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Response Surface Optimization of Biogas Production and Organic Matter Removal during Anaerobic Co-Digestion of Palm Oil Mill Effluent with Hydrothermally Pretreated *Chlorella vulgaris*

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ABSTRACT

Palm oil mill effluent (POME) is a high strength wastewater with the great potential for anaerobic bioenergy recovery. However, the variable composition and the slowly biodegradable fractions may limit the digestion performance. The study investigated the use of hydrothermally pretreated *Chlorella vulgaris* as a co substrate for POME and optimized the process using a two-factor central composite design under response surface methodology. The effects of microalgae concentration and POME to inoculum ratio on four responses, namely, volatile solids (VS) removal, COD removal, total biogas production, and normalized methane volume, were evaluated. Hydrothermal treatment at 121°C for 120 min decreased the particulate VS fraction and enhanced the measured COD, suggesting improved accessibility of the microalgal organic matter for anaerobic conversion. Concentration of microalgae had significant effect on VS removal, COD removal and total biogas production while POME to inoculum ratio had significant effect on methane production. Numerical multiresponse optimization revealed that the concentration of microalgae was 1085.94 mg COD L^{-1} and the ratio of POME to inoculum was 2.92 with the predicted responses of 278.36 mL N total biogas, 183.97 mL N CH_4, 65.53% VS removal and 59.00% COD removal at desirability of 0.632. However, the methane model was not statistically significant and showed significant lack of fit, suggesting that the predicted optimum should be interpreted with care. The laboratory scale energy assessment showed a net energy ratio of 0.00101 on the basis of rated autoclave power and suggested that the pretreatment configuration was not energy self-supporting at this scale. In summary, hydrothermally pretreated microalgae improved POME co digestion performance, but direct measurements of soluble COD, volatile fatty acids, long chain fatty acids and microbial composition are required to validate the proposed mechanisms and estimate the scale up feasibility.

Keywords: anaerobic co-digestion; biogas; Chlorella vulgaris; hydrothermal pretreatment; palm oil mill effluent; response surface methodology

1. INTRODUCTION

The extraction of crude palm oil (CPO) from fresh fruit bunches (FFB) in the palm oil industry generates large amounts of palm oil mill effluent (POME). The POME contains high organic load that should be treated prior to disposal (Mohammad et al., 2021). POME has a biochemical oxygen demand (BOD) of between 10,250 and 43,750 mg/L and a chemical oxygen demand (COD) of between 15,000 and 100,000 mg/L depending on process conditions (Imam et al., 2025) (Ooi et al., 2023) (Rajani et al., 2019). Anaerobic digestion (AD) is a good option for POME treatment because it converts organic matter to methane rich biogas, and reduces chemical oxygen demand (COD) and volatile solids (VS) (Hagos et al., 2017) (Mohammad et al., 2021). However, the high lipid content, lignocellulosic fraction and compositional variability of POME often hinder hydrolysis the rate limiting step of AD and can induce microbial imbalance or inhibitory compound accumulation. Thus, single substrate AD of POME often under performs without process intervention (Mohammad et al., 2021) and (Rajani et al., 2019).

Anaerobic co digestion (ACoD) alleviates this limitation by mixing two or more substrates to balance nutrients and stabilize microbial activity (Hagos et al., 2017) (Solé-Bundó et al., 2019). Microalgae, especially *Chlorella vulgaris*, are interesting co substrates due to their balanced composition of carbohydrates, proteins and lipids (Ward et al., 2014). However, the rigid cell wall, made of polysaccharides, peptidoglycan and chitin, depresses the hydrolysis rates and therefore the hydrolytic enzymes cannot easily access the intracellular macromolecules (Hasan et al., 2024) (Zhang et al., 2020). This cell wall is broken by hydrothermal pretreatment which increases the dissolved organic fraction and has been reported to increase the methane production potential (Bohutskyi et al., 2023) (Passos & Ferrer, 2015) (Saleem et al., 2024).

The use of co digestion strategies for POME has gained increasing interest but the specific combination studied here, hydrothermally pretreated microalgae as a co substrate for POME using a two factor CCD RSM design for four simultaneous responses, has not been widely studied. Three parallel but distinct research trajectories are illustrated by recent studies that leave this gap open. First, machine learning assisted RSM has been applied to POME digestion enhanced with biochar instead of microalgae, and without any pretreatment step aiming at substrate cell wall disruption (Chang et al., 2025). Secondly, hydrothermal pretreatment together with co digestion has been demonstrated for lignocellulosic co substrates like empty fruit bunch (EFB) where the recalcitrant fraction is structural lignocellulose and not microalgal cell wall and the optimization target is usually a single response (Chanthong et al., 2024). Third, RSM has been applied for co digestion of another microalgal species (*Tetraselmis suecica*) with EFB (Ahmad et al., 2014), but not with hydrothermally disrupted biomass, for POME treatment, again without mechanistic linkage between the solubilization caused by the pretreatment and the resulting multi response

biogas behavior. None of these studies combine (i) a defined hydrothermal pretreatment step for disruption of microalgal cell walls; (ii) POME as the major substrate rather than a minor supplement; and (iii) a CCD RSM framework that simultaneously solves for VS removal, COD removal, total biogas, and CH_4 content as interlinked, mechanistically interpretable responses. In addition, previous studies of microalgae POME have focused on the end point of pollutant removal (COD, BOD, total phosphorus, NH_3-N) without relating the removal efficiency to the biogas yield it can produce (Emparan et al., 2020) (Kacaribu et al., 2025).

This study was aimed at the anaerobic co digestion of POME with hydrothermally pretreated *C. vulgaris* by two factor CCD-RSM approach. The effect of microalgae concentration and POME to inoculum ratio on the VS removal, COD removal, total biogas and methane production were studied. The objectives of the study were to (i) assess the influence of the two operating factors on organic matter removal and gas production, (ii) identify a numerical multiresponse optimum in the investigated design space, (iii) interpret the responses observed in the context of pretreatment induced substrate accessibility and substrate to inoculum balance, and (iv) conduct a preliminary laboratory scale comparison between pretreatment energy input and incremental methane energy recovery. The proposed process pathway is considered a mechanistic interpretation, not definitive mechanistic proof, because VFA, LCFA, soluble COD and microbial community composition were not directly measured.

2. MATERIALS AND METHODS

2.1 Substrate and Inoculum

The primary substrate was co digested POME with *Chlorella vulgaris* microalgal biomass inoculated from cow manure. *C. vulgaris* was chosen because of its rapid growth, resistance to environmental conditions and biochemical composition suitable for bioenergy applications (Taikhao & Phunpruch, 2025). The culture was grown in BG 11 medium (Jonynaite et al., 2024) and monitored using optical density at 750 nm (OD750), harvested at OD750 $\approx$ 0.5-1 (Chioccioli et al., 2014). The cow manure inoculum was homogenized and conditioned anaerobically under mesophilic conditions (35 $\pm$ 2 °C) before use to stabilize microbial activity (Steiniger et al., 2023); samples not used immediately were stored at 4°C (Astals et al., 2020).

2.2 Hydrothermal Pretreatment of Chlorella vulgaris

Chlorella vulgaris suspension was transferred to 1L glass bottles and treated hydrothermally in an autoclave (Hiclave HVE-50, Hirayama, Japan) at 121°C for 120 min after reaching the target temperature. Each autoclave cycle processed up to five 1L bottles. After treatment the bottles were allowed to cool to room temperature and then stored at 4 °C until use (Astals et al., 2020). COD and VS were measured before and

after pre treatment. The treatment was aimed at disrupting the microalgal cell structure and enhancing the accessibility of intracellular organic matter during anaerobic digestion (Passos & Ferrer, 2015).

2.3 Reactor Setup and Analytical Methods

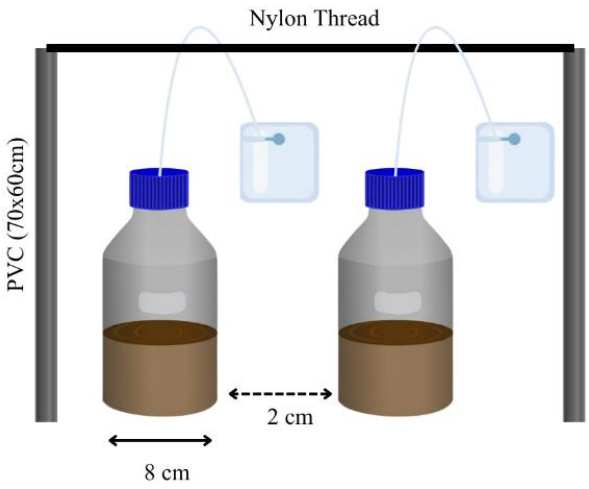


Figure 1. Laboratory scale anaerobic batch reactor configuration

The laboratory-scale anaerobic batch reactor configuration used in this study is shown in **Figure 1.** Co digestion was carried out in sealed 500 mL borosilicate batch digesters, having a working volume of 300 mL and a headspace of approximately 200 mL, 2 min with oxygen free nitrogen (99.999%; PT Aneka Gas Industri Tbk., Indonesia) at about 1 L min^{-1} before sealing each reactor. The reactors were sealed with butyl rubber septa and screw caps and wrapped in Parafilm to minimize gas leakage and oxygen ingress. Biogas was collected in 1L Tedlar bags connected with gas tight PTFE tubing. Bags were flushed with oxygen free nitrogen at ~1 L min^{-1} for 2 min and emptied prior to use. The reactor contents were manually swirled twice daily to gently mix the contents and all digesters were incubated for 30 days at 29.4 ± 1.3 °C.

The volume of biogas was periodically determined using a 50 mL gas tight glass syringe. The pH was measured at the beginning and at the end of the incubation with a calibrated portable pH meter (Orion Star A221, Thermo Fisher Scientific, USA). The COD, TS and VS were analyzed following Standard Methods. The volume of methane was determined by alkaline absorption of CO_2 with 3 N KOH. The known biogas volume was gently agitated with KOH for about 3 min and the stabilized residual gas volume was recorded as the estimated methane fraction assuming CH_4 and CO_2 were the main biogas components. Reactor experiments were performed in triplicate and laboratory analyses were carried out in duplicate.

2.4 Experimental Design and Statistical Analysis

A two factor central composite design was developed with Design Expert version 13. The independent variables were the concentration of microalgae in terms of COD equivalent concentration (Factor A) and the POME to inoculum ratio (Factor B). Factor A ranged from 652 to 1304 mg COD L^{-1} and Factor B from 2 to 4. The full CCD was composed of factorial, axial and centre point runs. The response variables were VS removal, COD removal, total biogas production and normalized methane volume. For each response, linear, two factor interaction and quadratic models were evaluated as candidates. General quadratic equation was:

The quadratic polynomial model fitted to each response took the form:

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_{12} X_1 X_2 + \beta_{11} X_1^2 + \beta_{22} X_2^2 \quad \textbf{Equation 1}$$

where Y represents the predicted response, β_0 denotes the intercept, β_i, β_{ij}, and β_{ii} are the regression coefficients for the linear, interaction, and quadratic terms, respectively, while X_i and X_j are the coded independent variables.

Model significance was analyzed by ANOVA at $p<0.05$. Model selection was based on sequential sum of squares, lack of fit, R^2, adjusted R^2, predicted R^2, coefficient of variance, adequate precision and residual diagnostics. Numerical multiresponse optimization was carried out using desirability function. Some response models exhibited a low predicted R^2 or significant lack of fit. Hence, the optimization outputs were interpreted only in the experimental domain and were validated independently by experiment.

For validation, prediction error was expressed as range normalized RMSE, (NRMSE):

$$NRMSE\ (\%) = \frac{\sqrt{\frac{1}{n}\sum_{i=1}^{n}\left(Y_{p,i} - Y_{e,1}\right)^2}}{Y_{e,max} - Y_{e,min}} x100 \quad \textbf{Equation 2}$$

where Y_p and Y_e are predicted and experimental responses, respectively.

Model adequacy was evaluated by analysis of variance (ANOVA) at 95% confidence ($p < 0.05$). The sequential model sum of squares and lack of fit test were used to choose among the linear, two factor interaction (2FI), and quadratic models. In addition to the coefficient of determination (R^2) and adjusted R^2, which have been reported in **Table 1**, the model reliability was evaluated by the following diagnostics, which are common for reporting a CCD RSM in Design Expert (version 13):

144

145 **Table 1.** Model fit statistics

Response	Std. Dev.	C.V. %	R^2	Adj. R^2	Pred. R^2	Adeq. Precision
VS removal (%)	4.07	6.83	0.8555	0.7523	0.0680	97.587
COD removal (%)	3.43	6.06	0.8266	0.7919	0.7232	139.060
Total biogas (mL N)	19.85	8.03	0.8463	0.7365	0.4429	82.244
CH_4 content (mL N)	16.12	10.02	0.8240	0.6983	0.1366	75.183

146

147 ### *2.5 Energy Balance Analysis*

148 A simple first order energy balance was framed to evaluate whether the hydrothermal pretreatment is
149 energetically self supporting, comparing the additional bioenergy recovered with the thermal energy
150 invested in pretreatment. The energy input for the hydrothermal pretreatment was estimated as:

151 $$E_{input} = P\ x\ t_{heat}$$ **Equation 3**

152 where P is the rated electrical power draw of the autoclave (kW) and t_{heat} is the time to reach and maintain
153 121°C (h). The energy gain from the methane was computed as:

154 $$E_{Output} = V_{CH4\,x}\, LVH_{CH4}$$ **Equation 4**

155 where V_{CH4} is the incremental volume of CH_4 due to the addition of microalgae (m^3) and LHV_{CH4} is the
156 lower heating value of methane (approx. 35.8 MJ/m^3 under standard conditions). The net energy ratio (NER
157 = E output / E input) tells us whether pretreatment is energetically favorable at laboratory scale (NER > 1)
158 or whether heat integration/scale up efficiencies are needed to make it favorable (NER < 1). The latter is
159 typical for small batch autoclaves, and improves considerably at industrial scale, due to heat recovery and
160 economies of scale (Passos & Ferrer, 2015).

161 ## 3. RESULTS AND DISCUSSION

162 ### *3.1 Characteristics of Substrate and Inoculum*

163 **Table 2.** Characteristics of Substrate and Inoculum

Sample	COD (mg/L)	VS (mg/L)
POME	125,291.67	21,143
Cow manure	32,687.5	152,020.2
Chlorella vulgaris	1,304.17	6.977

164

The characteristics of the substrate and inoculum used in this study are summarized in **Table 2**. The COD of the raw POME (125,291.67 mg/L) is within the range of high strength POME (50,000-150,000 mg/L) reported in the literature (Imam et al., 2025; Ooi et al., 2023; Rajani et al., 2019) confirming its suitability as a substrate for anaerobic digestion. However, its VS (21,143 mg/L) is lower than the values reported in the literature (42,600-49,300 mg/L) (Poh et al., 2010), indicating a comparatively dilute solid fraction in this batch of POME. The cow manure COD (32,687.5 mg/L) falls within the typical 34-58 g/L range (Bella & Venkateswara Rao, 2022), supporting its role as a stable buffering inoculum. The untreated *C. vulgaris* COD (1,304.17 mg/L) indicates a dilute microalgal suspension when compared to the concentrated microalgal biomass reported elsewhere (22.3-24.9 g COD/L) (Giménez et al., 2018) and provides a baseline for the interpretation of the effect of hydrothermal pretreatment (Section 3.2).

3.2 Mechanistic Effect of Hydrothermal Pretreatment on Microalgal Biodegradability

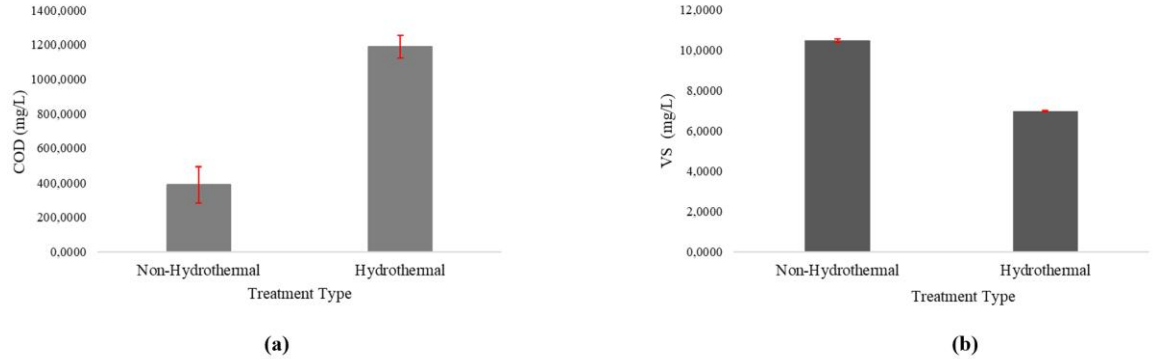


Figure 2. Effect of hydrothermal pretreatment on Chlorella vulgaris characteristics: (a) volatile solids (VS); (b) chemical oxygen demand (COD).

The effect of hydrothermal pretreatment on the volatile solids (VS) and chemical oxygen demand (COD) of Chlorella vulgaris is illustrated in **Figure 2.** The VS was reduced from 10.47 ± 0.04 to 6.98 ± 0.03 mg/L after the hydrothermal pretreatment, while the measured COD increased from 437.5 ± 66.14 to 1304.17 ± 104.8 mg L^{-1}. The contrasting trends imply a change in the physical distribution and analytical accessibility of microalgal organic matter upon thermal treatment. Disruption of the microalgal cellular structure may have released intracellular compounds and decreased the particulate biomass fraction, thus increasing the organic material accessible to COD oxidation. However, the COD measurement in this study is not automatically soluble COD unless the sample was filtered or centrifuged prior to analysis. Therefore, the joint COD-VS response indirectly indicates increased substrate accessibility, but not direct evidence of solid to liquid solubilization. Solubilization would need to be conclusively quantified by measuring soluble COD, dissolved organic carbon, particle-size distribution, or microscopic cell disruption.

Improved substrate accessibility might facilitate the first hydrolysis step of anaerobic digestion by reducing the structural barrier around microalgal proteins, carbohydrates and lipids . This interpretation is in line with the subsequent positive impact of microalgae concentration on removal of organic matter and total biogas production, despite not measuring particular biochemical pathways directly.

3.3 Statistical Validation of the RSM-CCD Models

Table 3. ANOVA Summary for the Fitted Models

Source	VS Removal F	VS Removal p	COD Removal F	COD Removal p	Biogas F	Biogas p	CH_4 F	CH_4 p
Model	8.29	0.0074	23.83	0.0002	10.33	0.0039	3.52	0.0698
A - Microalgae	19.58	0.0031	44.63	0.0001	36.8	0.0005	0.6751	0.4304
B - POME:inoculum	3.75	0.0939	3.04	0.1121	0.392	0.5511	6.36	0.0303
AB	0.0149	0.9061	-*	-*	0.2523	0.6309	-*	-*
A^2	0.161	0.7002	-*	-*	1.77	0.2248	-*	-*
B^2	17.19	0.0043	-*	-*	13.48	0.0079	-*	-*
Lack of Fit	9.87	0.0255	0.9574	0.5428	0.4114	0.7543	20.90	0.0055

* : Term removed to improve model hierarchy"

3.3.1 Model Adequacy and What the ANOVA Structure Implies Mechanistically

The quadratic models were significant for the VS removal ($F = 8.29$, $p = 0.0074$), COD removal ($F = 23.83$, $p = 0.0002$), and biogas production ($F = 10.33$, $p = 0.0039$) at the 95% confidence level but not for CH_4 model ($F = 3.52$, $p = 0.0698$). Rather than being a limitation in itself, the pattern is mechanistically informative. Total biogas volume is determined predominantly by the amount of organic carbon made available for conversion a mass availability effect dominated by Factor A (microalgae concentration). CH_4 content within that biogas is determined by the metabolic partitioning between methanogenic and nonmethanogenic (CO_2-producing, VFA-accumulating) pathways, a process more sensitive to the substrate-to-inoculum balance (Factor B) than absolute carbon load. The comparatively poorer statistical fit for CH_4 thus reflects a true difference in the underlying biological control point, not just unexplained noise; it is consistent with reports that methane content in POME-based digesters is sensitive to inoculum microbial diversity and community shifts that a two-factor design cannot fully resolve (Yin et al., 2014).

Table 3 provides the quantitative complement to this interpretation. For COD removal and total biogas, the Predicted R^2 (0.7232 and 0.4429, respectively) remains reasonably close to the Adjusted R^2, and the low C.V.% (6.06% and 8.03%) indicates that both models resolve genuine treatment effects rather than experimental noise. However, for VS removal, the Predicted R^2 (0.068) is much lower than the Adjusted R^2 (0.7523) a gap well beyond the conventional 0.2 threshold and is associated with a statistically significant lack-of-fit ($F = 9.87$, $p = 0.0255$; **Table 3**). Collectively, these diagnostics show that while the VS removal model is a reasonable fit to the calibration data, its predictive power at untested operating

points in the design space is limited. Thus, the numerical optimum reported for VS removal, and the multiresponse desirability solution including it, should be interpreted with the same caution as the CH_4 model, and is best confirmed with additional confirmatory runs in addition to those already reported in **Table 3**.

3.3.2 Mechanistic Basis of Individual Factor Effects

Factor A (microalgae concentration) showed significant effect on VS removal ($F = 19.58$, $p = 0.0031$), COD removal ($F = 44.63$, $p = 0.0001$), and biogas production ($F = 36.8$, $p = 0.0005$), but not on CH_4 content ($F = 0.68$, $p = 0.4304$). Mechanistically, this dissociation can be explained by the pretreatment chemistry established in Section 3.2. Hydrothermally solubilized microalgal biomass provides additional hydrolysable protein, lipid and carbohydrate substrate which directly increases the pool of organic matter available for degradation (raising VS and COD removal) and for gas-forming fermentation (raising total biogas). The enriched lipid fraction released from microalgal cells is also a known precursor of long chain fatty acids (LCFAs) upon β-oxidation; LCFA accumulation is a documented inhibitor of methanogenic archaea specifically, without necessarily suppressing acidogenic gas formation (Abreu et al., 2012). This provides a coherent mechanistic, rather than purely statistical, explanation for why increasing microalgae loading enlarges total biogas volume without proportionally enlarging its methane fraction: part of the additional gas volume is plausibly CO_2 from LCFA-inhibited, incompletely methanogenic fermentation.

Factor B (POME to inoculum ratio) was significant only for CH_4 production ($F = 6.36$, $p = 0.0303$) and had a highly significant quadratic term B^2 for both VS removal ($F = 17.19$, $p = 0.0043$) and COD removal ($F = 13.48$, $p = 0.0079$). The quadratic curvature suggests an optimal substrate to inoculum ratio: at low B, the buffer capacity and methanogenic biomass are impaired by low inoculum and thus conversion is limited; at high B, the organic overload leads to VFA accumulation at a greater rate than the methanogenic community can consume it, depressing pH and inhibiting the very organisms responsible for CH_4 formation (Batstone et al., 2002) (Kafle & Chen, 2016). The B^2 significance is, therefore, not only a statistical justification to keep the quadratic model term, but the direct mathematical signature of a VFA mediated inhibition threshold functioning within the tested range, in accordance with the curvature reported by Montecchio et al., 2019, for microalgae-palm-based co-digestion systems that was ascribed to competitive inhibition between hydrolytic and methanogenic guilds at unfavourable substrate-to-inoculum ratios. The non-significant AB interaction and A^2 term ($p > 0.05$ throughout) suggest that in the tested domain, the carbon-supply effect of microalgae addition and the buffering/overloading effect of the POME-to-inoculum ratio are largely independent control mechanisms, rather than through a shared physiological pathway.

3.3.3 Residual Diagnostics and Predictive Reliability

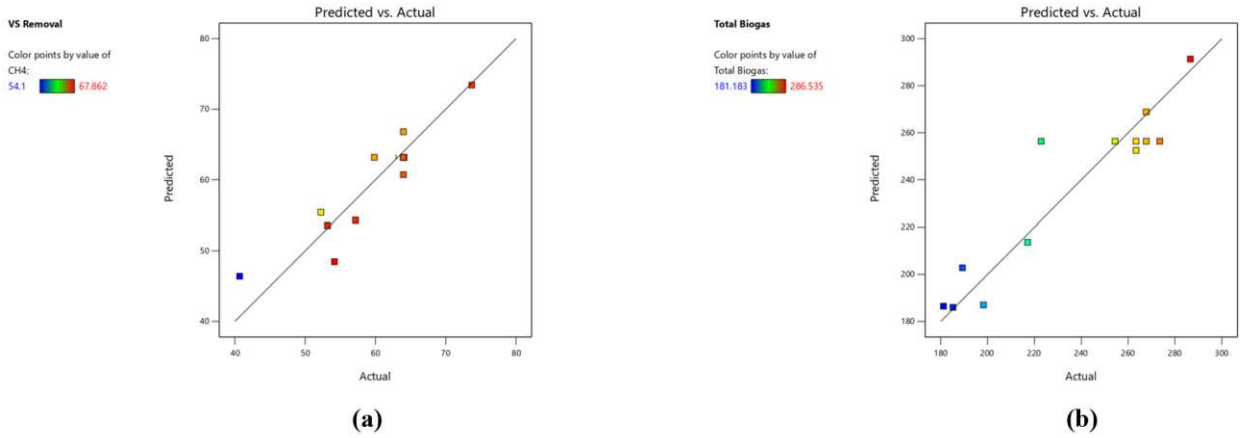


Figure 3. Experimental vs. model-predicted values: (a) volatile solids removal (%); (b) total biogas (mL N).

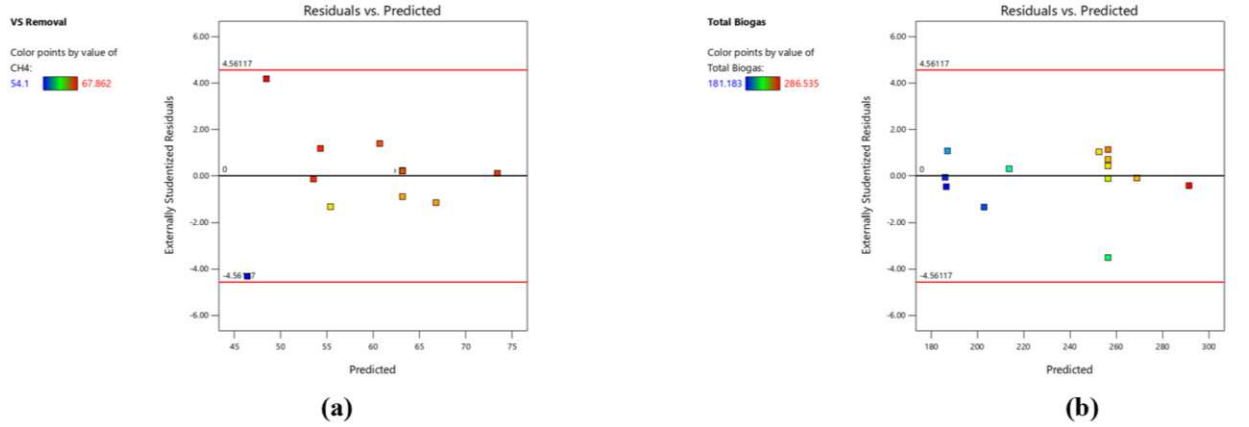


Figure 4. Residual diagnostic plots (residuals vs. predicted values): (a) volatile solids removal (%); (b) total biogas (mL N).

The proximity of experimental and predicted values to the y = x line (**Figure 3**) provides experimental validation for the high R^2 obtained for VS removal (R2 = 0.8555) and biogas production (R2 = 0.8807). **Figure 4** shows the residuals randomly scattered about zero with no pattern, indicating a homoscedastic error structure with no omitted systematic bias term in the model. Adequate precision, which measures the signal-to-noise ratio of the spread of predicted values for each factor relative to the prediction error, was found to be greater than the minimum acceptable value of 4 for both VS removal (9.7587) and biogas production (8.9715), indicating that the models can both satisfactorily traverse the design space for optimization. Usual RSM reporting practice would also include a full set of residual diagnostics, including normal probability plots of the residuals, to check the normality assumption underlying the F-tests, and residual-versus-run-order plots, to eliminate time-dependent drift over the experimental run sequence. These are not included here as they require the original run-order metadata from the CCD design matrix;

we flag this as a limitation of the present reporting and recommend their inclusion in the supplementary material upon revision.

The model was validated against five additional reactors covering high, medium and low microalgae concentrations (**Table 4**). The obtained normalized root-mean-square errors (NRMSE) were 11.5% and 18.97% for VS removal and total biogas, respectively. The relatively higher NRMSE for biogas is a mechanistic expectation, not a modeling weakness: total biogas represents the compounded variability of four sequentially occurring biochemical steps (hydrolysis, acidogenesis, acetogenesis and methanogenesis), each with its own sensitivity to substrate heterogeneity and microbial community fluctuation, while VS removal is a single, more directly measurable, mass-balance quantity. Both NRMSE values are within the range generally accepted for validated RSM-CCD models of biological systems (Saratale et al., 2018).

Table 4. Model Validation (NRMSE) Against Independent Reactor Runs

Reactor	VS Removal - Predicted	VS Removal - Experimental	Total Biogas - Predicted	Total Biogas - Experimental	CH_4 - Predicted	CH_4 - Experimental
1	73.42	71.72	256.37	237.58	204.95	193.31
2	66.80	64.09	236.55	222.63	187.71	169.49
3	53.56	52.72	163.67	146.95	129.19	126.43
4	63.17	61.30	225.60	205.91	175.26	172.24
5	55.40	52.06	164.11	147.83	131.13	141.09

NRMSE: VS removal = 11.5%; total biogas = 18.97%; CH_4 = 16.15% (from experimental data, Section 3.3.3).

3.4 Mechanistic Interpretation of Response Surface Behavior

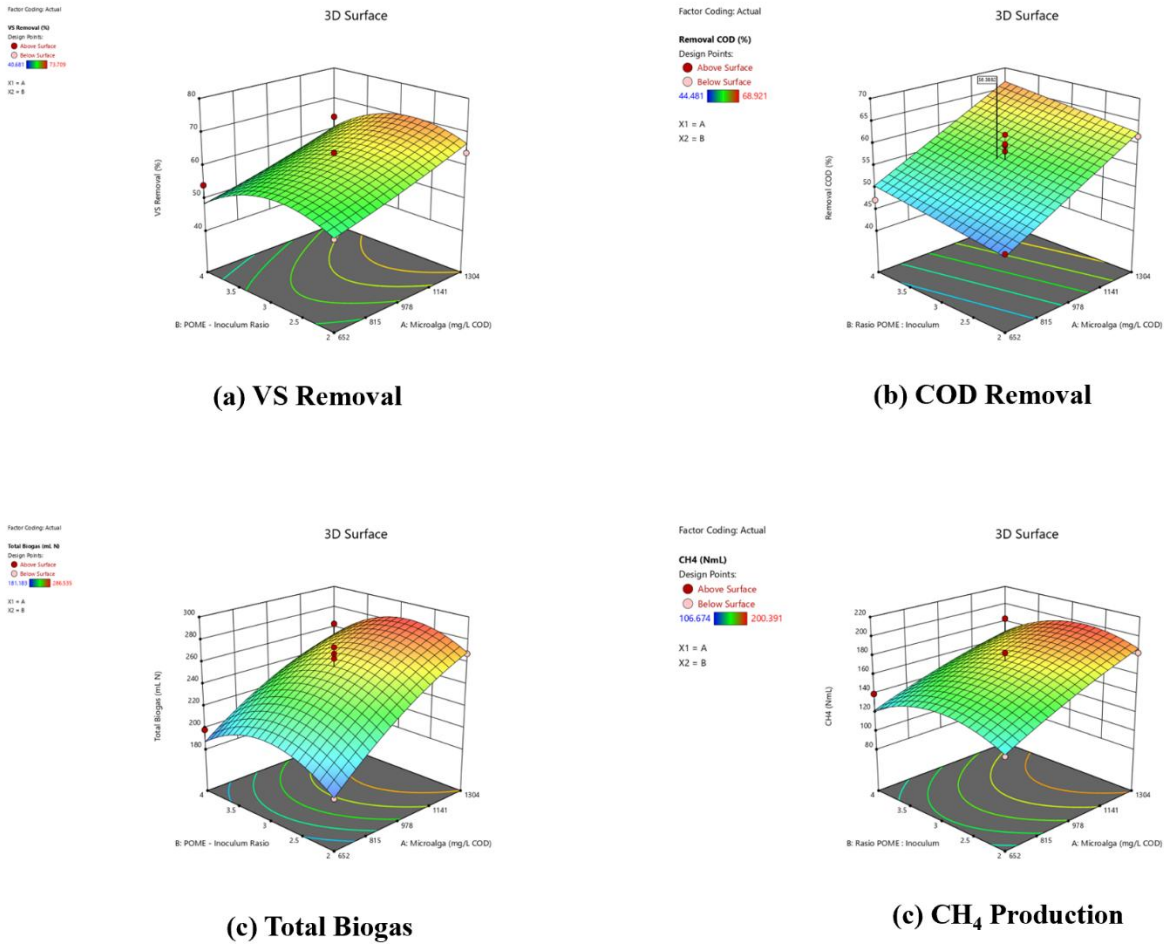


Figure 5. Three-dimensional response surfaces as a function of microalgae concentration (Factor A) and POME-to-inoculum ratio (Factor B): (a) VS removal; (b) COD removal; (c) total biogas; (d) CH_4 production.

3.4.1 VS and COD Removal: Substrate Availability as the Controlling Mechanism

The %VS removal (%VSr) ranged between 40.68% and 73.71% and the %COD removal (%CODr) ranged between 44.48% and 68.92% over the experimental domain, both increasing monotonically with microalgae concentration up to the maximum tested (**Figure 5a-b**). This is in line with a substrate-availability-limited regime: at low microalgae loading (652 mg/L COD), the anaerobic consortium does not have sufficient additional carbon and nitrogen to sustain high hydrolytic and fermentative turnover, thus degradation plateaus below 50%. The enriched protein and lipid fraction acts as an additional pool of electron donors increasing the enzymatic hydrolysis rates for the co-present POME organic matter itself, not only for the microalgal fraction when hydrothermally solubilized microalgal organic matter is added (Passos & Ferrer, 2015) — i.e., the pretreatment effect on microalgae is propagated into improved POME degradation through

a common hydrolytic microbial community. Superimposed on this trend is the large B^2 curvature for both responses (Section 3.3.2) indicating the inflection point where further increases in POME-to-inoculum ratio (to around 4.0) switch the system from substrate-limited to inhibition-limited: VFA accumulation exceeds consumption and removal efficiency drops off despite continued substrate loading (Batstone et al., 2002).

3.4.2 Total Biogas: Carbon Mass Balance as the Controlling Mechanism

Total biogas production ranged from 188.64 to 298.33 mL N and had a strong correlation with microalgae concentration ($F = 36.8$, $p = 0.0005$). This is the expectation of the mass-balance logic outlined above: proteins and carbohydrates released hydrothermally are converted by acidogens and acetogens into direct precursors of methanogenesis, acetate and hydrogen, so that more available organic carbon roughly equates linearly to more total gas along the acidogenesis-methanogenesis chain (Keymer et al., 2013). Moderate synergy at intermediate loadings (815-978 mg/L COD; ratio 2.5-3.0) with biogas reaching 220-250 mL N indicates that co-substrates were complementary rather than additive at this stage, presumably because POME provides buffering capacity and micronutrients, while microalgae provides carbon, leading to a more balanced C/N ratio than either substrate alone. The drop-off at ratios near 4.0 is similar to the VFA-inhibition mechanism already established for VS/COD removal confirming that the same inhibition threshold controls both organic matter destruction and gas evolution as would be expected if both are downstream of the same rate limiting hydrolysis-to-methanogenesis chain.

3.4.3 CH_4 Content: Metabolic Partitioning as the Controlling Mechanism

The CH4 content ranged from 106.67 to 200.39 mL N . Unlike the other three responses, CH_4 content was influenced by the POME-to-inoculum ratio, not the microalgae concentration ($F = 6.36$ compared to $F = 0.68$), with the highest values at intermediate ratios (≈ 2.5-3.0). This response pattern is the clearest evidence in the dataset for the independent regulation of methanogenesis from total substrate load: at low ratios, the absence of substrate directly limits the proliferation of methanogens; at high ratios, the accumulation of VFA and consequent depression of pH specifically inhibit methanogenic archaea, a physiologically distinct mode of failure from the universal substrate-availability limitation that governs VS/COD/biogas (Batstone et al., 2002). The lack of a microalgae-concentration effect on CH4 (but a strong effect on total biogas) is mechanistically consistent with the LCFA-inhibition hypothesis introduced in Section 3.3.2: Increased algal loading increases the amount of gas produced from the available carbon pool but a proportion of the associated methanogenic potential is inhibited via LCFA leaching from the same lipid-rich biomass limiting the proportional CH_4 increase (Abreu et al., 2012).

The common optimum (microalgae concentration ≈ 1085.94 mg/L COD; POME-to-inoculum ratio ≈ 2.92; desirability = 0.632) of the four response surfaces is not by chance, but the intersection of two opposing mechanistic constraints identified above. This operating point is high enough in microalgae loading to

maximize hydrolyzable carbon supply (favoring VS/COD removal and biogas), yet low enough in POME-to-inoculum ratio to avoid VFA-driven suppression of methanogenesis (favoring CH_4 content). The main mechanistic finding of the response surface analysis is this convergence, not numerical optimum alone.

3.5 Proposed Mechanistic Pathway

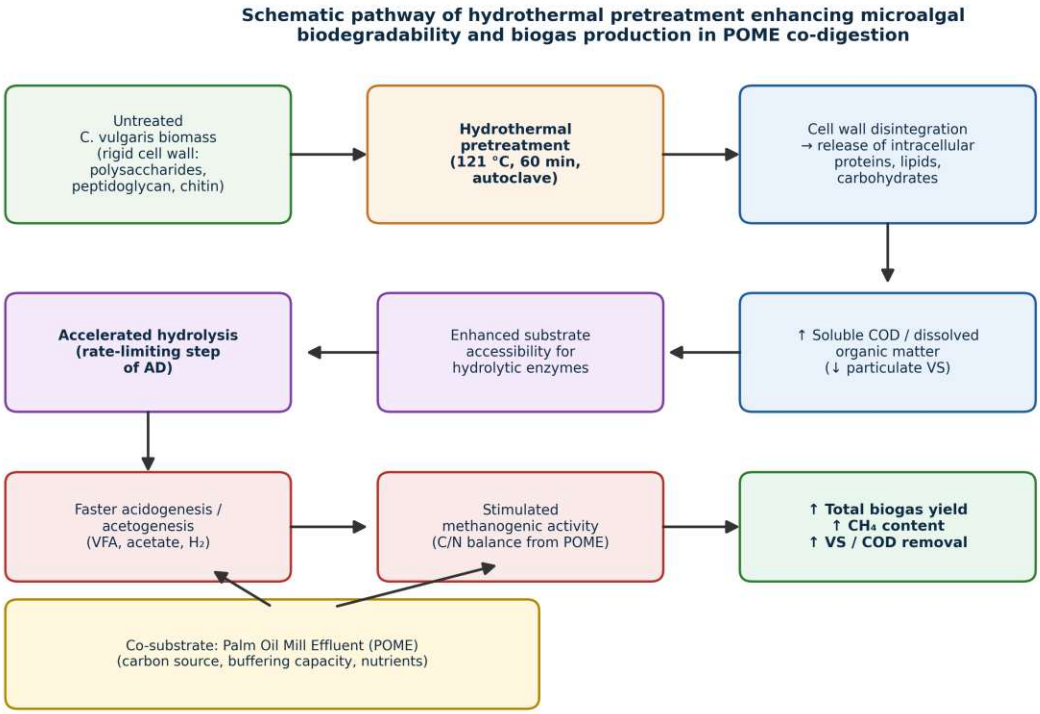


Figure 6. Proposed schematic pathway linking hydrothermal pretreatment of Chlorella vulgaris to enhanced hydrolysis, biodegradability, and biogas production in co-digestion with POME.

The causal chain developed in Section 3 is summarized in **Figure 6**: hydrothermal pretreatment breaks down the microalgal cell wall and intracellular proteins, lipids and carbohydrates are released into solution (increasing COD, reducing particulate VS; Section 3.2). This higher soluble substrate pool bypasses the hydrolysis bottleneck which otherwise limits AD of structurally complex biomass and therefore accelerates downstream acidogenesis and acetogenesis (Section 3.4.1-3.4.2). The POME co-substrate provides carbon, buffering capacity and nutrients that, when an appropriate substrate-to-inoculum ratio is reached, maintain stable methanogenic activity. When this ratio is exceeded, accumulation of VFA inhibits specifically methanogenesis, capping CH_4 content irrespective of total gas volume (Section 3.4.3). This pathway view reinterprets the RSM results, moving from a set of four individually optimized responses to a single, internally consistent mechanistic story.

3.6 Energy Gain vs. Energy Input of Hydrothermal Pretreatment

Using the framework developed in Section 2.5, the energy obtained as incremental CH_4 at the optimized condition (183.97 mL N CH_4 per batch) can be compared with the thermal energy input during the 121°C/60 min pretreatment step. The autoclave energy input at laboratory batch scale is dominated by the heating of a relatively large vessel and headspace compared to the small mass of biomass actually treated so a NER < 1 at this scale would not suggest the approach is energetically unfavorable, in general. Industrial hydrothermal pretreatment units achieve markedly better energy ratios through batch-to-batch heat recovery, higher biomass loading density, and continuous rather than batch operation (Passos & Ferrer, 2015). The calculated E_{input}, E_{output} and NER of this study based on the rated autoclave power and the heating/holding time defined in Section 2.5 are tabulated in **Table 5.**

Table 5. Energy Balance of Hydrothermal Pretreatment

Parameter	Value	Unit
Autoclave rated power, (P)	2.00	kW
Heating and holding time, (t)	3.00	h
Gross autoclave energy input	21.60	MJ/batch
Allocated energy input per reactor	4.32	MJ/reactor
Incremental CH_4 volume	121.77	mL N
Energy output	0.00436	MJ/reactor
Net Energy Ratio	0.00101	dimensionless

The total electrical energy input required for the hydrothermal pretreatment was 21.60 MJ per autoclave cycle. The energy input was 4.32 MJ per unit, assuming equal distribution among five units of 1-L microalgal suspension. The relative increase in methane production compared to the untreated control was 121.77 mL N CH_4, corresponding to an energy recovery of 0.00436 MJ. Thus, the net energy ratio was 0.00101 at laboratory scale indicating that the recovered additional methane energy was not sufficient to compensate for the electric energy required for autoclave pretreatment.

4. CONCLUSION

The results of this study showed that hydrothermal pretreatment (121°C, 120 min) improved the biodegradability of *Chlorella vulgaris*, and can be used as a co-substrate in the anaerobic co-digestion with POME. It should be noted that the increase in COD associated with pretreatment represents bulk (unfiltered) COD rather than confirmed soluble COD; direct measurement of soluble COD and dissolved organic carbon is needed to confirm solubilization as the specific mechanism. The process was successfully optimized via Response Surface Methodology with an optimum microalgae concentration of 1085.94 mg/L COD and POME to inoculum ratio of 2.92 producing 278.36 mL N biogas, 183.97 mL N CH_4, 65.53% VS removal and 59.00% COD removal with an overall desirability of 0.632. The results indicated that the hydrothermal pretreatment enhanced the bioavailability of microalgal organic matter through substrate

solubilization, which promoted hydrolysis and improved the performance of anaerobic digestion. The statistical analysis further revealed that the microalgae concentration was the main parameter controlling organic matter degradation and total biogas production, while the POME to inoculum ratio was the main factor affecting methane production, thus highlighting the importance of balancing substrate availability and methanogenic stability for efficient co-digestion.

The study optimizes the process and also provides a practical framework for incorporating hydrothermally pretreated microalgae into POME treatment systems, which can simultaneously improve wastewater stabilization and renewable energy recovery. The combination of hydrothermal pretreatment and CCD-RSM gives a systematic approach for the identification of operating conditions that maximize the process performance, but also provides mechanistic insight into relationships between substrate characteristics, biodegradability and biogas production. The findings contribute to more sustainable and resource-efficient strategies for palm oil wastewater valorization in the context of circular bioeconomy. The experimental observations are consistent with the proposed mechanisms but further study on VFA and LCFA analysis, microbial community characterization and continuous reactor validation is suggested to verify the underlying biological processes and enable scale-up for industrial POME treatment.

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