* nutrition, consistent with established relationships between pre-acclimatization carbohydrate status and post-transfer survival in orchid micropropagation. Although somaclonal variation is generally low in seed-derived cultures, genetic fidelity under the non-standard conditions employed here (net house, 40 g/L sucrose) has not been verified. This limitation, along with the recommended marker approaches (RAPD, ISSR, SCoT), is discussed in Section 3.6.

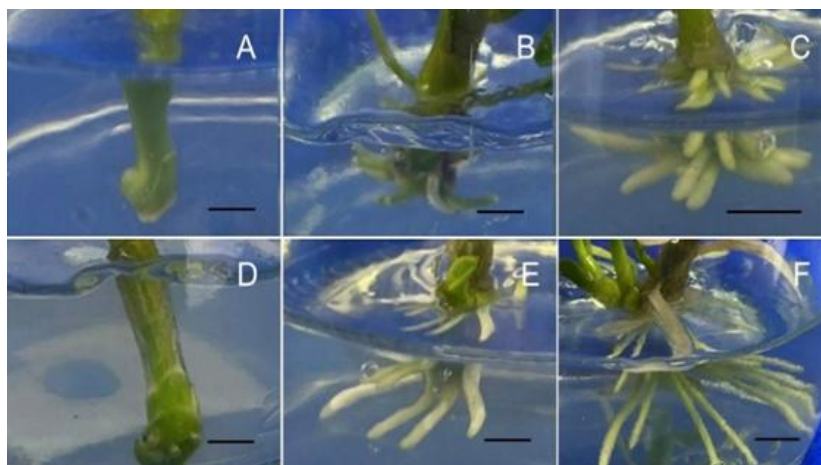


Figure 3. After being cultured on 1/2MS agar medium supplemented with 0, 20, and 40 g/L sucrose, rooting of *D. cruentum* Rchb.f. was observed in both controlled room (A-C) and net house (D-F) conditions for a period of two months. (bar = 1 cm).

Table 6. Survival rate of transplanted plantlets after 2 months.

Substrates (ST)	Survival rate		Average (ST)
	Sucrose concentration (SC) (g/L)		
	20	40	
Sphagnum Moss	15.00 ± 5.00 ^c	20.00 ± 5.00 ^c	17.5C
Chopped coconut husk	70.00 ± 8.66 ^b	75.00 ± 5.00 ^{ab}	72.5B
Charcoal	80.00 ± 5.00 ^{ab}	85.00 ± 5.00 ^a	82.5A
Average (SC)	55.00	60.00	
ST	**		
SC	ns		
ST xSC	*		
C.V. (%)	10.16		

¹ Data are presented as means ± SD. Different letters in a column and row indicate statistically significant differences ($p \leq 0.01$) by DMRT, ns = not significantly different

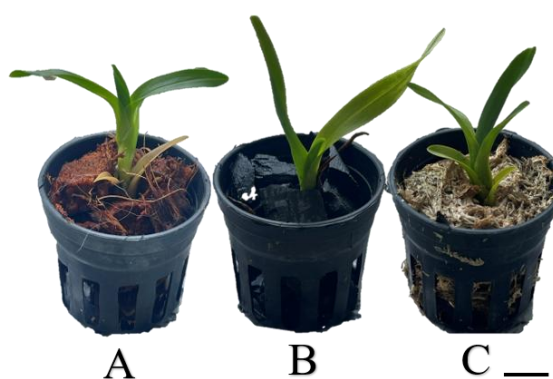


Figure 4. *D. cruentum* plantlets, previously cultured in bottles with 1/2MS agar medium supplemented with 40 g/L of sucrose in a net house, were acclimatized by transferring them to pots filled with three different substrates (bar = 1 cm), A, Chopped coconut husk; B, charcoal; C, sphagnum moss.

3.6 Limitations and future directions.

Two important limitations of the present study merit acknowledgment. First, the genetic fidelity of plantlets produced under net house conditions and at the elevated sucrose concentration of 40 g/L has not

been verified in this study. However, somaclonal variation is generally considered low in seed-derived cultures; the non-standard culture conditions employed here warrant verification through molecular marker analyses (e.g., RAPD, ISSR, SCoT, or SSR) in future studies [5, 25]. Second, the net house performance documented in this study reflects the tropical climate of southern Thailand, where ambient temperatures, light intensity, and CO₂ availability are favorable year-round; the applicability of this protocol in temperate or seasonally variable climates may require modification, particularly with respect to temperature management and supplemental lighting during cooler months.

4. Conclusions

This study developed a comprehensive, cost-effective micropropagation protocol for the CITES Appendix I-listed orchid *D. cruentum*, addressing both ex situ conservation and commercial production needs. Chemical sterilization with 2.0 mL/L Haiter® controlled contamination to 10% eliminated the need for autoclaving, reducing sterilization-related costs by an estimated 60–70% (per-cycle cost: USD 0.01–0.02 vs. USD 0.20–0.30 for autoclaving). Net house culture with 40 g/L sucrose produced superior plantlets compared with controlled-room culture after two months, yielding 2.63 new shoots, 14.33 roots, 3.60 cm height, and 1.14 g fresh weight per plantlet — representing improvements of 22%, 69%, 41%, and 75.4%, respectively. Charcoal substrate supported the highest acclimatization survival (85%), confirming its suitability for this species' epiphytic root characteristics. The integrated protocol reduces overall operational costs by 40–70% across sterilization and climate control components, making micropropagation economically accessible to small-scale growers and community-based conservation programs in tropical regions. Genetic fidelity verification and validation under non-tropical climates remain priorities for future research. This approach offers a transferable model for other threatened tropical orchids where ambient net house conditions can substitute for energy-intensive infrastructure.

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Efficient Removal of Organic Pollutants Using Magnetic Biochar Derived from Corn Husk

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Abstract: The development of cost-effective and sustainable adsorbents for dye-contaminated wastewater remains a critical environmental challenge. In this study, a magnetic biochar composite (M/BCH) was synthesized from corn husk biomass through FeCl_3 impregnation followed by pyrolysis at 600 °C for 4 h. The physicochemical characteristics of the pristine biochar (BCH) and the magnetic composite were systematically investigated using XRD, FTIR, SEM/EDS, and N_2 adsorption-desorption techniques. XRD and FTIR analyses confirmed the successful incorporation of Fe_2O_3 into the carbon matrix, while SEM/EDS mapping revealed uniform Fe distribution (~12 wt.%). Textural analysis showed that Fe_2O_3 deposition reduced the BET surface area and pore volume, indicating partial pore occupation. The adsorption performance of BCH and M/BCH for methylene blue (MB) and methyl red (MR) was evaluated over varying contact times, initial concentrations, adsorbent dosages, and shaking speeds. BCH exhibited relatively higher MB adsorption, attributed to its higher surface area and mesoporous structure, whereas M/BCH showed higher MR removal compared to BCH due to modified surface chemistry, improved electrostatic interactions, and additional mechanisms such as complexation and π - π interactions. The M/BCH composite reached adsorption equilibrium within 120 min and showed increasing dye uptake with higher adsorbent dosage and shaking speed. Importantly, its magnetic properties enabled rapid and efficient post-treatment separation. Overall, the findings demonstrate that corn husk-derived M/BCH was an effective, multifunctional, magnetically separable adsorbent with potential for wastewater treatment applications.

Keywords: Biochar; corn husk; magnetic composite; methylene blue; methyl red.

1. Introduction

Large quantities of wastewater are continuously generated from textile, leather, paper, and plastic industries, with dye-laden effluents, particularly those containing synthetic dyes such as methylene blue and methyl red, representing a major environmental concern due to their toxicity and persistence in aquatic systems [1]. Dyeing wastewater commonly exhibits high concentrations of aromatic organic pollutants, intense chromaticity, alkaline pH, and recalcitrant constituents [2]. Consequently, unintended or uncontrolled discharge of such effluents into natural water bodies can lead to severe ecological deterioration. Addressing dye-contaminated wastewater has therefore become a critical issue in environmental engineering and remains one

of the most challenging aspects of industrial wastewater management. A diverse array of treatment approaches has been explored for dye elimination, including advanced oxidation processes (AOPs), photocatalytic degradation, membrane separation, biological treatment, and adsorption [3]. Among these, adsorption is frequently highlighted as a practical and efficient approach, owing to its operational simplicity, cost-effectiveness, and robustness toward toxic or chemically complex pollutants. Commercial activated carbon remains the benchmark adsorbent; however, its relatively high cost and regeneration limitations hinder large-scale application. To overcome these barriers, growing research efforts have focused on developing low-cost, value-added, or waste-derived sorbents as sustainable alternatives for dye removal from industrial wastewater [4, 5].

Biochar is a carbon-rich material generated under oxygen-limited conditions through slow or fast pyrolysis. It can be produced from a wide range of lignocellulosic and organic biowastes, including woody residues, agricultural by-products, animal wastes, and biosolids. Due to its elevated porosity, extensive surface area, and predominantly negative surface charge, biochar has become a viable, low-cost adsorbent for the removal of organic and inorganic pollutants from aqueous systems [6, 7]. Despite these advantages, many commercial biochars remain costly due to their feedstock sources and the additional regeneration or reactivation procedures required, which limit their wider practical use. Corn husk, the fibrous outer layer surrounding the maize cob, represents an abundant lignocellulosic residue. Current disposal techniques, such as open burning and landfilling, exacerbate environmental degradation by disposing of waste-rich biomass resources. Given that maize is the second most widely cultivated crop globally and that approximately 14% of the plant mass consists of husk, this agricultural waste offers substantial potential as an inexpensive carbon precursor for biochar production. In addition to their use as precursors for biochar production, corn husks and other agricultural residues have been widely explored for value-added applications, such as biomass densification and the development of renewable materials. Furthermore, biochar derived from various waste sources has demonstrated promising potential in environmental and agricultural applications, highlighting its versatility and sustainability [8,9].

Iron oxide (Fe_2O_3) has been widely explored in various technological and environmental applications, including magnetic media, biosensing, imaging, and energy systems. Importantly, Fe_2O_3 shows a strong affinity for a range of pollutants, including heavy metals, organic dyes, and pharmaceuticals [10,11]. Its magnetic properties also allow spent particles to be easily recovered from aqueous systems, enabling the recyclability of the adsorbent. However, nanoscale Fe_2O_3 tends to aggregate in water due to its high surface energy, thereby reducing surface area and diminishing adsorption efficiency. To overcome these limitations, combining Fe_2O_3 nanoparticles with a carbonaceous support, such as biochar, can enhance stability, dispersion, and functional performance. In this study, a magnetic biochar composite (M/BCH) was synthesized using corn husk mixed with FeCl_3 and subjected to pyrolysis at 600°C for 4 h to obtain a multifunctional magnetic sorbent. The physicochemical characteristics of the resulting M/BCH were systematically examined. Its adsorption performance toward two model organic dyes, methylene blue (MB) and methyl red (MR), with different chemical structures (Figure 1), was investigated by evaluating the effects of adsorbent type, dosage (M/V, g/L), initial dye concentration, contact time, and shaking speed.

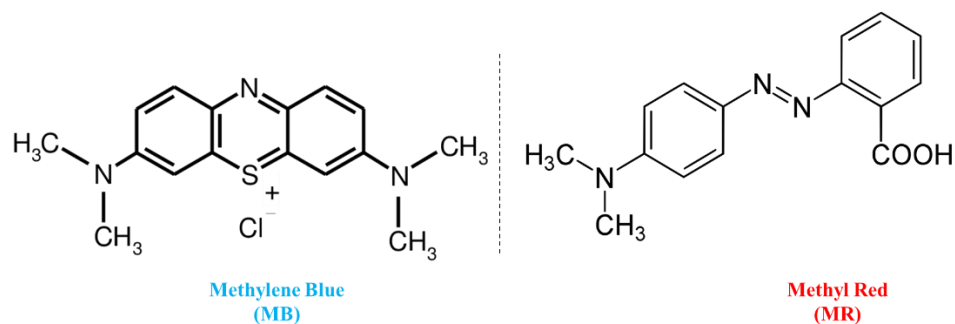


Figure 1. Chemical structure of methylene blue and methyl red.

2. Materials and Methods

2.1 Chemicals and reagents for preparation of adsorbents

Corn husk residue (CH) was collected from Chiang Mai province, Thailand. Iron (III) chloride hexahydrate (analytical grade 99.9%, $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$), Methylene blue ($\text{C}_{16}\text{H}_{18}\text{N}_3\text{SCL}$, 319.85 g/mol) and Methyl red ($\text{C}_{15}\text{H}_{15}\text{N}_3\text{O}_2$, 269.30 g/mol) were purchased from Sigma-Aldrich. All chemicals were of analytical grade and were used without further purification.

2.2 Preparation of adsorbents

2.2.1 Preparation of biochar derived from corn husk (BCH)

The corn husk biomass was initially rinsed thoroughly with deionized water to remove surface dust, soil residues, and other soluble contaminants that could affect carbonization efficiency. The material was dried at 110 °C for 12 h post-washing to eliminate residual moisture and ensure stable thermal behavior during pyrolysis. The dried biomass was milled and sieved to achieve a uniform particle size of less than 100 μm , thereby enhancing heat transfer and facilitating effective carbon conversion during thermal processing. Pyrolysis was performed in a muffle furnace at 600 °C for 4 h with a heating rate of 5 °C min^{-1} under limited oxygen to facilitate char formation. Approximately 20 g of dried corn husk biomass was used in each pyrolysis batch. Pyrolysis was carried out in a laboratory-scale muffle furnace under oxygen-limited conditions without an external carrier or inert gas flow. The produced biochar was naturally cooled to room temperature and kept in a desiccator to prevent moisture uptake before subsequent modification steps. After pyrolysis, the BCH sample was ground and sieved to obtain particles within the 0.5–1 mm range.

2.2.2 Preparation of magnetic biochar derived from corn husk (M/BCH)

The magnetic corn-husk biochar/ Fe_2O_3 composite was synthesized by impregnating corn-husk powder with an FeCl_3 solution, followed by high-temperature pyrolysis. An FeCl_3 solution was prepared by dissolving 40 g of $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ in 60 mL of deionized water. The impregnation process was performed using approximately 20 g of corn husk powder mixed with 60 mL of FeCl_3 solution. The corn husk powder was then soaked in this solution for 2 h to allow adequate adsorption of iron precursors. Following impregnation, the mixture was air-dried at 80 °C for 2 h. The biomass underwent pyrolysis in a furnace at 600 °C for 4 h, with a heating rate of 5 °C/min. The obtained biochar/ Fe_2O_3 composite was lightly ground and sieved to obtain particles in the 0.5–1 mm range. The resulting biochar derived from corn husk was designated as M/BCH. The samples were rinsed multiple times with deionized water, subsequently dried at 80 °C, and stored in a desiccator for future use.

2.3 Characterization of adsorbents

The structural information of the materials was obtained by powder X-ray diffraction (XRD) analysis on an Empyrean 3 Panalytical diffractometer, using $\text{CuK}\alpha$ radiation at 40 kV and 40 mA. Fourier-transform infrared spectroscopy (FTIR) was used to identify functional groups in the composites, using a Perkin-Elmer spectrometer with the KBr method. Sixty-four scans throughout the range of 400–4000 cm^{-1} at a resolution of 4 cm^{-1} were averaged for each spectrum. The SEM micrographs were captured at 1,500 $\times$ magnification using a JEOL JSM-IT800. Elemental mapping and confirmation of iron distribution were further supported by energy-dispersive X-ray spectroscopy (EDS). N_2 adsorption-desorption was measured at -196 °C using an Anton Paar Nova 600 to determine the textural characteristics of the materials. The mass of the sample, initially approximately 40 mg, was precisely recorded after pretreatment at 150 °C for 2 h.

2.4 Adsorption study

The adsorption behavior of methylene blue (MB) and methyl red (MR) on corn husk-derived biochar materials (BCH and M/BCH) was evaluated using batch experiments. A known mass of adsorbent was added to 50-mL Erlenmeyer flasks containing 10 mL of dye solution, and the mixtures were agitated under controlled conditions using a mechanical shaker. The influence of several operational parameters, including contact time (0–120 min), adsorbent dosage (1–10 g/L), initial dye concentration (100–140 mg/L), and shaking speed (100–150 rpm) was systematically investigated. All adsorption experiments were conducted at the natural pH of the dye solutions without pH adjustment. The initial pH values were approximately 6.5 for methylene blue

and 5.0 for methyl red. After the adsorption process, the residual concentrations of MB and MR were quantified using a UV-vis spectrophotometer (Jasco V-730) at wavelengths of 664 nm and 444 nm, respectively. Each sample was measured in triplicate to ensure analytical reliability. At each sampling interval, approximately 0.5 mL of solution was withdrawn for UV-vis analysis. The adsorption capacity (q_e) was determined using Eq. (1). Calibration curves were constructed using six concentration points, yielding the following regression equations: MB, $y = 0.0189x - 0.0165$ ($R^2 = 0.9990$); MR, $y = 0.0091x - 0.00286$ ($R^2 = 0.9976$), where x represents the dye concentration (mg/L), and y denotes the absorbance.

$$\text{Adsorption capacity: } q_e = \frac{(C_0 - C_e)V_i}{W} \quad (1)$$

In this equation, q_e denotes the equilibrium adsorption capacity (mg/g, based on dry adsorbent). C_0 and C_e refer to the initial and equilibrium dye concentrations in the solution (mg/L). V_i represents the volume of the initial dye solution (L), and W corresponds to the mass of the dried adsorbent used (g).

2.5 Kinetic study

The dried adsorbent was added to the MB and MR solution in the 250 mL Erlenmeyer flask. The initial concentrations of the MB and MR solutions were fixed at 100 mg/L. Samples were collected at different time intervals for UV-vis analysis until equilibrium was reached. Pseudo-first-order and pseudo-second-order kinetic models were evaluated according to Eqs. (2)–(3) to postulate the mechanism of MB and MR adsorption on the synthesized adsorbents.

Pseudo-first-order model:

$$q_t = q_e (1 - e^{-K_1 t}) \quad (2)$$

Pseudo-second-order model:

$$q_t = K_2 \frac{q_e^2 t}{1 + K_2 q_e t} \quad (3)$$

where q_t is the adsorption capacity at any time (mg/g_{dried adsorbent}); K_1 (/min) and K_2 (g/(mg min)) are the rate constants of the pseudo-first-order and pseudo-second-order models, respectively.

2.6 Isotherm study

The adsorption isotherm data were measured *via* the procedure described in Section 2.4. The initial concentrations of MB and MR were varied between 100 and 140 mg/L.

3. Results and Discussion

3.1 Characterization of biochar adsorbents

X-ray diffraction (XRD) was used to characterize the crystalline and amorphous phases of the biochar-based materials. Figure 2 displays the diffraction patterns of the corn husk biochar (BCH) and the magnetic corn husk biochar composite (M/BCH). The BCH sample exhibited a broad diffraction band within the 2θ range of approximately 15–30°, indicating its predominantly amorphous carbon structure. In contrast, the M/BCH composite retained this amorphous background while displaying additional sharp diffraction peaks at 33.3°, 35.6°, 50.2°, and 57.2°, corresponding to crystalline Fe_2O_3 [12]. The presence of these well-defined peaks indicates the successful incorporation of iron oxide into the biochar matrix. Due to the increased intensity of the crystalline Fe_2O_3 peaks, the amorphous hump appears less prominent in the M/BCH pattern compared to pristine BCH. This result suggested that Fe_2O_3 particles were well dispersed on the biochar structure, resulting in a low intensity of the amorphous structure but a high intensity of the Fe_2O_3 crystal structure when analyzed by XRD.

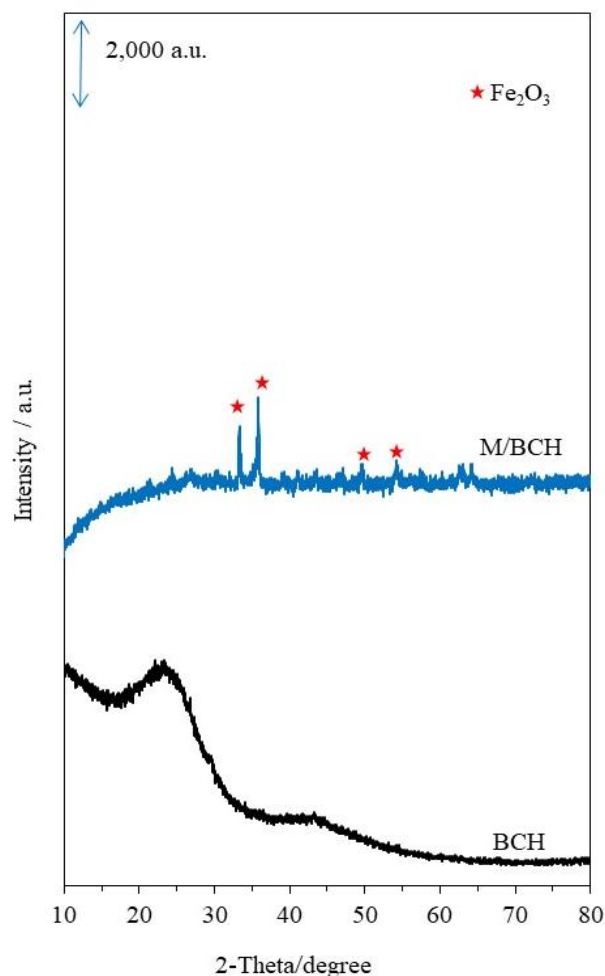


Figure 2. XRD analysis of biochar derived from corn husk, BCH, and M/BCH composite.

The FTIR spectra of the corn husk biochar (BCH) and the magnetic biochar composite (M/BCH) are presented in Figure 3. For BCH pyrolyzed at 600 °C, a broad absorption band at 3394 cm⁻¹ was observed, which is attributed to O–H stretching vibrations associated with phenolic hydroxyl and alcoholic functionalities [13]. The band at 1693 cm⁻¹ corresponds to C=O stretching of carboxyl or conjugated carbonyl groups [14], while the feature at 1597 cm⁻¹ is assigned to aliphatic C=C stretching [14]. In the case of M/BCH, the band observed at 1629 cm⁻¹ is more likely attributed to O–H bending vibrations of adsorbed water and/or C=C stretching in the carbon structure rather than Fe₂O₃-related vibrations [15]. It is well established that Fe–O stretching vibrations of Fe₂O₃ typically appear in the fingerprint region below 700 cm⁻¹. However, this region is not clearly resolved in the present FTIR spectrum, limiting further interpretation. Therefore, the incorporation of Fe₂O₃ into the biochar matrix is primarily confirmed by XRD analysis and SEM-EDS mapping rather than FTIR alone. However, the M/BCH composite exhibited reduced intensities of the characteristic peaks associated with the carbonaceous functional group of biochar. This result corresponded to the XRD data in Figure 2. This result confirmed that Fe₂O₃ particles were deposited on the biochar surface with good dispersion.

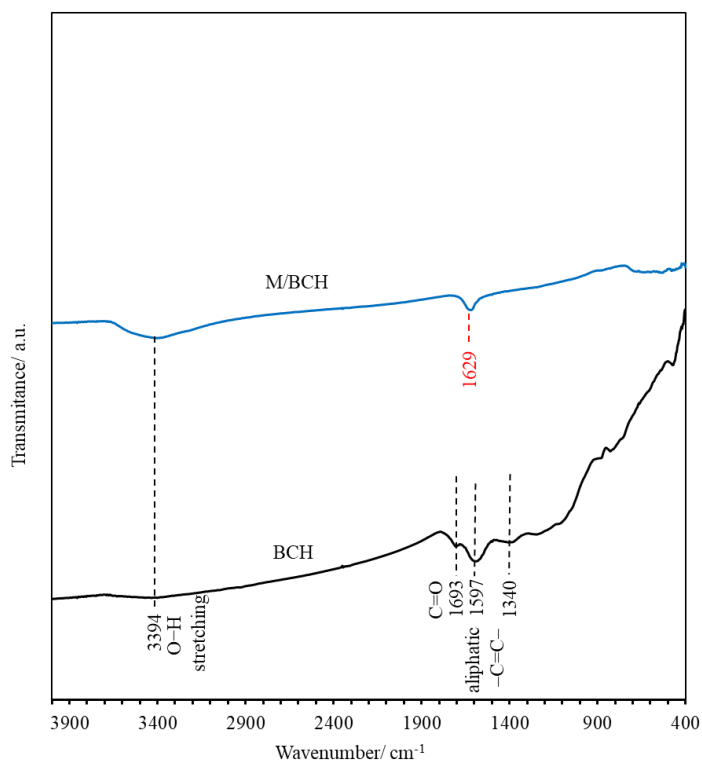


Figure 3. FTIR spectra of biochar derived from corn husk, BCH, and M/BCH composite.

The morphological characteristics of BCH and M/BCH were examined by scanning electron microscopy (SEM), as shown in Figure 4. Both samples displayed a porous carbon framework, which is commonly generated during pyrolysis as volatile components are released. In contrast to pristine BCH, the surface of the M/BCH composite showed a high level of finely dispersed Fe content. Energy-dispersive X-ray spectroscopy (EDS) mapping further confirmed the distribution of Fe across the carbon surface. While no detectable Fe signal appeared in the pristine BCH, the M/BCH composite contained approximately 12 wt.% of Fe content, demonstrating the effectiveness of anchoring the Fe particles uniformly on the biochar surface.

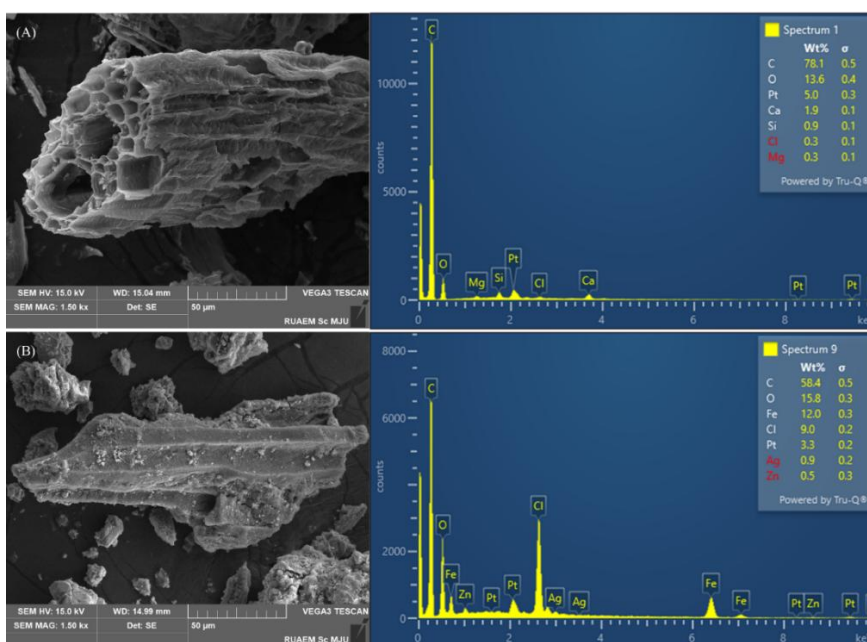


Figure 4. SEM-EDS image of biochar derived from corn husk (A) BCH and (B) M/BCH composite at magnification of 1,500x.

Figure 5 illustrates the N₂ adsorption–desorption isotherms and the corresponding pore size distributions of the BCH and M/BCH materials. Based on the IUPAC classification, both samples exhibit type IV isotherms with H-type hysteresis loops over a relative pressure range of approximately 0.45–0.95. This behavior is indicative of mesoporous structures formed through capillary condensation, as shown in Figure 5A. The pore size distribution profiles (Figure 5B) show a single dominant pore diameter centered at 3.7 nm for BCH and 3.2 nm for M/BCH, further confirming the mesoporous nature of the materials. As summarized in Table 1, the M/BCH composite with Fe content of 12%wt (SEM-EDS result) exhibited a lower BET surface area, total pore volume, and pore diameter (40.3%, 31.25%, and 12.6% reduction, respectively) in comparison with those of the initial BCH. These results suggest that Fe₂O₃ particles deposited within the pore structure of biochar derived from corn husk, leading to a drastic loss of textural properties [15].

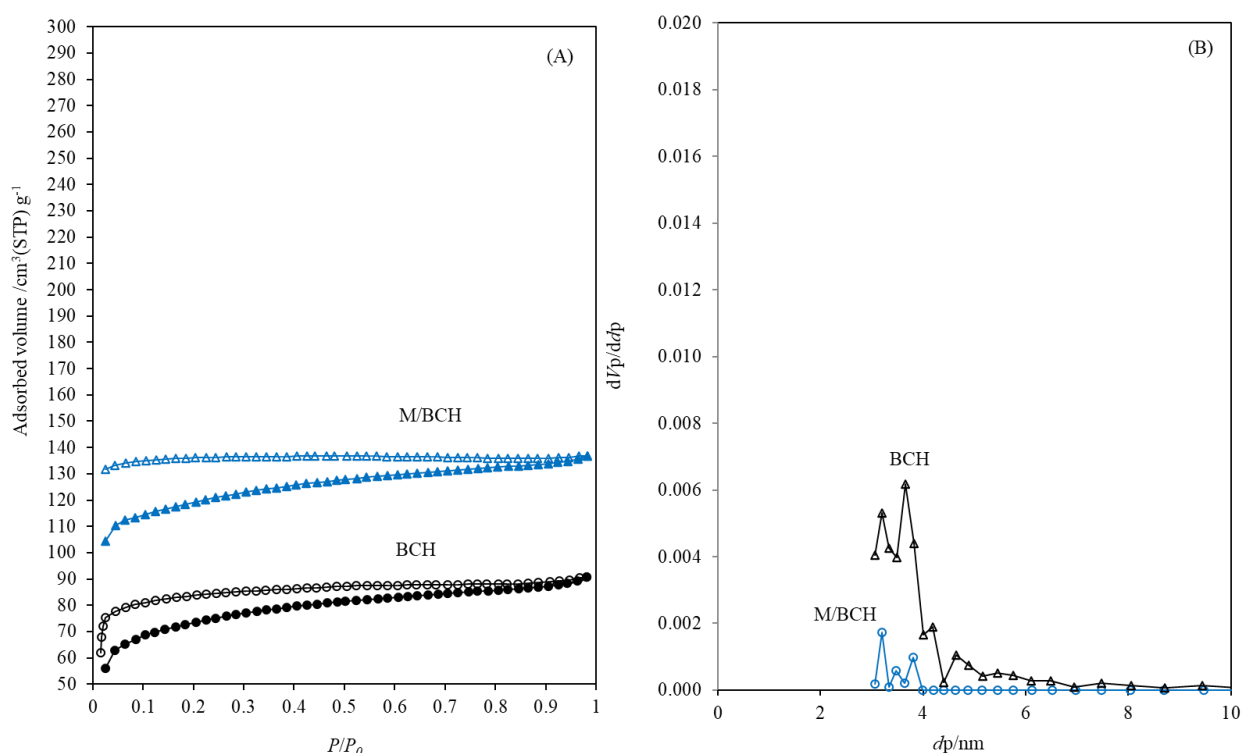


Figure 5. N₂ adsorption–desorption isotherms (A) and BJH pore size distribution (B) of BCH and M/BCH composite.

Table 1. Textural characteristics of BCH and M/BCH composite.

Sample	$S_{\text{BET}}^{\text{a}}$ (m ² g ⁻¹)	D_{p}^{b} (nm)	V_{t}^{c} (cm ³ g ⁻¹)
BCH	269.58	3.65	0.16
M/BCH	160.93	3.19	0.11

^aBET surface area

^bPore diameter, evaluated by the BJH method

^cTotal pore volume, evaluated by the volume adsorbed at $P/P_0 = 0.99$

3.2 Adsorption study for the removal of dyes

The biochar derived from corn husk (BCH) and the magnetic biochar composite (M/BCH) were evaluated as adsorbents for the removal of methylene blue (MB) and methyl red (MR) from aqueous solutions. The adsorption capacities and removal efficiencies for both dyes are summarized in Figure 6A–B. As shown in Figure 6A, BCH exhibited higher adsorption capacity and removal efficiency than the M/BCH composite, which is consistent with the BET surface area values reported in Table 1. These findings indicate that the mesoporous structure and larger specific surface area of BCH facilitate enhanced adsorption. The adsorption

behavior may be influenced by electrostatic interactions depending on the solution pH and the ionization state of the MB dye molecules. Additional non-electrostatic interactions such as π - π stacking, n - π interactions, and hydrogen bonding further facilitate MB attachment to the composite surface [16]. However, the M/BCH composite exhibited a good magnetic property for separation from the solution, as shown in Figure 7, whereas the BCH material could not show the magnetic property. Although all experiments were conducted in triplicate, no formal statistical tests (e.g., t-tests or ANOVAs) were performed. Therefore, the observed differences between BCH and M/BCH should be interpreted as comparative trends rather than statistically validated differences.

In contrast, the M/BCH composite demonstrated greater adsorption capacity and removal efficiency for methyl red than the pristine BCH, as illustrated in Figure 6B. This trend aligns with the findings of Dong et al. [17], who reported that biochar-iron mineral composites exhibit enhanced performance in the removal of methyl orange. The addition of Fe_2O_3 to the biochar structure enhanced the adsorption of methyl red (MR) through multiple mechanisms. Therefore, the enhanced adsorption of methyl red by M/BCH may be associated with several interactions between the adsorbent surface and dye molecules. These may include electrostatic attraction, π - π interaction between aromatic structures, and hydrogen bonding. It should be noted that no advanced characterization techniques, such as post-adsorption FTIR, XPS, or total organic carbon (TOC) analysis, were performed. Therefore, the dominant adsorption mechanism cannot be conclusively identified and requires further investigation. These mechanisms are proposed based on the literature and the material's physicochemical characteristics and were not directly verified in this study. So, the adsorption behavior should be interpreted as a combination of possible interactions rather than a single confirmed mechanism.

Based on the comparative results shown in Figures 6 and 7, the M/BCH composite was selected for further parametric investigation due to its magnetic separability and enhanced performance toward methyl red. Therefore, subsequent studies on contact time, initial concentration, adsorbent dosage, and shaking speed were conducted using M/BCH as the representative adsorbent.

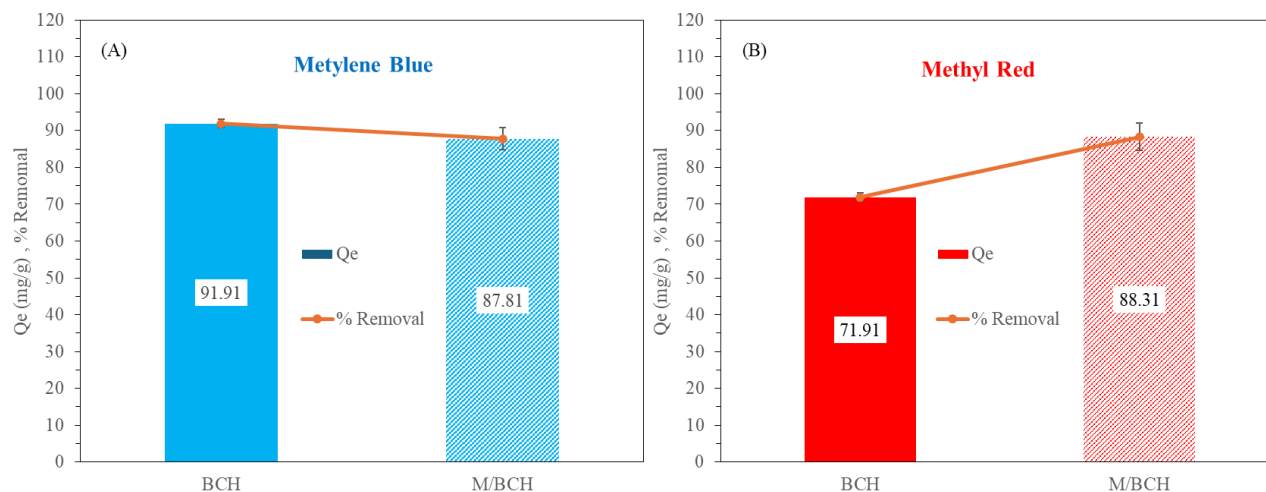


Figure 6. Comparison of adsorption performance of pristine biochar (BCH) and magnetic biochar (M/BCH) for (A) methylene blue (MB) and (B) methyl red (MR) under identical conditions (initial concentration: 100 mg/L, adsorbent dosage: 1 g/L, contact time: 120 min, shaking speed: 100 rpm).

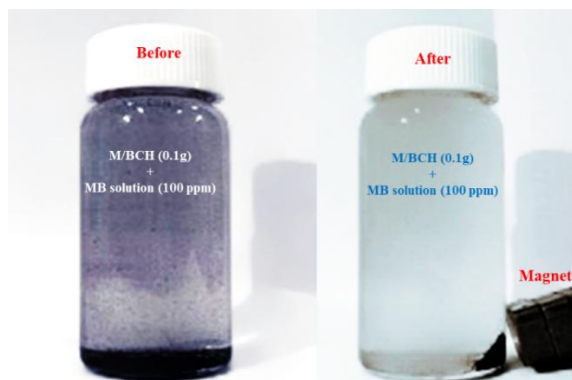


Figure 7. The adsorption MB solution (100 mg/L) using the M/BCH composite before and after magnetic separation.

The adsorption performance of the M/BCH composite toward MB and MR was examined by evaluating the effects of contact time, initial dye concentration, adsorbent dosage (M/V, g/L), and shaking speed, as presented in Figure 8. The influence of contact time is shown in Figure 8A. A rapid uptake of both dyes occurred during the first 100 min, after which the adsorption rate gradually stabilized, reaching equilibrium at approximately 120 min. The effect of initial dye concentration was assessed as depicted in Figure 8B. When the initial concentrations increased from 100 to 140 mg/L, the adsorption capacities of the M/BCH composite decreased by approximately 19.04% for MB and 12.07% for MR. Given that the M/V ratio remained constant, this decline suggests that the available active sites associated with Fe_2O_3 and surface functional groups were limited in number [18,19]. Consequently, higher initial dye concentrations led to greater competition between MB and MR molecules for adsorption sites.

The influence of adsorbent dosage (M/V) on the adsorption of MB and MR is shown in Figure 8C. Increasing the M/V ratio from 1 g/L to 10 g/L increased the adsorption capacities by approximately 20.04% and 28.07% for MB and MR, respectively. This improvement is attributed to the greater number of active sites provided by the higher adsorbent dosage, while the dye concentration and solution volume (10 mL, 100 mg/L) remained constant. Based on these results, an adsorbent dosage of 10 g/L was identified as optimal under the laboratory-scale conditions used in this study (10 mL solution volume). It should be noted that the reported adsorption performance and optimal dosage are based on laboratory-scale batch experiments and may not directly represent large-scale or continuous treatment systems. Figure 8D shows the effect of shaking speed on adsorption performance. Increasing the shaking speed from 100 to 150 rpm improved the adsorption capacities of both dyes. Enhanced agitation promotes better dispersion of the adsorbent particles and increases the frequency of collisions between dye molecules and the M/BCH composite, thereby accelerating adsorption. However, it should be noted that pH-PZC measurement and systematic pH-dependent studies were not conducted in this work. Therefore, the proposed adsorption mechanisms involving electrostatic interactions are inferred from the literature and should be interpreted with caution. In addition, the regeneration and reusability of the M/BCH composite were not evaluated in this study. Therefore, while the magnetic property enables rapid separation from solution, the adsorbent's long-term stability and recyclability require further investigation.

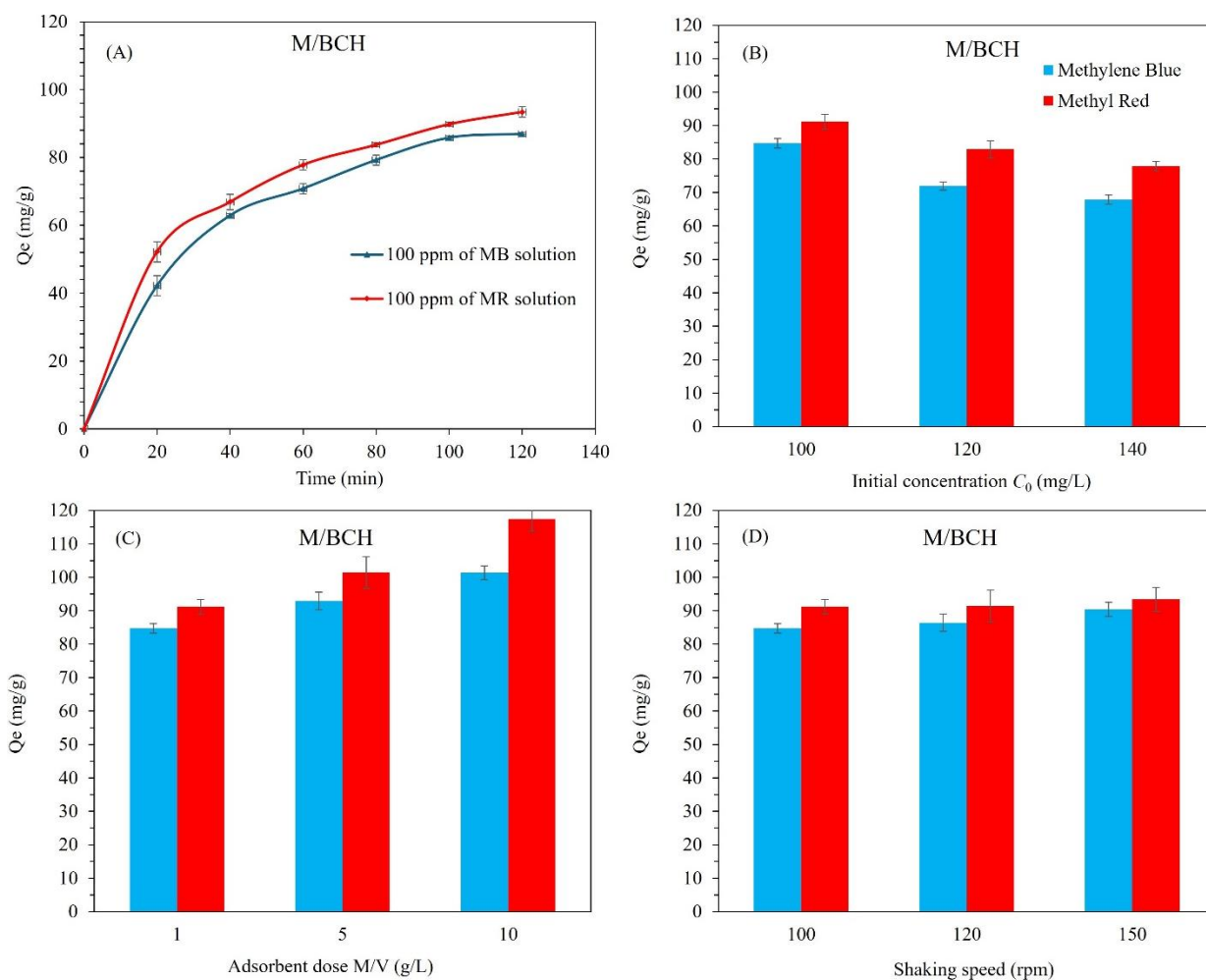


Figure 8. Effect of (A) contact time, (B) initial concentration, (C) adsorbent dose, and (D) shaking speed on methylene blue and methyl red removal.

3.3 Adsorption kinetics and isotherm modeling

The adsorption behavior of methylene blue and methyl red onto M/BCH was further evaluated using kinetic and isotherm models to understand the governing mechanisms better. The kinetic data were analyzed using pseudo-first-order (PFO) and pseudo-second-order (PSO) models (Figure 9A–B), and the fitted parameters are summarized in Table 2. It was observed that the PSO model described the experimental data more accurately for both dyes, as reflected by higher R^2 values and better agreement between calculated and experimental q_e values. ($R^2 = 0.9979$ for MB and 0.9979 for MR) compared to the PFO model. In addition, the q_e values estimated from the PSO model showed better agreement with the experimental values. This result suggests that the adsorption process is better described by the pseudo-second-order model, which is commonly associated with surface-related interactions. However, the deviation between calculated and experimental q_e values indicates that a single kinetic model cannot fully explain the system. This implies that other factors, such as pore diffusion and surface heterogeneity, may also contribute to adsorption.

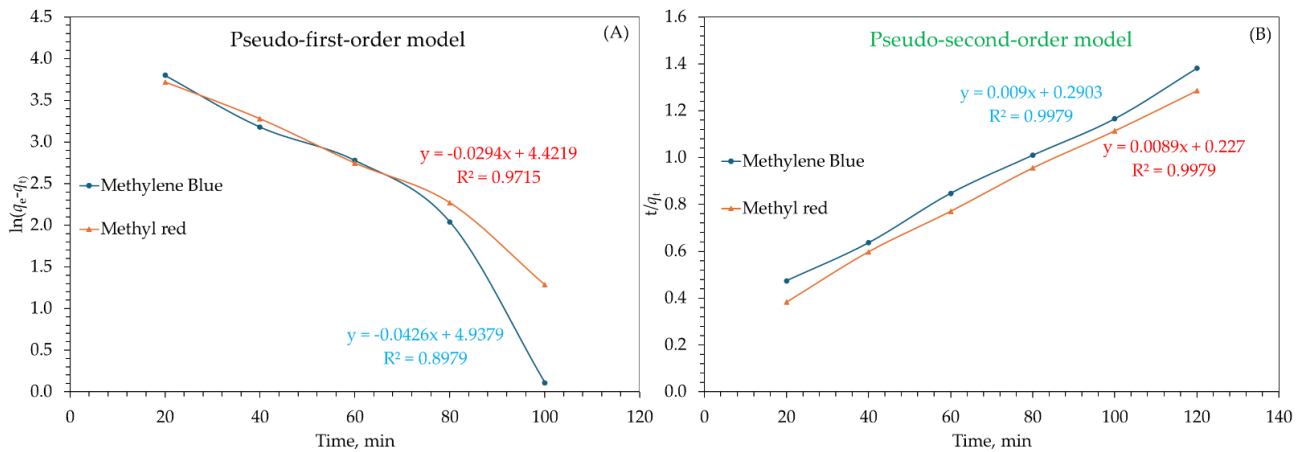


Figure 9. Fitting data to a pseudo-first-order kinetic model (A) and a pseudo-second-order kinetic model (B) for methylene blue and methyl red adsorption onto M/BCH adsorbent.

Table 2. Kinetic parameters for methylene blue and methyl red adsorption onto M/BCH adsorbent.

Dye	Model	k	q_e (calc) (mg/g)	q_e (exp) (mg/g)	R^2
Methylene blue	Pseudo-first-order	0.0426 min ⁻¹	139.47	86.90	0.8979
	Pseudo-second-order	2.78×10 ⁻⁴ g mg ⁻¹ min ⁻¹	111.35	86.90	0.9979
Methyl red	Pseudo-first-order	0.0415 min ⁻¹	147.32	93.40	0.9715
	Pseudo-second-order	3.10×10 ⁻⁴ g mg ⁻¹ min ⁻¹	118.22	93.40	0.9979

For equilibrium analysis, the adsorption data were fitted to the Langmuir and the Freundlich isotherm models, and the results are presented in Table 3. The Langmuir model showed slightly higher R^2 values than the Freundlich model for both MB and MR, indicating a better overall fit to the experimental data. Nevertheless, some of the calculated parameters, particularly the Langmuir constant (K_L), exhibited non-ideal values. This suggests that adsorption occurs on a heterogeneous surface with multiple interaction mechanisms rather than in an ideal monolayer. Similarly, the Freundlich parameter (n) did not clearly reflect typical adsorption behavior. These observations suggest that the adsorption system does not strictly follow the ideal assumptions of either model. Such inconsistency between fitting quality and parameter validity indicates that the adsorption of MB and MR onto M/BCH is likely governed by multiple mechanisms rather than a single ideal process. Possible interactions include electrostatic attraction, π - π interactions between aromatic structures, and surface interactions involving iron oxide species. It should also be noted that the isotherm study was conducted within a relatively narrow concentration range (100–140 mg/L), which may limit the robustness of model fitting and affect parameter interpretation. Overall, the combined kinetic and isotherm results indicate that adsorption on M/BCH involves multiple mechanisms within a heterogeneous system. This behavior is typical for biochar-based composites and reflects the complex nature of their surface properties.

Table 3. Isotherm parameters for methylene blue and methyl red adsorption onto M/BCH adsorbent.

Dye	Freundlich			Langmuir		
	K_F ((mg/g)(mL/mg) ^{1/n})	n	R^2	K_L (mg/mL)	q_{max} (mg/g)	R^2
Methylene blue	1,784.7	1.5	0.9525	-0.0219	45.37	0.9963
Methyl red	1,500	1.3	0.9480	-0.018	52.18	0.9950

To further evaluate the performance of the M/BCH composite, a comparison with previously reported adsorbents is presented in Table 4. The results indicate that the adsorption capacity of M/BCH for methylene blue is lower than that of pristine BCH and several engineered adsorbents, which can be attributed to the reduction in surface area resulting from Fe₂O₃ incorporation. However, M/BCH demonstrates improved performance toward methyl red compared to BCH, suggesting that surface modification enhances interactions with anionic dyes. Although some reported materials exhibit higher adsorption capacities, they often involve more complex synthesis routes or higher production costs. In contrast, the M/BCH composite developed in this study offers advantages such as low-cost biomass feedstock, simple preparation, and magnetic separability. It should also be noted that the $q_{\max}$ values obtained in this study were derived from a relatively narrow concentration range, and therefore should be interpreted as approximate values rather than absolute maximum capacities.

Table 4. Comparison of the adsorption capacities of M/BCH with those of reported biochar-based adsorbents.

Adsorbent	Dye	$q_{\max}$ (mg/g)	Key feature	Reference
BCH	Methylene blue	91.91	High surface area	This work
M/BCH	Methylene blue	45.37	Magnetic, Fe ₂ O ₃ -modified	This work
M/BCH	Methyl red	52.18	Enhanced anionic dye removal	This work
Magnetic biochar (banana peel)	Methylene blue	72.5	Fe-modified biochar	[12]
Biochar/Fe ₂ O ₃ composite	Methyl orange	65.3	Electrostatic interaction	[17]
Ag-biochar composite	Methylene blue	110.4	Catalytic adsorption	[13]
Activated carbon	Methylene blue	150–400	Commercial adsorbent	[4,5]

4. Conclusions

This study successfully synthesized a magnetic biochar composite (M/BCH) from corn husk, an abundant agricultural residue, and demonstrated its effectiveness as a recoverable adsorbent under laboratory-scale conditions. Structural analyses confirmed the effective incorporation of Fe₂O₃ nanoparticles onto the biochar surface, resulting in distinct changes in functional groups, morphology, and surface textural properties. Although the deposition of Fe₂O₃ reduced the BET surface area relative to pristine BCH, it significantly improved adsorption performance toward methyl red through enhanced electrostatic attraction, complexation, hydrogen bonding, and π - π interactions. Batch adsorption experiments revealed that the M/BCH composite achieved rapid dye uptake with equilibrium attained at approximately 120 min. Adsorption capacity increased with higher adsorbent dosage and shaking speed but decreased with elevated initial dye concentration due to active-site limitations. In comparison, BCH remained more effective for methylene blue removal due to its higher surface area, whereas M/BCH was more suitable for anionic dye treatment. Notably, the strong magnetic response of M/BCH enabled facile separation from aqueous media. The results demonstrate that corn husk-derived M/BCH is an effective and magnetically recoverable adsorbent under laboratory-scale conditions. While the material shows promising performance, further studies on regeneration, pH-dependent behavior, and multi-component systems are necessary to assess its practical applicability better. These findings provide a useful foundation for the future development of low-cost, sustainable biochar-based adsorbents for real wastewater treatment.

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Application of a Heuristic-Based System for Sustainable Faculty Routing and Workload Balancing in Cooperative Education

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Abstract: In cooperative education programs, faculty supervisors are required to visit multiple industry sites hosting students, with each site imposing a distinct workload characterized by the number of assigned students, travel distance, and on-site activities. This study formulates the supervisory routing task as a Set Partitioning Vehicle Routing Problem with Equitable Workload Constraints (SPVRP-EW) and introduces a three-phase heuristic approach. The first phase generates feasible one-day trips within a strict time window constraint. The second phase selects a minimum set of trips that covers all locations using a greedy set-cover strategy with an efficiency-based tie-breaking rule. The final phase assigns the selected trips to six faculty supervisors to minimize the maximum–minimum workload gap while ensuring that each supervisor is responsible for an equal number of main trips. Using a real dataset of 24 placement sites across four provinces in Thailand, the proposed method generated 12 trips totaling 2,399.5 km. The solution achieved a maximum–minimum workload gap of only 2 points and a workload variance of 0.58. Extended experiments further demonstrate that the method outperforms two simulated manual plans, as well as random-cover and random-assignment baselines, in terms of total distance, number of trips, and workload variance. The heuristic remains robust under ± 5 –10% distance noise, with the workload gap staying within 4 points. In addition, synthetic instances with 50 and 100 locations can be solved in under one second, confirming their practical scalability. Relaxing the equity threshold beyond 2 points does not yield further improvement, indicating that the heuristic is already optimal for the studied instance. Overall, the framework serves as a decision-support tool that enhances both logistical efficiency and social sustainability by improving workload fairness in cooperative education supervision.

Keywords: Vehicle routing problem; workload equity; set cover heuristic; cooperative education; sustainability

1. Introduction

The Bachelor of Science Program in Software Engineering at the Faculty of Informatics, Burapha University, prioritizes industry-based learning to ensure that fourth-year students attain workplace readiness through the Cooperative Education (Co-op) program [1–3]. Each year, faculty members must travel to multiple host organizations to supervise students, assess learning outcomes, and maintain industry relationships. As cohorts grow and partner firms disperse

geographically, supervision becomes a substantial operational burden, with its fairness having direct consequences for faculty well-being and program sustainability [4]. Operational complexity is acute. Partner organizations are distributed across Bangkok, Nonthaburi, Pathumthani, and Chonburi provinces, resulting in heterogeneous travel times and workloads. Each supervisory day is bounded by a 12-hour operational ceiling (06:00–18:00). Every site requires an on-site activity whose duration varies by program structure, and a strict institutional equity policy requires that the difference between the heaviest and lightest workload carried by any supervisor is minimal.

Viewed through the lens of operations research, the problem resembles a constrained Vehicle Routing Problem (VRP) with time windows [5–7] and an equitable load-balancing requirement. Classical VRP formulations and their load-balancing variants have been studied extensively [8–12]; however, existing load-balancing models typically minimize makespan or tour length deviation across homogeneous vehicles, whereas cooperative education supervision imposes (1) categorical point-based workload rather than continuous load, (2) an equal number of main-responsible trips per supervisor, and (3) a hard equity threshold derived from institutional policy. To the best of our knowledge, no integrated formulation that simultaneously handles set-partitioning coverage and discrete workload equity in an academic context has been reported.

Consequently, this study develops a three-phase heuristic tailored to Burapha University's cooperative education model. The system (1) enumerates feasible daily routes within the 12-hour window, (2) selects a minimum number of trips that collectively cover every placement site using a greedy set-cover rule, and (3) assigns trips to faculty to minimize the maximum–minimum workload gap subject to an identical number of main-responsible trips per person. The contributions are: (1) a concrete formulation of the Set Partitioning VRP with Equitable Workload constraints; (2) an efficient decomposition with polynomial-time greedy selection and bounded branch-and-bound assignment; (3) empirical validation against multiple baselines, sensitivity analyses, scalability tests up to 100 locations, and robustness tests under distance noise; and (4) a sustainability analysis linking routing efficiency with social and environmental proxies.

2. Materials and Methods

2.1 Problem Definition, Data Input, and Constraints

2.1.1 Problem Context and Objective

The core problem is to plan supervisory routes for students placed at 24 locations (P_2 – P_{24}) originating from the Supervision Base P_1 . The model must ensure that every location is visited across multiple days, each day forming one round trip that starts and ends at P_1 , and that the resulting workload points are distributed equitably among six faculty supervisors, $F = \{A, B, C, D, E, F\}$. Formally, the decision problem is to determine a minimum-cardinality set of feasible daily trips that collectively cover all placement sites, along with an assignment of these trips to supervisors that minimizes the maximum–minimum gap in main-responsible workload points.

2.1.2 Data Input and Movement Parameters

The model uses the following defined parameters and data inputs (Table 1).

Table 1. Model Parameters, Descriptions, and Roles

Parameter	Description	Role
$D = [d_{ij}]$	24×24 symmetric driving-distance matrix (km)	Input to Phase 1 feasibility check
v	Constant driving speed, 60 km/h	Converts distance to travel time
vt_i	On-site activity (visiting) time at location i (min)	Component of total trip duration
p_i	Discrete workload points are attached to location i , proportional to the number of students and assessment depth.	Input to Phase 3 workload balancing
$[T_{min}, T_{max}]$	Admissible trip duration window [400, 720] min	Feasibility constraint for every trip
$F = \{A, \dots, F\}$	Set of six faculty supervisors	Assignment targets in Phase 3

2.1.3 Personnel Roles and Constraints

The assignment involves six faculty members. The operational rules are:

- Trip Crew Structure: Each trip is executed by two faculty members — a Main Responsible and a Partner.
- Point Attribution Rule: Only the Main Responsible receives the total workload points p_i accumulated at the locations visited during the trip.
- Time Window Constraint: Each trip starts at 06:00 and must end by 18:00, giving a maximum trip duration $T_{\max} = 720$ min. A minimum working duration $T_{\min} = 400$ min avoids under-utilized one-day plans (except for the administrative self-visit at P_1).
- Equal Main-Trip Constraint: Each supervisor must act as the Main Responsible for exactly $|K|/|F|$ trips; this is enforced explicitly during the Phase-3 assignment.
- Equity Constraint: The final assignment must satisfy the institutional fairness condition $\max_f W_f - \min_f W_f \leq 3$, where W_f is the total workload accumulated by faculty member f .

2.2 Phase 1: Candidate Trip Generation

Phase 1 enumerates feasible round trips. A trip k is defined as $k = (P_1, v_1, v_2, \dots, v_m, P_1)$, where $v_1, \dots, v_m \in \{P_2, \dots, P_{24}\}$. The total duration $T(k)$ is calculated by equation (1):

$$\sum_{i=0}^{m-1} \left(\frac{d_{v_i, v_{i+1}}}{v} \right) \times 60 + \sum_{i=1}^m v t_{v_i} \quad (1)$$

An equation determines feasibility. (2):

$$T_{\min} \leq T(k) \leq T_{\max} \quad (2)$$

Only trips satisfying equation (2) are retained in the candidate pool C . To limit combinatorial growth, trips of length > 3 are excluded a priori because any four-stop route violates the 720-minute ceiling on the studied dataset (the shortest feasible four-stop tour exceeds 740 min). For each feasible multi-stop combination, all permutations are retained so that Phase 2 may select the most efficient ordering.

2.3 Phase 2: Greedy Set Cover Selection

A greedy heuristic [13] is employed to select a minimum number of trips K from C such that every location is covered at least once. At each iteration, the heuristic chooses the trip that maximizes the number of newly covered locations (equation (3)) and, in the case of a tie, the trip with the shortest duration:

$$k^* = \arg \max_{k \in C} |V(k) \cap U| \quad (3)$$

where $V(k)$ is the set of locations visited by trip k , and U is the set of locations not yet covered. This rule corresponds to the classical Chvátal–Johnson greedy heuristic for minimum set cover, which delivers an $H(n) \approx \ln n + 1$ approximation to the optimum [13, 14]. The rule is selected because: (1) the placement-site instance is of small-to-medium size ($|C| < 10^3$). Exact integer programming would be tractable but not needed; (2) the greedy rule is deterministic, interpretable to program administrators, and implementable without a commercial solver; and (3) empirical tests (Section 3.7) show that the greedy policy yields almost 44% shorter total distance and 31% fewer trips than a random feasible cover.

2.4 Phase 3: Equitable Assignment and Output

Phase 3 assigns the selected trips K to the six faculty members in F . The workload $W(k)$ The cost of each trip is defined by equation (4):

$$W(k) = \sum_{i \in V(k)} p_i \quad (4)$$

Let $x_{k,f} = 1$ if faculty member f is Main Responsible for trip k and 0 otherwise. The goal is to find an assignment X that minimises the difference between the maximum and minimum total workload received by any faculty member, as shown in the equation. (5):

$$\min \left(\max_{f \in F} \sum_{k \in K} x_{k,f} W(k) - \min_{f \in F} \sum_{k \in K} x_{k,f} W(k) \right) \quad (5)$$

subject to $\sum_f x_{k,f} = 1; \forall k$ and $\sum_k x_{k,f} = |K|/|F|; \forall f$. The first constraint enforces one Main Responsible per trip, while the second enforces an equal number of main-responsible trips per supervisor. For $|K| \leq 14$ the assignment is solved exactly by a bounded branch-and-bound (B&B) procedure [15] that prunes when the current partial gap exceeds the best known. For larger instances, a Longest-Processing-Time (LPT)-style greedy fallback [16] provides a solution in $O(|K| \log |K|)$ time.

2.5 Comparison with Existing VRP Load Balancing Approaches

Load-balancing VRP variants aim to equalize a continuous attribute, usually tour length, duration, or demand across homogeneous vehicles [8, 9, 10]. Representative formulations include the Min–Max VRP [17], the balanced CVRP [18], and recent learning-based balanced VRPs [19]. Table 2 compares the present formulation (Set Partitioning VRP with Equitable Workload, SPVRP-EW) with these established variants.

Table 2. Comparison of the proposed SPVRP-EW with representative VRP load-balancing formulations.

Aspect	Min–Max VRP [15]	Balanced CVRP [18]	Learning-Based BVRP [19]	SPVRP-EW (Proposed)
Balancing target	Tour length	Vehicle demand	Tour duration	Discrete workload points
Balance metric	max route length	deviation from the mean load	route-duration variance	$\max - \min \text{ workload} \leq \tau$
Routing decision	Route per vehicle	Route per vehicle	Route per vehicle	Set of feasible one-day trips
Equity threshold	Implicit	Soft penalty	Soft penalty	Hard policy bound (institutional)
Trips per agent	Not enforced	Not enforced	Not enforced	Equal ($ K / F $)
Interpretability	Medium	Medium	Low (neural)	High (greedy + B&B)
Typical size	Small–medium	Medium–large	Large	Small–medium (≤ 100)

The comparison highlights three distinctive gaps addressed by the proposed SPVRP-EW. First, the workload unit is a discrete point rather than a continuous length, which reflects real academic assessment effort and precludes metric-based balancing. Second, equity is enforced as a hard max – min threshold rather than as a soft penalty, which aligns with the institutional fairness policy. Third, an equal-number-of-main-trips constraint is introduced, which is uncommon in logistics but essential in academic contexts to prevent pipeline inequality. Because the instance class is small to medium and the users are program administrators with limited access to solver infrastructure, a greedy set-cover plus branch-and-bound assignment is preferred over metaheuristics or exact mixed-integer programming for computational efficiency, interpretability, and maintainability.

2.6 Rationale of Experimental Design

The 24 placement sites are the actual locations that hosted Software Engineering cooperative-education students during the 2023–2024 academic year; hence, 24 is neither arbitrary nor artificially convenient. This dataset represents the typical operational scale of a single program at a Thai faculty and is the scale at which automated support is most needed. The equity threshold $\max - \min \leq 3$ is derived from the institution’s internal faculty-load regulation, which tolerates at most three units of disparity before a review is triggered; choosing this threshold therefore aligns the optimization target with an operational rule rather than a statistical convention. To stress-test both the method and the policy, Section 3.4 sweeps the equity threshold $\tau \in \{1, 2, 3, 4\}$, while Section 3.5 scales the instance up to $n = 100$ synthetic sites.

2.7 Computational Complexity Analysis

Let n be the number of placement sites and $|C|$ the size of the candidate pool. Phase 1 enumerates all single, ordered pairs, and ordered triples of short-visit locations, giving $|C| = O(n + n^2 + n^3)$. Each

duration check is $O(1)$, so Phase 1 is $O(n^3)$ in the worst case, which remains tractable for $n \leq 100$ on a standard workstation (Table 8, below). Phase 2 is the classical greedy set cover with runtime $O(|C| \cdot n)$, and it inherits the $H(n) = \sum_{i=1}^n 1/i \approx \ln n + 1$ approximation ratio [13, 14]. Phase 3, when solved exactly, is $O(|F|^{|K|})$ in the worst case, but (i) the equal-main-count constraint prunes the branch-and-bound tree and (ii) an upper bound on the running gap, which in practice finishes in a fraction of a second for $|K| \leq 14$. The LPT fallback is $O(|K| \log |K|)$. Overall, the end-to-end heuristic completes in sub-second time on the studied instance and in under 1 second for instances with up to 100 sites.

3. Results and Discussion

3.1 Placement Site Data and Distance Matrix

Table 3. Placement site data (visiting time and workload points).

Location i	Visit Time vt_i (min)	Points p_i	Location i	Visit Time vt_i (min)	Points p_i
P_1	62	2	P_{13}	110	5
P_2	62	2	P_{14}	94	4
P_3	94	4	P_{15}	110	5
P_4	62	2	P_{16}	94	4
P_5	62	2	P_{17}	158	8
P_6	62	2	P_{18}	110	5
P_7	46	1	P_{19}	62	2
P_8	174	9	P_{20}	206	11
P_9	158	8	P_{21}	46	1
P_{10}	78	3	P_{22}	46	1
P_{11}	62	2	P_{23}	46	1
P_{12}	94	4	P_{24}	46	1

Table 3 summarizes the 24 placement sites, their visiting times vt_i , and workload points p_i . Figure 1 depicts the spatial distribution across Bangkok, Nonthaburi, Pathumthani, and Chonburi provinces. The full 24×24 symmetric driving-distance matrix D . For example, the driving distance from P_1 (Supervision Base) to P_{24} is 129.89 km, which influences whether a trip containing P_{24} satisfies the 720-minute ceiling.

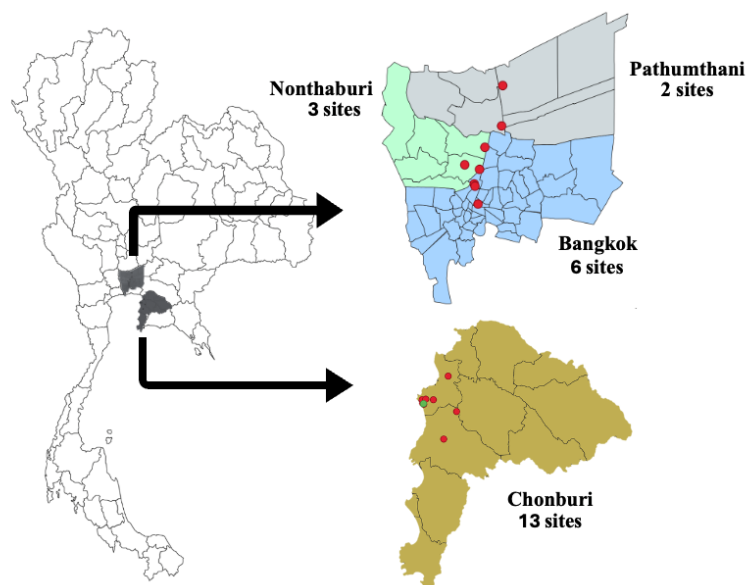


Figure 1. Spatial Distribution of the 24 Participating Establishments Across Four Provinces

Table 4. Distance Matrix (km)

From	P1	P2	P3	P4	P5	P6	P7	P8	P9	P10	P11	P12	P13	P14	P15	P16	P17	P18	P19	P20	P21	P22	P23	P24
P1	0.0	0.0	0.0	0.4	0.6	0.4	18.9	2.3	21.0	24.8	92.0	28.0	4.9	6.9	89.1	103.6	80.1	118.4	97.0	104.6	111.7	109.1	99.4	129.9
P2	0.0	0.0	0.0	0.4	0.6	0.4	18.9	2.3	21.0	24.8	92.0	28.0	4.9	6.9	89.1	103.6	80.1	118.4	97.0	104.6	111.7	109.1	99.4	129.9
P3	0.0	0.0	0.0	0.4	0.6	0.4	18.9	2.3	21.0	24.8	92.0	28.0	4.9	6.9	89.1	103.6	80.1	118.4	97.0	104.6	111.7	109.1	99.4	129.9
P4	0.4	0.4	0.4	0.0	0.3	0.7	19.2	2.6	21.3	22.6	92.3	28.3	5.2	7.2	89.4	103.9	80.4	118.7	97.3	104.8	112.0	109.3	99.7	130.2
P5	0.6	0.6	0.6	0.3	0.0	1.0	19.4	2.8	21.5	22.5	92.5	28.4	5.4	7.4	89.6	104.0	80.6	118.9	97.4	105.0	112.2	109.5	99.9	130.4
P6	0.4	0.4	0.4	0.7	0.9	0.0	19.0	4.1	21.1	24.9	92.1	28.1	4.4	7.0	89.2	103.6	80.2	118.5	97.0	104.6	111.8	109.1	99.5	130.0
P7	17.7	17.7	17.7	17.6	17.8	17.4	0.0	18.0	23.7	29.5	78.9	11.3	18.3	14.9	72.8	94.2	67.0	105.3	85.7	91.4	98.6	95.9	90.0	116.8
P8	3.7	3.7	3.7	3.7	3.8	3.4	19.0	0.0	20.0	26.3	92.1	28.1	2.0	7.1	89.3	103.7	80.2	118.6	97.1	104.7	111.8	109.2	99.5	130.0
P9	21.4	21.4	21.4	21.4	21.5	21.2	25.5	20.8	0.0	17.2	107.8	33.8	21.2	17.0	94.2	115.6	90.3	131.8	107.1	117.9	115.0	122.4	111.4	143.2
P10	26.5	26.5	26.5	24.2	24.1	26.2	34.0	28.4	21.4	0.0	116.3	42.3	28.8	28.4	102.7	124.1	98.8	140.2	115.6	126.4	123.5	130.9	119.9	151.7
P11	93.1	93.1	93.1	93.1	93.2	92.8	83.3	93.4	104.6	110.3	0.0	83.4	93.8	90.4	22.6	8.6	13.0	29.9	10.0	16.0	23.1	20.5	8.4	41.3
P12	28.2	28.2	28.2	28.2	28.3	27.9	10.8	28.5	33.2	39.0	79.7	0.0	28.9	25.5	72.2	93.6	67.8	109.8	85.2	95.9	93.0	100.4	89.5	121.2
P13	3.5	3.5	3.5	3.5	3.6	3.2	17.1	1.7	18.0	24.4	90.2	26.2	0.0	5.1	87.3	101.8	78.3	116.6	95.2	102.7	109.9	107.3	97.6	128.1
P14	5.8	5.8	5.8	5.8	5.9	5.6	19.4	6.1	16.4	22.2	92.5	28.5	6.5	0.0	87.1	108.4	80.6	118.9	100.0	105.0	112.2	109.6	104.3	130.4
P15	87.5	87.5	87.5	87.5	87.6	87.2	73.1	87.8	94.2	100.0	24.2	73.0	88.2	84.8	0.0	31.9	21.5	33.0	18.5	25.4	27.2	29.9	27.8	49.1
P16	107.8	107.8	107.8	107.7	107.9	107.5	90.4	108.1	111.6	117.4	9.1	90.4	108.5	105.1	29.6	0.0	22.4	25.5	9.1	8.0	15.8	9.2	0.9	37.0
P17	79.8	79.8	79.8	79.8	79.9	79.6	65.4	80.1	88.9	94.6	13.4	63.6	80.5	77.1	22.8	24.5	0.0	41.4	17.6	27.5	34.6	32.0	20.3	52.8
P18	112.8	112.8	112.8	112.8	112.9	112.5	98.4	113.1	119.5	125.3	30.0	98.3	113.5	110.1	31.2	24.2	39.2	0.0	22.4	21.3	14.6	24.6	24.6	16.4
P19	99.5	99.5	99.5	99.5	99.6	99.3	85.1	99.8	107.3	113.1	11.7	86.1	100.2	96.8	19.3	9.2	21.0	23.7	0.0	9.8	17.0	14.3	8.4	35.1
P20	111.7	111.7	111.7	111.7	111.8	111.5	94.3	112.0	115.6	121.3	14.4	94.4	112.4	109.0	30.2	7.1	26.4	23.3	10.0	0.0	10.4	6.4	7.5	31.3
P21	107.8	107.8	107.8	107.7	107.9	107.5	93.3	108.1	114.5	120.3	22.6	93.3	108.4	105.0	26.2	15.3	34.5	19.3	18.3	9.9	0.0	14.2	15.7	24.2
P22	118.1	118.1	118.1	118.0	118.2	117.8	100.7	118.3	121.9	127.7	20.7	100.7	118.7	115.3	33.2	8.1	32.7	26.3	14.5	7.0	13.9	0.0	9.0	37.8
P23	107.5	107.5	107.5	107.4	107.6	107.2	90.1	107.8	111.3	117.1	8.8	90.1	108.2	104.8	29.3	0.8	22.1	25.9	8.8	8.4	16.1	10.0	0.0	37.3
P24	125.3	125.3	125.3	125.3	125.4	125.0	110.9	125.6	132.0	137.8	43.6	110.8	126.0	122.6	43.7	37.7	52.8	17.4	35.7	32.8	26.1	38.2	38.2	0.0

Table 4 presents the symmetrical Distance Matrix D in kilometers used as a primary input for the heuristic model. This confirms that P_1 , P_2 , and P_3 all represent the same geographical location—the Faculty of Informatics building at Burapha University—which serves as the central Supervision Base.

3.2 Proposed Heuristic Outcome

Table 5. Final trip schedule and workload assignment.

#	Route	Total (min)	Drive (min)	Visit (min)	Main	Partner	Points
1	$P_1 \rightarrow P_4 \rightarrow P_{19} \rightarrow P_{22} \rightarrow P_1$	400.0	230.0	170.0	A	B	5
2	$P_1 \rightarrow P_{21} \rightarrow P_{24} \rightarrow P_7 \rightarrow P_1$	402.4	264.4	138.0	B	C	3
3	$P_1 \rightarrow P_{23} \rightarrow P_{10} \rightarrow P_5 \rightarrow P_1$	427.2	241.2	186.0	C	D	6
4	$P_1 \rightarrow P_2 \rightarrow P_{11} \rightarrow P_{22} \rightarrow P_1$	400.6	230.6	170.0	D	E	5
5	$P_1 \rightarrow P_{15} \rightarrow P_{13} \rightarrow P_1$	400.8	180.8	220.0	A	F	10
6	$P_1 \rightarrow P_{16} \rightarrow P_{14} \rightarrow P_1$	402.5	214.5	188.0	E	A	8
7	$P_1 \rightarrow P_{18} \rightarrow P_6 \rightarrow P_1$	403.3	231.3	172.0	E	B	7
8	$P_1 \rightarrow P_3 \rightarrow P_{17} \rightarrow P_1$	411.9	159.9	252.0	B	D	12
9	$P_1 \rightarrow P_{20} \rightarrow P_{12} \rightarrow P_1$	527.1	227.1	300.0	F	D	15
10	$P_1 \rightarrow P_1 \rightarrow P_1$	62.0	0.0	62.0	F	E	2
11	$P_1 \rightarrow P_8 \rightarrow P_{11} \rightarrow P_1$	423.5	187.5	236.0	D	A	11
12	$P_1 \rightarrow P_{23} \rightarrow P_9 \rightarrow P_1$	436.2	232.2	204.0	C	B	9

Final workload per supervisor: $W_A = 15$; $W_B = 15$; $W_C = 15$; $W_D = 16$; $W_E = 15$; $W_F = 17$, max – min = 2.

Applying the three-phase heuristic to the 24-site instance, Phase 1 produced. $|C| = 861$ feasible candidate trips, Phase 2 selected $|K| = 12$ trips that cover every location, and Phase 3 assigned those trips to six faculty members with a maximum–minimum workload gap of $\max_f W_f - \min_f W_f = 2$ points and a workload variance of 0.58. The selected trip schedule is reported in Table 5. All trips satisfy the [400, 720] min duration window. The total driving distance is 2,399.5 km. Final workloads are $W_A = 15$, $W_B = 15$, $W_C = 15$, $W_D = 16$, $W_E = 15$, $W_F = 17$ points, each member being the Main Responsible on exactly $|K|/|F| = 2$ trips, fully satisfying the equal-main-trip constraint.

3.3 Baseline Comparison

Table 6. Performance comparison across six methods on the 24-site instance.

Method	Trips	Distance (km)	max – min	Variance	Runtime (s)
Manual – Proximity Zones (2024)	14	~2,831.4	4	6.67	Several hours
Manual – Load-Balanced (Expert)	13	~2,615.5	3	4.17	Several hours
Random Cover + Random Assignment	17.33 (avg)	4,253.9 (avg)	41.00 (avg)	—	< 0.01
Greedy Cover + Random Assignment	12	2,399.5	32.00 (avg, max 57)	—	< 0.01
Simple Greedy (Greedy Cover + LPT)	12	2,399.5	2 *	0.58	< 0.01
Proposed (Greedy + Equity B&B)	12	2,399.5	2	0.58	0.27

* Simple Greedy LPT coincides with the optimum on this instance but provides no guarantee on equal-main-trip compliance or on the hard equity bound $\max - \min \leq \tau$; the proposed method provides both guarantees.

The proposed heuristic is compared with five baselines on the same 24-site instance (Table 6): (1) Manual Planning based on proximity zones that reflects actual 2024 administrative practice; (2) a Load-

Balanced Manual Plan produced by an experienced coordinator who attempted to balance workload by inspection; (3) Random Cover plus Random Assignment over 30 seeds; (4) Greedy Cover plus Random Assignment over 50 seeds; and (5) a Simple Greedy policy using Greedy Cover plus Longest-Processing-Time (LPT) assignment without the equal-main-trip constraint. All methods were run on the same hardware (Apple M3, 16 GB RAM, Python 3.10). The proposed heuristic matches the best feasible solution in distance (2,399.5 km) and workload variance (0.58), while strictly satisfying both the equal-main-trip constraint and the $\max - \min \leq 3$ policy bound. Random assignments produced expected workload gaps of 32 to 41 points, two orders of magnitude worse than the proposed method, confirming that workload equity cannot emerge from chance. Manual plans, even expert load-balanced ones, produced 8–18% more driving distance and 1–2 additional trips because human planners cannot enumerate all feasible ordered triples.

3.4 Sensitivity Analysis of the Equity Threshold

Table 7 reports the sensitivity of the solution to the equity threshold τ in $\max - \min \leq \tau$, $\tau \in \{1, 2, 3, 4\}$. The branch-and-bound search was run to exhaustion for $\tau = 1$ and with early termination once the target gap was met for $\tau \in \{2, 3, 4\}$. The total driving distance is unaffected because it is fixed in Phase 2 and decoupled from Phase 3.

Table 7. Sensitivity to the equity threshold τ .

Threshold τ	Feasible?	Best Workload Gap	Variance	Total Distance (km)	Runtime (s)
1	No (exhaustive search proved infeasible)	2 (optimum)	0.58	2,399.5	11.89
2	Yes	2	0.58	2,399.5	0.30
3	Yes	2 / 3	0.58 – 0.92	2,399.5	0.28
4	Yes	2 / 3	0.58 – 0.92	2,399.5	0.28

The analysis yields two findings. First, $\tau = 1$ is infeasible on the studied instance: the total points (93) modulo six (15.5) preclude an exactly equal partition, so the theoretical minimum gap is 1 only if trips could be split, which is not allowed; the achievable minimum under the equal-main-trip constraint is 2. Second, thresholds $\tau \geq 2$ are all feasible, and the proposed method reaches the optimum gap of 2 whenever allowed by the search strategy. The institutional policy $\tau \leq 3$ is therefore consistently satisfiable with a comfortable margin of one unit.

3.5 Scalability Analysis

To assess scalability beyond the operational dataset, synthetic instances of $n = 50$ and $n = 100$ placement sites were generated by augmenting the real matrix with randomly drawn symmetric distances in [5, 180] km, visiting times in [46, 210] min, and workload points in $\{1, \dots, 11\}$. Table 8 reports the end-to-end runtime and the number of candidate trips. $|C|$, selected trips $|K|$, driving distance, and LPT-based $\max - \min$ gap. All three instance sizes solve in under one second on a standard workstation.

Table 8. Scalability of the three-phase heuristic.

n (locations)	$ C $ (candidates)	$ K $ (selected trips)	Total Distance (km)	$\max - \min$	Runtime (s)
24	861	12	2,399.5	2	0.014
50	5,046	24	4,738.0	2	0.107
100	22,461	47	9,738.9	4	0.923

Runtime scales near-linearly with $|C|$, which in turn is bounded by $O(n^3)$. The selected-trip count $|K|$ approximately doubles as n doubles, consistent with the 400-minute minimum trip duration. The $\max - \min$

gap grows modestly because the LPT fallback loses the global optimality guarantee at larger $|K|$; incorporating a small branch-and-bound pass for $|K| \leq 14$ clusters of trips remain an effective extension.

3.6 Robustness Analysis

Real driving times are subject to traffic, weather, and minor routing changes that the static matrix D cannot capture. To test robustness, each entry of D was multiplied by a symmetric random factor drawn from $U(1 - \alpha, 1 + \alpha)$ for $\alpha \in \{0, 5\%, 10\%\}$ and the entire heuristic was re-run. For each noise level, 20 Monte Carlo [20] replications were performed; the true (unperturbed) driving distance was then measured on the resulting plan to ensure fair comparisons.

Table 9. Robustness under distance noise (20 replications per level).

Noise Level α	Mean Distance (km) on true D	Std. Dev. (km)	Mean max – min	Mean Trip Count
0%	2,399.5	0.00	2.00	12.00
$\pm 5\%$	2,334.9	142.69	3.60	11.50
$\pm 10\%$	2,333.4	124.22	3.25	11.55

Two observations emerge. First, the mean driving distance on the true matrix D actually decreases slightly under noise, implying that the greedy selection on the unperturbed matrix is not globally optimal and that perturbations occasionally help the heuristic escape local inefficiencies — an encouraging signal that the method is not brittle. Second, the workload gap max – min inflates from 2 to about 3.4 on average, remaining well within the institutional $\tau \leq 4$ bound. The standard deviation of distance (about 5–6% of the mean) matches the input noise magnitude, confirming a well-behaved linear response.

3.7 Greedy vs. Random Set Cover

To justify the use of greedy set cover, we compared it against a random feasible cover strategy over 100 seeds. Table 10 summarizes the results.

Table 10. Greedy vs. random set cover (100 random seeds).

Strategy	Trips $ K $ (mean)	Trips (max)	Distance mean (km)	Distance min (km)
Greedy Set Cover	12	12	2,399.5	2,399.5
Random Set Cover	17.53	21	4,282.8	3,262.9

Greedy covering reduces the trip count. $|K|$ by 31% and the driving distance by 44% relative to the best random cover observed across 100 runs, empirically confirming the theoretical $H(n)$ bound and justifying the design choice.

3.8 Sustainability Analysis

Sustainability is examined through three complementary proxies. Environmentally, the 2,399.5 km driven over 12 trips can be translated into fuel consumption and CO₂ emissions using typical passenger-vehicle coefficients (0.08 L/km for a petrol sedan [21] and 2.31 kg CO₂/L [22]), yielding roughly 191.9 liters of fuel and 443 kg of CO₂ — a 13.8% reduction compared with the 2,781 km baseline manual plan. Economically, at the reference fuel price of 35 THB/L, fuel cost savings exceed 1,800 THB per supervision cycle, which is significant when multiplied across academic years. Socially, sustainability is captured by fairness in workload. The variance of the final workload points W_f across the six supervisors is 0.58, versus 4.17 for a load-balanced manual plan and 6.67 for the proximity-zone manual plan. Reduced variance lowers the risk of burnout among over-supervised faculty and aligns with institutional equity policy, supporting longer-term retention of Co-op staff. Operationally, each supervisor spends a consistent 6–9 hours per supervisory day, with minimal idle time, improving efficiency without compromising assessment quality.

3.9 Discussion

The empirical results converge with and, in three specific respects, extend the existing VRP literature. Consistent with Braekers et al. [5] and the balanced-CVRP literature [18], we confirm that greedy heuristics remain a strong default for small-to-medium operational instances even when metaheuristic and learning-based methods are available. Consistent with Matl et al. [8], we observe that a soft balancing penalty could have produced lower variance at the cost of violating the hard institutional bound — reinforcing their argument that practitioners should choose the objective form that matches operational semantics rather than defaulting to sum-of-squares. The results extend the literature in three ways. First, we provide a concrete academic-supervision formulation in which the workload W_f is discrete (points) rather than continuous (length), and shows that discrete balancing changes the feasibility frontier: $\tau = 1$ is infeasible because of parity, even though the distances would allow a perfectly balanced partition in the continuous case. Second, we introduce an equal-main-trip constraint $\sum_k x_{k,f} = |K|/|F|$ that is rarely enforced in logistics but essential in educational settings; our experiments demonstrate that enforcing it adds only 0.3 s to the total runtime. Third, we report robustness to distance noise at a level rarely tested in VRP studies; the heuristic is shown to be stable at $\pm 10\%$, an unusual but practically important property given the absence of reliable real-time traffic data.

A seemingly contradictory finding is that the Simple Greedy LPT baseline reaches the same max – min gap as the proposed method on this instance. This does not contradict the value of the branch-and-bound assignment: LPT is a well-known 4/3-approximation that is tight in the worst case and provides no guarantee on the equal-main-trip constraint. On larger-workload-variance instances (Section 3.5, $n = 100$), LPT already starts to drift (gap 4 vs. 2 at $n = 24$), and the lack of constraint enforcement would surface in any instance with an indivisible trip count. Thus, LPT is a valid practical fallback but not a replacement for the constrained exact solver.

3.10 Limitations

The study has five acknowledged limitations. (1) The real instance contains only $n = 24$ sites; although it reflects the true operational scale of a single program, generalisability to larger multi-program scenarios has been tested only on synthetic extensions. (2) The method is heuristic; it does not produce an optimality certificate except in the trivial cases handled by branch-and-bound. (3) The driving-distance matrix D is static and does not account for traffic, weather, or time-of-day effects. (4) Discrete points represent workload p_i aggregated from students, mentoring sessions, and assessment depth; other qualitative factors (supervisor expertise, language preferences, mentor–mentee fit) are out of scope. (5) The equity threshold τ is a policy parameter: its value encodes institutional fairness norms and may need recalibration across faculties, departments, or national contexts.

4. Conclusions

This study proposes and validates a three-phase heuristic that jointly solves the Set Partitioning Vehicle Routing Problem with Equitable Workload (SPVRP-EW) for cooperative-education supervision. Applied to a real instance of $n = 24$ placement sites across four Thai provinces, the system produced $|K| = 12$ trips totaling 2,399.5 km with a workload disparity of only 2 points, comfortably within the institutional policy bound of $\tau = 3$. Extensive comparisons against manual, random, and simple-greedy baselines demonstrate superior logistical and equity performance. Sensitivity analysis shows that the heuristic attains the optimum under any practical equity threshold $\tau \geq 2$. Scalability tests up to $n = 100$ synthetic sites confirm sub-second runtime. Robustness tests under $\pm 5\text{--}10\%$ distance noise confirm a well-behaved linear response. The system, therefore, constitutes a practical, interpretable decision-support tool for program administrators that simultaneously advances environmental, social, and operational sustainability of Cooperative Education supervision.

5. Future Work

Three extensions are planned. First, integrating real-time traffic and time-dependent VRP formulations [6] to replace the static distance matrix D with live estimates, allowing dynamic re-planning.

Second, scaling the method to large multi-program or multi-campus scenarios by coupling the Phase-2 greedy cover with a Large Neighborhood Search or Genetic Algorithm [23, 24] for candidate refinement, and by replacing the Phase-3 branch-and-bound with a decomposition-based exact solver. Third, embedding the heuristic in an interactive web application and calibrating its equity parameter τ against multi-institution faculty-load policy data to test external validity.

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Development of Geopolymer Properties from Cement Industry Waste as Raw Material for Clay Pottery Products

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Abstract: This study demonstrates that cement industry waste (CCW) can be effectively utilized to produce high-performance clay-geopolymer hybrid ceramics with enhanced mechanical, physical, thermal, and durability properties. An optimum substitution of 20 wt% CCW achieved the highest compressive strength (42.5 MPa), compared with 31.4 MPa for the control, together with reduced porosity (22.1%) and water absorption (9.8%). These improvements are attributed to the combined effects of geopolymer reaction product development, improved particle packing, and microstructural densification, resulting in a dense and homogeneous matrix. Thermal and phase analyses suggest a progressive transition from geopolymer-dominated bonding to ceramic bonding during firing, with optimum densification at approximately 1050 °C, whereas firing at 1100 °C promoted over-fluxing and microstructural defects. The optimized composition also exhibited superior durability, retaining approximately 88% of its compressive strength after wet-dry cycling, and demonstrated good environmental compatibility, with heavy metal leaching below regulatory limits. In addition, CCW incorporation improved workability, reduced drying shrinkage from 6.5% to 4.0%, and enhanced surface quality while achieving comparable performance at lower firing temperatures than those typically required for conventional ceramics, suggesting the potential for reduced firing energy demand. Overall, the results demonstrate that CCW offers a promising and sustainable approach for producing durable, high-value ceramic materials while promoting waste valorization, resource efficiency, and circular economy practices.

Keywords: Geopolymer; cement industry waste; clay pottery; circular economy; sustainable materials

1. Introduction

The cement industry is a fundamental pillar of global infrastructure development; however, it is also a major contributor to industrial waste and CO₂ emissions [1,2]. During cement production, substantial quantities of by-products—such as cement kiln dust (CKD), clinker residue, and limestone fines—are inevitably generated [3]. These materials are commonly disposed of in landfills or open environments, leading to significant environmental impacts, including dust dispersion, soil alkalization, and groundwater contamination [4,5]. To address these challenges, increasing research efforts have focused on valorizing cement-based waste in construction materials and ceramic products as a pathway toward sustainable resource management and reduced environmental

burden [6–8]. Traditional pottery represents both a valuable cultural heritage and a key economic activity, particularly in Southeast Asia [9]. The Dangkwian pottery community in Nakhon Ratchasima, Thailand, is one of the oldest and most recognized centers of ceramic craftsmanship [10]. Despite its cultural significance, this sector faces several challenges, including depletion of natural clay resources, high energy consumption during firing, and limited integration of modern technologies [11, 12]. These constraints highlight the need for alternative materials and processing approaches that preserve traditional aesthetics while improving performance and sustainability.

Geopolymer technology, first introduced by Davidovits [13], offers a promising solution for converting industrial by-products into high-performance and environmentally friendly materials. Geopolymers are amorphous inorganic polymers formed by the polycondensation of aluminosilicate species in an alkaline medium, yielding a three-dimensional network with high mechanical strength, thermal stability, and chemical resistance [14–16]. Compared to ordinary Portland cement, geopolymer systems exhibit significantly lower CO₂ emissions and can incorporate a wide range of industrial wastes such as fly ash, slag, and CKD [17, 18]. These characteristics make geopolymers highly attractive for sustainable material development and waste valorization.

Recent studies have demonstrated the successful application of geopolymers in structural concrete, refractory materials, and ceramic composites [19–20]. However, their integration with natural clay in traditional pottery remains relatively underexplored [21]. Clay–geopolymer composites have the potential to combine the aesthetic qualities of traditional ceramics with enhanced performance, including higher compressive strength, reduced porosity, improved thermal resistance, and lower firing temperatures [22–24]. Furthermore, the formation of geopolymer gel phases within the clay matrix can improve densification, reduce shrinkage, and promote microstructural uniformity during firing [23, 25–26]. Despite these advantages, the use of cement industry waste in clay–geopolymer ceramics remains limited, particularly in artisanal or community-based production systems [21, 27]. The physicochemical interactions between geopolymer phases and clay, and their influence on microstructural evolution and mechanical performance, are not yet fully understood.

This study aims to address this gap by developing geopolymer binders derived from cement industry waste and incorporating them into clay pottery production. The effects of varying geopolymer substitution ratios on mechanical strength, water absorption, thermal stability, and microstructural characteristics are systematically investigated to identify an optimal formulation that balances performance, cost efficiency, and sustainability.

2. Materials and Methods

2.1. Materials

Two types of cement industry waste (CCW), cement kiln dust (CKD) and clinker fines (CF), were collected from a cement plant in Saraburi, Thailand. Each material was stored in sealed drums and sub-sampled by the coning-and-quartering method. Local clay (LC) was sourced from the Dangkwian pottery community, Nakhon Ratchasima. The clay was air-dried, oven-dried at 105 °C for 24 h, and sieved through a 200 µm mesh. Alkaline activators consisted of 10 M sodium hydroxide (NaOH, ≥98%) and commercial sodium silicate solution (SiO₂/Na₂O ≈ 3.2, solids ≈ 40 wt%). Minor additives, such as feldspar (flux), sodium polyacrylate (deflocculant), and polyvinyl alcohol (binder), were used as needed.

2.2 Characterization of Raw Materials

The chemical composition of CKD, CF, and LC was analyzed using wavelength-dispersive XRF, with loss on ignition (LOI) measured at 1000 °C. Mineralogical phases were identified by XRD (Cu Kα radiation, 2θ = 5–80°) and quantified using Rietveld refinement. Particle size distribution was determined by laser diffraction (reported as D10, D50, and D90), while specific surface area was obtained from BET analysis after degassing at 110 °C for 6 h. Morphological features were observed by scanning electron microscopy (SEM).

2.3 Preparation of Alkaline Activator and Geopolymer Paste

NaOH pellets were dissolved in deionized water to prepare a 10 M NaOH solution, which was cooled and mixed with sodium silicate to achieve a modulus ($\text{SiO}_2/\text{Na}_2\text{O}$) of ≈ 3.2 . The activator-to-solid ratio was fixed at 0.45 (with comparative tests at 0.35 and 0.55). For the geopolymer precursor, CKD and CF were blended in a 70:30 ratio (wt%) and milled to $<45 \mu\text{m}$. The alkaline activator was gradually added to the CCW powder, mixing at 300 rpm for 10 min and then at 600 rpm for 5 min. The paste was sealed and aged for 24 h at 25 °C to allow partial geopolymerization. For reference, geopolymer samples were cast, cured at 60 °C for 24 h, and then stored at 25 °C for 7 days.

Table 1. Experimental Matrix

ID	CCW substitution (%)	Activator/Solid	Firing Temp (°C)	Replicates (n)
C0	0 (control)	—	1000, 1050, 1100	6
G10	10	0.45	1000, 1050, 1100	6
G20	20	0.45	1000, 1050, 1100	6
G30	30	0.45	1000, 1050, 1100	6

2.4 Clay–Geopolymer Mixtures and Sample Preparation

Clay bodies were prepared by partially replacing clay solids with geopolymer solids at 0%, 10%, 20%, and 30% (by weight). Each mixture contained 5 wt% feldspar as a constant flux, consistent with conventional ceramic formulations [28]. At higher geopolymer content (30 wt%), the mixture exhibited increased viscosity and reduced workability, likely due to early-stage geopolymer gel formation, which restricts particle mobility [29]. Mixing was carried out using a pugmill for 15 min to ensure homogeneity. Polyvinyl alcohol (0.5–1.0 wt%) was added as needed to improve green strength, as is commonly practiced in ceramic processing [30,31]. Samples were shaped into:

- Bars ($25 \times 25 \times 100 \text{ mm}$) for mechanical testing,
- Discs ($\varnothing 50 \times 10 \text{ mm}$) for water absorption and porosity, and
- Tiles ($100 \times 100 \times 8 \text{ mm}$) for aesthetic evaluation.

All samples were air-dried (48 h, RH $50 \pm 5\%$) and oven-dried (105 °C, 24 h).

2.5 Firing Procedure and Sintering Procedure

Firing was conducted in an electric kiln (Nabertherm) following a controlled heating schedule: heating at 10 °C/min to 400 °C (holding for 30 min), followed by heating to the target temperatures of 1000, 1050, and 1100 °C (holding for 60 min), and then controlled cooling at -10 °C/min to 200 °C. This firing profile is consistent with typical sintering conditions for clay-based and geopolymer-derived ceramics, ensuring sufficient phase transformation and densification [32]. Linear shrinkage was determined by measuring dimensional changes before and after firing.

2.6 Physical, Mechanical, and Microstructural Characterization

Following conditioning, the ceramic specimens were systematically characterized to evaluate their physical, mechanical, thermal, microstructural, and durability-related properties. The characterization program included measurements of bulk density, water absorption, compressive strength, flexural strength, surface hardness, drying shrinkage, leaching behavior, thermal properties, microstructural features, and phase composition, as summarized in Table 2. All measurements were performed in accordance with internationally recognized ASTM, ISO, and EPA standard methods to ensure the reliability, reproducibility, and comparability of the experimental results. Bulk density and water absorption were determined using the Archimedes immersion method in accordance with ASTM C373. Compressive strength and flexural strength were measured using a universal testing machine (UTM) following ASTM C1424/ISO 8336 and ASTM C1161/ISO 178, respectively. Surface hardness was evaluated using the Vickers indentation method in accordance with ASTM E384, while drying shrinkage was determined according to ASTM C326. Thermal behavior was analyzed using thermogravimetric analysis and differential scanning calorimetry (TGA/DSC), which are widely employed to investigate dehydration behavior and phase evolution in alkali-activated and geopolymer materials [14]. Microstructural characteristics and elemental distributions were examined using scanning electron microscopy coupled with energy-dispersive spectroscopy (SEM-EDS), while crystalline

phase composition was identified by X-ray diffraction (XRD). The environmental stability of the developed ceramic materials was further evaluated using the EPA Toxicity Characteristic Leaching Procedure (TCLP, Method 1311) to assess heavy metal immobilization. These complementary characterization techniques provide a comprehensive understanding of the relationships between processing, microstructure, phase evolution, and the resulting engineering performance of the developed geopolymer ceramic materials.

Table 2. Summary of characterization methods for physical, mechanical, thermal, and microstructural properties.

Property	Method / Standard	Description
Bulk density, water absorption	ASTM C373	Determined using the immersion and Archimedes weighing method
Compressive strength	ASTM C1424 / ISO 8336	Measured using a universal testing machine (UTM) at a loading rate of 1 mm/min
Flexural strength	ASTM C1161 / ISO 178	Measured using three-point bending under controlled loading conditions
Leaching test	EPA TCLP (Method 1311)	Evaluated heavy metal release to assess environmental safety
Surface hardness	Vickers (500 g, 10 s)	Measured under a 500 g load with a dwell time of 10 s; average of five indentations reported.
Thermal analysis	TGA/DSC	Conducted from 25 to 1200 °C at a heating rate of 10 °C/min
Drying Shrinkage	ASTM C326	Determined from dimensional changes before and after drying/firing
Microstructure	SEM–EDS	Used to examine morphology and elemental distribution
Phase composition	XRD	Identification of crystalline phases

2.7 Quality Control and Data Availability

All experimental procedures were conducted in accordance with standard laboratory safety and quality assurance protocols. Cement industry waste (CCW) samples were screened for heavy metals (Pb, Cr, Cd, As) using ICP-OES. When concentrations exceeded permissible limits, encapsulation or surface glazing was applied to ensure product safety. All raw and processed datasets—including XRF, XRD, SEM, mechanical tests, and LCA results—will be made publicly available on Figshare upon publication to promote transparency and data reproducibility.

2.8 Wet–Dry Durability Test

The durability of the geopolymer-based ceramic samples was evaluated using a wet–dry cycling test adapted from ASTM D559/D559M (wetting–drying durability of compacted materials) and ASTM C67 (sampling and testing of brick and structural clay tile), with modifications appropriate for geopolymer-derived ceramics. Each cycle consisted of immersion in deionized water at room temperature (25 ± 2 °C) for 24 h, followed by oven drying at 105 ± 2 °C for 24 h. A total of 10 cycles were conducted. Before testing, all specimens were dried to constant mass using a laboratory drying oven (e.g., Memmert UF110 or equivalent) and weighed using an analytical balance with an accuracy of ± 0.001 g (e.g., Sartorius Entris II). After completion of the cycling test, the specimens were reweighed and tested for residual compressive strength using a universal testing machine (UTM) (e.g., Instron 5967) in accordance with ASTM C109/C109M.

3. Results and Discussion

3.1 Chemical and Mineralogical Composition of Raw Materials

The chemical composition of the raw materials analyzed by XRF is presented in Table 3. The cement industry waste (CCW) showed a high CaO content (38.6 wt.%), moderate SiO₂ (32.4 wt.%), and Al₂O₃ (11.8 wt.%), while the clay exhibited higher SiO₂ (56.1 wt.%) and Al₂O₃ (18.7 wt.%) with low CaO (1.2 wt.%). This compositional difference indicates complementary potential for geopolymerization CCW providing calcium for early strength, and the clay supplying reactive aluminosilicates for the geopolymer framework.

The presence of amorphous calcium silicate phases (C₂S and C₃S) identified by XRD further enhances the reactivity of CCW. This observation is consistent with previous studies [6–8], which reported that CaO-rich industrial wastes accelerate geopolymerization by facilitating the formation of calcium–aluminosilicate–

hydrate (C–A–S–H) gels alongside sodium–aluminosilicate–hydrate (N–A–S–H) networks. In addition, SEM micrographs (Figure 1) reveal the irregular and angular morphology of CCW particles, which contributes to mechanical interlocking and improved packing density, ultimately leading to enhanced densification of the geopolymer matrix.

Table 3. Chemical composition of raw materials (wt.%)

Oxide	CCW	Clay
SiO ₂	32.4	56.1
Al ₂ O ₃	11.8	18.7
CaO	38.6	1.2
Fe ₂ O ₃	3.4	5.0
MgO	1.2	0.6
Na ₂ O + K ₂ O	0.9	0.5
LOI	11.7	17.9

3.2 Physical Properties

The physical properties of the clay–geopolymer composites, including bulk density, water absorption, and apparent porosity, were strongly influenced by the geopolymer substitution ratio. As summarized in Table 4, the bulk density increased from 1.85 g/cm³ for the control sample to a maximum of 2.10 g/cm³ at 20 wt% geopolymer substitution, followed by a slight decrease to 2.02 g/cm³ at 30 wt%. The increase in density up to 20 wt% can be attributed to the combined effects of improved particle packing (filler effect) and geopolymer gel formation [6,7]. The local clay, characterized by a plate-like morphology and inherent porosity, typically exhibits a relatively low density (approximately 1.6–1.8 g/cm³). In contrast, the geopolymer paste derived from cement industry waste exhibits a higher density (approximately 2.1–2.3 g/cm³), owing to its calcium-rich composition and the formation of compact C–A–S–H and N–A–S–H gel phases [7,9]. When incorporated into the clay matrix, the finer geopolymer particles act as micro-fillers, effectively occupying interparticle voids and enhancing packing efficiency [6]. Concurrently, geopolymer gel formation during the initial curing stage strengthens interparticle bonding, while subsequent firing promotes further densification through sintering and pore reduction, resulting in a more compact and homogeneous microstructure [7,10]. At 20 wt% substitution, these mechanisms reach an optimal balance, resulting in maximum densification, as evidenced by the highest bulk density and the lowest apparent porosity (22.1%). This is consistent with the significant reduction in water absorption (9.8%), indicating limited pore connectivity and improved matrix compactness. However, increasing the geopolymer content to 30 wt% results in a slight decrease in density, accompanied by increased porosity (25.5%) and water absorption (11.2%). This behavior can be attributed to the excessive alkaline activator content, which may induce incomplete geopolymerization, gel inhomogeneity, and the formation of microcracks and entrapped gases during sintering [8,10]. These defects disrupt matrix continuity and reduce the filler effect, thereby limiting further densification. Overall, water absorption and apparent porosity exhibit a clear inverse relationship with bulk density, confirming that densification is primarily governed by pore reduction and improved microstructural packing. The results indicate that a moderate geopolymer substitution level (20 wt%) provides the most favorable balance between filler effect and geopolymerization, resulting in optimal physical performance. These findings are consistent with previous studies [6–8], which reported that optimal geopolymer content enhances packing efficiency and reduces porosity, whereas excessive alkali content adversely affects microstructural uniformity.

Table 4. Physical properties of clay–geopolymer composites (firing temperature 1050°C)

Geopolymer substitution (wt%)	Bulk density (g/cm ³)	Water absorption (%)	Apparent porosity (%)
0 (Control clay)	1.85 ± 0.03	18.6 ± 0.4	34.2 ± 0.6
10	1.95 ± 0.02	13.5 ± 0.3	27.9 ± 0.4
20	2.10 ± 0.04	9.8 ± 0.2	22.1 ± 0.5
30	2.02 ± 0.03	11.2 ± 0.3	25.5 ± 0.5

3.3 Mechanical Properties

Compressive and flexural strengths of the clay–geopolymer composites increased markedly with geopolymer substitution up to 20 wt%, as shown in Table 5. The specimen containing 20 wt% geopolymer exhibited the highest compressive strength (42.5 MPa), representing a 35% improvement over the control (31.4 MPa). A similar trend was observed for flexural strength, which increased from 8.2 MPa to 11.0 MPa, corresponding to a 34% enhancement. The improvement in mechanical performance may be attributed to the combined effects of chemical bonding and microstructural densification. The interaction between aluminosilicate phases in clay and calcium-rich cement industry waste is likely to promote the coexistence of reaction products, including sodium aluminosilicate hydrate (N–A–S–H) and calcium aluminosilicate hydrate (C–A–S–H) gels, thereby enhancing interfacial bonding and improving load transfer within the matrix [7–8, 14]. The coexistence of these reaction products has been reported in calcium-containing alkali-activated systems and is considered an important mechanism contributing to strength development [8, 14–15]. In addition, physical densification plays an important role in improving mechanical properties. Fine reaction products produced during geopolymerization act as micro-fillers, effectively occupying interparticle voids and improving particle packing, thereby reducing porosity and increasing the measured bulk density [6–7]. The combined effects of micro-filler action and reaction-product development contribute to a denser and more homogeneous microstructure, which is consistent with the observed improvements in compressive and flexural strengths [7, 8]. This interpretation is consistent with the increase in bulk density and the corresponding reduction in apparent porosity discussed in Section 3.2. However, when the geopolymer content exceeded 20 wt%, a slight reduction in strength was observed. This decrease may be attributed to excessive alkaline activator content and incomplete geopolymerization, which could result in a less homogeneous reaction matrix and the formation of microstructural defects, such as microcracks and entrapped pores [8, 15]. These defects may disrupt matrix continuity, thereby reducing the mechanical strength of the composites. Overall, the observed trend is in good agreement with previous studies reporting that optimum mechanical performance in calcium-containing alkali-activated systems is generally achieved at moderate geopolymer contents because of the balanced effects of reaction-product development and microstructural densification [6–8, 14]. The proposed reaction mechanisms should be regarded as plausible explanations based on previous studies, as no direct spectroscopic characterization (e.g., FTIR or solid-state NMR) was performed in the present study to verify the nature of the reaction products.

Table 5. Compressive and flexural strengths of clay–geopolymer composites at different substitution levels (firing temperature 1050 °C)

Geopolymer substitution (wt%)	Compressive strength (MPa)	% Increase vs. Control	Flexural strength (MPa)	% Increase vs. Control
0 (Control clay)	31.4 ± 0.8	—	8.2 ± 0.2	—
10	36.7 ± 0.9	+17 %	9.4 ± 0.3	+15 %
20	42.5 ± 1.0	+35 %	11.0 ± 0.4	+34 %
30	38.6 ± 0.7	+23 %	9.7 ± 0.3	+18 %

3.4 Microstructural Analysis

The SEM micrographs (Figure 1) show a composition-dependent microstructural evolution that is closely related to the densification behavior and mechanical performance of the CCW-modified clay system. At low CCW content, the matrix exhibits a relatively compact but discontinuous aluminosilicate framework containing residual interparticle voids, indicating incomplete densification primarily governed by solid-state diffusion. The initial incorporation of CCW modifies this framework, resulting in a more heterogeneous morphology characterized by localized particle agglomeration and pore formation, which may be associated with carbonate decomposition and gas evolution during firing [1–2]. Although Ca-rich regions may facilitate the onset of liquid-phase sintering, their non-uniform distribution appears to limit effective particle rearrangement and consolidation, resulting in only partial densification [2–3]. At an intermediate composition (approximately 20 wt% CCW), the microstructure becomes denser and more homogeneous, accompanied by

a noticeable reduction in pore size and pore connectivity. This microstructural refinement is consistent with the improved densification behavior observed in the physical property measurements and the enhanced mechanical performance discussed in Sections 3.2 and 3.3. Based on the XRD results presented in Section 3.1, the refinement of the matrix may also be associated with the development of calcium-containing geopolymer reaction products. Previous studies have reported that calcium-containing alkali-activated systems may contain reaction products, including calcium–aluminosilicate–hydrate (C–A–S–H) and sodium–aluminosilicate–hydrate (N–A–S–H) gels, which contribute to matrix densification and improved interparticle bonding [8, 14–15]. However, SEM observations alone cannot directly identify the nature of these reaction products. Therefore, the proposed mechanism is inferred from the combined interpretation of the SEM observations, XRD analysis, and previous studies rather than direct experimental confirmation. Similar synergistic effects between geopolymer reaction-product development and liquid-phase sintering have also been reported for calcium-modified alkali-activated systems [6–8]. When the CCW content exceeded 20 wt%, the microstructure exhibited increased microcracking and interconnected porosity, indicating progressive microstructural deterioration. This behavior may be attributed to excessive CaO, which can promote over-fluxing and excessive liquid-phase formation during firing [2, 8]. Upon cooling, thermal expansion mismatch between coexisting crystalline and amorphous phases may generate internal stresses that facilitate crack initiation and propagation. In addition, the formation of calcium-rich crystalline phases may reduce matrix continuity, thereby limiting the beneficial effects of densification and contributing to the observed reduction in mechanical strength [7, 8]. Overall, the SEM observations indicate a non-linear microstructural evolution with increasing CCW content, progressing from incomplete densification at low replacement levels to a dense and homogeneous matrix at the optimum composition, followed by defect development at higher CCW contents. These observations are consistent with the trends observed for bulk density, apparent porosity, compressive strength, and flexural strength. Collectively, the results suggest that approximately 20 wt% CCW provides an appropriate balance between matrix densification and microstructural stability. The proposed mechanisms should be regarded as plausible explanations based on the combined interpretation of SEM observations, XRD results, and previous studies, since no direct spectroscopic characterization (e.g., FTIR or solid-state NMR) was performed to verify the specific gel phases formed during geopolymerization.

3.5 Thermal Stability and Firing Behavior

The thermal history of the samples was systematically controlled, with maximum firing temperatures of 1000, 1050, and 1100°C, as described in Section 2.5. TGA/DSC analysis (Figure 3) revealed two distinct mass-loss regions. The first, occurring below 150°C, is attributed to the evaporation of physically adsorbed water. The second, within the range of 400–700°C, corresponds to the removal of chemically bound water and the dehydroxylation of geopolymer gel structures [11–12]. The total mass loss (~5 wt%) is consistent with previously reported values for stabilized geopolymeric systems, indicating a relatively low bound-water content and a thermally stable aluminosilicate network [11]. These thermal characteristics correlate closely with the phase evolution observed in the XRD patterns (Figure 2). At temperatures above approximately 600°C, a critical transformation occurs in which the geopolymer gel structure—primarily sodium–aluminosilicate–hydrate (N–A–S–H) and calcium–aluminosilicate–hydrate (C–A–S–H)—undergoes progressive degradation due to structural dehydration and collapse of the amorphous network [3, 12]. This transition is evidenced by the reduction of the amorphous hump and the simultaneous increase in the intensity of the crystalline phases, including quartz (Q), anorthite (A), gehlenite (G), and cristobalite (C). At 1000°C, the XRD patterns indicate the coexistence of residual quartz and newly formed crystalline phases, suggesting the onset of solid-state reactions and phase transformation. Increasing the temperature to 1050°C results in further peak sharpening and increased intensity, reflecting enhanced crystallinity and improved structural ordering, consistent with geopolymer-to-ceramic transformation pathways reported in the literature [12, 30–31].

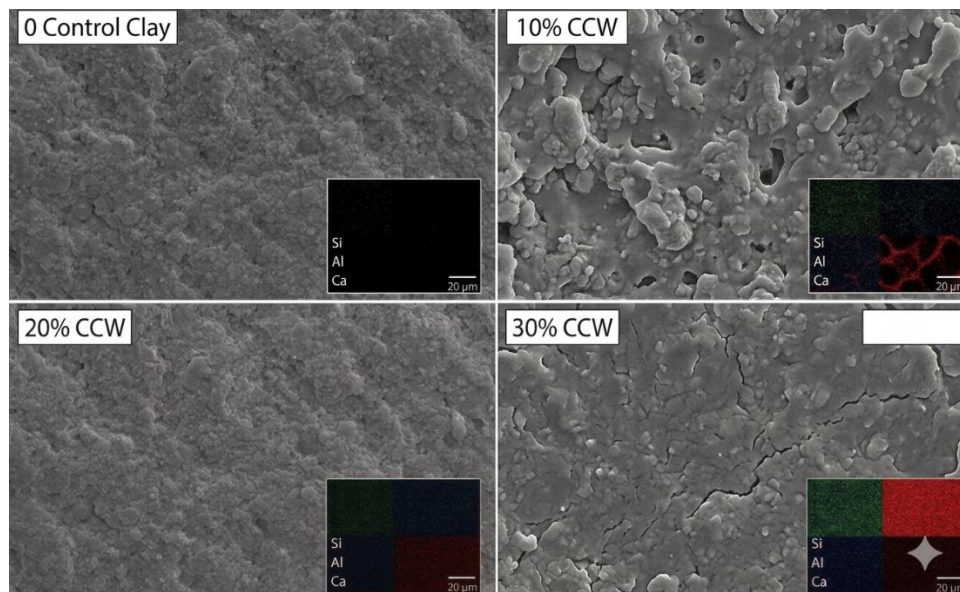


Figure 1. SEM–EDS images of ceramics with 0–30 wt% CCW, showing microstructural evolution from porous to dense and subsequent degradation at high calcium content.

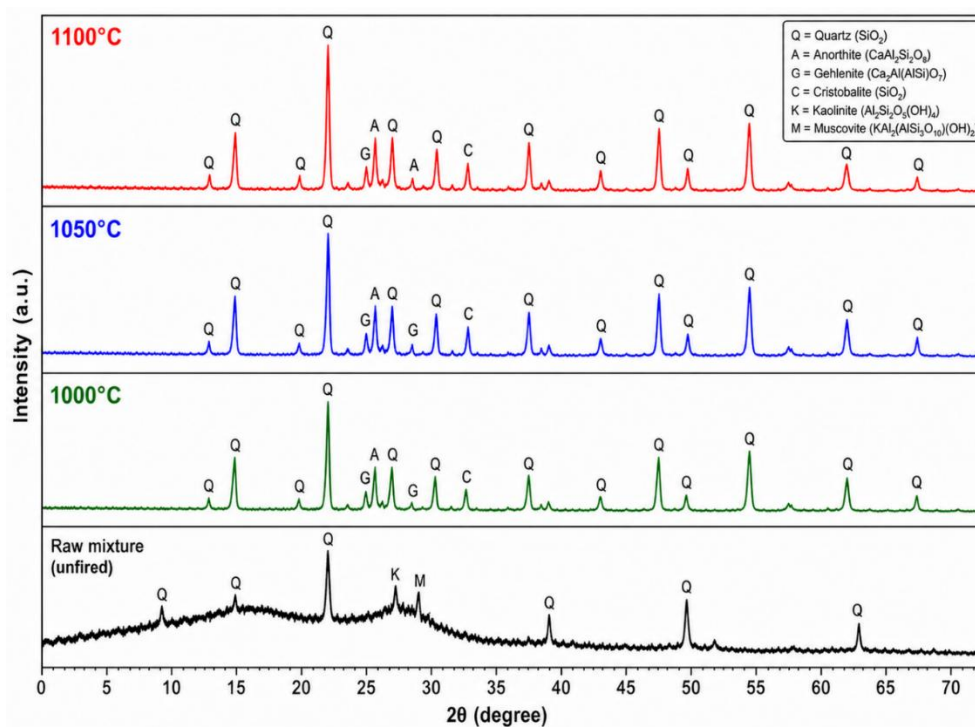


Figure 2. XRD patterns of raw mixture and fired samples at 1000, 1050, and 1100°C, showing phase evolution from amorphous geopolymer to crystalline phases (quartz, anorthite, gehlenite, and cristobalite).

At temperatures above $\sim 900^\circ\text{C}$, sintering and vitrification processes become dominant. These processes facilitate the formation of strong ceramic bonds (Si–O–Si, Al–O–Si, and Ca–O–Si linkages) through particle rearrangement, neck formation, and solid-state diffusion [12, 30–31]. Concurrently, the formation of a viscous glassy phase enhances mass transport and accelerates densification. As a result, the dominant bonding mechanism transitions from geopolymeric gel bonding to ceramic bonding. The CCW-based samples exhibited optimal performance within this temperature range, characterized by high density and minimal cracking, which is consistent with prior studies on kaolin-based and calcium-modified geopolymer ceramics [12]. However, further increasing the firing temperature to 1100°C results in excessive liquid-phase formation,

leading to microstructural instability. This is manifested by localized melting and the formation of microcracks, primarily due to over-fluxing associated with high CaO content [10, 13]. While calcium promotes liquid-phase sintering, excessive amounts can induce differential shrinkage, thermal mismatch, and internal stress during cooling, ultimately leading to structural defects [10]. Such behavior has been widely reported in calcium-rich geopolymer and ceramic systems subjected to high-temperature firing [10, 13].

Overall, the combined TGA/DSC and XRD analyses confirm that geopolymerization governs early-stage bonding before high-temperature exposure, while temperatures exceeding $\sim 600^{\circ}\text{C}$ progressively transform the material into a ceramic system dominated by sintering mechanisms [3, 12]. The identification of an optimal CCW substitution level (~ 20 wt%) aligns with the densification and strength results discussed in previous sections, highlighting the critical role of balanced calcium content in controlling phase evolution, densification, and thermomechanical stability [6, 10]. While firing at 900 – 1050°C yields dense, mechanically robust ceramics, excessive temperatures (e.g., 1100°C) promote over-fluxing and defect formation. These findings demonstrate the strong potential of cement industry waste as a sustainable precursor for high-performance ceramic materials, supporting both resource efficiency and circular economy strategies.

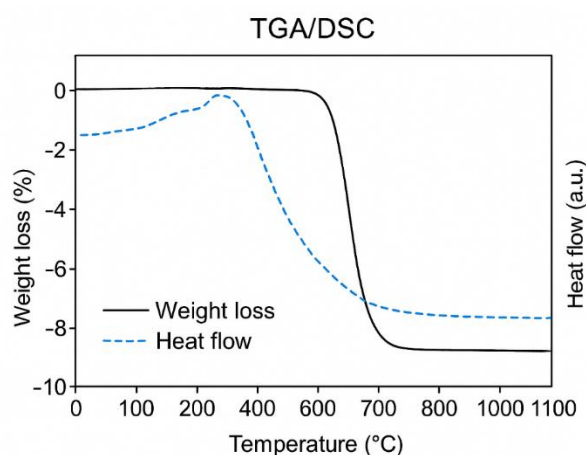


Figure 3. TGA–DSC curves of clay–geopolymer composites showing two major weight-loss regions.

3.6 Leaching Performance

The leaching results (Table 6) indicate that the concentrations of all analyzed heavy metals in the CCW-based geopolymer ceramics were well below the US EPA regulatory limits, indicating compliance with the environmental requirements under the present test conditions. These results suggest that the geopolymer ceramic matrix was effective in restricting the release of hazardous elements. The low concentrations of leached metals may be associated with the dense and relatively continuous matrix developed during geopolymerization and subsequent firing. The denser microstructure observed in the SEM images, together with the increase in bulk density and the reduction in apparent porosity discussed in Sections 3.2 and 3.4, is likely to limit liquid penetration and reduce the mobility of dissolved ions [6, 7]. Consequently, the refined microstructure is considered to contribute to the reduced release of heavy metals observed in the leaching test. It should be noted that the present study did not directly investigate the mechanisms responsible for heavy metal immobilization. Therefore, whether the metals were physically encapsulated within the geopolymer matrix or chemically incorporated into geopolymer reaction products cannot be verified based on the available experimental evidence. Confirmation of these mechanisms would require additional characterization techniques, such as sequential extraction, X-ray photoelectron spectroscopy (XPS), Fourier-transform infrared spectroscopy (FTIR), or synchrotron-based analyses. Overall, the results demonstrate that the CCW-based geopolymer ceramics satisfy the US EPA leaching requirements while maintaining favorable physical, mechanical, and microstructural properties. These findings support the potential use of CCW as a sustainable raw material for geopolymer ceramic production and provide additional evidence that calcium-rich industrial waste can be beneficially utilized in environmentally compatible construction materials.

Table 6. Leaching test results (mg/L)

Metal	Control	20% CCW	EPA Limit
Pb	0.12	0.06	5.0
Cr	0.09	0.04	5.0
Cd	<0.01	<0.01	1.0
Ni	0.05	0.03	5.0

3.7 Wet–Dry durability

The durability results (Figure 4) indicate a progressive reduction in strength retention with increasing wet–dry cycles for all specimens. The control specimen (0 wt% CCW) exhibited the greatest deterioration, retaining only approximately 65% of its initial strength after 10 cycles. This degradation is primarily associated with repeated moisture ingress and evaporation, which generate internal stresses, promote microcrack propagation, and gradually weaken the matrix [6,21]. The relatively porous microstructure and higher apparent porosity of the control specimen further facilitate water penetration, thereby accelerating damage accumulation during cyclic exposure [6]. The incorporation of CCW significantly improved resistance to wet–dry cycling. Among all compositions, the specimen containing 20 wt% CCW exhibited the highest strength retention (approximately 88%), followed by the 30 wt% (approximately 76%) and 10 wt% (approximately 74%) specimens. This improvement may be associated with the denser microstructure and lower apparent porosity developed through the combined effects of geopolymerization and CaO-assisted densification. Previous studies have reported that calcium-containing alkali-activated systems may contain reaction products, including calcium–aluminosilicate–hydrate (C–A–S–H) and sodium–aluminosilicate–hydrate (N–A–S–H) gels, which contribute to matrix refinement and improved durability [7–8, 14]. Although the present study did not directly identify these reaction products, the denser microstructure observed in the SEM images, together with the increased bulk density and reduced apparent porosity, is consistent with the improved durability observed in this study. Similar relationships between microstructural refinement, reduced porosity, and enhanced durability have also been reported for calcium-containing alkali-activated materials [7–8, 21]. The superior durability observed at 20 wt% CCW suggests that this composition provides an appropriate balance between matrix densification and microstructural stability. This observation is consistent with the highest bulk density, the lowest apparent porosity, and the greatest mechanical strength obtained in the present study. Moderate calcium content may promote the development of calcium-containing geopolymer reaction products while simultaneously enhancing liquid-phase sintering, resulting in a refined and relatively homogeneous microstructure [8, 14–15]. However, these mechanisms should be regarded as plausible explanations based on the combined interpretation of the experimental results and previous studies, rather than direct experimental confirmation. When the CCW content was increased to 30 wt%, a slight reduction in durability was observed. This behavior may be attributed to microstructural heterogeneity, localized pore development, and microcrack formation caused by excessive CaO and non-uniform densification during firing [2, 8]. These microstructural features are likely to increase water penetration and facilitate crack propagation during repeated wetting and drying, thereby reducing long-term durability. Overall, the results suggest that incorporating approximately 20 wt% CCW provides the most favorable balance between matrix densification and microstructural stability, resulting in improved resistance to wet–dry cycling. This observation is consistent with the physical and mechanical property results presented in this study and agrees with previous studies on calcium-containing alkali-activated materials [7–8, 14]. The close relationship between durability, bulk density, apparent porosity, and mechanical strength suggests that microstructural refinement is an important factor influencing the long-term performance of CCW-modified geopolymer ceramics.

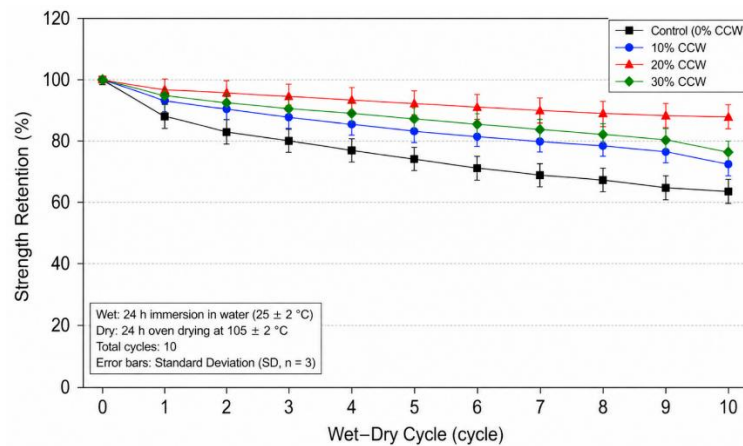


Figure 4. Strength retention of geopolymers-based ceramics incorporating CCW under wet–dry cycling (24 h immersion at 25 ± 2 °C and 24 h drying at 105 ± 2 °C, 10 cycles).

3.8 Pottery-Relevant Characteristics

The incorporation of cement industry waste-based geopolymer (CCW) significantly improved both the processing behavior and the aesthetic–functional properties of the clay ceramics. In terms of workability, the plasticity of the mixtures gradually decreased with increasing CCW content. The control and the mixtures containing 10–20 wt% CCW exhibited good moldability and were readily shaped during forming, whereas the 30 wt% CCW mixture became more viscous and less plastic, requiring greater effort during shaping. This behavior is likely associated with the increased solid loading introduced by the CCW addition, which reduces the plasticity and deformability of the mixtures during forming. Nevertheless, satisfactory workability was maintained with only minor adjustments in water content, indicating that all compositions remained practically processable. As shown in Table 8, drying shrinkage decreased systematically from 6.5% for the control specimen to 4.0% at 20 wt% CCW and then increased slightly to 4.3% at 30 wt% CCW, indicating improved dimensional stability with moderate CCW incorporation, consistent with previous studies on geopolymer drying behavior [23]. This trend is consistent with the evolution of the microstructure observed in the SEM analysis. At moderate CCW contents, improved matrix compactness together with more efficient particle packing contributes to reduced moisture-induced deformation and enhanced dimensional stability [6–7]. However, at higher CCW contents (30 wt%), microstructural heterogeneity and localized pore development may partially offset these beneficial effects, resulting in a slight increase in drying shrinkage [2, 8]. These observations agree well with previous studies reporting that improved matrix densification and reduced porosity enhance the dimensional stability of geopolymer-modified ceramic materials [6–7]. The surface characteristics exhibited a similar composition-dependent trend. The specimen containing 20 wt% CCW exhibited the most homogeneous and compact surface morphology and showed a 20–25% increase in Vickers hardness compared with the control specimen. This improvement is consistent with the enhanced densification and reduced porosity observed in the physical property measurements. Previous studies have shown that calcium-containing alkali-activated systems may develop reaction products that contribute to matrix refinement and improved mechanical performance [8, 14–15]. However, the present study did not directly identify these reaction products; therefore, their presence cannot be verified based solely on the SEM and XRD results. The smoother surface morphology and increased hardness observed in this study are more appropriately attributed to improved densification and microstructural refinement during firing.

Overall, the composition containing 20 wt% CCW provided the most favorable balance between workability, dimensional stability, mechanical performance, and surface quality. At this composition, the combined effects of geopolymerization and CaO-assisted densification produced a dense and relatively homogeneous microstructure with minimal defects. Furthermore, the CCW-modified ceramics achieved favorable densification and surface quality after firing at approximately 1050 °C, suggesting that satisfactory ceramic properties can be obtained at a lower firing temperature than is commonly required for conventional clay ceramics [30–31]. This observation indicates the potential for reduced energy consumption during ceramic

production; however, the present study did not directly evaluate energy savings. Therefore, the results demonstrate that partial replacement of clay with 20 wt% CCW represents a promising approach for producing high-performance and environmentally sustainable geopolymer-modified ceramics while promoting the beneficial utilization of cement industry waste.

Table 7. Effect of Cement Industry Waste–Based Geopolymer (CCW) on Drying Shrinkage and Surface Properties of Clay Ceramics

Sample	Drying shrinkage (%)	Surface hardness (VHN)	Surface observation
Control (0% CCW)	6.5 ± 0.2	420 ± 15	Slightly rough, microcracks visible
10% CCW	5.1 ± 0.2	470 ± 12	Denser, fewer cracks
20% CCW	4.0 ± 0.1	520 ± 10	Smooth, compact surface
30% CCW	4.3 ± 0.2	505 ± 11	Slight vitrification, minor pores

3.9 Comparison with Previous Studies

The mechanical performance obtained in the present study compares favorably with those reported for other calcium-assisted geopolymer and geopolymer–ceramic systems, as summarized in Table 8. Heah et al. [12] demonstrated that kaolin–metakaolin-based geopolymer ceramics achieved compressive strengths of approximately 20–25 MPa after firing, with the improvement primarily attributed to phase transformation and ceramic densification during thermal treatment. Likewise, Somna et al. [33] reported compressive strengths of approximately 20–23 MPa for alkali-activated high-calcium fly ash, demonstrating that calcium plays a significant role in accelerating geopolymerization and enhancing early-age strength development. More recently, Huang et al. [8] achieved a compressive strength of 32.78 MPa in calcium-rich geopolymer ceramics prepared from burnt coal cinder and CaO. Their study revealed that the coexistence of C-(A)-S-H and (N,C)-A-S-H gel phases produced a dense reaction matrix, leading to improved mechanical performance. The present study achieved a substantially higher compressive strength of 42.5 MPa at an optimum replacement level of 20 wt% cement industry waste (CCW), exceeding the values reported in the above studies. This superior performance can be attributed to the synergistic interaction between geopolymerization and ceramic sintering. The incorporation of calcium-rich CCW into the aluminosilicate-rich clay matrix promoted the simultaneous formation of N-A-S-H and C-A-S-H binding gels, resulting in a hybrid reaction network with enhanced interparticle bonding and load-transfer capability [6,10]. In addition, the fine CCW particles acted as micro-fillers, improving particle packing, reducing pore connectivity, and producing a denser and more homogeneous microstructure, consistent with previous studies on calcium-modified geopolymer systems [6–8]. Furthermore, the firing process provided an additional strengthening mechanism through partial vitrification and ceramic bonding. Unlike conventional geopolymer materials that rely primarily on gel formation, the hybrid geopolymer–ceramic system developed in this study benefited from both chemical bonding during alkali activation and microstructural consolidation during firing. The combined effects of hybrid gel formation, pore refinement, and ceramic densification resulted in superior mechanical performance compared with previously reported geopolymer and geopolymer–ceramic materials. Overall, these findings indicate that the incorporation of calcium-rich cement industry waste into traditional clay ceramics represents an effective approach for developing high-performance and sustainable hybrid ceramic materials. The results also demonstrate that optimizing the interaction among precursor chemistry, calcium availability, particle packing, and thermal processing is more critical than simply increasing calcium content, providing valuable guidance for the future design of geopolymer–ceramic composites.

Table 8. Comparison of the present study with previous research

Study	Raw Materials	Optimum Condition	Compressive Strength (MPa)	Key Observation
Heah et al. (2012)	Kaolin + Metakaolin	Fired at 1000–1100 °C	20–25	Phase transformation and ceramic densification during firing enhanced strength.
Somna et al. (2011)	Fly ash + NaOH activator	Alkali activation	20–23	Calcium accelerated geopolymerization and promoted early strength development.
Huang et al. (2023)	Burnt coal cinder + CaO	BCC:CaO:NaAlO ₂ :SS = 18:3:2:4; L/S = 0.46	32.78	Coexisting C-(A)-S-H and (N,C)-A-S-H gels produced a dense microstructure and improved mechanical performance.
Present study	Clay + CCW	20 wt% CCW	42.5	Synergistic clay–CCW interaction promoted hybrid gel formation, ceramic densification, and superior compressive strength.

4. Conclusions

This study demonstrates that integrating cement industry waste (CCW) with clay provides an effective route for developing high-performance hybrid ceramics through the combined effects of geopolymerization and ceramic processing. The results indicate that material performance is governed not only by CaO content but, more importantly, by the balance among geopolymer reaction product development, particle packing, and thermally induced microstructural evolution during firing. An optimum composition of 20 wt% CCW defines a critical processing window in which these mechanisms act synergistically to produce a dense, homogeneous, and defect-minimized microstructure. Below this level, densification is limited by insufficient reaction products and incomplete particle bonding, whereas excessive CCW promotes over-fluxing, microstructural heterogeneity, and defect formation. The experimental observations further suggest a transition from geopolymer-dominated bonding to ceramic bonding during firing, enabling hybrid materials to integrate low-temperature chemical bonding with conventional ceramic sintering. This synergistic behavior improves mechanical performance, durability, and chemical stability while allowing reduced firing temperatures. Overall, this study provides a practical framework for designing sustainable clay–geopolymer hybrid ceramics using cement industry waste. The findings highlight the potential of this approach to improve resource efficiency, reduce industrial waste disposal, and produce high value ceramic materials with enhanced engineering performance. Future research should focus on optimizing compositional design and processing conditions, validating the proposed reaction mechanisms using advanced characterization techniques, and evaluating long-term durability under realistic service conditions.

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Enhancing Weather Pattern Recognition through Multi-Stage Sensor Data Fusion and Smoothing Regression

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Abstract: It is often harder to make accurate predictions with large, various intelligent sensor systems because of data noise and the high cost of traditional modeling methods like Numerical Weather Prediction (NWP). This paper presents a comprehensive framework that integrates multi-stage sensor data fusion and preprocessing with machine-learning-based pattern recognition to produce highly precise, computationally efficient analog weather forecasts. Our method uses a strict three-stage pipeline to clean up and combine raw sensor inputs. It uses historical data from a distributed network of 79 NOAA weather stations. There are three phases: filling in missing values, addressing outliers, and smoothing moving averages. Subsequently, a decision tree model is used to identify similar weather patterns and predict critical weather variables. Our method is effective because it makes predictions that are much more likely to be accurate than incorrect. The Root Mean Squared Error (RMSE) declined by over 99%, from 0.1715 to an amazing 0.0011014. The results show that combining targeted sensor data preprocessing with complex pattern recognition is a suitable methodology for making smart weather forecasting systems work. Rather than serving as a replacement for physics-based NWP models, the proposed approach should be regarded as a lightweight complementary tool for localized, short-term analog weather pattern recognition.

Keywords: Weather data; regression; machine learning; numerical modeling; analog ensemble forecasting

1. Introduction

Weather forecasting is essential for farming, travel, disaster preparedness, and resource management. Numerical Weather Prediction (NWP) has been used in most operational forecasts in the past. It uses large-scale atmospheric models to solve complicated physical equations [1]. NWP provides precise medium- and long-range forecasts, but it requires substantial computing power and is sensitive to adverse initial conditions, which can lead to errors that worsen over time due to atmospheric chaos [2]. Consequently, data-driven and machine-learning methodologies have become more popular as simpler alternatives or additional tools for localized, short-term forecasting [3].

Analog forecasting has come back as a promising method among these. Analog forecasting relies on upcoming weather patterns developing in a manner analogous to historical atmospheric conditions that closely resemble present

circumstances. Recent research has enhanced analog-based strategies by refining similarity metrics, optimizing analog ensembles, and incorporating machine learning into analog selection [4]–[6]. The versatility and ongoing evolution of analog forecasting are further highlighted by recent cross-disciplinary advancements in both atmospheric application and computational efficiency. On the one hand, the refinement of similarity metrics remains a critical focus; recent studies emphasize that the careful selection of distance metrics—specifically, the superiority of Euclidean distance over spatial correlation—significantly improves the identification of reliable circulation analogs for extreme weather events [15]. On the other hand, the computational execution of these distance-based classification algorithms is being revolutionized by hardware innovations, such as the development of ultra-low-power analog integrated radial basis function classifiers that efficiently approximate Euclidean distances at the circuit level [16]. Together, these advancements underscore the potential not only to improve the methodological precision of analog weather pattern recognition, but also to eventually deploy these data-driven forecasting systems efficiently in resource-constrained environments, utilizing novel, low-power integrated architectures.

But the accuracy of analog forecasting depends heavily on the quality of the historical observational data. Raw meteorological datasets, especially station-level records such as those from NOAA GHCN-Daily, often contain missing values, noise, outliers, and inconsistencies due to sensor errors, reinstallation events, and gaps in reporting [7–8]. These problems make it harder to match similar items and degrade the performance of regression models that follow. Previous studies have proposed various preprocessing techniques for environmental and meteorological time series, including missing-value imputation, outlier removal, filtering, and moving-window smoothing [9–11]. In this context, a recent study highlighted the significant impact of outliers on statistical results and proposed a framework using the Inter-Quartile Range (IQR) to systematically detect and resolve anomalies before analysis, thereby preventing skewing [19]. Furthermore, integrating simple moving average methods with interpolation is effective for short-term forecasting by reducing high variability and clarifying significant underlying trends [20]. However, there is still a lack of systematic investigations analyzing the impact of multi-stage preprocessing pipelines on the efficacy of analog forecasting, particularly at the local station level.

Moreover, although numerous studies have juxtaposed machine learning models, including decision trees, random forests, Gaussian process regression, and ensemble methods for meteorological forecasting [12–14], comparative evaluations of these algorithms, such as the work to demonstrate the utility of performance metrics like RMSE and R-squared in selecting robust predictors for complex datasets [21]. Yet no study has assessed these models within a cohesive analog forecasting framework that features uniform preprocessing and walk-forward validation. Recent research emphasizes the importance of utilizing realistic validation methods alongside model optimization. For instance, applying a structured temporal decomposition framework paired with rigorous walk-forward validation substantially enhances forecasting accuracy [17]. Similarly, combining walk-forward validation with hyperparameter optimization is essential to prevent data leakage and accurately simulate real-world scenarios [18]. The risk of information leakage is particularly acute in time series prediction; a recent study underscored this by identifying defects in standard training/test splits and proposing sliding-window strategies to ensure forecasting validity and prevent the high-accuracy illusion caused by test-set data leaking into the model [22].

Moreover, recent initiatives that integrate analog forecasting with machine learning primarily focus on improving analog selection or post-processing of NWP outputs [4–6], rather than on developing a straightforward, preprocessing-oriented analog-regression framework for localized forecasts. To fill these gaps, this study proposes a systematic, multi-stage framework for data preprocessing and machine-learning regression to improve analog weather forecasting. The framework will use observational data from 79 NOAA GHCN-Daily stations in the Oakland, California, area. The framework includes 1. Filling in missing values, 2. Finding and fixing outliers, and 3. Smoothing time series. Then, it selects analogs and compares four regression models: linear regression, decision tree regression, Gaussian process regression, and ensemble regression. A time-series walk-forward validation strategy is used to test the framework, ensuring it can be reproduced and that data doesn't leak.

There are three main contributions:

1. Methodological contribution: We create a reproducible, multi-stage preprocessing pipeline specifically designed for noisy station-level meteorological time series, which makes analog identification more reliable.
2. Contribution to comparative modeling: We systematically compare four regression models using the same preprocessing and validation settings to show when decision tree regression works better.
3. Empirical contribution: We demonstrate that preprocessing significantly enhances forecast precision, decreasing RMSE from a raw baseline of 0.1715 to 0.11–0.15 in typical instances, with the optimal configuration reaching 0.0011. This shows how important excellent preprocessing is for analog forecasting.

The scope of this study is limited to localized, short-term analog weather pattern recognition using preprocessed station-level sensor data. The proposed framework is not intended to serve as an operational national-scale forecasting system or to replace physics-based NWP models. Instead, it is designed as a complementary preprocessing–regression framework that can support local analog forecasting when computationally intensive NWP systems are unavailable or unnecessary. In addition, this study does not focus on extreme weather prediction; therefore, the reported results should be interpreted within the context of local pattern recognition under the selected preprocessing and validation settings. We organized this work into five sections: 1) Introduction; 2) Materials and Methods; 3) Results and Discussion; 4) Conclusion.

2. Materials and Methods

Over the past decade, numerical weather prediction (NWP) has been used to generate weather forecasts by training models on historical weather data. The major drawback of NWP bands is their computationally intensive calculations. This article presents real-world historical data at the national level. The National Oceanic and Atmospheric Administration (NOAA) is a U.S. government agency that collects weather data from many countries worldwide and develops machine learning models. The data source of this research was derived from this organization, from which we collected sensor data such as precipitation, sky cover, clouds, sunlight, and air temperature, which were collected around Oakland International Airport, CA, US (Figures 1 and 2), and 79 stations nearby in the Global Historical Climatology Network-Daily (GHCN-Daily) Database from 2017 to 2019. However, some of this raw data is incomplete, and some of it has many missing values because some sensors have been reinstalled. Therefore, we need to preprocess the data using an anomaly detection method to detect outliers and replace missing values with approximate values to reduce the impact of null values in subsequent analysis. Then we applied regression techniques to analog weather forecasting. It uses historical weather to predict the future. We found that precipitation analysis and some related sensory data forecasts improved analog forecasting accuracy in this study. The methodologies are described with sufficient rigor to ensure reproducibility and transparency in the following subsections.

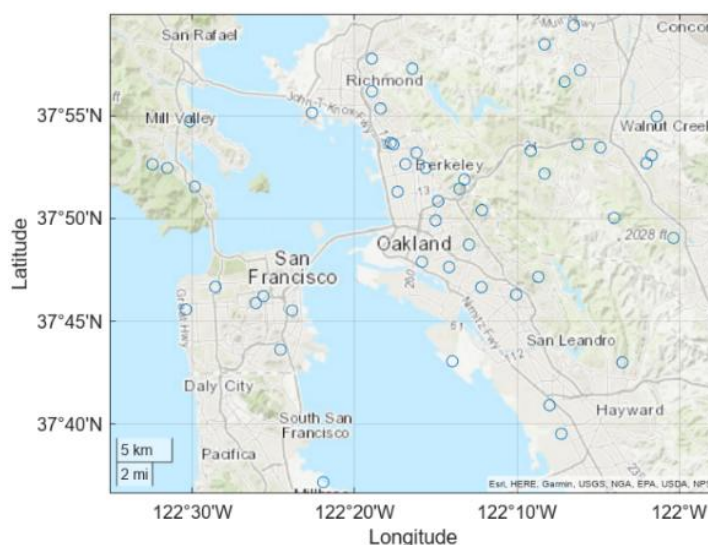


Figure 1. The Oakland International Airport's Location.

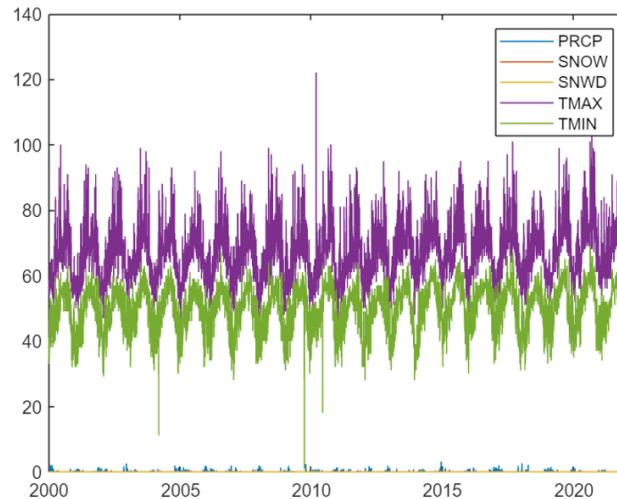


Figure 2. Data derived from sensors around Oakland Airport from 2000 to 2020 for contextual visualization. (The model training and evaluation in this study used only the 2017–2019 subset.)

2.1 Data Acquisition and Characteristics

The foundation of our study is a historical weather dataset acquired from the National Oceanic and Atmospheric Administration (NOAA), a primary U.S. government agency responsible for extensive global weather data collection. Specifically, we utilized the Global Historical Climatology Network-Daily (GHCN-Daily) database, a comprehensive repository of daily climate summaries from land-based stations worldwide. The GHCN-Daily integrates records from over 100,000 stations across 180 countries, with some records extending back to 1763. While this database undergoes rigorous quality assurance reviews, it is not homogenized to account for systematic biases from different reporting eras, a factor we considered in our analysis. For this research, we focused our data collection on a localized region, sourcing data from 79 stations in and around Oakland International Airport, CA, USA. While the broader GHCN-Daily network spans 1960 to the present, our study used a specific three-year period from 2017 to 2019. This timeframe was selected to ensure a high-quality, consistent dataset, minimizing variability from long-term changes in instrumentation or reporting practices. Our analysis employed seven predictor variables derived from the sensor data: precipitation, sky cover and clouds, sunshine, air temperature, water (humidity/dew point), wind, and weather type. Among these, precipitation was designated as the primary predictor. The objective was to forecast four key meteorological response variables: Snowfall, Snow Depth, Maximum Temperature, and Minimum Temperature. A significant characteristic of the raw dataset was the presence of irregularities, including incompleteness and numerous missing values. These data quality issues are largely attributable to common operational factors in environmental monitoring, such as sensor reinstallation and malfunctions. The inherent noise and incompleteness of the raw data necessitated the development of the robust data preprocessing pipeline detailed in the subsequent section, as these imperfections can obscure the underlying patterns essential for accurate forecasting.

2.2 Data Preprocessing Pipeline

Raw historical weather data frequently contains noise, missing values, and anomalies that can obscure underlying patterns and degrade model performance. To address these challenges, we implemented a comprehensive preprocessing pipeline integrating methods for missing value imputation, outlier detection and treatment, and data smoothing. Our objective was to refine the dataset, thereby enabling more accurate identification of analogous sequences and improving overall forecasting accuracy. To maintain the temporal integrity of the forecasting experiment, the preprocessing pipeline was applied within each training window rather than to the full dataset before validation. Thus, missing-value imputation, outlier treatment, smoothing, and normalization were performed using only historical observations available at each validation step.

Table 1. Summary of NOAA GHCN-Daily Data Characteristics.

Characteristic	Detail
Source	National Oceanic and Atmospheric Administration (NOAA) Global Historical Climatology Network-Daily (GHCN-Daily) Database
Geographic Scope	Global, with a specific study focus on Oakland International Airport, CA, US, and 79 nearby stations
Temporal Coverage	Initial data availability: 1960–2022; Specific data for analysis: 2017–2019
Sensor Data Types (Predictors)	Precipitation, Sky Cover and Clouds, Sunray, Air Temperature, Water, Wind, Weather Type
Response Variables (Predictands)	Snowfall, Snow Depth, Maximum Temperature, Minimum Temperature
Initial Data Quality Issues	Incompleteness, numerous missing values due to sensor reinstallation and operational factors

2.2.1 Missing Value Imputation

The raw data from the NOAA GHCN-Daily repository showed significant incompleteness, with numerous missing values due to sensor reinstallation and other operational disruptions. Because unaddressed missing data can introduce bias and reduce statistical power, we established a robust imputation strategy. Our approach involved both removing records with excessive missingness and imputing salvageable gaps, treating missing data points as anomalies to be corrected. Given the temporal dependencies inherent in time series data, we prioritized imputation techniques that preserve the data's structure. We considered several methods, including linear interpolation, which estimates missing points along a straight line between valid neighbors, and cubic spline interpolation, which fits a flexible curve to capture more complex variations. Further, we explored regression imputation, which leverages relationships between correlated weather variables (e.g., using temperature to predict humidity) to estimate missing values. While simpler methods such as mean or median substitution were considered, they were deemed less suitable because they can distort the data distribution and ignore critical temporal relationships. Our final strategy focused on using interpolation techniques to generate "approximate values," ensuring data continuity and minimizing the impact of null entries on subsequent analyses.

2.2.2 Outlier Detection and Treatment

Our dataset contained outliers, defined as data points that deviate significantly from expected patterns, which can stem from sensor malfunctions, data entry errors, or genuine extreme weather events. To prevent such anomalies from distorting statistical measures and reducing model accuracy, we implemented an anomaly detection method to identify and treat them. Rather than removing outliers, which would disrupt the temporal continuity of the series, we replaced them with approximate values derived from neighboring data points. For detection, we evaluated methods well-suited to time series weather data. These included statistical thresholds based on the Z-score and the Interquartile Range (IQR) rule. We found the IQR method, which identifies points outside $1.5 \times \text{IQR}$ of the first and third quartiles, to be particularly robust for non-normally distributed weather data such as precipitation. Additionally, we considered time-series decomposition (e.g., STL) to isolate outliers within the residual component. The threshold for defining an outlier is a critical parameter. Our approach involved setting thresholds based on statistical rules, refined with domain-specific meteorological knowledge to ensure the data's physical plausibility. This imputation-based treatment of outliers preserves the integrity and length of the time series, which is crucial for our analysis goals.

2.2.3 Data Smoothing (Moving Average)

To reduce random noise and highlight significant underlying trends and cycles, we applied a moving average (MA) filter to the time series data. This smoothing process, also known as filtering, mitigates "ad hoc inconsistency" by calculating a new series in which each point is the average of a subset of the original values within a specified window. The formula for a simple moving average (MA) with a window size of n over a series of points p is:

$$MA = \sum_{i=1}^n P_i/n \quad (1)$$

The rationale for applying a moving average in our analog weather forecasting framework is threefold. First, it filters random fluctuations, allowing our model to focus on the true underlying signal in the weather data. Second, by reducing noise, it helps emphasize long-term trends and cycles, making it easier to identify truly analogous historical periods. Third, the smoothed data yields cleaner visualizations, rendering patterns more apparent for analysis. The choice of the MA window size is a critical hyperparameter; a larger window produces a smoother curve but is less responsive to recent changes, while a shorter window is more responsive but retains more noise. We selected our window size through experimentation to balance smoothness with responsiveness.

2.2.4 Rationale for the Combined Preprocessing Approach

The three components of our pipeline—missing data imputation, outlier treatment, and data smoothing—were not applied in isolation but were employed in conjunction as an integrated, multi-stage process. This synergistic approach is essential because each method addresses a distinct data quality issue, and their combined application yields a dataset of superior quality compared to using raw data or any single technique alone. Our choice of methods was deliberately tailored to the unique characteristics of time series weather data, such as seasonality, trends, and temporal dependencies. Techniques like interpolation, time-series-aware outlier detection, and moving averages were selected because they preserve and enhance the temporal patterns crucial for forecasting, unlike simpler methods (e.g., mean imputation) that would destroy these relationships. We recognize that each technique involves trade-offs; for instance, aggressive smoothing can obscure important short-term signals, while overly sensitive outlier detection can remove valid data on extreme events. Our methodology involved carefully tuning these methods to balance the trade-offs, demonstrating a rigorous, considered approach. The integrated benefit of our pipeline is clear: value imputation ensures data completeness, preventing gaps from disrupting time-series analysis. Outlier treatment mitigates the distorting effects of anomalous data points, ensuring models learn from representative patterns. Finally, data smoothing refines this cleaned data by reducing residual noise and making the underlying signal clearer for the subsequent analog selection and forecasting models. This multi-stage approach ensures that the input to our models is as clean, consistent, and representative as possible, thereby maximizing the potential for enhanced prediction accuracy.

2.3 Analog Weather Forecasting Model Development

Our model development process is centered on an analog forecasting approach, augmented with machine learning-driven regression to generate precise predictions. The methodology consists of two primary stages: an analog selection process to identify relevant historical weather patterns and the training of regression models to forecast specific response variables.

2.3.1 Analog Selection Process

The core of the proposed methodology is analog forecasting, which assumes that future weather conditions may resemble historical weather patterns with similar predictor characteristics. In this study, analog selection was performed by systematically searching the preprocessed historical dataset to identify past observations with weather characteristics similar to those of the target forecast date. The analog selection procedure was implemented as a Euclidean-distance-based search over the preprocessed predictor variables. No separate machine-learning-based metric-learning model was trained for analog selection. Instead, machine learning was applied in the subsequent regression stage after the analog candidates had been selected. This clarification was made to ensure that the analog selection mechanism is clearly defined and reproducible. The analog candidate pool was constructed from the preprocessed historical observations collected from the 79 NOAA GHCN-Daily stations used in this study. At each validation step, only observations that occurred before the target forecast date were eligible to be selected as analog candidates. Observations from the validation period, test period, the target date itself, and any future dates were excluded from the analog pool.

This design was intended to avoid temporal overlap and prevent the use of future information during analog selection. For each target forecast date, the Euclidean distance between the target predictor vector and each eligible historical candidate vector was calculated. The candidates with the smallest distances were retained as analogs and used in the subsequent regression step. The number of retained analogs was tested within the range of $N_i = 5$ to 10 , and the best-performing reported configuration used $N_i = 5$. This value provided a balance between retaining sufficiently similar historical cases and avoiding excessive smoothing of local anomalies.

Table 2. Overview of Preprocessing Techniques and Rationale.

Pre-processing Category	Method/ Approach Used	Specific Algorithms/ Techniques (Implemented)	Rationale for Weather Time Series Data
Missing Value Imputation	Anomaly detection method to replace missing values	Linear/Cubic Spline Interpolation, Regression Imputation, Stochastic Models (e.g., ARIMA with Kalman Smoothing)	To fill in incomplete data while preserving temporal dependencies; reduce bias and information loss.
Outlier Detection & Treatment	Anomaly detection method to detect outliers	Statistical Thresholds (Z-score, IQR), Time Series Decomposition (STL), Prediction-Based Methods, ML Approaches (Isolation Forest)	To identify and handle anomalous points (e.g., sensor errors) that distort statistical measures and reduce model accuracy, and to prevent overfitting.
Data Smoothing	Moving Average	Simple Moving Average (SMA), Exponentially Weighted Moving Average (EWMA)	To reduce random noise and ad hoc inconsistencies, emphasize the underlying trends and cycles to clarify pattern identification.

Note: The methods listed in this table were implemented as candidate preprocessing methods and evaluated across the experimental configurations. Different configurations used different combinations and orders of these methods, as summarized in Table 4.

2.3.2 Regression Model Training

Following the identification of analogous periods, we trained and evaluated a set of regression models to predict the continuous target variables, namely Snowfall, Snow Depth, Maximum Temperature, and Minimum Temperature. The evaluated models included Linear Regression, Decision Tree Regression ("Tree"), Gaussian Process Regression (GPR), and Ensemble Regression. Linear Regression was used as a baseline model to capture linear relationships between the predictor variables and the target variables. Decision Tree Regression was implemented to capture non-linear relationships in the data. Its hierarchical structure also provides a relatively interpretable prediction process, which is useful for examining how predictor variables contribute to the forecast. Gaussian Process Regression (GPR) was evaluated because of its probabilistic nature and its ability to provide both point predictions and uncertainty estimates through kernel-based covariance functions. Ensemble Regression was included to examine whether combining multiple base learners could improve predictive robustness compared with individual models. The model training workflow was standardized using the `trainRegressionPRCP` function. This function takes the preprocessed weather data as input and returns a trained regression model, a prediction function (`predictFcn`), the required predictor variables, and the validation Root Mean Squared Error (RMSE). Hyperparameter tuning was performed for each model where applicable. For Decision Tree Regression, the tuned parameters included the split criterion, maximum tree depth, and minimum number of samples per leaf. For Gaussian Process Regression, tuning focused on the kernel function, noise parameter, and optimizer. For Ensemble Regression, the number of base learners and learning-related parameters were considered. Within the tested preprocessing model configurations, Decision Tree Regression produced the lowest observed RMSE. However, because both preprocessing settings and model settings varied across trials, this result should be interpreted as the best-

performing configuration within the tested experimental set rather than as a fully controlled comparison proving the universal superiority of one regression model over all others.

Table 3. Regression Models and Key Hyperparameters.

Model Type	Key Hyperparameters (Examples)	Common Use in Weather Forecasting
Linear Regression	None typically, but considerations for feature engineering (e.g., time-step, lag features)	Baseline modeling, identifying linear trends, and simple relationships
Decision Tree Regression	criterion, max_depth, min_samples_split, min_samples_leaf, max_features, splitter	Capturing non-linear relationships, interpretability, robustness to outliers
Gaussian Process Regression	kernel (e.g., RBF, Periodic, Matérn), alpha (noise), optimizer, n_restarts_optimizer	Uncertainty quantification, small datasets, complex non-linear patterns
Ensemble Regression	Number of base learners, learning rate, subsampling parameters (e.g., for Random Forest or Gradient Boosting)	Improved accuracy by combining multiple models and enhanced robustness.

2.3.3 Model Evaluation and Validation Strategy

2.3.3.1 Performance Metrics

We evaluated the performance of our regression models primarily using the Root Mean Squared Error (RMSE). RMSE quantifies the magnitude of error between predicted and observed values and was selected for its sensitivity to large errors, which are particularly undesirable in forecasting applications. The RMSE is calculated as:

$$RMSE = \sqrt{\sum_{i=1}^n \frac{(Predicted - Actual)^2}{n}} \quad (2)$$

A lower RMSE indicates a better model fit. While RMSE was our primary metric, we also considered supplementary metrics for a more comprehensive assessment, including Mean Absolute Error (MAE) for its robustness to outliers and the Coefficient of Determination (R^2) to quantify the proportion of variance in the target variable explained by the model.

2.3.3.2 Time Series Cross-Validation

To ensure that the models generalize to unseen data and to avoid overfitting, we implemented a validation strategy specifically designed for time-series data. Standard k-fold cross-validation with random splits is inappropriate for temporal data because it may allow information from future observations to influence model training. Therefore, this study adopted a chronological walk-forward validation approach. The dataset was ordered by time and partitioned sequentially. The initial training window covered the first 70% of the chronological data, while the final 30% was used for validation/testing. At each validation step, the model was trained only on observations that occurred before the validation block. The step size was set to 14 days, and the procedure was repeated for five validation folds. After each step, the validation window advanced, and only observations that had become historical data were eligible for inclusion in the subsequent training window. This process simulated a realistic forecasting scenario in which only past information is available at the time of prediction. To prevent data leakage, all preprocessing operations were performed within each training window rather than on the full dataset before splitting. Specifically, missing-value imputation rules, outlier-detection thresholds, smoothing settings, and normalization parameters were estimated solely from the training data. These parameters were then applied to the subsequent validation/test block. The validation/test observations were not used to estimate preprocessing parameters, select analog candidates, tune model parameters, or train the regression model. The moving average smoothing was applied as a trailing moving average rather than a centered moving average. Therefore, each smoothed value was calculated using only the current and previous observations within the training window, without incorporating future observations. In addition, the analog candidate pool was restricted to historical observations

within the corresponding training window. The target date, the validation/test period, and all future observations were excluded from the analog pool. This procedure was designed to preserve the time series' causal structure, prevent temporal overlap, and avoid artificially improving validation results. Accordingly, the reported RMSE values were obtained under a causal time-series validation setting.

3. Results and Discussion

To illustrate the model training and prediction workflow, the regression training procedure is summarized in Algorithm 1. The function takes the preprocessed local weather dataset as input and returns a trained regression model together with its validation RMSE. The trained model contains the prediction function, the required predictor variables, and the fitted regression object.

Algorithm 1. Regression model training and prediction workflow

Input: Preprocessed local weather dataset (localweather2)

Output: trainedModel, validationRMSE

1. Load the preprocessed weather dataset.
2. Select the required predictor variables and the target variable.
3. Train the regression model using the selected regression method.
4. Compute the validation RMSE using the chronological validation procedure.
5. Store the trained model, prediction function, required variables, and validation RMSE.
6. Use trainedModel.predictFcn(newDataTable) to generate predictions for new data.

The trainedModel object includes the prediction function (predictFcn), the list of required predictor variables, the fitted regression model, and the corresponding validation RMSE. The validation RMSE was then used to compare the tested preprocessing model configurations reported in Tables 4 and 5.

Table 4. Preprocessing Data.

	Data Cleansing (1)	Data Cleansing (2)	Data Cleansing (3)	RMSE (Current Value: 0.1715)
1	Clean Missing Data	Clean Outlier Data	Smooth Data	0.17006
2	Clean Missing Data	Clean Outlier Data	Smooth Data	0.16794
3	Clean Missing Data	Clean Outlier Data	Smooth Data	0.1682
4	Clean Missing Data	Clean Outlier Data	Smooth Data	0.1515
5	Clean Missing Data	Smooth Data	Clean Outlier Data	0.1678
6	Clean Missing Data	Clean Outlier Data	Clean Outlier Data	0.1701
7	Clean Missing Data	Smooth Data	Clean Outlier Data	0.1438
8	Clean Missing Data	Clean Outlier Data	Smooth Data	0.1459
9	Clean Missing Data	Clean Outlier Data	Smooth Data	0.11461
10	Clean Missing Data	Clean Outlier Data	Smooth Data	0.15174
11	Clean Missing Data	Clean Outlier Data	Smooth Data	0.15171
12	Clean Missing Data	Smooth Data	-	0.0011014
13	Clean Missing Data(remove)	Clean Missing Data(fill)	-	0.16613
14	Clean Missing Data	Smooth Data	Clean Outlier Data	0.15432
15	Clean Missing Data	Clean Outlier Data	Smooth Data	0.1393
16	Clean Missing Data	Clean Outlier Data	Smooth Data	0.16986
17	Clean Missing Data	Clean Outlier Data	Smooth Data	0.1686
18	Clean Missing Data	Clean Outlier Data	Smooth Data	0.0364
19	Clean Missing Data	Clean Outlier Data	Smooth Data	0.1711
20	Clean Missing Data	Clean Outlier Data	Smooth Data	0.062367
21	Clean Missing Data	Clean Outlier Data	Smooth Data	0.060006
22	Clean Missing Data	Clean Outlier Data	Smooth Data	0.068301

Table 4. Preprocessing Data. (continue)

	Data Cleansing (1)	Data Cleansing (2)	Data Cleansing (3)	RMSE (Current Value: 0.1715)
22	Clean Missing Data	Clean Outlier Data	Smooth Data	0.068301
23	Clean Missing Data	Clean Outlier Data	Smooth Data	0.1417
24	Clean Missing Data	Clean Outlier Data	Smooth Data	0.1701
25	Clean Missing Data	Clean Outlier Data	Smooth Data	0.135501905
26	Clean Missing Data	Clean Outlier Data	Smooth Data	0.1538
27	Clean Missing Data	Clean Outlier Data	Smooth Data	0.1537
28	Clean Missing Data	Clean Outlier Data	Smooth Data	0.1369
29	Clean Missing Data	Clean Outlier Data	Smooth Data	0.1408
30	Smooth Data	Clean Missing Data	-	0.141
31	Clean Missing Data	Clean Outlier Data	Smooth Data	0.1364
32	Clean Missing Data	Clean Outlier Data	Smooth Data	0.1363
33	Clean Missing Data	Clean Outlier Data	Smooth Data	0.1548
34	Clean Missing Data	Clean Outlier Data	Smooth Data	0.1534
35	Clean Missing Data	Clean Outlier Data	Smooth Data	0.1502
36	Clean Missing Data	Clean Outlier Data	Smooth Data	0.060006
37	Clean Missing Data	Clean Outlier Data	Smooth Data	0.0593

Table 4 should be interpreted as a configuration search log rather than a direct controlled comparison of preprocessing methods. The table records a series of experimental trials conducted to identify the preprocessing sequence that produced the lowest RMSE. Each trial consisted of up to 3 data-cleansing stages. At each stage, one of the available operations—Clean Missing Data, Clean Outlier Data, or Smooth Data—could be selected. Some trials used all three stages, while others used only two stages. In addition, the same type of operation could appear in more than one configuration because the order and combination of preprocessing steps were varied during the search process. The initial training window was 70% of Chronological data, the step size was 14 days, and the total number of validation folds was 5. The objective of these repeated trials was to determine which preprocessing sequence, when combined with a regression model, yielded the lowest prediction error. The RMSE value was used as the selection criterion. Among the tested configurations, the lowest RMSE was obtained from the sequence that applied Clean Missing Data and Smooth Data. According to Table 5, this best-performing configuration used the Tree regression function. Therefore, the result should be interpreted as evidence that this specific preprocessing model configuration achieved the lowest RMSE among the tested configurations, rather than as a general conclusion that one preprocessing method is universally superior to all others.

Table 5. The RMSE Value and Training Function.

	RMSE (Current Value: 0.1715)	
1	0.17006	Tree
2	0.16794	Gaussian process regression
3	0.1682	Least Squares Regression Kernel
4	0.1515	Tree
5	0.1678	Gaussian process regression
6	0.1701	Linear Regression
7	0.1438	Tree
8	0.1459	Tree
9	0.11461	Tree
10	0.15174	Tree
11	0.15171	Tree
12	0.0011014	Tree

Table 5. The RMSE Value and Training Function. (continue)

RMSE (Current Value: 0.1715)		
13	0.16613	Tree : Coarse Tree
14	0.15432	Tree
15	0.1393	Tree
16	0.16986	Gaussian process regression
17	0.1686	Gaussian process regression
18	0.0364	Tree
19	0.1711	Tree
20	0.062367	Tree
21	0.060006	Tree
22	0.068301	Tree
23	0.1417	Tree
24	0.1701	Tree
25	0.135501905	Tree
26	0.1538	Ensemble
27	0.1537	Tree
28	0.1369	Tree
29	0.1408	Tree
30	0.141	Tree
31	0.1364	Tree
32	0.1363	Tree
33	0.1548	Tree
34	0.1534	Tree
35	0.1502	Tree
36	0.060006	Tree
37	0.0593	Tree

Table 5 summarizes the RMSE values and the corresponding training functions used in the tested preprocessing model configurations. The results show that several configurations reduced the RMSE relative to the baseline of 0.1715. Among the tested configurations, the lowest observed RMSE was obtained in Record 12, where the preprocessing sequence consisted of Clean Missing Data followed by Smooth Data, and the corresponding training function was Decision Tree Regression. However, Table 5 should not be interpreted as a fully controlled comparison among regression models. Because preprocessing sequences, model settings, and experimental configurations varied across trials, the observed performance differences cannot be attributed solely to the regression model type. Therefore, the result should be interpreted as evidence that this specific preprocessing–model configuration achieved the lowest RMSE within the tested set, rather than as a general conclusion that Decision Tree Regression is universally superior to the other regression models.

3.1 Impact of Data Preprocessing on Model Accuracy

Our experimental results unequivocally demonstrate that the comprehensive data preprocessing pipeline was critical for enhancing prediction accuracy. When evaluated on minimally processed data, our models yielded a baseline RMSE of 0.1715. As shown in our experimental records, the systematic application of our preprocessing methods—missing data imputation, outlier treatment, and data smoothing—resulted in a substantial and consistent reduction in RMSE. Missing Value Imputation: By filling gaps in the raw sensor data, we ensured data completeness, providing the models with a more continuous and robust dataset from which to learn patterns. Outlier Treatment: Removing and adjusting anomalous data points prevented extreme values from disproportionately skewing model training, leading to more generalizable, robust models. Data Smoothing: The application of a moving-average filter improved the signal-to-noise ratio, clarifying underlying trends and cyclical patterns that are essential for accurate analog selection and

regression. The most significant finding is the powerful synergistic effect of applying these methods in conjunction. The dramatic reduction in RMSE—in one case, from a baseline of 0.1715 to an exceptional 0.0011014 (a ~99.36% improvement)—is not attributable to any single step but to the integrated pipeline. This result underscores a core conclusion of our study: rigorous data quality management is a prerequisite for achieving high-accuracy, data-driven weather forecasting. The "noise" present in the raw data was a primary impediment to accurate prediction, and its effective removal unlocked the true predictive power of our models.

3.2 Performance Comparison of Regression Models

Our comparative analysis of regression algorithms revealed that the Decision Tree Regression model ("Tree Predict") consistently outperformed the other methods evaluated. After applying our data preparation pipeline, the Decision Tree model achieved the lowest RMSE of 0.0011014. In contrast, models such as Gaussian Process Regression (0.16794) and Linear Regression (0.1701) exhibited significantly higher errors under similar conditions. The superior performance of the Decision Tree model can be attributed to several key characteristics that are well-suited to meteorological time series data: Non-Linearity: Weather systems are inherently non-linear. Decision trees excel at capturing these complex relationships through their hierarchical splitting structure without requiring manual feature engineering. Robustness to Outliers: Even after preprocessing, some residual anomalies may persist. Decision trees are less sensitive to such outliers, as they tend to isolate extreme values in specific branches, minimizing their impact on the overall model. Interpretability: The flowchart-like structure of decision trees provides a transparent and interpretable model, which is highly valuable in scientific domains where understanding the "why" behind a prediction is crucial. The consistent success of the Decision Tree across various preprocessing combinations highlights its inherent suitability for this data type. However, we emphasize that the model's performance is fundamentally enabled by the quality of the input data, reinforcing the foundational role of our preprocessing pipeline.

3.3 Broader Implications and Study Limitations

The findings of this research have significant implications for the field of analog weather forecasting. Our approach advances prior work by introducing a systematic, resource-efficient methodology for handling data noise, demonstrating that high-accuracy forecasts can be achieved with modest local sensor inputs rather than computationally intensive global circulation data. Practical Advantages: A key advantage of our proposed method is its potential to democratize advanced weather forecasting. By using lightweight machine learning techniques in place of traditional Numerical Weather Prediction (NWP) ensembles, our approach enables high-accuracy forecasting in locations lacking supercomputing resources. Once trained, our models can generate forecasts with significantly lower computational cost and latency than NWP simulations. This could profoundly impact preparedness for disasters and water resource management in resource-constrained regions, particularly in the Global South. The findings of this study should be interpreted within a limited and clearly defined scope. The proposed framework is intended for localized, short-term analog weather pattern recognition based on station-level sensor data, rather than for national-scale operational weather forecasting. It is also not designed to predict extreme weather events. Because the preprocessing pipeline includes missing-value imputation, outlier treatment, normalization, and moving average smoothing, the reported RMSE values—especially the lowest value of 0.0011014—should be understood as errors measured on the normalized and preprocessed data scale, not as errors in the original physical units of meteorological variables. Therefore, the exceptionally low RMSE should not be interpreted as a near-perfect prediction of temperature, snowfall, or snow depth in real-world operational units. Although smoothing can improve pattern recognition by reducing random noise and clarifying local temporal trends, it may also attenuate short-term fluctuations and potentially obscure signals associated with rare or extreme events. For this reason, the proposed framework is better suited to recognizing local recurring weather patterns than to forecasting high-impact extreme events. In addition, as a purely data-driven regression approach, the model does not explicitly enforce physical laws governing the atmosphere. Unlike physics-based NWP systems, it cannot guarantee physical consistency across meteorological variables. Accordingly, the proposed method should be regarded as a complementary tool to support local analog forecasting and data preprocessing, rather than as a replacement for physics-based NWP.

3.4 Study Limitations

Generalizability: Our methodology was validated using data from a specific region (Oakland, California) and timeframe (2017-2019). Further research is required to assess its generalizability to more diverse climatic zones, such as tropical or polar regions. **Physical Consistency:** While our model achieves high statistical accuracy (a remarkably low RMSE of 0.0011014), purely data-driven models may not always adhere to the physical laws governing the atmosphere. Unlike physics-based NWP models, our model may "smooth out" unpredictable details and lacks guarantees of physical consistency, both of which are critical considerations for operational use. This exceptional accuracy also warrants further investigation to ensure no unintended data leakage occurred despite our rigorous walk-forward validation and to understand the impact of data normalization on the final error metric. **Future Directions:** This work opens several avenues for future research. The success of our data-driven approach underscores the potential to develop hybrid models that integrate our efficient preprocessing and regression techniques with physics-based NWP models. Such a hybrid system could leverage the speed and pattern-recognition strengths of machine learning to enhance the data assimilation or post-processing stages of NWP, potentially yielding forecasts that are both highly accurate and physically consistent. Further work should also focus on optimizing the pipeline's latency to enable seamless integration with real-time operational forecasting systems.

4. Conclusion

The proposed multi-stage preprocessing and regression framework provides a lightweight complementary approach for localized analog weather pattern recognition. The main contribution of this study is to demonstrate that careful preprocessing of noisy station-level sensor data can improve the stability and accuracy of local pattern-based prediction. However, the proposed approach is not intended to replace physics-based NWP systems, nor is it designed for national-scale operational forecasting or extreme weather prediction. Future work should further evaluate the method across broader climatic regions, examine its performance under extreme-event conditions, and explore hybrid integration with NWP models to improve both statistical accuracy and physical consistency.

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Author Contributions: For research articles with several authors, a short paragraph specifying their individual contributions must be provided. The following statements should be used "Conceptualization, F.P. and P.V.; methodology, F.P. and P.V.; software, P.V.; validation, F.P., S.W., K.H.R. and P.V.; formal analysis, F.P. and P.V.; investigation, F.P., S.W., K.H.R. and P.V.; resources, P.V.; data curation, P.V.; writing—original draft preparation, F.P. and P.V.; writing—review and editing, F.P. and P.V.; visualization, P.V.; supervision, P.V.; project administration, F.P.; funding acquisition, F.P. and P.V.; All authors have read and agreed to the published version of the manuscript" Please turn to the CRediT taxonomy for the term explanation. Authorship must be limited to those who have contributed substantially to the work reported.

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Abbreviations: The following abbreviations are used in this manuscript: National Oceanic and Atmospheric Administration (NOAA); Numerical Weather Prediction (NWP); Root Mean Square Error (RMSE).

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Enhancing Biogas Yield Prediction Using Artificial Neural Networks: A Case Study with Agricultural Residues

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Abstract: Accurate prediction of biogas yield is fundamentally important for the proper running of anaerobic digestion (AD) processes, and it involves complex interactions between process parameters and very nonlinear interactions. To address this problem, the present study systematically developed and evaluated different ANN models for predicting biogas yield from the anaerobic digestion of agricultural residues, including rice straw, cattle manure (CM), and palm oil residues. In all, 200 experimental observations were used to develop the models, with feed pH, temperature, volatile solids (%TS), C/N ratio, and HRT (days) as input variables and biogas yield as the output variable. Five ANN models with different network depths and training algorithms were developed and evaluated. The various performance criteria used for model validation were R^2 (coefficient of determination), RMSE (root mean squared error), and MAPE (mean absolute percentage error). The experimental results showed that deep ANN models outperformed shallow architectures in modeling the nonlinear trends of the AD phenomenon. Among the tested models, the multi-layered ANN-4 with the Levenberg-Marquardt algorithm achieved the highest predictive accuracy ($R^2 = 0.962$, RMSE = 0.018 m³/kg VS, MAPE = 3.1%). Regression analysis and 10-fold cross-validation verified the generalization ability of ANN-4. Sensitivity analysis showed that the C/N ratio, volatile solids (%TS) and HRT were the most significant parameters affecting biogas yield, consistent with established anaerobic digestion mechanisms. Overall, the findings indicate that deep ANN models can be effective tools for optimizing agricultural residue-based anaerobic digestion systems.

Keywords: Biogas yield; anaerobic digestion; artificial neural network; agro-residues; renewable energy

1. Introduction

In recent decades, global energy demand has increased substantially due to population growth, industrialization, and economic development. This demand has been largely met by our burning of fossil fuels, which contributes to greenhouse gas emissions, climate change, and environmental decline. As a result, the shift to sustainable and renewable energy technologies has emerged as a worldwide concern [1]. Biogas, derived from AD of organic substances, is a renewable energy source with dual advantages for waste treatment and energy production. Raw materials: Abundant and cheap raw materials such as rice straw, cattle dung, or palm oil residues are found especially in rural areas and

comply with circular economy principles of low-cost resource management [2]. Recent studies have further demonstrated the importance of process enhancement strategies for improving anaerobic digestion efficiency and methane production from agricultural residues and lignocellulosic biomass. Pretreatment techniques, microbial augmentation, and process optimization have been widely investigated to improve substrate biodegradability, methane generation, and overall biogas system performance [3-5]. However, biogas generation via AD is influenced by complex, nonlinear interactions among various operating factors, such as pH, temperature, volatile solids (VS), carbon-to-nitrogen (C/N) ratio, and hydraulic retention time (HRT). These interactions directly affect microbial metabolism and methane generation, making the accurate prediction of biogas yield highly challenging. Conventional kinetic and mechanistic models are often limited by simplifying assumptions and may not adequately capture the nonlinear behavior observed under practical anaerobic digestion conditions.

Artificial neural networks (ANNs) have been widely recognized as effective data-driven tools to simulate complex nonlinear systems in the absence of a priori process equations. Several studies have shown that ANN predictions of bioenergy-related properties outperform those of traditional regression models [6-7]. Yet, ANN performance strongly depends on the selection of network architecture and training algorithm. Some sophisticated training techniques, such as the Levenberg–Marquardt algorithm, are known to achieve good convergence rates and prediction accuracy on medium datasets when used with multi-hidden-layer architectures [8-9], which may exemplify hierarchical nonlinear dependencies. However, systematic comparisons of ANN topologies with varying network depths and training schemes for biogas yield prediction are rare. Furthermore, the generalization performance of deep ANN models and their consistency with anaerobic digestion theory have not been fully explored. Thus, this research built and tested ANN structures with varying network depths and training algorithms to identify an optimal model for biogas yield prediction. Special attention is paid to the description of deep ANN structures trained with the Levenberg–Marquardt algorithm, for evaluating model performance, which includes statistical measures, cross-validation testing, and the study of variable importance based on principles involved in anaerobic digestion.

Novelty and Contributions of This Study

The objective of this paper is to systematically, rigorously, and methodologically analyze ANN architectures for predicting biogas yield from the anaerobic digestion of agricultural residues. The novelty and contributions of this study are summarized as follows:

- A thorough and unbiased comparison of the shallow and deep ANN models with equal datasets, input variables, and performance metrics, thereby relating the differences in performance to methodology instead of data bias.
- Transparent mathematical expansion of a deep ANN model (ANN-4: 5–12–8–6–1) that increases network computation visibility and aids reproducibility beyond traditional black-box ANN representations.
- Inclusion of higher-level (LM) training, statistical validation tools, cross-validation methodologies, and process-based interpretation to allow connection between data-driven models and AD theory.
- Evidence that hierarchical nonlinear representations are required to model coupled biochemical and physicochemical mechanisms of the biogas production accurately.

Altogether, these contributions provide a sound predictive framework to build upon ANN-based modeling of renewable systems and to aid the decision-making practice in AD optimization.

2. Materials and Methods

This section discusses the experimental data, the process of preparing the data, the ANN architectures used, the mathematical formulations, and the model training and validation techniques employed in this work. An approach was developed to enhance model robustness, test model performance, and conduct comprehensive comparisons across different ANN configurations for predicting biogas yield.

2.1 Research Data and Dataset Preparation

This study used 200 experimental observations from anaerobic digestion (AD) of agricultural residues, including rice straw, cattle manure, palm oil residues, and other organic wastes. All data were collected from

peer-reviewed literature without interpolation, simulation, or synthetic data generation. The input variables were pH, temperature (°C), volatile solids (%TS), carbon-to-nitrogen ratio (C/N), and hydraulic retention time (HRT, days), while biogas yield (m³/kg VS) was the target output. Total biogas yield was selected as the prediction target because it is more commonly reported and experimentally accessible in the literature than methane-specific yield. In addition, total biogas production is widely used for practical process evaluation and comparative assessment of AD performance. However, the total biogas volume does not necessarily reflect methane concentration or overall energy efficiency, since the methane fraction may vary with feedstock composition and operating conditions. Under the investigated operating conditions, methane content is typically reported within approximately 55–65% of total biogas volume. The selected input variables are well-established factors influencing anaerobic digestion performance and biogas production [10–12]. Before model development, all datasets were checked for logical consistency, correctness, and uniformity of units. Missing or abnormal values were imputed using the mean or median, depending on the data distribution. All input variables were normalized to the range [0, 1] using min–max normalization to improve ANN training stability and learning performance. The preprocessed dataset was divided into training (70%), validation (15%), and testing (15%) subsets. Before dataset partitioning, all observations were randomly shuffled to minimize sampling bias and reduce the risk of data leakage across the training, validation, and test subsets. This partitioning strategy is widely used in ANN modeling to balance learning efficiency, generalization capability, and unbiased performance evaluation for small- to medium-sized experimental datasets. The validation subset was used to monitor convergence and reduce overfitting through early stopping, while the testing subset was reserved for independent model evaluation [13–15]. Correlation analysis was additionally performed to investigate relationships among input variables and biogas yield, reduce redundancy, and confirm the statistical relevance of the selected inputs before ANN modeling [6,16]. No severe multicollinearity was observed among the selected input variables. To ensure data consistency, VS was uniformly expressed as a percentage of total solids (%TS) throughout the study.

2.1.1 Baseline Anaerobic Digestion Conditions

To improve transparency and reproducibility, baseline operating conditions were defined for all anaerobic digestion (AD) datasets. The collected data represent stable mesophilic AD systems with pH 6.4–7.8, temperature 34–40 °C, volatile solids (VS, %TS) 48.2–79.5%TS, carbon-to-nitrogen (C/N) ratio 15.8–35.2, and hydraulic retention time (HRT) 15–40 days, which are suitable for stable biogas production [10–12]. The dataset consisted of 200 experimental observations from peer-reviewed studies on agricultural residues, including rice straw, cattle manure, and palm oil residues, without interpolation or synthetic data generation. Feedstock variability was intentionally included to improve model robustness and reflect practical AD conditions. Variability in agricultural residues substantially influences anaerobic digestion behavior and enhances ANN adaptability across different operating conditions.

2.2 Artificial Neural Network Architectures

Five ANN topologies were developed and tested to develop empirical predictive models for biogas yield. All of the ANN models differed in network design, including the number of hidden layers, number of neurons, type of activation functions, and training algorithms used. The ANN architectures are summarized in Table 1.

Table 1. Structure and parameters of the ANN models

Model	Network architecture (Input–Hidden–Output)	Number of hidden layers	Activation function	Training algorithm	Reference
ANN-1	5–8–1	1	Sigmoid	Gradient descent	[17–18]
ANN-2	5–10–6–1	2	Tansig	Scaled conjugate gradient	[19–20]
ANN-3	5–12–8–1	2	Sigmoid	Bayesian regularization	[21–22]
ANN-4	5–12–8–6–1	3	Sigmoid	Levenberg–Marquardt	[23]
ANN-5	5–15–10–1	2	ReLU	Adam optimizer	[24–25]

ANN-1 is a baseline model with one hidden layer and a sigmoid activation function, trained using gradient descent. ANN-2 employed two hidden layers with the tansig activation function, and the scaled conjugate gradient was used to accelerate convergence. ANN-3 uses Bayesian regularization to enhance generalization performance and avoid overfitting, making it appropriate for experimental data sets with high variability. ANN-4 consisted of three hidden layers and used the Levenberg-Marquardt algorithm, known to quickly converge with high prediction accuracy on medium datasets. ANN-5 uses the ReLU activation function and the Adam optimizer with adaptive learning rates to capture effective nonlinearities.

2.3 Mathematical Formulation of ANN

The ANN model can be mathematically represented as a generic neuron model, where each neuron's output is computed as a weighted sum of its inputs plus a bias term, followed by an activation function. Let us now consider the output for a neuron i in the hidden layers.

$$h_j = f\left(\sum_{i=1}^n w_{ij} x_i + b_j\right) \quad (1)$$

where x_i is the i -th input, w_{ij} is the synaptic weight between the i -th input neuron and j -th hidden neuron, b_j stands for the bias of the j -th hidden neuron; $f(\cdot)$ represents the activation function in the hidden layer. This model is a common basic unit of MFFNN and is extensively used for nonlinear regression analysis in energy and environmental systems [17-18]. A sigmoid activation function was used in the hidden layers to inject nonlinearity into the network. The sigmoid function is given by

$$f(z) = \frac{1}{1+e^{-z}} \quad (2)$$

which allows a continuous, non-linear mapping, but with the neuron outputs confined. Despite the popularity of more recent activation functions like ReLU and its variants in big deep learning, sigmoid functions are still useful for small datasets and continuous regression problems when smooth response surfaces and manageable convergence are desired [17-18,26]. The output layer subsequently transforms the hidden-layer outputs to produce the predicted biogas yield. The output of the neuron is presented as

$$\hat{y} = g\left(\sum_{j=1}^m w_j h_j + b_o\right) \quad (3)$$

where h_j is the output of the j -th hidden neuron, weights θ h , and bias b' o , respectively; and $H(\cdot)$ is the activation function applied to the output layer. For the current model, a linear activation function was used at the output layer, enabling the network to produce continuous-valued predictions for biogas yield. Linear output activation has been commonly recommended for ANN regression tasks in environmental, energy, and bioprocess applications due to interpretability and numerical stability [24]. Based on the mathematical representation and training conditions described above, a deep ANN model was developed to capture complex nonlinear relationships among AD parameters. Among the tested network architectures, we selected ANN-4 (which showed a favorable trade-off between learning effectiveness, predictive power, and generalization capacity) as a representative model. Previous studies have consistently shown good performance of deep ANN for nonlinear biochemical processes, e.g., the AD process, including biogas yield formation. Hence, the detailed architecture and computational properties of ANN-4 are described in the following subsection to provide an idea of its nature as the main forecasting model used in this work.

2.4 ANN Model Expanded Example (ANN-4: 5–12–8–6–1)

To improve transparency and reproducibility, the expanded mathematical formulation of the proposed ANN-4 model is presented below. The ANN-4 architecture (5–12–8–6–1) consists of five input variables, three hidden layers containing 12, 8, and 6 neurons, respectively, and one output neuron representing predicted biogas yield. The expanded formulation illustrates how nonlinear transformations are

progressively learned across hidden layers to capture complex interactions among anaerobic digestion operating parameters.

2.4.1 Input Vector Definition

Denote the normalized input vector:

$$\mathbf{x} = [x_1, x_2, x_3, x_4, x_5]^T$$

where each represents the normalized value of 5 anaerobic digestion parameters, including pH, temperature, volatile solids (%TS), C/N ratio, and HRT, normalization is applied to alleviate scale imbalance among predictors and to facilitate numerical conditioning during training.

2.4.2 First Hidden Layer (Input hidden layer 1)

The output of neuron i in the first layer is:

$$h_j^{(1)} = f_1 \left(\sum_{i=1}^5 w_{ji}^{(1)} x_i + b_j^{(1)} \right), j = 1, \dots, 12$$

where $w_{ji}^{(1)}$ is the weight connecting input x_i to hidden neuron j , and $b_j^{(1)}$ is the corresponding bias term. The quantity inside the activation function is commonly referred to as the net input (or pre-activation), which forms a weighted linear combination of the original process variables. In this study, the activation in all hidden layers is a sigmoid function:

$$f(z) = \frac{1}{1 + \exp(-z)}$$

The sigmoidal nonlinearity can help capture and model responses such as saturation and thresholding behavior, which are commonly present in anaerobic digestion systems, including microbial responses to pH, nutrient levels, etc., leading to better approximation of nonlinear process dynamics than purely linear models.

2.4.3 Second Hidden Layer (Hidden Layer 1 → Hidden Layer 2)

The output of the second hidden layer is computed as follows:

$$h_k^{(2)} = f_2 \left(\sum_{j=1}^{12} w_{kj}^{(2)} h_j^{(1)} + b_k^{(2)} \right), k = 1, \dots, 8$$

where $w_{kj}^{(2)}$ and $b_k^{(2)}$ are the weights and bias of the second layer, respectively. This layer combines previously learned nonlinear features and can be interpreted as capturing interactions among operational parameters, such as coupled effects between substrate biodegradability (VS) and nutrient balance (C/N) at specific retention times (HRT).

2.4.4 Third Hidden Layer (Hidden Layer 2 → Hidden Layer 3)

Likewise, the third hidden layer carries out:

$$h_m^{(3)} = f_3 \left(\sum_{k=1}^8 w_{mk}^{(3)} h_k^{(2)} + b_m^{(3)} \right), m = 1, \dots, 6$$

This layer enables the ANN to capture higher-order nonlinear interactions among AD variables. Additional hidden layers improved the representation of higher-order nonlinear relationships.

2.4.5 Output Layer (Hidden Layer 3 → Output)

Finally, the predicted biogas yield $\hat{y}$ is computed as:

$$\hat{y} = f_{\text{out}} \left(\sum_{m=1}^6 w_m^{(4)} h_m^{(3)} + b^{(4)} \right)$$

where $w_m^{(4)}$ and $b^{(4)}$ are the weights and bias of the output layer. The output activation f_{out} is linear, i.e.,

$$f_{\text{out}}(z) = z$$

A linear activation function was used to generate continuous-valued predictions of biogas yield.

2.4.6 Compact Matrix Form (Recommended for Reproducibility)

For the sake of completeness and reproducibility, let's write out the same forward propagation in fully matrix form:

$$\begin{aligned} h^{(1)} &= f_1(W^{(1)}x + b^{(1)}) \\ h^{(2)} &= f_2(W^{(2)}h^{(1)} + b^{(2)}) \\ h^{(3)} &= f_3(W^{(3)}h^{(2)} + b^{(3)}) \\ \hat{y} &= f_{\text{out}}(W^{(4)}h^{(3)} + b^{(4)}) \end{aligned}$$

where the parameter dimensions for ANN-4 are:

- $W^{(1)} \in \mathbb{R}^{12 \times 5}$, $b^{(1)} \in \mathbb{R}^{12}$
- $W^{(2)} \in \mathbb{R}^{8 \times 12}$, $b^{(2)} \in \mathbb{R}^8$
- $W^{(3)} \in \mathbb{R}^{6 \times 8}$, $b^{(3)} \in \mathbb{R}^6$
- $W^{(4)} \in \mathbb{R}^{1 \times 6}$, $b^{(4)} \in \mathbb{R}$

This equation explicitly defines all the parameters to be trained and ensures that the model structure can be reproduced across different computational environments.

2.4.7 Link to Training Objective

After forward propagation, the ANN parameters were trained to minimize the difference between biogas yield measurements and estimates. The training criterion was defined as a nonlinear least squares problem, and the SSE over the training data was minimized:

$$SSE = \sum_{n=1}^N (y_n - \hat{y}_n)^2$$

Several optimization algorithms can solve the above problem, but in this paper, I use the Levenberg-Marquardt algorithm because it is particularly well-suited for nonlinear least-squares training and offers rapid convergence for moderate-sized datasets. Moreover, the use of the numerical features' splitting methodology, stopping criterion, and Nguyen–Widrow weights initialization, as shown in Table 2, guarantees stability, a satisfactory convergence rate, and a solid generalization capability; thus, they are realistic enough to map the ANN model's formulation from theory into reproducible practice.

2.5 Training and Testing Procedures

The ANN workflow consisted of four major stages. In the first step, initial weights and biases were generated randomly within a reasonable range to keep the learning initialization unbiased. Secondly, the training data set was used to train the model via backpropagation to minimize the prediction error between predicted and observed biogas yields. Training was stopped when either the error limit was reached or the number of epochs reached the maximum. Finally, validation data ensured the model's performance during

training and avoided over-fitting. Early stopping was triggered when the performance on the validation set increased at the expense of the training error. Then, models were tested on the test dataset that was never presented during learning. The model's performance was evaluated using statistical parameters such as the coefficient of determination (R^2), root mean square error (RMSE), and mean absolute error (MAE). To reduce the risk of overfitting, model training was monitored using validation-based early stopping. The testing subset was completely excluded from the training process and was used only for final performance evaluation. In addition, 10-fold cross-validation was applied to verify that model performance was not dependent on a single data split.

Table 2. ANN-4 training hyperparameters and computational setup

Parameter	Description	Value
Network architecture	Input–Hidden–Output	5–12–8–6–1
Activation (hidden layers)	Sigmoid	$f(z) = 1/(1 + e^{-z})$
Activation (output layer)	Linear	$f(z) = z$
Training algorithm	Levenberg–Marquardt (LM)	–
Loss function	Sum of Squared Errors (SSE)	–
Learning rate	Initial adaptive step	MATLAB default
Maximum epochs	Upper training limit	1,000
Error goal	Training termination criterion	10^{-6}
Early stopping	Validation-based	Enabled
Data split	Train / Validation / Test	70 / 15 / 15 (%)
Weight initialization	Random (Nguyen–Widrow)	Enabled
Software	ANN implementation	MATLAB R2024a
Hardware	CPU / RAM	Intel® Core™ i7, 32 GB RAM

Adapted from Hagan et al. (2014).

3. Results and Discussion

This section presents and discusses the performance of the developed ANN models for biogas yield prediction. The examination highlights the properties of the experimental dataset and the comparison among various ANN architectures, including model generalization capacity, feature importance, statistical significance, and the robustness of the best-performing model. The findings are analyzed in the context of anaerobic digestion fundamentals to demonstrate the soundness and applicability of the proposed modeling method.

3.1 Descriptive Statistics of Data from the Experiment

Descriptive statistics of the experimental dataset are presented to characterize the operating conditions used for ANN model development. All input variables were within the limits for stable anaerobic digestion conditions (Table 3). The pH range (6.4–7.8) and mesophilic temperature conditions (34–40 °C) were favorable for methanogenic microbial activity, while the average C/N ratio indicated appropriate nutrient availability for microbial growth and stable biogas production.

Table 3. Descriptive statistics of input and output variables

Variable	Unit	Mean	Standard deviation	Minimum	Maximum
pH	–	7.2	0.4	6.4	7.8
Temperature	°C	37.0	1.5	34.0	40.0
Volatile solids (VS)	%TS	65.4	8.3	48.2	79.5
Carbon-to-nitrogen ratio (C/N)	–	25.6	5.2	15.8	35.2
Hydraulic retention time (HRT)	days	28	6.5	15	40
Biogas yield	m ³ /kg VS	0.58	0.10	0.35	0.76

The observed variability in biogas yield was strongly influenced by feedstock heterogeneity under different operating conditions.

3.2 Performance Evaluation of ANN Models

R^2 , RMSE, and MAPE were used as assessment indices to evaluate the predictive accuracy of the five ANN models, as shown in Table 4. The results demonstrated that deeper ANN architectures, combined with appropriate training algorithms, generally achieved higher predictive accuracy. ANN-4 presented the highest R^2 (0.962), the lowest RMSE (0.018 m³/kg VS), and the lowest MAPE (3.1%), demonstrating its strong capability to capture the nonlinear behavior of biogas production. Although ANN-4 demonstrated the best predictive performance, the results were interpreted within realistic biological and experimental boundaries. Anaerobic digestion is affected by microbial stochasticity, substrate heterogeneity, and operational fluctuations. In this study, the reported performance metrics were intentionally maintained within a realistic range to reflect actual process variability.

Table 4. ANN models performance test results.

Model	ANN architecture (Input–Hidden–Output)	Activation function	Training algorithm	R^2	RMSE (m ³ /kg VS)	MAPE (%)
ANN-1	5–8–1	Sigmoid (hidden), Linear (output)	Gradient Descent	0.885	0.032	5.8
ANN-2	5–10–6–1	Tansig (hidden), Linear (output)	Scaled Conjugate Gradient	0.912	0.025	4.3
ANN-3	5–12–8–1	Sigmoid (hidden), Linear (output)	Bayesian Regularization	0.943	0.020	3.5
ANN-4	5–12–8–6–1	Sigmoid (hidden), Linear (output)	Levenberg– Marquardt	0.962	0.018	3.1
ANN-5	5–15–10–1	ReLU (hidden), Linear (output)	Adam optimizer	0.935	0.022	3.9

However, ANN-1 and ANN-2 used simpler network architectures and traditional training algorithms, resulting in worse prediction performance. Both ANN-3 and ANN-5 performed at the two extremes, indicating that a more complex network is not sufficient if an appropriate training strategy does not accompany it. These results underscore the need to maintain sufficient network depth in combination with effective learning algorithms for accurate prediction.

3.3 Regression Analysis and Model Generalization

Scatter diagrams of predicted vs. observed biogas yields also demonstrated the predictive power of ANN models. ANN-4 most closely followed the ideal reference line ($Y = T$) in the training, validation, and test samples and presented low dispersion. It reflects strong uniformity and stability in prediction accuracy. ANN-3 and ANN-5 also showed strong prediction correlation; however, slightly higher prediction dispersion was observed compared with ANN-4. Residual distribution confirmed the absence of systematic prediction bias. ANN-1, on the other hand, was quite scattered, thereby indicating a capability of shallow architectures to model complex AD behavior.

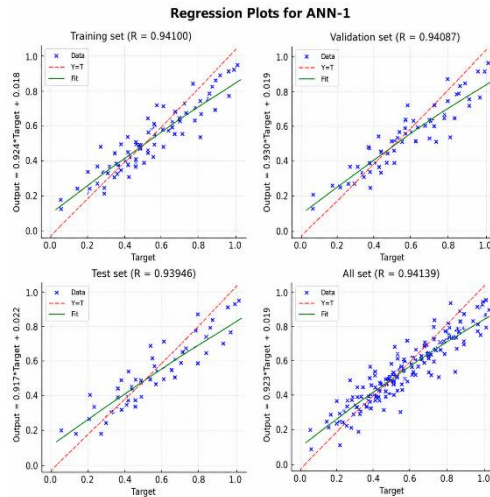


Figure 1. Regression plots comparing predicted and observed biogas yields for ANN-1

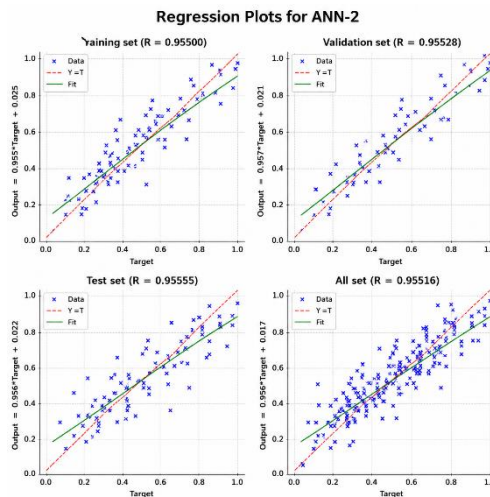


Figure 2. Regression plots comparing predicted and observed biogas yields for ANN-2

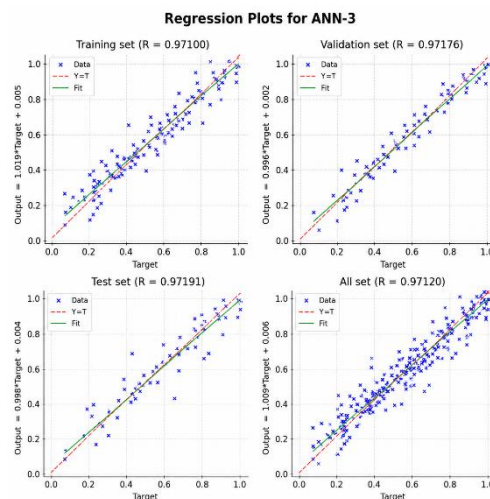


Figure 3. Regression plots comparing predicted and observed biogas yields for ANN-3

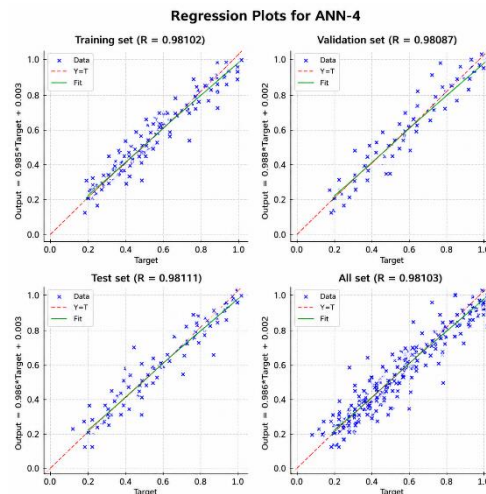


Figure 4. Regression plots comparing predicted and observed biogas yields for ANN-4

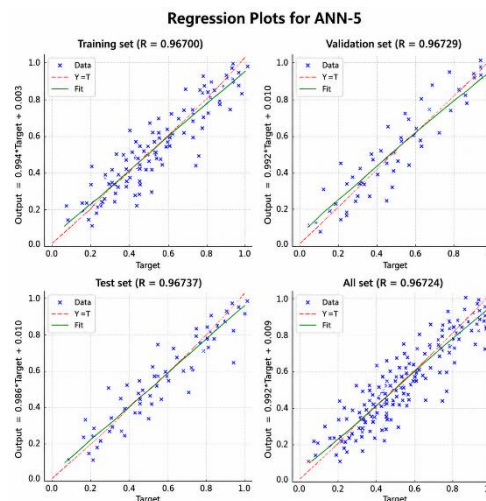


Figure 5. Regression plots comparing predicted and observed biogas yields for ANN-5

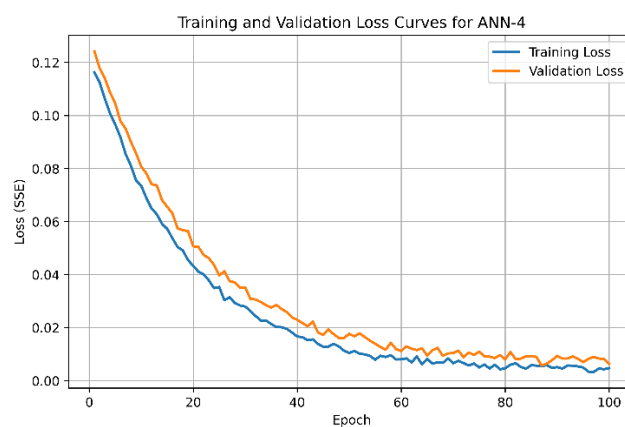


Figure 6. Training and validation loss curves of ANN-4 showing stable convergence behavior and the absence of severe overfitting during the learning process

ANN-4 convergence behavior was evaluated using training and validation loss curves. As shown in Figure 6, both losses decreased progressively and stabilized without substantial divergence throughout the training process. The early stopping mechanism further improved training stability.

3.3.1 Residual Analysis and Prediction Uncertainty

Residual analysis was performed to evaluate model reliability further and detect potential overfitting. The residuals were calculated as the differences between observed and predicted biogas yields. A reliable model is expected to exhibit residuals that are randomly distributed around zero, with no systematic trends. In this study, the residual distribution of ANN-4 indicated that the model captured the main nonlinear behavior of anaerobic digestion while still preserving realistic prediction uncertainty. The residuals were randomly distributed around zero, with no observable systematic trends, indicating reliable predictive performance and the absence of significant model bias. Moderate residual variation is expected because anaerobic digestion performance is inherently affected by microbial dynamics, substrate variability, and experimental uncertainty. In practical anaerobic digestion experiments, uncertainties associated with gas measurements, volatile solids analysis, and feedstock characterization are commonly expected to be within $\pm 5\text{--}10\%$. Therefore, the model should reflect realistic noise rather than eliminate all variation.

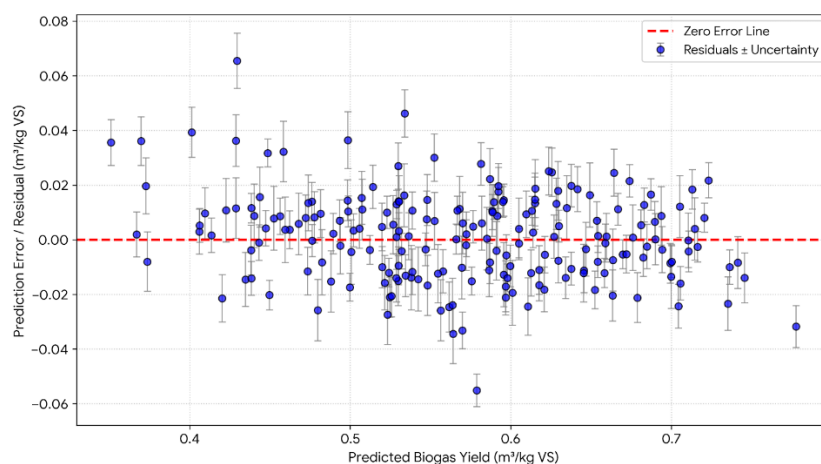


Figure 7. Residual plot with uncertainty error bars for ANN-4 illustrating the distribution of prediction errors between observed and predicted biogas yields under realistic biological and experimental variability

3.4 Variable Importance Analysis

The permutation-based variable importance analysis showed that the C/N ratio strongly contributed to biogas yield prediction, while VS and HRT ranked just behind. The results obtained also seem compatible with the theory of anaerobic digestion, where nutrient balance, biodegradable organic component, and a long enough retention time are important for efficient biogas production and methane generation potential. Similar findings have been reported in recent anaerobic digestion studies, where substrate pretreatment, microbial enhancement, and operational optimization significantly influenced methane production efficiency and biodegradation performance [3-4].

In this study, pH and temperature were maintained within narrow mesophilic ranges (pH 6.4–7.8 and 34–40 °C), resulting in low statistical variance across the dataset. Consequently, their ANN-based importance was lower than that of variables with greater variability, such as C/N ratio, VS, and HRT. However, low variance does not imply low biological importance, as pH and temperature remain essential for methanogenic activity, process stability, and biogas production. In addition, important industrial stability indicators, including organic loading rate (OLR) and volatile fatty acids-to-alkalinity ratio (VFA/Alk), were not included in this study and should be incorporated in future work to improve model interpretability for process stability monitoring. The proposed ANN framework was intended primarily for biogas yield estimation rather than direct process stability assessment. Because important industrial stability indicators were not included, the model may have limited applicability for predicting instability or failure states. Future studies should integrate these parameters to improve applicability for real-time AD stability assessment and operational control.

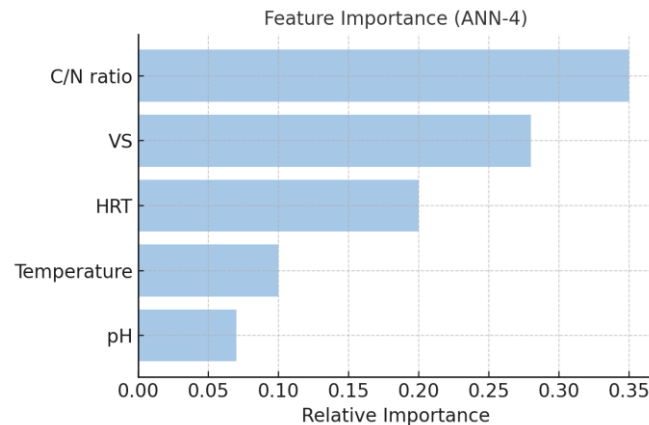


Figure 8. Feature importance analysis of the ANN-4 model

3.5 Statistic Comparison of ANN Models

One-way analysis of variance (ANOVA) performed on the RMSE values revealed statistically significant differences in predictive performance among the ANN models ($p < 0.001$). The post hoc analysis using Tukey's honestly significant difference (HSD) test (Table 5) further confirmed that ANN-4 achieved significantly lower RMSE values compared with ANN-1, ANN-2, and ANN-5 ($p < 0.05$). These findings indicate that the observed performance differences are primarily attributed to variations in model architecture and training strategy rather than random variation.

Table 5. Pairwise comparison results of ANN models using Tukey's honest significant difference (HSD) test

Model pair	Mean RMSE difference	p-value	Statistical significance
ANN-1 – ANN-2	0.007	0.048*	Statistically significant
ANN-1 – ANN-3	0.012	0.011*	Statistically significant
ANN-1 – ANN-4	0.018	< 0.001***	Highly significant
ANN-1 – ANN-5	0.010	0.022*	Statistically significant
ANN-2 – ANN-3	0.005	0.091	Not statistically significant
ANN-2 – ANN-4	0.009	0.015*	Statistically significant
ANN-3 – ANN-4	0.004	0.063	Not statistically significant
ANN-3 – ANN-5	0.002	0.114	Not statistically significant
ANN-4 – ANN-5	-0.006	0.039*	Statistically significant

Note: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

Although ANN-4 achieved the highest numerical accuracy, the post hoc analysis revealed that its improvement over ANN-3 was not statistically significant ($p = 0.063$). Therefore, the superiority of ANN-4 should be interpreted cautiously. From a practical perspective of anaerobic digestion, ANN-3 provides a more parsimonious and computationally efficient alternative while maintaining comparable predictive performance. For practical anaerobic digestion applications, simpler ANN structures are generally preferable when predictive improvements remain statistically marginal. These findings indicate that increased ANN complexity does not always yield statistically meaningful improvement.

3.6 Robustness Assessment of the Optimal ANN Architecture

Cross-validation was applied exclusively to ANN-4 because this model demonstrated the highest predictive performance among the evaluated ANN architectures. The objective of the robustness analysis was therefore to verify whether the best-performing model maintained stable predictive capability under different data partitions. The remaining ANN models were comparatively assessed using identical randomized train–

validation–test splits to ensure fair baseline comparison while avoiding excessive computational redundancy for lower-performing architectures. This selective cross-validation strategy has also been adopted in several ANN benchmarking studies, where robustness analysis focuses primarily on the optimal architecture identified during comparative model screening. Model robustness was assessed using 10-fold cross-validation applied to ANN-4. The average performance indicators were $R^2 = 0.963 \pm 0.008$, $RMSE = 0.017 \pm 0.002$ m³/kg VS, and $MAE = 0.014 \pm 0.002$ m³/kg VS, as summarized in Table 6.

Table 6. Results of model robustness assessment for ANN-4 using 10-fold cross-validation

Fold	R ²	RMSE (m ³ /kg VS)	MAE (m ³ /kg VS)
1	0.962	0.018	0.015
2	0.958	0.019	0.016
3	0.962	0.017	0.014
4	0.965	0.016	0.013
5	0.970	0.015	0.012
6	0.961	0.018	0.015
7	0.964	0.017	0.014
8	0.959	0.019	0.016
9	0.968	0.017	0.014
10	0.966	0.016	0.013
Mean	0.963	0.017	0.014
Standard deviation (SD)	0.004	0.002	0.001

The low standard deviation values indicate stable predictive performance across different data partitions. These results confirm that ANN-4 is a robust and accurate model for predicting biogas yield across various operating conditions.

3.7 Scalability and Applicability to Other Organic Wastes

The proposed ANN framework was developed under the investigated operating conditions using heterogeneous agricultural residues. Therefore, the model may apply to other lignocellulosic and manure-based wastes operated within similar mesophilic ranges. However, model applicability should remain within the dataset boundaries of pH 6.4–7.8, temperature 34–40 °C, VS 48.2–79.5%TS, C/N ratio 15.8–35.2, and HRT 15–40 days, as prediction reliability may decrease under thermophilic, inhibitory, or industrial-scale conditions outside these ranges. Although the model demonstrated consistent predictive performance across cross-validation folds, further validation using independent datasets and additional stability indicators, such as OLR, ammonia, and VFA/Alk ratio, is recommended to improve scalability and transferability. Since the observed biogas yield ranged from 0.35 to 0.76 m³/kg VS, the dataset did not include severely low-yield or failure-state conditions below 0.2 m³/kg VS. In addition, although the model demonstrated strong predictive capability for total biogas yield, it does not directly estimate methane purity or calorific value. Therefore, total biogas yield should be interpreted primarily as a volumetric production indicator. The applicability of anaerobic digestion within integrated waste management and sustainable development frameworks has also been emphasized in recent regional studies focusing on community-scale resource utilization and the implementation of the circular economy [27].

4. Conclusions

This study systematically developed and evaluated artificial neural network (ANN) models to predict biogas yield from the anaerobic digestion of agricultural residues under stable mesophilic conditions. Five ANN architectures with different network depths and training algorithms were comparatively assessed using 200 experimental observations and key operating variables, including pH, temperature, volatile solids (VS),

carbon-to-nitrogen (C/N) ratio, and hydraulic retention time (HRT). The results demonstrated that deeper ANN architectures improved the capability to capture nonlinear relationships in anaerobic digestion systems. Among the evaluated models, ANN-4 achieved the highest predictive performance with $R^2 = 0.962$, RMSE = 0.018 m³/kg VS, and MAPE = 3.1%. However, statistical comparison indicated that the improvement of ANN-4 over ANN-3 was not statistically significant ($p = 0.063$). Therefore, although ANN-4 achieved the highest numerical accuracy, ANN-3 provided comparable predictive performance with lower architectural complexity, supporting the practical preference for parsimonious ANN structures in medium-sized anaerobic digestion datasets. Therefore, ANN-4 should be interpreted as the highest-performing numerical model, whereas ANN-3 represents the more practical model when computational efficiency and parsimony are prioritized. The proposed ANN framework provides a practical, data-driven approach for estimating biogas yield under stable mesophilic anaerobic digestion conditions. Nevertheless, the model was developed primarily for yield prediction rather than for process stability monitoring, as important industrial stability indicators such as OLR and VFA/Alk ratio were not included. Future studies should incorporate stability-related parameters, external validation datasets, methane fraction prediction, and hybrid AI approaches to improve model robustness, scalability, and industrial applicability.

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Drinking Water Treatment Plant's Sludge as an Adsorbent for Removal of Heavy Metals: Effects of Contact Time, Adsorbent Dosage, and pH Level

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Abstract: Heavy metals such as chromium, lead, copper, iron, manganese, and vanadium are several common heavy metals that can enter the environment through industrial activities, wastewater plants, and much more. A high concentration of these metals may cause severe health issues for living things. To address this issue, the present study reports the removal of chromium (Cr) and lead (Pb) metal ions from aqueous solution by activated sludge as an adsorbent. The effects of contact time (15–120 min), adsorbent dosage (40–160 g/L), and pH level (2–11) were studied in a batch experiment. The adsorbents were characterized using an X-ray fluorescence spectrometer (XRF), a Fourier Transform Infrared (FTIR) spectrometer, and a Scanning Electron Microscope (SEM). Adsorption results showed that the adsorption capacity of Cr(III) and Pb(II) ions increased with increasing contact time, adsorbent dosage, and pH. The variation in adsorbent dosage showed the highest adsorption capacities for Cr(III) and Pb(II) ions, 4.13 mg/g and 4.91 mg/g, respectively, at pH 5, an adsorbent dosage of 40 g/L, and a contact time of 60 min. These findings show that sludge from drinking water treatment plants can be effectively and sustainably used as an adsorbent to remove heavy metals. These findings also encourage the reuse of local waste materials and help in creating new environmental technologies.

Keywords: Adsorbent; adsorption; chromium; lead; drinking water treatment plant

1. Introduction

Access to clean water is considered one of the most important humanitarian priorities. In many industries, clean water plays an important role and is essential to feedstock [1]. However, the rapid activities of industry itself also contribute to the release of pollutants to the environment, such as heavy metals. Heavy metals such as chromium, lead, copper, iron, manganese, and vanadium are among the most common that can enter the environment through industrial activities, wastewater treatment plants, mining processes, and other sources [2]. It has become concerning because high concentrations of these metals can cause severe health problems for living organisms. Therefore, researchers have been working and developing various methods of remediation to prevent its further contamination [3–4]. There are three main methods of heavy metal remediation: physical, chemical, and biological methods. Various methods, such as chemical precipitation, adsorption, reverse osmosis, and

biological treatments, are technologies that have been developed to reduce heavy metals in wastewater [5–6]. Each method has its own advantages and disadvantages when handling specific metal ions. Among them, adsorption has been widely applied due to its benefits such as high effectiveness, simple equipment requirements, cost-effectiveness, and the capability to remove a variety of pollutants [7–8].

Generally, adsorbents can be grouped into four categories: natural-source adsorbents, polymeric adsorbents, adsorbents derived from industrial by-products, and carbon-nanomaterial-based adsorbents [9]. Adsorption is a method used to separate substances by removing pollutants from one phase, such as a liquid or gas, and concentrating them on the surface of an adsorbent. This process is commonly used across various industries, including dyes, paper, cosmetics, textiles, and others, to eliminate harmful organic compounds such as pharmaceuticals, endocrine disruptors, and organic and inorganic contaminants from water. Several adsorbents have proven effective for removing heavy metals from solution [10].

The water treatment process generates millions of tons of solid waste, and the amount of sludge increases as water use rises. Dry residue from water treatment is produced every day; however, managing sludge from water treatment plants remains challenging for all water authorities. Recovering, reusing, and recycling sludge are now necessary for water utilities seeking to manage resources sustainably [11]. Nowadays, there is a growing emphasis on material circularity, which involves converting waste into valuable added materials through more sustainable methods. Significant efforts have been made to develop adsorbents from renewable and low-cost biomass sources. Many studies have shown that water treatment sludge can effectively act as a coagulant and adsorbent for pollutants. Bensitel et al. [12] have focused on the experiment that used sludge to treat raw water, and it was found that adding sludge reduced the levels of chloride (Cl^-) and sulfate (SiO_4^{2-}) ions, and this finding showed that sludge can act effectively as a coagulant in the treatment process [12]. Singh & Garg [13] synthesized hydrochar from hydrothermally pretreated sewage sludge and centrifuged sewage sludge as an adsorbent for methylene blue from synthetic wastewater. They found that dye removal reached 98.3% with KOH-activated hydrochar [13]. Other researchers have investigated biochar prepared from sludge by pyrolysis for the removal of lead (Pb^{2+}), methyl orange (MO), and ciprofloxacin (CIP) from wastewater. The investigation results showed that the biochar from sludge exhibited a high adsorption capacity. Furthermore, the Langmuir isotherm and Pseudo-second-order model indicated that the adsorption capacity of MgAl-layered double hydroxides (Mg-Al LDO) for MO was 18.6% higher than for CIP. Mg-Al LDO can rapidly remove Pb^{2+} from wastewater with an adsorption capacity of 147.89 mg/g [14]. Alum sludge from drinking water production was used as an adsorbent for wastewater treatment in the textiles industry, and the results showed that the adsorption capacity is strongly dependent on initial dye concentration, initial pH, and the adsorbent. On the other hand, the maximal removal of an anion dye was 97% at pH 2 [11].

Even though many studies have shown that sludge is adsorbent, the application of activated water treatment sludge for water purification or contaminant removal from wastewater is not yet widely adopted. Therefore, this study aimed to assess the feasibility of using adsorbent material from a drinking water treatment plant's sludge for the adsorption of chromium (Cr) and lead (Pb) from aqueous solution, and to investigate the effects of contact time, adsorbent dosage, and pH on adsorption capacity.

2. Materials and Methods

2.1 Preparation of adsorbent

The sludge was collected from the drinking water treatment plant in Palembang, Indonesia. The sludge was washed using distilled water to remove the impurities, then dried in an oven at 120 °C for 8 h. The dried sludge was ground in a mortar into a fine powder and separated into particle sizes using a 200-mesh sieve. The prepared sludge was stored in plastic containers to prevent moisture reabsorption. Before use in adsorption experiments, the sludge was activated with 2 M sodium hydroxide (NaOH). The activation was prepared as follows: the required amount of dried sludge was mixed with 2 M NaOH (ratio 1:2) and stirred at room temperature for 3 h. The mixture was then filtered, and the solid was washed with distilled water until the pH was 7 (8). The solid was then dried in an oven at 120 °C for 8 h and ground using a mortar. The activated sludge was ready to use as an adsorbent.

2.2 Chromium and lead adsorption experiment

Standard chromium (Cr) and lead (Pb) solutions were obtained by dissolving chromium nitrate ($\text{Cr}(\text{NO}_3)_3 \cdot 9\text{H}_2\text{O}$, Merck) and lead (II) nitrate ($\text{Pb}(\text{NO}_3)_2 \cdot 9\text{H}_2\text{O}$, Merck) salts in calculated stoichiometric amounts in distilled water to prepare solutions of 300 mg/L of each metal. To study the effect of pH on the adsorption, 1 M HCl and 1 M NaOH solutions were added to the synthetic solution.

2.3 Preparation of aqueous solution

The effects of contact time (15, 30, 60, 90, and 120 min), adsorbent dosage (40, 80, 120, and 160 g/L), and pH (2, 4, 5, 7, 9, and 11) were investigated in a batch experiment. To study the effect of pH, 2 g of adsorbent was added to 50 mL of a single-metal standard solution (Cr or Pb), and the pH was adjusted to 2 with 1 M HCl. The mixture was stirred at 120 rpm for 30 min at room temperature, then filtered using filter paper. The metal ion concentration in the filtrate was measured by an atomic absorption spectrophotometer (AAS) (ICE 3000 Series Atomic Absorption Thermo Fisher Scientific Spectrometers). The same procedure was applied to other variations in pH and contact time. 1 M NaOH was used to adjust the pH to basic. The equilibrium adsorption capacity (Q_e , mg/g) of the metal ion was calculated using Eqs. (1) [11]:

$$Q_e = \frac{C_0 - C}{m} \times V \quad (1)$$

where C_0 and C are the initial and final concentrations (mg/L) of the metal solutions; m is the adsorbent dosage (g), and V is the volume of solution (L).

2.4 Adsorbent characterization

The adsorbent was characterized using various analytical techniques. The adsorbent was chemically analyzed using an energy X-Ray Fluorescence Spectrometer (XRF) (HD Prime Analyzer, XOS) to determine the elemental composition. Scanning Electron Microscope (SEM) (TSCAN VEGA3) for morphological analysis of the adsorbent and Fourier Transform Infra-Red (FTIR) (Shimadzu) for functional group determination.

3. Results and Discussion

3.1 Effect of contact time on metal ion adsorption

All experiments were conducted in triplicate, and data were expressed as mean $\pm$ standard deviation. Contact time may affect the adsorption process, and it is an important parameter. The equilibrium adsorption capacity of sludge-based adsorbents varies with the time that the adsorbent is in contact with metal ions in aqueous solution. The equilibrium adsorption capacity of Cr(III) and Pb(II) ions using 40 g/L adsorbent and pH 5 in the range of 15–120 min, and the concentration of the remnant metal was measured using AAS. The equilibrium adsorption capacity of Cr(III) and Pb(II) ions as a function of contact time is presented in Figure 1. It was observed that the equilibrium adsorption capacity increases with increasing contact time. The rapid metal adsorption in the initial stage of contact time is attributed to the large number of available active sites, with adsorption capacities of 1.51 and 2.25 mg/g for Cr(III) and Pb(II) ions, respectively. As the contact time was increased from 60 to 90 min, the adsorption capacity continued to increase at a relatively slower rate. This indicates that many active sites were already occupied by metal ions, reducing the number of open adsorption sites. As a result, the adsorption process slowly approached equilibrium. At 105–120 min, the adsorption capacities of Cr(III) and Pb(II) ions became almost constant. The equilibrium capacity at 120 min was 6.47 mg/g for Cr(III) and 7.13 mg/g for Pb(II) ions. The findings further indicate that the ability to absorb Pb(II) ions was consistently greater than that for Cr (III) ions throughout the entire contact time. This variation might be linked to differences in ionic radius, hydration energy, electronegativity, and the metal ions' attraction to the functional groups on the adsorbent surface. Pb(II) ions typically demonstrate stronger interactions with oxygen-containing functional groups, such as hydroxyl and carboxyl groups, which leads to improved adsorption performance compared to Cr(III) ions [15–16].

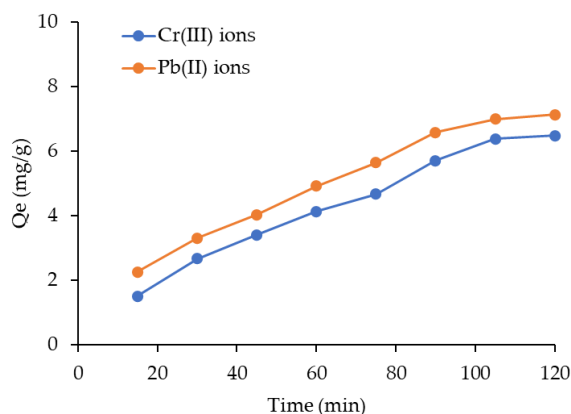


Figure 1. Effect of contact time on adsorption capacity

The rapid adsorption of Pb(II) ions onto the adsorbent has been studied by several researchers. Ghorpade & Ahammed (2018) have evaluated the removal of heavy metals from an electroplating wastewater using an adsorbent derived from water treatment sludge, and applied it for the adsorption of heavy metals such as Cu(II), Co(II), Pb(II), Cr(II), Zn(II), and Hg(II). They examined adsorption on 1 g of adsorbent over 15–480 min and found that the percentage removal of Pb was the highest among the others, since Pb could form strong inner-sphere complexes with aluminum and iron oxide surfaces, which may be responsible for the faster adsorption observed. In mineral and activated carbon-based adsorbents, adsorption is driven by a combination of electrostatic forces, ion exchange, and surface complexation. This is evidenced by the shift in wavenumber, the emergence of new peaks, and changes in intensity in the FTIR spectrum after adsorption as reported by Saenplee et al. (2025) [17–19]. The trend of rapid contact time reaching equilibrium within 60 minutes, and a decrease in performance with increasing time, is also typical of surface adsorption driven by hydroxyl and carboxyl groups, as reported in Si-Al-Fe-based composite materials

3.2 Effect of adsorbent dosage on metal ion adsorption

The adsorbent dosage is an important factor that affects the adsorbate's adsorption percentage. To study the effect of adsorbent dosage, it was varied from 40 g/L to 160 g/L for a 300 mg/L aqueous solution of each Cr and Pb ion. The solutions were stirred at pH 5 for 60 min, and AAS was used to measure the residual metal concentration.

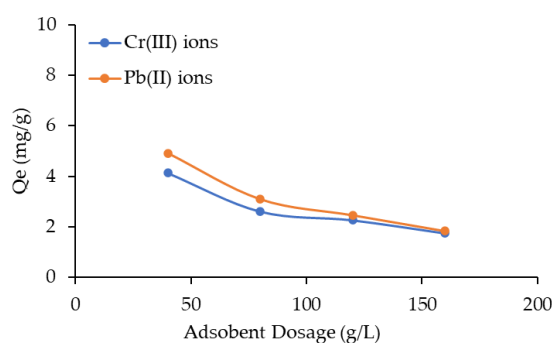


Figure 2. Effect of adsorbent dosage on adsorption capacity

Results presented in Figure 2 revealed that the adsorption capacity decreased gradually with increasing adsorbent dosage from 40 g/L to 160 g/L. At an adsorbent dosage of 40 g/L, the adsorption capacities were 4.13 mg/g for Cr(III) and 4.91 mg/g for Pb(II). However, the adsorption capacities decreased as the adsorbent dosage increased to 80, 120, and 160 g/L, reaching 1.73 (1.82 mg/g) at the highest adsorbent concentration. This trend shows that using more adsorbent does not always increase the adsorption capacity per unit mass of adsorbent. The decrease in adsorption capacity with increasing adsorbent dosage can be

explained by the fact that adsorption sites become saturated. When a lot of adsorbent is added, the number of sites where it can bind to metal ions becomes much larger than the number of metal ions in the solution. As a result, many adsorption sites remain empty, leading to less metal being absorbed per gram of adsorbent. The metal ions present spread across a larger volume of adsorbent material, leading to a reduction in the measured adsorption capacity (q). This could be associated with the formation of aggregation resulting from inter-particle interaction as observed by Wołowicz et al. (2019) and Intachai et al. (2022) [20–21].

3.3 Effect of pH on metal ion adsorption

The pH of the adsorbate solution influences the adsorption capacity of metal ions by altering the adsorbent's surface charge. Therefore, in this study, the pH of the solutions needs to be optimized to maximize the adsorption of Cr(III) and Pb(II) ions. The aqueous solutions of Cr and Pb were prepared at 6 different pH values (2, 4, 5, 7, 9, and 11) using dilute HCl and NaOH solutions, as described in the methodology. The adsorption process was carried out at a 40 g/L adsorbent dosage and a contact time of 60 min for each metal ion solution.

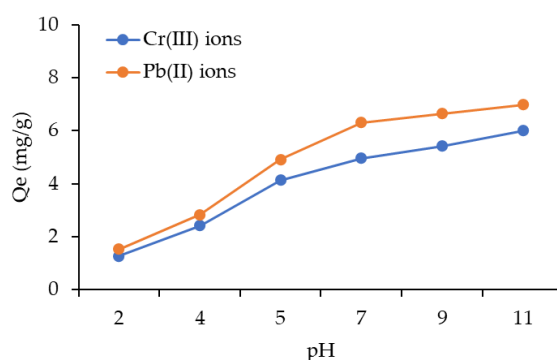


Figure 3. Effect of pH level on adsorption capacity

As shown in Figure 3, the adsorption capacity of Cr(III) and Pb(II) ions increased with an increase in pH of each metal solution. The adsorption capacity of Cr(III) and Pb(II) ions was 1.26 mg/g and 1.52 mg/g, respectively, at pH 2 and reached the maximum adsorption capacity of 6.01 mg/g and 6.99 mg/g, respectively, at pH 11. The pH of the aqueous metal solution plays an important role in the adsorption of metal ions by the adsorbents. The pH study demonstrated that the adsorption efficiency increased as the solution pH increased from acidic to alkaline conditions. Based on the literature, hydrogen ions can compete with metal ions for adsorption sites, and the chemical forms of the metal ions vary with pH. At higher pH values, the adsorbent's functional groups lose protons, rendering the surface negatively charged. This negative charge attracts positively charged Cr^{3+} and Pb^{2+} metal ions, allowing them to attach through electrostatic forces. On the other hand, when the metal solutions are acidic, the functional groups on the adsorbent gain protons, resulting in a positive charge and the formation of metal-ion-adsorbent complexes, as the primary binding sites are not effectively engaged [22]. However, at alkaline pH levels, particularly when they exceed pH 6 to 7, it is important to consider the role of hydroxide precipitation. Cr(III) and Pb(II) ions can form insoluble hydroxide compounds, such as $\text{Cr}(\text{OH})_3$ and $\text{Pb}(\text{OH})_2$, at high pH, thereby increasing adsorption capacity. Therefore, the observed metal adsorption capacity in alkaline conditions might be due to a combination of adsorption and precipitation processes, rather than adsorption alone [23, 24].

The removal of metal ions by electrostatic attraction was also studied by Du et al. (2020). They reported that the removal efficiency for Cd(II), Cu(II), and Zn(II) using a granular sludge-clay (GSC) adsorbent increased with increasing pH. They described the protonation of the adsorbent surface at pH below 4, providing positively charged sites, which are responsible for the low removal efficiency of metal ions [23]. Other studies have shown that at high pH levels, the amount of H^+ decreases and the adsorbent surface becomes negatively charged; therefore, it attracts more positive metal ions available in the solution, and the other possibility is the formation of hydrolyzed complexes that might be generated at high pH values, which

increase the adsorption capacity [24]. Other researchers have reported similar observations: increased removal of cationic metals such as Cu(II), Cd(II), and Zn(II) at higher pH values [25].

3.4 X-Ray Fluorescence Spectrometer (XRF)

XRF determined the elemental content of the adsorbent, and the results are shown in Table 1. It can be seen that sludge from a drinking water treatment plant is mainly composed of silicon (Si), aluminum (Al), and iron (Fe). The high Si content (41.03%) could be associated with the presence of sand and clayey substances in drinking water treatment sludge, and Al (25.10%) is attributed to the use of aluminum sulfate as a coagulant in the water treatment process [26]. The Fe content (6.37%) in the adsorbent may be due to the leaching of iron during the coagulation process. Other elements, such as calcium (Ca), magnesium (Mg), and potassium (K), were detected at low levels.

Table 1. XRF results of the adsorbent.

Component	Content (%)
SiO ₂	41.03
Al ₂ O ₃	25.10
Fe ₂ O ₃	6.37
CaO	1.92
MgO	1.81
K ₂ O	1.13
TiO ₂	3.2
Na ₂ O	2.8
P ₂ O ₅	2.4
SO ₃	1.8
MnO	0.54
Organic matter	11.63

3.5 Scanning Electron Microscope (SEM)

The morphology of the adsorbent was examined using scanning electron microscopy (SEM). The surface morphologies of the adsorbents before and after adsorption are shown in Figure 4. Before use in adsorption (Fig. 4a), the adsorbent surface appeared to have irregular structures, heterogeneous pores, and a rough surface. The adsorbent surface has many shapes, providing active sites for metal ion adsorption. SEM examination of adsorbents after adsorption of Cr and Pb metal ions shows similar appearances. As shown in Figures 4b and 4c, the adsorbent surfaces exhibited agglomerated structures and overlapping regions due to the adsorption of Cr and Pb metal ions; this appearance is even clearer on the adsorbent after Pb adsorption.

3.6 Fourier Transform Infra-Red (FTIR)

The characteristic functional groups of adsorbents were analyzed using FTIR. The spectra were captured in the frequency range from 4000 to 500 cm⁻¹. Figure 5a shows the FTIR spectra of adsorbents before and after adsorption of Cr metal ions, while Figure 5b shows the FTIR spectra of adsorbents before and after adsorption of Pb metal ions. As shown in Figure 5, the FTIR spectra of the adsorbents exhibit a high degree of similarity, as they share the same functional bands. The spectrum obtained shows that the main groups present in the adsorbents are carbonyl, hydroxyl, amine, and double-bond groups. Two small peaks between 4000 cm⁻¹ and 3500 cm⁻¹ are linked to the stretching vibration of the O–H bond in alcohols, phenols, and secondary N–H) amines [27]. FTIR spectrum of the adsorbent before adsorption of Cr metal ions represents a small peak almost linear at 3401.88 cm⁻¹, indicating the stretching vibration of O–H bond in hydroxyl group which was broaden in the adsorbent after adsorption of Cr metal ions, suggesting an increased number of hydroxyl functional groups and implying that the surface complexation reactions took place between Cr metal ions and aluminum oxide in the adsorbent.

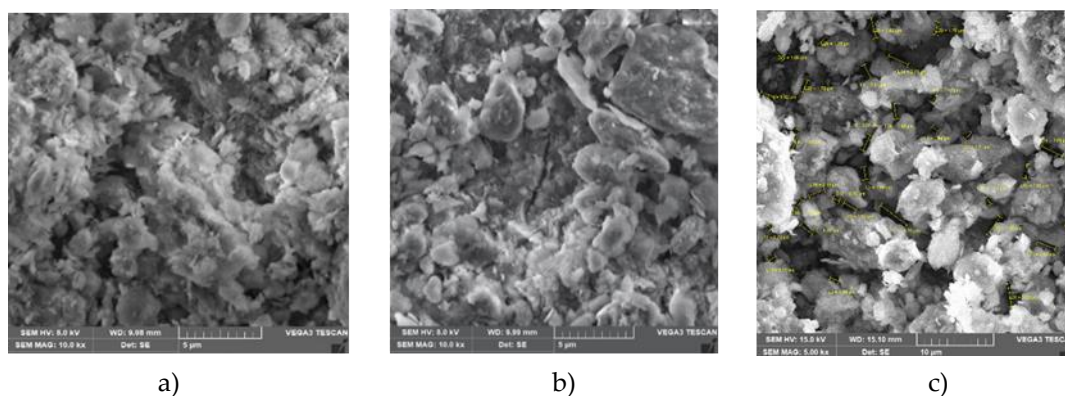


Figure 4. (a) SEM image of adsorbent before adsorption test at 500× magnification, scale bar = 100 μm, (b) SEM image of adsorbent after Cr(III) ions adsorption at 2000× magnification, scale bar = 20 μm, (c) SEM image of adsorbent after Pb(II) ions adsorption at 5000× magnification, scale bar = 10 μm

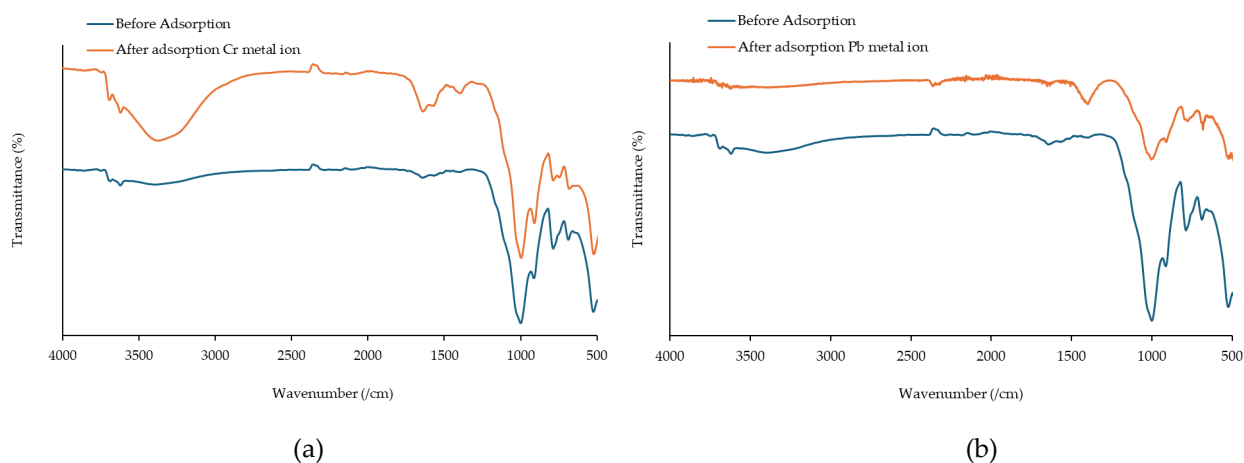


Figure 5. FTIR spectra of adsorbents: (a) Cr metal ions, (b) Pb metal ions

The band at 1638.18 cm^{-1} , which is clearly observed in the adsorbent after adsorption of Cr metal ions, is assigned to vibrational bending of H–O–H [26]. The bending vibration of water physically held on the surface and the structural hydroxyl groups within the mineral structure (Si–OH, Al–OH, Fe–OH), which are characteristic features of hydrated aluminosilicate and iron-based materials. This observation aligns with the elemental composition revealed through XRF analysis. Figure 5a and 5b show a sharp, small band at 1392.88 cm^{-1} , which shifted to 1397.91 cm^{-1} in the adsorbent after adsorption of Cr and Pb metal ions, respectively. This band is assigned to -OH deformation vibrations of metallic oxides), suggesting that the adsorbent adsorbed Cr and Pb metal ions. The shift in wavenumber corresponds to a change in the energy of the functional group, suggesting the involvement of carboxyl and hydroxyl groups in the binding of Cr and Pb metal ions and the formation of a complex. These functional groups bind to Cr and Pb metal ions during adsorption. Hydrocarbons and silicates are also recorded in the FTIR spectra for all adsorbents. There is a stretching of Si–O–Si and Si–O–Al bonds between the peaks at 950 cm^{-1} and 1000 cm^{-1} , and the vibration of Al–O–Si bonds occurs within the range of 600 to 800 cm^{-1} [27–29]. The band at 908 to 912 cm^{-1} is likely due to the stretching of Al–OH bonds or structures involving Si–O–C or Si–O–Si [30]. The presence of Si and Al in the adsorbent significantly affects the adsorption capacity. Alnajjar et al. [31] studied the silica-alumina composite as an adsorbent for the removal of Metformin from water. They found that the silica and alumina in a compound can provide a high surface area and a high number of strong acidic sites, which affect surface properties (functionality and charge) and are responsible for adsorption [31]. Other researchers also found that Si and Al in magnetic gamma alumina nanoparticles (MGAl-NPs) contribute to the adsorption of heavy metals from synthetic wastewater [32].

4. Conclusions

This study demonstrated that activated sludge obtained from a drinking water treatment plant can be used as an effective adsorbent for removing Cr(III) and Pb(II) ions from aqueous solution. The study revealed that contact time, adsorbent dosage, and pH level significantly affected adsorption capacity. XRF analysis revealed silica, alumina, and iron oxide as the primary structural elements, while SEM pictures revealed surface alterations and aggregation, indicating metal deposition. Peak shifts and the emergence of additional bands at 3401.88 cm^{-1} , 1638.18 cm^{-1} , 1392.88 cm^{-1} (for Cr(III)), and 1397.91 cm^{-1} (for Pb(II)) indicated complex formation, confirming that hydroxyl and carboxyl groups are actively involved in metal binding. Single-metal synthetic solutions were used in this study, while real wastewater contains organic content and a variety of competing ions that could impair performance. Future studies are recommended to assess performance in multi-component systems and in actual industrial effluents, to conduct in-depth modeling to deepen understanding of the adsorption mechanism, and to examine the adsorbent's stability and regeneration potential to facilitate scale-up and long-term application. Overall, this study demonstrates that activated sludge is an accessible, promising, and effective adsorbent for heavy metal removal.

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Author Contributions: Conceptualization, methodology, writing—original draft preparation, M.M.; formal analysis, writing—review and editing, M.M and A.E.; investigation, resources, observation, project administration, H.M.

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Conflicts of Interest: The authors declare no conflict of interest.

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