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Experimental Assessment of Mesophilic and Thermophilic Batch Fermentative Biohydrogen Production from Palm Oil Mill Effluent Using Response Surface Methodology

Azam Akhbari*,†, Shaliza Ibrahim,†, Low Chin Wen**, Afifi Zainal***, Noraziah Muda***, Liyana Yahya***, Onn Chiu Chuen****, Farahin Mohd Jais**** and Mohamad Suffian bin Mohamad Annuar*****

*Higher Institution Centre of Excellence (HICoE), UM Power Energy Dedicated Advanced Centre (UMPEDAC), Level 4, Wisma R&D, University of Malaya, Jalan Pantai Baharu, 59990, Kuala Lumpur, Malaysia
**Institute of Ocean and Earth Sciences (IOES), University of Malaya, Kuala Lumpur 50603, Malaysia
***TNB Research Sdn.Bhd., No 1, Lorong Ayer Itam, Kawasan Institusi Penyelidikan, 43000, Kajang Selangor
****Department of Civil Engineering, Faculty of Engineering, University of Malaya, Kuala Lumpur 50603, Malaysia
*****Institute of Biological Sciences, University of Malaya, Kuala Lumpur 50603, Malaysia*

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Abstract – The present work evaluated the production of biohydrogen under mesophilic and thermophilic conditions through dark fermentation of palm oil mill effluent (POME) in batch mode using the design of experiment methodology. Response surface methodology (RSM) was applied to investigate the influence of the two significant parameters, POME concentration as substrate (5, 12.5, and 20 g/l), and volumetric substrate to inoculum ratio (1:1, 1:1.5, and 1:2, v/v.%), with inoculum concentration of 14.3 g VSS/l. All the experiments were analyzed at 37 ℃ and 55 ℃ at an incubation time of 24 h. The highest chemical oxygen demand (COD) removal, hydrogen content (H_2%), and hydrogen yield (HY) at a substrate concentration of 12.5 g COD/l and S:I ratio of 1:1.5 in mesophilic and thermophilic conditions were obtained (27.3, 24.2%), (57.92, 66.24%), and (6.43, 12.27 ml H_2/g COD_{rem}), respectively. The results show that thermophilic temperature in terms of COD removal was more effective for higher COD concentrations than for lower concentrations. Optimum parameters projected by RSM with S:I ratio of 1:1.6 and POME concentration of 14.3 g COD/l showed higher results in both temperatures. It is recognized how RSM and optimization processes can predict and affect the process performance under different operational conditions.

Key words: Biohydrogen production, Mesophilic, Thermophilic, Dark fermentation, Palm oil mill effluent

1. Introduction

Palm oil mill effluent (POME) as a renewable resource for biogas production has received attention in Malaysia. It is projected for each ton of crude palm oil, around 3.5 tons of POME are produced [1,2]. POME is a complex thick brownish effluent discharged from the palm oil mill industry. It is not toxic, but due to its high organic content, is considered particularly contaminating [3]. Its characterization might vary according to the operational process and raw material used. This industrial effluent has been projected as a potential substrate for biohydrogen production due to its large quantity, low cost, and reliability [4]. Among different biological methods, dark fermentation is the most studied and promising method for biohydrogen production [4]. In this process, microorganisms in pure or mixed culture are responsible for treating wastewater and producing biohydrogen simultaneously [1]. Biohydrogen has been considered as the most capable energy carrier among all the current fuels. To produce hydrogen, it is required to remove H_2-consuming bacteria (HCB) from the anaerobic sludge as a mixed culture medium, avoiding methanogenesis bacteria [1]. Several types of pre-treatments can be preferred considering the microflora in the inoculum [5]. To develop H_2 production efficiency, some key parameters need to be studied. The factors that mostly affect the hydrogenase enzymes activity are pH, temperature, types of substrate and substrate concentration [6].

It is essential to control pH value in an optimum range to preserve hydrogen production. During the building up the hydrogen through dark fermentation process, volatile fatty acids such as butyric and acetic acids with high molecular weight are accumulated, resulting in a pH drop in the system. Hence, it is necessary to control the pH in the desired range, mostly between 5 to 6; otherwise it will confine microbes from growing and stop hydrogen production [1,3]. Biohydrogen production process through dark fermentation could be produced in a different range of temperatures, including mesophilic, thermophilic, and extreme thermophilic. From economical and technological points of view, the mesophilic condition is desirable to the thermophilic condition; however, thermophilic dark fermentation, due to a higher amount of hydrogen yield and production rate, shows more potential than the mesophilic conditions [7]. Moreover, the rate of biochemical processes is influenced by temperature due to the effects of the enzymatic activity. Whereas substrate concentration affects the metabolic pathways of microbial community structures.

†To whom correspondence should be addressed.
E-mail: Shaliza@um.edu.my, azamakhbari@um.edu.my

The ideal temperature for this process using mixed culture differs generally due to the complex bacterial communities, while in some cases the process under thermophilic conditions was found to be more favorable [8]. Therefore, to additionally improve biohydrogen production, these factors should be optimized. To the best of our knowledge, there is a lack of research concerning the application of the modeling techniques in the dark fermentation process and pre-design of the experiments before the lab study. More importantly, the importance of the operational factors should be studied to back up the experimental design and optimization of operating conditions. Hence, statistical modeling is a useful tool that delivers a superior consideration of how diverse variables can affect biological methods. Response surface methodology has been recognized as an efficient method to evaluate optimal conditions. It considers statistical approaches for experimental design, examining the effect of experimental factors and probing for the optimum conditions [9]. The benefit of using RSM is to reduce the test run numbers to assess some factors and relations. Moreover, this less time-consuming method is capable of studying several factors simultaneously. The present study considered the application of the RSM on hydrogen production from POME at mesophilic and thermophilic conditions and recognized the best approximation of the operating variables affecting this process by dark fermentation.

2. Materials and Methods

The collection location of anaerobic sludge, POME, and methods that were used to prepare the samples are given in our previous published paper [1]. The palm oil mill effluent pond was shown in Fig. 1. The characteristics of the anaerobic sludge used were as follows: pH = 8.55 ± 0.1, total suspended solids (TSS) = 40.2 g/l, and volatile suspended solids (VSS) = 14.3 g/l. The samples were allowed to settle before use due to the high amounts of suspended solids. Hence, the supernatant was used as a substrate. The POME substrate with a COD concentration of 50.63 g/l was diluted to prepare the three different COD concentrations of 5, 12.5, and 20 g/l. Triplicates of each evaluated condition were included, as well as endogenous control (without substrate). The necessary amount of inoculum was added to each serum bottle to maintain the F/M ratio (substrate/inoculum, ml POME ml VS), considering a working volume of max 120 ml with three volumetric ratios 1:1, 1:1.5, 1:2. The initial pH was adjusted to 5.5 using $NaHCO_3$ 2.0 g/l. The bottles were incubated at 37 ℃ and 55 ℃ with an orbital shaking of 150 rpm. Table 1 provides the POME characterization. The analytical method used for the characterization of POME can be found in our previous study [1].

Fig. 1. Palm oil mill effluent (POME) pond for sampling.

2-1. Experimental Design and Mathematical Model

Response surface methodology (RSM) comprises statistical approaches that are used to evaluate, design, create models, and study the correlation between the input parameters and responses. In this study, RSM was used to determine which variables have a significant impact on the process. RSM includes three main steps conducting the statistical design of the experiments, evaluating the mathematical modes coefficients, predicting the responses, and investigating the model fitness. Central composite design (CCD) is the most often used strategy under RSM to study the effect of the input parameters and output responses in the optimization process.

Table 1. POME characteristics [1]

Parameter	Concentration	Regulatory discharge limits	Unit
Major characteristics			
Temperature	80-90	40	℃
pH	4.6 ± 0.5	6-9	-
Oil and Grease (O&G)	4100 ± 20	1	mg/l
Biochemical oxygen demand (BOD_5)	25000 ± 1000	100	mg/l
Total Chemical oxygen demand (TCOD)	56000 ± 2000	50	mg/l
Total solid (TS)	42000 ± 1000	-	mg/l
Total suspended solids (TSS)	19000 ± 500	50	mg/l
Total volatile solids (TVS)	34000 ± 700	-	mg/l
NH_3-N	33 ± 1	-	mg/l
Total kjelhdal nitrogen (TKN)	725 ± 5	-	mg/l
Turbidity	680 ± 5	-	NTU

Table 2. Selected parameters optimized by RSM experiments

RSM parameters	Unit	Tested range Low (-1)	Middle (0)	High (+1)
A: Substrate concentration	g/l	5	12.5	20
B: S: I ratio	(v/v,%)	1:1	1:1.5	1:2

Incubation time: 24h

The CCD is according to the multi-variant non-linear model for the optimization process, and from appropriate trials the regression model equations can be estimated [9,10]. A set of two parameters were optimized using RSM-CCD to validate the interaction influence of the nominated range on the responses. From the experiment, a design

Table 3. ANOVA results of mesophilic condition

Response	Source	Sum of squares	DF	F-value	Prob>F	R^2	Adj-R^2	Adeq-Precision	SD
COD removal	Model	170.71	4	7.19	0.0092	0.78	0.67	8.13	2.44
	A	117.18	1	19.75	0.0022				
	B	15.53	1	2.62	0.1444				
	A^2	26.13	1	4.40	0.0691				
	AB	11.88	1	2.0	0.1948				
Lack of fit		37.72	4	3.87	0.1090				
Pure error		9.74	4	-	-				
Model Equation +27.62-24.42*A-1.61*B-5.80*A^2-2.84*B^2-1.72*A*B									
H_2 content	Model	2039.90	4	8.15	0.0063	0.80	0.70	8.69	7.91
	A	785.93	1	12.56	0.0076				
	B	22.12	1	0.35	0.5685				
	A^2	1231.82	1	19.69	0.0022				
	B^2	183.28	1	2.93	0.1253				
Lack of fit		348.87	4	2.30	0.2197				
Pure error		151.60	4	-	-				
Model Equation +42.45+11.44*A-1.92B-21.12*A^2+8.15*B^2									
HY	Model	12.97	5	7.73	0.0091	0.84	0.73	9.08	0.58
	A	6.2	1	18.48	0.0036				
	B	2.65E-003	1	7.92E-003	0.9316				
	A^2	0.69	1	2.05	0.1958				
	B^2	3.33	1	9.94	0.0161				
	AB	0.71	1	2.12	0.1891				
Lack of fit			3	0.44	0.7381				
Pure error			4	-					
Model Equation +8.28+1.02*A+0.021*B-0.5*A^2-1.1*B^2-0.42AB									

Table 4. ANOVA results of Thermophilic condition

Response	Source	Sum of squares	DF	F-value	Prob>F	R^2	Adj-R^2	Adeq-Precision	SD
COD removal	Model	58.28	3	4.16	0.0419	0.58	0.44	5.7	2.16
	A	0.59	1	0.13	0.7310				
	B	12.24	1	2.62	0.1401				
	A^2	45.45	1	9.72	0.0124				
Lack of fit		34.31	5	3.53	0.1225				
Pure error		7.77	4	-	-				
Model Equation +25.98+0.31*A-1.43*B-3.75*A^2									
H_2 content	Model	2854.01	3	9.07	0.0044	0.75	0.66	6.58	10.2
	A	908.97	1	8.66	0.0164				
	B	0.45	1	4.27E-003	0.9493				
	A^2	1944.59	1	18.54	0.0020				
Lack of fit		528.64	4	1.02	0.5073				
Pure error		415.49	3	-	-				
Model Equation +53.86+12.31*A-0.27B-24.53*A^2									
HY	Model	17.21	3	1.31	0.3287	0.30	0.07	3.768	2.09
	A	0.78	1	0.18	0.6818				
	B	11.43	1	2.62	0.1400				
	A^2	5.00	1	1.15	0.3124				
Lack of fit		35.28	5	7.07	0.0407				
Pure error		3.99	4	-	-				

Significant lack of fit was bad, the model could not be fit that consisted of 13 runs with five replications at the middle level was generated. The independent operating variables including A: Substrate concentration and B: S:I ratio were optimized on hydrogen content, hydrogen yield (HY), and COD removal as responses. Each run was conducted three times at their related HRT. Each factor was evaluated at three different levels of low (-1), center (0), and high (+1). The coded and the actual values of the variables tested are provided in Table 2. The batch performance was analyzed according

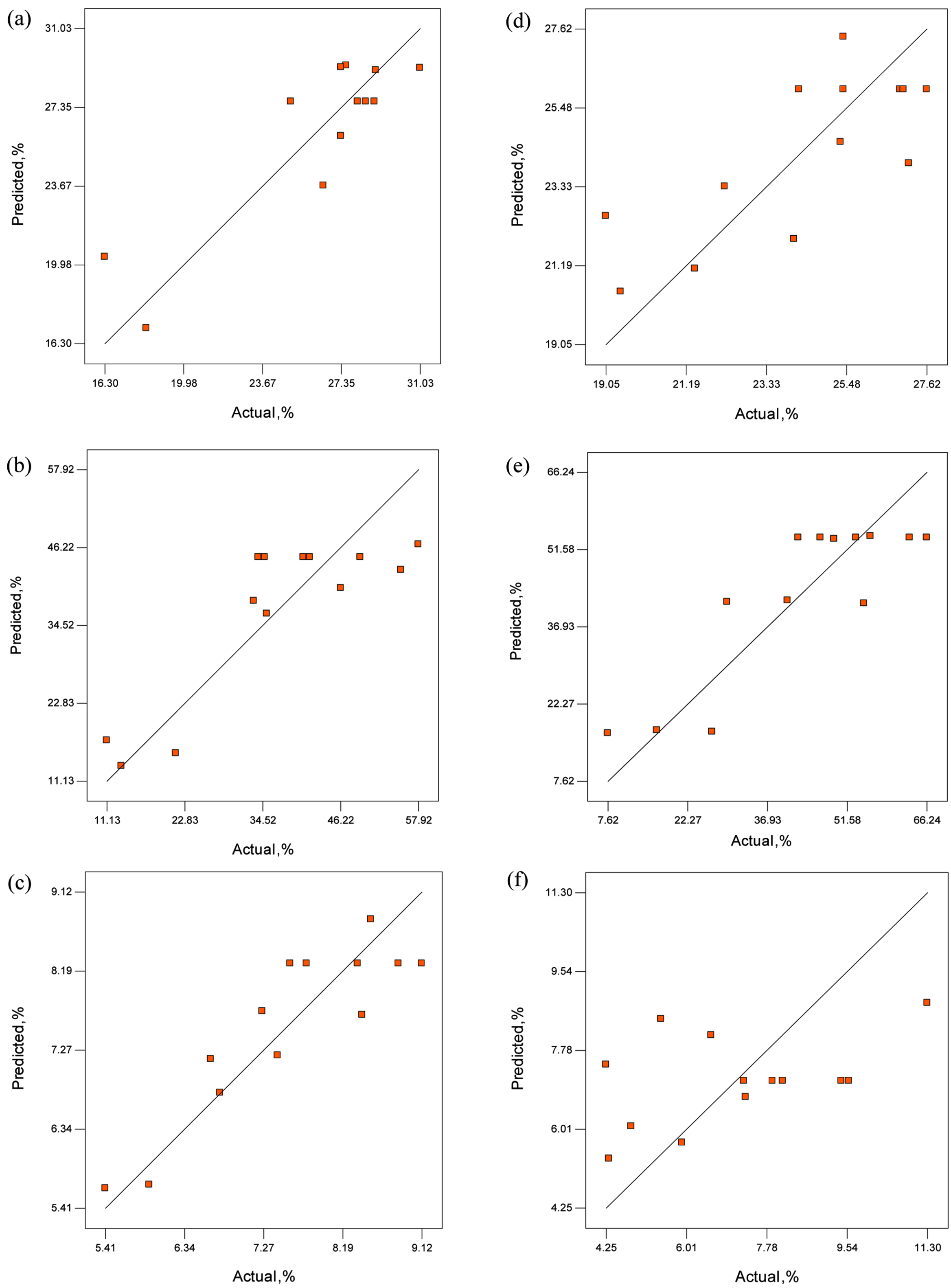

Fig. 2. Predicted vs. actual values plots for the mesophilic condition: (a) COD removal, (b) H_2 content, (c) HY; and thermophilic condition: (d) COD removal, (e) H_2 content, (f) HY.

to the CCD experimental design provided in Table 3 and Table 4. After setting the experiments, the polynomial model coefficients were calculated using the polynomial equation. The insignificant interactions of model terms were removed, and models were selected according to the probability value (p-value) with a confidence level of 95%. As shown in Table 3 and Table 4, the results were assessed by analysis of variance (ANOVA). The three-dimensional (3D) plots were depicted based on the influence of the two-variable level.

2-2. Analytical analysis

Hydrogen volume was measured using the water displacement method. Biogas content was measured using Perkin Elmer Pte Ltd Gas Chromatography (600 Series). The details of the GC were described in the previous study [1,3]. Other chemical parameters were measured according to the APHA Standard Method [11].

3. Results and Discussion

3-1. Statistical analysis

The design of experiment (DoE) models were considered statistically using analysis of variance (ANOVA). The summary of ANOVA results from mesophilic and thermophilic temperature are provided in Table 3 and Table 4, respectively. The coefficient of determination (R^2) was between 0.87-0.84 in mesophilic conditions, and 0.58-0.75 in thermophilic, which shows a relatively good degree of correlation between the experimental and predicted values in mesophilic but not considerable in thermophilic conditions. The model for all responses in mesophilic condition was significant, and the lack of fit was not significant. Meanwhile, the predicted R^2 and adjusted R^2 were within reasonable agreement; while the statistical results for hydrogen content in thermophilic condition were in good agreement, the other two responses could not be fitted well by the model. In this study, quadratic models were used to fit the data at a higher degree of polynomial equations. Here, the quadratic process order of the models was statistically significant confirmed by the F-values and p-values given in Table 3, and Table 4. Figure 2 shows the predicted vs. actual plots for the optimization stage for the responses to demonstrate the ANOVA's suitability for mesophilic and thermophilic conditions. Data points are located more closely to the trend line, which indicates the errors have been distributed normally.

3-2. Modeling and process analysis

The fundamentals of Design-Expert Software, RSM, and analysis of variance (ANOVA) can be found in our previous published paper [8]. The two effective variables, i.e., substrate concentration (A), and volumetric S:I ratio (B), were used to determine the responses. The variable ranges selected were (5, 12.5, and 20; g/l), (1:1, 1:1.5, and 1:2; v/v%), respectively. The RSM results for mesophilic and thermophilic conditions are provided in Table 5. The components of generated biogas were H_2, CO_2, and N_2, without CH_4 detection. The effects of parameters are shown as three-dimensional graphs in Figure 3. A batch study on biohydrogen production using high POME concentration as a substrate with a COD concentration of 32-86 g COD/l reported a COD removal of approximately 37% with the maximum hydrogen yield of 5.98 L H_2 L-med at 10% POME sludge [12]. In another study using POME at a low COD concentration of 3-10 g/l as substrate reported the highest hydrogen yield of 124.48 mmol H_2 g-1 COD removed with COD removal of 54.2% [13]. In this study, the substrate concentrations of POME were varied from a low concentration of 5 g COD/l to a high concentration of 20 g COD/l. From Figures 3(a) and (d) at mesophilic temperature more COD removal efficiency was achieved with a decrease in substrate concentration from 20 g/l to 12.5 g/l. From the plots, COD removal improved slightly with a decrease in S:I ratio from 1:2 to 1:1. The analysis of three-dimensional plots for thermophilic conditions shows an elongation diagonally in both directions. Maximum COD removal was obtained 28.96% for a POME concentration of 5 g COD/l at mesophilic and 27.77 for 12.5 g COD /l under thermophilic conditions. It seems that at higher substrate concentrations organic acids accumulate, which results in a pH drop and might stop the metabolism of the

Table 5. Experimental condition and results at the mesophilic and thermophilic condition

	Input Factors		Responses Mesophilic Temperature			Responses Thermophilic Temperature		
Run No.	Substrate conc., g/l	S:I, %V/V	H_2 Content, %	COD removal, %	Hydrogen Yield, ml H_2/g COD_{rem}.l	H_2 Content, %	COD removal, %	Hydrogen Yield, ml H_2/g COD_{rem}.l
1	12.50	1:1.5	41.6	25.00	9.12	66.20	24.20	9.57
2	12.50	1:1.5	49.2	28.50	7.58	63.10	27.80	7.90
3	12.50	1:1.5	40.6	28.12	8.37	53.30	26.90	9.41
4	12.50	1:2	55.3	27.34	7.43	49.20	25.30	5.45
5	20.0	1:1	46.3	26.52	8.42	40.60	27.10	4.31
6	20.0	1:1.5	33.2	16.30	8.52	29.60	19.00	7.31
7	20.0	1:2	35.2	18.24	7.25	54.70	21.40	6.55
8	12.50	1:1.5	33.9	28.12	8.84	46.70	25.40	8.12
9	5.0	1:1	11.1	28.96	5.41	16.60	22.20	4.80
10	5.0	1:2	13.3	27.58	5.92	7.60	19.40	11.30
11	5.0	1:1.5	21.5	31.03	6.75	26.80	24.10	4.24
12	12.50	1:1	57.9	27.34	6.64	55.90	25.40	5.91
13	12.50	1:1.5	34.9	28.90	7.77	42.60	27.60	7.27

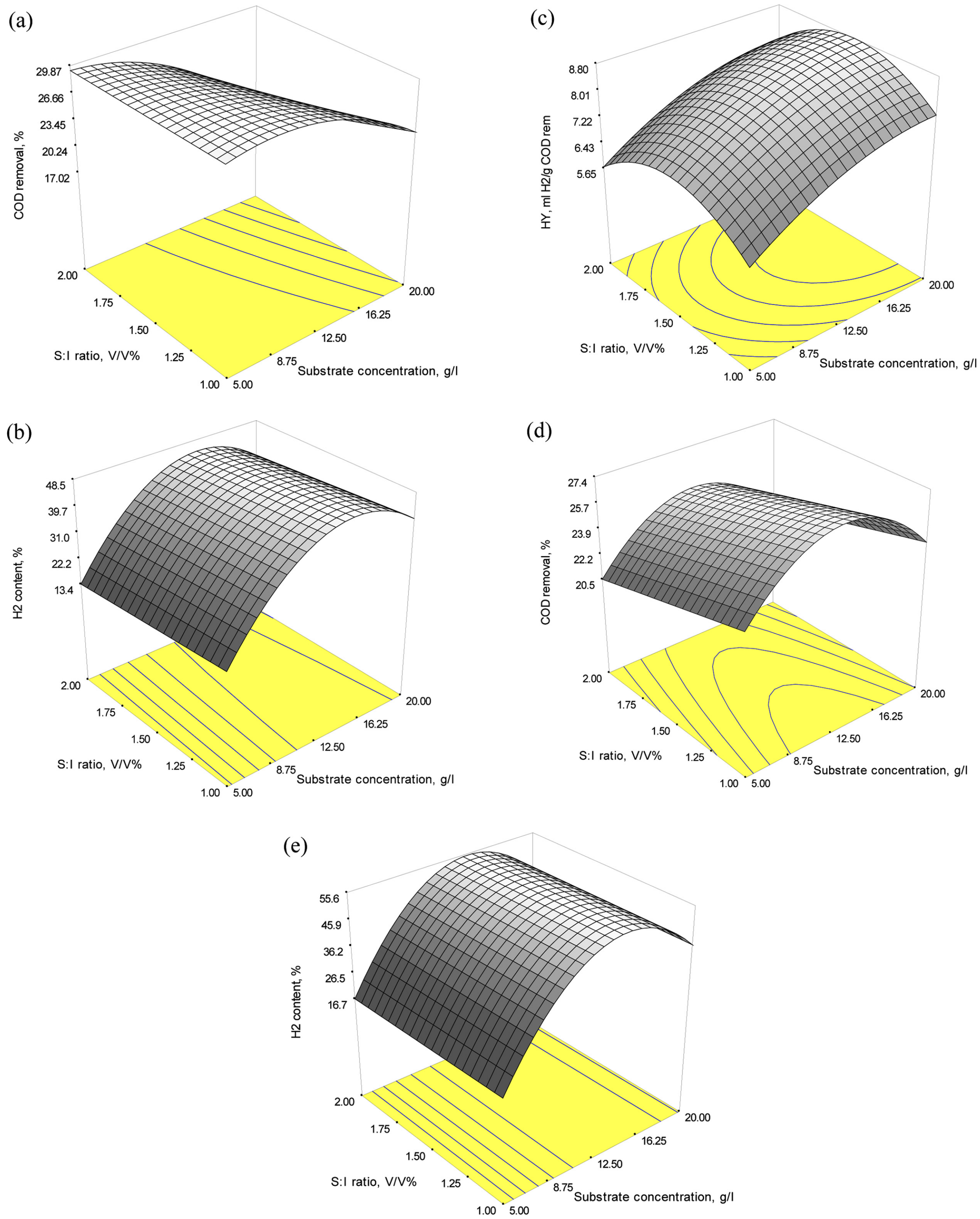

Fig. 3. Three-dimensional plots of the Substrate concentration and S:I for responses; Mesophilic condition (a) COD removal, (b) H_2 Content, (c) HY; Thermophilic condition, (d) COD removal, (e) H_2 Content.

HPB. The COD removal increment could be due to the formation of simple intermediates extended from the degradation of complex components in POME. From the results, it can be seen that at a higher temperature more COD removal can be achieved at a higher POME concentration, which might be due to more bacteria activity under thermophilic conditions. The effect of substrate concentration and S:I ratio on hydrogen content showed the same trend for both mesophilic and thermophilic conditions, which indicates the response increases with increasing substrate concentration from 5 g/l to 12.5 g/l and S:I from 1:1.5 to 1:2. Maximum H_2 content obtained was

55.3% at 37 ℃ at 12.5 g/l and S:I of 1:2, over incubation time of 24h, while the highest amount achieved for the thermophilic condition was 66.24% at 12.5 g/l and S:I ratio of 1:1.5. The mesophilic and thermophilic conditions in anaerobic treatment give different effects on the COD removal, biohydrogen production yield of POME. The effects of temperature (25-40 ℃) on hydrogen production were examined by Oh *et al*. [14] which concluded the increase in temperature from 25 to 36 ℃ improved the cell growth rate and hydrogen production rate. Another study reported that a temperature above 35 ℃ may inhibit the growth of the granular sludge [15]. Meanwhile, with the thermophilic conditions, it was concluded that this condition is good for POME to be converted to biohydrogen, as it has less variety of end-products, and thermodynamic conditions as well. In this study, increasing the temperature from 37 ℃ to 55 ℃ improved the H_2 content (Figure 3(b) and (e)). Maximum hydrogen yield was obtained 9.12 ml H_2/g COD_{rem} in the middle point of substrate concentration and S:I ratio at mesophilic temperature. The design of the experiment could not provide a graph for thermophilic temperature due to the low regression coefficient (R^2) and bad significant lack of fit. However, thermophilic conditions provided a higher yield and hydrogen content in comparison with mesophilic ones, which might be due to the enhanced hydrogen producer activity.

3-3. Process optimization

In a process involving various responses, finding areas where necessities concurrently come across the critical features is required [8]. Hence, an overlay plot from a graphical optimization is plotted to show the region of possible response values in the space. Figure 4 clarifies the overlay plot of the optimized responses. To endorse the reliability of the models' estimations, three maximum points for confirmation tests were selected within the optimal area (yellow color at S:I ratio 1:1.21, 1:1.58, and 1:1.81 v/v%, with corresponding substrate concentrations of 15.6, 14.3, and 15.4 g/l, respectively). Experimental data were compared to its corresponding estimated value provided in Table 6. The optimum value for the mesophilic condition with S:I ratio 1:1.6 v/v% and substrate concentration 14.3

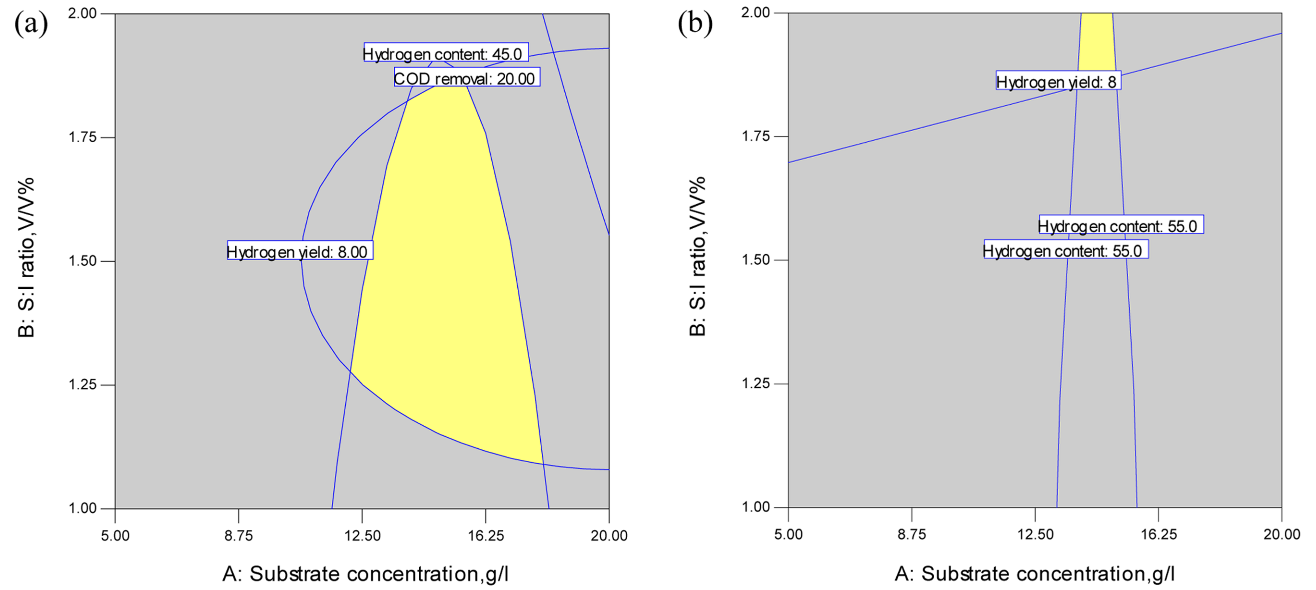

Fig. 4. Overlay plot of the optimization process for a) Mesophilic, and b) Thermophilic conditions.

Table 6. Confirmation experiments at optimum conditions for Mesophilic and Thermophilic conditions

Run conditions			Mesophilic condition		
			H_2 content, %	COD Removal, %	HY, ml H_2/g COD_{rem}
1	S:I= 1:1.21 Substrate concentration=15.6	Experimental values Predicted values -	53.0 47.54 STDEV = ±3.86	25.20 26.64 STDEV = ±1.01	8.32 8.22 STDEV = ±0.07
2	S:I=1:1.6 Substrate concentration=14.3	Experimental values Predicted values -	60.0 46.18 STDEV = ±9.77	24.2 26.05 STDEV = ±1.3	8.80 8.47 STDEV = ±0.23
3	S:I= 1:1.8 Substrate concentration=15.5	Experimental values Predicted values -	52.0 45.28 STDEV = ±4.75	23.50 23.99 STDEV = ±0.34	8.35 8.18 STDEV = ±0.12
Run conditions			Thermophilic condition		
			H_2 content, %	COD Removal, %	HY, ml H_2/g COD_{rem}
1	S:I= 1:1.9 Substrate concentration=14.3	Experimental values Predicted values -	72.0 55.16 STDEV = ±11.9	25.3 24.59 STDEV = ±0.5	8.60 8.19 STDEV = ±0.28

g/l resulted in hydrogen content, COD removal, and HY of 60%, 24.2%, and 8.8 ml H_2/g COD_{rem}, respectively. For the thermophilic condition, one point was selected in the yellow area for optimizing the hydrogen content corresponding to the input variables. The standard deviation was calculated to check the accuracy of the optimized responses. The experiment and prediction data were almost close. This verified that the optimization of the biohydrogen production in the optimization stage using RSM for mesophilic conditions was more applicable.

4. Conclusions

The optimization of operating parameters--POME concentration and the substrate-to-inoculum ratio at mesophilic and thermophilic temperature by RSM--allowed for maximizing the biohydrogen production. From the results, it can be realized how POME concentration and the volumetric size of the inoculum and substrate affect the responses. Operation of the batch serum bottles through dark fermentation process at such optimal conditions showed higher results at both temperatures. Maximum process responses for mesophilic conditions, including hydrogen content, hydrogen yield, and COD removal, were obtained at a substrate concentration of 14.3 g/l and S:I ratio 1:1.6. Thermophilic temperature in terms of COD removal was more effective for higher COD concentrations than lower concentrations, and more hydrogen content was obtained compared to the mesophilic temperature. The results obtained indicate that the POME concentration and the operational temperature have a significant influence in affecting the conversion of POME into biohydrogen.

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Authors

Azam Akhbari: Lecturer, Higher Institution Centre of Excellence (HICoE), UM Power Energy Dedicated Advanced Centre (UMPEDAC), Wisma R&D, University of Malaya, Kuala Lumpur 59990, Malaysia; azamakhbari@um.edu.my
Shaliza Ibrahim: Professor, Institute of Ocean and Earth Sciences (IOES), University of Malaya, Kuala Lumpur 50603, Malaysia; shaliza@um.edu.my
Low Chin Wen: Ph.D. Candidate, Institute of Ocean and Earth Sciences (IOES), University of Malaya, Kuala Lumpur 50603, Malaysia; geoffrey.low@hotmail.com
Afifi Zainal: Researcher, TNB Research Sdn.Bhd., No 1, Lorong Ayer Itam, Kawasan Institusi Penyelidikan, 43000, Kajang Selangor; afifi.zainal@tnb.com.my
Noraziah Muda: Researcher, TNB Research Sdn.Bhd., No 1, Lorong Ayer Itam, Kawasan Institusi Penyelidikan, 43000, Kajang Selangor; noraziah.omar@tnb.com.my

Liyana Yahya: Researcher, TNB Research Sdn.Bhd., No 1, Lorong Ayer Itam, Kawasan Institusi Penyelidikan, 43000, Kajang Selangor; liyana.yahya @tnb.com.my
Onn Chiu Chuen: Associate Professor, Department of Civil Engineering, Faculty of Engineering, University of Malaya, Kuala Lumpur 50603, Malaysia; onnchiuchuen@um.edu.my
Farahin Mohd Jais: Senior Lecturer, Department of Civil Engineering, Faculty of Engineering, University of Malaya, Kuala Lumpur 50603, Malaysia; arahin.mohdjais@um.edu.my
Mohamad Suffian bin Mohamad Annuar: Professor, Institute of Biological Sciences, University of Malaya, Kuala Lumpur 50603, Malaysia; suffian_annuar@um.edu.my