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Thermophilic biohydrogen production from optimized enzymatic pretreatment of palm oil mill effluent via box-behnken design

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ABSTRACT

The demand for energy is increasing continuously due to the vast developments in many ways of life. Palm oil mill effluent (POME) seems to be a good carbon feedstock for hydrogen generation in fermentation processes. A biological pretreatment was applied to degrade the lignocellulose biomass in the anaerobic digestion process and improve the bioenergy production yield; one of the techniques is to use enzymes. In this study, enzymatic pretreatment was applied to POME to determine the optimum process parameters for producing reducing sugars (RS) to be used as a substrate in biohydrogen production. The Box-Behnken design (BBD) was used to construct an experiment to optimize the pretreatment variables, such as reaction time (h), enzyme concentration (% w/v), and pH. The optimum experimental conditions were found to be 12 hr of reaction time, an enzyme concentration of 3.76% w/v, and pH 5. The result showed that POME treated with the optimal enzymatic pretreatment increased the RS content by 182%. Next, thermophilic biohydrogen production using a pretreated substrate was carried out at a temperature of 55 °C, mixing speed of 150 rpm, Chemical Oxygen Demand (COD) concentration of 29,100 mg/L, seed content of 18.2%, and initial pH 7.14. The biohydrogen production potential (H_{max}) was significantly increased by 145% (177 mL H_2/g reducing sugar) by using enzymatically pretreated POME as a substrate. This result indicated that the recovery of RS from recalcitrant POME via enzymatic pretreatment could enhance biohydrogen production. Hence, it is a useful proposal for further application in bioenergy conversion from organic waste.

Introduction

Agricultural residue provides a large amount of lignocellulosic biomass generated during the harvesting and processing of different crops, such as corn, sugarcane, rapeseed, soybean, maize, and palm oil [7]. World palm oil production has increased to 70.29 million metric ton per year since 1980, accounting for 36.4% of the total world vegetable oil production. Other vegetable oil production, such as soybean oil, rapeseed oil, and sunflower oil, accounts for 27.5%, 13.1%, and 10%, respectively [41].

Palm oil industries generate lignocellulosic biomass wastes such as 23% of empty fruit bunch (EFB), 12% of mesocarp fiber, 5% of shell, and 60% of palm oil mill effluent (POME) from each tonne of fresh fruit bunch (FFB) processed [32]. More than half of the waste is turned into POME which is a liquid by-product produced from the sterilization and milling process of fresh fruit bunch (FFB) [14]. The fresh POME or anaerobically digested sludge of POME has a considerable amount of organic materials. The dried fresh POME contains 38.4% cellulose, 23.2% hemicellulose, and 26.7% lignin, while the dried digested sludge comprises 10.45% cellulose, 6.01% hemicellulose, and 48.13% lignin [4]. Cellulose has recalcitrant characteristics in terms of its rigidity, complexity, and arranged crystallinity [34,36]. The hemicellulose causes additional recalcitrance by limiting access to cellulose by binding to lignin from the attachment of ferulates and arabinoxylans [9,48]. The complex structure of POME inhibits hydrolysis during the anaerobic digestion (AD) [20] and would limit the release of the intercellular materials in the cell wall [24]. The AD process takes longer time with untreated POME. Pretreatment is thus needed for the isolation of polysaccharides, reduction of crystallinity, breakdown of cell wall structures, and the increase of accessibility and porosity to make the raw material consumable by the microbial group [3,15,23].

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The RS are the reactive molecules that can be converted into fuels, chemicals, food, and animal feed via a biological or chemical process [47]. As in the natural ecosystem, RS is often stored in the polysaccharide form of grains like corn, wheat, and rice. Palm oil mill effluent contains complex cellulose and polysaccharides, which can be further converted into RSs that favor biohydrogen production [35]. Khadaroo et al. [25] employed thermal pretreatment at 120 ˚C on POME, which increased methane yield by as much as 9 times. The maximum biogas production of 1471 mL was achieved using thermal pretreatment at a 20:80 solid/liquid sample ratio. The ratio was calculated after the pretreated samples were allowed to settle, where the solid part is the settled suspension and the liquid part is the clear liquor phase. Chaiprapat and Laklam [5] observed that the pretreatment of POME by ozone yielded higher COD removal in an anaerobic sequencing batch reactor.

(ASBR) at an organic load of 6.52–9.04 kg COD/m^3/d. A similar study using ASBR reported an increase in methane yield of 21.5% and a maximum organic degradation of 96% at 30 ˚C [19]. Mahmod et al. [28] studied acid pretreatment, which yielded increases in hydrogen yield by as much as 97% and 65% using phosphoric and nitric acids, respectively. At 0.8% w/v of phosphoric acid and 18.47 g/L of the initial RS, they achieved a hydrogen yield of 1.24 mol H_2/mol glucose, which corresponded to the maximum hydrogen production of 0.181 mmol/L/h.

Among the aforementioned pretreatment methods, enzymatic pretreatment has been recognized as a useful pretreatment for POME, since it does not corrode equipment but uses less energy [24,49]. The most common enzymes used in the pretreatment of lignocellulosic materials are cellulase [33], xylanase [37], lipase [44], vinasse [52] and laccase [39].

The response surface methodology (RSM) is an efficient procedure and robust in making an approximation and optimization of the stochastic models [10]. Among the common RSMs used in process optimization are Central Composite Design (CCD), Doehlert Design, and Box-Behnken Design (BBD) [26,31]. The BBD design is slightly more efficient than CCD. In terms of model calculation, BBD is known about 11.5% more efficient than CCD, which means the former requires fewer experimental runs than the latter [11]. Relative to the one factor in time-based optimization, in the case of monitoring four variables at four different levels, the BBD design of the experiment requires just 29 experimental runs, including the 5 center points, while a full four-level factorial design needs 256 experimental runs [13]. The advantages of RSM are requiring less effort, expense, and time derived from fewer experiments while giving all the necessary information to design a process of interest [17].

Thermophilic biohydrogen is suitable to treat POME since the latter is discharged at a high temperature ranging between 70 and 80 ˚C [6,21]. The thermophilic process can facilitate a higher biogas production and higher substrate degradation relative to the mesophilic process [18,22]. Besides, the concentration of volatile fatty acids (VFAs) as the main inhibitor of biohydrogen production remains low under the thermophilic condition [30]. Therefore, this study aimed to optimize the RS recovery from POME as a platform to enhance the biohydrogen production through the enzymatic pretreatement using Cellulase Novozym 50199. The Box-Behnken design (BBD) was utilized to design experiments and determine the optimal working parameters, including reaction time (in hours), pH, and enzyme concentration (% w/v). The reducing sugar (RS) content of the pretreated POME was determined using the dinitrosalycyclic acid (DNS) method, and a dataset was constructed based on these measurements. Using the experimental data from the BBD run points, an enzymatic pretreatment model was developed to detect the optimal conditions and reveal the relationship between independent variables and outputs. The optimization process resulted in higher RS recovery, which ultimately led to enhanced biohydrogen production via the thermophilic process. We believe that this technique has the potential to improve the economic feasibility of bioenergy production by lowering the cost of enzymatic pretreatment.

Materials and methods

The POME that was collected and sealed in a tight container was obtained from Felda Global Ventures Mill, Kemahang, Tanah Merah, Kelantan. The sample was then shipped to the University of Seoul, South Korea, and stored at under 4 ˚C until use. Sodium hydroxide and hydrochloric acid (Duksan Chemical, Seoul, Korea) were used to adjust the POME pH during the treatment process. Cellulase Novozym 50199 (Novozymes, Copenhagen, Denmark) was used for enzymatic pretreatment.

Inoculum

The inoculum was obtained from the bottom of an acetogenesis reactor which has been used for processing food waste at a local waste treatment plant (Jungnang, Seoul, Korea). The use of inoculum from an acetogenesis reactor is based on the fact that it provides a culture of microorganisms that specialize in producing hydrogen from organic compounds. During the acetogenesis process, complex organic molecules such as sugars, amino acids, and fatty acids undergo a series of enzymatic reactions to produce simpler compounds such as hydrogen, carbon dioxide, and acetic acid. Inoculum from an acetogenesis reactor contains microorganisms that are already acclimated to the specific conditions required for this process. The characteristics of raw sludge are as follows: pH 7.2, total chemical oxygen demand (TCOD) 64 g/L, total suspended solid (TSS) 49.6 g/L, total solids (TS) 47.8 g/L, total volatile solids (TVS) 39.4 g/L, total volatile suspended solid (TVSS) 45.4 g/L, fixed total solids (FTS) 8.4 g/L, and total organic carbon (TOC) 13.9 g/L. The sludge had undergone a heat-shock treatment for 1 hr to inhibit non-hydrogen producers such as mesophile microbes, acetogens, and methanogens [1]. The seed sludge and POME characteristics were analyzed following the Standard Method (APHA, 2005) (Federation & Association, 2005).

Enzymatic pretreatment

Enzymatic hydrolysis of raw POME was performed in a 500 mL laboratory screw cap bottle and was applied in a Box-Behnken design with five replicates at the center (Design-Expert, Minneapolis, MN,USA). The working volume was 250 mL. The reaction mixture consisted of 1–5% w/v Cellulase Novozym 50199 with enzymes activity of 1000 BHU-2/g (e.g., 1 mL enzyme per 100 mL of POME). The mixture was then incubated in a rotary incubating shaker at 50 ˚C, 150 rpm for 2–12 hr. The pH range for the reactions was 5–5.5. The center value for the independent variables was 1% enzyme concentration, pH 5.25, and a 7-hr reaction time. The samples were further heated at 100 ˚C to eliminate enzymatic activity and then cooled to room temperature. The aliquots were withdrawn from the sample after centrifugation (Labogene 1236 R, Seoul, Korea) at 4500 rpm for 15 min and then analyzed for the released sugars via the dinitrosalycyclic acid (DNS) method. The total RS detection was obtained using a UV–visible spectrophotometer (Shimadzu UV-2600i, Kyoto, Japan). The wavelength used for RS analysis was 540 nm.

Thermophilic biohydrogen production

A 500 mL batch reactor with a working volume of 400 mL was used as a batch reactor for thermophilic biohydrogenation, 10% of which was filled with enriched mixed cultures (inoculum). The rest of the working volume was filled with enzymatic POME hydrolysate (substrate). Nitrogen gas was sparged into the system for 15 min to eliminate oxygen. The hydrolysate was diluted with DI water to obtain a 29 100 mg/L COD content. The temperature was set at 55 ˚C and the initial

pH was set at pH 7.14 using sodium hydroxide or hydrochloric acid. The other experimental condition was a seed composition of 18.2% and a mixing speed of 150 rpm. The cumulative hydrogen production (CHP) and gas composition were measured and analyzed every 12 hr, while VFA content, VSS, TOC, and COD were analyzed on a daily basis. Gas composition was analyzed using gas chromatography (Shimadzu GC-2010, Kyoto, Japan) equipped with a TCD detector using argon of 99.99% purity as the carrier gas. The injection temperature was set at 250 ˚C and the initial oven temperature was set at 25 ˚C. The samples were injected manually using a gas-tight syringe. The VFAs were analyzed using a GC with a flame ionization detector and helium as a carrier gas. An additional batch reactor was run with raw POME as the control. All the samples were run in triplicates.

The kinetics of biohydrogen production were studied using the optimized pretreated enzymatic substrate. The modified Gompertz model was applied in the kinetics study, as shown in Equation 1.

$$H(t) = H_{\max}.\ \exp\left\{-\exp\left[\frac{R_m.\ e}{H_{\max}}(\lambda - t) + 1\right]\right\} \quad (1)$$

$H(t)$: cumulative biohydrogen (mL) at time, t (h).
H_{max}: biohydrogen production potential (mL).
R_m: biohydrogen production rate (mL/hour).
λ: lag-phase time (h).
e: Euler's number (2.71828).

The Gompertz model's parameters, including H_{max}, R_m, and λ, were obtained by fitting the model to the actual cumulative biohydrogen production data using the least-squares method. This was accomplished using Microsoft Excel (MS Excel, Seattle, WA, USA), which utilized the Solver add-in function to minimize the sum of squared errors (SSE) between observed and predicted values by adjusting the parameters H_{max}, R_m, and λ. The predicted values with the lowest SSE were then compared to the observed data.

Results and discussion

Palm oil mill effluent (POME) requires treatment before being released into the environment. Organic processing methods are highly recommended due to their advantages in terms of environmental friendliness, cost-effectiveness, and practicality. Different microorganisms have been connected to the POME treatment handle, such as parasites, microscopic organisms, and microalgae.

Parametric studies have been conducted for the effect of pretreatment on reducing sugar yield, the optimum conditions for enzymatic pretreatment on POME, and the effect of pretreated POME with enzymatic pretreatment on hydrogen yield.

Effect of pretreatment condition on reducing sugar yield

The amount of RS produced from the enzymatically pretreated POME was determined and compared with that from untreated POME. The enzymatically pretreated POME produced 9590 mg/L, which is a 182% increase in RS content relative to untreated POME, which produced 3400 mg/L. From the results, it was found that POME could be efficiently transformed to RS after the enzymatic pretreatment via the Box-Behnken design. Table 1 shows the experimental designs generated by the BBD for the enzymatic pretreatment containment; a total of 17 experiments were designed and run. Table 1 presents the measured and predicted RS amounts produced after enzymatic pretreatment under different operation conditions, i.e., pH (A), reaction time (B), and enzyme concentration (C). In all 17 experiments, different RS yields could be obtained. The highest RS amounts produced were 9550 mg/L from Run 8 (experimental condition: pH 5, 12-hr reaction time, and enzyme concentration of 3.0%), and the lowest RS (i.e., 4420 mg/L) was obtained from Run 13 (experimental condition: pH 5.25, 2-hr pretreatment time, and enzyme concentration of 1.00%).

It is observed that RS production increased after an extended period of pretreatment at higher enzyme concentrations and the lowest pH value. The predicted RS was calculated based on the coefficients generated from the analysis of variance (ANOVA) (Table 2) as shown in Equation 2. Y represents the reducing sugar yield and pH (A), reaction time (B), and C as Cellulase Novozym 50199 concentration represent the process variables. The actual lowest and maximum RS values obtained from experiments Run 13 and Run 8 were compared with those from the computation. As shown in Table 1, the coded values for each parameter were set to − 1, 0, and 1, which indicate the minimum, center, and maximum values.

$$Y = 7743.65 - 58.32A + 875.53B + 1390.19C - 103.18AB + 182.86AC - 308.45BC + 312.66A^2 + 274.52B^2 - 1174.37C^2 \quad (2)$$

An analysis of variance for the generated quadratic model with the coefficients (Table 2) for the enzymatic pretreatment of POME was performed to test the significance of the model parameters for the RS production (Table 3). The calculated F-value of 31.5 suggested that the model would be significant and suitable for extracting RS from POME via enzymatic pretreatment. The coefficient of determination (R^2) of the model fit was 0.9759. In addition, the coefficient of variation was found to be very low (i.e., 4.24), indicating the reliability and accuracy of the experiment. The calculated signal-to-noise ratio (i.e., 21.0) also indicated that the precision of the model would be high enough to reasonably reflect the experiment; a score greater than 4 indicates an adequate signal. The linear terms of the model for the reaction time and enzyme concentration were found to be significant; their p-values were 0.0001 and < 0.0001, respectively. However, the linear term for pH was insignificant (i.e., p = 0.6188). All the interaction terms were not significant, but the quadratic term of C^2 was significant (i.e., p = 0.0001).

Fig. 1 shows the contour and response surface plots between the process parameters for the enzymatic pretreatment of POME (cellulase concentration, pH, and reaction time). As shown in Figs. 1(a) and 1(d), the RS yield was varied between 7500 mg/L and 9000 mg/L when the reaction time and pH were varied with the cellulase concentration fixed. The RS yield was found to be significantly affected by reaction time; when it was increased to 12 hr, the RS yield was predicted to be over 9200 mg/L. In short, the highest RS yield was achieved at pH 5 and a duration of 12 h. The predicted value was 9370 mg/L, which was very close to the observed value (9550 mg/L). By looking at the contour plot in Fig. 1(a), an extended operation time was needed to achieve the maximum value in the pH range from 5.15 to 5.48; this is outside of the experiment boundary. The pH value has no significant effect on both reaction time and cellulase concentration, as it was observed that the higher RS yield was acquired in the studied pH range (pH 5.0 to pH 5.5) (Figs. 1(b) and 1(e)). This finding was in good agreement with the result of the ANOVA test; the interaction term related to pH (0.5357 and 0.2864) was insignificant.

Figs. 1(c) and 1(f) show that the interaction between cellulase concentration and reaction time had a significant effect on the RS yield. The RS content was increased with an increase in cellulase concentration, and reaction time. The maximum RS production was observed at a reaction time of 11.4 hr and 3.94% cellulase concentration. The minimum RS content was observed at a reaction time of < 6 hr and a cellulase concentration of 1.37%. At this range, the highest predicted RS yield was observed at 5000 mg/L, while the predicted maximum RS content was about 9000 mg/L based on the interaction between the cellulase concentration (3.22–4.62%) and the reaction time (11.44 hr to 12 hr), respectively. This result indicated that the enzymatic hydrolysis rate of POME had increased with the increased enzyme dosage, where the strength of the hydrogen bond inside the polysaccharide molecules in POME was weakened as the hydroxyl activity was improved [3]. Thus, extended reaction time promoted interaction between the enzyme and substrate complex, which would favor polysaccharide degradation and result in more production of RS [2].

Table 1
The experimental design of enzymatic pretreatment was generated by Box-Behnken design.

	Enzymatic pretreatment parameters						Response	
Run	A: pH	A: Coded Value	B: Time (Hr)	B: Coded Value	C: Conc. (%)	C: Coded Value	RS (Actual) mg/L	RS (Predicted) mg/L
1	5.25	0	7	0	3	0	7966.32	7743.65
2	5.50	1	7	0	1	-1	5275.88	5250.58
3	5.25	0	7	0	3	0	7808.29	7743.65
4	5.50	1	12	1	3	0	8962.65	9044.87
5	5.25	0	12	1	1	-1	6694.49	6637.59
6	5.25	0	7	0	3	0	8137.32	7743.65
7	5.50	1	7	0	5	1	8632.98	8396.68
8	5.00	-1	12	1	3	0	9547.26	9367.85
9	5.25	0	7	0	3	0	7497.01	7743.65
10	5.50	1	2	-1	3	0	7320.76	7500.17
11	5.25	0	7	0	3	0	7309.3	7743.65
12	5.00	-1	7	0	1	-1	5496.61	5732.92
13	5.25	0	2	-1	1	-1	4423.75	4269.63
14	5.25	0	2	-1	5	1	7610.01	7666.91
15	5.00	-1	2	-1	3	0	7492.64	7410.43
16	5.25	0	12	1	5	1	8646.96	8801.07
17	5.00	-1	7	0	5	1	8122.27	8147.58

Table 2
Coefficients in terms of coded factors determined from ANOVA.

Factor	Coefficient estimate
Intercept	7743.65
A-pH	-58.32
B-Time	875.53
C-Concentration	1390.19
AB	-103.18
AC	182.86
BC	-308.45
A^2	312.66
B^2	274.52
C^2	-1174.37

The other process parameter was temperature, which was held at 50 °C throughout all the experiments since the Novozym 50199 enzymolysis is known to be efficient at 50 °C. As the temperature approached 50 °C, the enzymolysis reaction was accelerated, probably due to the increased contact between cellulase and POME due to the promoted molecular motions. When the temperature exceeds 50 °C, however, energy absorption by the enzyme is possibly exaggerated, thus leading to a weakened enzyme activity and low degradation efficiency [46].

Optimum conditions for enzymatic pretreatment on POME

The predicted value for RS (mg/L) was validated experimentally. As aforementioned, the optimal conditions for the extraction of RS with the enzymatic pretreatment of POME were a pH of 5, a 12 hr reaction time, and a 3.76% w/v cellulose concentration (Table 4). With these conditions, the obtained RS yield from the experiment was 9590 mg/L, which is very close to the value predicted by the model (9540 mg/L).

The amount of RS extracted from POME with the enzymatic pretreatment was compared with that obtained without the pretreatment (i.e., control). The former was 9590 mg/L while the latter was 3400 mg/L, indicating the enzymatic method produced 180% higher RS production. This confirmed that the enzymatic pretreatment of POME using Novozym 50199 could considerably increase RS production via the Box-Behnken experimental design.

Similar studies using POME material have been reported. Uddin et al. [44], for example, reported that the use of lipase had enhanced free fatty acid content and improved the RS concentration up to 67% for a lipase dose of 12.3 U/mL at pH 4.5. Garritano et al. [12] worked on biohydrogen production from POME using a commercial enzyme (Lipomod 34 MDP) in a two-step process; they observed a 12% increase in biohydrogen yield (2.09 ± 0.05 mmol H_2 $g_{COD}{}^{-1}$). Prasertsan et al. [37] employed xylanase in the biogas production from POME and observed a 3-fold increase in the biogas yield relative to the untreated

Table 3
Analysis of variance (ANOVA) and fit statistics for the quadratic model used for enzymatic pretreatment of POME.

Source	Sum of Squares	df	Mean Square	F-value	p-value	Fit statistics	
Model	2.85×10^7	9	3.16×10^6	31.49	< 0.0001	**R^2**	**0.9759**
A	2.72×10^4	1	2.72×10^4	0.2709	0.6188 *	Adjusted R^2	0.9449
B	6.13×10^6	1	6.13×10^6	61.06	0.0001	Predicted R^2	0.8412
C	1.55×10^7	1	1.55×10^7	153.94	< 0.0001	Adeq Precision	20.9747
AB	4.26×10^4	1	4.26×10^4	0.424	0.5357 *	Std. Dev.	316.92
AC	1.34×10^5	1	1.34×10^5	1.33	0.2864 *	Mean	7467.32
BC	3.81×10^5	1	3.81×10^5	3.79	0.0926 *	C.V.%	4.24
A^2	4.12×10^5	1	4.12×10^5	4.1	0.0826 *		
B^2	3.17×10^5	1	3.17×10^5	3.16	0.1187 *		
C^2	5.81×10^6	1	5.81×10^6	57.82	0.0001		
Residual	7.03×10^5	7	1.00×10^5				
Lack of Fit	2.45×10^5	3	81609.28	0.7124	0.5936		
Pure Error	4.58×10^5	4	1.15×10^5				
Cor Total	2.92×10^7	16					

*insignificant value
A-pH, B-Time (hour) C-Concentration (% w/v)

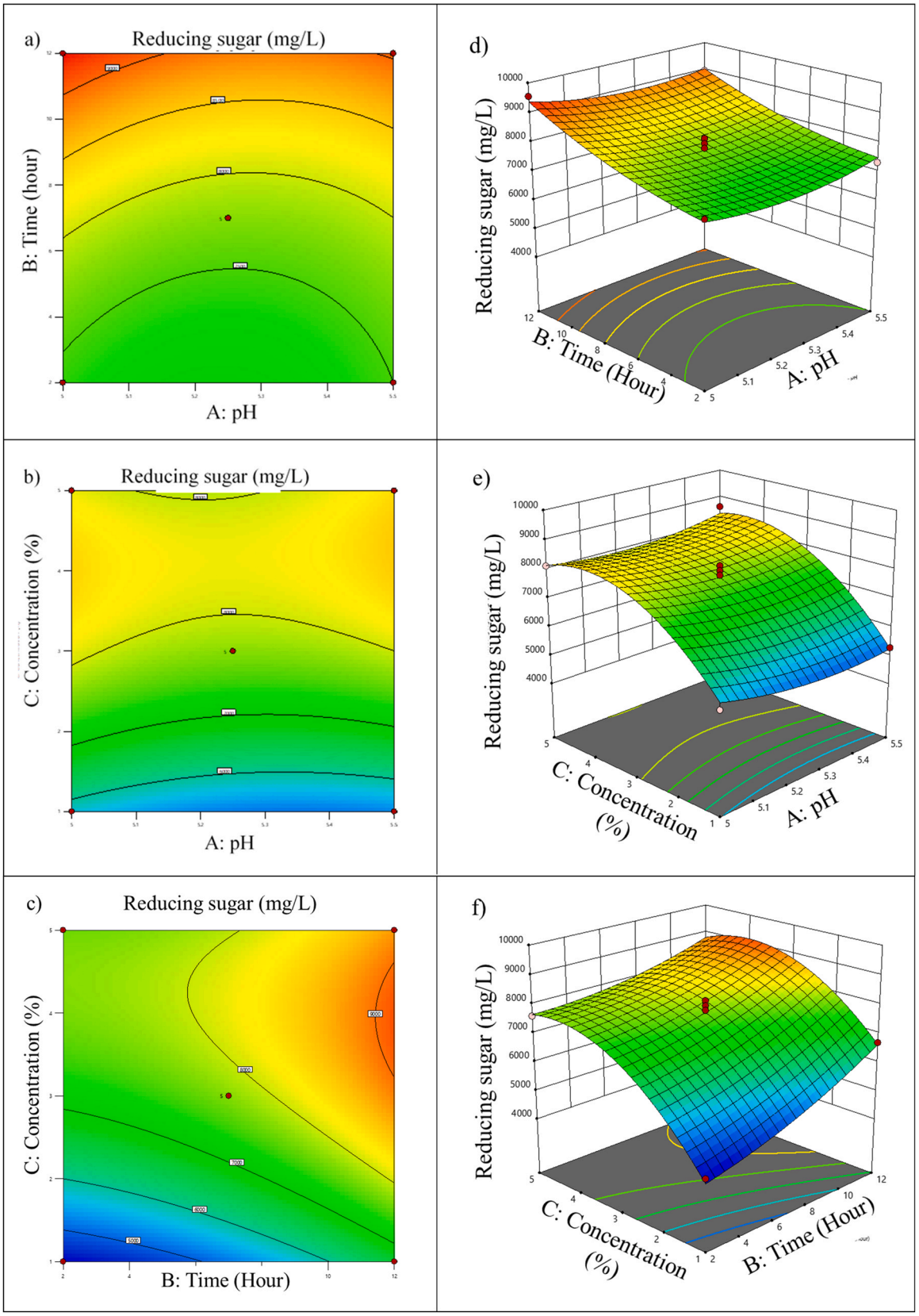

Fig. 1. The contour (left side) and response surface plot (right side) of the effects of Novozym cellulase concentration (C), pH (A) and time (B) for enzymatic pretreatment.

Table 4
The reducing sugar yield for the predicted and experimental and the optimal condition determined by BBD.

Reducing sugar yield (mg/L)		Optimal condition		
Predicted value	Experimental value	pH	Time (Hr)	Concentration (%)
9539.86	9588.46	5	12	3.76487

Table 5
The characteristics of treated and untreated POME.

	Raw POME	Pretreated with cellulase
tCOD (mg/L)	57,700	42,500
pH	4.3	5.7
TOC (mg/L)	5010	6870
TS (g/L)	39	33
VS (g/L)	35	28
TSS (g/L)	31	16
Reducing sugar (mg/L)	3400	9590

substrate. Therefore, the results of this study were consistent with the aforementioned reports where the enzymatic pretreatment had degraded the polysaccharide structure in POME for higher RS or bioenergy production [38,40].

Effect of pretreated POME with enzymatic pretreatment on hydrogen yield

The pretreated POME was then subjected to biohydrogen production with the dark fermentation method. Table 5 illustrates the characteristics of the enzymatically pretreated and untreated POME. The POME pretreated with the cellulase showed lower COD, TS, VS, and TSS, but higher TOC, and RS. This is because the enzyme added had degraded some structures in POME. As stated above, the RS content of the pretreated POME was 181% higher than that of untreated POME. The higher RS concentration in the substrate facilitated higher hydrogen production since the monosaccharide form would be easily metabolized by bacterial strains [16].

The pretreated POME hydrolysate was mixed with seed sludge for biohydrogen production. Fig. 2 shows the profile of cumulative biogas and biohydrogen during a 120-hr fermentation. Cumulative biogas production from POME hydrolysate rose to over 650 mL within 60 hr of fermentation. This amount is 80% higher than that from untreated POME. The cumulative biohydrogen production (CHP) for POME hydrolysate was 156-mL H_2 at a fermentation time of 53 hr, while the CHP for untreated POME was 68-mL H_2; Hence, a 128% higher CHP could be

Table 6
Biohydrogen production from untreated POME and POME pretreated with cellulase.

	Untreated POME	Pretreated POME
Maximum H_2 content (%)	28	38
Yield (mL/g COD)	18	41
λ (h)	3.5	16
H_{max} (mL)	83	200
R_{max} (mL h^{-1})	19	19
R_{max}' (mL h^{-1} g^{-1} VS)	1.9	2.1
Acetic acid (mg/L)	30	22
Propionic acid (mg/L)	13	34
Butyric acid (mg/L)	76	111
Valeric acid (mg/L)	13	22
HBu / HAc ratio	2.5	5.0

obtained from the pretreated POME, compared to that from untreated POME. This result indicates that enzymatic pretreatment favors biohydrogen production over biogas production, with 48% differences. At the end of fermentation (after 113 hr), the cumulative biogas amounts from untreated POME and POME hydrolysate were 428 mL and 784 mL, respectively, while the CHP productions from untreated POME and POME hydrolysate were 84-mL H_2 and 200-mL H_2, respectively.

The curve fitting process for CHP using the Gompertz equation is shown in Table 6; the fitted Gompertz models for biohydrogen production using untreated POME and pretreated POME are shown in Fig. 3. The coefficients of determination for the fits to the cumulative H_2 gas produced with pretreated POME and that produced with untreated POME were 0.9937 and 0.9782, respectively, indicating that the Gompertz model can reasonably well explain the experimental data [51].

As shown in Table 6, the H_2 contents of the biogas produced by untreated POME and treated POME were 28% and 38%, respectively. Therefore, the enzymatic pretreatment of POME increased the biohydrogen content by 34%. Methane was not detected in any experiment, indicating that the heat treatment on seed sludge had successfully inhibited the growth of methanogens. The biohydrogen production

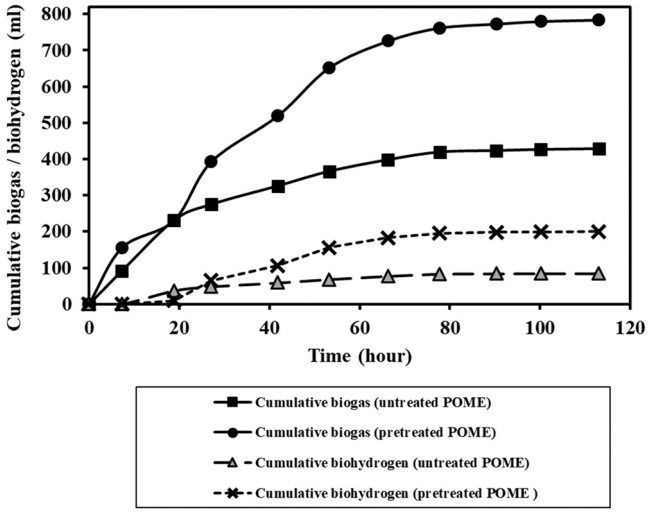

Fig. 2. Cumulative thermophilic biogas and biohydrogen production curve for treated (enzymatic pretreatment) and untreated POME (raw).

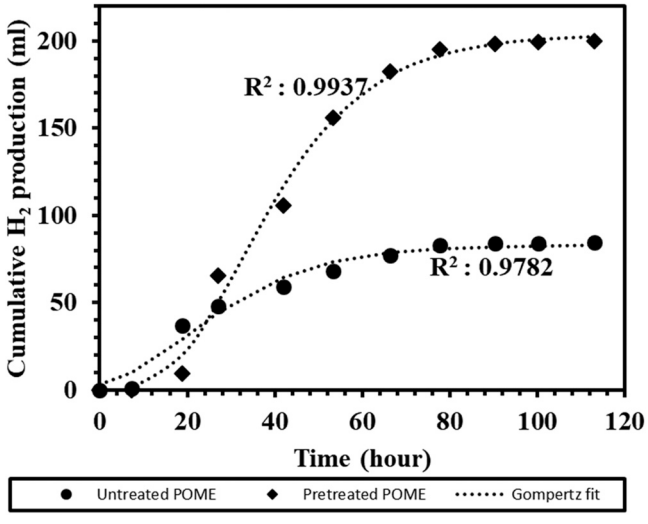

Fig. 3. Cumulative biohydrogen production (mL) for untreated and pretreated POME (enzymatic pretreatment) vs. Gompertz fit.

potential of H_{max} was significantly improved after the enzymatic pretreatment. The hydrogen production rates of the pretreated and pretreated POME were almost the same: 18.8 mL h^{-1} versus 18.9 mL h^{-1}. In addition, the lag-phase time (λ) value for the pretreated substrate (16.2 hr) was longer than that of the untreated POME (3.5 hr). This indicated that the enzymatic pretreatment had improved the RS loading rate, which in turn affected the production rate of the biohydrogen and caused microorganisms to take a longer time adapting to the higher loading rate [42,50]. The specific hydrogen production rate ($R_{max'}$) had slightly increased from 1.9 to 2.1 mL h^{-1} g^{-1} VS after pretreatment. This result is in agreement with that reported by Tanikkul, Juntarakod, et al. [43]. In their study with a mesophilic process, the ozone-pretreated POME showed improved $R_{max'}$ and biohydrogen yield.

As illustrated in Table 6, acetic, butyric, propionic, and valeric acids are the main VFAs detected in both samples, while ethanol was not detected. The enzymatic pretreatment decreased the acetic acid production by 100% but increased the butyric acid production by 46.1%. The decrease in acetic acid content was accompanied by the production of butyrate, propionate, and valeric acid in the fermentation effluent. The butyric acid concentration was high in the pretreated sample. In fact, the high level of butyric acid should have inhibited ethanol formation, which would be advantageous for biohydrogen production. Ethanol can act as an NADH free-electron consumer, resulting in reduced hydrogen production [8]. However, the propionic content was higher in the pretreated sample, indicating the inhabitation of hydrogenetic bacterial activity and thus lowering the hydrogen production rate (R_{max} of 18.9 mL h^{-1}) [45]. The presence of propionic acid can also be advantageous since it can inhibit methanogenic archaeal activity [29]. This finding was supported by zero-detection of methane in all cases. The mixture of butyric and propionic acids also had a significant interaction with biodegradation efficiency, and their effect was more significant, compared to the butyric acid effect alone [29]. The higher butyrate-to-acetate ratio (HBu/HAc) of 5.045 in enzymatically pretreated POME also indicated a higher potential for biohydrogen production. The HBu/HAc ratio of > 1 indicates that the hydrogen-producing bacteria would be more dominant than the hydrogen-consuming bacteria [27]. Overall, the accumulation of VFAs increased following the increased RS concentration, which was higher in the enzymatically pretreated POME that accommodated the higher yield of hydrogen.

Conclusions

This study investigated the effects of pretreatment on reducing sugar yield, optimum conditions for enzymatic pretreatment on POME, and the effect of pretreated POME with enzymatic pretreatment on hydrogen yield. The present study indicated that the enzymatic pretreatment of POME using cellulase (Novozym 50199) could significantly improve biohydrogen production efficiency. The optimal conditions determined via BBD were a cellulase concentration of 3.76% (w/v), reaction time of 12 hr, and pH 5. The predicted value of RS obtained by the quadratic response surface model was 9540 mg/L, which was experimentally validated. From the thermophilic biohydrogen production conducted using POME pretreated with the cellulase at the aforementioned optimal conditions, the value of H_{max} and the cumulative biohydrogen production could be significantly increased: 145% and 138%, respectively. In conclusion, the optimized process parameters via the response surface methodology were able to improve RS content in the substrate and hence improve the thermophilic biohydrogen production.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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References

[1] M.F. Abdullah, J. Md Jahim, P.M. Abdul, S.S. Mahmod, Effect of carbon/nitrogen ratio and ferric ion on the production of biohydrogen from palm oil mill effluent (POME), Biocatal. Agric. Biotechnol. 23 (2020) 101445, https://doi.org/10.1016/j.bcab.2019.101445

[2] A.C. Assabjeu, E. Noubissié, S.C.Z. Desobgo, A. Ali, Optimization of the enzymatic hydrolysis of cellulose of triplochiton scleroxylon sawdust in view of the production of bioethanol, Sci. Afr. 8 (2020) e00438, https://doi.org/10.1016/j.sciaf.2020.e00438

[3] M.M. Aziz, K.A. Kassim, M. ElSergany, S. Anuar, M.E. Jorat, H. Yaacob, Arifuzzaman, Recent advances on palm oil mill effluent (POME) pretreatment and anaerobic reactor for sustainable biogas production, Renew. Sustain. Energy Rev. 119 (2020) 109603, https://doi.org/10.1016/j.rser.2019.109603

[4] A.S. Baharuddin, L.S. Hock, M. Yusof, N.A.A. Rahman, U. Shah, M.A. Hassan, Y. Shirai, Effects of palm oil mill effluent (POME) anaerobic sludge from 500 m 3 of closed anaerobic methane digested tank on pressed-shredded empty fruit bunch (EFB) composting process, Afr. J. Biotechnol. 9 (16) (2010) 2427–2436.

[5] S. Chaiprapat, T. Laklam, Enhancing digestion efficiency of POME in anaerobic sequencing batch reactor with ozonation pretreatment and cycle time reduction, Bioresour. Technol. 102 (5) (2011) 4061–4068, https://doi.org/10.1016/j.biortech.2010.12.033

[6] Y. Chan, M. Chong, C. Law, Effects of temperature on aerobic treatment of anaerobically digested palm oil mill effluent (POME), Ind. Eng. Chem. Res. (2010) *49*, https://doi.org/10.1021/ie901952m

[7] Y. Chen, W. Wang, D. Zhou, T. Jing, K. Li, Y. Zhao, J. Xie, Biodegradation of lignocellulosic agricultural residues by a newly isolated Fictibacillus sp. YS-26 improving carbon metabolic properties and functional diversity of the rhizosphere microbial community, Bioresour. Technol. 310 (2020) 123381, https://doi.org/10.1016/j.biortech.2020.123381

[8] P.S. Chong, J.M. Jahim, S. Harun, S.S. Lim, S.A. Mutalib, O. Hassan, M.T.M. Nor, Enhancement of batch biohydrogen production from prehydrolysate of acid treated oil palm empty fruit bunch, Int. J. Hydrog. Energy 38 (22) (2013) 9592–9599.

[9] E. Donev, M.L. Gandla, L.J. Jönsson, E.J. Mellerowicz, Engineering non-cellulosic polysaccharides of wood for the biorefinery, Front. Plant Sci. 9 (2018) 1537.

[10] Eren Şenaras, A. (2019). Chapter 8 - Parameter optimization using the surface response technique in automated guided vehicles. In K. Kumar, D. Zindani, & P. Davim (Eds.), *Sustainable Engineering Products and Manufacturing Technologies* (pp. 187–197): Academic Press. Standard methods for the examination of water and wastewater, (2005).

[11] S.L.C. Ferreira, R.E. Bruns, H.S. Ferreira, G.D. Matos, J.M. David, G.C. Brandão, W.N.L. dos Santos, Box-Behnken design: An alternative for the optimization of analytical methods, Anal. Chim. Acta 597 (2) (2007) 179–186, https://doi.org/10.1016/j.aca.2007.07.011

[12] A. d N. Garritano, L.R.V. de Sá, É.C.G. Aguieiras, D.M.G. Freire, V.S. Ferreira-Leitão, Efficient biohydrogen production via dark fermentation from hydrolized palm oil mill effluent by non-commercial enzyme preparation, Int. J. Hydrog. Energy 42 (49) (2017) 29166–29174, https://doi.org/10.1016/j.ijhydene.2017.10.025

[13] E.O. Geiger, Chapter 21 - statistical methods for fermentation optimization, in: H.C. Vogel, C.M. Todaro (Eds.), Fermentation and Biochemical Engineering Handbook, *third* ed., William Andrew Publishing, Boston, 2014, pp. 415–422.

[14] A. Geng, Upgrading of oil palm biomass to value-added products, in: K.R. Hakeem, M. Jawaid, U. Rashid (Eds.), Biomass and Bioenergy: Applications, Springer International Publishing, Cham, 2014, pp. 187–209.

[15] A.A. Houfani, N. Anders, A.C. Spiess, P. Baldrian, S. Benallaoua, Insights from enzymatic degradation of cellulose and hemicellulose to fermentable sugars– a review, Biomass-.-. Bioenergy 134 (2020) 105481, https://doi.org/10.1016/j.biombioe.2020.105481

[16] B.-B. Hu, M.-Y. Li, Y.-T. Wang, M.-J. Zhu, High-yield biohydrogen production from non-detoxified sugarcane bagasse: Fermentation strategy and mechanism, Chem. Eng. J. 335 (2018) 979–987, https://doi.org/10.1016/j.cej.2017.10.157

[17] D. Humbird, Q. Fei, Chapter 20 - scale-up considerations for biofuels, in: C.A. Eckert, C.T. Trinh (Eds.), Biotechnology for Biofuel Production and Optimization, Elsevier, Amsterdam, 2016, pp. 513–537.

[18] E. İnce, M. İnce, G. Önkal Engin, Comparison of thermophilic and mesophilic anaerobic treatments for potato processing wastewater using a contact reactor, Glob. NEST J. 19 (2) (2017) 318.

[19] M.H. Isa, L.-P. Wong, M.J.K. Bashir, N. Shafiq, S.R.M. Kutty, I.H. Farooqi, H.C. Lee, Improved anaerobic digestion of palm oil mill effluent and biogas production by ultrasonication pretreatment, Sci. Total Environ. 722 (2020) 137833, https://doi.org/10.1016/j.scitotenv.2020.137833

[20] I. Ismail, M.A. Hassan, N.A.A. Rahman, C.S. Soon, Thermophilic biohydrogen production from palm oil mill effluent (POME) using suspended mixed culture, Biomass Bioenergy 34 (1) (2010) 42–47.

[21] S. Ismail, I. Idris, Y. Ng, A. Ahmad, Coagulation of palm oil mill effluent (POME) at high temperature, J. Appl. Sci. 14 (12) (2014) 1351–1354.
[22] J.-Y. Jeong, S.-M. Son, J.-H. Pyon, J.-Y. Park, Performance comparison between mesophilic and thermophilic anaerobic reactors for treatment of palm oil mill effluent, Bioresour. Technol. 165 (2014) 122–128.
[23] K. Karimi, M.J. Taherzadeh, A critical review of analytical methods in pretreatment of lignocelluloses: Composition, imaging, and crystallinity, Bioresour. Technol. 200 (2016) 1008–1018, https://doi.org/10.1016/j.biortech.2015.11.022
[24] E. Kendir Çakmak, A. Ugurlu, Enhanced biogas production of red microalgae via enzymatic pretreatment and preliminary economic assessment, Algal Res. 50 (2020) 101979, https://doi.org/10.1016/j.algal.2020.101979
[25] S.N.B.A. Khadaroo, P. Grassia, D. Gouwanda, P.E. Poh, The impact of thermal pretreatment on various solid-liquid ratios of palm oil mill effluent (POME) for enhanced thermophilic anaerobic digestion performance, J. Clean. Prod. 261 (2020) 121159, https://doi.org/10.1016/j.jclepro.2020.121159
[26] A.I. Khuri, S. Mukhopadhyay, Response surface methodology, Computational Statistics 2 Wiley Interdisciplinary Reviews, 2010, pp. 128–149.
[27] R.M. Maaroff, J. Md Jahim, A.M. Azahar, P.M. Abdul, M.S. Masdar, D. Nordin, M.A. Abd Nasir, Biohydrogen production from palm oil mill effluent (POME) by two stage anaerobic sequencing batch reactor (ASBR) system for better utilization of carbon sources in POME, Int. J. Hydrog. Energy 44 (6) (2019) 3395–3406, https://doi.org/10.1016/j.ijhydene.2018.06.013
[28] S.S. Mahmod, J.M. Jahim, P.M. Abdul, Pretreatment conditions of palm oil mill effluent (POME) for thermophilic biohydrogen production by mixed culture, Int. J. Hydrog. Energy 42 (45) (2017) 27512–27522, https://doi.org/10.1016/j.ijhydene.2017.07.178
[29] C. Mamimin, P. Prasertsan, P. Kongjan, S. O-Thong, Effects of volatile fatty acids in biohydrogen effluent on biohythane production from palm oil mill effluent under thermophilic condition, Electron. J. Biotechnol. 29 (2017) 78–85, https://doi.org/10.1016/j.ejbt.2017.07.006
[30] C. Mamimin, A. Singkhala, P. Kongjan, B. Suraraksa, P. Prasertsan, T. Imai, O. Sompong, Two-stage thermophilic fermentation and mesophilic methanogen process for biohythane production from palm oil mill effluent, Int. J. Hydrog. Energy 40 (19) (2015) 6319–6328.
[31] M.D. Morris, A class of three-level experimental designs for response surface modeling, Technometrics 42 (2) (2000) 111–121, https://doi.org/10.1080/00401706.2000.10485990
[32] G. Najafpour, A. Zinatizadeh, A. Mohamed, M.H. Isa, H. Nasrollahzadeh, High-rate anaerobic digestion of palm oil mill effluent in an upflow anaerobic sludge-fixed film bioreactor, Process Biochem. 41 (2) (2006) 370–379.
[33] S. Niglio, A. Procentese, M.E. Russo, A. Piscitelli, A. Marzocchella, Integrated enzymatic pretreatment and hydrolysis of apple pomace in a bubble column bioreactor, Biochem. Eng. J. 150 (2019) 107306, https://doi.org/10.1016/j.bej.2019.107306
[34] C.K. Nitsos, P.A. Lazaridis, A. Mach-Aigner, K.A. Matis, K.S. Triantafyllidis, Enhancing lignocellulosic biomass hydrolysis by hydrothermal pretreatment, extraction of surface lignin, wet milling and production of cellulolytic enzymes, ChemSusChem 12 (6) (2019) 1179–1195.
[35] B. Nurul-Adela, A.-B. Nasrin, S.-K. Loh, Palm oil mill effluent as a low-cost substrate for bioflocculant production by Bacillus marisflavi NA8, Bioresour. Bioprocess. 3 (1) (2016) 20, https://doi.org/10.1186/s40643-016-0096-6
[36] A. Peciulyte, J. Kiskis, P.T. Larsson, L. Olsson, A. Enejder, Visualization of structural changes in cellulosic substrates during enzymatic hydrolysis using multimodal nonlinear microscopy, Cellulose 23 (3) (2016) 1521–1536.
[37] P. Prasertsan, W. Khangkhachit, W. Duangsuwan, C. Mamimin, S. O-Thong, Direct hydrolysis of palm oil mill effluent by xylanase enzyme to enhance biogas production using two-steps thermophilic fermentation under non-sterile condition, Int. J. Hydrog. Energy 42 (45) (2017) 27759–27766, https://doi.org/10.1016/j.ijhydene.2017.05.140
[38] N.F.A.A. Rizal, M.F. Ibrahim, M.R. Zakaria, S. Abd-Aziz, P.L. Yee, M.A. Hassan, Pretreatment of oil palm biomass for fermentable sugars production, Molecules 23 (6) (2018) 1381.
[39] M. Schroyen, H. Vervaeren, H. Vandepitte, S.W.H. Van Hulle, K. Raes, Effect of enzymatic pretreatment of various lignocellulosic substrates on production of phenolic compounds and biomethane potential, Bioresour. Technol. 192 (2015) 696–702, https://doi.org/10.1016/j.biortech.2015.06.051
[40] H. Silvamany, S. Harun, T. Mumtaz, M. Jamaliah, Recovery of fermentable sugars from palm oil mill effluent via enzymatic hydrolysis, J. Teknol. (2015) *77*, https://doi.org/10.11113/jt.v77.7016
[41] Y.-L. Tan, B.-H. Tay, R.M. Shafi, N.H.M. Ghani, Selected review on climate change and palm oil prices, Int. J. Bus. Econ. 2 (2) (2020) 61–74.
[42] P. Tanikkul, S. Boonyawanich, M. He, N. Pisutpaisal, Thermophilic biohydrogen recovery from palm oil mill effluent, Int. J. Hydrog. Energy 44 (11) (2019) 5176–5181, https://doi.org/10.1016/j.ijhydene.2018.10.005
[43] P. Tanikkul, P. Juntarakod, N. Pisutpaisal, Optimization of biohydrogen production of palm oil mill effluent by ozone pretreatment, Int. J. Hydrog. Energy 44 (11) (2019) 5203–5211, https://doi.org/10.1016/j.ijhydene.2018.09.063
[44] M.N. Uddin, M.A. Rahman, J. Taweekun, K. Techato, M. Mofijur, M. Rasul, Enhancement of biogas generation in up-flow sludge blanket (UASB) bioreactor from palm oil mill effluent (POME), Energy Procedia 160 (2019) 670–676, https://doi.org/10.1016/j.egypro.2019.02.220
[45] L. Wang, Q. Zhou, F. Li, Avoiding propionic acid accumulation in the anaerobic process for biohydrogen production, Biomass-.-. Bioenergy 30 (2) (2006) 177–182.
[46] Q. Wu, D. Qin, H. Cao, Y. Bai, Enzymatic hydrolysis of polysaccharide from Auricularia auricula and characterization of the degradation product, Int. J. Biol. Macromol. 162 (2020) 127–135, https://doi.org/10.1016/j.ijbiomac.2020.06.098
[47] C. Wyman, B. Dale, Producing biofuels via the sugar platform, Chem. Eng. Prog. 111 (2015) 45–51.
[48] B. Yang, Z. Dai, S.-Y. Ding, C.E. Wyman, Enzymatic hydrolysis of cellulosic biomass, Biofuels 2 (4) (2011) 421–449.
[49] Y. Yao, M. Wang, Y. Liu, L. Han, X. Liu, Insights into the improvement of the enzymatic hydrolysis of bovine bone protein using lipase pretreatment, Food Chem. 302 (2020) 125199.
[50] K. Zhang, N.-Q. Ren, A.-J. Wang, Fermentative hydrogen production from corn stover hydrolyzate by two typical seed sludges: Effect of temperature, Int. J. Hydrog. Energy 40 (10) (2015) 3838–3848.
[51] W. Zhao, J. Zhang, H. Zhang, M. Yang, L. Zang, Comparison of mesophilic and thermophilic biohydrogen production amended by nickel-doped magnetic carbon, J. Clean. Prod. 270 (2020) 122730, https://doi.org/10.1016/j.jclepro.2020.122730
[52] K. Ziemiński, M. Kowalska-Wentel, Effect of enzymatic pretreatment on anaerobic co-digestion of sugar beet pulp silage and vinasse, Bioresour. Technol. 180 (2015) 274–280, https://doi.org/10.1016/j.biortech.2014.12.035