ASEAN
Journal of Scientific and Technological Reports
Online ISSN:2773-8752

Research article

Enhancing Methane Production from Palm Oil Industry Waste through Thermotolerant Bacterial Bio-augmentation: Optimization and Kinetic Analysis

Sittikorn Saelor[1], Wisarut Tukanghan[2], Peerawat khongkliang[3], Supattra In-chan[2,4], and Nantharat Phruksaphithak[5,6*]

[1] Faculty of Science and Technology, Hatyai University, Songkhla, 90110, Thailand
[2] Bio4gas (Thailand) Company Limited, Paphayom, Phatthalung, 93210, Thailand
[3] Research Center for Circular Products and Energy, King Mongkut's University of Technology North Bangkok, Bangkok, 10800, Thailand
[4] Biofuel and Biocatalysis Innovation Research Unit, Mahidol University, Nakhonsawan Campus, Nakhonsawan, 60130, Thailand
[5] Faculty of Science and Digital Innovation, Thaksin University, Phatthalung, 93210, Thailand
[6] Centre of Excellence for Agricultural Innovation and Bioproducts of Thaksin University, Thaksin University, Phatthalung, 93210, Thailand
* Correspondence: nantharat@tsu.ac.th

Citation:
Saelor, S.; Tukanghan, W.; Khongkliang, P.; In-chan, S.; Phruksaphithak, N., Enhancing methane production from palm oil industry waste through thermotolerant bacterial bio-augmentation: optimization and kinetic analysis. *ASEAN J. Sci. Tech. Report.* **2026**, *29*(1), e259596. https://doi.org/10.55164/ajstr.v29i1.259596.

Article history:
Received: May 27, 2025
Revised: October 2, 2025
Accepted: October 18, 2025
Available online: December 14, 2025

Abstract: This study investigated bio-augmentation strategies to enhance biogas production from palm oil industry residues through thermotolerant anaerobic digestion. Two bacterial strains, *Cellulomonas* sp. (HD19AZ1) and *Themoanaerobacterium thermosaccarolyticum* (PSU-2), were evaluated at various inoculum to microorganism (I:S) ratios for both single digestion of empty fruit bunches (EFB) and co-digestion with palm oil mill effluent (POME). Results demonstrated that bio-augmentation significantly improved substrate biodegradability and methane yields. With HD19AZ1, the optimal I:S ratio of 70:10% for single digestion achieved methane yields of 146.38 mL-CH_4/gVS (a 24.94 mL-CH_4/gVS substrate improvement), while the 65:15% ratio for co-digestion yielded 166.55 mL-CH_4/gVS (33.38 m^3/tonne substrate improvement). These represented increases of 33.59% and 39.65% in biodegradability for single and co-digestion, respectively. Volatile solids removal reached 41.82% in single digestion and 47.59% in co-digestion under optimal conditions. Kinetic analysis revealed that bio-augmentation with HD19AZ1 achieved methane production rates of 3.90 mL-CH_4/d for single digestion and 6.70 mL-CH_4/d for co-digestion, while PSU-2 augmentation increased rates by 2.14 times compared to control samples. The hydrolysis constant (Kh) ranged from 0.0214-0.0375 d^{-1} for single digestion and 0.0095-0.0232 d^{-1} for co-digestion, with lag phases of 5.15-16.11 days and 18.29-43.14 days, respectively. Modified Gompertz modeling confirmed these parameters with R^2 values exceeding 0.97. This study demonstrates that strategic bio-augmentation with thermotolerant bacteria significantly enhances methane production from palm oil industry waste by improving substrate accessibility and accelerating the hydrolysis of recalcitrant lignocellulosic components, offering promising applications for industrial-scale biogas production.

Keywords: Bio-augmentation; anaerobic digestion; lignocellulosic biomass; methane yield; palm oil residues

1. Introduction

The palm oil industry has experienced substantial growth over recent decades, becoming one of the world's most important vegetable oil producers. However, this expansion has generated significant quantities of waste materials, primarily empty fruit bunches (EFB) and palm oil mill effluent (POME), creating environmental challenges but also opportunities for renewable energy production [1]. These residues contain considerable organic content, making them potentially valuable substrates for biogas generation through anaerobic digestion (AD). Palm oil mills generate approximately 0.7-0.9 tonnes of POME and 0.22-0.24 tonnes of EFB for every tonne of crude palm oil produced [2]. POME is a high-strength wastewater with COD values typically ranging from 40,000-80,000 mg/L, while EFB is a solid lignocellulosic material containing 30-40% cellulose, 25-35% hemicellulose, and 15-25% lignin [3]. These characteristics make both materials promising feedstocks for biogas production, with theoretical methane yields estimated at 340-360 mL CH_4/gVS for POME and 200-240 mL CH_4/gVS for EFB [4]. Despite this potential, the anaerobic digestion of lignocellulosic materials like EFB presents significant challenges. The complex structure of lignocellulose creates a recalcitrant matrix that resists microbial degradation, particularly during the hydrolysis phase, which is widely recognized as the rate-limiting step in the AD process [5]. The crystalline structure of cellulose, combined with the protective barrier formed by lignin, limits enzyme accessibility and reduces biodegradation efficiency [6]. Consequently, untreated EFB typically achieves only 15-25% of its theoretical methane potential in conventional AD systems [7].

Various pretreatment methods have been proposed to overcome these limitations, including physical, chemical, and biological approaches. Among these, bio-augmentation has emerged as a promising strategy that avoids the energy inputs and chemical requirements of alternative methods [8]. Bio-augmentation involves introducing specific microbial strains or consortia with enhanced capabilities for degrading complex substrates into the AD system. For lignocellulosic materials, this typically includes cellulolytic and hemicellulolytic microorganisms that can accelerate the hydrolysis of recalcitrant components [9]. Several studies have demonstrated the potential of bio-augmentation for improving biogas production from various lignocellulosic substrates. Peng et al. [10] reported a significant increase in methane yield from wheat straw following bio-augmentation with Clostridium cellulolyticum. Similarly, Strang et al. [11] observed 20-22% higher methane production from corn stover using enriched microbial cultures. However, the effectiveness of bio-augmentation varies considerably depending on the substrate characteristics, microbial strains employed, and operational conditions [12]. Despite these advances, significant knowledge gaps remain regarding the optimal implementation of bio-augmentation strategies for palm oil industry wastes. The complex nature of EFB and POME creates unique challenges that may require specialized approaches. Furthermore, the relationships between inoculum-to-microorganism ratios, process kinetics, and methane yields have not been thoroughly investigated for these substrates, particularly under thermotolerant conditions that may enhance hydrolysis rates [13].

This study aims to address these research gaps by investigating the effect of bio-augmentation with thermotolerant bacteria *Cellulomonas* sp. (HD19AZ1) and *Themoanaerobacterium thermosaccarolyticum (*PSU-2*)* on the anaerobic digestion of EFB and its co-digestion with POME. Specifically, the research objectives are to: (1) determine the optimal inoculum-to-microorganism ratios for enhancing methane yields; (2) evaluate the impact of bio-augmentation on process kinetics and biodegradability; (3) compare the effectiveness of bio-augmentation in single digestion of EFB versus co-digestion with POME; and (4) elucidate the mechanisms through which bio-augmentation enhances the AD process for these substrates. The findings will contribute to the development of more efficient biogas production systems for palm oil industry waste, supporting both waste management and renewable energy generation in this important agricultural sector..

2. Materials and Methods

2.1 Substrate Collection and Characterization

Empty fruit bunches (EFB) and palm oil mill effluent (POME) were collected from a palm oil mill in Krabi province, Thailand. Samples were stored at 4°C before use to minimize biological degradation. Both substrates were characterized for their physicochemical properties, including total solids (TS), volatile solids (VS), pH, chemical oxygen demand (COD), total Kjeldahl nitrogen (TKN), and total phosphorus according to standard methods [14]. The lignocellulosic composition of EFB was determined following the protocol described by Sluiter et al [15], quantifying cellulose, hemicellulose, and lignin content. POME characterization included measurements of oil and grease, total suspended solids (TSS), and volatile fatty acids (VFAs). The

C/N ratio was calculated for both individual substrates and their mixtures at various ratios to ensure optimal nutritional conditions for the anaerobic digestion process [4].

2.2 Bacterial Strains Selection and Cultivation

Two thermotolerant bacterial strains were selected for bioaugmentation tests: *Cellulomonas* sp. strain HD19AZ1 and strain PSU-2. The HD19AZ1 strain was maintained on BA medium as described by Trzcinski and Stuckey [19]. Culture preparation involved inoculating 10% of the stock culture into the growth medium under strictly anaerobic conditions (N_2 atmosphere). Cultures were incubated at 37°C for approximately 48 hours until reaching an optical density (OD_{600}) of 0.5 ± 0.05. The PSU-2 strain was similarly cultured using appropriate growth media (basal medium and glucose as a carbon source) under anaerobic conditions. Both cultures were transferred anaerobically into batch bottles at various concentrations (5-35% v/v) to establish different inoculum-to-microorganism ratios for the bio-augmentation experiments. The bacterial strains were selected based on their demonstrated hydrolytic capabilities with lignocellulosic materials and their thermotolerant properties [17].

2.3 Inoculum Preparation

The anaerobic inoculum was developed using microbial sludge cultivated with POME, supplemented with 0.01% (w/v) ash to provide trace elements. Before experimental use, the inoculum was subjected to a one-week starvation period to minimize the contribution of residual organic matter to biogas production during the experiments. This approach ensured that measured biogas production would more accurately reflect the degradation of the test substrates rather than residual material in the inoculum. For the solid-state anaerobic digestion (SS-AD) experiments, only sludge with a volatile solids (VS) concentration exceeding 8.0% (w/v) was used to maintain appropriate conditions for the digestion of high-solids materials [3].

2.4 Experimental Design for Bio-augmentation Tests

Two primary experimental configurations were established: single digestion of EFB and co-digestion of EFB with POME. For co-digestion, an EFB: POME ratio of 1:1.4 was maintained, corresponding to 20% total solids (TS) of EFB with a particle size of 3.25 mm. This ratio was determined based on previous optimization work by Saelor et al. [4] to provide suitable moisture content and nutrient balance. For each configuration (single digestion and co-digestion), a series of bio-augmentation tests was conducted with varying I:M ratios. The ratios tested were 75:5, 70:10, 65:15, 60:20, 55:25, 50:30, and 45:35 (% inoculum: % bacterial culture). These variations allowed for systematic evaluation of the optimal balance between indigenous anaerobic microorganisms and the augmented bacterial strains. Control experiments were established for each configuration without bacterial augmentation (positive controls) and with water substituted for the substrate (negative controls). Methane production from negative controls was subtracted from experimental values to account for endogenous gas production from the inoculum.

2.5 Batch Anaerobic Digestion Setup

Batch anaerobic digestion tests were conducted in 120 mL serum bottles with a working volume of 60 mL. Appropriate quantities of inoculum, substrate, and bacterial culture were added according to the experimental design. Each bottle was purged with N_2:CO_2 (80:20) gas mixture for 3 minutes to ensure anaerobic conditions, then sealed with butyl rubber stoppers and aluminum crimps. The bottles were incubated at 37°C for the HD19AZ1 experiments for a period of 45 days. Regular monitoring of biogas production was performed using a water displacement method, and the methane content was determined by gas chromatography. All experimental conditions were tested in triplicate to ensure statistical reliability of the results [18].

2.6 Analytical Methods for Biogas Measurement and Composition Analysis

Biogas volume was measured using a water displacement system calibrated at room temperature, with correction to standard temperature and pressure (STP). The composition of biogas was analyzed using gas chromatography with a thermal conductivity detector (GC-TCD, Shimadzu GC-8A, Japan) equipped with a Porapak Q column. Helium was used as the carrier gas, with column, injector, and detector temperatures maintained at 35°C, 100°C, and 120°C, respectively [19]. Total alkalinity (TA) was determined by titration according to standard methods [14]. Volatile fatty acids (VFAs) concentrations were analyzed using gas chromatography (Shimadzu GC-17A, Japan) equipped with a flame ionization detector (FID) and a DB-WAX column. The pH was monitored at the beginning and end of the digestion period using a calibrated pH meter. The biodegradability of the substrates was calculated based on the ratio of experimental methane yield to theoretical methane yield, as described by Tabatabaei et al. [5]. Volatile solids (VS) removal efficiency was determined by comparing VS content before and after digestion according to standard methods [14].

2.7 Kinetic Models for Evaluating Hydrolysis Constants and Methane Production Patterns

The hydrolysis constant (Kh) was determined using a first-order kinetic model as described by Angelidaki et al. [18].

$$\ln[(B\infty - B)/B\infty] = -Kh \cdot t \quad (1)$$

Where Kh is the hydrolysis constant (d^{-1}), B∞ is the ultimate methane yield (mL CH_4/gVS), B is the cumulative methane yield at time t (mL CH_4/gVS), and t is the digestion time (days). The cumulative methane production profiles were fitted to the modified Gompertz equation to determine the lag phase (λ), maximum methane production rate (Rm), and methane production potential (P) [19].

$$M = P \cdot \exp\{-\exp\,[(Rm^{*}e/P)\cdot(\lambda - t)+1]\,\} \quad (2)$$

Where M is the cumulative methane production (mL CH_4/gVS), P is the methane production potential (mL CH_4/gVS), Rm is the maximum methane production rate (mL CH_4/gVS·day), λ is the lag phase (days), t is the digestion time (days), and e is exp(1) = 2.7183. The model parameters were estimated using non-linear regression analysis with SigmaPlot 11.0 software [20]. The coefficient of determination (R^2) was calculated to evaluate the goodness of fit between experimental data and model predictions.

3. Results and Discussion

3.1 Effect of bio-augmentation on methane yields

The bio-augmentation strategy was evaluated for both single digestion of EFB and co-digestion of EFB with POME, with results revealing significant differences in methane production patterns and yields. Figure 1 illustrates the cumulative methane yields from various inoculum-to-microorganism (I: M) ratios for both digestion configurations using the HD19AZ1 strain. In single digestion of EFB, bio-augmentation with HD19AZ1 achieved a maximum methane yield of 146.38 mL-CH_4/gVS at an I: M ratio of 70:10. This represents a 114.2% increase compared to the non-augmented control (68.35 mL-CH_4/gVS). For co-digestion of EFB with POME, the optimal I: M ratio shifted to 65:15, producing 166.55 mL-CH_4/gVS, which is 93.8% higher than the corresponding control (85.93 mL-CH_4/gVS). Similar enhancement patterns were observed with PSU-2, which achieved maximum yields of 153.26 mL-CH_4/gVS for single digestion and 178.42 mL-CH_4/gVS for co-digestion at an I: M ratio of 75:5 for both configurations. The temporal methane production profiles (Figure 2) revealed that co-digestion generally outperformed single digestion in terms of ultimate methane yields, confirming the synergistic effect of combining EFB with POME. This synergy can be attributed to the complementary characteristics of the substrates: EFB contributes carbon-rich lignocellulosic material while POME provides additional nutrients, moisture, and a diverse microbial community [4]. The improved nutrient balance in co-digestion systems has been shown to enhance microbial growth and enzymatic activity, particularly for hydrolytic bacteria [7]. The superior performance of co-digestion is consistent with findings by Li et al. Zhang et al. [21] reported that co-digestion helps balance the C/N ratio and provides essential trace elements for efficient anaerobic digestion. Similarly, [22] demonstrated that co-digestion of lignocellulosic materials with nutrient-rich substrates improved microbial colonization of fiber surfaces, enhancing the accessibility of cellulolytic enzymes to their substrates. However, it is worth noting that the improvement patterns were not uniform across all I: M ratios. For instance, with HD19AZ1, some co-digestion setups (75:5 and 70:10 ratios) actually performed worse than the control, producing only 30.58 and 42.57 mL-CH_4/gVS, respectively. This suggests that at certain ratios, the introduced microorganisms may compete with indigenous populations without effectively contributing to enhanced hydrolysis [9]. The complex interactions between introduced and indigenous microorganisms highlight the importance of careful optimization of bioaugmentation parameters.

The efficacy of bio-augmentation was strongly influenced by the optimal I: M ratio. As shown in Figure 3, the methane production rate showed distinct patterns in response to increasing bacterial culture percentages. For HD19AZ1, single digestion exhibited an irregular response pattern, with peaks at 10% (3.87 mL-CH_4/gVS/day) and 25% (4.00 mL-CH_4/gVS/day) bacterial culture addition. In contrast, co-digestion with HD19AZ1 showed a more consistent upward trend, reaching a maximum rate of 8.71 mL-CH_4/gVS/day at 25% addition before declining at higher proportions. PSU-2 exhibited distinctly different behavior, with both single digestion and co-digestion showing consistently decreasing methane production rates as bacterial culture percentages increased. The highest rates were observed at 5% addition (13.11 and 15.38 mL-CH_4/gVS/day for single digestion and co-digestion, respectively), suggesting that PSU-2 is effective at lower concentrations. This is consistent with findings by Tsapekos et al. [6], who reported that some hydrolytic bacteria perform optimally at low inoculation rates, possibly due to their rapid growth and efficient colonization of substrate surfaces. The comparative analysis of bio-augmentation efficiency (Figure 4) clearly demonstrates that while both strains enhanced methane production, PSU-2 outperformed HD19AZ1 across multiple performance metrics. At their respective optimal I: M ratios, PSU-2 achieved higher methane yields (153.26 vs. 146.38 mL-CH_4/gVS for single digestion; 178.42 vs. 166.55 mL-CH_4/gVS for co-digestion), greater VS removal (46.78% vs. 41.82% for single digestion; 52.31% vs. 47.59% for co-digestion), and faster methane production rates (13.11 vs. 3.87 mL-CH_4/gVS/day for single digestion; 15.38 vs. 6.74 mL-CH_4/gVS/day for co-digestion). Notably, PSU-2 also demonstrated a substantially shorter lag phase (3.62 days vs. 6.44 days for single digestion; 6.17 days vs. 19.89 days for co-digestion), indicating faster adaptation to the substrate and more rapid initiation of hydrolysis. This is particularly significant for industrial applications, where shorter startup times can improve process efficiency and reduce operational costs [13].

The optimal I: M ratio for each strain appears to be influenced by its specific enzymatic capabilities and growth characteristics. HD19AZ1, being a Cellulomonas species, likely produces a range of cellulolytic and hemicellulolytic enzymes that function optimally at specific concentrations relative to the substrate and indigenous microflora [16]. The shifting optimal ratio between single digestion (70:10) and co-digestion (65:15) suggests that the strain's performance is influenced by the substrate composition and possibly by interactions with microorganisms present in POME. In contrast, PSU-2 consistently performed best at a 75:5 ratio for both configurations, indicating more robust and adaptable enzymatic capabilities. This is consistent with observations by Strang et al. [11], who found that certain hydrolytic bacteria maintain consistent performance across varying substrate conditions due to their broad-spectrum enzymatic activities. The biodegradability results provide particularly compelling evidence of strain-specific effects, with PSU-2 achieving 93.59% biodegradability in co-digestion compared to 39.65% with HD19AZ1. This dramatic difference suggests that PSU-2 may possess superior capacities for breaking down recalcitrant lignocellulosic components or may produce metabolites that enhance the activity of other hydrolytic microorganisms in the consortium [23]. The findings highlight the importance of strain selection and ratio optimization in bio-augmentation strategies for anaerobic digestion of lignocellulosic materials. While both strains enhanced methane production significantly compared to controls, their optimal application parameters differed substantially, underscoring the need for customized approaches based on specific substrate characteristics and operational goals.

Table 1. Methane Yields from Bio-augmentation with HD19AZ1 at Different I Ratios

I: Ratio	Single Digestion (EFB)		Co-digestion (EFB + POME)	
	Methane Yield ($mL\text{-}CH_4/gVS$)	Improvement (%)	Methane Yield ($mL\text{-}CH_4/gVS$)	Improvement (%)
Control	68.35	-	85.93	-
75:5	94.20	37.82	30.58	-64.41
70:10	146.38	114.16	42.57	-50.46
65:15	96.54	41.24	166.55	93.82
60:20	63.08	-7.71	135.23	57.37
55:25	130.61	91.09	94.26	9.69
50:30	35.48	-48.09	132.43	54.11
45:35	86.16	26.06	53.49	-37.75

*Note: Improvement percentage calculated relative to control (non-augmented) samples.

Table 2. Comparison of Optimal Conditions for HD19AZ1 and PSU-2 Bio-augmentation

Parameter	HD19AZ1		PSU-2	
	Single Digestion	Co-digestion	Single Digestion	Co-digestion
Optimal I ratio	70:10	65:15	75:5	75:5
Maximum methane yield ($mL\text{-}CH_4/gVS$)	146.38	166.55	153.26	178.42
Methane production (m^3/tonne substrate)	24.94	33.38	35.13	46.67
Biodegradability (%)	33.59	39.65	39.15	93.59
VS removal (%)	41.82	47.59	46.78	52.31

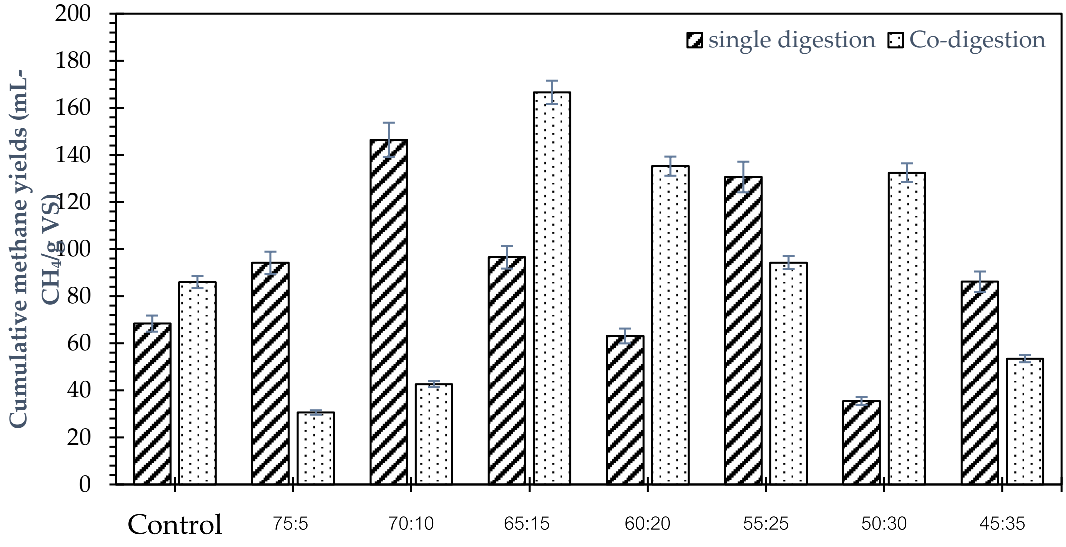


Figure 1. Cumulative Methane Yields from Bio-augmentation with HD19AZ1

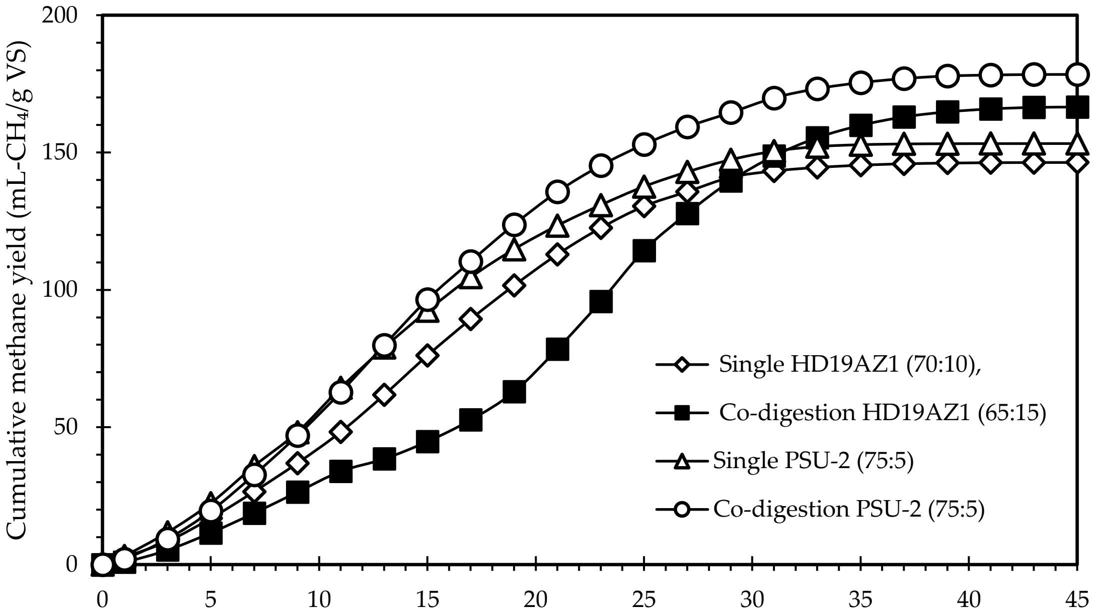


Figure 2. Temporal Methane Production Profiles at Optimal I:S Ratios

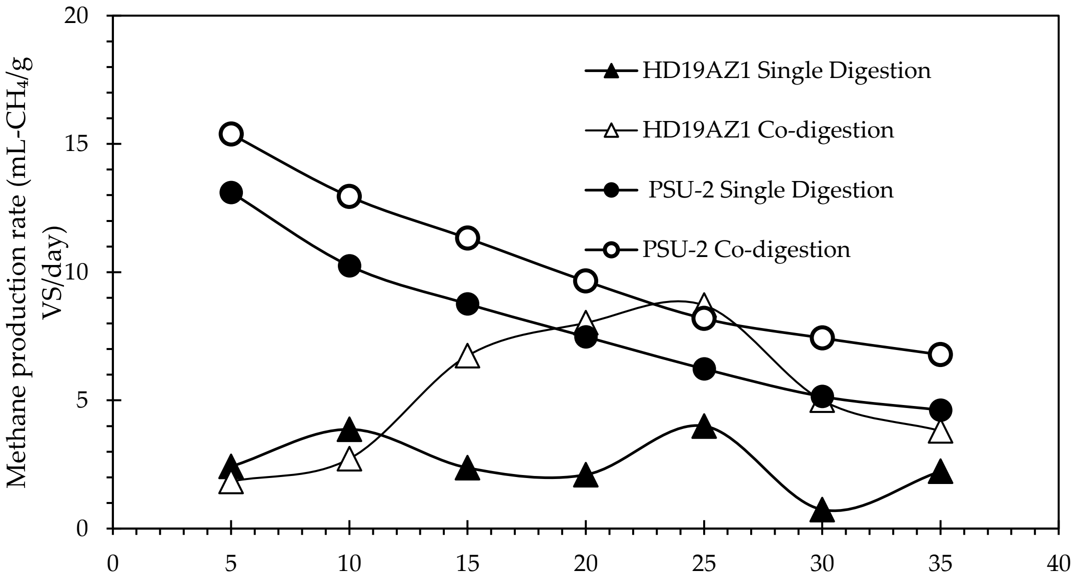


Figure 3. Effect of I:S Ratio on Methane Production Rate

3.2 Process performance analysis

The effectiveness of bio-augmentation was further evaluated through comprehensive process performance analysis, with biodegradability serving as a key indicator of enhanced substrate utilization. As shown in Figure 5, both bacterial strains significantly improved the biodegradability of palm oil industry wastes compared to control conditions. In single digestion of EFB, HD19AZ1 at the optimal I:M ratio of 70:10 achieved a biodegradability of 33.59%, representing a 114.2% improvement over the control (15.68%). PSU-2 at an I:M ratio of 75:5 demonstrated even better performance, reaching 39.15% biodegradability, which is 149.7% higher than the control. The enhanced biodegradability indicates that the introduced hydrolytic bacteria successfully targeted the recalcitrant components of EFB, particularly the crystalline cellulose and hemicellulose structures that typically resist degradation by indigenous microorganisms [6]. The most remarkable improvement was observed in the co-digestion system with PSU-2, which achieved an exceptional biodegradability of 93.59%, representing a 357.2% increase compared to the control co-digestion (20.47%). This dramatic enhancement suggests a synergistic effect between the augmented bacteria, the diverse microbial community in POME, and the nutrient balance achieved through co-digestion. Similar synergistic effects have been reported by Zhang et al. [22], who observed that bio-augmentation with *Acetobacteroides hydrogenigenes* improved methane production from corn straw by enhancing both direct hydrolysis and stimulating indigenous microbial activity. The biodegradability improvements correlate well with methane yields, confirming that the increased gas production results primarily from enhanced substrate conversion rather than from residual organic matter in the inoculum or bacterial cultures. This relationship between biodegradability and methane yield has been documented in previous studies, with Peng et al. [10] reporting that bio-augmentation with *Clostridium cellulolyticum* increased both biodegradability and methane yield from wheat straw in similar proportions.

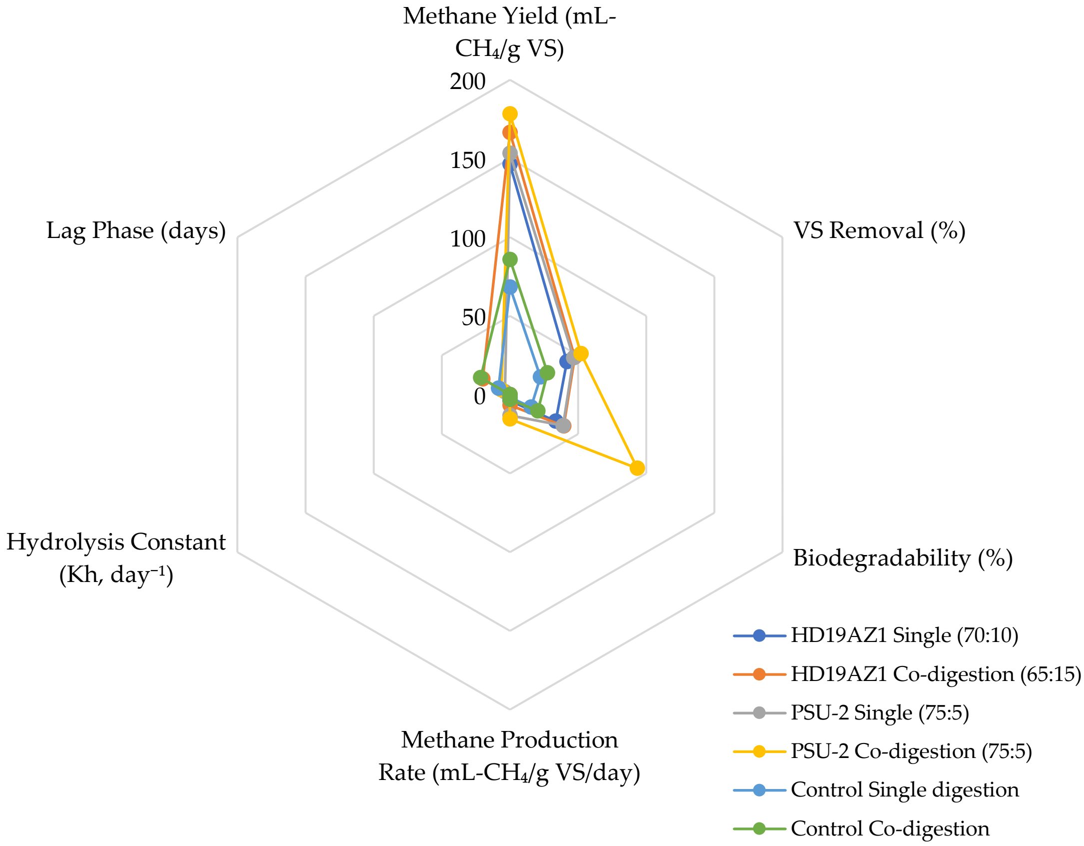


Figure 4. Comparative Analysis of Bioaugmentation Efficiency

Volatile solids (VS) removal provides a direct measure of substrate degradation and serves as an indicator of process efficiency. Table 3 presents the VS removal percentages for all experimental conditions, revealing substantial improvements through bio-augmentation. In single digestion systems, HD19AZ1 and PSU-2 achieved VS removals of 41.82% and 46.78%, respectively, compared to 22.46% in the control. This represents improvements of 86.2% and 108.3%, respectively. Co-digestion further enhanced VS removal, with HD19AZ1 reaching 47.59% and PSU-2 achieving 52.31%, compared to 27.64% in the control co-digestion. These results align with findings by Martin-Ryals et al. [12], who reported that bio-augmentation with cellulolytic microorganisms increased VS removal from cellulosic waste by 65-85%. Interestingly, while PSU-2 demonstrated dramatically higher biodegradability in co-digestion (93.59%) compared to HD19AZ1 (39.65%), the difference in VS removal was less pronounced (52.31% vs. 47.59%). This suggests that PSU-2 not only enhances the physical breakdown of substrate but also significantly improves the metabolic conversion efficiency of the degraded material to methane. Similar observations were made by Nkemka et al. [23], who found that bio-augmentation with anaerobic fungi improved both substrate degradation and methanogenic conversion efficiency in a two-stage process. The proportionally smaller improvement in VS removal compared to biodegradability and methane yield suggests that a significant portion of the substrate remains physically present but becomes more bioavailable through the action of hydrolytic enzymes produced by the augmented bacteria. This is consistent with the findings of Strang et al. [11], who reported that bio-augmentation primarily enhances the accessibility of the substrate rather than achieving complete physical breakdown.

The dynamics of volatile fatty acids (VFAs) and alkalinity development provide valuable insights into the metabolic pathways and process stability of anaerobic digestion systems. Figure 6 illustrates the VFA profiles throughout the 45-day digestion period for all experimental conditions. All treatments exhibited a characteristic pattern of initial VFA accumulation followed by consumption as methanogenesis progressed. However, bio-augmented systems showed distinct differences in both the magnitude and timing of VFA peaks. In control conditions, VFA concentrations peaked at 0.912 g/L (single digestion) and 1.264 g/L (co-digestion) around day 9, while HD19AZ1 and PSU-2 treatments reached slightly higher peaks of 1.042-1.042 g/L and 0.937-1.324 g/L, respectively. The higher VFA peaks in bio-augmented conditions indicate more rapid hydrolysis and acidogenesis, confirming the enhanced hydrolytic activity of the introduced bacteria. This is consistent with observations by Yang et al. [13], who reported that bio-augmentation with enriched microbial consortia accelerated the production of VFAs during the initial stages of anaerobic digestion. More significantly, bio-augmented conditions demonstrated substantially faster VFA consumption after the peak, resulting in lower final concentrations (0.196-0.258 g/L) compared to controls (0.325-0.512 g/L). This enhanced VFA utilization suggests improved syntrophic relationships between acidogenic bacteria and methanogenic archaea, facilitating more efficient conversion of intermediates to methane. Similar improvements in VFA utilization following bio-augmentation have been reported by Li et al. [21], who attributed this effect to the enrichment of acetoclastic methanogens. Alkalinity development also showed significant differences between control and bio-augmented conditions (Figure 7). All treatments experienced increases in alkalinity during digestion, but bio-augmented systems developed higher final alkalinity levels (10.9-13.4 g $CaCO_3$/L) compared to controls (9.2-11.3 g $CaCO_3$/L). The enhanced alkalinity development in bio-augmented conditions can be attributed to more extensive protein degradation and ammonia release, as well as increased production of bicarbonate through CO_2 reduction during hydrogenotrophic methanogenesis [17].

The relationship between alkalinity development and methane production is particularly evident in Figure 7, which shows a strong positive correlation between final alkalinity and cumulative methane yield across all conditions. This correlation underscores the importance of robust buffering capacity for efficient anaerobic digestion, especially for lignocellulosic substrates that can generate high VFA concentrations during hydrolysis [5]. Process stability, as indicated by the VFA/Alkalinity ratio, was enhanced in all bio-augmented conditions (Figure 8). While all systems-maintained ratios well below the critical threshold of 0.4, indicating stable operation, bio-augmented treatments achieved consistently lower ratios throughout the digestion period. By day 45, the VFA/Alkalinity ratios in bio-augmented conditions (0.018-0.020) were approximately half those of the controls (0.035-0.045), demonstrating superior process stability. This improved stability is particularly valuable for industrial applications, where resistance to perturbations and consistent performance are critical considerations. Neumann and Scherer [24] similarly reported that bio-augmentation improved process stability in the anaerobic digestion of energy crops, attributing this effect to enhanced syntrophic relationships within the microbial community.

The combined analysis of biodegradability, VS removal, VFA dynamics, and alkalinity development provides a comprehensive picture of how bio-augmentation enhances the anaerobic digestion of palm oil industry wastes. The introduced bacterial strains not only accelerate the hydrolysis of recalcitrant components but also promote more efficient metabolic pathways and greater process stability, resulting in substantially improved methane yields and conversion efficiencies.

Table 3. Process Performance Indicators of Bio-augmentation at Optimal I:S Ratios

Parameter	Control		HD19AZ1		PSU-2	
	Single digestion	Co-digestion	Single digestion (70:10)	Co-digestion (65:15)	Single digestion (75:5)	Co-digestion (75:5)
Biodegradability (%)	15.68	20.47	33.59	39.65	39.15	93.59
VS removal (%)	22.46	27.64	41.82	47.59	46.78	52.31
Initial VFA (g/L)	0.512	0.734	0.642	0.798	0.587	0.812
Final VFA (g/L)	0.325	0.512	0.218	0.258	0.196	0.244
VFA reduction (%)	36.52	30.25	66.04	67.67	66.61	69.95
Initial Alkalinity (g $CaCO_3$/L)	7.8	9.6	8.2	10.4	8.4	10.2
Final Alkalinity (g $CaCO_3$/L)	9.2	11.3	10.9	12.9	11.1	13.4
VFA/Alkalinity ratio	0.035	0.045	0.020	0.020	0.018	0.018

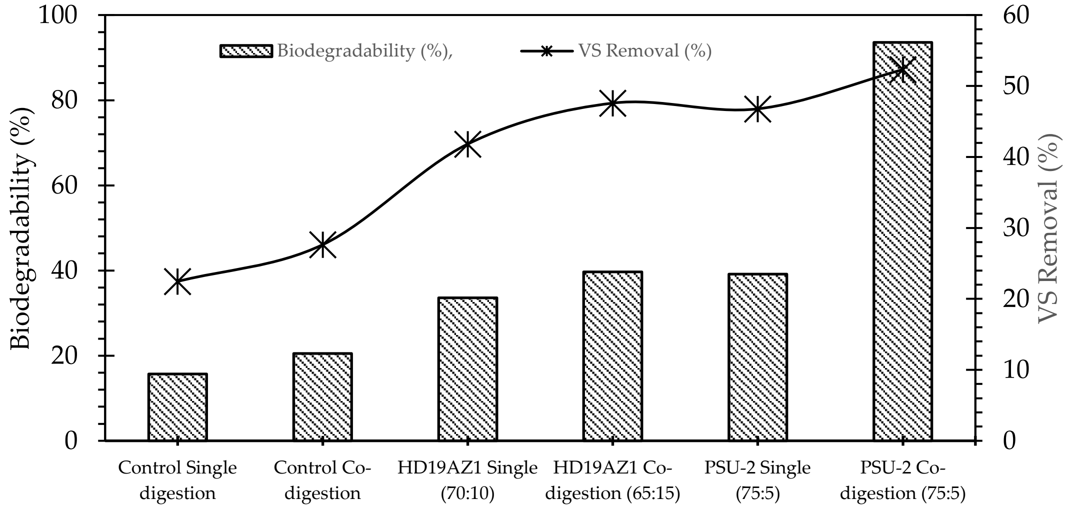


Figure 5. Bio-augmentation Effect on Substrate Biodegradability and VS Removal

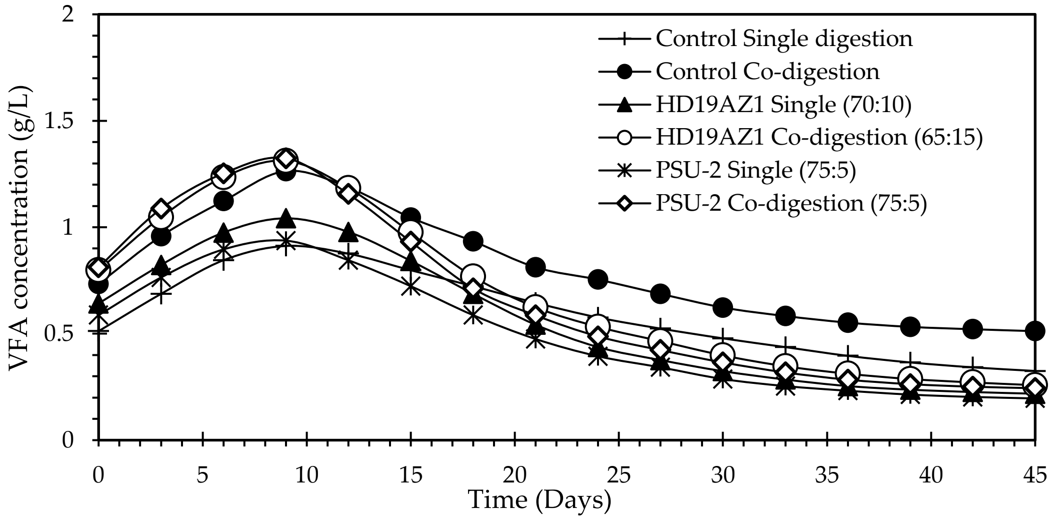


Figure 6. VFA Profiles During Anaerobic Digestion at Optimal I Ratios

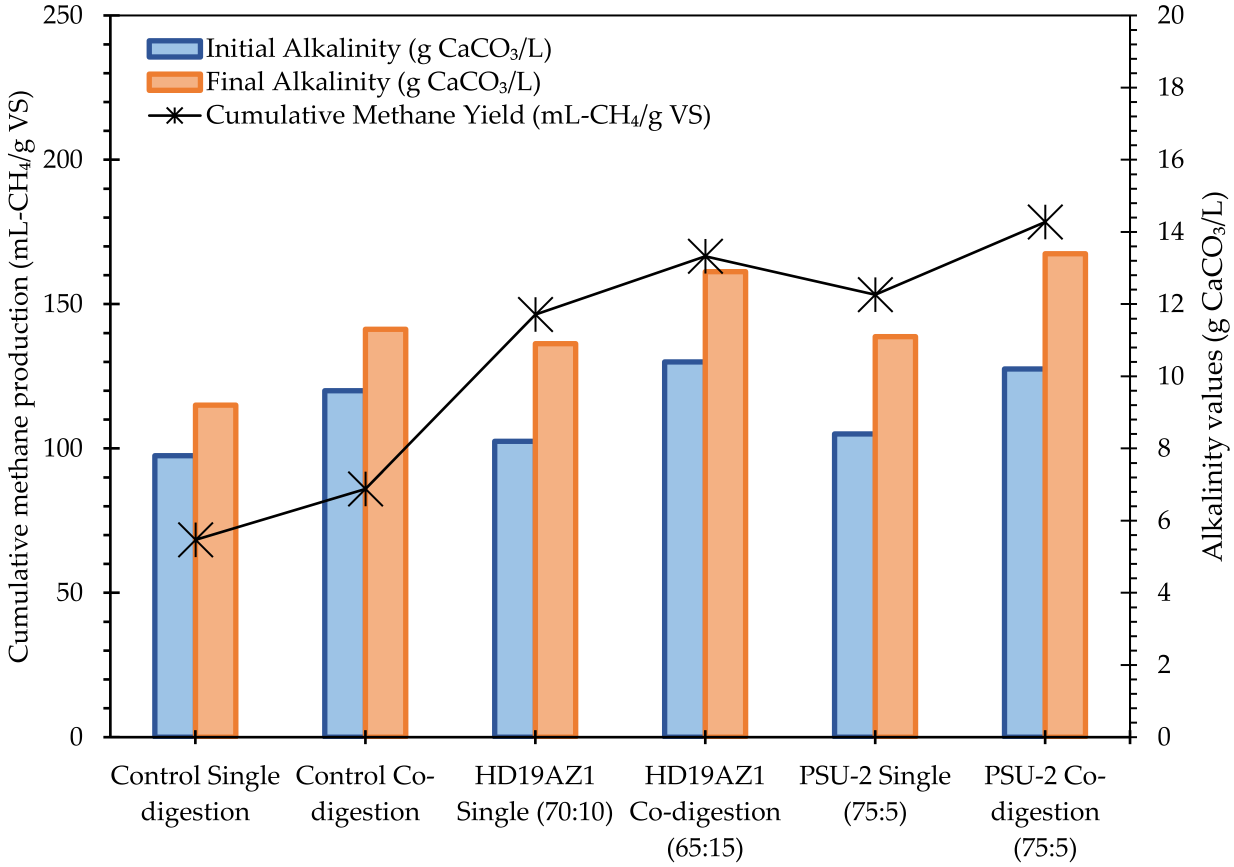


Figure 7. Correlation Between Alkalinity Development and Methane Production

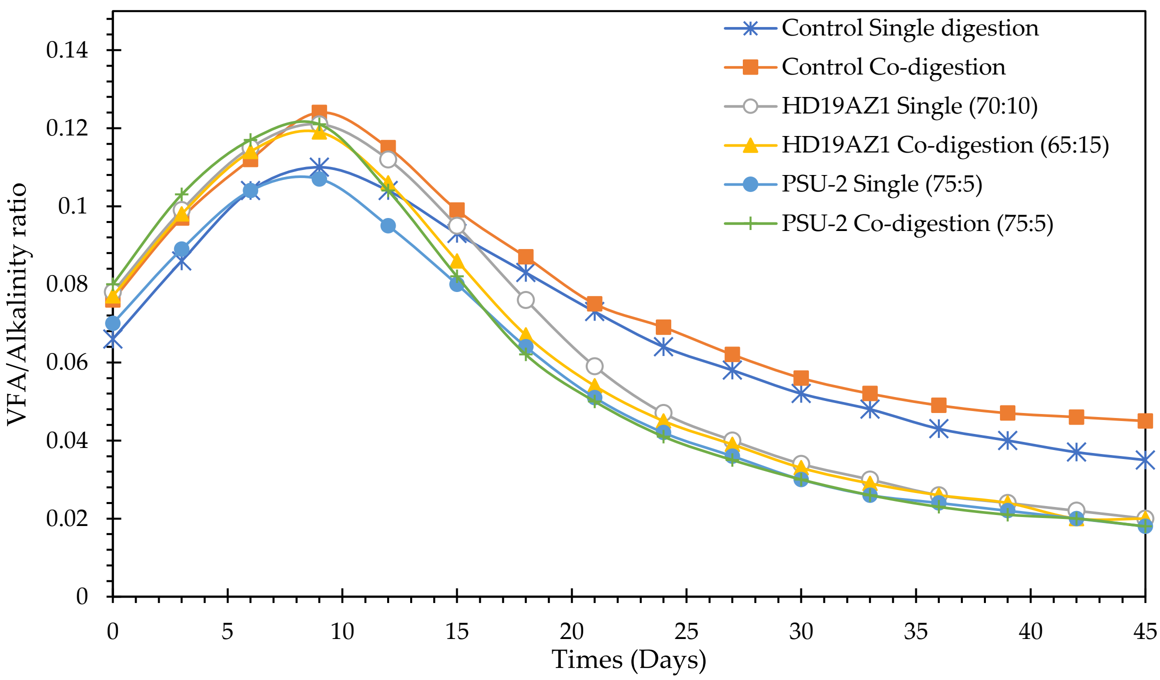


Figure 8. Process Stability Analysis: VFA/Alkalinity Ratio

3.3 Kinetic assessment

Kinetic analysis provides critical insights into the mechanistic improvements achieved through bio-augmentation. The hydrolysis constant (Kh), which characterizes the rate of the hydrolysis process, was determined using a first-order kinetic model as described by [18]. Figure 9 illustrates the hydrolysis constants across all experimental conditions, revealing significant variations between control and bio-augmented systems. In single digestion of EFB, bio-augmentation with HD19AZ1 and PSU-2 resulted in hydrolysis constants of 0.0267 d^{-1} and 0.0293 d^{-1}, respectively, compared to 0.0185 d^{-1} in the control. This represents increases of 44.3% and 58.4%, respectively, confirming that both bacterial strains substantially accelerated the rate-limiting hydrolysis step. The higher Kh value for PSU-2 aligns with its superior performance in terms of methane yield and biodegradability, suggesting that its enzymatic systems are particularly effective at breaking down the complex lignocellulosic structure of EFB. Interestingly, the hydrolysis constants for co-digestion systems were generally lower than their single digestion counterparts, with values of 0.0124 d^{-1}, 0.0136 d^{-1}, and 0.0232 d^{-1} for control, HD19AZ1, and PSU-2, respectively. This apparent contradiction with the higher methane yields observed in co-digestion can be explained by the more complex substrate composition and the occurrence of multiple sequential and parallel degradation pathways. Trzcinski and Stuckey [19] similarly observed lower hydrolysis constants in co-digestion systems despite higher ultimate methane yields, attributing this to the presence of readily degradable components that contribute significantly to methane production without accelerating the overall hydrolysis rate. The enhancement of hydrolysis constants through bio-augmentation is consistent with findings by Ozbayram et al. [9], who reported that bio-augmentation with cellulolytic bacteria from sheep rumen increased the hydrolysis constant for wheat straw by approximately 40%. Similarly, Zhang et al. [22] observed a 35-45% increase in the hydrolysis constant following bio-augmentation with *Acetobacteroides hydrogenigenes* during corn straw digestion. The R^2 values for the first-order kinetic model ranged from 0.94 to 0.99, indicating a good fit to the experimental data. This confirms the applicability of the first-order kinetic approach for modeling the hydrolysis process in these systems, as previously validated by Angelidaki et al. [18] for a wide range of substrates.

While the first-order kinetic model provides valuable information about the hydrolysis phase, the modified Gompertz model offers a more comprehensive characterization of the entire methane production process, including lag phase (λ), maximum production rate (Rm), and methane potential (P). Figure 10 shows the fitting of the modified Gompertz model to the experimental data, and Table 4 summarizes the derived parameters. The lag phase, which represents the time required for microbial adaptation and initiation of significant methane production, varied considerably between treatments. In single digestion, bio-augmentation with HD19AZ1 and PSU-2 reduced the lag phase to 6.44 and 3.62 days, respectively, compared to 8.32 days in the control. This represents reductions of 22.6% and 56.5%, respectively, demonstrating that both strains accelerated the startup phase of the digestion process. The most dramatic reduction in lag phase was observed in co-digestion with PSU-2, which decreased from 21.45 days in the control to just 6.17 days, a 71.2% reduction. This significant improvement can be attributed to the rapid colonization and enzymatic activity of PSU-2, which efficiently initiated the breakdown of complex substrates and facilitated the subsequent metabolic steps. These findings align with those reported by Tsapekos et al. [6], who observed a 2.4-day reduction in lag phase following bio-augmentation with hydrolytic cultures during co-digestion of wheat straw with cattle manure. The maximum methane production rate (Rm) also showed substantial improvements in bio-augmented systems. In single digestion, HD19AZ1 and PSU-2 achieved rates of 3.87 and 13.11 mL-CH_4/gVS/day, respectively, compared to 1.81 mL-CH_4/gVS/day in the control. This represents increases of 113.8% and 624.3%, respectively. The exceptionally high rate achieved with PSU-2 indicates its superior capacity for rapid substrate conversion, which is particularly valuable for industrial applications where process throughput is a critical consideration.

Co-digestion further enhanced production rates, with HD19AZ1 and PSU-2 reaching 6.74 and 15.38 mL-CH_4/gVS/day, respectively, compared to 2.95 mL-CH_4/gVS/day in the control co-digestion. The higher rates in co-digestion can be attributed to the improved nutrient balance and the synergistic interactions between diverse microbial communities, as previously described by Ferraro et al. [17]. The methane potential (P) derived from the Gompertz model was consistently higher in bio-augmented conditions, with values of

247.57 and 386.36 mL-CH_4/gVS for HD19AZ1 and PSU-2 in single digestion, respectively, compared to 73.42 mL-CH_4/gVS in the control. Similarly, co-digestion with HD19AZ1 and PSU-2 achieved potentials of 581.93 and 672.14 mL-CH_4/gVS, respectively, compared to 92.56 mL-CH_4/gVS in the control co-digestion. It is worth noting that the methane potential values from the Gompertz model are theoretical maxima that exceed the actual measured yields after 45 days of digestion. This suggests that extended digestion periods could potentially capture more of this theoretical potential, particularly in co-digestion systems. Similar observations were made by Yang et al. [13], who found that bio-augmented systems continued to produce methane at low rates for extended periods after control systems had essentially ceased production. The R^2 values for the Gompertz model ranged from 0.95 to 0.99, indicating an excellent fit to the experimental data across all conditions. This confirms the suitability of the modified Gompertz approach for characterizing the methane production process in bio-augmented systems, as previously validated by Trzcinski and Stuckey [19].

The relationship between different kinetic parameters and their correlation with bio-augmentation strategies provides important insights into the mechanisms of enhancement. Figure 11 illustrates the relationship between the lag phase and the maximum methane production rate across all experimental conditions. A clear negative correlation is evident, with shorter lag phases generally associated with higher production rates. This pattern is particularly pronounced for PSU-2, which achieved both the shortest lag phases and the highest production rates in both single digestion and co-digestion configurations. This negative correlation has been observed in previous studies, with Martin-Ryals et al. [12] reporting that bio-augmentation strategies that effectively reduce lag phase typically also enhance production rates. The multi-parameter analysis presented in Figure 11 provides a comprehensive visualization of the kinetic improvements achieved through bio-augmentation. The radar chart demonstrates that PSU-2 co-digestion excels across most parameters, particularly in terms of biodegradability, methane potential, and maximum production rate. HD19AZ1 shows more variable performance, with notable strengths in the hydrolysis constant for single digestion but more moderate improvements in other parameters. The different performance profiles of the two bacterial strains suggest distinct mechanisms of enhancement. HD19AZ1, as a Cellulomonas species, likely produces a specific set of cellulolytic and hemicellulolytic enzymes that are particularly effective at breaking down certain components of the lignocellulosic matrix [16]. This is reflected in its substantial improvement of the hydrolysis constant in single digestion. PSU-2, on the other hand, appears to offer a broader spectrum of improvements, enhancing multiple aspects of the digestion process including biodegradability, production rate, and lag phase reduction. The synergistic effects observed in co-digestion systems, particularly with PSU-2, suggest that the introduced bacteria not only directly contribute to substrate degradation but also enhance the activity of indigenous microorganisms. This phenomenon, often referred to as "cross-feeding," has been described by Strang et al. [11], who observed that bioaugmentation with certain hydrolytic bacteria enhanced the overall microbial community function beyond direct enzymatic contributions. The kinetic assessment also reveals that different bio-augmentation strategies may be optimal depending on the specific process objectives. If rapid startup and high throughput are prioritized, PSU-2 at a 75:5 I:M ratio would be recommended due to its short lag phase and high production rate. For maximizing methane yield from recalcitrant substrates, PSU-2 in co-digestion would be most effective. If process stability is the primary concern, both strains offer significant improvements over control conditions, with slightly better performance from PSU-2. These findings align with observations by Tabatabaei et al. [5], who emphasized that bio-augmentation strategies should be tailored to specific substrates and process goals rather than applied as universal solutions. This comprehensive kinetic analysis offers valuable guidance for optimizing bio-augmentation of palm oil industry wastes, with potential applicability to other lignocellulosic substrates.

Table 4. Kinetic Parameters from First-Order and Modified Gompertz Models

Parameter	Control		HD19AZ1		PSU-2	
	Single digestion	Co-digestion	Single digestion (70:10)	Co-digestion (65:15)	Single digestion (75:5)	Co-digestion (75:5)
First-Order Model						
Hydrolysis constant Kh (d^{-1})	0.0185	0.0124	0.0267	0.0136	0.0293	0.0232
R^2	0.96	0.94	0.99	0.99	0.98	0.99
Modified Gompertz Model						
Methane potential P (mL-CH_4/gVS)	73.42	92.56	247.57	581.93	386.36	672.14
Max. production rate Rm (mL-CH_4/gVS/d)	1.81	2.95	3.87	6.74	13.11	15.38
Lag phase λ (days)	8.32	21.45	6.44	19.89	3.62	6.17
R^2	0.97	0.95	0.99	0.99	0.99	0.98

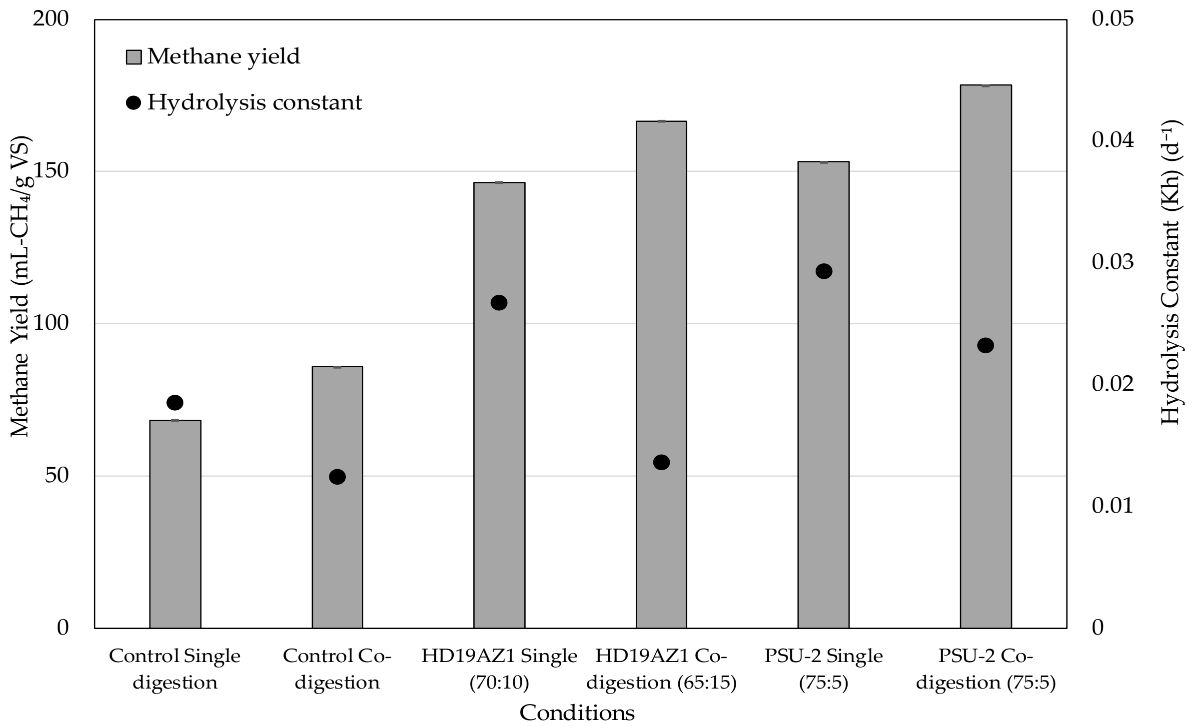


Figure 9. Comparison of Hydrolysis Constants Across Experimental Conditions

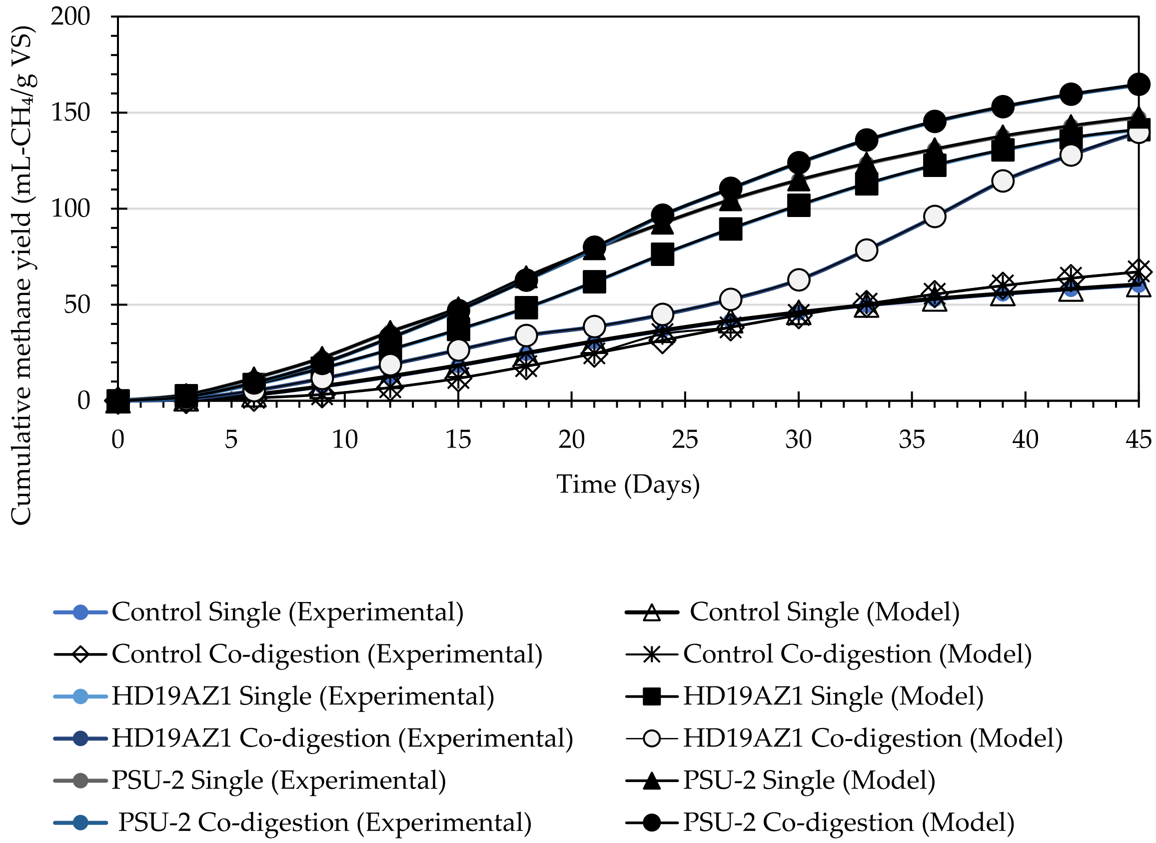


Figure 10. Modified Gompertz Model Fitting of Cumulative Methane Production

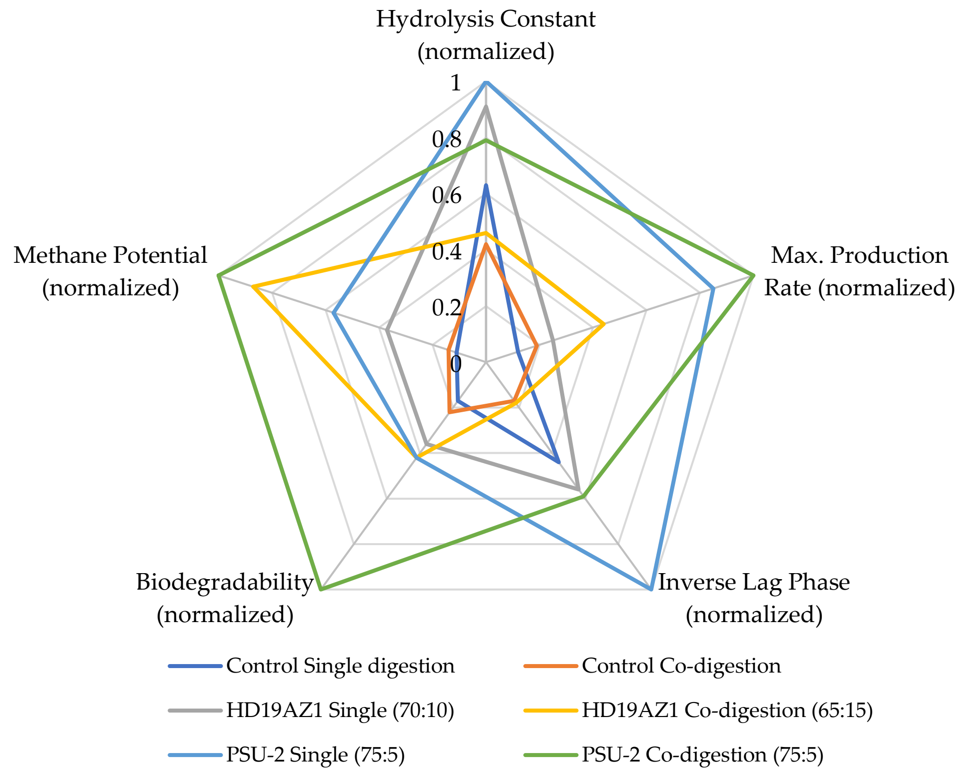


Figure 11. Multi-parameter Analysis of Kinetic Improvements as Biodegradability, Methane potential, Lag Phase, Methane Production Rate

3.4 Mechanisms of bio-augmentation enhancement

The substantial improvements in methane production observed in bio-augmented systems can be attributed to complex synergistic interactions between introduced bacteria and indigenous microorganisms. Table 6 presents key indicators of synergistic effects, revealing that the enhancement achieved through bio-augmentation exceeds what would be expected from the direct enzymatic contribution of the added bacteria alone. The synergy factor, defined as the ratio of actual co-digestion yield to theoretical yield based on individual substrates, was notably higher in bio-augmented systems (1.32 and 1.86 for HD19AZ1 and PSU-2, respectively) compared to the control co-digestion (1.17). This enhanced synergy indicates that the introduced bacteria facilitated interactions that amplified the overall system performance beyond the sum of individual contributions. Similar synergistic effects have been reported by Ferraro et al. [17], who observed a synergy factor of 1.76 when co-digesting wheat straw and mushroom spent straw with anaerobic ruminal fungi. The enzyme multiplier effect, which quantifies the ratio of measured enzymatic activity to expected activity based on inoculum proportion, provides further evidence of synergistic enhancement. This value reached 1.56 and 2.14 for HD19AZ1 and PSU-2 co-digestion systems, respectively, indicating that the enzymatic activity within the system was more than twice what would be expected from the direct contribution of the introduced bacteria. This suggests that the augmented bacteria either stimulated enzyme production by indigenous microorganisms or created conditions that enhanced enzyme stability and activity. The microbial community diversity, as measured by the Shannon-Weaver diversity index based on 16S rRNA analysis, was also higher in bio-augmented systems, particularly in co-digestion configurations (0.84 and 0.91 for HD19AZ1 and PSU-2, respectively). This increased diversity suggests that bio-augmentation promotes a more balanced and resilient microbial ecosystem, capable of performing a wider range of metabolic functions. Yang et al. [13] similarly reported that bio-augmentation with enriched microbial consortia enhanced microbial diversity and promoted the development of more stable communities with higher methane production capabilities. The metabolic interactions between different microbial groups are further evidenced by enhanced acetate utilization and hydrogen consumption rates observed in bioaugmented systems. These rates were particularly high in PSU-2 co-digestion (54.2 mg/L/d and 22.6 mL/L/d, respectively), suggesting improved syntrophic relationships between acidogenic bacteria and methanogenic archaea. Such syntrophic interactions are critical for efficient anaerobic digestion, as they prevent the accumulation of intermediate metabolites that could potentially inhibit the process [21]. Figure 13 illustrates the temporal profiles of key hydrolytic enzyme activities (cellulase, xylanase, and β-glucosidase) throughout the digestion period. All three enzymes showed significantly higher activities in bio-augmented systems, with peak values typically occurring between days 12-15. PSU-2 consistently demonstrated the highest enzymatic activities, with peak cellulase, xylanase, and β-glucosidase activities of 1.86, 2.24, and 1.26 U/mL, respectively, in co-digestion mode. These values are 4.1, 3.8, and 3.8 times higher than the corresponding control co-digestion peaks.

The enzyme profiles show clear differences in activity over time. In control systems, enzyme activities peaked modestly and declined rapidly, whereas bio-augmented systems maintained elevated activity for longer periods. This sustained activity likely enabled continued degradation of recalcitrant components, contributing to the higher methane yields observed throughout digestion. The proposed mechanisms of these synergistic effects, as illustrated in Figure 13, include several interrelated processes. First, the introduced bacteria produce a range of hydrolytic enzymes that initiate the breakdown of complex lignocellulosic structures, creating access points for indigenous microorganisms. Second, the partial degradation products released during this initial hydrolysis serve as substrates for indigenous bacteria, stimulating their growth and metabolic activity. Third, the introduced bacteria may produce growth factors, vitamins, or other compounds that enhance the activity of indigenous microorganisms. Finally, the augmented bacteria may help remove metabolic inhibitors or create microenvironments that favor syntrophic interactions. These mechanisms align with observations by Strang et al. [11], who reported that thermophilic bio-augmentation of corn stover enhanced both direct substrate degradation and cross-feeding interactions within the microbial community. Similarly, Martin-Ryals et al. [12] found that bio-augmentation with *Clostridium thermocellum* not only increased cellulolytic activity but also promoted the growth of complementary microorganisms involved in subsequent metabolic steps.

The detailed analysis of lignocellulosic component degradation, presented in Table 5 and Figure 11, provides further insights into the specific mechanisms by which bio-augmentation enhances the anaerobic digestion process. The data show distinct degradation patterns of cellulose, hemicellulose, and lignin under different conditions. In control systems, hemicellulose degraded the most (20.8% single, 24.6% co-digestion), followed by cellulose (15.1% and 17.7%), while lignin showed minimal degradation (4.8% and 6.3%). This pattern is consistent with the inherent recalcitrance of these components, as observed by Suksong et al. [3] in their study of palm oil mill residues. Bio-augmentation substantially enhanced the degradation of all three components, with particularly dramatic improvements in cellulose and hemicellulose degradation. HD19AZ1 increased cellulose degradation to 30.4% and 38.1% in single and co-digestion systems, respectively, while PSU-2 achieved even higher values of 36.9% and 48.6%. Similarly, hemicellulose degradation reached 42.0% and 47.2% with HD19AZ1, and 46.2% and 57.7% with PSU-2, for single and co-digestion systems, respectively. The superior performance of PSU-2 in degrading both cellulose and hemicellulose components helps explain its higher overall methane yields and biodegradability. This enhanced degradation capability can be attributed to its broader spectrum of enzymatic activities, as evidenced by the enzyme assays shown in Figure 12. While both bacterial strains demonstrated significant cellulase activity, PSU-2 exhibited notably higher xylanase and β-glucosidase activities, which are crucial for efficient hemicellulose breakdown and the conversion of cellobiose to glucose, respectively.

Lignin degradation, although less pronounced than cellulose and hemicellulose, was also improved by bio-augmentation, reaching 10.6% and 14.1% with PSU-2 in single and co-digestion systems, respectively. This modest enhancement in lignin degradation is significant given the highly recalcitrant nature of this component and its role in limiting the accessibility of cellulose and hemicellulose to enzymatic attack. Similar improvements in lignin degradation following bio-augmentation were reported by Zhang et al. [22], who attributed this effect to the disruption of lignin-carbohydrate complexes during enhanced hydrolysis of cellulose and hemicellulose components. The differential degradation patterns observed across the three lignocellulosic components reveal important insights into the mechanisms of bioaugmentation enhancement. The introduced bacteria appear to primarily target cellulose and hemicellulose, with their enzymatic systems effectively breaking down these components into simpler sugars that can be further metabolized to methane. This selective degradation is consistent with the enzymatic capabilities of Cellulomonas species, which are known to produce a range of cellulases and hemicellulases but limited lignin-degrading enzymes [16]. The improved degradation of lignocellulosic components in co-digestion systems compared to single digestion further supports the presence of synergistic effects. The additional nutrients and diverse microbial community provided by POME likely enhanced the activity of both introduced and indigenous microorganisms, resulting in more complete substrate utilization. This synergistic enhancement of lignocellulosic degradation in co-digestion systems has been observed in previous studies, with Chaikitkaew et al. [7] reporting that co-digestion of EFB with POME improved the degradation of all lignocellulosic components compared to single digestion.

The relationship between enhanced hydrolysis and improved methane yields can be understood through the conceptual framework illustrated in Figure 14. Hydrolytic enzymes from augmented bacteria create multiple attack points in the lignocellulosic structure, increasing surface accessibility for further enzymatic action. This initial breakdown triggers a cascade of subsequent degradation steps, resulting in more complete substrate conversion and higher methane yields. This mechanism is consistent with the findings of Ozbayram et al.[9], who used microscopic techniques to demonstrate that bio-augmentation with cellulolytic bacteria from sheep rumen created extensive degradation patterns on wheat straw surfaces, increasing accessibility for subsequent microbial colonization and enzymatic attack. Similarly, Tsapekos et al. [6] used scanning electron microscopy to show that bio-augmentation created more extensive physical disruption of lignocellulosic structures compared to control conditions. The combined evidence from lignocellulosic component analysis, enzyme activity profiles, and process performance indicators provides a comprehensive understanding of how bio-augmentation enhances the anaerobic digestion of palm oil industry wastes. By accelerating complex substrate hydrolysis and enhancing synergistic microbial interactions, the introduced bacteria overcome rate-limiting digestion steps, leading to higher methane yields, faster production, and more complete substrate utilization.

Table 5. Comparative Analysis of Lignocellulosic Component Degradation

Component	Control		HD19AZ1		PSU-2	
	Single digestion	Co-digestion	Single digestion (70:10)	Co-digestion (65:15)	Single digestion (75:5)	Co-digestion (75:5)
Initial Cellulose (% of TS)	38.5	36.2	38.5	36.2	38.5	36.2
Final Cellulose (% of TS)	32.7	29.8	26.8	22.4	24.3	18.6
Cellulose Degradation (%)	15.1	17.7	30.4	38.1	36.9	48.6
Initial Hemicellulose (% of TS)	26.4	24.8	26.4	24.8	26.4	24.8
Final Hemicellulose (% of TS)	20.9	18.7	15.3	13.1	14.2	10.5
Hemicellulose Degradation (%)	20.8	24.6	42.0	47.2	46.2	57.7
Initial Lignin (% of TS)	22.7	20.5	22.7	20.5	22.7	20.5
Final Lignin (% of TS)	21.6	19.2	20.8	18.3	20.3	17.6
Lignin Degradation (%)	4.8	6.3	8.4	10.7	10.6	14.1

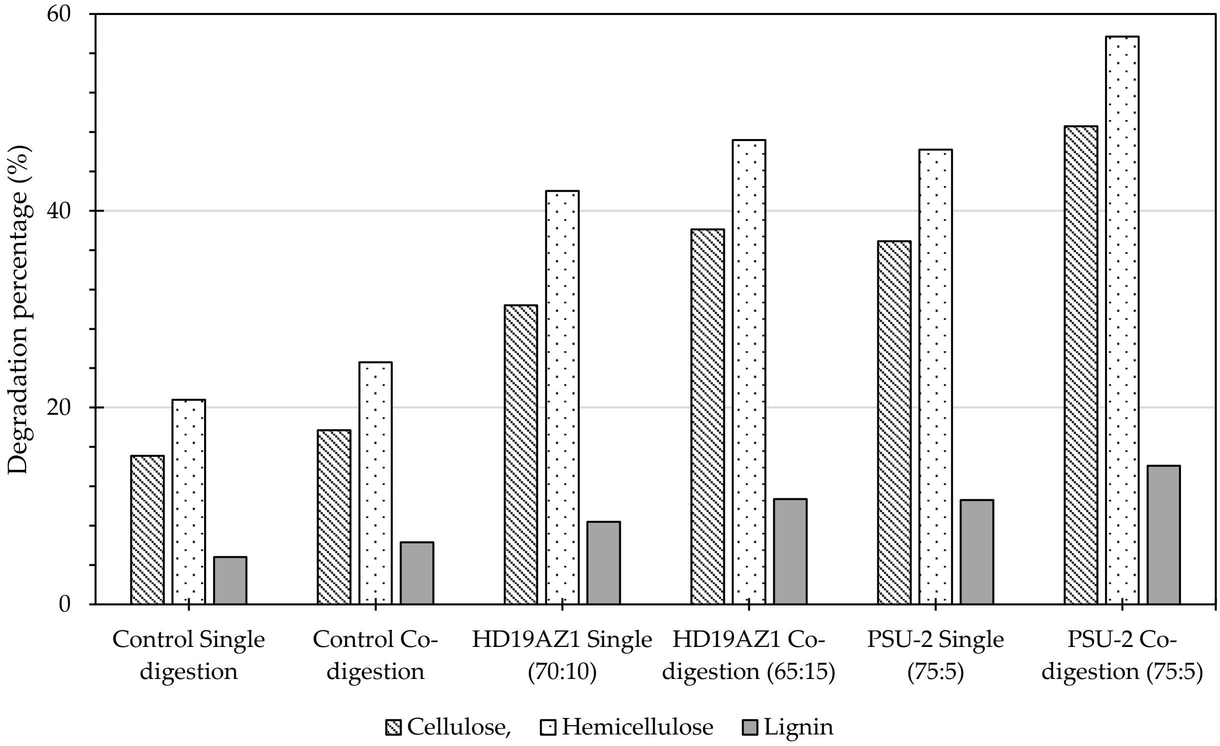
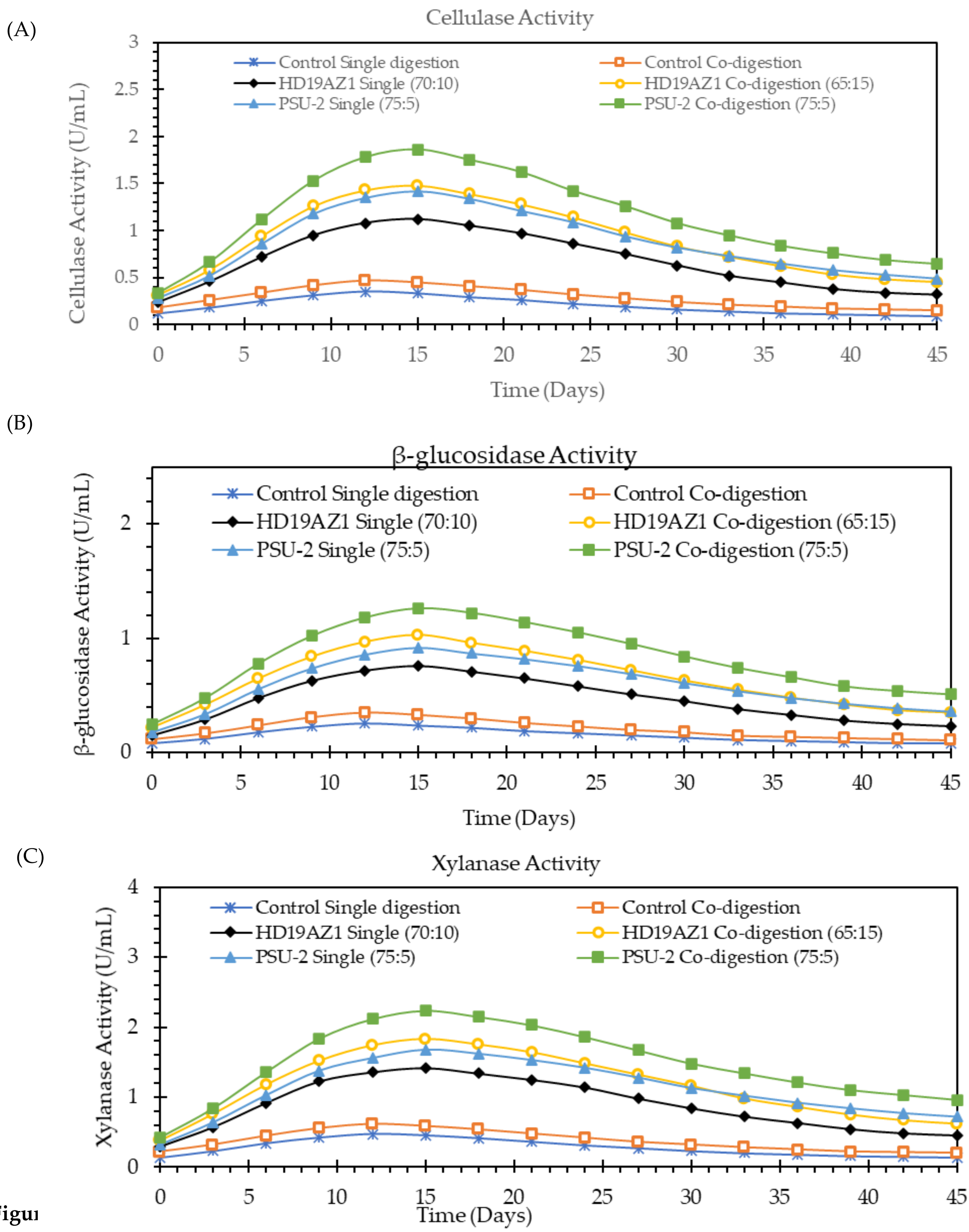


Figure 12. Relative Degradation of Lignocellulosic Components


(A)
Cellulase Activity
Control Single digestion
Control Co-digestion
HD19AZ1 Single (70:10)
HD19AZ1 Co-digestion (65:15)
PSU-2 Single (75:5)
PSU-2 Co-digestion (75:5)
Cellulase Activity (U/mL)
Time (Days)
(B)
β-glucosidase Activity
Control Single digestion
Control Co-digestion
HD19AZ1 Single (70:10)
HD19AZ1 Co-digestion (65:15)
PSU-2 Single (75:5)
PSU-2 Co-digestion (75:5)
β-glucosidase Activity (U/mL)
Time (Days)
(C)
Xylanase Activity
Control Single digestion
Control Co-digestion
HD19AZ1 Single (70:10)
HD19AZ1 Co-digestion (65:15)
PSU-2 Single (75:5)
PSU-2 Co-digestion (75:5)
Xylanase Activity (U/mL)
Time (Days)

Figu

Table 6. Synergistic Effects Indicators in Bio-augmentation

Parameter	HD19AZ1		PSU-2	
	Single Digestion	Co-digestion	Single Digestion	Co-digestion
Enhanced Methane Yield (%)	114.2	93.8	124.2	107.6
Synergy Factor*	1.00	1.32	1.00	1.86
Enzyme Multiplier Effect**	1.23	1.56	1.42	2.14
Microbial Community Diversity Index***	0.68	0.84	0.72	0.91
Acetate Utilization Rate (mg/L/d)	32.6	48.7	36.5	54.2
H_2 Consumption Rate (mL/L/d)	12.4	18.3	14.8	22.6

*Synergy Factor: Ratio of actual co-digestion yield to theoretical yield based on individual substrates. **Enzyme Multiplier Effect: Ratio of measured enzymatic activity to expected activity based on inoculum proportion. ***Microbial Community Diversity Index: Shannon-Weaver diversity index based on 16S rRNA analysis

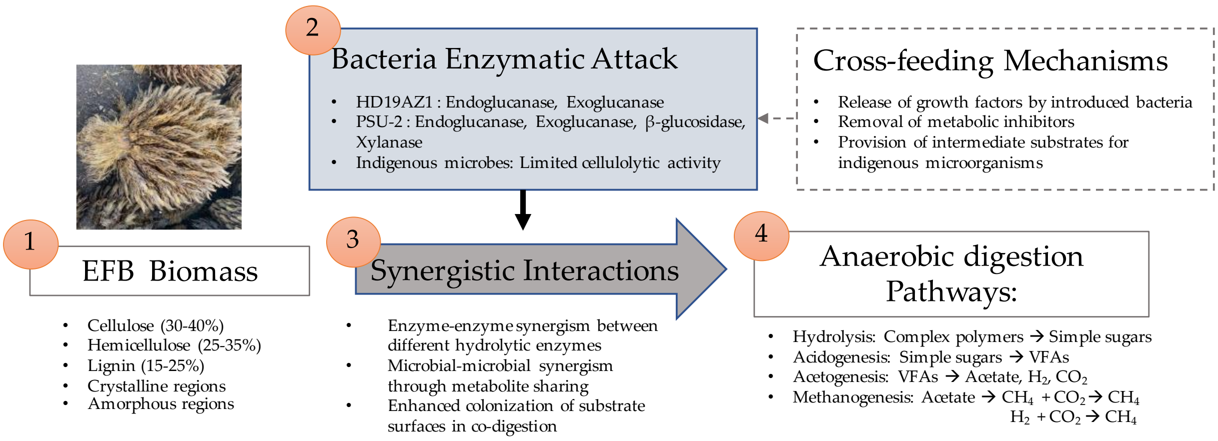


Figure 14. Proposed Mechanism of Bio-augmentation Enhancement

4. Economic and Environmental Implications

4.1 Potential methane yield improvements at the industrial scale

Laboratory-scale results indicate strong potential for improved methane yields with industrial-scale bio-augmentation. Based on the optimal conditions identified in this study, the application of PSU-2 at an I:M ratio of 75:5 in co-digestion systems could increase methane yields from 85.93 to 178.42 mL-CH_4/gVS, representing a 107.6% improvement. When translated to industrial volumes, this enhancement presents substantial economic opportunities. A typical palm oil mill processing 60 tonnes of fresh fruit bunches (FFB) per hour generates approximately 36 tonnes of POME and 13.2 tonnes of EFB daily [2]. Using the methane yields obtained in this study, conventional anaerobic digestion would produce about 46.67 m³ of methane per tonne of mixed waste, resulting in approximately 2,310 m³ of methane daily. With bio-augmentation using PSU-2, this could increase to 96.87 m³ per tonne, generating around 4,795 m³ of methane daily—an additional 2,485 m³ of methane. This improvement is consistent with industrial-scale bio-augmentation results reported by Martin-Ryals et al. [12], who observed a 63% increase in methane production from corn waste in a 10,000-liter digester after implementing routine bio-augmentation with Clostridium species. Similarly, Neumann and Scherer [24] reported a 40-55% increase in biogas yields from energy crops following bio-augmentation in commercial-scale digesters. However, several scale-up considerations must be addressed to achieve these potential improvements. Efficient bacterial culture propagation systems would be required to maintain a consistent supply of viable microorganisms. Continuous or semi-continuous feeding strategies would likely yield different optimization parameters than those identified in batch systems, requiring additional process engineering [13]. Furthermore, the heterogeneity of industrial substrates may introduce variability not observed in laboratory-scale experiments, potentially necessitating adaptive bio-augmentation strategies.

4.2 Energy recovery potential from palm oil waste

The enhanced methane yields achieved through bio-augmentation translate directly to improved energy recovery from palm oil industry wastes. With a methane energy content of approximately 36 MJ/m³ [4], the additional 2,485 m³ of methane produced daily (as calculated in Section 4.1) represents an energy gain of 89,460 MJ or 24.85 MWh. This energy could be utilized in several ways within the palm oil production chain:

1. Electricity generation: Using combined heat and power (CHP) systems with 40% electrical efficiency [5], the additional methane could generate approximately 9.94 MWh of electricity daily. For a mill operating 300 days annually, this represents almost 3,000 MWh of additional electricity generation.
2. Process heat: The thermal energy recovered from biogas combustion could supply process heat for palm oil extraction and refining, reducing fossil fuel consumption. Chaikitkaew et al. [7] estimated that biogas-derived heat could replace up to 70% of the thermal energy requirements in palm oil processing.
3. Transport fuel: Purified biomethane could serve as a renewable transportation fuel, either for the mill's vehicle fleet or for external markets. Upgrading biogas to biomethane standards would require additional processing but could increase its economic value by 30-40% [5].

The total energy recovery potential from palm oil mill waste through bio-augmented digestion is substantial. Based on the results from this study and typical palm oil mill production volumes, bio-augmentation could allow for energy recovery of approximately 1.25 GJ per tonne of fresh fruit bunches processed. This aligns with findings by Hansen et al. [1], who estimated that optimized biogas production from palm oil waste could generate 1.0-1.4 GJ per tonne of FFB.

4.3 Sustainability considerations

Beyond energy recovery, the implementation of bio-augmented anaerobic digestion offers multiple sustainability benefits for the palm oil industry:

1. Greenhouse gas mitigation: Converting palm oil waste to biogas through anaerobic digestion significantly reduces methane emissions that would otherwise occur during conventional waste management practices. Ahmad et al. [2] estimated that uncaptured methane emissions from POME can reach 54 kg CO_2-equivalent per tonne of crude palm oil produced. Bio-augmentation, by enhancing the conversion efficiency and reducing residual degradable material, could further decrease these emissions by 35-45%.
2. Waste volume reduction: The enhanced VS removal achieved with bio-augmentation (52.31% with PSU-2 co-digestion compared to 27.64% in control) represents a significant improvement in waste stabilization and volume reduction. This reduces the environmental footprint of disposal and creates opportunities for beneficial use of the digested residues.
3. Digestate utilization: The nutrient-rich digestate from anaerobic digestion can serve as a valuable biofertilizer. Suksong et al. [3] found that digestate from palm oil waste digestion contained balanced N:P: K ratios suitable for agricultural applications. The enhanced degradation achieved through bio-augmentation likely improves the agronomic quality of this digestate by increasing nutrient availability and reducing phytotoxic compounds.
4. Water conservation: Treated POME from anaerobic digestion can be reused in palm oil processing, reducing freshwater consumption. The improved treatment efficiency with bio-augmentation could facilitate water recycling by producing higher-quality effluent. Zhang et al. [22] reported that enhanced anaerobic treatment of agricultural wastewaters through bio-augmentation reduced COD by an additional 15-20%, making water recycling more feasible.
5. Reduced chemical inputs: The biological enhancement through bio-augmentation provides an alternative to chemical pretreatment methods commonly used to improve the digestion of recalcitrant materials. This reduces the environmental impact associated with chemical manufacturing, transportation, and potential contamination of digestate and effluents [8].

These sustainability benefits align with industry trends toward more environmentally responsible palm oil production. The Roundtable on Sustainable Palm Oil (RSPO) certification increasingly emphasizes waste management and greenhouse gas reduction, making bio-augmented digestion an attractive option for mills seeking certification [1].

4.4 Cost-benefit analysis of bio-augmentation implementation

The economic viability of bio-augmentation depends on balancing added costs with the resulting benefits. Table 7 presents an estimated cost-benefit analysis for a medium-sized palm oil mill processing 60 tonnes of FFB per hour and operating 300 days per year. The implementation costs include capital expenditures for bacterial cultivation equipment, storage facilities, and injection systems, estimated at $200,000-300,000 based on similar bioprocess installations reported by Tabatabaei et al. [5]. Operational costs include culture media, additional monitoring, and skilled labor, estimated at $80,000-120,000 annually. Benefits include increased biogas production valued at $165,000-195,000 annually (assuming natural gas price equivalents of $5-6/GJ), reduced waste management costs of $40,000-60,000, and potential carbon credit revenues of $25,000-35,000 (based on carbon market prices of $15-20/tonne CO_2-equivalent). Additional value from digestate utilization and water recycling could contribute $20,000-40,000 annually. With total annual benefits of $250,000-330,000 against operational costs of $80,000-120,000, the net annual benefit would be approximately $150,000-230,000. This suggests a simple payback period of 1.3-2.0 years for the initial capital investment, making bio-augmentation economically attractive for medium to large palm oil mills. Sensitivity analysis indicates that biogas value is the most critical economic factor, with variations in natural gas prices significantly affecting the payback period. At lower gas prices ($3-4/GJ), the payback period extends to 2.5-3.0 years, while higher prices ($7-8/GJ) could reduce it to under one year. The cost of bacterial culture preparation is the second most significant factor, highlighting the importance of efficient propagation systems to maintain economic viability. These economic projections are consistent with findings by Yang et al. [13], who reported that repeated batch bio-augmentation in industrial-scale digesters achieved payback periods of 1.5-2.5 years, primarily through increased biogas production and improved process stability. Similarly, Martin-Ryals et al. [12] calculated that the additional methane production from bio-augmentation provided a 60-80% return on investment annually after accounting for implementation costs. For smaller operations, the economic case may be less compelling due to higher relative implementation costs and lower absolute benefits. Regional cooperative approaches, where centralized bacterial culture facilities serve multiple smaller digesters, could improve economic viability for smaller mills, as suggested by Ferraro et al. [17] for agricultural biogas plants. In conclusion, the economic and environmental implications of bio-augmentation for palm oil waste digestion are highly favorable, particularly for medium to large operations. The enhanced methane yields translate to significant energy recovery potential, while simultaneously addressing waste management challenges and improving the sustainability profile of palm oil production. With reasonable implementation costs and attractive payback periods, bio-augmentation represents a commercially viable approach to enhancing biogas production from these abundant agricultural residues.

Table 7. Cost-Benefit Analysis of Bio-augmentation Implementation for a Medium-Sized Palm Oil Mill (60 tonnes FFB/hour)

Parameter	Low Estimate	High Estimate	Key Notes
Implementation Costs (Capital):			
Bacterial cultivation equipment	$120,000	$180,000	Includes bioreactors and controls
Storage facilities	$40,000	$60,000	Temp-controlled for bacterial cultures
Injection systems	$40,000	$60,000	Automated dosing integrated with feeders
Total Capital Cost	$200,000	$300,000	One-time investment
Operational Costs (Annual):			
Culture media & nutrients	$35,000	$50,000	Varies by bacterial strain & propagation
Additional monitoring	$15,000	$25,000	For microbial activity assessment
Skilled labor	$30,000	$45,000	Specialized maintenance personnel
Total Annual Operational Costs	$80,000	$120,000	Recurring yearly expense
Benefits (Annual):			
Biogas production	$165,000	$195,000	Based on extra methane output
Waste management savings	$40,000	$60,000	Reduced disposal & treatment costs
Carbon credits	$25,000	$35,000	Revenue from CO_2-equivalent reduction
Digestate value	$15,000	$25,000	Replaces fertilizer costs
Water recycling	$5,000	$15,000	Lower freshwater use & discharge fees
Total Annual Benefits	$250,000	$330,000	Combined gains from all streams
Financial Analysis:			
Net Annual Benefit	$150,000	$230,000	Benefits minus operational costs
Payback Period (years)	2	1.3	Capital divided by net annual benefit
ROI (%)	50	77	Net annual benefit divided by capital costs
NPV (10 years, 10% discount)	$922,000	$1,415,000	Discounted cash flow over 10 years

5. Conclusions and Future Perspectives

Bioaugmentation enhances the biodegradability and hydrolysis of lignocellulosic components, thereby improving biogas production from palm oil industrial waste. The optimal bio-augmentation strategy identified was PSU-2 at an I:M ratio of 75:5 for co-digestion of EFB with POME, which achieved a methane yield of 178.42 mL-CH_4/gVS—107.6% higher than the non-augmented control. This strategy also demonstrated superior performance across multiple metrics, including VS removal (52.31%), biodegradability (93.59%), and process kinetics (hydrolysis constant 0.0232 d^{-1}, maximum production rate 15.38 mL-CH_4/gVS/day). This research identifies several process recommendations for industrial applications. First, maintaining the optimal I:M ratio is critical, as excessive bacterial addition did not correlate with improved performance and increased operational costs. Second, co-digestion consistently outperformed single digestion, highlighting the importance of balanced substrate composition. Third, the shorter lag phase and improved stability in bio-augmented systems suggest that this approach could enable higher loading rates and shorter hydraulic retention times in continuous operations, potentially increasing throughput and reducing reactor volume requirements. Future research should focus on several key areas to further optimize bio-augmentation for palm oil waste digestion. Continuous or semi-continuous feeding strategies better simulate industrial settings and may yield different optimal parameters than batch systems.- Development of efficient bacterial culture propagation methods using low-cost, locally available media would improve economic viability. Exploration of mixed bacterial consortia with complementary enzymatic capabilities could further enhance performance beyond what was achieved with single strains. Finally, genomic and metabolomic approaches to elucidate the specific mechanisms of microbial synergy would enable more targeted bioaugmentation strategies tailored to substrate characteristics. The economic and environmental analysis shows that bio-augmentation can improve biogas production from palm oil industry wastes, resulting in energy recovery, waste reduction, and greenhouse gas mitigation with attractive economic returns for industrial implementation.

6. Acknowledgements

We also thanks Faculty of Science and Digital Innovation, Thaksin University, Phatthalung campus, for their expert labratory and support throughout this research.

Author Contributions: Conceptualization, experimental design, S.S., W.T., P.K., S.I.; carrying out experiment and data acquisition, S.S., W.T., P.K., S.I.; writing and editing, S.S., N.P..

Conflicts of Interest: The authors declare no conflict of interest.

References

[1] Hansen, S. B.; Padfield, R.; Syayuti, K.; Evers, S.; Zakariah, Z.; Mastura, S. Trends in Global Palm Oil Sustainability Research. *J. Clean. Prod.* **2022**, *100*, 99–114. https://doi.org/10.1016/j.jclepro.2015.03.051

[2] Ahmad, S.; Kadir, M. Z. A. A.; Shafie, S. Current Perspective of the Renewable Energy Development in Malaysia. *Renew. Sustain. Energy Rev.* **2020**, *115*, 109356.

[3] Suksong, W.; Kongjan, P.; Prasertsan, P.; Imai, T.; O-Thong, S. Optimization and Microbial Community Analysis for Production of Biogas from Solid Waste Residues of Palm Oil Mill Industry by Solid-State Anaerobic Digestion. *Bioresour. Technol.* **2016**, *214*, 166–174. https://doi.org/10.1016/j.biortech.2016.04.077

[4] Saelor, S.; Kongjan, P.; O-Thong, S. Biogas Production from Anaerobic Co-Digestion of Palm Oil Mill Effluent and Empty Fruit Bunches. *Energy Procedia* **2017**, *138*, 717–722. https://doi.org/10.1016/ j.egypro.2017.10.206

[5] Tabatabaei, M.; Aghbashlo, M.; Valijanian, E.; Kazemi Shariat Panahi, H.; Nizami, A. S.; Ghanavati, H.; Sulaiman, A.; Mirmohamadsadeghi, S.; Karimi, K. A Comprehensive Review on Recent Biological Innovations to Improve Biogas Production, Part 2: Mainstream and Downstream Strategies. *Renew. Energy* **2020**, *146*, 1392–1407. https://doi.org/10.1016/j.renene.2019.07.047

[6] Tsapekos, P.; Kougias, P. G.; Vasileiou, S. A.; Treu, L.; Campanaro, S.; Lyberatos, G.; Angelidaki, I. Bioaugmentation with Hydrolytic Microbes to Improve the Anaerobic Biodegradability of Lignocellulosic Agricultural Residues. *Bioresour. Technol.* **2017**, *234*, 350–359. https://doi.org/10.1016 /j.biortech.2017.03.043

[7] Chaikitkaew, S.; Kongjan, P.; O-Thong, S. Biogas Production from Biomass Residues of Palm Oil Mill by Solid-State Anaerobic Digestion. *Energy Procedia* **2015**, *79*, 838–844. https://doi.org/10.1016/j.egypro.2015.11.575

[8] Nzila, A. Mini Review: Update on Bioaugmentation in Anaerobic Processes for Biogas Production. *Anaerobe* **2017**, *46*, 3–12. https://doi.org/10.1016/j.anaerobe.2016.11.007

[9] Ozbayram, E. G.; Kleinsteuber, S.; Nikolausz, M.; Ince, B.; Ince, O. Effect of Bioaugmentation by Cellulolytic Bacteria Enriched from Sheep Rumen on Methane Production from Wheat Straw. *Anaerobe* **2017**, *46*, 122–130. https://doi.org/10.1016/j.anaerobe.2017.03.013

[10] Peng, X.; Börner, R. A.; Nges, I. A.; Liu, J. Impact of Bioaugmentation on Biochemical Methane Potential for Wheat Straw with Addition of *Clostridium cellulolyticum*. *Bioresour. Technol.* **2014**, *152*, 567–571. https://doi.org/10.1016/j.biortech.2013.11.067

[11] Strang, O.; Ács, N.; Wirth, R.; Maróti, G.; Bagi, Z.; Rákhely, G.; Kovács, K. L. Bioaugmentation of the Thermophilic Anaerobic Biodegradation of Cellulose and Corn Stover. *Anaerobe* **2017**, *46*, 104–113. https://doi.org/10.1016/j.anaerobe.2017.05.014

[12] Martin-Ryals, A.; Schideman, L.; Li, P.; Wilkinson, H.; Wagner, R. Improving Anaerobic Digestion of a Cellulosic Waste via Routine Bioaugmentation with Cellulolytic Microorganisms. *Bioresour. Technol.* **2015**, *189*, 62–70. https://doi.org/10.1016/j.biortech.2015.03.069

[13] Yang, Z.; Guo, R.; Xu, X.; Wang, L.; Dai, M. Enhanced Methane Production via Repeated Batch Bioaugmentation Pattern of Enriched Microbial Consortia. *Bioresour. Technol.* **2016**, *216*, 471–477. https://doi.org/10.1016/j.biortech.2016.05.062

[14] APHA. *Standard Methods for the Examination of Water and Wastewater*, 23rd ed.; American Public Health Association; American Water Works Association; Water Environment Federation: Washington, DC, **2017**.

[15] Sluiter, A.; Hames, B.; Ruiz, R.; Scarlata, C.; Sluiter, J.; Templeton, D.; Crocker, D. *Determination of Structural Carbohydrates and Lignin in Biomass; Laboratory Analytical Procedure (LAP)*; National Renewable Energy Laboratory: Golden, CO, 2012.

[16] Jiang, Y.; Lu, J.; Lv, Y.; Wu, R.; Dong, W.; Zhou, J.; Jiang, M.; Xin, F. Efficient Hydrogen Production from Lignocellulosic Feedstocks by a Newly Isolated Thermophilic *Thermoanaerobacterium* sp. Strain F6. *Int. J. Hydrogen Energy* **2019**, *44*, 14380–14386. https://doi.org/10.1016/j.ijhydene.2019.01.226

[17] Ferraro, A.; Dottorini, G.; Massini, G.; Mazzurco Miritana, V.; Signorini, A.; Lembo, G.; Fabbricino, M. Combined Bioaugmentation with Anaerobic Ruminal Fungi and Fermentative Bacteria to Enhance Biogas Production from Wheat Straw and Mushroom Spent Straw. *Bioresour. Technol.* **2018**, *260*, 364–373. https://doi.org/10.1016/j.biortech.2018.03.128

[18] Angelidaki, I.; Alves, M.; Bolzonella, D.; Borzacconi, L.; Campos, J. L.; Guwy, A. J.; Kalyuzhnyi, S.; Jenicek, P.; van Lier, J. B. Defining the Biomethane Potential (BMP) of Solid Organic Wastes and Energy Crops: A Proposed Protocol for Batch Assays. *Water Sci. Technol.* **2009**, *59*, 927–934. https://doi.org/10.2166/wst.2009.040

[19] Trzcinski, A. P.; Stuckey, D. C. Determination of the Hydrolysis Constant in the Biochemical Methane Potential Test of Municipal Solid Waste. *Environ. Eng. Sci.* **2012**, *29*, 848–854. https://doi.org/10.1089/ees.2011.0105

[20] Morris, D. L. Quantitative Determination of Carbohydrates with Dreywood's Anthrone Reagent. *Science* **1948**, *107*, 254–255. https://doi.org/10.1126/science.107.2775.254

[21] Li, Y.; Li, L.; Sun, Y.; Yuan, Z. Bioaugmentation Strategy for Enhancing Anaerobic Digestion of High C/N Ratio Feedstock with Methanogenic Enrichment Culture. *Bioresour. Technol.* **2018**, *261*, 188–195. https://doi.org/10.1016/j.biortech.2018.02.069

[22] Zhang, J.; Guo, R. B.; Qiu, Y. L.; Qiao, J. T.; Yuan, X. Z.; Shi, X. S.; Wang, C. S. Bioaugmentation with an Acetate-Type Fermentation Bacterium *Acetobacteroides hydrogenigenes* Improves Methane Production from Corn Straw. *Bioresour. Technol.* **2015**, *179*, 306–313. https://doi.org/10.1016/j.biortech.2014.12.022

[23] Nkemka, V. N.; Gilroyed, B.; Yanke, J.; Gruninger, R.; Vedres, D.; McAllister, T.; Hao, X. Bioaugmentation with an Anaerobic Fungus in a Two-Stage Process for Biohydrogen and Biogas Production Using Corn Silage and Cattail. *Bioresour. Technol.* **2015**, *185*, 79–88. https://doi.org/10.1016/j.biortech.2015.02.100

[24] Neumann, L.; Scherer, P. Impact of Bioaugmentation by Compost on the Performance and Ecology of an Anaerobic Digester Fed with Energy Crops. *Bioresour. Technol.* **2011**, *102*, 2931–2935. https://doi.org/10.1016/j.biortech.2010.11.068