Effect of temperature and hydraulic retention time on hydrogen production from palm oil mill effluent (POME) in an integrated up-flow anaerobic sludge fixed-film (UASFF) bioreactor

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ABSTRACT

The current state of palm oil mill wastewater treatment focuses solely on open ponding or closed lagoon systems for biogas production. However, efforts to convert this wastewater into biohydrogen are limited. Therefore, this research investigates the feasibility of converting palm oil mill effluent (POME) for biohydrogen production via dark fermentation. Temperature and hydraulic retention time (HRT) effects on biohydrogen production and COD removal efficiency in an up-flow anaerobic sludge fixed-film (UASFF) bioreactor were investigated. The experiment was carried out and analysed using a central composite design (CCD) and the Response Surface Methodology (RSM). The hydrogen (H_2) yield, H_2 production rate (HPR), and COD removal efficiency were investigated as responses. HPR increased significantly by 28.8 folds as temperature increased from 37 °C to 53.5 °C (transition from mesophilic to thermophilic) at HRT of 3 h. Meanwhile, the COD removal efficiency significantly increased from 24.76% to 33.33% between 4 to 9 h of HRT. Maximum H_2 yield of 0.95 L H_2 g^{-1} $COD_{removed}$, HPR of 10.39 L H_2 d^{-1}, and 35.9% COD removal were reported at the optimum HRT and temperature of 7 h and 57 °C, respectively. This study indicates that under the thermophilic condition and short HRT, POME could be treated while producing biohydrogen using the UASFF bioreactor.

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1. Introduction

Malaysia is the world's second-largest palm oil producer. About 50 million m^3 of palm oil mill effluent (POME) is produced annually. Most palm oil mills (over two-thirds) in Malaysia use open-ponding/lagoon to treat POME. The raw POME is a brown and viscous liquid at a pH of 4–5. The typical chemical oxygen demand (COD) of POME is in the range of 50 to 120 g L^{-1}; thus, it is not suitable for direct environmental discharge (Nawaz et al., 2021; Lokman et al., 2021). Open-ponding/lagoon treatment of POME is not efficient and can result in significant fugitive methane emissions, which is a major contributor to global warming.

POME contains proteins, fatty acids, lipids, carbohydrates, and minerals (Habib et al., 1997), making it a valuable biomass resource. It also has a high organic carbon content; thus, it can be an excellent substrate for biogas (Hosseini and Wahid, 2013) and biohydrogen production (Loh et al., 2014). In Malaysia, anaerobic systems for treating POME are usually operated at mesophilic (30–40 °C) temperature with a hydraulic retention time (HRT) of 30–60 days. Organic matter decomposition in POME produces 30%–40% carbon dioxide (CO_2) and 60–70% methane (CH_4), with a trace amount of hydrogen sulphide (H_2S) (Lokman et al., 2021; Loh et al., 2014).

HRT is a critical parameter for continuous hydrogen production. Very low HRT causes active biomass washout. In contrast, suitable HRT yields abundant hydrogen. HRT is related to the varying and specific growth rates of hydrogen-producing bacteria (HPB). Low HRT facilitated methanogen washout, thereby guaranteeing the survival of hydrogen producers. Thus, slightly acidic medium and low HRT represent the best conditions for hydrogen production, while increasing it could shift the hydrogen fermentation pattern to methanogenic. Temperature is another crucial parameter for biohydrogen production. There are three temperature ranges in which hydrogen-producing microorganisms can be found: psychrophilic (0–20 °C), mesophilic (20–42 °C), and thermophilic (42–75 °C) (David et al., 2019).

POME is an ideal substrate for hydrogen production with high organic content. However, to date, no Malaysian oil palm millers with the anaerobic digestion system have attempted to capture hydrogen (Mohammadi et al., 2011; Jeong et al., 2014). This is because POME has high lignin and cellulosic content; thus, it cannot be effectively treated under mesophilic conditions, especially at low HRT. As a result, prior to the fermentation and digestion processes, a pretreatment must be applied (Mohammadi et al., 2011). Recent works have shown that two-stage fermentation-anaerobic digestion of POME could reduce total COD by more than 70% and simultaneously produce hydrogen and methane under thermophilic conditions (Jeong et al., 2014; Khemkhao et al., 2012). Other biohydrogen production sources from palm types, such as using coconut milk wastewater as a substrate and coconut milk sludge as an inoculum, Wongthanate and Khumpong (2015) reported the highest cumulative hydrogen production of 0.33 L H_2 L^{-1} wastewater, with 64.98% of COD removal efficiency. However, COD removal was reported to be 23.19% without the enriched bacteria. Meanwhile, Swathy et al. (2020) reported that waste date palm seeds as inoculum were valorised for cellulase production. They reported high productivity of 187.44 mmol/L when using cellulase for biohydrogen production. Nonetheless, COD removal was not reported.

Raw POME from the mill is released at a high temperature (80–90 °C), which is compatible with anaerobic thermophilic treatment without additional heat (Zainal et al., 2020a). Several studies have shown that treating POME under thermophilic conditions results in more substrate degradation than mesophilic anaerobic digestion (Jeong et al., 2014; Khemkhao et al., 2012). When treating wastewater at high temperatures, the hydrogen evaluation rate could be increased (Ramos et al., 2019; Porca et al., 2018). This is due to low partial pressure in the liquid phase and suppression of propionate formation (Cheong and Hansen, 2007).

Meanwhile, many researchers have used a two-stage system for hydrogen (Maaroff et al., 2019) and methane production, treating POME in various bioreactor configurations under various operating conditions (O-Thong et al., 2016; Krishnan et al., 2016a). They used continuous stirred tank reactor (CSTR) (Krishnan et al., 2016a), an integrated system of up-flow anaerobic sludge blanket (UASB) (Mohammadi et al., 2017), sequencing batch fermenters (Mishra et al., 2016), and anaerobic sequencing batch reactor (ASBR) — UASB in their study (Mamimin et al., 2015b). UASB reactors are deliberately developed for anaerobic digestion and can withstand higher organic loading rate (OLR) and short HRT digestion of many organic wastes with high undissolved solids. However, there are some limitations — this reactor cannot be used to treat pharmaceutical waste because it contains toxic chemicals and antibiotics that anaerobic treatment cannot treat. Moreover, using this type of reactor consumes much power and is not cost-effective.

CSTR, on the other hand, operates in a steady state with a continuous flow of reactants and products. It has a high methane recovery rate, good temperature control, and continuous operation. Nevertheless, although microbial cultures in a CSTR are evenly suspended in the liquor, resulting in lower mass transfer resistance, a CSTR cannot maintain a high cell inventory due to its mixed operation and inherent reactor construction. At short HRTs, cell washout may occur, causing a reduction in hydrogen production (Show et al., 2019).

Meanwhile, the primary distinction between batch and continuous fermentation is that in the former process, fermentation occurs one after the other, whereas, in continuous, the fermentation process runs for a longer period and never stops, with the feeding of fresh media containing harvesting products and at nutrients regular intervals. Batch fermentation has a low turnover rate because nutrients are only added once, and the environmental conditions are not natural (Anon, 2022). A comprehensive study on a single-stage fermentation process to produce hydrogen (Mohammadi et al., 2017) or methane (Zinatizadeh and Mirghorayshi, 2017) from POME using an integrated up-flow anaerobic sludge blanket-fixed film (UASFF or UASB-FF) bioreactor has also been published. They reported that under mesophilic conditions,

Table 1

Differences between raw POME and digested POME used in this study act as substrate and inoculum for biohydrogen production.

Properties	Unit	POME	
Physicochemical		Raw (substrate)	Digested (inoculum)
Colour	–	Brownish	Black
Odour	–	Earthy-cake smell	Pungent
Temperature	°C	80–90	30–40
pH	–	5.01	7.42
Total suspended solids (TSS)	mg L^{-1}	36,670	50,000
Volatile Suspended solids (VSS)	mg L^{-1}	4400	27,250
Soluble chemical oxygen demand (SCOD)	mg L^{-1}	28,000	16,033

more than 50% of COD removal efficiency was obtained with 0.31 L H_2 g^{-1} COD removed. Meanwhile, for biomethane production from POME, 94% COD removal and 0.331 CH_4 g^{-1} COD removed day^{-1} was achieved at 50 °C (thermophilic).

The hydrogen production potential of POME in a two-stage anaerobic digestion system has been documented using different reaction temperatures and longer HRT (more than one day) (Krishnan et al., 2016a; Mamimin et al., 2015b). Nevertheless, little is known concerning the effects of shorter HRT on converting POME to hydrogen using mixed cultures. Most studies looked at HRT between 96–36 h (Badiei et al., 2011) and 6 h (Krishnan et al., 2016b) for biohydrogen production. However, research on the effects of starting a bioreactor at 3 h of HRT for hydrogen production using POME is scarce. If the reactor is appropriately controlled, its performance may improve when reduced HRT. However, biomass washout occurs at faster dilution rates, failing the operation (Kumar et al., 2016; Lin et al., 2012).

To date, optimising HRT and temperature using a UASFF bioreactor for biohydrogen production in treating POME has gained attention. Although the UASFF bioreactor's reactor stability and efficiency depend on the feed flow rate, up-flow velocity, internal packing, and effluent recycle ratio, it has higher biomass retention and requires a shorter start-up for sludge granulation (Zinatizadeh et al., 2006). Moreover, gas production is less efficient at short HRT and high OLR when compared to CSTR, which is less expensive and easier to manage.

As a result, the current work investigates the effects of temperature and low HRT to determine the optimum conditions for maximum biohydrogen production using POME. This study concentrated on biohydrogen production in the integrated UASFF bioreactor. At various conditions, parameters such as hydrogen production rate, yield, and COD removal efficiency were investigated. Previous batch and start-up studies conducted under various operating, process, and environmental conditions provided preliminary results and findings supporting this study's experimental setup.

2. Materials and methods

2.1. Inoculum and substrate preparation

This study used the remaining acclimatised inoculum from the previous UASFF start-up treating POME. The sludge was collected from the Jugra Palm Oil Mill's mesophilic anaerobic open-ponding system in Banting, Selangor. This literature (Zainal et al., 2018a) contains specifics on inoculum preparation. Meanwhile, raw POME was obtained from the Palm Oil Mill in Dengkil, Selangor, and used as a substrate to produce biohydrogen. Raw POME's total COD and soluble COD are 38 g L^{-1} and 28 g L^{-1}, respectively, with a pH of 5.01. Table 1 shows inoculum and substrate characteristics in detail.

Before use, the substrate was stored in a cold room (4 °C). Suspended solids of raw POME were left to settle before being applied to the integrated UASFF bioreactor. Pre-settled POME (liquid part) was taken and diluted with tap water to obtain the desired influent COD concentration of 20,000 mg L^{-1}. After placing the diluted substrate in a closed container, nitrogen gas was purged at a rate of 10 ml min^{-1} for 10–15 min to create an anaerobic environment.

2.2. UASFF system

A schematic diagram of the integrated UASFF bioreactor is presented in Fig. 1. In the context of this study, the bioreactor is referred to as the H_2-UASFF. The details of the reactor configuration were also explained in a previous article (Zainal et al., 2018a). H_2-UASFF has a 2.5 L capacity, and the pH in the bioreactor was kept between 5 to 5.5. For pH adjustment, 0.1 N sulphuric acid (H_2SO_4, 95%–98%, A. R, Brand R&M) and 0.1 N hydrochloric acids (HCL, 37% A. R, Brand R&M) were used. For the feed rate of COD to VSS (F/M) ratio, recycle flow rate (Q_R), feed flow rate (Q_F), and up-flow velocity (V_{up}), the initial reactor conditions were 1.03 d^{-1}, 54.49 L d^{-1}, 2.51 L d^{-1}, and 1 m h^{-1}, respectively (peristaltic pump (EYELA, model: MP-1000, Japan). The H_2-UASFF unit's temperature was controlled using a water bath (Lab. Companion, model: CW-05G, Korea). The HRT and V_{up} calculations can be found in this literature (Zainal et al., 2018a).
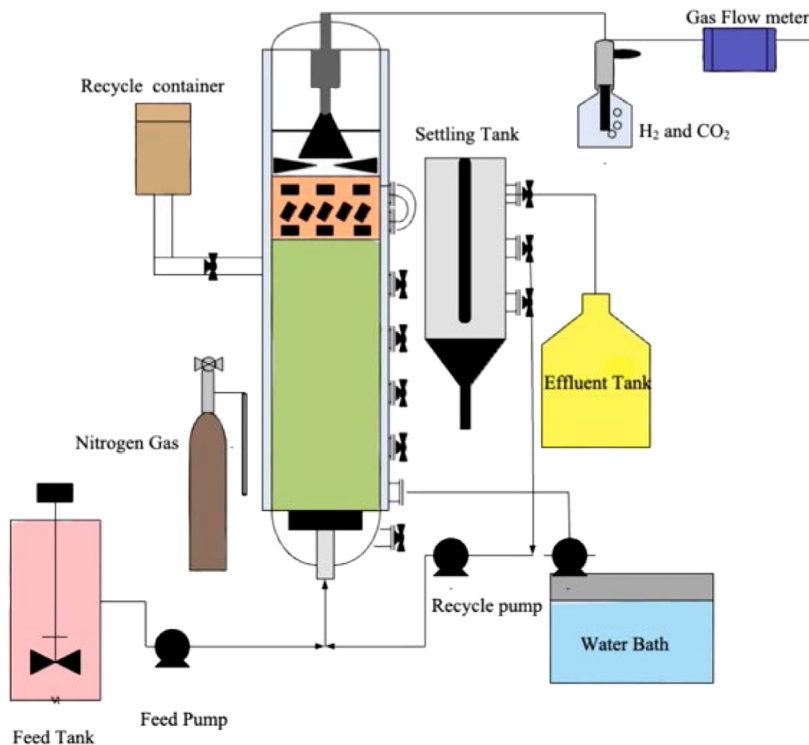
Fig. 1. A high-rate integrated anaerobic bioreactor.

2.3. Optimisation design

According to the findings of the UASFF start-up study, a model for the H_2-UASFF unit was developed in Design-Expert 10 using central composite design (CCD) and analysed using response surface methodology (RSM). In this study, three levels (low, middle, and high) and two-factor CCD (temperature and HRT) with five replications at centre points led to a total number of observations of 17. Three issues were analysed, as shown in Table 2. As a result, two factors were selected and evaluated at low (37 °C, 3 h), high (70 °C, 9 h), and five centre points experiment to evaluate the pure error. This procedure exists to determine the optimum number of centre points of a K-factor design (Clark and Williges, 1973; Box and Hunter, 1957). The interpretation of effects of the variables and responses studied were analysed by using ANOVA. The responses were H_2 yield (L H_2 g^{-1} COD$_{removed}$), HPR (L H_2 L^{-1} d^{-1}), and COD removal efficiency (%). This study aimed to determine the best temperature and HRT and estimate the experiment's variability, design, and results.

In this study, 17 experimental data sets were developed. Temperature and HRT were set in a range while all three responses were maximised using numerical optimisation. The temperature ranged from 37 to 70 °C because the results could classify the preferences of hydrogen-producing bacteria (HPB) in ambient, mesophilic, or thermophilic conditions. One method of producing hydrogen is fermentation, known as thermophilic biohydrogen production, particularly at high temperatures. Higher temperatures (60 °C) are more energetically favourable for biological H_2 production (Stams, 1994), allowing thermophiles to achieve higher yields than mesophiles (Schgnheit and Schafer, 1995). Meanwhile, HRT was set between 3 and 9 h because, according to preliminary results from the start-up study, it takes 4 h to produce biohydrogen using POME (Zainal et al., 2018a). Based on previous experience during start-up, higher HRT is avoided to minimise the formation of methanogens in the UASFF bioreactor.

2.4. Analytical analysis

COD analysis followed APHA Standard Method 5220 D (Show et al., 2019), while the H_2 yield (Υ_{H_2}) and COD removal efficiency (%) (COD$_{rem.eff}$) calculations are as follows.

$$\Upsilon H_2 = \frac{Q\ H_2}{QF\ (COD_{in} - COD_{out})} \tag{1}$$

$$COD_{rem.eff} = \frac{COD_{in} - COD_{out}}{COD_{in}} \times 100 \tag{2}$$

Table 2
Central composite design experimental condition.

Run	Factor 1 A: Temperature	Factor 2 B: HRT	Response 1 HPR	Response 2 COD removal eff.	Response 3 H$_2$ Yield
	°C	h	L H$_2$ L^{-1} d	%	L H$_2$ g^{-1} COD$_{removed}$
1	53.5	6	8.51	35.57	0.45
2	53.5	6	8.49	23.90	0.67
3	70	9	1.55	14.29	0.21
4	37	6	0.51	18.57	0.05
5	70	3	1.64	41.75	0.07
6	53.5	6	11.93	22.38	1.01
7	37	3	0.41	10.48	0.07
8	53.5	6	7.90	41.43	0.36
9	70	9	5.14	16.03	0.61
10	37	9	0.19	24.76	0.01
11	53.5	3	11.81	4.92	1.17
12	37	9	0.22	21.43	0.02
13	37	3	0.45	16.38	0.05
14	53.5	9	13.07	33.33	0.74
15	70	6	7.68	11.27	1.30
16	70	3	5.50	42.14	0.25
17	53.5	6	7.58	38.60	0.37

Table 3
Analysis of bacteria from untreated and heat-treated POME digested sludge as inoculum.

Sample	Parameters	Methods Used (Method Reference)	Unit
Untreated POME	Heterotrophic plate count	APHA 9215B (Anon, 1999)	CFU/mL
	Bacteria Identification	API 50 CHL (API Kit Manual)	–
Heat-treated POME	Anaerobic plate count	In-house No. M078 is based on the Merck Manual.	CFU/mL
	Total coliform	APHA 9222B (Anon, 1999)	CFU/mL
	Escherichia coli	APHA 9222G (Anon, 1999)	CFU/mL
	Bacillus spp.	In-house M038 based on AOAC 980.31 & APHA 9222B.	CFU/mL
	Clostridium perfringens	Enumeration of *Clostridium perfringens* by membrane filtration, National Standard Method W 5 Issue 3 (2004).	CFU/mL
	Bacteria identification method	Microscopic Examination.	–

Q$_{H_2}$ is the hydrogen gas flow rate (L d^{-1}), Q$_F$ is the feed flow rate (L d^{-1}), COD$_{in}$ is the initial substrate concentration, and COD$_{out}$ is an effluent concentration after treatment. A gas flow meter was used to determine the volume of gas produced. Meanwhile, the gas (5 ml) was collected from the gas port at the top of the H$_2$-UASFF unit using a gastight syringe (2500 microL Hamilton, USA) before being stored in a closed bottle using the water displacement method to determine the percentage of hydrogen and carbon dioxide. A gas chromatograph (GC) was then used to analyse the gas (Perkin Elmer, Gas Chromatograph, 600 Series LINK). Supelco (10 ft × 1/8 in., MR2924D, 40/80 carboxen 1000) pack GC column, thermal conductivity detector (TCD), and argon (carrier gas, 30 ml min^{-1}) were used in the GC. The injector, detector, and oven temperatures were set to 150 °C, 200 °C, and 100 °C, respectively. A 2500 µl gas syringe was used to inject 0.1 ml of biogas from the bottle into the GC (Hamilton, USA).

The treated effluent was filtered using glass microfibre filters GF/CTM (D = 47 mm), CAT No. 1822-047 for the preparation and analysis of volatile fatty acids (VFAs). 5 g of the filtered samples were stored in 15 ml vials. The rubber and silver cap provided by Perkin Elmer Co. Ltd. was used to seal the vials tightly. VFA analysis was performed using a Headspace Gas Chromatograph (Perkin Elmer, Clarus$^{®}$ 680) and a Headspace Sampler (Perkin Elmer, Turbomatrix 40 Trap) with column type Elite-1, 0.25 umdf, 0.25 mm internal diameter, and 30-metre long. The initial oven temperature was 40 °C, with injector and flame ionisation detector (FID) temperatures of 200 °C and 250 °C, respectively. With 1.2 ml min^{-1}, helium gas was used as a carrier. The purified air and hydrogen flow rates were set to 450 ml min^{-1} and 45 ml min^{-1}, respectively. The needle temperature, carrier gas pressure, column, and oven temperatures for the Headspace Sampler were 90 °C, 20 psi, 120 °C, and 75 °C, respectively.

2.5. Microbiological analysis

2.5.1. Culture-dependent method

Permulab Sdn Bhd performed preliminary bacterial identification on heat-treated and untreated POME sludge to detect the presence of culturable bacteria. The bacteria were chosen based on previous research that discovered *Clostridium* spp., *Bacillus* spp., and *E. coli* (Chong et al., 2009; Mamimin et al., 2015a; Rosales-Colunga et al., 2010). Table 3 lists the parameters and methods that were used in those studies.

2.5.2. Culture-independent method: PCR-DGGE analysis

2.5.2.1. DNA isolation and PCR amplification. DNA was extracted from untreated and heat-treated POME digested sludge samples using (NucleoSpin Soil, Macherey-Nagel, Germany) following the manufacturer's instructions. The 16S rRNA genes were amplified by nested PCR using Veriti Thermal Cycler (Applied Biosystems, UK). The reaction mixture (25 μL) comprised 1 μL of DNA template, 5 μl of 5x MyTaq Red Reaction Buffer, 10 μM of each 27F and 1492R primers, and 2.5 U of MyTaq DNA Polymerase (Bioline, UK). The initial denaturation was at 95 °C for 3 min, followed by 30 cycles of 95 °C for 45 s, 56 °C for 45 s, and 72 °C for 1 min, with a final elongation step at 72 °C for 5 min. A subsequent amplification with GC clamp primers was performed with 10 μM of the 341F-GC and 517R primers, 10 μl of 5x MyTaq Red Reaction Buffer, and 2.5 U of MyTaq DNA Polymerase (Muyzer et al., 1993). The initial denaturation was at 95 °C for 4 min, followed by 35 cycles of 95 °C for 30 s, 56 °C for 40 s, and 72 °C for 40 s, with a final elongation step at 72 °C for 7 min.

2.5.2.2. Denaturing Gradient Gel Electrophoresis (DGGE). DGGE was conducted using the CBS-DGGE 2001 system (C.B.S. Scientific Co., Inc, USA). The gel consisted of 40% acrylamide/Bis (37.5:1) in 1X TAE with a 30%–70% linear gradient denaturant urea. Electrophoresis was conducted at a constant voltage (90 V) and temperature (60 °C) for 16 h. Next, gels were stained for 30 min in 1.25X TAE containing 1X SYBR Gold (Invitrogen, USA) before being visualised using UV-Transilluminator (Cleaver Scientific, UK). Banding patterns were analysed using BioNumerics 7.5 (Applied Mathds, Belgium). Clear, distinct bands were cut and sent for sequencing at Apical Scientific Sdn. Bhd. The obtained sequences were compared to corresponding sequences in GenBank using Nucleotide BLAST (NCBI, 1988).

3. Results and discussion

The effect of temperature and HRT on simultaneous hydrogen production and COD removal efficiency was analysed using a factorial central composite experimental design. Seventeen runs were conducted with the studied responses, and the parameters are shown in Table 2.

3.1. Effects of temperature and HRT on hydrogen production rate (HPR)

The studied temperature range was successfully investigated between 37 °C and 70 °C. Table 4 demonstrates a reduced quadratic model for the HPR using Analysis of Variance (ANOVA), which revealed a significant relationship between HPR and temperature. Based on the attained second-order polynomial equation, Sqrt (HPR) represents the predicted values of HPR, while A is a coded temperature value. A determination coefficient (R^2) of 0.87 explains 87% of the variability in the response, and the adjusted R^2 of 86% suggests the model's significance. Moreover, a very low probability ($P < 0.05$) obtained from the ANOVA proved that the model was significant. HPR increased when the temperature increased from 37 °C to 56.8 °C (transition from mesophilic to thermophilic) (Fig. 2(A)). The highest HPR of 13.07 L H_2 day^{-1} was obtained at 53.5 °C, i.e., under thermophilic conditions. Thermophilic conditions significantly increase biogas production and the endurable OLR value compared to mesophilic conditions (Li et al., 2017). Nonetheless, higher temperatures (56.8 °C to 70 °C) demonstrated diminishing HPR.

Meanwhile, the difference in HRT used in this study did not affect HPR using mixed cultures. At HRT of 3 to 9 h, the lowest HPR was reported at 37 °C, while the highest was at 53.5 °C. Yang et al. (2019) reported that HRT demonstrates the hydraulic load ability of the anaerobic digestion reactor while OLR shows the organic load. Hence, as HRT increases, the OLR decreases, affecting system stability.

A thermophilic temperature can generate more hydrogen than a mesophilic temperature (Khan et al., 2018). This is because thermophiles in the sludge may require a longer lag time, even if they convert more substrate into hydrogen at a slower rate. According to van de Werken et al. thermophiles can degrade cellulose, hemicellulose, and pectin-containing biomass (Van De Werken et al., 2008). In a different study (Groenestijn et al., 2002), thermophiles produced more hydrogen than mesophiles. Because the system's entropy has increased, a higher thermodynamic temperature favours hydrogen production, resulting in a more energetic process. Our findings could also imply that different microbial communities, such as thermophiles, were activated as fermentation temperatures varied.

3.2. Effects of temperature and HRT on COD removal efficiency (%)

The second response evaluated in this study was COD removal efficiency (%). Based on the attained second-order polynomial equation, Sqrt (COD removal eff.) represents the predicted values of COD removal efficiency, whereby A is a temperature-coded value and B represents the HRT. An R^2 of 0.84 shows 84% of the variability in the response, and the adjusted R^2 of 71% explains the model's significance. Additionally, a very low probability ($P < 0.05$) obtained from the ANOVA proved that the model was significant. The COD is an essential measurement in wastewater treatment for oxygen required to break down water pollutants (organic substances). As shown in Fig. 2 A, the maximum COD removal efficiency was detected at 70 °C, 3 h HRT (42.14%). The 3D plot revealed that COD removal efficiency increased as temperature increased at 3 h HRT. This demonstrated that microbes in the inoculum degraded raw POME more quickly at lower HRT and preferred thermophilic conditions. When HRT was increased from 3 to 9 h (at 37 °C), COD removal efficiency decreased between 3 and 4 h before gradually increasing from 4 to 9 h. COD removal increased (from 24.76% to 33.33%) between 37 °C–56.8 °C before decreasing (from 33.33% to 14.29%) when the temperature reached 70 °C.

Table 4

Analysis of Variance (ANOVA) for Response Surface Reduced Quadratic Model for HPR and Response Surface Reduced Quartic Model for COD removal Efficiency (%) and H_2 Yield.

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F	
1. Hydrogen Production Rate (HPR)						
Model	18.87	2.00	9.43	48.92	0.00000	Significant
A-Temperature	4.87	1.00	4.87	25.24	0.00019	
A^2	14.00	1.00	14.00	72.60	0.00000	
Residual	2.70	14.0	0.19			
Lack of Fit	1.30	6.00	0.22	1.24	0.37861	Not significant
R-Squared	0.87					
Adj R-Squared	0.86					
Equations	Sqrt (HPR) = 3.13 + 0.70A − 1.84A^2					
2. COD removal Efficiency (%)						
Model	22.01	7.00	3.14	6.52	0.00603	Significant
A-Temperature	0.84	1.00	0.84	1.74	0.21982	
B-HRT	6.32	1.00	6.32	13.11	0.00556	
AB	7.01	1.00	7.01	14.55	0.00412	
A^2	4.70	1.00	4.70	9.75	0.01228	
B^2	3.89	1.00	3.89	8.08	0.01932	
A^2B	7.28	1.00	7.28	15.11	0.00369	
A^2B^2	4.80	1.00	4.80	9.95	0.01165	
Residual	4.34	9.00	0.48			
Lack of Fit	1.47	1.00	1.47	4.08	0.07804	Not significant
R-Squared	0.84					
Adj R-Squared	0.71					
Equations	Sqrt (COD removal eff.) = 5.65 + 0.29A + 1.78B −0.94AB −1.81A^2− 1.65B^2− 2.13A^2B + A^2B^2					
3. H_2 Yield						
Model	27.71	5.00	5.54	12.10	0.00036	Significant
A-Temperature	11.77	1.00	11.77	25.69	0.00036	
B-HRT	0.13	1.00	0.13	0.28	0.60457	
AB	2.55	1.00	2.55	5.56	0.03792	
A^2	7.79	1.00	7.79	17.00	0.00169	
B^2	0.33	1.00	0.33	0.71	0.41640	
Residual	5.04	11.0	0.46			
Lack of Fit	2.86	3.00	0.95	3.50	0.06971	Not significant
R-Squared	0.85					
Adj R-Squared	0.78					
Equations	Ln (H_2 yield) = −0.38 + 1.08A − 0.11B + 0.56AB − 1.06A^2− 0.33B^2					

The enzymes involved in the hydrolysis process may be temperature sensitive, resulting in a decrease in hydrolysis rate and a reduction in substrate degradation efficiency (Rizvi et al., 2015). At higher temperatures, substrates decompose more quickly, and substrate solubilisation to produce SCOD improves, increasing the mixture's biodegradability. Meanwhile, Zhang et al. discovered that using a low HRT (6 h) could increase hydrogen yield, resulting in higher degradation efficiency (Zhang et al., 2006). The shorter HRT may reduce microorganism diversity (with dominant species remaining), resulting in propionate production suppression.

3.3. Effects of temperature and HRT on hydrogen yield

The temperature has a relatively significant impact on digestion products and biogas yield. ANOVA showed a significant relationship between temperature and hydrogen yield using Response Surface Reduced Quartic Model as in Table 4. Regression analysis was used based on data in Table 4 using the quadratic equation stated below to evaluate the results, complying with a second-degree polynomial function.

$$Ln\ (H_2\ Yield) = -0.38 + 1.08A - 0.11B + 0.56AB - 1.06A^2 - 0.33B^2 \qquad (3)$$

Based on Eq. (1), A and B are coded values for temperature and HRT, respectively. The model showed an R^2 of 0.85, indicating 85% of the variability in the response. The adjusted R^2 denotes the model's goodness; hence the value for adjusting R^2 in this study was 0.78. Meanwhile, the Model F-value of 12.10 demonstrates a high significance of the fitted model. The individual P-value translated the significance of all the coefficients, with a Prob>F value of 0.00036 (less than 0.05) indicating the significance of the model and model terms, respectively.

The 3D counterplot in Fig. 2 showed that H_2 yield increased (0.25–0.61 L H_2 g^{-1} $COD_{removed}$) when HRT increased from 3–9 h at 70 °C. When the temperature increased from 37 °C to a temperature between 56.8–63.4 °C at 3 h HRT, the H_2
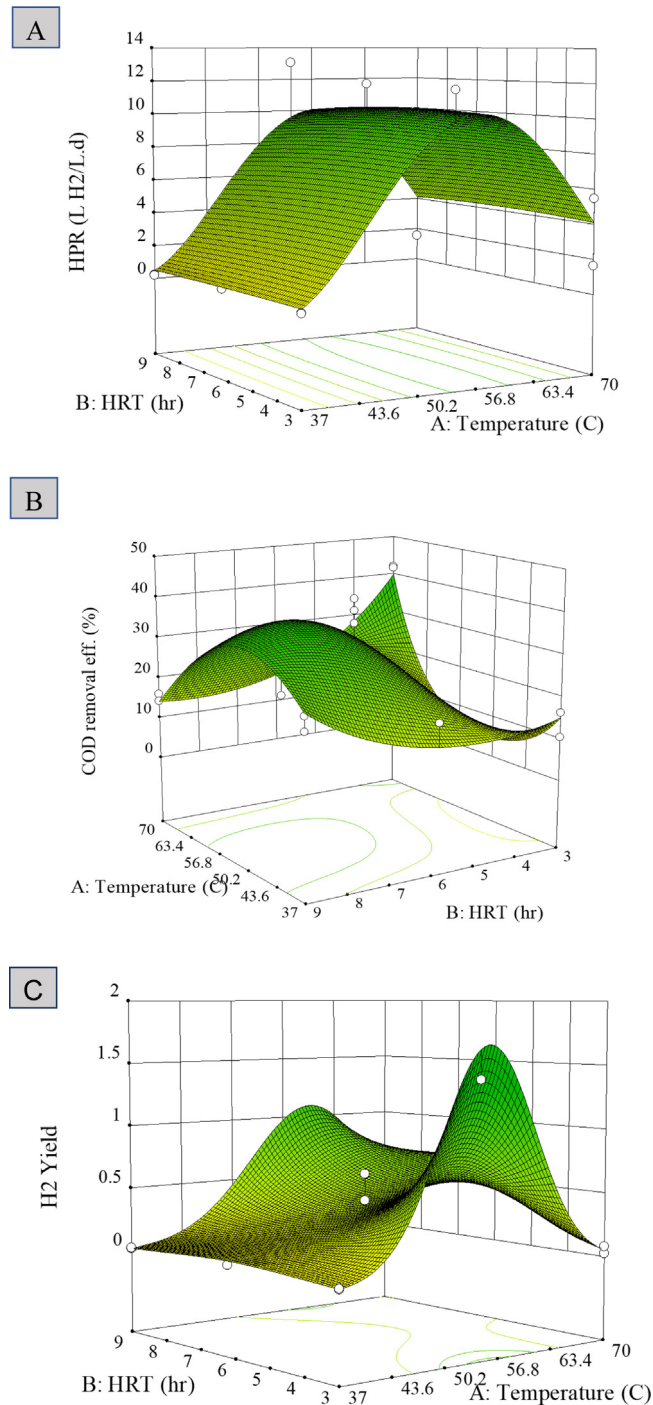
Fig. 2. 3D surface of effects of temperature and HRT on three responses; (A) HPR, (B) COD removal efficiency (%), and (C) H_2 Yield.

yields increased (from 0.05–1.17 L H_2 g^{-1} $COD_{removed}$) before decreasing when the temperature reached 70 °C (0.25 L H_2 g^{-1} $COD_{removed}$). Based on the previous start-up operation of the UASFF bioreactor, this trend indicates that the mixed cultures in POME digested sludge are already adapted to thermophilic conditions at lower HRT. Lin et al. obtained a similar result when they used municipal sewage sludge as inoculum and a gradual increase in hydrogen gas production at 50 °C due to mixed microflora adaptation at a new HRT of 12 h with strict enrichment of cultivation (Lin et al., 2008).

Fermentative hydrogen production is determined by nitrogen, phosphorus, carbon source, temperature, seed microorganisms, and pH (O-thong et al., 2008). In their study using Clostridium-rich compost from food wastes to investigate
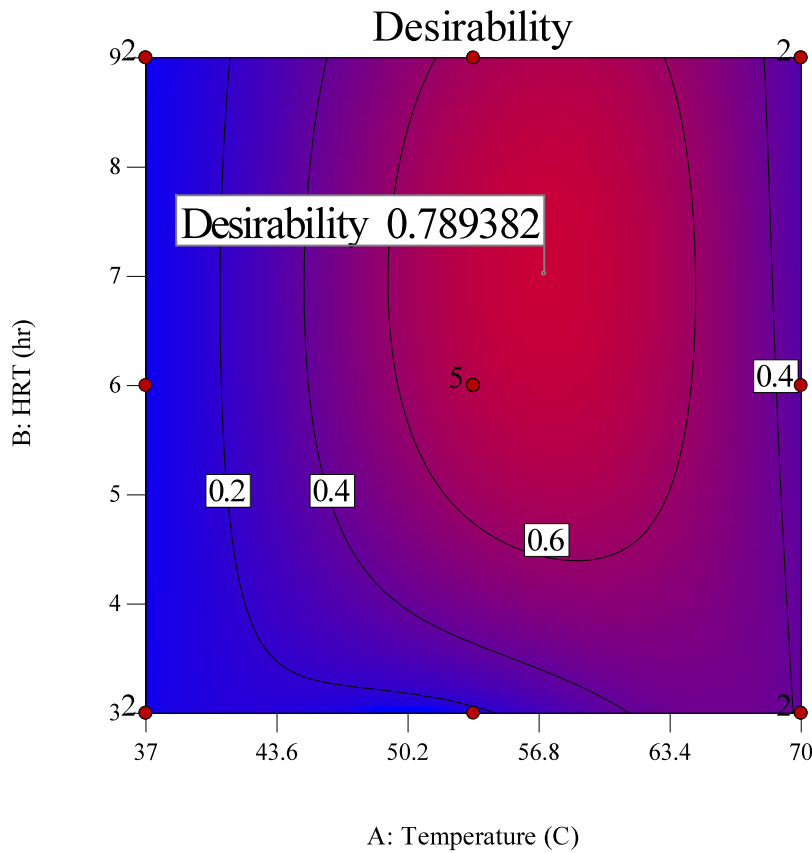
Fig. 3. Counterplot with 78.90% desirability at optimum HRT and temperature of 7 h and 57 °C, respectively.

factors affecting hydrogen production, Lay et al. (2005) discovered that hydrogen-producing Clostridium species were dominant after a heat-shock treatment at 80 °C for 3 h. This is because the temperature has killed all other non-spore-forming competitors and/or hydrogenotrophic organisms. This study used a heat-treated (90 °C, 1 h) POME sludge as inoculum, which may have resulted in gram-negative rod bacteria. The study by Lay and colleagues also revealed that the hydrogen production potential of carbohydrate-rich organics is 20 times greater than that of protein- and fat-rich organics (Lay et al., 2003).

3.4. Optimisation

The optimal conditions for the highest output were discovered through optimisation. These findings were used in the CH$_4$-UASFF study (Zainal et al., 2020b) to investigate the interactions between the optimal conditions used in the H$_2$-UASFF unit and the influence of two factors in the CH$_4$-UASFF unit, which produces biomethane (i.e., temperature and dark fermentation effluent). With this condition, only one solution with 78.90% desirability was obtained (Fig. 3). The highest HPR, hydrogen yield, and COD removal efficiency were found to be at thermophilic (57 °C) with 7 h HRT, with values of 10.39 L H$_2$ d^{-1}, 0.951 L H$_2$ g^{-1} COD$_{removed}$ d^{-1}, and 35.88%, respectively.

COD removal efficiencies in a dark fermentative hydrogen production process were reported to be 20%–40% (Zainal et al., 2018b; Vijayaraghavan and Ahmad, 2006). This is because POME lacks nutrients such as phosphorus, iron, and nitrogen, resulting in a low COD removal efficiency (O-thong et al., 2008).

The temperature at which biohydrogen is produced is linked to a shift in the microbial community and the metabolic pathways (Balachandar et al., 2013). Along these lines, studies have shown that understanding the temperature dependence of the microbial community is critical for improving hydrogen production systems. Because of its natural high temperature after sterilisation and purification, thermophilic-anaerobic fermentation for POME is more effective than mesophilic (Zeidan and van Niel, 2010). In this study, fermentative bacteria, specifically acidogenic bacteria, converted POME into hydrogen gas. As a result, the dark fermentation process is more efficient, cost-effective, and energy-efficient (Vijayaraghavan and Ahmad, 2006; Atif et al., 2005). Furthermore, the partial pressure of hydrogen gas could be reduced by controlling pH and nitrogen purging, resulting in a high hydrogen yield. In this study, the pH was kept between 5 and 5.5 with no added buffer (sodium bicarbonate), indicating the bioreactor's stability.

Table 5
Bacterial identification and plate count for untreated and heat-treated POME sludge.

Sample	Parameters	Unit	Results
Untreated POME sludge (control)	Heterotrophic plate count	CFU/mL	2.4×10^7
	Bacterial Identification	–	*Lactobacillus acidophilus*
Heat-treated POME sludge (90 °C, 1 h) used in this study	Anaerobic plate count	CFU/mL	2.5×10^7
	Total coliform	CFU/mL	n.d (<1)
	Escherichia coli	CFU/mL	n.d (<1)
	Bacillus spp.	CFU/mL	n.d (<1)
	Clostridium perfringens	CFU/mL	n.d (<1)
	Bacterial Identification	–	Gram-negative rod

n.d. = not detected.
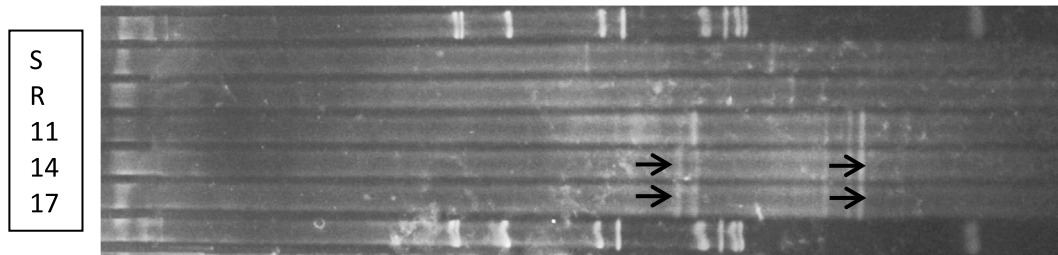
Fig. 4. DGGE banding patterns for samples S, R, 11, 14, 17. Only bands indicated by arrows gave reliable sequences. (S = POME sludge; R = raw POME, 11 = Run 11; 14 = Run 14; 17 = Run 17).

3.5. Bacterial identification and Volatile Fatty Acids (VFAs) composition

After the dark fermentation process, a small amount of heat-treated POME sludge from the H_2-UASFF bioreactor was inoculated onto agar media. Gram-negative rod-shaped bacteria had the highest count in heat-treated POME, with 2.5×10^7 CFU/mL. The hydrolysis of long-chain carbon compounds is followed by acidogenesis and acetogenesis for hydrogen production in the POME degradation process (Tan et al., 2015). Table 5 shows the bacteria found in both untreated and treated POME sludge. Putative *Lactobacillus* species (Gram-positive facultative anaerobes) were detected in untreated POME sludge, and their presence is probably due to the nature of the treatment process at the mill. Meanwhile, *Lactobacillus* spp. is a significant part of the lactic acid group that could convert sugars to lactic acid.

Based on Table 5, there was no presence of *Escherichia coli, Bacillus* spp., and *Clostridium perfringens* in heat-treated sludge. This contrasts with a study by Yossan et al. (2012), who found *Clostridium* spp. to be a dominant species after heat-shock sludge treatment with POME for biohydrogen production. This could be due to the different treatment systems used in the respective palm oil mills, weather conditions in the area, and the inoculum heat-treatment conditions used. However, DGGE analysis revealed that samples 14 and 17 had similar DGGE banding profiles, and the partial 16S rRNA gene sequences from all four distinct bands were 95% similar to *Acetobacter* spp (Fig. 4). Acetic acid bacteria, such as *Acetobacter* spp., is an organic compound that converts glucose to acetic acid during fermentation. Our finding suggests that these bacteria may be abundant in the system. This finding is consistent with another study in which *Acetobacter* spp. predominate in a biohydrogen co-digestion process (García-Depraect et al., 2017). Table 6 summarises the effluent characteristics and bioreactor performance in various studies that used POME as a substrate (see Fig. 4).

Meanwhile, the presence of VFA is one of the factors influencing process stability in the dark fermentation system. POME was fermented to VFAs and hydrogen as a substrate during the hydrolysis stage. Because of the system's stability, no buffer (alkalinity) was added, as previously stated. Zinatizadeh and Mirghorayshi (2017) reported that the high total VFA reduced treatment efficiency. As a result, the TVFA/alkalinity ratio can be used to assess process stability in anaerobic digestion systems.

Based on Table 6, there was a presence of propionic, butyric, and acetic acid. The acetic acid content was comparable to Badiei et al. (2011). On the other hand, propionic acid was accumulated, indicating that methanogenesis failed. This is a good indicator for biohydrogen production in this study because the process's primary goal was to maximise HPR and yield. It has been reported that propionic acid concentrations greater than 0.95 g L^{-1} inhibit the growth of methanogenic archaea (Demirel and Yenigu, 2002). It is also important to note that the pH of the medium influences the presence of methanogenic bacteria.

4. Conclusions

Results from this study demonstrated the performance of an integrated UASFF bioreactor for biohydrogen production from POME under the dark fermentation process. Raw POME and POME sludge are excellent sources of biohydrogen.

Table 6

A comparison study of dark fermentation effluent characteristics in H_2-reactor and its performance.

Parameters	Krishnan et al. (2016a)	Mamimin et al. (2015b)	Badiei et al. (2011)	This study
Bioreactor/Substrate	UASB/POME	ASBR/POME	ASBR/POME	UASFF/POME
Temperature (°C)	55	55	37	57
HRT (h)	48	48	72	7
Acetic Acid (mg L^{-1})	4750	4866	710	0–762
Propionic acid (mg L^{-1})	146	150	80	69–6959
Butyric Acid (mg L^{-1})	5600	7855	60	68–437
TVFA (g COD L^{-1})	15.49	19.73	2.27	0.3–10.7
Types of bacteria in hydrogen reactor	*Thermoanaerobacterium* spp.	*Thermoanaerobacterium thermosaccharolyticum*	N/A	Gram-negative rods
HPR (L H_2 L^{-1} d^{-1})	1.92	1.84	6.7	10.39
H_2 Yield	215 L H_2 kg^{-1} COD	2.10 ml H_2 g^{-1} COD	0.34 L H_2 g^{-1} $COD_{feeding}$	0.951 L H_2 g^{-1} $COD_{removed}$
COD removal efficiency (%)	42	38	37	35.88

N/A = not analysed.

The best reactor performance was achieved under thermophilic conditions at temperatures and HRT of 57 °C and 7 h, respectively, with maximum hydrogen production rate and yield of 10.4 L H_2 day^{-1} and 0.95 L H_2 g^{-1} $COD_{removed}$ with 35.9% COD removal. *Acetobacter* spp. predominated in the H_2-UASFF unit under thermophilic conditions. Temperature also has a significant impact on HPR and hydrogen yield, while HRT has a significant impact on COD removal efficiency. Our findings also demonstrated that once the microbes were acclimatised to such temperatures, the H_2-UASFF reactor could tolerate temperature changes in the 37–70 °C range without affecting process stability.

CRediT authorship contribution statement

Bidattul Syirat Zainal: Conceptualization, Formal analysis, Software, Validation, Microbial, Research investigation, Resources, Writing – original draft. **Kartini Gunasegaran:** Formal analysis, Microbial, Research investigation. **Geok Yuan Annie Tan:** Formal analysis, Microbial, Resources, Writing – review & editing. **Mahmoud Danaee:** Formal analysis, Software, Validation, Resources. **Nuruol Syuhadaa Mohd:** Writing – review & editing. **Shaliza Ibrahim:** Resources. **Ong Hwai Chyuan:** Writing – review & editing. **Long D. Nghiem:** Writing – review & editing. **T.M. Indra Mahlia:** Supervision.

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.

Data availability

Data will be made available on request.

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