



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.

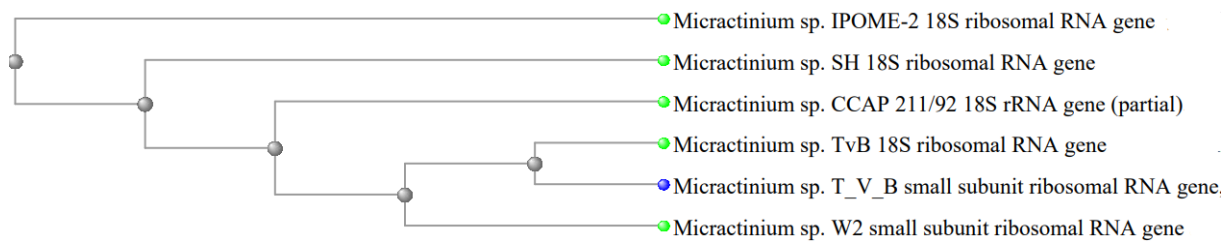


Figure 1. Phylogenetic tree of *Micractinium* sp. strain in the NCBI database.

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 μ m 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.

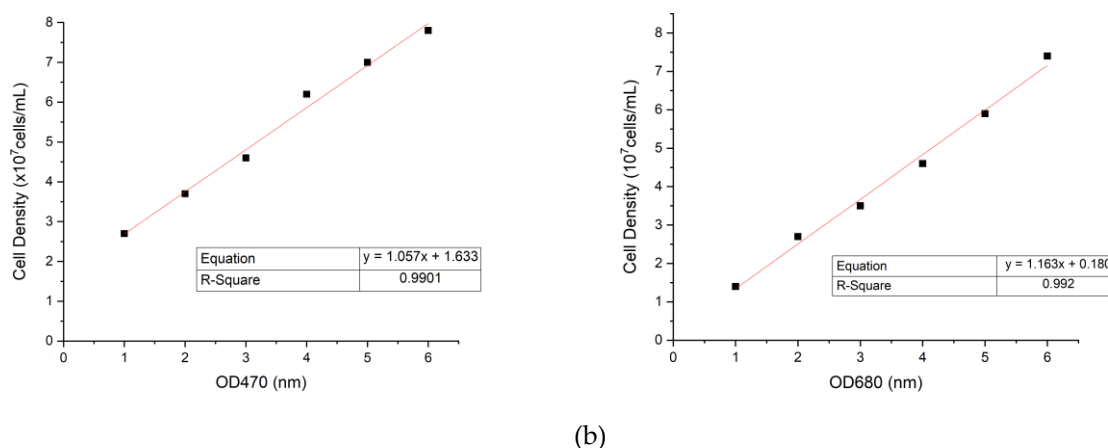


Figure 2. Calibration curve of (a) cell density vs. OD470 and (b) cell density vs. OD680.

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].

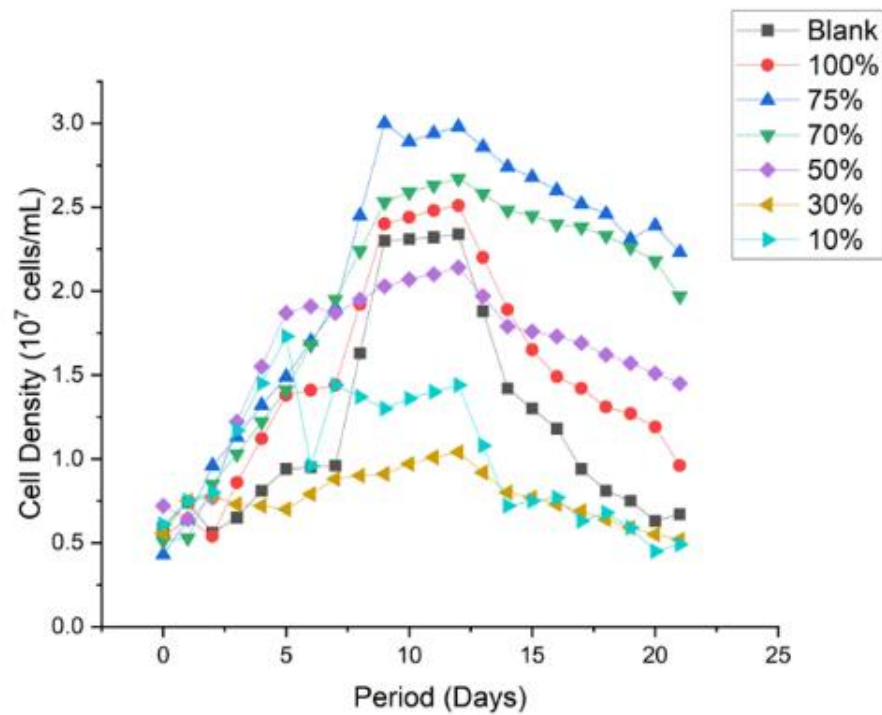


Figure 3. The growth of *Micractinium* sp. at different percentages of wastewater dilution.

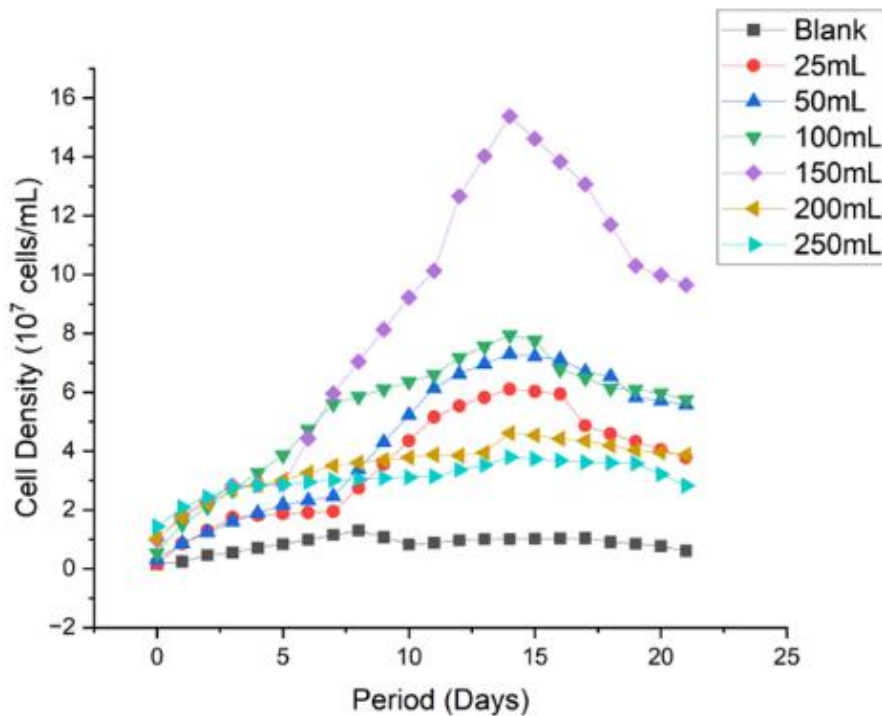


Figure 4. The growth of *Micractinium* sp. at different volumes of microalgae seed.

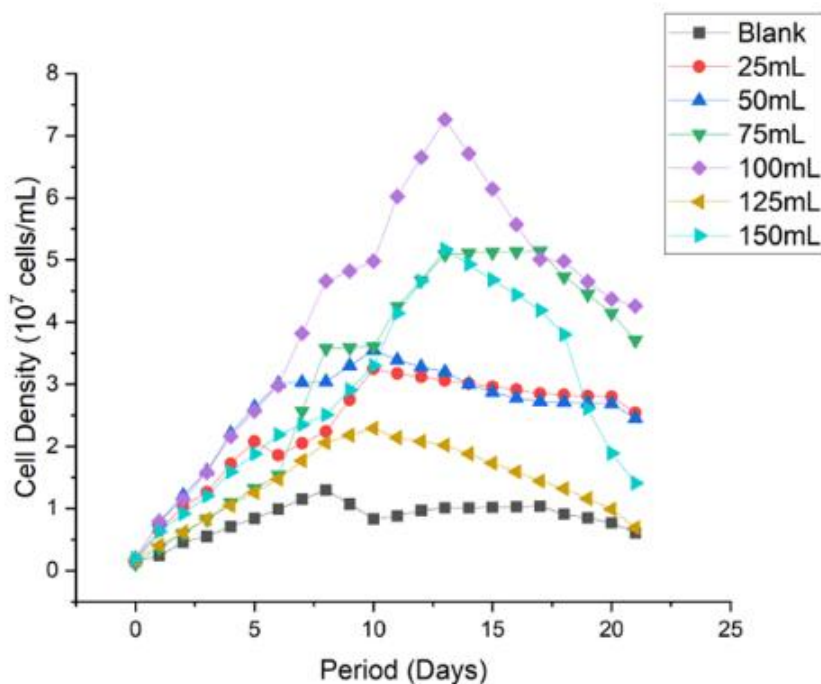


Figure 5. The growth of *Micractinium* sp. at different concentrations of NPK nutrients.

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].

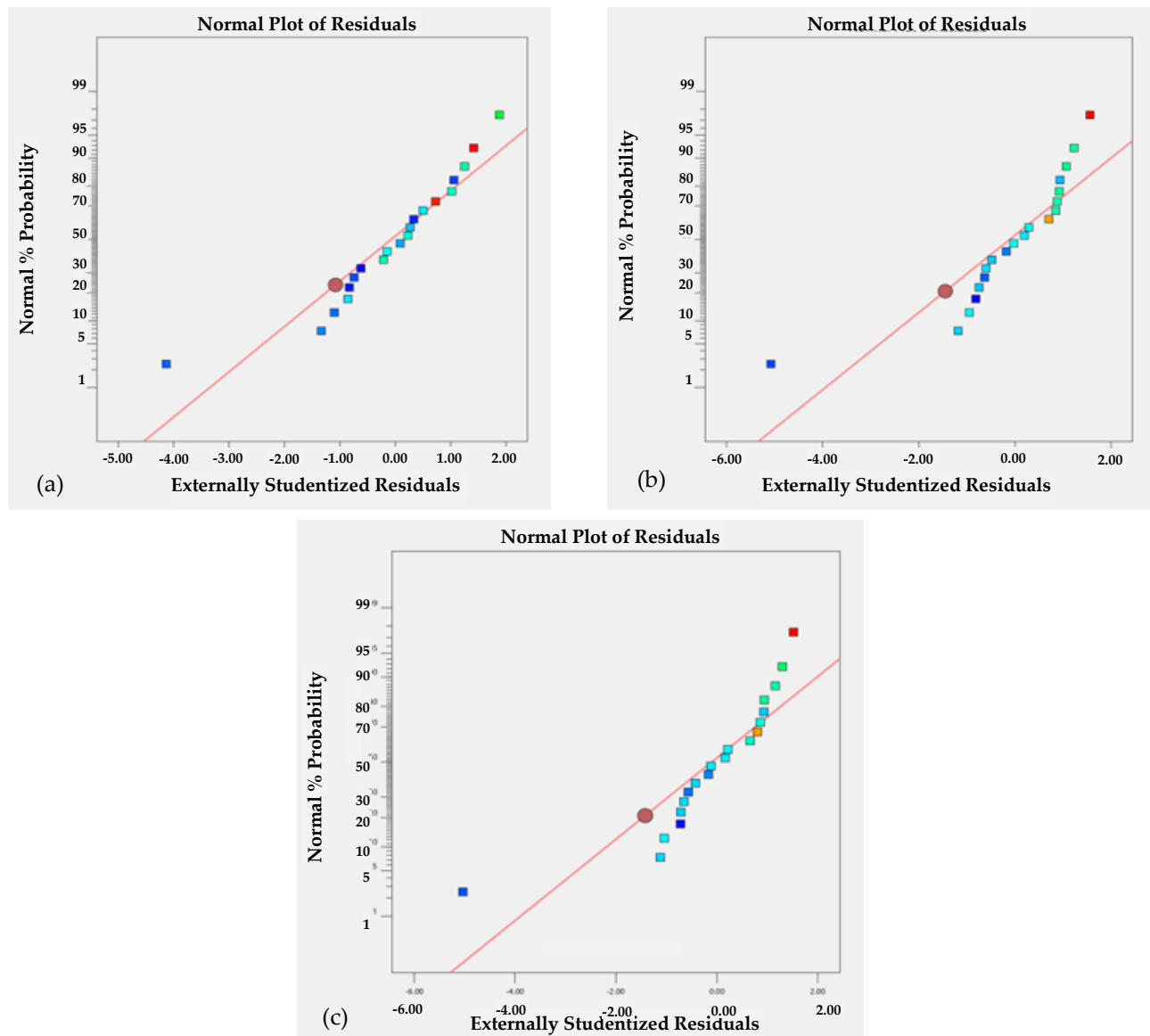


Figure 6. Design expert plot (domestic wastewater); normal probability plot of the internally standardized residual for cultivation response (a) cell density, (b) growth, and (c) biomass nutrient.

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].

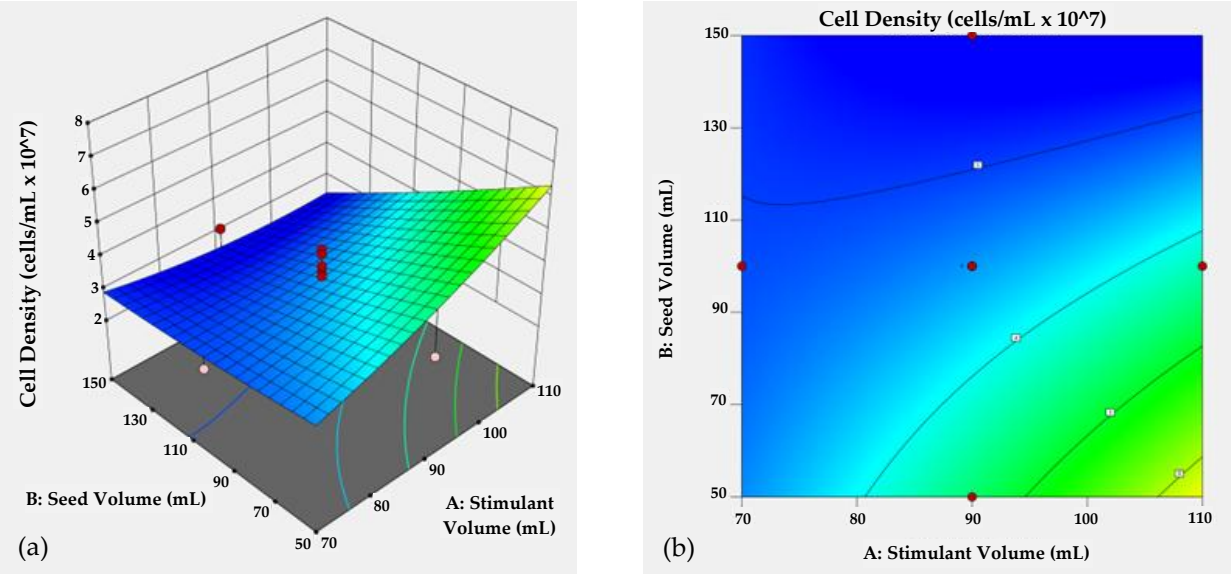


Figure 7. Design expert plot; (a) 3D surface and (b) contour for interaction between seed and stimulant volume.

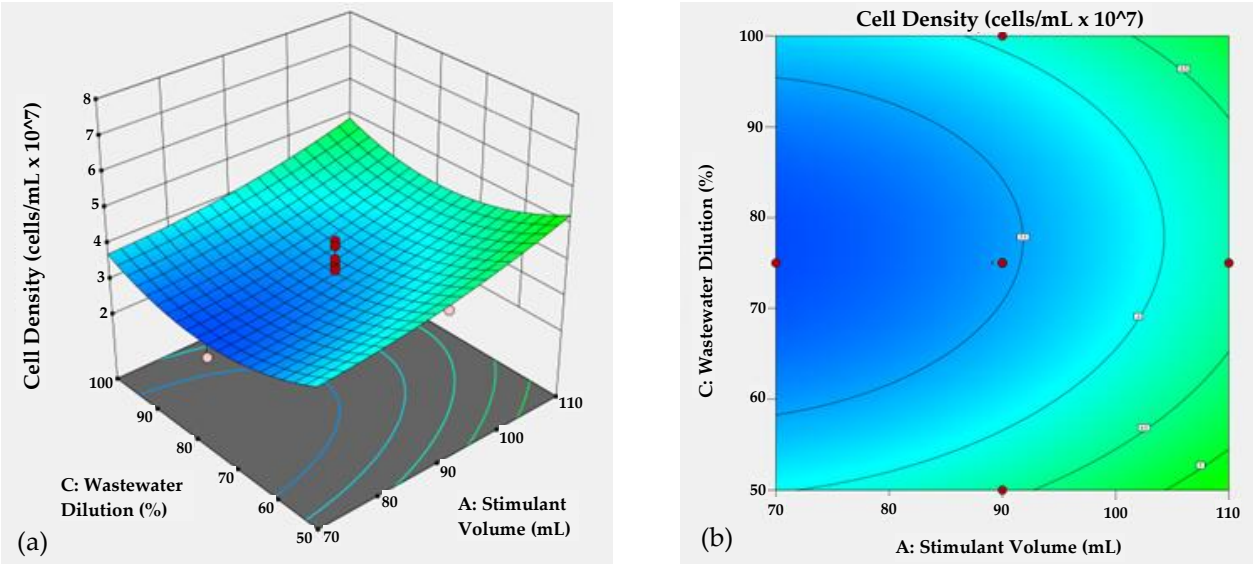


Figure 8. Design expert plot; (a) 3D surface and (b) contour for interaction between wastewater dilution and stimulant volume.

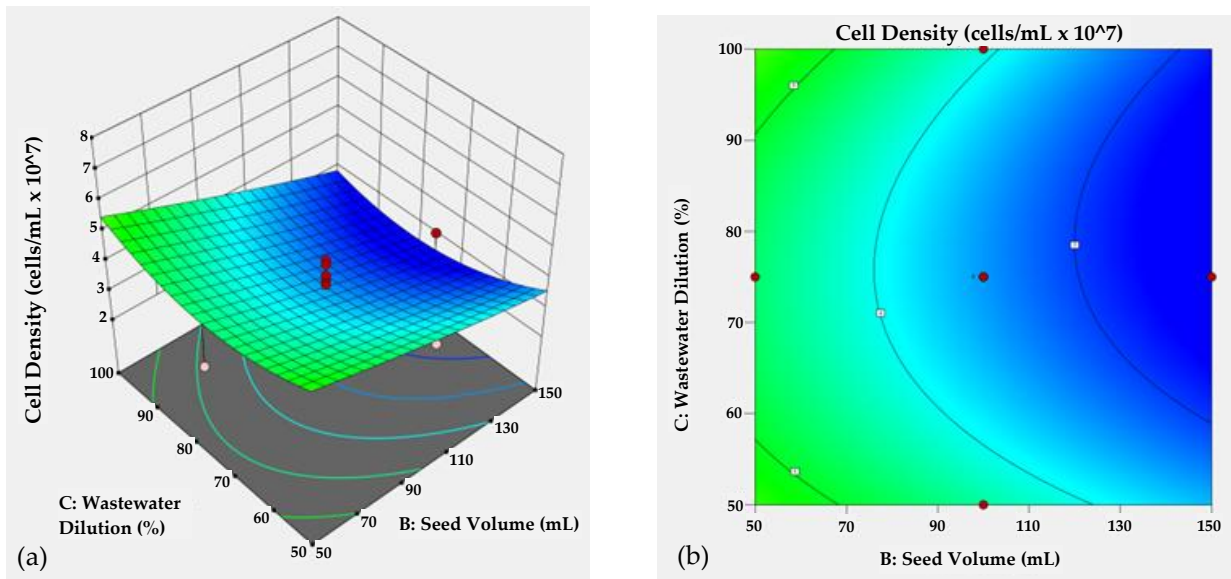


Figure 9. Design expert plot; (a) 3D surface and (b) contour for interaction between wastewater dilution and seed volume.

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.

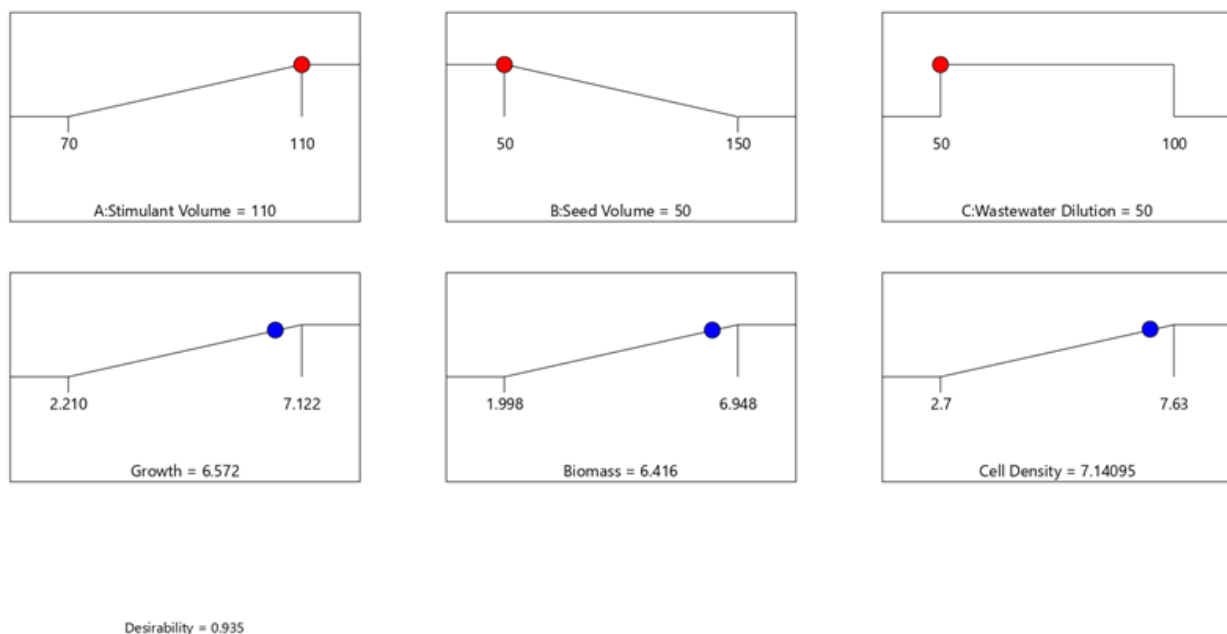


Figure 10. Design Ramps of the factors (wastewater dilution, seed and stimulant volume) and response (cell density, growth, and biomass) optimization.

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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