HIGH-RATE BIOLOGICAL HYDROGEN AND METHANE PRODUCTION FROM POME IN A TWO-STAGE ANAEROBIC HYBRID REACTOR

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ABSTRACT

HIGH-RATE BIOLOGICAL HYDROGEN AND METHANE PRODUCTION FROM POME IN A TWO-STAGE ANAEROBIC HYBRID REACTOR

This study was divided into three phases, *viz*. i) batch study, ii) start-up study using up-flow anaerobic sludge fixed film (UASFF) bioreactor, and iii) optimization study. UASFF bioreactor is a type of bioreactor configuration similar to the type used by industries for the treatment of wastewaters. It is a hybrid system that combines two compartments for providing granular and fixed biomasses in a single bioreactor. In this study, a 2.5 L and 3.5 L of H_2-UASFF and CH_4-UASFF bioreactor units, respectively, were successfully operated for palm oil mill effluent (POME) treatment. An initial experiment was done to evaluate the nature of POME wastewater by conducting a batch study using a 160-mL serum bottle, under anaerobic condition. A batch study for biohydrogen production was conducted using raw POME and POME sludge as a feed and inoculum respectively. Response Surface Methodology (RSM) was used to design the experiments. Experiments were conducted at different reaction temperatures (30-50°C), inoculum size to substrate ratios (I:S) and reaction times (HRT) (8-24 h). Although the highest COD removal efficiency was 49.09% at 24 h, 50°C and 10:90 (I:S), however, based on the optimization study using RSM, the optimum condition of biohydrogen production was achieved with COD removal efficiency of 21.95% with hydrogen yield of 28.47 mL H_2 g^{-1} COD removed (2.22 mg H_2 g^{-1} COD removed). The I:S ratio was 40:60, with a reaction temperature of 50°C at 8 h of reaction time. The next experiment was done in a two-stage UASFF bioreactor in order to study its performance. A start-up study was conducted to produce biohydrogen and biomethane from POME. During this period of continuous operation, the HRT and temperature were adjusted in order to optimize the condition for biogas production. After 59 days of operation, using 100% raw POME led to a total COD

removal of 83.70%, average gas production rates of 5.29 L H_2 d^{-1} (57.11% H_2) and 9.60 L CH_4 d^{-1} (94.08% CH_4), in H_2-UASFF unit and CH_4-UASFF unit, respectively. This work concludes that the two-stage UASFF bioreactor operating at a final HRT of 4 h and temperature of 43°C in H_2-UASFF unit and 24 h HRT, 43°C in CH_4-UASFF unit has taken a period of two months for start-up. The third phase was done in order to find the optimum conditions for two-stage UASFF bioreactor in treating POME. Two variables, i.e. temperature (37-70°C) and HRT (3-9 h) was examined in H_2-UASFF unit and the same temperature and dark fermentation effluent was used as a substrate (12 – 20 g COD L^{-1}) in CH_4-UASFF unit. At optimum temperature and HRT of 57°C and 7 h, respectively, maximum hydrogen production rate of 10.39 L H_2 d^{-1}, hydrogen yield of 0.95 L H_2 g^{-1} $COD_{removed}$ and 35.88% of COD removal were observed. In CH_4-UASFF unit, at 24 h HRT, 76% of total COD removal efficiency was achieved with methane production rate of 15.63 L CH_4 d^{-1}, methane yield of 0.803 L CH_4 g^{-1} $COD_{removed}$, COD removal efficiency of 66.28%, and 93.31% of CH_4 content at optimum temperature and substrate concentration of 54°C and 12 g COD L^{-1}, respectively. These findings proved that the integrated system can enhance biogas production rate, yield and efficiently treating POME wastewater under a short period of time with low substrate concentration and thermophilic condition.

Keywords: biohydrogen, biomethane, POME, UASFF bioreactor, anaerobic process.

ABSTRAK

PENGHASILAN HIDROGEN DAN METANA BERKADAR TINGGI SECARA BIOLOGI DARIPADA POME DI DALAM DUA PERINGKAT REAKTOR HIBRID ANAEROBIK

Kajian ini dibahagikan kepada tiga fasa, iaitu. i) kajian kumpulan, ii) kajian permulaan menggunakan reaktor hibrid anaerobik dua-peringkat (UASFF), dan iii) kajian pengoptimuman. Bioreaktor UASFF ialah bioreaktor yang digunakan oleh industri sekarang untuk rawatan sisa air buangan. Ia adalah sistem hibrid yang menggabungkan dua bahagian untuk menghasilkan biomas berbutir dan tetap dalam satu bioreaktor tunggal. Dalam kajian ini, 2.5 L H_2-UASFF dan 3.5 L CH_4-UASFF unit bioreaktor telah berjaya dikendalikan untuk rawatan efluen kilang minyak sawit (POME). Percubaan awal dilakukan untuk menilai sifat sisa air POME dengan melakukan kajian kumpulan menggunakan botol serum 160 mL, dalam keadaan anaerob. Kajian kumpulan untuk pengeluaran biohidrogen dilakukan menggunakan POME mentah dan enapcemar POME, masing-masing sebagai makanan dan inokulum. Response Surface Methodology (RSM) telah digunakan untuk merekabentuk eksperimen. Eksperimen dijalankan pada suhu reaksi yang berbeza (30-50°C), saiz inokulum kepada nisbah substrat (I:S) dan masa reaksi (HRT) (8-24 jam). Walaupun kecekapan penyingkiran COD tertinggi didapati sebanyak 49.09% pada 24 jam masa tindak balas, suhu 50°C dan nisbah I:S adalah 10:90, namun, berdasarkan kajian optimum menggunakan RSM, keadaan optimum pengeluaran biohidrogen dicapai dengan kecekapan penyingkiran COD sebanyak 21.95% dengan hasil hidrogen sebanyak 28.47 mL H_2 g^{-1} COD dikeluarkan. Nisbah I:S adalah 40:60, dengan suhu tindak balas 50°C pada 8 jam masa tindak balas. Percubaan seterusnya dilakukan dalam bioreaktor UASFF dua-peringkat untuk mengkaji prestasinya pada peringkat permulaan. Kajian awal dijalankan untuk menghasilkan biohidrogen dan biometana dari POME. Dalam tempoh operasi berterusan ini, masa reaksi dan suhu

diselaraskan untuk mengoptimumkan keadaan pengeluaran biogas. Selepas 59 hari, penggunaan 100% POME mentah menyebabkan jumlah penyingkiran COD sebanyak 83.70%, purata kadar pengeluaran gas 5.29 L H_2 d^{-1} (57.11% H_2) dan 9.60 L CH_4 d^{-1} (94.08% CH_4), dalam unit masing-masing. Kerja-kerja ini menyimpulkan bahawa bioreaktor UASFF dua peringkat yang beroperasi pada HRT akhir sebanyak 4 jam dan suhu 43°C di dalam H_2-UASFF unit telah mengambil masa dua bulan untuk permulaan. Fasa ketiga dilakukan untuk mencari keadaan optimum bioreaktor dua peringkat UASFF dalam merawat POME. Dua pembolehubah, iaitu suhu (37-70 ° C) dan HRT (3-9 jam) diperiksa dalam unit H_2-UASFF dan suhu yang sama dan efluen penapaian gelap digunakan sebagai substrat (12 - 20 g COD L^{-1}) dalam unit CH_4-UASFF. Pada suhu optimum dan HRT 57°C dan 7 jam, kadar pengeluaran hidrogen maksimum 10.39 L H_2 d^{-1}, hasil hidrogen 0.95 L H_2 g^{-1} CODremoved dan 35.88% penyingkiran COD dicapai. Dalam unit CH_4-UASFF, pada 24 jam HRT, 76% daripada kecekapan penyingkiran COD dicapai dengan kadar pengeluaran metana sebanyak 15.63 L CH_4 d^{-1}, hasil metana 0.803 L CH_4 g^{-1} $COD_{removed}$, kecekapan penyingkiran COD daripada 66.28%, dan 93.31% kandungan CH_4 pada suhu optimum dan kepekatan substrat masing-masing 54°C dan 12 g COD L^{-1}. Penemuan ini membuktikan bahawa bioreaktor hibrid mampu meningkatkan kadar pengeluaran biogas, menghasilkan dan dengan berkesan merawat air sisa POME dalam tempoh yang singkat di bawah kepekatan substrat yang rendah dan keadaan termophilic.

Kata kunci: biohidrogen, biometana, POME, bioreaktor UASFF, proses anaerobik.

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF SYMBOLS AND ABBREVIATIONS

ANOVA	:	Analysis of variance
ASBR	:	Anaerobic sequencing batch reactor
BOD	:	Biological oxygen demand
CH_4	:	Methane
CO_2	:	Carbon dioxide
COD	:	Chemical oxygen demand
CSTR	:	Continuous stirred tank reactor
FBR	:	Fluidized bed reactor
GC	:	Gas Chromatography
GC-MS	:	Gas Chromatography-Mass Spectrometry
GPR	:	Gas production rate
H_2	:	Hydrogen
H_2SO_4	:	Sulphuric acid
HCl	:	Hydrochloric acid
HPB	:	Hydrogen-producing bacteria
HPR	:	Hydrogen production rate
HRT	:	Hydraulic retention time
MLSS	:	Mixed liquor suspended solids
MLVSS	:	Mixed liquor volatile suspended solids
MPR	:	Methane production rate
NaOH	:	Sodium hydroxide
OLR	:	Organic loading rate
POME	:	Palm oil mill effluent

RSM	:	Response surface methodology
TCOD	:	Total chemical oxygen demand
TSS	:	Total suspended solids
UASB	:	Up-flow anaerobic sludge blanket
UASFF	:	Up-flow anaerobic sludge fixed-film
VFA	:	Volatile fatty acids
VSS	:	Volatile suspended solids
$Balk_{in}$	:	Initial bicarbonate alkalinity
COD_{eff}	:	Effluent substrate concentration
COD_{in}	:	Initial substrate concentration
F/M	:	Feed rate of COD to VSS
pH_{in}	:	Initial pH
Q_{CH4}	:	Methane flow-rate
Q_F	:	Feed flow-rate
Q_{H2}	:	Hydrogen flow-rate
Q_R	:	Recycle flow-rate
R^2	:	Correlation coefficient
T	:	Temperature
V_R	:	Reactor volume
V_{up}	:	Up-flow velocity
Y_{CH4}	:	Methane yield
Y_{H2}	:	Hydrogen yield

LIST OF APPENDICES

CHAPTER 1: INTRODUCTION

1.1 General background

Renewable energy is an energy that can be obtained from natural resources such as fuels, minerals, water, natural vegetation and forests. Hydropower, solar energy and bioenergy utilized more than one source of renewable energy. The renewable energy forms include biomass, wind, and biofuels.

In Malaysia, the government is presently heading to apply green technology especially for industrial sector. Green technology portrays the reduction in greenhouse gas (GHG) emissions, promotes utilization of renewable energy resources, energy conservation and use of natural resources (Ng, Yew, Basiron, & Sundram, 2011). Factually, Malaysia is the second biggest palm oil producer in the world after Indonesia, therefore, the biggest biomass and wastewater created each year is originating from oil palm plantations. The most recent report in 2017 obviously demonstrated that oil palm planted zone achieved 5.81 million hectares, an expansion of 1.3% in comparison to 5.74 million hectares recorded in the previous year (Din, 2018). This was mainly because of the expansion of a newly planted area in Sarawak (currently 1.56 million hectares as the biggest oil palm planted state in Malaysia), followed by 1.55 and 2.70 million hectares in Sabah and Peninsular Malaysia, respectively.

Palm Oil Mill Effluent (POME) is discharged after sterilization, clarification and separation process. It could create a mass amount of methane gas from the anaerobic process that has 23 times Global Warming Potential (GWP) compared to carbon dioxide (Vijaya, Ma, & Choo, 2010). The wastewater treatment facility is among the most vital

segment in the palm oil process flow. This is on account that the ponding system is to treat POME that is being created in vast volume amid the generation of crude palm oil (CPO). Because of the chemical compound and physical properties of POME, the most productive treatment utilized in the underlying phase of the wastewater plant is anaerobic treatment.

1.1.1 Palm Oil Mill Effluent (POME)

POME is the main pollutant produced in palm oil mills in Malaysia. For one ton of crude palm oil processing, it is estimated that 3.05 m^3 of POME produced (Loh & Choo, 2013). If there is no proper effluent management, POME will be the main source of air and water pollution in the future. POME contains a high nutrient, organic and carbon contents despite having high BOD and COD content (Table 1.1). It also possess huge potential for the production of biogas (Hosseini & Wahid, 2013). During POME decomposition of organic matters, there are 60-70% of methane and 30-40% of CO_2 produced, with the remaining consists of a trace amount of H_2S (Loh et al., 2014).

Table 1.1: Palm Oil Mill Effluent (POME) characteristics.

Parameter	Raw POME
Potassium (K)	2270 mg L^{-1}
Magnesium (Mg)	615 mg L^{-1}
Calcium (Ca)	439 mg L^{-1}
Zinc (Zn)	2.3 mg L^{-1}
Iron (Fe)	46.5 mg L^{-1}
Copper (Cu)	0.89 mg L^{-1}
Total Kjedahl Nitrogen (TKN)	750 mg L^{-1}
Ammoniacal Nitrogen (NH_3-N)	35 mg L^{-1}
Total Volatile Solids (TVS)	34,000 mg L^{-1}
Total Suspended Solid (TSS)	18,000 mg L^{-1}
Total Solid (TS)	40,000 mg L^{-1}
Biological Oxygen Demand (BOD_3)	25,000 mg L^{-1}
Chemical Oxygen Demand (COD)	50,000 mg L^{-1}
pH	4.7
Temperature	80-90°C
Oil & Grease	4000 mg L^{-1}

1.1.2 Palm Oil Wastes Generation

There are two types of wastes generated from oil palm mill, namely, liquid and solid wastes. POME is a liquid-type waste that produced approximately 53 million m^3 per year in Malaysia (based on 14.8 million ton of oil palm production) (Ahmad et al., 2016). About 60%, 36% and 4% of POME mixtures come from clarification, sterilization and hydrocyclone units, respectively (Rupani, Singh, Ibrahim, & Esa, 2010). Raw POME, on the other hand, is a colloidal matter that contains water (95-96%), total solids (4-5%) that contains 2-4% of suspended solids and oil (0.6-0.7%). The presence of suspended solids are mainly from palm fruit mesocarp that underwent sterilizer condensate, sludge separator and finally hydrocyclone waste (Najafpour, Zinatizadeh, Mohamed, Hasnain Isa, & Nasrollahzadeh, 2006).

On the other hand, most of the solid wastes are in the form of trunks, shell, palm oil mill sludge, oil palm empty fruit bunch, decanter cake and palm kernel cake. They are normally generated during harvesting, replanting or milling processes. Generally, the process of retrieving palm oil is almost similar in Indonesia, Malaysia or Thailand. The difference is probably some of the palm oil industry uses biogas from palm oil process, thus having a closed reactor for methane capture.

1.2 Environmental Regulations of Effluent Discharge

In 1978, Environmental Quality Regulations enactment was proposed for POME discharge standards with the focus on BOD. From 25,000 mg L^{-1} of untreated POME, the discharge standard limit was reduced to 5000 mg L^{-1} in the first generation, down to the current BOD of 100 mg L^{-1} (Malaysian Palm Oil Board, 2015). Initiatives are in progress to decrease the BOD level to 50 mg L^{-1}, and in places where release into conduits is required. Research and Development (R&D) is effectively sought after to decrease the

BOD load to 100 mg L^{-1}. Table 1.2 represents POME discharge standards since 1978 until 2015 (Malaysian Palm Oil Board, 2015).

Table 1.2: POME Discharge Limit from 1978 to 1984 and thereafter.

Parameter	Limits required based on the period of discharge					
	1st July 1978 – 30th June 1979	1st July 1979 – 30th June 1980	1st July 1980 – 30th June 1981	1st July 1981 – 30th June 1982	1st July 1982 – 31st Dec 1983	1st Jan 1984 onwards
pH	5 - 9	5 - 9	5 - 9	5 - 9	5 - 9	5 - 9
Temperature (°C)	45	45	45	45	45	45
Oil and Grease (mg L^{-1})	150	100	75	50	50	50
Total Solids (mg L^{-1})	4,000	2,500	2,000	1,500	-	-
Suspended Solids (mg L^{-1})	1,200	800	600	400	400	400
Total Nitrogen (mg L^{-1})	200	100	75	50	-	-
Ammoniacal Nitrogen (mg L^{-1})	25	15	15	10	150	100
COD (mg L^{-1})	10,000	4,000	2,000	1,000	-	-
BOD (mg L^{-1})	5,000	2,000	1,000	500	250	100

Palm oil and rubber mills effluent discharge standard was first introduced by Malaysia. In 1977, the Department of Environment (DoE) announced the discharge standard for POME. Before the regulation was implemented by all palm oil mills, crude palm oil seems to be the worst main source of pollution. The daily discharge was more than 300% increased from 1965 until 1977. Hence, the regulation was made in order to reduce pollution without hindering the growth of oil palm industries.

1.3 POME Treatment Systems in Malaysia

Anaerobic process has become the most suitable method in treating POME due to its high organic properties. The high concentration of lipid, nitrogenous compounds, carbohydrates, protein and minerals in POME can be converted to valuable products by using microbial process (Habib, Yusoff, Phang, Ang, & Mohamed, 1997). Because of that, treating POME using ponding system has been used in an earlier stage for the palm oil industry.

Despite the fact that POME is non-lethal, there is a concern that economic expansion, environmental protection and sustainable development need to be balanced due to the fact that POME is a potential cause of pollution (Rupani et al., 2010). To ensure that this industry remains to be sustainable and environmentally friendly, POME needs to be well taken care of and cannot directly be discharged into a water body as it can contaminate the water and endanger aquatic ecosystem (Vijaya et al., 2010).

Therefore, a lot of studies have been done by researchers to treat POME using alternative methods. This is because conventional methods such as aerobic/anaerobic system, open decomposing tank, anaerobic system, closed anaerobic decomposition tank and advanced ventilation system requires extensive land area and producing a foul odor, which resulting in environmental pollution (Chin, Poh, Tey, Chan, & Chin, 2013; Poh & Chong, 2009).

Ahmad et al., (2003) reported that due to the presence of untreated palm oil residue, raw POME consists of a high value of degradable organic matter. Biological treatment with the aerobic, anaerobic or facultative process is the most suitable method to degrade/treat POME. This is because biological treatment requires less energy demand, does not liberate foul odour, can minimize sludge accumulation and can produce

hydrogen and methane gas by anaerobes under fermentation and digestion processes. Moreover, the methane gas produced can further be used for electricity generation.

However, the open ponding system could cause methane gas being released into the atmosphere. This contributes to the thinning of the ozone layer that resulted in greenhouse gas (GHG) effect. Even though less operational energy and small capital are required, an open ponding system involves longer retention time (20-60 days) and large area (Loh & Choo, 2013). The implementation of a closed anaerobic system has drawn many changes towards the regulatory standard. It was reported that covered lagoon or closed-tank anaerobic digester has been widely used to treat POME (Wang et al., 2015).

On the other hand, a hybrid system which combines the conventional and alternative methods such as anaerobic filter (Bello & Abdul Raman, 2017), up-flow anaerobic sludge blanket (UASB) (Khemkhao, Nuntakumjorn, & Techkarnjanaruk, 2011), sequencing batch reactor (SBR) (Chan et al., 2011), up-flow anaerobic sludge fixed-film reactor (UASFF) (Najafpour et al., 2006) and anaerobic fluidized bed reactor (AFBR) (Borja, Banks, & Sinchez, 1996) were studied and used to obtain higher efficiency and ensure lesser processing time. These hybrid reactors were proven to reduce hydraulic retention time (HRT) when studied on a laboratory scale.

Above all, all palm oil millers must meet the standard requirement provided by the DoE, as shown in Table 1.2. The transition of the treatment method makes conventional POME treatment system becomes outdated and the new requirement for BOD discharge limit of 20 mg L^{-1} seems hard to be fulfilled by the respective mills. However, a lot of POME treatment technologies have been studied as an alternative to the above-mentioned problem.

1.4 POME as A Renewable Energy

Open ponding or lagoon system in palm oil mills in Malaysia has been used to treat POME since the beginning of 1982. It has been reported that more than 85% of palm oil mills uses ponding system, with 50% of total 442 mills uses anaerobic pond while the rest uses various digesters (Zainal, Jalani, & Mamat, 2017). Open ponding system becomes favourable to most oil palm millers due to its simple operation and low-cost system.

However, a new regulation has been implemented by the Malaysian Palm Oil Board for biogas to be captured for all mills in Malaysia. An introduction of close anaerobic/aerobic digestion tank for POME treatment is compulsory to comply with the discharge standard/limits set by the Department of Environment (DoE) Malaysia. Nevertheless, anaerobic digestion that employed by mills for POME treatment has created biogas as a by-product. The amount of biogas produced is depending on the type of the treatment used.

For every ton of POME treated, approximately 36% of biogas (average 5.5 kg of methane) was produced from open digesting tanks (Yacob, Hassan, Shirai, Wakisaka, & Subash, 2006). In average, 5.4 L min^{-1} m^2 biogas was produced and 518.9 kg day^{-1} of total methane emission per one open digesting tank was recorded (Madaki & Seng, 2013).

The number of studies on biogas production from waste and the development of renewable energy for sustainable power generation has increased due to the concern of the depletion of fossil fuels. POME, a waste that contains high organic carbon has the potential to boost up the renewable energy sector and become a promising source for biogas production. Thus, being one of the leading crude palm oil producer in the world, Malaysia is well positioned for biogas development with a high amount of POME generated every year (Chin et al., 2013).

Bumibiopower, a developer of a renewable energy power plant in Malaysia has set up a plant utilizing POME for power generation and methane extraction near Pantai Remis, Manjung, Perak (west coast of Peninsular Malaysia). A closed anaerobic system has been installed to consistently produce and collect high-quality methane-rich biogas. 1 and 1.5 MW generators are also installed and included in this project (Abdullah & Sulaiman, 2013).

1.5 Problem Statement

Palm Oil Mill Effluent (POME) is the wastewater discharged from the sterilization process, crude oil clarification process and cracked mixture separation process (Ahmed, Yaakob, Akhtar, & Sopian, 2015). There are three types of POME, namely raw POME and digested POME from the anaerobic or the aerobic treatment process, with each type has different chemical composition. Despite its negative impacts on the environment if its huge annual production is not managed properly, POME can be used as an important biomass resource due to its high organic, carbon and nutrients content (Kamyab et al., 2014). POME also has a potential for biogas production, i.e. through the organic matter decomposition.

POME has high lignin content; thus, a pre-treatment is necessary before further treatment for hydrogen (H_2) and methane (CH_4) production. Different pre-treatment methods such as acid pre-treatment, chemical pre-treatment, heat pre-treatment and base pre-treatment has extensively studied (Mohammadi, Ibrahim, & Mohamad Annuar, 2012b). For different types of POME that has different chemical compositions, raw POME is a potential substrate for H_2 and CH_4 production. To save the cost and energy for pre-treatment, a pre-settled POME can be used instead. POME sludge in anaerobic and aerobic pond might as well be a good source of inoculum. The mixed consortia in the

sludge is varies such as mesophiles and thermophiles, dependable on the temperature, oxygen level and pH of the sludge.

Using a two-stage microbial fermentation, H_2 and CH_4 are produced through acidogenesis and methanogenesis processes, respectively. In these processes, the difference between the acidogens (i.e. microorganisms responsible for hydrogen production during acidogenesis) and methanogens (i.e. microorganisms responsible for methane production during methanogenesis) is significant; especially for their nutritional needs, growth kinetics, physiology and environmental sensitivity (e.g. temperature) (Khongkliang, Kongjan, & O-Thong, 2015). In the first phase of the anaerobic digestion process, hydrolysis and acidogenesis take place with an optimal hydraulic retention time (HRT) of 1-3 days at optimum pH of 5-6 (Kongjan, O-thong, & Angelidaki, 2011). Under acetate and butyrate pathways, carbohydrate will then be converted to hydrogen and organic acids. In the second phase, the remaining organic acids will be converted to methane under anaerobic conditions with optimum pH of 7-8 (Zabranska & Pokorna, 2017) and 15-20 days HRT (Chonticha Mamimin, Singkhala, Kongjan, et al., 2015).

In spite that studies on the treatment of high concentration of POME using two-stage systems are quite extensive, the study on biohydrogen and biomethane production using two-stage UASFF reactor from POME in Malaysia has not been done. Hence, prior to this study, a start-up study which utilizes an exploratory methodology was done to observe the characteristics and performance of POME in a two-stage UASFF reactor before being operated into the optimization system. Factors affecting process stability and biogas production were examined. Therefore, the present study focusses on waste processing technology, particularly to a method for producing biogas from POME.

In the next stage of this study, a lab-scale two-stage UASFF bioreactor of 2.5 L and 3.5 L capacities were used to increase biohydrogen and biomethane productions. Acclimatized POME sludge from an anaerobic pond is used as inoculum and raw POME from acidification ponds is used as the substrate. Trials were done to investigate the feasibility of utilizing POME for hydrogen and methane production in discrete reactors (H_2-UASFF and CH_4-UASFF). The performance of the reactors under a short HRT was also tested, as this has significant implications on full-scale operations for treatment of large volumes of wastewater.

1.6 The Scope of the Study

In this study, an integrated two-stage UASFF bioreactor is derived from up-flow anaerobic sludge blanket (UASB) and up-flow fixed film (UFF) reactor. A two-stage UASFF bioreactor was selected as it can accomplish better biodegradation productivity and higher substrate loading rate that can be applied in separate process (Zupancic & Grilc, 2012).

For the first objective, a batch experiment was conducted to study the effects of important operational, process and environment parameters on biohydrogen production in treating POME biologically. The study was initiated to study biohydrogen production of POME in a dark fermentation process. Raw POME was used as a substrate and POME anaerobic sludge as an inoculum. The inoculum sizes (10:90 - 40:60), reaction times (8 - 24 h) and mesophilic to thermophilic conditions (30 - 50°C) were varied to study their effects on biohydrogen production and its COD removal efficiency. For the optimization study, (RSM) was applied for hydrogen yield and COD removal efficiency. In this study, the substrate concentrations were varied from a low concentration of <20 g L^{-1} to high concentration of >20 g L^{-1} CODin.

The study was continued in a scaled-up of two-stage UASFF bioreactor by running a start-up experiment for the second objective. Although studies on treating high concentration of POME using two-stage system are extensive, study on biohydrogen and biomethane production using two-stage UASFF reactor from POME is rarely found. Thus, the start-up study which uses an exploratory approach was done to observe the behavior and performance of POME wastewater in a two-stage UASFF reactor. Factors affecting process stability and biogas production were examined. Several attempts such as using different temperatures, applying inoculum and substrate pre-treatment and change the source of substrate were done to increase hydrogen production in H_2-UASFF unit.

The last phase was the optimization study in a two-stage UASFF bioreactor. To date, the optimization study of HRT and temperature using UASFF bioreactor for biohydrogen production in treating POME is rarely studied. Therefore, the effects of temperature and low HRT are investigated to determine the optimum conditions for maximum biohydrogen production utilizing POME. This study focused on biohydrogen production in one part of identical two-stage UASFF bioreactor called H_2-UASFF unit. Parameters at different conditions were analyzed, including hydrogen production rate and yield and COD removal efficiency.

In CH_4-UASFF unit, three different operating temperature (min. 37°C, max. 70°C) and effluent COD from H_2-UASFF unit (also known as dark fermentation effluent) (lowest: 12,150 mg L^{-1}, highest: 19,967 mg L^{-1}) were designed using historical data in response surface methodology (RSM). Five responses were studied and analysed, *viz*. COD removal efficiency (%) and methane percentage (%) in CH_4-UASFF unit, methane production rate (MPR), methane yield, and total COD (in two-stage UASFF reactor). All responses were analysed using polynomial whereby quadratic was used in the designed

model. At the end of the experiment, the optimum parameters were chosen based on the highest responses when effluent COD from H_2-UASFF unit and temperature were set in-ranged.

1.7 Research Objectives

In this investigation, the practicality of treating POME by using UASFF bioreactor was examined. As for industrial applications in Malaysia, some palm oil mills have started to produce their own power from biogas plants. The fundamental goal of this exploration is to generate biogas from POME, by studying the reactor performance and additionally, the quantity and quality of the yield. The inoculum and substrate used were from palm oil wastes itself, as to associate it with the palm oil mills. The objectives of this study are:

1. To study effects of operational, process and environmental parameters on the generation of biohydrogen utilizing POME in batch fermentation.
2. To study the feasibility of utilizing POME as a substrate in a scale-up UASFF bioreactor.
3. To determine the optimum condition in H_2-UASFF and CH_4-UASFF units for biogas production by utilizing POME wastewater.

1.8 Thesis Organization

This is an article-style thesis. Chapters 1 and 2 describe the general introduction and some literature review on previous and current studies utilizing different kinds of organic wastes for biohydrogen/biomethane production under different operating conditions and bioreactors, respectively. The importance of POME as renewable and sustainable energy and its potential as biogas is also discussed. Chapter 3 describes the general materials and methods used in this study. Chapter 4 presents the preliminary (batch) study utilizing

POME for biohydrogen production using 160 mL serum bottles. The purpose was to investigate the feasibility of using POME as a potential substrate and inoculum for biohydrogen production in a studied process, environment and operational conditions. From the batch study results, a scale-up UASFF bioreactor utilizing POME under controlled pH and temperature was started-up and discovered in Chapter 5. During this start-up period, different approaches such as using different temperatures, pH and source of substrates have been considered in order to achieve highest possible biogas production, yield, and total substrate degradation (total COD removal). Chapters 6 and 7 are the continuity from Chapter 5 where optimum conditions are defined by designing and analyzing the two-stage UASFF bioreactor using Response Surface Methodology (RSM). 17 runs were obtained under different temperature and hydraulic retention time (HRT). These two important factors were chosen in two-stage UASFF bioreactor utilizing POME based on the start-up study, whereby different HRT and temperature were used. The optimum parameters are necessary for the prediction of biohydrogen and biomethane production. Finally, Chapter 8 concludes the findings that were obtained from batch study, start-up study and optimization study using two-stage UASFF bioreactor. Results showed that it is possible to reduce COD level of high-strength wastewater, particularly POME. Inoculum heat-treatment must be considered to suppress methanogenic bacteria for hydrogen production. This study also concluded that thermophilic temperature and short HRT were found to be the optimal conditions for highest hydrogen and methane production.

CHAPTER 2: LITERATURE REVIEW

2.1 Introduction

Strategies to produce renewable energy from organic waste have become a high priority topic in any energy, bioconversion, bioresource and sustainability conferences in the world. Conversion of organic and inorganic wastes into useful and valuable end products like biohydrogen, biomethane and bio alcohols are increasingly studied each year as many nations progressively working towards sustainable world development. This is due to the fact that biohydrogen gas is a clean energy alternative and it acts as a good source of fuel to apply in fuel cells for electricity generation. Meanwhile, biomethane, another clean energy alternative for electricity and transportation, is produced from the anaerobic digestion process. Bio alcohols that include biomethanol, bioethanol and biopropanol, that are produced by the action of enzymes and microorganism through fermentation, would also be used as fuels for internal combustion engines.

Renewable energy is an energy that can be replaced, sustainable and does not harm the environment as it is derived from non-nuclear and non-fossil sources (Elbeshbishy, Dhar, Nakhla, & Lee, 2017). Due to its high energy efficiency, hydrogen (H_2) is considered one of the preferable biofuels among various renewable energy sources (Jung et al., 2013). It is considered the best and most effective fuels for transportation. This is because, when H_2 is combusted (only water vapour is produced with the absence of CO), the energy yields are 2.75 times higher (122 kJ g^{-1}) than hydrocarbon fuels (Jung, Kim, Kim, & Shin, 2011). This can minimize environmental problems and makes H_2 a future fuel which has drawn significant attention to the world.

Various biotechnologies such as dark fermentation (DF) can be used to generate H_2 in a green and environmental-friendly way using renewable resources (Lay et al., 2005; Wang & Zhao, 2009). Through the activities of fermentative hydrogen producing-bacteria (HPB) (obligate anaerobes and facultative anaerobes), DF process could utilize various types of wastewaters and organic wastes as a feedstock to produce H_2. As compared to photo-fermentation, DF process is independent on weather conditions and produce relatively higher H_2 production rate. On the other hand, in anaerobic digestion (AD) process, organic materials were converted into biogas, nutrients and some refractory organic matter under anaerobic condition by a mixture of symbiotic microorganisms (Wilkie, 2008). This process could reduce pollution and odour as well as produce renewable energy in an effective waste treatment due to the microbial conversion. Compared to fossil fuels, renewable methane does not contribute to carbon dioxide (CO_2) emissions in the atmosphere (Wilkie, 2005).

2.2 Biohydrogen Production via Dark Fermentation

Hydrogen is naturally produced by varieties of organisms under anaerobic conditions. Dark fermentation is known to be involved in hydrogen production while dark fermentative microorganisms are those associated with the process. These microorganisms can be distinguished based on their sensitivity to temperature and oxygen. Obligate anaerobes are those that favour anaerobic conditions while facultative anaerobes are those that can survive in both aerobic and anaerobic environments.

Pure microbial species or mixed cultures can both produce hydrogen. In their community, some of the microorganism can act as hydrogen-producing bacteria (HPB) while some may act as hydrogen-consuming bacteria (HCB) for their energy. In most of the biohydrogen studies, researchers were using either mixed cultures or pure culture in

a laboratory or scale-up bioreactor (Mohammadi et al., 2011; Norfadilah, Raheem, Harun, & Ahmadun, 2016).

2.2.1 Dark Fermentative Bacteria

2.2.1.1 Obligate anaerobic bacteria

Obligate anaerobic bacteria are used in most biohydrogen studies because of their ability to utilize the various type of wastewaters and carbohydrate. In addition, they are also able to produce a higher rate of hydrogen production, compared to facultative anaerobes. Hydrogen production is mainly occurred during the exponential growth phase. During stationary phase, microorganism metabolism are shifted from hydrogen/acid production to solvent production such as acetone, butanol and ethanol (Han & Shin, 2004).

2.2.1.2 Mixed cultures

Mixed cultures are normally applied when the complex substrate is used, for example, raw POME. Mixed cultures can boost substrate consumption compared to using pure cultures. According to Guwy, Hawkes, Hawkes, & Rozzi, (1997), pure cultures are easily contaminated with HCB. Compared to mixed cultures, the operation in industries is normally under nonsterile conditions as they have been designated for growth and dominance. Therefore, this makes them robust to environmental changes such as temperature and pH.

The choice of mixed cultures for hydrogen production as inocula can be obtained from anaerobic digester of municipal sewage, sludge from digested POME of an anaerobic pond or fermented soybean meal. However, the presence of methanogens or HCB becomes a major bottleneck in selecting these mixed cultures. Therefore, in some cases, several researchers will pretreat these mixed cultures in order to suppress the activity of

methanogens and remove HCB (Chen, Lin, & Lin, 2002; Shaw, Jenney, Adams, & Lynd, 2008). Zhang, Liu, & Fang, (2003) reported that *Clostridium* species are normally present in mixed cultures. Therefore, at high temperature, mixed cultures would be favourable to reaction kinetics, thus, contamination by HCB could be avoided.

2.2.1.3 Thermophiles

Most thermophiles are obligate anaerobes. Thermophiles can utilize a various type of substrates such as lignin, hemicellulose and cellulose, as well as pectin-containing biomass (Van De Werken et al., 2008). According to O-thong, Prasertsan, Intrasungkha, Dhamwichukorn, & Birkeland, (2007), nutrient addition helped in promoting the growth of HPB, i.e. *Thermoanaerobacterium thermosaccharolyticum* when POME was treated under thermophilic conditions. Other studies include thermophiles for hydrogen production are *Thermoanaerobacterium* sp. (O-thong, Prasertsan, Karakashev, & Angelidaki, 2008), *Caldicellulosiruptor saccharolyticus* (Niel et al., 2002) and *Thermotoga* sp. (Schroder, Selig, & Schonheit, 1994).

2.2.2 Dark Fermentation

Under anaerobic condition, fermentation (metabolic) process occurs to regenerate the cell's energy currency (ATP). The tricarboxylic acid cycle is also blocked under this condition. When reduced metabolic end products (e.g. alcohol and acids) formed, fermentation will dispose of the excess cellular reductant. Similarly, the cellular redox potential is maintained by the production of hydrogen that acts as a reduced metabolic end product as well.

For the fermentation process, carbohydrates are the preferred carbon source that contains mainly glucose, which can predominantly increase acetic and butyric acids along with hydrogen gas. Under glycolysis pathway, one mole of glucose would be converted

to 2 moles of pyruvates. Subsequently, pyruvate may be involved in the formation of hydrogen in two different biochemical reactions (Balachandar, Khanna, & Das, 2013).

Pyruvate will be oxidized to acetyl coenzyme A (acetyl-CoA) in Clostridia (McCord, Keele, & Fridovich, 1971) as obligate anaerobes and thermophilic bacteria (Zeikus, 1977) by pyruvate-ferredoxin oxidoreductase (Kosaku & Rabinowitz, 1970). Next, acetyl-CoA will be converted to acetyl phosphate, along with the production of ATP and acetate. Reduction of ferredoxin (Fd) is required for oxidation of pyruvate to acetyl-CoA. [Fe-Fe]-hydrogenase will oxidize the reduced Fd and catalyzes H_2 formation. The overall reaction is shown in the equations below.

$$\text{Pyruvate} + \text{CoA} + 2\text{Fd (ox)} \longrightarrow \text{Acetyl-CoA} + 2\text{Fd (red)} + CO_2$$

Equation 2.1

$$2H^+ + \text{Fd (red)} \longrightarrow H_2 + \text{Fd (ox)}$$

Equation 2.2

When pyruvate is oxidized to acetate as the sole metabolic end product, four moles of hydrogen per mole of glucose is formed (Mohan & Pandey, 2013). However, when pyruvate is oxidized to butyrate, only two moles of hydrogen produced per mole of glucose. Therefore, in the mixed acid pathway, higher acetate to butyrate ratio is critical for higher hydrogen production (Khanna, Kotay, Gilbert, & Das, 2011). Overall biochemical reaction with acetic and butyric acid as metabolic end products is shown in the next equations, respectively.

$$C_6H_{12}O_6 + 2H_2O \longrightarrow 2CH_3COOH + 2CO_2 + 4H_2$$

Equation 2.3

$$C_6H_{12}O_6 \longrightarrow CH_3CH_2CH_2COOH + 2CO_2 + 2H_2$$

Equation 2.4

2.3 Biomethane Production via Anaerobic Digestion

2.3.1 Anaerobic Digestion

In the absence of oxygen, a process by which microorganisms will breakdown biodegradable material is called anaerobic digestion. Since it can provide a significant reduction in the mass of the input material (substrate), therefore, anaerobic digestion is mostly used for wastewater treatment or any organic wastes.

Seghezzo (2004) reported that in the anaerobic digestion process of organic polymeric materials, there are seven sub-processes involved (Figure 2.1). At first, complex organic materials will be hydrolyzed by the fermentative bacteria (I), followed by fermentation of amino acids and sugars in the second phase. Next, the oxidation process occurred in long-chain fatty acids and alcohols. In the fourth phase, short-chain fatty acids take place in anaerobic oxidation (except acetate), followed by the production of acetate from carbon dioxide and hydrogen in the fifth phase. Later, acetate will be converted to methane by acetoclastic methanogens. The last phase is the production of methane by carbon dioxide and the hydrogen reduction process (Seghezzo, 2004).

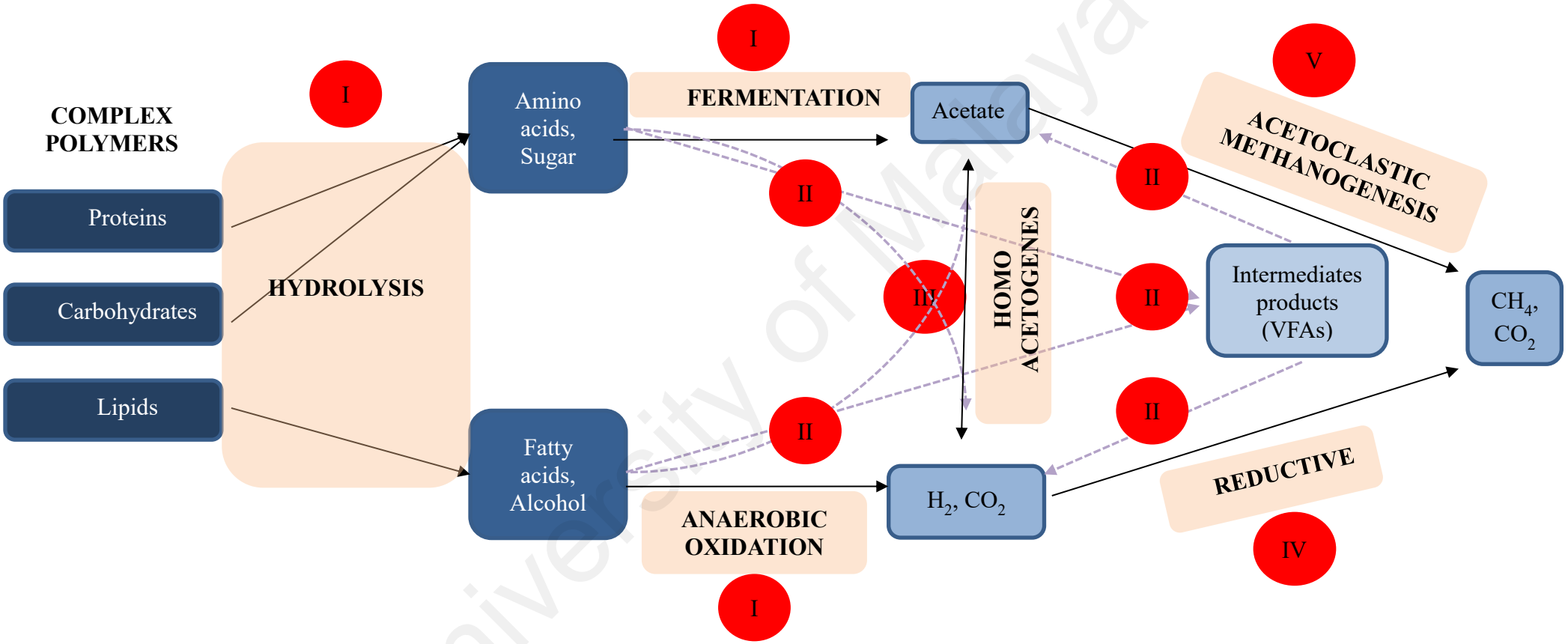


Figure 2.1: Anaerobic digestion process. I: fermentative bacteria; II: hydrogen-producing acetogenic bacteria; III: hydrogen-consuming acetogenic bacteria; IV: carbon dioxide-reducing methanogens; V: acetoclastic methanogens.

However, even though there are seven sub-processes mentioned, the principal of bacteria classes is only divided into three categories (Seghezzo, 2004);

I – Bacteria that responsible for hydrolysis. These bacteria hydrolyze the substrate and breakdown the insoluble organic polymers (e.g. carbohydrates) and make them accessible for other bacteria.

II – Acid-producing bacteria. There are two acid-producing bacteria involves in this pathway. The first one is acidogenic bacteria while the other is acetogenic bacteria. The former will convert sugars and amino acids into CO_2, H_2, ammonia and organic acids while the latter will then convert the produced organic acid into acetic acid (along with ammonia, H_2 and CO_2).

III – Methane-producing bacteria. At the end, these bacteria convert the aforementioned products into CH_4 and CO_2. Methane formation is strictly under anaerobic condition in this phase and the reaction is exergonic. It is also reported that not all methanogens will degrade the substrates (Chandra, Takeuchi, & Hasegawa, 2012). Substrates that acceptable for methanogenesis process are divided into three groups as mention below:

(i) Acetoclastic methanogenesis - will convert acetate to $CH_4 + CO_2$

Chemical equation: $CH_3\text{-}COOH \rightarrow CH_4 + CO_2$

Equation 2.5

(ii) Hydrogenotrophic methanogenesis – will convert $H_2 + CO_2$ to CH_4

Chemical equation: $CO_2 + H_2 \rightarrow CH_4 + CO_2$

Equation 2.6

(iii) Methylotrophic methanogenesis – will convert methanol to $CH_4 + H_2O$

Chemical equation: $HCOOH + 2H_2 \rightarrow CH_4 + H_2O$

Equation 2.7

Chemical equation: $CH_3OH + H_2 \rightarrow CH_4 + H_2O$

Equation 2.8

There are two biochemical components for methanogens that makes them unique; the mechanism of H_2 oxidation and CO_2 reduction. Methanogens utilize H_2 with acetate, formate, CO_2 and methanol as substrates under methanogenesis process (Zeikus, 1977). Meanwhile, the methanogens use CO_2 as thermal electron acceptor which later produce CH_4 (Chandra et al., 2012).

2.4 Bioreactor Configuration and Operation

Studies on biohydrogen and/or biomethane production can be operated in a batch, semi-continuous or continuous mode. Most of biohydrogen/biomethane studies were done in a batch or continuous operation. Batch mode is normally done for an optimization study, however, in the industries, continuous operation is preferable. A lot of studies used continuous stirred tank reactor (CSTR) due to its simple operation and construction, effective mixing, and can be operated under certain HRT so that microbial growth rate can be controlled. Besides that, the fluidized bed reactor, up-flow anaerobic sludge bed reactor (UASB), fixed bed bioreactor and anaerobic sequencing batch reactor (ASBR) have been extensively studied while producing high production yields.

In order to find a suitable bioreactor, types of feedstock must be taken into consideration. This is because the feedstock must be able to be converted into organic acids, alcohols and biogas, with the help of microorganisms. A developed bioreactor is

also needed in order to meet certain criteria such as resistant to short-term fluctuations, a stable performance over a long period of time and more robust for biohydrogen/biomethane production. Most of the challenges in bioreactor design lie on the mixing and aeration since large fermentation process requires oxygen. However, under anaerobic condition, the design and construction are simpler without agitation or sparging.

2.4.1 Up-flow Anaerobic Sludge Blanket (UASB) Reactor

In UASB reactor, biogas is produced by degradation of organic materials in the wastewater within a sludge bed in the tank due to a digestion process. The wastewater will be fed from the bottom of the bioreactor (up-flow). While at the top part, biogas and effluent of the treated wastewater are released (Figure 2.2). At the upper part in the bioreactor, above the sludge bed, some suspended biomass particles will form a blanket zone. This zone acts as a separation zone between the suspended biomass with water flowing up. Because of this, sludge production is low. For instance, in a 4m height of UASB bioreactor, only one time is required in a month to discharge the sludge (Seghezzo, 2004).

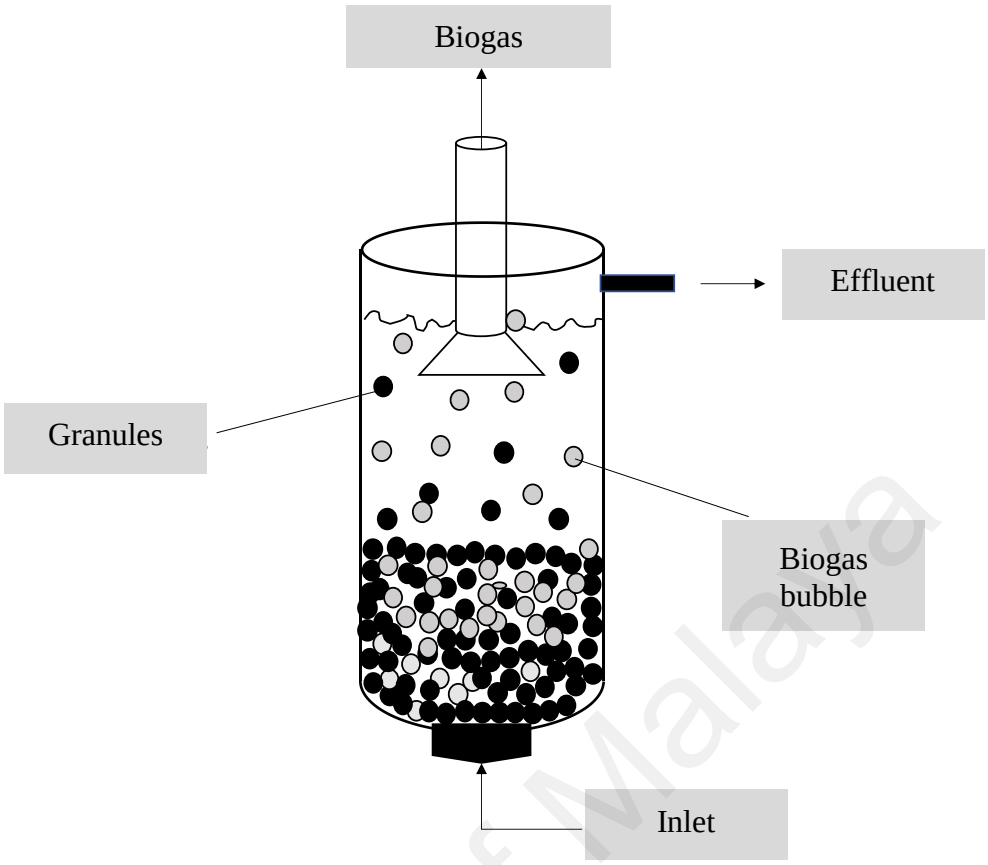


Figure 2.2: Schematic diagram of a UASB bioreactor.

In tropical countries, the application of UASB bioreactor is preferable. This is because UASB bioreactor operated well under mesophilic condition. UASB bioreactor is normally suitable to treat a high organic load of wastewater, thus, in food industries, UASB is widely used. Formation of granules is easier without the need for inert material. UASB bioreactor has been widely and successfully used for various industrial effluents including those with high organic content that capable of inhibiting digestion (Buitrón, Kumar, Martinez-Arce, & Moreno, 2014; Jung, Kim, Lee, & Shin, 2012; Rizvi et al., 2015). In POME, the suspended organic solids have high biogas potential. This makes UASB acts as a driving force as the conversion technology is economically feasible. Normally, during POME treatment using UASB, high content of VFA cause POME wastewater overloading, thus makes the process to be epileptic for about 15 days of use (Ohimain & Izah, 2014). However, two-stage UASB has been proposed for POME

treatment to inhibits the form of granules at high organic loading rate without biomass washout (Borja et al., 1996; Poh & Chong, 2009).

2.4.2 Fixed Bed Reactor

With respect to its excellent capacity in retaining microorganism in a support media, fixed bed anaerobic reactor (Figure 2.3) has been successfully employed for wastewater treatment. The system is more stable, has high degradation efficiency and is controllable after some improvements being made in the configurations when operated under low HRT, as biomass concentration was elevated with longer cellular retention time (Lima, Ribeiro, Foresti, & Zaiat, 2005).

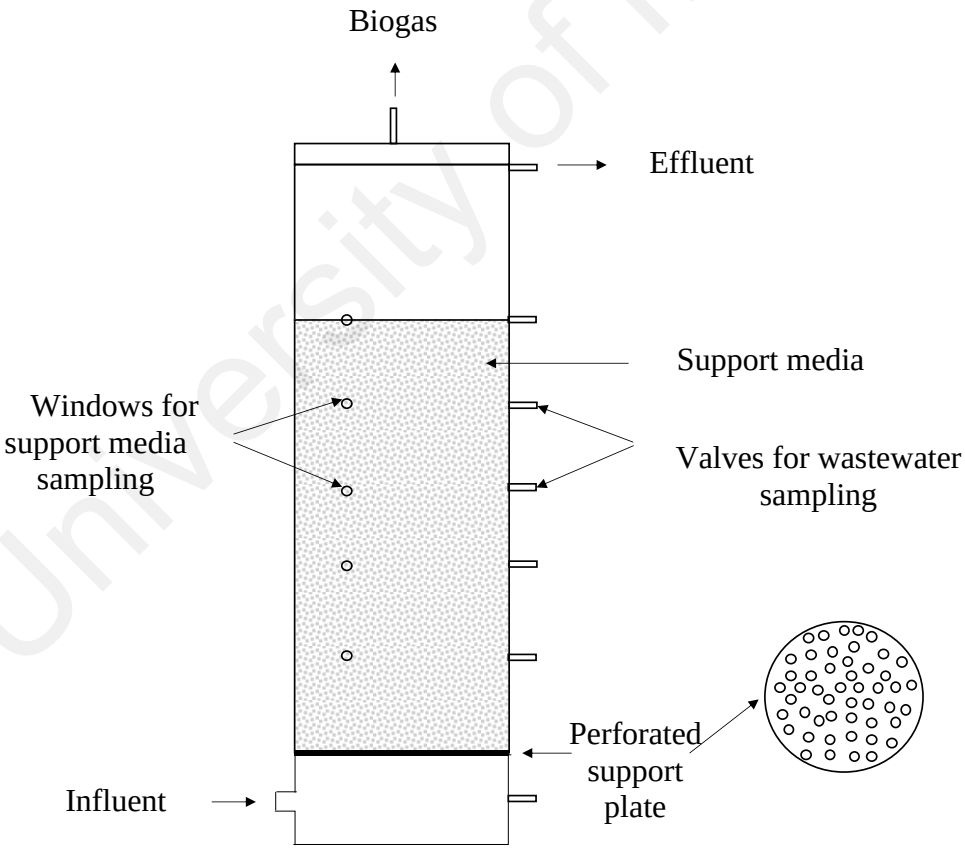


Figure 2.3:A schematic diagram of a fixed-bed reactor.

Studies on treating POME using a fixed bed reactor are very limited. However, Fia et al., (2010) in their study, used a fixed bed reactor to treat a high-pollution-potential coffee wastewater. The effectiveness of a fixed bed reactor was investigated in treating coffee grain wastewater with different support media (i.e. polyurethane foam, blast furnace slag, #2 crushed stone with different porosities). 80% of COD removal efficiency was achieved with greater fixation and retention of biomass (*viz*. total volatile solids of 1301 mg g^{-1}). They also concluded that even though microbial development using blast furnace slag was not stable, but various microbial morphologies were present for anaerobic treatment even under lower substrate concentrations.

2.5 Factors Influencing Biohydrogen/Biomethane Production

2.5.1 Temperature

Levin, Pitt, & Love, (2004) reported that dark hydrogen fermentation can be operated at four different temperatures; mesophilic (25-40°C), thermophilic (40-65°C), extreme thermophilic (65-80°C) or hyperthermophilic (>80°C). Li & Fang, (2007) found that more than 50% of laboratory studies on biohydrogen production was conducted under mesophilic condition. This demonstrates that temperature significantly affects hydrogen production. The microorganism specific growth rate and rate of substrate conversion had been affected during hydrogen production.

In the meantime, a higher temperature may prompt to thermal inactivation of the enzymes for the fermentative hydrogen production process. Various batch studies about utilizing mixed cultures have demonstrated the reliance of hydrogen production on the operational temperature. Lin, Wu, & Hung (2008) used mixed consortia for biohydrogen production under different temperatures (30-55°C) using chemostat-type reactor. They found out that the highest hydrogen production was obtained at 45°C.

Metabolic pathways and changes in a microbial community were correlated with various temperature used for hydrogen production. In this way, it is critical to understand the temperature reliance of the microbial community in order to optimize the hydrogen production systems. It has been reported that information on the effect of temperature on microbial dynamics is scarce in bioreactor systems (Sinha & Pandey, 2011). Table 2.1 summarizes a wide variety of microorganisms involved in hydrolysis and fermentation processes (Tan, Liew, Muda, & Kassim, 2015).

Table 2.1:**Reference summary of microorganisms involved in hydrolysis/fermentation process isolated from POME.**

Type of bacteria (Reference)	Description	Role/function	Carbon/Energy Source
Hydrolytic bacteria			
Bacillus sp. *B. licheniformis* *B. firmus*	Rod-shaped, gram positive	Degrade plant dry matter	Lignocellulosic biomass
Clostridium sp. (Khemkhao, Techkarnjanaruk, & Phalakornkule, 2015)	Rod-shaped, gram positive	Produce xylanase and cellulose for hydrolysis	Hemicellulose, cellulose
Cellulomonas sp.	Rod-shaped, flagellated, gram positive	Synthesized acid from glucose	Cellulose
Micrococcus lutues	Coccus, gram-positive	Hydrolyzed lipids into fatty acids and glycerol	Lipids, cellulose
Pseudomonas sp. (Ohimain & Izah, 2013)	Rod-shaped, gram negative, flagellated	Produce xylanase and cellulose for hydrolysis	Hemicellulose, cellulose
Fermentative/Acidogenic bacteria			
Clostridium butyricum *C. paraptrificum* *C. beijerinckii PS-3* (Chong, Abdul, Shirai, &	Gram positive, rod-shaped		Carbohydrates, cellulose

Ali, 2009; Yossan, O-Thong, & Prasertsan, 2012)			
Enterobacter asburiae *Enterobacter cloacae* (Wong, Teng, & Ong, 2014)	Gram negative, rod-shaped	Produce hydrogen gas from fatty acids and carbohydrates	Carbohydrates, fatty acids
Thermoanaerobacterium sp. *T. aciditolerans* *T.polysaccharolyticum* *T. aotearoense* *T. thermosaccharolyticum* (Chonticha Mamimin et al., 2012)	Gram positive, rod-shaped	Will produce hydrogen, butyric acid	Cellulose
Enterococcus gallinarum (Wang, Ai, Hu, & Zhang, 2011)	Gram negative, coccus	Convert sugars into lactic acid through fermentation	Glucose
Escherichia coli *E. fergusonii* (Ohimain, Daokoru-olukole, Izah, Eke, & Okonkwo, 2012)	Gram negative, rod-shaped		

Based on Table 2.1, the Gram-negative bacteria mostly found in POME were *Pseudomonas sp.*, *Enterobacter sp.* or *Escherichia coli* (Ohimain & Izah, 2013; Elijah I Ohimain et al., 2012; Wong et al., 2014). These bacteria species tend to produce xylanase and cellulase for hydrolysis of cellulose, production of hydrogen and butyric acid.

On the other hand, the biological activity of some anaerobic bacteria, especially methane-forming bacteria, will be inhibited if the temperature in a digester varies/changes, even a few degrees. Most of the methane-forming bacteria are active at two temperature ranges, i.e. mesophilic (30-35°C) and thermophilic (50-60°C). It is

reported that the temperature between 40-50°C caused inhibition of the bacteria (Deublein & Angelika, 2008). This is because the transition from mesophilic to thermophilic happens between this temperature.

Psychrophilic (10-20°C), mesophilic (30-35°C) and thermophilic (50-60°C) methanogens convert organic substrates into CH_4. The former will produce CH_4 when reaction temperature reaches 20°C and less quantity of biodegradable volatile solids will be converted, thus, a low amount of biogas is produced (Park, 1988). Meanwhile, between 20-45°C, mesophilic methanogens come into play. This condition maximizes biogas production when the temperature is maintained around 35°C (Pain, West, Oliver, & Hawkes, 1984). It is reported that the thermophilic anaerobic process has an acceleration effect on the biochemical reactions. In comparison with mesophilic process, thermophilic conditions have higher degradation efficiency of organic matter. Higher production of methane also have been reported when the study was operated under thermophilic condition, compared to mesophilic (Watts, Hamilton, & Keller, 2006).

Converting POME into hydrogen under high temperatures is also favorable due to low hydrogen partial pressure inhibition, more thermodynamics condition and less variety of fermentation end-products (C. Mamimin, Singkhala, & Kongjan, 2015). It has been reported that microbial fermentation was successfully converted POME into hydrogen under thermophilic condition. At 60°C, a continuous hydrogen production rate was achieved by *Thermoanaerobacterium*-rich sludge using anaerobic sequencing batch reactor (ASBR) (Prasertsan, O-Thong, & Birkeland, 2009).

2.5.2 pH

One of the factors influencing metabolic pathways and hydrogen yield is pH. During glycolysis, most facultative anaerobes will breakdown glucose to pyruvate to produce

hydrogen (Antonopoulou, Stamatelatou, Venetsaneas, Kornaros, & Lyberatos, 2008). In studies where mixed cultures are used as an inoculum, pH regulations become crucial in suppressing hydrogen-consuming methanogens' activity (Ginkel, Sung, & Lay, 2001). At low pH (<5), hydrogen production decreased due to the increase of acidic metabolites formation, which disturb the cell ability to maintain internal pH (Bowles & Ellefson, 1985).

Several studies have demonstrated that a pH of media in the range of 5 and 6 was appropriate to get a higher hydrogen yield (Cao & Zhao, 2009; Ginkel et al., 2001; Ma, Ke, & Chen, 2008). pH other than the ideal (i.e. between 5 – 6) has been appeared to stifle the hydrogen yields. Along these lines, it is vital to control the pH with the end goal to create a higher hydrogen generation.

In the methanogenesis process, pH is an important parameter which can significantly affect the growth and performance of microorganisms throughout the process. By having an optimal organic loading rate, the desired pH can be kept within the digester. A pH outside the range of 6.0 – 8.5 will be toxic to methanogens population (Chandra et al., 2012). During fermentation, the rate of intermediates formed will determine the pH of the system. According to Dague (1968), methanogens activity will be adversely affected if pH drops below 6.6, while at pH 6.2, it will become toxic. Acid is continuously producing because acidogens are producing acid and cause pH to drops until 4.5-5.0.

pH range of 6.8 – 7.2 is found to be a suitable condition for most of the anaerobic bacteria to perform well, including methane-forming bacteria. When volatile fatty acids (VFA) were produced, pH in anaerobic digester will normally decrease below 6.0, as a great deal of CO_2 is being produced (Chandra et al., 2012). After some time, the pH will increase to 7.0 - 8.0 and above. However, when methane-forming bacteria consume the

VFA, more CH_4 and CO_2 will be produced, thus increase the alkalinity which later stabilizes the digester (John Fry, 1974; Michael H. Gerardi, 2003).

2.5.3 Hydraulic Retention Time (HRT)

In choosing microbial populations, fermentation time is considered important as microbial growth rates are required to withstand the mechanical dilution formed by a continuous volumetric flow. Hydrogen-producing bacteria has a specific growth rate of 0.172 h^{-1} while methane-producing bacteria is even lower, i.e. 0.0167-0.01 h^{-1} (Lo et al., 2009). Therefore, a shorter HRT is preferable for mixed cultures as it can inhibit the growth of methanogens in the reactor. This eventually leads to a bigger formation of HPB. In a study done by Zhang, Ann, & Logan (2006), they have successfully increased the hydrogen production and hydrogen yield when HRT was adjusted from 8 h to 6 h. This diminished the generation of propionate, bringing about higher hydrogen yields.

In anaerobic digester with steady mixing, the substances in the bioreactor have a similar retention time. Under a short HRT, system failure will usually happen. Even though high yield of methane could be achieved, the growth rate of important microbial community might have caused this problem. A study done by Najafpour et al., (2006) found that 71.9% of CH_4 gas production rate was achieved at 3 days of HRT. According to another study, HRT enhanced metabolic shift in concurrent to extended fermentation time, organic loading rate, pH and nature of the effluent (Mohan, 2008).

Short HRT could lead to low biodegradation efficiency of organic matter although the biogas production rate is high. Studies were done by Atif et al., (2005) and Vijayaraghavan & Ahmad, (2006). They reported 57% of hydrogen content at 7 days HRT with average biogas generation of 0.42 L g^{-1} COD removed by using isolated microbes in POME digested sludge. According to Ohimain & Izah, (2014), the optimum

HRT is mainly dependent on the type of bioreactor configuration. Further studies need to be done in order to optimize biogas production from POME under short HRT.

2.5.4 Source of inoculum and substrates

Biomass that contain proteins, carbohydrates and fats, as well as any biodegradable organic materials are suitable substrates for biohydrogen/biomethane production. However, the choice of substrates will be selective for techno-economical purposes. Biogas composition is greatly affected by the compositions of fats, protein and carbohydrates contents of the substrate. Recently, wastewaters from natural activities are being considered as a potential substrate/feedstock for harnessing renewable energy. In order to meet sustainable nature, it is important to reduce the treatment cost of wastewaters and finding ways to produce value-added products from the respective treatments.

In wastewater treatment, biological processes are preferable as they are simpler, feasible, eco-friendly and economical. The biological process also can control pollution while converting the negative-valued organic waste into useful forms of energy. In Malaysia, POME wastewater is abundant, which is composed of a reasonably good biodegradable carbon fraction, associated with essential net-positive energy. Biohydrogen generated from renewable wastewater treatment is able to reduce the cost of overall effluent treatment and simultaneously makes the whole process environmentally sustainable (Mohan, Babu, & Sarma, 2007; Mohan, Chandrasekhar, Chiranjeevi, & Babu, 2013).

2.6 Lignocellulosic Biomass

Lignocellulosic biomass such as POME consists of mainly cellulose, hemicellulose, lignin, and inorganic materials, together with smaller amounts of protein, pectin and

extractives including chlorophyll, sugars and waxes as a soluble non-structural material (Chandra et al., 2012). As shown in Figure 2.4, the primary building block of a cell wall is lignocellulose that comprises of large fraction such as in crop residues, forest residues, municipal solid wastes and many energy crops.

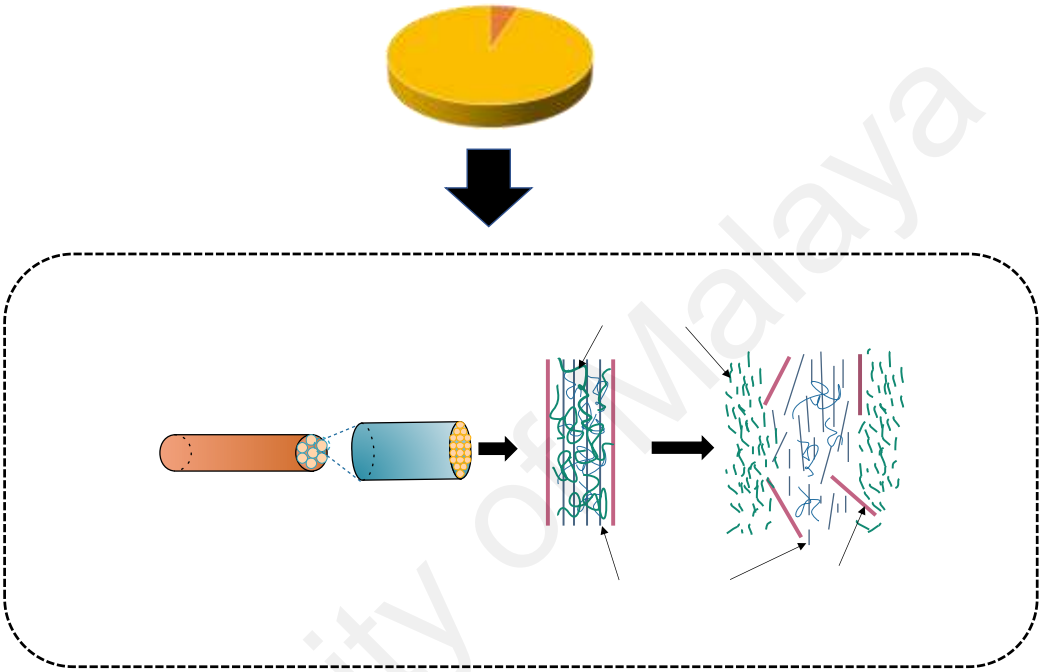


Figure 2.4: Schematic diagram of pre-treatment of lignocellulosic materials.

Cellulose is mostly found in the fibrous structure and is the main structural constituents in plant cell walls. It is present in both amorphous and crystalline forms where the latter comprises the major proportion of cellulose, which later forms amorphous cellulose from a small percentage of unorganized cellulose (Chandra et al., 2012). In long-chain cellulose polymers, hydrogen and van der Waals bonds linked them together, which is known as ‘elementary and micro-fibrils’. Micro-fibrils are normally in the form of

bundles, whereby the fibrils will be attached together by hemicellulose and bonded together by lignin (Delmer & Amor, 1995).

On the other hand, lignin is a very complex and large molecule. Herbaceous plants such as grasses have the lowest lignin contents while softwoods have the highest. Lignin can structurally support the plant and is almost resistant to certain conditions. For example, in fermentation, the major drawback of using lignocellulosic material is that it is resilient to chemical and biological degradation (Palmqvist & Barbel, 2000; Taherzadeh & Filtration, 2016). The presence of lignin protects the biomass and prevents the cell from destructions by enzymes, fungi or bacteria.

In the conversion of biomass to fuel, both cellulose and hemicellulose must be degraded into simple monomers (e.g. sugars) for microorganisms to utilize them under biological pathway for energy conversion process. Therefore, pre-treatment is important for the breakdown of lignin layer, before microorganisms can hydrolyze the cellulose and hemicellulose and then convert them into simple sugars (Chandra et al., 2012).

2.7 Biogas production from POME

About 53 million tons of palm oil and 13 million tons of empty fruit bunches were produced annually in Malaysia (Foo & Hameed, 2009). This phenomenon has pulled in researchers and investigators to deal with energy production from POME (Zakaria et al., 2008). To date, majority of oil palm processes in Malaysia has applied POME as a feedstock for biogas generation (Basri et al., 2010). Production of biohydrogen using digested POME as inoculum was examined by Mamimin, Chaikitkaew, Niyasom, Kongjan, & O-Thong, (2015) using anaerobic sequencing batch reactor (ASBR). The impacts of hydraulic retention time (HRT), temperature and organic loading rate (OLR) were explored for process stability in ASBR in a continuous process. In their study, they

used a thermophilic condition to enhance biohydrogen production. They found out that there was a significant increase in biohydrogen production under the thermophilic condition, as compared to mesophilic temperature. This is because thermophilic bacteria were present in POME sludge (inoculum) due to a long adaptation time, thus making it more favourable for biohydrogen production. Different studies on the effects of volatile fatty acids (VFAs) (Chonticha Mamimin, Prasertsan, Kongjan, & O-Thong, 2017), pH (Yossan et al., 2012), and organic loading rate (OLR) (Mohammadi et al., 2017) using POME were conducted, either using single stage or integrated reactors. Studies using single stage reactor revealed that biogas production rate could be accomplished at HRT of 1.5 days and the system was capable to effectively treat POME (Najafpour et al., 2006). On the other hand, several researchers also reported higher efficiency in energy recovery and increased process stability using integrated system as compared to using a single stage process (Liu et al., 2013). These findings showed that an integrated system using two-stage bioreactor is better in terms of COD removal efficiency, stability and give a significant impact on biogas production and yield, in comparison to a single stage reactor.

2.8 Current anaerobic treatment methods using hybrid reactors

A large amount of water is consumed during palm oil mill processing. This contributes to the mass production of POME wastewater that leads to water contamination because of its high BOD and COD content. However, through anaerobic digestion, POME has become one of the potential and valuable sources of bioenergy, *viz.* biohydrogen and biomethane. Lam & Lee (2011) suggested that every oil palm industry in Malaysia should consider having a renewable and sustainable bioenergy strategy, as well as in-house wastewater treatment system. The production of methane and CO_2 by the action of active microorganism requires multi-stage processes for organic matter degradation, i.e. hydrolysis, acidogenesis, acetogenesis and methanogenesis (Borja et al., 1996). During

the early stage of hydrolysis and acidogenesis, acid-forming bacteria will convert fresh raw POME to volatile fatty acids (VFAs), before converting to CH_4 and CO_2 in methanogenesis under anaerobic digestion process (Wong et al., 2013). This will lead to the formation of biohydrogen and biomethane from POME which helps in stabilizing the system through sludge diminishing. Nowadays, anaerobic digestion systems are springing up like a mushroom. For treating POME, the most recommended digestion process include UASB, UASFF, anaerobic sequencing batch reactor (ASBR) and continuously stirred tank reactor (CSTR) (Ahmed et al., 2015). Table 2.2 and Table 2.3 show some comparison studies using single stage bioreactor and integrated bioreactor for POME treatment, respectively, and Table 2.4 summarizes the preferences and drawbacks of each bioreactor.

Table 2.2: POME treatment using single-stage bioreactor for biohydrogen/biomethane production.

Types of Waste	Inoculum	Bioreactor	HPR (L H_2 L^{-1} d^{-1})	MPR (L CH_4 L^{-1} d^{-1})	COD removal efficiency (%)	References
Raw POME	Digested POME	500 mL serum bottle	5.99 ± 0.5*	-	42	(Norfadilah et al., 2016)
Raw POME	Digested POME	UASFF	-	4.40	94	(Zinatizadeh & Mirghorayshi, 2017)
Raw POME	Digested POME	Integrated Baffled Reactor	-	-	79	(Malakahmad, Abd Lahin, & Yee, 2014)
Raw POME	Digested POME	AnaEG[a]	-	3.29	94	(Tabassum, Zhang, & Zhang, 2015)
Raw POME	Digested POME	CSTR	1.16	-	<30	(Mansor, Jahim, Mumtaz, Rahman, & Mutalib, 2016)

Raw POME	POME	UMAS[b]	-	92[**]	87.22	(Abdurahman & Chandra, 2015)
Raw POME	Digested POME	UASFF	4.61	-	40-54	(Mohammadi, Ibrahim, & Mohamad Annuar, 2014)
Raw POME	Digested POME	500 mL serum bottles	-	-	86	(Mohammadi et al., 2011)
Raw POME	Digested POME	50-L bioreactor	-	992	>90	(Basri et al., 2010)
Raw POME	Digested POME	160 mL serum bottle	-	-	21.95	This study
Raw POME	Pre-settled POME	UASFF	-	-	>90	(Zinatizadeh et al., 2009)

[*] L H_2 L^{-1} medium; [**] in a volume percent (%) [a] anaerobic expanded granular sludge bed; [b] ultrasonic membrane anaerobic system

Table 2.3: Comparison studies of dark fermentation coupled with anaerobic digestion for biogas production from POME using two-stage systems.

Types of Waste	**Inoculum**	**Integrated system used**	**HPR (L H_2 L^{-1} d^{-1})**	**MPR (L CH_4 L^{-1} d^{-1})**	**COD removal efficiency (%)**	**References**
Raw POME	Anaerobic seed sludge	DF-AD (UASB-UASB)	1.75	3.25[a]	85	(Krishnan, Singh, Sakinah, Thakur, Wahid, & Alkasrawi, 2016)
POME	Decanter cake	DF-AD (two-stage fermenter)	1.48	51.59[b]	62	(Suksong, Kongjan, & O-thong, 2015)
Raw POME	POME sludge	DF-AD (UASFF-UASFF)	5.29	9.60	83.70	This study
Raw POME	POME sludge	DF-AD (ASBR-UASB)	1.80	2.61	95	(Chonticha Mamimin, Singkhala, Kongjan, et al., 2015)

Raw POME	POME sludge	DF-AD (UASB-CSTR)	3.80	14.00	93	(O-thong et al., 2016)

UASFF - up-flow anaerobic sludge blanket (UASB)-fixed film (FF); AD - Anaerobic digester; ASBR - anaerobic sequencing batch reactor; POME – palm oil mill effluent. [a] mL g^{-1}MLVSS d^{-1}; [b] L L^{-1} waste.

Table 2.4: The pros and cons of the anaerobic treatment system commonly used for POME treatment

Anaerobic treatment system	Advantages	Disadvantages	References
UASB	High COD removal efficiency and CH_4 emission rate	High dependable on sludge settle stability	(Borja et al., 1996)
UASFF	Higher biomass retention, shorter start-up for sludge granulation	Reactor stability and efficiency depend on feed flow rate, internal packing, up-flow velocity and effluent recycle ratio	(Zinatizadeh et al., 2006)
ASBR	Simple operation, flexible and no separate clarifiers needed.	Low proficiency at higher OLR	(O-thong et al., 2007)
CSTR	Inexpensive and easy to handle	At high OLR and short HRT, gas production is less proficient	(Tong & Jaafar, 2006)

2.9 Challenges using POME wastewater

Raw POME is composed of lignocellulosic material types, that make it hard to degrade. A biological pre-treatment, either using specific bacteria or mixed culture will take a longer time compared to using chemical pre-treatment. A study on pre-treatment of brewery seed sludge for biohydrogen production using raw POME as substrate was done with the end goal to determine the best pre-treatment strategy for biohydrogen efficiency (Mohammadi et al., 2012b). Among all the studied strategies, heat-shock pre-treatment was found to produce the highest cumulative hydrogen with highest COD removal efficiency. This is because homoacetogens in the seed sludge (inoculum) had

been suppressed during the heat-shock, thus enable hydrogen-producing bacteria (HPB) to grow. Mohammadi et al., (2012b) likewise uncovered that their results were higher than a study done by Mohan, Babu, & Sarma (2008) using dairy wastewater as a substrate, regardless of the pre-treatment method used. It showed that even though the hydrogen production using raw POME is not as high as another study (Ren, Li, Li, Wang, & Liu, 2006), but its carbohydrate-rich material contains a large amount of starch, simple sugars and cellulose (O-thong et al., 2007). This makes it a suitable substrate for biogas production, especially in Malaysia. Considering the above matter, dark fermentation is by all accounts the key innovation for producing hydrogen from agricultural wastes, especially POME. Such waste, which is complex substrates, can be biologically degraded by complex microbial ecosystems. Furthermore, biological pre-treatment is preferable as it is much cheaper compared to chemical pre-treatments.

Khemkhao, Nuntakumjorn, Techkarnjanaruk, & Phalakornkule, (2012) reported a long start-up period using POME. They needed 123 operating days for microbial adaptations and evaluated the performance of a single-stage up-flow anaerobic sludge batch (UASB) reactor during temperature shift. This is due to the reason that UASB reactor can treat high-strength wastewater that contains high suspended solids and can also deliver a high measure of biomethane. However, other study has demonstrated that high-rate anaerobic bioreactor could abbreviate the start-up period in 22 days for biohydrogen generation. By using single-stage up-flow anaerobic sludge fixed-film (UASFF) bioreactor, they found out that the start-up period could be shortened by initially acclimatized the digested POME and used fresh raw POME as a substrate (Mohammadi et al., 2014). However, the up-flow velocity in the bioreactor, influent and effluent flow rate, as well as internal packing material in the up-flow Fixed-Film (UFF) part, play important roles in reactor stability and efficiency.

The UASB reactor due to its ability to keep high microorganism concentration and high rate of waste stabilization in the reactor, is an alternative bioreactor to generate biological hydrogen. The long start-up period (2-4 months), high and very low up-flow velocities, and granules washout at hydraulic stresses are the major problems associated with UASB reactors. Therefore, the UASB process modification is needed to eliminate the existing problems as well as having high-performance hydrogen production from POME.

In an earlier study performed by this research group (Mohammadi et al., 2014), a combination of UASB and UFF in a single reactor was used as modified UASFF bioreactor to produce biohydrogen from POME. The high rate systems showed high POME mineralization as well as high methane yield compared to the conventional treatment systems. The suspended solids in the POME, however, may present unfavorable impact towards granule formation and sludge bed stability when the up-flow velocity is high in the UASB.

On the other hand, provision of a required up-flow velocity is very important to guarantee the granules stability. Therefore, to partially solve this problem and to balance between the favorable performance of the process and stable microbial granules population, an external settling tank is designed to settle out the suspended solids prior to recycling the effluent to the reactor. The use of UASFF reactor was a good strategy to accelerate anaerobic granulation and to achieve a high COD removal efficiency as well as H_2 yield in a short period of time. The reactor was efficient in the fermentation of pre-settled POME at high OLR and short HRT.

In Malaysia, current situation is not ready for the implementation of biohydrogen production technology from POME. The main problems lead to the constraints of scale-

up of biohydrogen production are HRT, storage and safety problems, and the reactor engineering (Ahmad et al., 2016). However, the conventional POME treatment does require wide land area, longer HRT, mass sludge production and low treatment efficiency. Therefore, the inexpensive high-rate anaerobic treatment, together with steady and well-organized bioreactor (in terms of biogas capture) rise an important consideration for oil palm industries.

2.10 Importance of biohydrogen and biomethane

Application of biohydrogen and biomethane, or their mixture (biohythane), has become an increasing interest for the industries as alternative renewable energy. The increment in energy demand and continuous usage of fossil fuels are vulnerable by the concerns of global warming due to the increase of carbon dioxide (CO_2) released into the atmosphere (Angeriz-campoy, Álvarez-gallego, & Romero-garcía, 2015). Hydrogen is present in high amount in nature, contrasted with fossil fuel (Ntaikou, Antonopoulou, & Lyberatos, 2010). When burning biohydrogen, water produced as a by-product, that has higher calorific value due to its higher energy value (Guo, Trably, Latrille, Carrre, & Steyer, 2010). This high energy (heating) value (142 kJ g^{-1}) makes biohydrogen applicable for combustion engines.

Pure biohydrogen can produce electricity in fuel cells. This criterion makes hydrogen the most environmentally friendly and an ideal alternative to fossil fuels (Piera, Martínez-Val, & José Montes, 2006). According to Redwood, Paterson-Beedle, & MacAskie, (2009), for future energy economy, hydrogen has become a key energy trajectory. Attentions have been focused on the fuel cell efficiency and technology for hydrogen storage for transport applications to meet commercial viability, by having a clean environment and reducing the pollution (Sharma & Krishna, 2015). In general, hydrogen

is applied in ammonia production (Lattin & Utgikar, 2007; Ramachandran, 1998), petroleum refining (Barreto, Makihira, & Riahi, 2003; Mueller-langer, Tzimas, Kaltschmitt, & Peteves, 2007) and metal refining (tungsten, copper, lead) (Eliaz, Eliezer, & Olson, 2000; Eliezer, Eliaz, Senkov, & Froes, 2000). Hydrogen is highly used for the synthesis of ammonia, hazardous waste hydrogenization, desulphurization (e.g. hydro-desulphurization and hydrogenation reactions) and refining, food preparation, chemical plants, rocket fuel, and high-temperature industrial furnace fuel (Dupont, 2007). In ammonia production, with 500 billion cubic meters (Bm^3) of hydrogen, 250 Bm^3 of hydrogen is consumed for ammonia production, 65 Bm^3 for other chemical products production and 185 Bm^3 of the hydrogen is for petrochemistry production (Balat, 2008; Dupont, 2007). Furthermore, there are significant hydrogen applications on cooking food, hydrogen-powered industries, electricity generation, jet planes, fuel for automobiles, hydrogen village and not to forget the domestic requirements (Jain, 2009).

Production of biohydrogen from organic waste is followed by the production of organic acids, which has become the source of substrate for methane production (Pagliaccia, Gallipoli, Gianico, Montecchio, & Braguglia, 2016). Biomethane has the potential to reduce fossil fuels demands, for example, coal, oil, and natural gas that provide power. In order to improve energy yields from other biofuel production processes (e.g. biohydrogen, bioethanol and biodiesel), biomethane production can be performed together. Digestion technology implementation at municipal, industrial as well as agricultural industries has allowed effective distribution and decentralized energy generation (Wilkie, 2008). Biomethane also can be produced from bioethanol production industries for electricity or fuel usage (Wilkie, 2008). Production of biomethane via anaerobic digestion can produce clean fuel, especially from renewable feedstocks. Instead of produce energy from fossil fuels, biomethane can also act as a source of energy that

can reduce the environmental impacts (i.e. global warming and acid rain) (Chynoweth, 2005). Applications of pure methane in appliances, industries, vehicles and power generation are increasing every year. However, according to Chynoweth (2005), different states of purity can also be applied especially in energy conversion and transportation compared to electricity.

CHAPTER 3: GENERAL MATERIALS AND METHODS

3.1 General Experimental Design

The general experimental flow chart is shown in Figure 3.1. The study was divided into three phases; (i) Batch study; (ii) A start-up study; (iii) Optimization study.

3.2 Sample preparation

3.2.1.1 Raw POME (substrate)

For the start-up study using two-stage UASFF bioreactor, two different sources of raw POME were used. The first substrate was taken from Jugra Palm Oil Mill, Banting, Selangor. After several attempts taken to increase biohydrogen production were failed, the source of raw POME changed to the second source. The second substrate was taken from Sri Ulu Langat Palm Oil Mill, Dengkil, Selangor, Malaysia. The substrates were kept in a closed container and stored in a 4°C cold room to inhibit the microbial activity. Suspended solids of raw POME were allowed to settle before applying the substrate into H_2-UASFF unit. To obtain a desired influent COD concentration of 20,000 mg L^{-1}, pre-settled POME (liquid part) was taken and diluted with tap water. Diluted substrate was then put in a closed container with nitrogen gas purging at 10 mL min^{-1} for 10-15 min to provide anaerobic condition.

pH, temperature, soluble COD (SCOD) and particulate COD (PCOD) were pre-determined before preparing a mother solution of desired concentration for the experiments.

3.2.1.2 POME sludge (Inoculum)

Biologically treated POME was taken from anaerobic pond in Jugra Palm Oil Mill, Banting, Selangor, Malaysia. The sludge remains throughout the study as it shows a good source of inoculum by producing methane. To proof the presence of methane, the sludge was first tested for biomethane production in CH_4-UASFF unit, before heat-treated (90°C, 1 h) for biohydrogen production in H_2-UASFF unit. Samples were pre-determined for total suspended solids (TSS), volatile suspended solids (VSS), pH, and temperature.

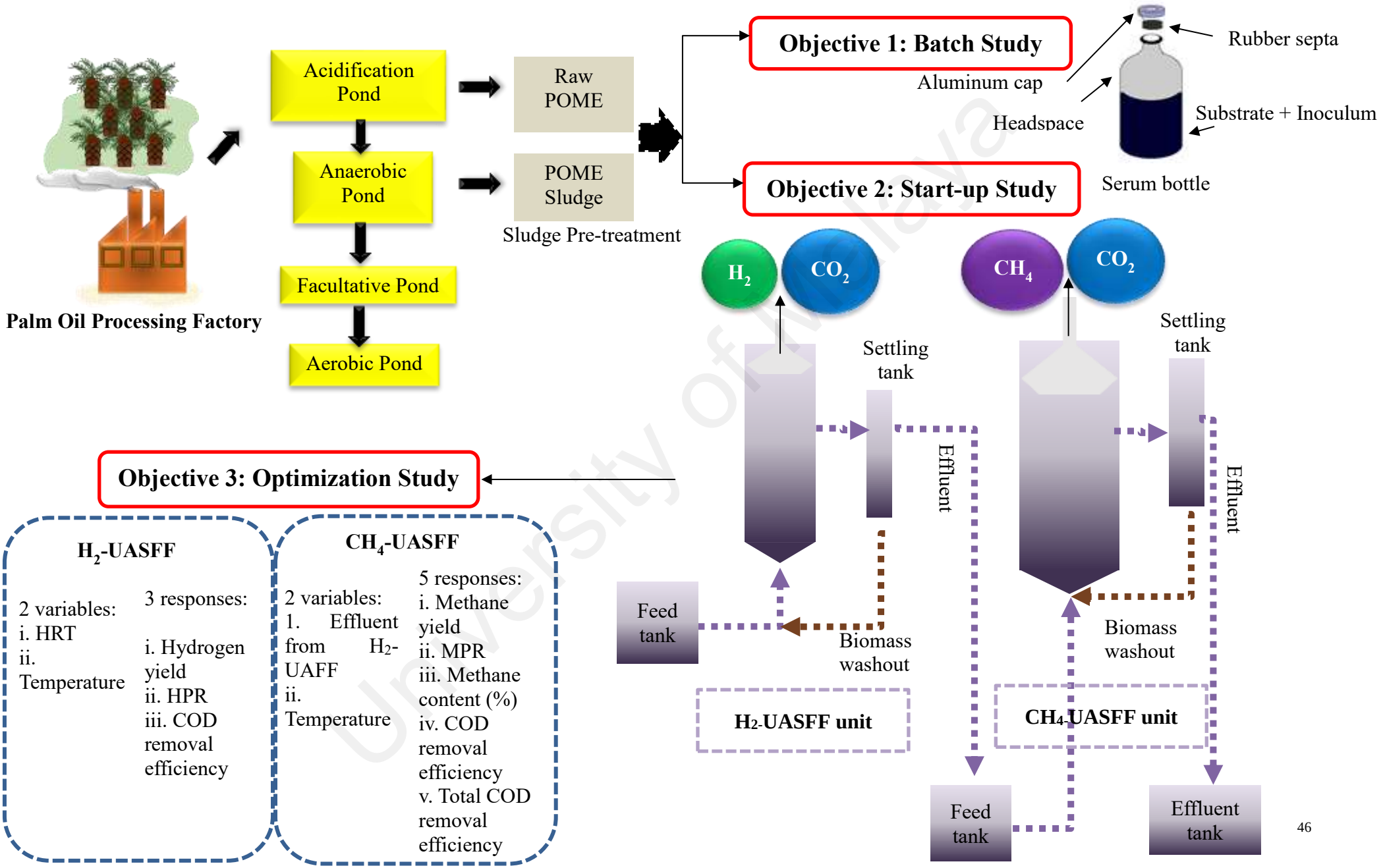


Figure 3.1: Experimental flow chart

3.3 Methodology

3.3.1 Characterization of raw POME and POME sludge

Substrate and inoculum were subjected to different analysis to determine their compositions before continuing with dark fermentation process, followed by anaerobic digestion process.

3.3.1.1 Physico-chemical characteristics of POME

COD measurement was carried by APHA Standard method 5220D, Closed Reflux, Colorimetric Method. After the sample was filtered using glass microfiber filters GF/C™ (D = 47 mm), CAT No. 1822-047, a 50 times dilution was made into a volumetric flask. 1.5 mL of potassium dichromate and 3.5 mL of sulphuric acid were added into COD vial followed by 2.5 mL of filtered sample. Standard potassium dichromate solution and sulphuric acid reagent were prepared as in APHA Standard Method 5220B (3(a) and 3(b)). The sample was then digested for 2 h by using digester (Spectroquant® TR420, MERCK). After the digesting process, the vial could cool down at room temperature before the COD was measured using spectrophotometer (Spectroquant® Pharo 100, MERCK) at 600 nm (*American Public Health Association (APHA). Standard Methods for the Examination of Water and Wastewater*, 1999).

For total suspended solids (TSS) and volatile suspended solids (VSS) measurement, APHA Standard Method 2540B and 2450C were applied. Samples were placed in a dry dish and dried in an oven for 1 h at 103-105°C. The weight of the dry dish was initially recorded. After 1 h, the dish was then cooled in a desiccator until a constant weight is obtained (i.e. <4%). For VSS measurement, the dish will be heated at 500 ±5 0°C for 15 mins in a furnace. The dish was stored in a desiccator and immediately weighed before used. The calculations for TSS and VSS are as follows (*American Public Health*

Association (APHA). Standard Methods for the Examination of Water and Wastewater, 1999):

(TSS) mg total solids/L = [(A - B) X 1000] sample volume (mL)

Equation 3.1

where:

A = weight of dried residue (mg) + dish (mg)

B = weight of dish (mg)

(VSS) mg volatile solids/L = [(A - B) X 1000] sample volume (mL)

Equation 3.2

where:

A = weight of residue (mg) + dish before ignition (mg)

B = weight of residue (mg) + dish or filter after ignition (mg)

3.3.1.2 Alkalinity

The alkalinity measurement was based on APHA Standard Method 2320B using titration method. 0.1 N sulphuric acid (H_2SO_4) was used for the titration and pH adjustment. The calculation for alkalinity is:

Alkalinity (mg $CaCO_3$ L^{-1}) = [A x N x 50,000] / mL sample

Equation 3.3

where:

A = mL standard acid used

N = normality of standard acid

3.3.2 Volatile Fatty Acids (VFA) Analysis

3.3.2.1 Preparation of the standards

The VFA consists of acetic acid (AA), propionic acid (PA) and butyric acid (BA). The preparation of the standards (AA, PA and BA) at different concentrations are shown in Figure 3.2. By using 100 mL volumetric flask, 1% (10,000 ppm) of pure AA, PA and BA were prepared each. In order to get the calibration curve with five points (50 ppm, 100 ppm, 200 ppm, 300 ppm and 1000 ppm) for each VFAs, 25 µL, 50 µL, 100 µL, 150 µL and 500 µL of diluted acids were added in 5 mL volumetric flask, respectively. Each of the standards were then injected into Gas Chromatography-Mass Spectrometry (GC-MS) and the calibration curve were then plotted. The method file was attached in Appendix.

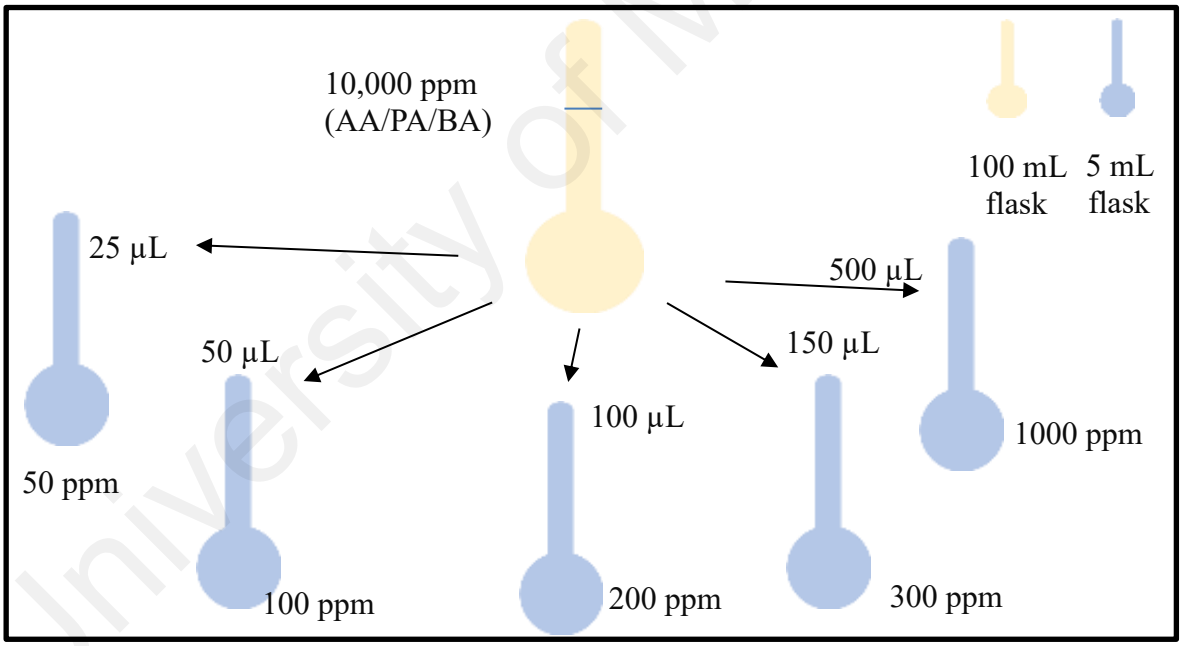


Figure 3.2: Standard preparation for calibration curve of acetic acid (AA), propionic acid (PA) and butyric acid (BA).

3.3.2.2 Sample preparation for VFA analysis

After dark fermentation process, the treated effluent was filtered using glass microfiber filters GF/C™ (D = 47 mm), CAT No. 1822-047. About 5 g of the filtered sample was kept in a 15 mL vial. The vial was closed tightly using a rubber and a silver cap provided by Perkin Elmer Co. Ltd. for VFA analysis using Headspace.

3.3.3 Biogas measurement and analysis

Gas volume was measured by using a gas flow meter.

(a) ***Water displacement method***

Water displacement method was used to keep the collected biogas for further analysis using Gas Chromatography (GC). A 15 mL vial was used and submerged in an acidic water (pH below than 2). After gas sampling using 5 mL syringe, the biogas was injected into the vial under the water to collect the biogas. The vial was sealed for further used.

(b) ***Gas Chromatography (GC)***

By using Gas Chromatography (Perkin Elmer, AutoSystem Gas Chromatograph, 600 Series LINK), the composition of the biogas was analyzed using a pack GC column Supelco, 40/80 carboxen 1000, MR2924D, 10' x 1/8' and thermal conductivity detector (TCD). At flowrate of 30 mL min^{-1}, carrier gas used was high purity argon. Oven, injector and detector's temperature were set to 100°C, 150°C and 200°C, respectively. A 0.5 mL gas tight syringe 2500 µL Hamilton, USA was used for gas sampling for injection purposes.

(c) ***Gas Chromatograph-Mass Spectrometry (GCMS)***

Gas Chromatograph (Perkin Elmer, Clarus® 680) and Headspace Sampler (Perkin Elmer, Turbomatrix 40 Trap) with column type Elite-1, 30-meter long, 0.25 mm internal

diameter, 0.25 umdf were used for VFA analysis. The initial oven temperature was 40°C, with injector and flame ionization detector (FID) temperature of 200°C and 250°C, respectively. Helium gas was used as a carrier with 1.2 mL min^{-1} of flowrate. Hydrogen and purified air flowrate were set to 45 mL min^{-1} and 450 mL min^{-1}, respectively. For the Headspace, the condition for needle temperature, carrier gas pressure, column and oven were 90°C, 20 psi, 120°C and 75°C, respectively.

3.3.4 General Bioreactor Design

Two-stage UASFF was designed and fabricated based on previous studies done by Mohammadi et al., (2014); Najafpour et al., (2006); and Zinatizadeh et al., (2017). Figure 3.3 and Figure 3.4 shows the engineering design and the actual design, respectively. In H_2-UASFF unit, the glass bioreactor column was fabricated with an internal diameter of 6 cm at the bottom and middle parts and 10 cm at top part. The bioreactor comprised of three sections. The lowest section of the UASB reactor's column has a height of 65 cm (granular sludge portion) while the middle section is a 15 cm in height (fixed film reactor). The top section of the bioreactor consists of a gas-solid separator and outlet zone for fermented POME. The use of packing media in the middle section could reduce channeling problem and loss of biomass due to flotation associated with poorly performing UASB reactors. Additionally, the packing material caused the flocculated biomass to precipitate over the sludge blanket to serve as suitable and natural hydrophobic core for the development of granular sludge.

In CH_4-UASFF unit, the glass bioreactor column was fabricated with an internal diameter of 7 cm at the bottom and middle parts and 11-12 cm at the top part. The lowest section of the UASB reactor's column has a height of 80 cm (granular sludge portion) while the middle section is 15 cm in height (fixed film reactor). Since this is an identical

two-stage UASFF bioreactor, the top section of the bioreactor is also consisting of a gas-solid separator and outlet zone for fermented POME. The middle part of H_2-UASFF unit and CH_4-UASFF unit was packed with 20 and 30 pall rings, respectively (diameter and height 16 mm; specific surface area 341 m^2/m^3). The sampling ports were designed at appropriate intervals along the height of the reactor.

The gas-liquid-solid separator at the top part is for the separation of the washed-out solids and the biogas from the liquid phase. To measure the biogas volume generated, a gas meter counter was connected to an inverted funnel and cylinder-shaped gas separator. To sample the biogas for the determination of its composition, a gas sampling port with tubing connector was provided.

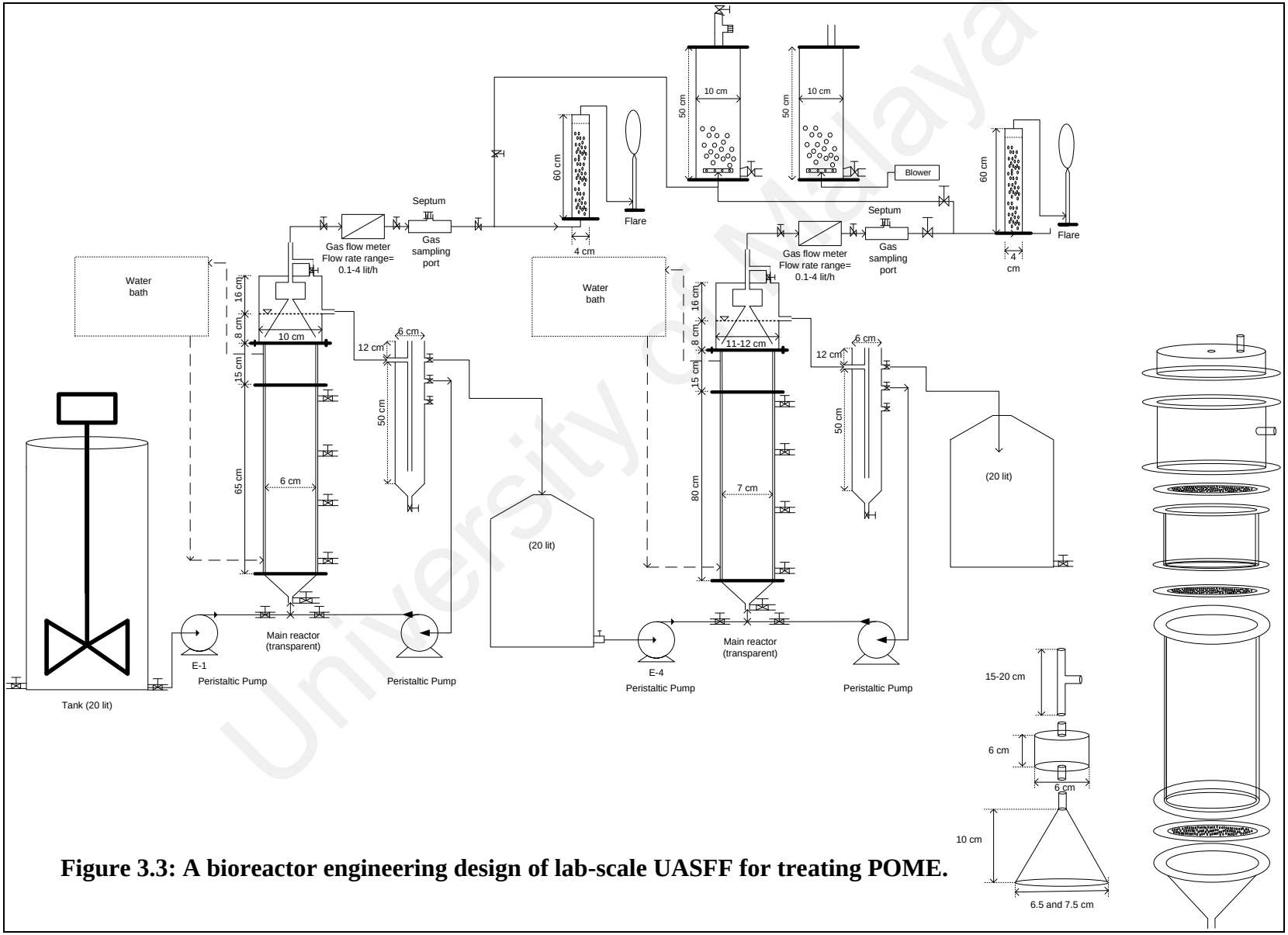


Figure 3.3: A bioreactor engineering design of lab-scale UASFF for treating POME.

Figure 3.4: A lab-scale UASFF bioreactor used in this study.

To ensure isothermal operation of the UASFF reactor at the selected temperature, water was circulated through the bioreactor jacket from a thermostat water bath equipped with a centrifugal pump (Lab. Companion, model: CW-05G, Korea). The feeding of substrate (POME) was carried out continuously into the bottom inlet of the reactor using a peristaltic pump (EYELA, model: MP-1000, Japan, 0.24-34.8 L d^{-1}) and the effluent went out from the top of the column. An influent liquid distributor was mounted at the base of the column to assist in distributing the feed uniformly into the reactor column.

A cylindrical settling tank was installed in order to settle the washed-out suspended solids from the reactor and to provide an effluent with low suspended solids for recycling into the reactor. The size of the settling tank is 50 cm x 6 cm (H x W). The effluent was continuously recycled using a peristaltic pump (EYELA, Japan). A manual recycling was done for the washed-out granular sludge (settled at the bottom of the settling tank) into the reactor. To ensure a homogenous substrate supply, the feed tank was purged with nitrogen gas in a closed container for 20 mins at 10 mL min^{-1} before starting the experiment.

3.3.5 Design of Experiment

Response Surface Methodology (RSM) is a collection of mathematical and statistical techniques that can be used for analyzing the effects of several independent variables on the response. It has an important application in the process design and optimization as well as the improvement of existing design. In this study, experiments were designed, analyzed and optimized by applying central composite design (CCD) in RSM.

CCD is the most commonly used response surface design experiment. It is a factorial or fractional factorial design with center points, augmented with a group of axial points that can estimate a curvature. CCD can be efficiently used to estimate first- and second-

order terms, as well as model a response variable with curvature by adding center and axial points to a previously-done factorial design. Compared to Box-Behnken Design which also one of the types of response surface design, it does not contain an embedded factorial or fractional factorial design. This caused it to be not suited for sequential experiments.

In this study, CCD with RSM was used for optimization process since it helps to test the validity of independent factors selected. Moreover, the use of CCD avoids misleading conclusion from factorial interaction and allow effect of factor to be estimated at several levels of other factors, yielding conclusions that are valid over a range of experimental conditions.

In a batch study, three-factor central-composite design suggested 6 number of replications at center points, thus increased the total number of observations to 20. Based on a research done by Clark & Williges (1973), this procedure exist in order to determine the optimum number of center points of a *K*-factor design (Box & Hunter, 1957). Previous study also used CCD for a three-different levels for hydrogen production treating POME (Mohammadi, Ibrahim, & Mohamad Annuar, 2012a).

Meanwhile, the interpretation of the effects of the variables and responses studied were analyzed by using ANOVA. Experiments were conducted based on the RSM data using Design Expert® Software (Stat-Ease Inc., version 7.0.0).

CHAPTER 4: EFFECTS OF PROCESS, OPERATIONAL AND ENVIRONMENTAL VARIABLES ON BIOHYDROGEN PRODUCTION USING PALM OIL MILL EFFLUENT (POME)

4.1 Introduction

In the last 15 years, a lot of research focused on producing biohydrogen using different types of wastewater; namely municipal wastewater, agricultural wastewater, and beverages wastewater (Assawamongkholsiri, Reungsang, & Pattra, 2013; Cai, Liu, & Wei, 2004; Cappai et al., 2014; De Gioannis, Muntoni, Polettini, & Pomi, 2013). This is possibly due to the fuel crisis resulting from fossil fuel resource depletion (Hosseini & Abdul Wahid, 2013). Other than the fuel crisis, the combustion of fossil fuel that lead to the emissions of toxic materials, which is also responsible for many environmental problems (Su, Kao, & Huang, 2012). In addition to that, it will indirectly contribute to other consequences such as the increase of greenhouse gases (GHGs), the rising of sea levels, the impact on climate change, and the diminishing biodiversity (Nigam & Singh, 2011).

One of the plausible resources for the biohydrogen production in Malaysia is from the treatment of palm oil-based industry's wastewater as Malaysia is among the world's biggest palm oil exporter (Ng et al., 2011). Regardless of its many useful products, there are some harmful aspects associated with the production. Tons of palm oil mill effluent (POME) produced everyday can endanger the environment. The biogas that is produced from POME during anaerobic treatment is a thoughtful challenge resulted from the current production processes (Hosseini & Abdul Wahid, 2013; Yacob et al., 2006).

POME is categorized as a very high contaminating wastewater that contains a biochemical oxygen demand (BOD) of 25,000 mg L^{-1} and chemical oxygen demand (COD) of 69, 500 mg L^{-1} (Abdullah, Ujang, & Yahya, 2011). Some other properties contain in POME that may harm the environment include glycerin, dissolved oil and fatty acids, crude oil solids and other soluble materials (Khemkhao et al., 2012). Therefore, direct discharge into the land is not encouraged. According to the Department of Environment Malaysia, in conjunction with the Environment Quality Act 1974, POME must be treated before it is directly released into the environment (Environmental Quality Act, 1974). Since raw POME normally is discharged at 80-90°C, therefore several researchers reported that treatment of POME can be done whether in mesophilic or thermophilic conditions (Mustapha, Ashhuby, Rashid, & Azni, 2003; Najafpour et al., 2006). Some other studies have suggested for treatment of POME which includes these evaporation ponds; thermal, physicochemical, and biological treatment. For wastewater to be treated by biological, its BOD/COD ratio needs to be greater than 0.5 (Tchobanoglous, Burton, & Stensel, 2003). Moreover, biological treatment is also preferable due to its cost-effectiveness as compared to chemical treatment.

Production of biogas from POME is widely known by using anaerobic digestion. Since its potential for treating wastes while producing renewable energy, it has become the most studied technology. Anaerobic digestion is a process where organic materials are decomposed in a condition where there is no oxygen present and useful biogas is produced, simultaneously. There are three stages of reactions involved in anaerobic digestion. The first stage is hydrolysis, followed by acidogenesis and lastly methanogenesis (O-thong et al., 2016; Yuzir, Chelliapan, & Sallis, 2012).

Hydrolysis is a process where complex organic compounds are converted (hydrolyze) by fermentative bacteria to simple monomers such as fatty acids, monosaccharides and amino acids. Next, in the acidogenesis process, these simple monomers included sugars will be degraded further to acetate, hydrogen (H_2) and carbon dioxide (CO_2) (Tchobanoglous et al., 2003). Acetate, H_2 and CO_2 will be the precursors for methane production in methanogenesis process.

For a normal process of microbial fermentation, it has been reported that organic wastes only have about 7.5 to 15% of energy to be converted to hydrogen, whilst the rest will remain in volatile fatty acids (VFA), i.e. acetic acids (AA), butyric acid (BA), propionic acid (PA) and lactic acid (LA) (Hallenbeck & Ghosh, 2009). VFA will then be converted to methane or any other suitable by-products through a process called methanogenesis (Liu et al., 2013). A basic dark fermentation process can be simplified using Equation 4.1 below (Hawkes et al., 2007):

$$\underset{\text{Glucose}}{C_6H_{12}O_6} + 2\,H_2O \longrightarrow \underset{\text{VFA}}{2\,CH_3COOH} + 4\,H_2 + 2\,CO_2 \qquad \textbf{Equation 4.1}$$

Biohydrogen production from POME is not new in this field. O-thong et al., (2007) showed that under thermophilic condition (60°C), a hydrogen yield of 4.2 L H_2 L^{-1}_{waste} and COD reduction of 37% was achieved when using POME of 85 g COD L^{-1}. Other studies reported a hydrogen yield of 4.5 L H_2/L_{waste} and a COD reduction of 40% using high concentration of POME (10 – 59.3 g L^{-1} COD) as substrate (Vijayaraghavan & Ahmad, 2006), a hydrogen yield of 0.27 L H_2 g^{-1} COD and 57% of a COD reduction under thermophilic condition (Prasertsan et al., 2009) and 51.5 mL H_2 g^{-1} COD of

hydrogen yield with 15.1% of a COD reduction using initial COD of 5 – 35 g L^{-1} COD at 55°C (Tanikkul & Pisutpaisal, 2014), respectively.

Whether it is mesophilic or thermophilic in anaerobic treatment, both conditions give different impacts on the COD removal, biohydrogen production yield and rate of POME. Oh, Seol, Rae, & Park (2003) in their study of effects of temperature (25-40°C) on hydrogen production concluded the increase of temperature from 25-36°C also improved the cell growth rate and hydrogen production rate. They also reported that at 36°C, maximum hydrogen yield was achieved (i.e. 2.49 mol H_2 mol^{-1} glucose). Chong, Sabaratnam, Shirai, & Hassan, (2009) in their study using *Clostridium sp.* extracted from the mixed cultures of POME anaerobic sludge at 37°C reported a total accumulated hydrogen gas was higher (i.e 3 L H_2 day^{-1}). Another study was done by Lee, Lin, & Chang (2006) reported that temperature above 35°C may inhibit the growth of the granular sludge. Meanwhile, with the thermophilic condition, Mamimin, Singkhala, & Kongjan, (2015) reported that this condition is good for POME to be converted to H_2 as it has less variety of end-products, thermodynamic condition as well as has low inhibition of hydrogen partial pressure.

The high production rate of hydrogen was produced in the dark fermentation process but with low hydrogen (Li & Fang, 2007; Saraphirom & Reungsang, 2013). The most recent batch study done by Norfadilah et al., (2016) used high concentration of raw POME as a substrate (initial COD concentrations were 32 g L^{-1} to 86 g L^{-1}), reported a COD removal of approximately 37% with the maximum hydrogen yield of 5.98 L H_2 L^{-1}-medium at 10% POME sludge. Additionally, a study was done by Mohammadi, Ibrahim, & Mohamad Annuar, (2012a) using pre-settled POME as substrate reported that the highest hydrogen yield calculated was 124.48 mmol H_2 g^{-1} COD removed with COD

removal of 54.2%. However, the study was done at low COD influent of 3 g L^{-1} COD – 10 g L^{-1} COD.

Based on the presented studies, most of the researchers were done at high substrate concentrations (>20 g L^{-1} COD). However, Poh & Chong, (2009) reported that in Malaysia, only mesophilic temperature conditions are conducted for anaerobic POME treatments.

Thus, current study was initiated to produce biohydrogen from POME using dark fermentation process in a batch mode. Raw POME was used as a substrate and POME anaerobic sludge as an inoculum. The inoculum sizes (10:90 – 40:60), reaction times (8 – 24 h) and mesophilic to thermophilic conditions (30-50°C) were varied to study their effects on biohydrogen production and its COD removal efficiency. For the optimization study, response surface methodology (RSM) was applied for hydrogen yield and COD removal efficiency. In this study, the substrate concentrations were varied from a low concentration of < 20 g L^{-1} to a high concentration of >20 g L^{-1} CODin.

4.2 Materials and methods

4.2.1 Inoculum preparation

The POME sludge was taken from an anaerobic pond and was obtained from Jugra Palm Oil Mill, Banting, Selangor, Malaysia and used as inoculum. Prior to its use, the sludge was heat-treated at 100°C for an hour to promote hydrogen-producing bacteria (HPB) and to suppress the hydrogenotrophic methanogens that could produce methane since the sludge is a base (pH 7.42) (Mohammadi et al., 2014). A 600 mL of mother solution was prepared for the heat-treated inoculum using the dilution equation ($M_1V_1 = M_2V_2$ where M is the molarity of the solution and V is the volume of the solution). The inoculum concentration calculated was based on volatile suspended solids (VSS) value.

A fixed concentration of 5.4 g MLVSS L^{-1} was then used throughout the study, corresponding to food-to-microbe ratio (F/M) of 3.4 (Nathao, Sirisukpoka, & Pisutpaisal, 2013). The chemical parameters of the seed sludge were presented in Table 4.1.

Table 4.1: Parameters studied of the seed sludge (inoculum) and raw POME (substrate).

Parameter	Unit	Heat-treated POME sludge (Inoculum)	Raw POME (substrate)	Pre-settled POME (substrate)
Total Suspended Solid (TSS)	mg TSS L^{-1}	27,611 ± 2,149	-	-
Volatile Suspended Solid (VSS)	mg VSS L^{-1}	19,167 ± 2,357	-	-
COD	mg COD L^{-1}	-	52,122 ± 96	28,122 ± 419
pH	-	7.42	4.99	4.99

4.2.2 Pre-settled POME (substrate) preparation

The substrate was the POME collected from Jugra Palm Oil Mill, Banting, Selangor, Malaysia after the acidification process. Prior to its use, raw POME could settle before the supernatant was extracted and used as a substrate. The supernatant was called pre-settled POME. The characteristics of the substrate were presented in Table 4.1. A mother solution was prepared for 2 L and calculated based on the COD of pre-settled POME using the dilution equation mentioned above. A fixed COD concentration of 18.5 g L^{-1} COD was used throughout the batch study.

4.2.3 Batch study

Based on a working volume of 100 mL, a batch study was conducted using 160 mL serum bottles (Choi & Ahn, 2015). The reaction starts right after the substrate was mixed with inoculum at initial COD of 32,320 mg L^{-1} for 10:90 inoculum to substrate ratio (I:S; v/v), initial COD of 28,720 mg L^{-1} for 20:80 (I:S; v/v) and initial COD of 16,720 mg L^{-1}

for 40:60 (I:S; v/v). Raposo et al., (2009) reported inoculum to substrate ratio between 0.5 - 3.0 under mesophilic temperature while Florio et al., (2017) reported an inoculum to substrate ratio between 0.01 - 0.14 for biohydrogen production. Therefore, this study intended to determine the optimum inoculum to substrate ratio between 0.11 – 0.67 based on POME used. The mixture of the inoculum that has pH 7.42 and pH substrate of 4.99 would have a pH of approximately 6 in the serum bottles. Therefore, the pH of the mixture was adjusted to pH 5.5 ± 0.2 with 1N HCl or 1N NaOH. Fang & Liu (2002) reported the optimal pH for hydrogen production was pH 5.5. The serum bottles were then purged with nitrogen gas for 10 mins at 10 mL min^{-1} in order to create anaerobic conditions. All batch tests were conducted based on the RSM data using Design Expert® Software (Stat-Ease Inc., version 7.0.0) shown in Table 4.2. The total biogas composition was monitored by gas chromatography. Several studies have reported high hydraulic retention time (HRT) of more than 24 h (Badiei, Jahim, Anuar, & Sheikh Abdullah, 2011; C. Mamimin, Chaikitkaew, Niyasom, et al., 2015). However, it is reported that 12 h HRT is the optimum to get 88.62% of sugar consumption in POME for hydrogen production (Muzhafar, Jahim, Shahbudin, & Nordin, 2018). Therefore, reaction time of 8 – 24 h were chose in this study and the liquid samples were analyzed for effluent COD at 8, 16 and 24 h intervals.

Table 4.2: Central Composite Design Experimental Conditions and Results (with 6 replicates at a central point).

	Variables			Responses	
Run	I:S	Temperature (°C)	Reaction time (h)	COD $_{\text{removal efficiency}}$ (%)	H_2 Yield (mL H_2/g $COD_{removed}$)
2	20:80	40	16	42.25	6.60
9	10:90	50	24	41.67	5.38
7	10:90	40	16	49.09	3.53
17	20:80	40	8	33.43	10.01
3	40:60	40	16	27.91	16.82
11	10:90	30	8	49.50	2.07
12	20:80	40	16	39.00	3.58
14	10:90	30	24	45.38	2.26
13	20:80	40	16	42.71	2.61
15	20:80	40	16	39.46	6.79
1	40:60	30	8	20.74	19.10
4	10:90	50	8	35.07	7.95
19	20:80	40	16	40.39	4.14
18	20:80	30	16	35.75	12.90
8	40:60	50	8	21.95	28.47
16	20:80	50	16	41.32	11.12
6	20:80	40	24	32.50	4.35
5	40:60	30	24	27.11	10.22
20	20:80	40	16	44.57	3.76
10	40:60	50	24	24.72	10.02

4.2.4 Analytical methods

Every 8 h interval, samples were collected and filtered for chemical oxygen demand (COD). Meanwhile, volatile suspended solids (VSS) and total suspended solids (TSS) were analyzed at the initial experiment. All the tests were conducted as stated in Sub-section 3.3.1. The composition of the biogas was analyzed as mentioned in Sub-section 3.3.3.

4.2.5 Design of Experiment

In this batch mode study, H_2 production from POME was analyzed and optimized by applying central composite design (CCD) in RSM. The reasons of using CCD was explained in Chapter 3, Sub-section 3.3.5. The suggested design mode was quadratic,

with 20 runs (six replicates at central points). The three factors, namely I:S, reaction temperature and fermentation time were used. Table 4.2 shows the experimental conditions based on CCD design for biohydrogen production from POME. Each factor in the design was assessed at low, high and central levels in order to estimate the experimental variability, design and corresponding experimental results. A three-dimensional (3D) plots are presented in Figures 4.1 and 4.2 to interpret the effects of the variables studied. The results were analyzed by using ANOVA. By using Equation 4.2, the coefficients of the polynomial model were obtained;

$$Y = \beta_0 + \beta_i X_i + \beta_j X_j + \beta_{ii} X_i^2 + \beta_{jj} X_j^2 + \beta_{ij} X_i X_j + \ldots \quad \textbf{Equation 4.2}$$

where β is the regression, i is linear and j is a quadratic coefficient, respectively. Based on Figure 4.1 and 4.2, the effects of the relationship between both variables and responses were generated. The values were presented using ANOVA are shown in Table 4.3.

Table 4.3: ANOVA for response surface reduced quadratic model.

1. Response	**COD removal efficiency**				
Source	Sum of Squares	Mean Square	F Value	Prob > F	
Model	1297.78	216.30	22.32	< 0.0001	significant
A-I:S	974.13	974.13	100.51	< 0.0001	significant
B-Temperature	20.10	20.10	2.07	0.1735	
C-Reaction time	12.35	12.35	1.27	0.2793	
AB	34.26	34.26	3.53	0.0827	
AC	6.29	6.29	0.65	0.4351	
C^2	250.65	250.65	25.86	0.0002	significant
R-Squared	0.9115				
Adj R-Squared	0.8707				
Lack of Fit	102.97	12.87	2.79	0.1363	not significant
2. Response	**H_2 Yield (mL H_2/g COD removed)**				
Source	Sum of Squares	Mean Square	F Value	Prob > F	
Model	17.25	3.45	14.24	< 0.0001	significant

A-I:S	10.43	10.43	43.02	< 0.0001	significant
B-Temperature	0.83	0.83	3.41	0.0861	
C-Reaction time	2.36	2.36	9.74	0.0075	significant
AC	1.06	1.06	4.36	0.0555	
B^2	2.58	2.58	10.65	0.0057	significant
R-Squared	0.8357				
Adj R-Squared	0.7770				
Lack of Fit	2.61	0.29	1.85	0.2577	not significant

4.3 Results and discussion

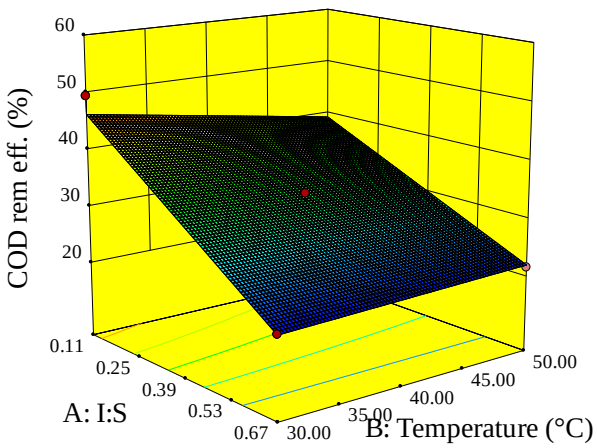
4.3.1 COD removal efficiency

Based on Table 4.3, Model F-value of 22.32 indicates that the model is significant. Values of Prob > F less than 0.05 represents that the model terms are significant. For Response on COD removal efficiency (COD rem. eff.), only variable A is a significant model term. Some of the insignificant model terms such as temperature (B) and reaction time (C) were removed to simplify the model. The reduced quadratic model equation for the response is reported as below:

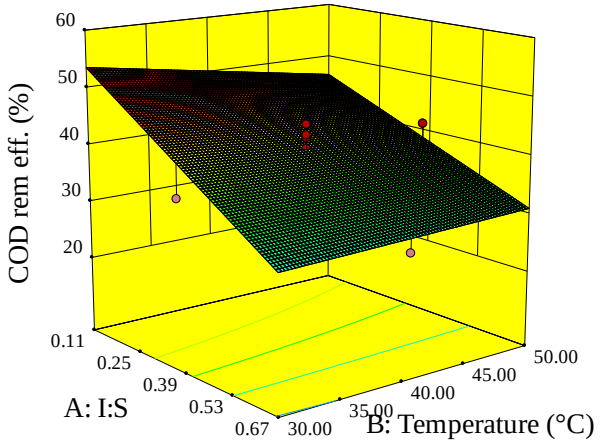
$$\text{COD rem. eff.} = 40.24 - 9.87A - 1.42B + 1.11C + 2.07AB + 0.89AC - 7.08C^2$$

Equation 4.3

8 h



16 h



24 h

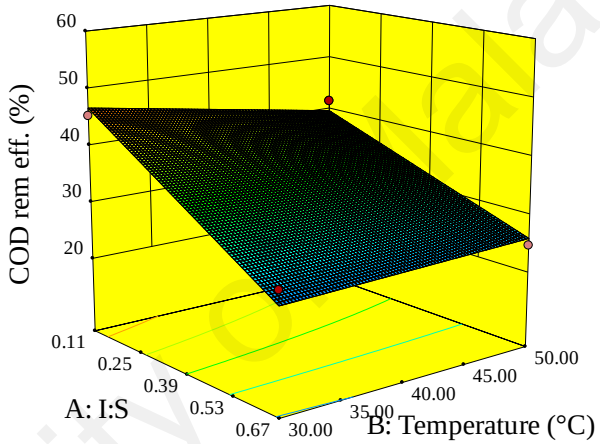


Figure 4.1: The 3D image of effects of I:S and reaction temperature (h) on COD removal efficiency (%) at 8 h reaction time, 16 h reaction time and 24 h reaction time.

From the 3D response surface plots (Figure 4.1), COD removal efficiency was highest at the lowest I:S of 0.11, at 30°C for 8, 16 and 24 h reaction time. However, COD removal efficiency decreases when temperature was increased to thermophilic condition (50°C). The decrease in COD removal efficiency at highest I:S might be due to the mineralization of components into water and carbon dioxide that present in POME. Meanwhile, high COD removal efficiency at lowest I:S may attributed to the formation of simple intermediates produced from the degradation of complex components in POME (Manickam et al., 2014).

Results also showed that highest COD removal efficiency was achieved at 30°C for 8, 16 and 24 h at 0.11 I:S. It is reported that high temperature will increase hydrogen yield and its enhancements were from the presence of thermophilic bacteria rather than mesophilic bacteria (Chong, Rahim, Shirai, & Hassan, 2009; Liu et al., 2009). Few studies have demonstrated that in a suitable range in fermentative hydrogen production, temperature can promote the ability of mixed cultures to utilize substrate with increasing temperature (Lee et al., 2006; Mu, Zheng, Yu, & Zhu, 2006; Zhang & Shen, 2006). However, Wang & Wan (2008) reported that increase in temperature from 20 - 40°C will increase the hydrogen production and substrate degradation efficiency. This is because the maximum biomass was observed at 35°C, thus the mixed cultures can produce hydrogen faster.

Figure 4.1 also showed that the higher the I:S ratio (<20 g L^{-1} COD), the lower the COD removal efficiency was. The highest COD removal efficiency of 49.50% reported being at 30°C for 8 h reaction time at the influent COD concentration of 32,320 mg L^{-1} with I:S of 10:90. A high amount of organic acids, carbohydrates, proteins and lipid in POME can make it a good source of substrate for the mixed cultures in the POME sludge. Therefore, temperature is very important in biohydrogen production as it can affect the HPB activity. HPB activity will influence the enzyme activity such as hydrogenase for fermentative hydrogen production (Wang & Wan, 2008).

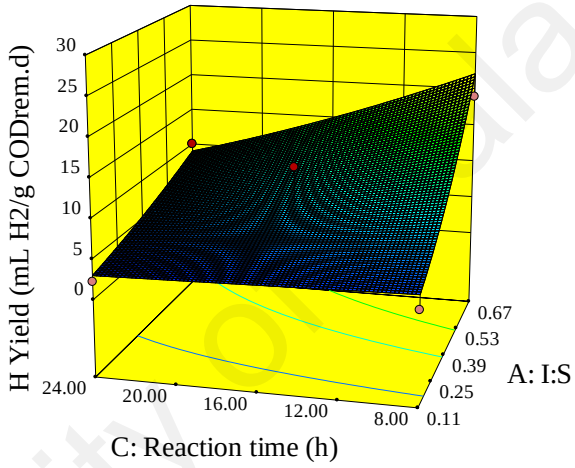
4.3.2 Hydrogen yield

From Table 4.3, as the model F value is equal to 14.24, hydrogen yield regression model is significant. The R-squared (R^2) value was reported at 0.84. After simplifying the quadratic model, the regression equation for the response is as follows:

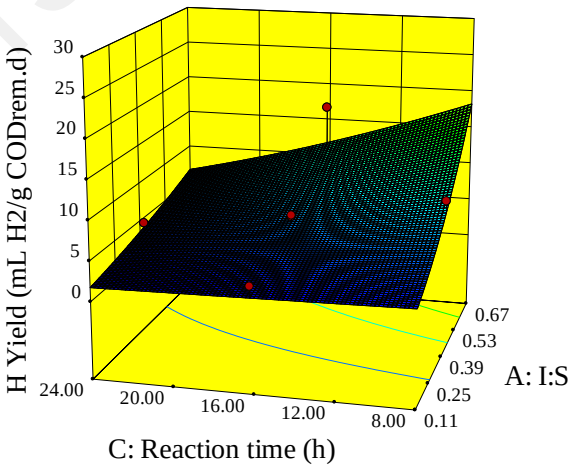
$$H_2 \text{ Yield} = 2.39 + 1.02A + 0.29B - 0.49C - 0.36AC + 0.72B^2 \quad \textbf{Equation 4.4}$$

The 3D response surface as shown in Figure 4.2 is based on Equation 4.3. The coded value for A, B and C represents I:S (A), temperature (B) and reaction time (C). From the response surface plots (Figure 4.2), the increment of hydrogen yield has been observed as the I:S decreased from 32,320 mg L^{-1} (10:90) to 16,720 mg L^{-1} (40:60) at the initial reaction time of 8 h. However, as the reaction time increased to 24 h, hydrogen yield decreased in all 30°C, 40°C and 50°C. These interactions implied that I:S and reaction time may significantly affect the hydrogen yield.

30°C



40°C



50°C

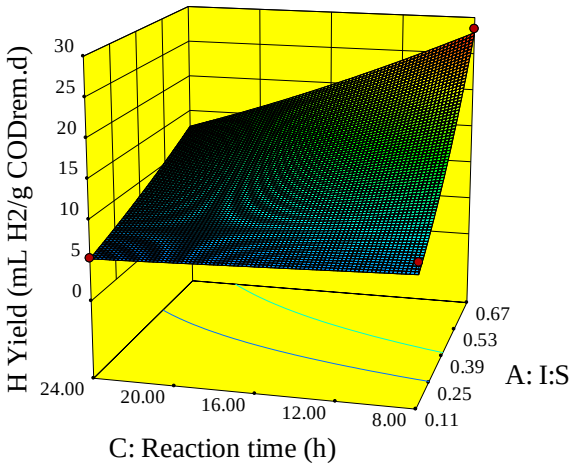


Figure 4.2: The 3D image of effects of I:S and reaction time (h) on hydrogen yield (mL H_2 g^{-1} COD removed d^{-1}) at a reaction temperature of 30°C, 40°C and 50°C.

This results agreed with the previous report that hydrogen yield increased with decreasing HRT (reaction time) (Shao, Peng, Teng, & Ju, 2008). Higher yield at 8 h might due to the activation of spore form bacteria in an existing appropriate condition. The population of hydrogen producing bacteria (HPB) was improved which could efficiently utilize the carbohydrates for H_2 production (Badiei et al., 2011). The reason why the yield decreased at higher reaction time is that probably by the generation of inhibitive by-products (i.e. volatile fatty acids (VFAs)). VFAs accumulation could prevent the fermentative bacteria in the sludge to effectively utilizing the POME (Stamatelatou, Vavilin, & Lyberatos, 2003).

Additionally, the designed test was monitored for a fermentation time of 24 h in order to understand the variation during the fermentation period in producing maximum hydrogen yield without shifting the process towards methanogenesis. Based on Figure 4.2, the maximum amount of hydrogen yield was 28.47 mL H_2 g^{-1} COD removed with I:S of 40:60 (initial COD concentration of 16,720 mg L^{-1}) at 8 h reaction time and 50°C

of reaction temperature (Table 4.2 and Figure 4.2). Subsequently, the yield was 15.84 mL H_2 g^{-1} COD removed at 50°C, 8 h with 20:80 I:S. In pre-settled POME, the concentration of organic acids might be high, thus a low hydrogen yield detected at a longer reaction time in the dark fermentation process. This might also inhibit the metabolism of specific HPB which then lower the responses. Despite of this, the HPB might favor the condition of high food-to-microbe (F/M) in a shorter reaction time. Lower reaction time have a positive effect on hydrogen yield since it has an influence on the competition of the bacteria (Ntaikou et al., 2009).

4.3.3 Optimization

A numerical optimization was chosen with the criteria of A (I:S), B (temperature) and C (reaction time) was set in the range, and the goal was to maximize hydrogen yield. Based on the desirability of 0.98, I:S ratio at 40:60 with 50°C and 8 h reaction time was the optimum condition to maximize hydrogen yield, with COD removal efficiency of 21.95%. The 3D plot of the optimization is shown in Figure 4.3.

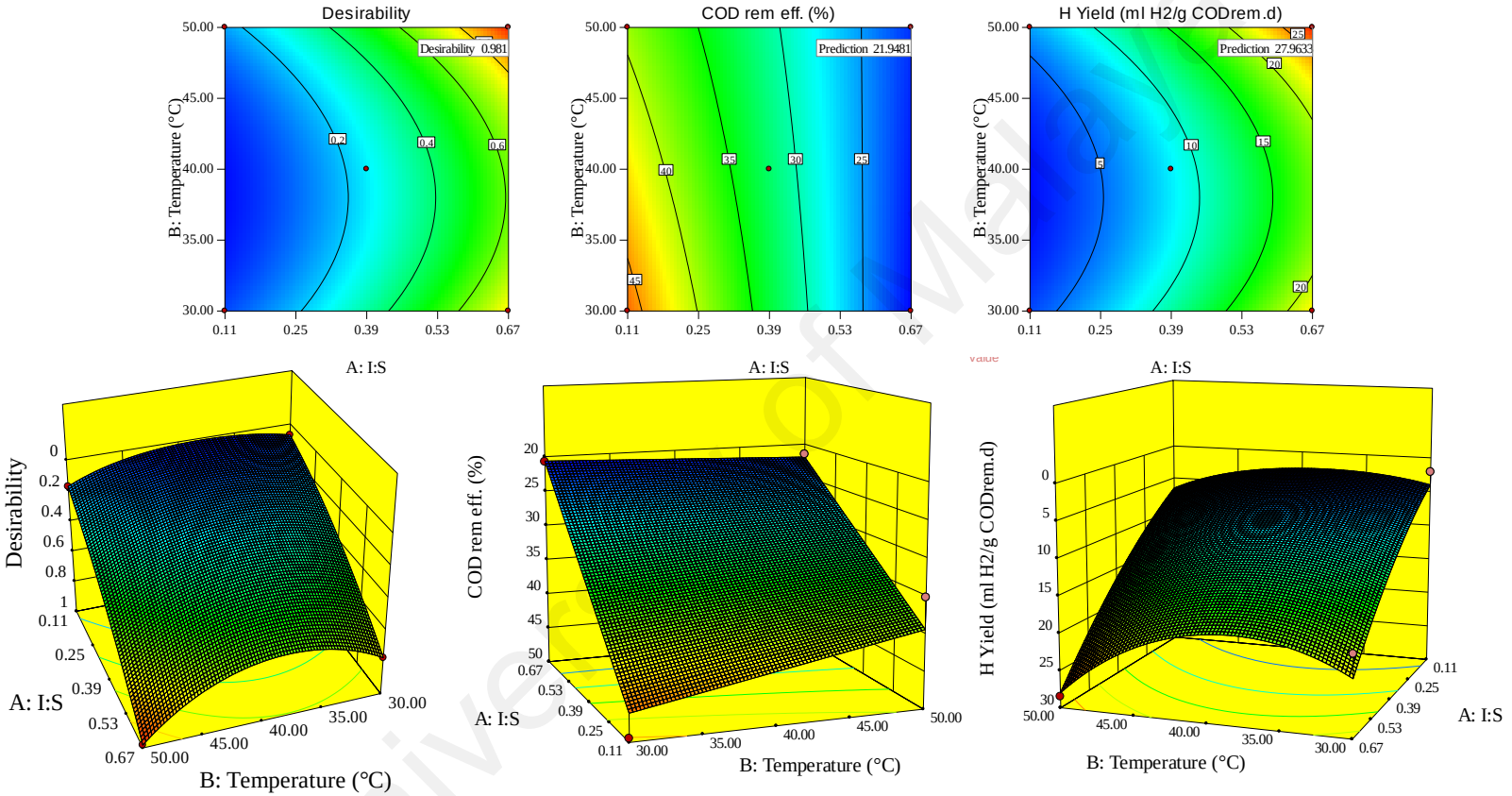


Figure 4.3: A counter-plot and the 3D surface of desirability, COD removal efficiency and hydrogen yield with a variation of I:S ratio and temperature. Reaction time held constant at 8 h.

Based on Figure 4.3, the highest desirability (0.98) was achieved when I:S ratio was 40:60 under thermophilic condition (50°C) with the lowest HRT of 8 h. The 3D surface showed the higher the I:S ratio, the lowest the desirability under 50°C. Meanwhile, the counterplot of I:S ratio versus temperature shows the highest COD removal efficiency (45.78%) can be achieved at I:S ratio of 10:90 under mesophilic condition (i.e. 30°C). The trend in its 3D plot can be distinguished by the highest the I:S ratio, the lowest the COD removal efficiency. In 3D surface response of hydrogen yield, at 50°C, the highest hydrogen yield (i.e. 3.69 mL H_2 g^{-1} COD rem.) reached I:S ratio of 40:60. The 3D plot showed that at 50°C, the maximum hydrogen yield shall be obtained when I:S ratio increased.

Lee et al., (2006) studied the effect of different temperatures (30 - 45°C) on biohydrogen production treating sucrose-based synthetic wastewater. They found that when temperature increase from 30°C to 40°C, hydrogen production rate and yield tend to increase. However, when temperature rose to 45°C, the rate and yield decreased. The study reveals that when temperature increased, the biomass content decreased. This is because at high temperature, the formation of granular sludge was inhibited.

Lin et al., (2008) reported the optimum temperature was 50°C for biohydrogen production using anaerobic mixed cultures for biohydrogen production. The average hydrogen content was achieved between 25-42% (v/v) with highest value was at 50°C. When conducting the thermodynamic analysis, they revealed that the biological reaction rates increase along with temperature until reach the growth limitation temperature. They also concluded that the difference in hydrogen production and yield at different

temperature might relate to the shifts in metabolic pathway or the microbial community presence.

4.4 Conclusions

Based on the experimental study, it can be concluded that POME sludge as inoculum and raw POME as a substrate are feasible for biohydrogen production. The main findings found in this study are:

i) Inoculum size and reaction time do have a significant effect on the hydrogen yield;

ii) Temperature and reaction time are not significant factors on the COD removal efficiency; however, it was found that inoculum size has a significant effect on it;

iii) The highest desirability (0.981) was achieved at a constant reaction time of 8 h. This is paralleled to a 21.95% of COD removal efficiency and highest hydrogen yield of 28.47 mL H_2 g^{-1} $COD_{removed}$ at 50°C with 40:60 I:S ratio.

Although inoculum size does not have a significant impact on hydrogen yield, however, substrate concentration that corresponds to F/M ratio affected hydrogen fermentation. At lower reaction time, the fermentative bacteria's activity to degrade complex organic components could be enhanced under thermophilic temperature.

To prove whether thermophilic temperature and short reaction time (HRT) are the optimal parameters for biohydrogen production treating POME, this study proceeds with Chapter 5 that will discuss on a scale-up of two-stage UASFF bioreactor treating POME. Several attempts have been made to increase the performance of the bioreactor such as using different temperatures, HRTs and source of substrates.

CHAPTER 5: UASFF START-UP FOR BIOHYDROGEN AND BIOMETHANE PRODUCTION FROM TREATMENT OF PALM OIL MILL EFFLUENT

5.1 Introduction

A continuous biohydrogen start-up through dark fermentation is a complex process. However, it is a key to the hydrogen production performance for a long-term successful operation. Biologically produced hydrogen is an emerging way forward, especially when operated under dark fermentation. This is because dark fermentation has several advantages such as the usability of wide range of feedstocks (e.g. agricultural residues, derivatives of biomass and waste streams) (Cheng et al., 2011; Guo, Trably, Latrille, Carrre, & Steyer, 2010b; Lin et al., 2012), and it also can be integrated with other process. For example, anaerobic digestion, photo-fermentation or membrane-based processes. The purpose of integrating processes is to increase or upgrade the biohydrogen or methane in order to have an energy efficient in a viable feedstock (Bakonyi, Nemestóthy, Simon, Béla, & Bélafi-Bakó, 2014).

To make biohydrogen and methane generation more attractive, several practical aspects need to be considered to increase hydrogen/methane yields and rates. They are the bioreactor configurations and their operations (Hallenbeck & Ghosh, 2009). Due to higher expectable efficiencies especially in the process, biohydrogen fermentation should be conducted in a continuous mode, rather than batch, regardless of the type of fermenter used (Jianlong Wang & Wan, 2009).

Single-stage anaerobic digester is prone to upset due to high production of volatile fatty acids (VFAs), thus lowering the pH. This cause the inhibition of methanogenesis

process and lead to process failure. Therefore, to overcome the operational problem, alternatively, a two-stage anaerobic processes have been developed (Pohland & Ghosh, 1971). Hydrolysis/acidogenesis and methanogenesis are spatially separated in two individual reactors in a two-stage anaerobic process. The acidogenic reactor is initially introduced to enables high acidogenesis rate to take place. This can reduce the total reactor volume when using a two-stage reactor, compared to single-stage. Meanwhile, two-stage anaerobic process has demonstrated to effectively treating complex wastewater from municipal and industrial with a proper enrichment of microbial communities (Fongastitkul, Mavinic, & Lo, 1994; Ghosh, Jerger, Henry, & Sajjad, 2001; Krishnan, Singh, Sakinah, Thakur, Wahid, & Alkasrawi, 2016; C. Mamimin, Singkhala, & Kongjan, 2015).

In establishing a proper community structure in the two-stage anaerobic treatment process or any biological treatments, a start-up is an important step. It has been reported that a long period of acclimation (Wu, Liu, Tseng, & Cheng, 2001) and ineffective in organic matter removal (Griffin, McMahon, Mackie, & Raskin, 1998) had occurred due to the poor start-up. To overcome the problem, one of the factors that need to consider is the operational of the two-stage bioreactor. Other factors that may contribute to the poor start-up are the process parameters used such as substrates, hydraulic retention time (HRT), temperature, anaerobic microorganisms, organic loading rate (OLR) and pH (Boodhun, Mudhoo, Kumar, Kim, & Lin, 2017).

The effects of process parameters on the generation of biohydrogen treating palm oil mill effluent (POME) in a batch study has been studied as presented in Chapter 4. To increase the production of hydrogen and additionally, methane, while increasing POME treatment efficiency, a start-up study using a scale-up of two-stage up-flow anaerobic

sludge fixed-film (UASFF) bioreactor has been proposed. Again, several process parameters such as temperature, pH and substrate were considered to increase the production rate of biohydrogen and methane.

A study by O-thong et al., (2016) on a two-stage fermentation of POME under thermophilic and mesophilic conditions found that the maximum hydrogen production was 42% higher with recirculation of methanogenic effluent in the hydrogen reactor, compared to none recirculation. In the second stage, methane production was also reported to be 14% higher in the same conditions. Biohythane gas consists of 54.4% CH_4, 13.3% H_2 and 32.2% CO_2 in the hydrogen reactor and was found to be a beneficial energy recovery from POME. Mamimin et al., (2015) reported that under thermophilic and mesophilic conditions, using POME under HRT of 2 days in hydrogen reactor and 15 days for methane reactor, they observed a 34% higher of energy yield using a continuous two-stage fermentation. The hydrogen and methane yield was reported at 210 L H_2 kg COD^{-1} and 315 L CH_4 kg COD^{-1}, respectively, with 4.4 L biogas L^{-1} (51% CH_4, 14% H_2 and 35% CO_2). Krishnan et al., (2016) in their study using POME in a two-stage thermophilic and mesophilic fermentation, 2 days HRT of Up-flow Anaerobic Sludge Blanket (UASB) reactor as a first stage and 5 days HRT of Continuous Stirred-Tank Reactor (CSTR) as a second stage, proved that a stable production of both hydrogen and methane was obtained in UASB reactor and CSTR reactor, respectively, after operation for 120 days, with highest production rate of 1.92 L H_2 d^{-1} and 3.2 L CH_4 d^{-1} (in 1 L POME). They also reported 94% total COD removal efficiency using both reactors. Additionally, a 35% H_2 with 2.1 L H_2 d^{-1} was achieved in UASB-H_2 reactor and 65% CH_4, 13 L d^{-1} CH_4 was obtained in CSTR-CH_4 reactor, with total COD removal efficiency of 85% by using POME under thermophilic conditions, as reported in another study (Krishnan, Singh, Sakinah, Thakur, Wahid, & Sohaili, 2016).

These studies proved that POME is a good and suitable substrate for biohydrogen and biomethane production in terms of high biogas production rate and COD removal efficiency. However, the production of biohydrogen and biomethane varies by changes in substrate characteristics, environmental and operating conditions.

Najafpour et al., (2006) and Zinatizadeh et al., (2006) used a single stage UASFF reactor to treat POME with HRT of 1.5 days. They concluded that UASFF reactor is a good system and could help in treating POME. However, there were also studies found using POME in a two-stage process with two different types of reactors (Mamimin et al., 2015; O-thong et al., 2016) and two different reaction temperatures (Krishnan et al., 2016). These studies showed that the two-stage process could obtain 34% greater energy yield as compared to a single stage process for methane production. They also reported that the energy in a two-stage process could be efficiently recovered for biohythane (biohydrogen and biomethane) production. According to Li & Yu (2011), substrate degradation efficiency could also be increased. Additionally, using two-stage system can enhance methane production rate from the rise of methanogens specific activity, increase total COD removal, and applying higher organic loading rate (OLR) would also increase process stability (Liu et al., 2013).

Although studies on treating high concentration of POME using the two-stage system are extensive, a study on biohydrogen and biomethane production using two-stage UASFF reactor from POME are rarely found. Thus, the present study which uses an exploratory approach was done to observe the behaviour and performance of POME in a two-stage UASFF reactor.

5.2 Materials and methods

5.2.1 Inoculum preparation

POME sludge was collected from an anaerobic pond in a palm oil mill in Banting, Selangor, Malaysia (i.e. digested POME). The sludge was acclimatized in a single stage UASFF bioreactor with 10 g L^{-1} glucose solution being fed as a substrate, to produce biogas until it stabilizes at a pH of between 7-8, methane percentage in the biogas was consistently at 70% or more, and final effluent COD removal of 76.34%. The portion of the acclimatized sludge to be used as inoculum in the H_2-UASFF system for biohydrogen production was heat-treated at 90°C for an hour, after being diluted with water to have MLSS and MLVSS of 10 g L^{-1} and 6 g L^{-1}, respectively. The remaining sludge from the single stage UASFF was used in the CH_4-UASFF unit without heat treatment but after being diluted for MLSS and MLVSS of 12.5 g L^{-1} and 8.75 g L^{-1}, respectively.

5.2.2 Feedstock preparation

There were two sources of raw POME used in this study. Initially, raw POME was collected from a palm oil mill located in Banting. After 40 days of start-up and the percentage of hydrogen continued to remain at a significantly low level, the second source of raw POME from a mill in Dengkil, Selangor was used. This was done based on a literature that reported good results using samples from the Dengkil mill (Yusoff, Hassan, Abd-Aziz, & Rahman, 2009). In both cases, the POME was taken from the acidification pond which is the first treatment pond (before the anaerobic pond). The characteristics of the inoculum and substrate are shown in Table 5.1 below. Samples were kept in a 4°C cold room prior to its use. Suspended constituents of the raw POME were allowed to settle before the raw POME was used for the next step. Only the top liquid part of the pre-settled raw POME was used in the feedstock.

The feedstock comprises a mixture of molasses and the raw POME at ratios ranging from 0% raw POME:100% molasses to 100% raw POME:0% molasses (v/v), at increments of 10% raw POME. Another parameter that was controlled in the feedstock was the organic loading rate (OLR), measured in units of g COD L^{-1} d^{-1}. Molasses and raw POME used in the feed were diluted separately with tap water to achieve the desired OLR before the two are mixed in the given v/v ratio.

Table 5.1: Initial characteristics of the inoculum and substrates used.

Parameter	Unit	POME sludge (Inoculum)[a]	Raw POME (substrate)[a]	Raw POME (substrate)[b]
Total Suspended Solid (TSS)	g TSS L^{-1}	50	n.a	n.a
Volatile Suspended Solid (VSS)	g VSS L^{-1}	27.25	n.a	n.a
Total COD	g COD L^{-1}	50.00	50.00	38.00
Particulate COD	g COD L^{-1}	39.00	28.00	10.00
Soluble COD	g COD L^{-1}	11.00	22.00	28.00
pH	-	7.42	4.90	5.01

[a]Palm Oil Mill, Banting; [b]Palm Oil Mill, Dengkil. (n.a = not applicable)

5.2.3 Reactor set-up and operation

The two-stage UASFF bioreactor was set up with the capacity of 2.5 L for the first unit (H_2-UASFF reactor) and 3.5 L for the second unit (CH_4-UASFF reactor). Both units consisted of a closed feed tank, main reactor, settling tank (to recycle the effluent and biomass washout) and effluent tank. The H_2-UASFF reactor was started using 20 g L^{-1} molasses as a feed (100% molasses) before raw POME (20 g L^{-1}) was slowly being added, at 10% increment until the whole feed was 100% POME. The operation of the CH_4-UASFF reactor was initiated using 20 g L^{-1} of molasses before using the effluent from the H_2-UASFF unit in the next day. The initial conditions of both reactors are shown

in Table 5.2. During the start-up operation, all pumps and silicone tubing were inspected to be in a good condition and without clogging. This was due to the fact that the continuous flow of the substrate may clog the silicone tubing due to the high amount of suspended solids in raw POME. The circulating water bath was continuously maintained at mesophilic or thermophilic condition depending on the type of digestion process. A gas flow meter was connected to H_2-UASFF and CH_4-UASFF reactor to record the gas volume. All silicone tubing and pumps used in this experiment were routinely replaced if necessary.

Table 5.2: Initial reactor conditions and parameters applied during start-up of H_2-UASFF and CH_4-UASFF reactors.

		H_2-UASFF (hydrolysis and acidogenesis)	**CH_4-UASFF (methanogenesis)**
I. Reactor conditions	**Unit**	**Value**	**Value**
Bicarbonate Alkalinity (BA)	mg L^{-1}	3000*	3000*
Initial Temperature (T)	°C	37	37
Inoculum Heat Treatment	°C/h	90/1	n.a
pH substrate	n.a	5-5.2	7-8
Working Volume	L	1.8	3.2
Initial HRT	d	1	1
II. Parameters			
MLSS	g L^{-1}	10	12.50
MLVSS	g L^{-1}	6	8.75
V_{up}	m h^{-1}	1	1
T	°C	37 - 43	37 - 43
V_R	L	2.5	3.5
Q_F	L d^{-1}	2.51	2.70
Q_R	L d^{-1}	54.49	76.93
HRT	h	24 - 4	24
$Balk_{in}$	mg $CaCO_3$ L^{-1}	3000	3000
F/M	d^{-1}	0.70	varies
pH_{in}	n.a	5-5.2	7-8
OLR	g $L^{-1}d^{-1}$	20	varies depends on H_2-UASFF effluent concentration

n.a = not applicable; * stop adding when reactor pH is stable.

5.2.3.1 Reactor size

H_2-UASFF reactor was used as the first stage of the system. This is where hydrolysis and acidogenesis took place. The effluent from the settling tank in the H_2-UASFF unit, that may contain organic acids, was continuously fed into the second unit, that is CH_4-UASFF reactor, at which the anaerobic process was allowed to progress to the methanogenesis step. Both reactors had 1.8 L and 3.2 L working volumes, internal diameters of 5.50 cm and 6.50 cm, and a height of 80 cm and 120 cm, respectively. The difference in size for H_2-UASFF and CH_4-UASFF units is because of their reaction rates. In order to operate both reactors in continuous mode with approximately the same feed flow rate, so H_2-UASFF must be smaller than CH_4-UASFF as the HRT of H_2-UASFF unit is almost half of HRT in CH_4-UASFF unit.

Table 5.2 presents all the parameters considered during start-up of UASFF system using a co-mixture of molasses and raw POME as a substrate. The substrate for the CH_4-UASFF reactor was the effluent from H_2-UASFF system. The schematic diagram of the two-stage UASFF bioreactor is presented in Figure 5.1.

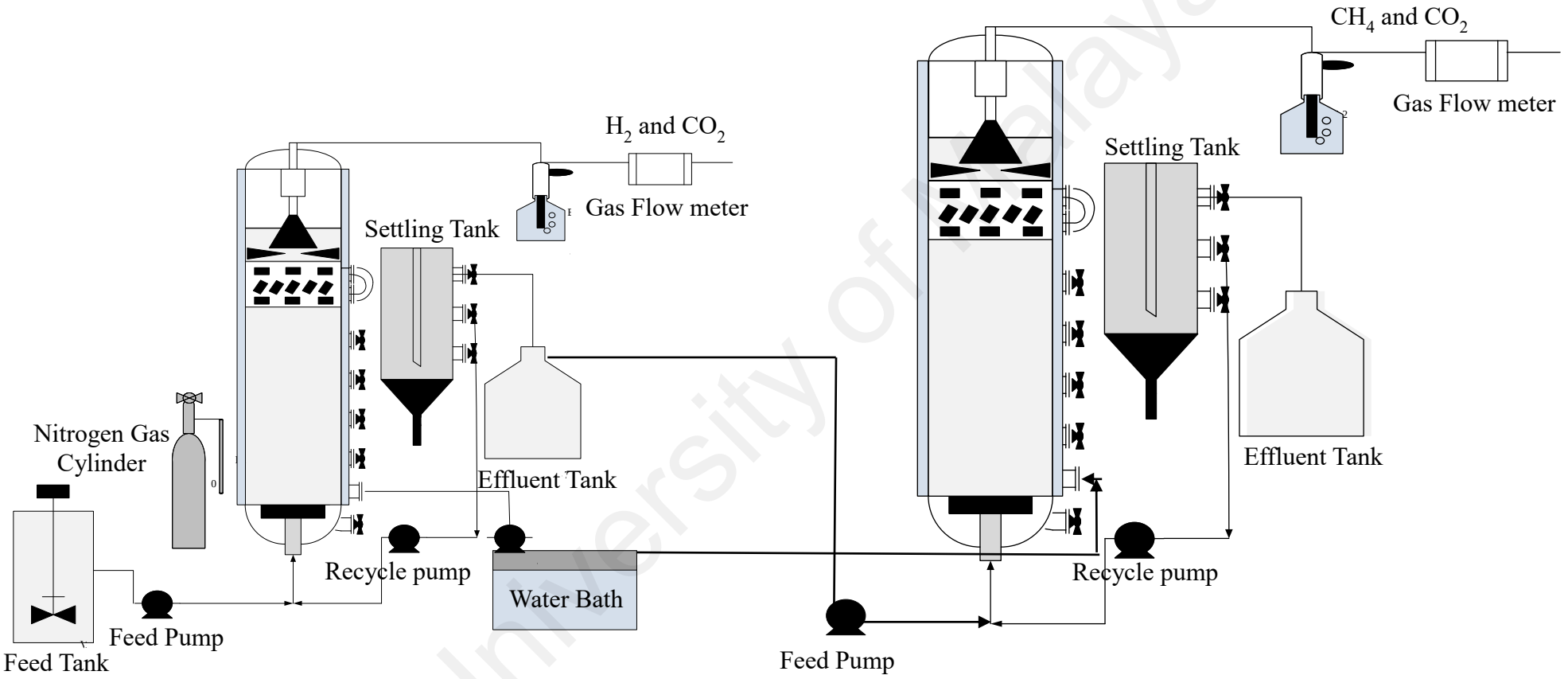


Figure 5.1: Two-stage UASFF bioreactor. A 2.5 L H_2-UASFF system that produced hydrogen and carbon dioxide (left) and 3.5 L CH_4-UASFF system that produced methane and carbon dioxide (right).

5.2.3.2 H_2-UASFF operation (Stage I)

The feed, whether it was 100% molasses, a mixture of molasses and POME at various percentages, or 100% raw POME was diluted with tap water to 20 g L^{-1} COD as feed to the H_2-UASFF reactor. Started with 100% molasses on day 1, 10% (v/v) raw POME was introduced into the diluted molasses on day 2, and the POME addition process continued with raw POME percentage increased 10% each time until the feed substrate consisted of 100% raw POME on the final day (day 59) of start-up monitoring. The feed concentration of raw POME must be added stage by stage for the start-up of the H_2-UASFF system, to prevent shock for hydrogen-producing bacteria (HPB), which may cause a reduction in biohydrogen production rate. Additionally, bicarbonate alkalinity was also added as a buffer until a stable pH was reached within the reactor. Nitrogen (N_2) gas was purged into the closed feed tank for 10-15 mins at the rate of 10 mL min^{-1}. The same procedure was repeated each time the feed was prepared.

5.2.3.3 CH_4-UASFF Operation (Stage II)

The inoculum used in this reactor was the portion of not heat-treated sludge, from the inoculum preparation described in subsection 5.2.1. The initial biomass concentration was 8.75 g MLVSS L^{-1}, and the CH_4-UASFF system was continuously fed with the effluent from H_2-UASFF effluent tank unit. The effluent was first added with 3 g L^{-1} sodium bicarbonate before being purged with nitrogen gas for 10-15 mins. The addition of sodium bicarbonate was stopped once the pH in the CH_4-UASFF system was stable. The effluent pH was then adjusted to pH 7-8 by slowly adding 6N NaOH. This is because the effluent pH was acidic (4.9-5.3) due to the presence of volatile fatty acids (VFAs). The feed tank was fully sealed with parafilm after being purged with N_2 to maintain the anaerobic condition.

5.2.4 Analytical Method

Total suspended solids (TSS), volatile suspended solids (VSS), and chemical oxygen demand (COD) tests were conducted before and after the experiment and in accordance to APHA Standard Methods 2540 D, 2540 G, and 5220 D, respectively (APHA, 1999). 6N HCl and 6N NaOH used for pH adjustment were initially prepared based on their molecular weight and used whenever necessary.

The biogas volume was measured using a gas flow meter. The biogas percentage was periodically measured by first collecting it with gas-tight syringe (with 0.1 mL injection volume from the gas port) and then analyzed with a gas chromatograph (Perkin Elmer, AutoSystem Gas Chromatograph (GC), 600 Series LINK) equipped with a thermal conductivity detector (TCD) and pack GC column Supelco, with 40/80 carboxen 1000, MR2924D, 10 ft x 1/8 in. Argon was used as a carrier gas at a flow rate of 30 mL min^{-1}. The temperatures of the oven, injector and detector were 100°C, 150°C and 200°C respectively. The amount of gas used for GC injection was 1.0 mL using gas syringe 2500 µL Hamilton, USA.

5.2.5 Parameters Calculations

Based on the value of the initial COD of substrate, the feed and recycle flow rate (Q_F and Q_R) and MLVSS of inoculum, other important parameters can be calculated, as shown in the equations below (Najafpour et al., 2006; Zinatizadeh, Mohamed, Mashitah, Abdullah, & Isa, 2007):

5.2.5.1 During start-up

Organic Loading Rate (OLR) (mg L^{-1} d^{-1})= S_0/HRT

Equation 5.1

Hydraulic Retention Time (HRT)(d) = V_R/Q_F

Equation 5.2

Feed rate of COD to VSS (F/M) (d^{-1})= $(Q_F.S_0)/(V_R.X)$

Equation 5.3

Up-flow Velocity (V_{up})(m h^{-1})= $(Q_F+ Q_R)/A$

Equation 5.4

where:

S_0 = initial substrate concentration [CODin (mg L^{-1})];

V_R = reactor volume (L);

Q_F = feed flow rate (L d^{-1});

Q_R = recycle flow rate (L d^{-1});

A = area (cm^2);

X = biomass concentration (mg MLVSS L^{-1}).

5.2.5.2 After treatment process

COD removal efficiency (%) = [(COD_{in} (mg L^{-1}) - COD_{eff} (mg L^{-1}))/COD_{in} (mg L^{-1})] x 100

Equation 5.5

Gas Production Rate, Q_{H2} or Q_{CH4} (L d^{-1}) = Q_{biogas} x H_2 or CH_4 percentage (%)

Equation 5.6

Gas Yield (Y_{H2} or Y_{CH4})(L gas $g^{-1}COD_{rem}$.) = (Rate of H_2 or CH_4 gas produced)/(Rate of COD removed)

Equation 5.7

Total COD removal efficiency (%) = [(COD_{in} (mg L^{-1}) in H_2 UASFF – COD_{eff} (mg L^{-1}) in CH_4 UASFF)/(COD_{in} (mg L^{-1}) in H_2 UASFF)] x 100

Equation 5.8

5.3 Results and discussion

5.3.1 Stage I (H_2-UASFF)

Stage I of H_2-UASFF was divided into three phases where Phase I was the initial phase of the start-up, followed by an adjustment phase in Phases II and III.

5.3.1.1 Phase I: 0-50% POME (Day 1-11)

Based on Figure 5.2, the percentage of biohydrogen decreased after adding 10% of raw POME, but the presence of molasses in the feed continued to produce hydrogen in the system. After approximately 10 days with 50% of raw POME in the feed, no hydrogen was detected such that the biogas only contained methane and carbon dioxide. Methane gas was detected at 13.01% before slowly increased up to 53.98% after adding 60% of raw POME. The observation shows that methanogenic bacteria were present in the H_2-UASFF reactor (O-thong et al., 2016), indicates that methanogenic bacteria already adapted from the beginning.

A study done by Sivagurunathan et al., (2017), showed that a repeated inoculum heat treatment could increase hydrogen production performance by 37%, as well as increase the population of the hydrogen-producing bacteria. Another study revealed that changes in temperature from 37°C to 47°C during the reactor performance showed an increase in hydrogen production rate of 13.58 L H_2 L^{-1} substrate d^{-1} (Sivagurunathan, Sen, & Lin, 2014). Based on these reports, the start-up in this work was continued with 60:40 (raw POME: Molasses) by using different approaches in the attempt to increase hydrogen production and minimize methane gas content in the biogas such as increase substrate

concentration intermittently, using different temperatures, control pH and change source of substrate used.

On day 6, pH measurement was ranging from 5.5 to 6.2 within the same day and the hydrogen percentage had dropped to almost 60%. The inoculum in the reactor was then taken out from the bottom of the reactor column and heat-treated again at 90°C for an hour to further suppress the methanogenic bacteria. While the inoculum was taken out, the substrate was left to remain in the reactor column, and when the operation was resumed with the pretreated inoculum, the substrate POME to molasses ratio used remained. After this step, pH in the H_2-UASFF reactor was adjusted to 5.5 by adding 6N HCl to promotes HPB, as this is the optimum pH for hydrogen production. On Day 7, despite the inoculum heat-treatment the hydrogen percentage continued to drop to 13.30%. The water bath was adjusted to 53.5°C in order to create a thermophilic condition (Alitalo, Niskanen, & Aura, 2015). This condition is needed for increasing the hydrogen percentage. However, the hydrogen percentage showed a further decrease from Day 7 to Day 11. The inoculum was taken out again for another heat-treatment to be repeated at the same condition (90°C for one hour).

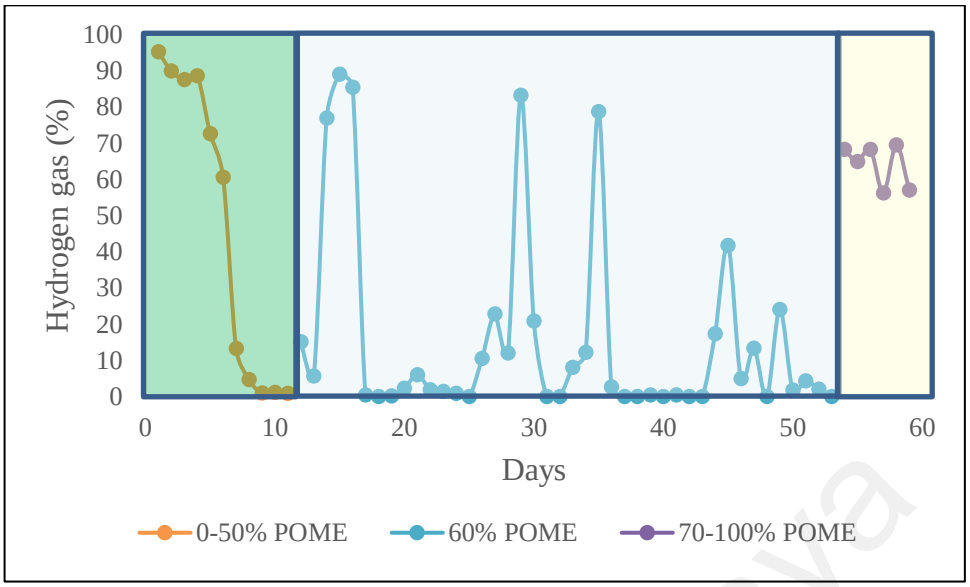


Figure 5.2: Three phases were divided during the start-up of UASFF system based on hydrogen percentage in Stage 1 (hydrolysis process).

5.3.1.2 Phase II: 60% POME (Day 12-53)

On day 12, the hydrogen percentage showed an increment to 15.16%. This is attributed to the inoculum heat re-treatment to 90°C conducted on the previous day. But during heat treatment, some of the methanogenic and lactic acid bacteria or their spores may still be able to tolerate the high temperature (Kim, Kim, Ko, Lee, & Shin, 2008). This could explain the decrease of hydrogen to 5.69% on day 13. This shows that propionic and lactic acid bacteria that are normally contained in mixed cultures will not be completely suppressed by the heat-treatment method. On another perspective, Sivagurunathan et al., (2014) suggested that intermittent temperature shift might be a good strategy to eliminate those bacteria and increase the hydrogen production rate. This strategy was successfully applied by Sivagurunathan et al., (2017) using mixed cultures where the hydrogen production increased by a decrease in propionic acid bacteria on the

33rd day of their UASBR operation. Table 5.3 summarizes the conditions applied in Phase I, Phase II and Phase III of this work.

Table 5.3: Conditions applied during a start-up period in H_2-UASFF.

Phase	Day	Conditions	Source of Substrate Used
I	1-7	Adjusted pH to 5.5, 24 h HRT, 37-53.5°C	Jugra Palm Oil Mill, Banting
	8-12	Inoculum heat-treated @ 90°C, 1 h, 24 h HRT, 53.5°C	Jugra Palm Oil Mill, Banting
II	13-16	12 h HRT, 53.5°C	40 g L^{-1} molasses
	17 -18	12 h HRT, 53.5°C	Jugra Palm Oil Mill, Banting
	19-21	substrate heat-treated @ 90°C for 1 h, 12 h HRT, 53.5°C	Jugra Palm Oil Mill, Banting
	22-25	Non-heat-treated substrate, 8 h HRT, 45°C	Jugra Palm Oil Mill, Banting
	26-27	OLR of 80 g COD L^{-1} d^{-1}, 6 h HRT, 55°C	Jugra Palm Oil Mill, Banting
	28	Non-heat-treated substrate, 6 h HRT, 55°C	Jugra Palm Oil Mill, Banting
	29-30	OLR 40 g COD L^{-1} d^{-1}, 6 h HRT, 55°C	Jugra Palm Oil Mill, Banting
	31-34	Non-heat-treated substrate 10 g $L^{-1,}$ 6 h HRT, 55°C	Jugra Palm Oil Mill, Banting
	35	10 h HRT, 55°C	Jugra Palm Oil Mill, Banting
	36-38	10 g L^{-1} substrate, 6 h HRT, 50°C	Jugra Palm Oil Mill, Banting
	39-42	6 h HRT, 55°C	Jugra Palm Oil Mill, Banting
	43	Sample analysis and characterization	Sri Ulu Langat Palm Oil Mill, Dengkil
	44-47	Using fresh raw POME, pH 5.5, 6 h HRT, 55°C	Sri Ulu Langat Palm Oil Mill, Dengkil

	48-49	6 h HRT, 43°C	Sri Ulu Langat Palm Oil Mill, Dengkil
	50-52	6 h HRT, 43°C	Sri Ulu Langat Palm Oil Mill, Dengkil
	53	4 h HRT, 43°C	Sri Ulu Langat Palm Oil Mill, Dengkil
III	54-59	pH 5, 4 h HRT, 43°C	Sri Ulu Langat Palm Oil Mill, Dengkil

In addressing the decreased hydrogen percentage on day 13, intermittent high COD concentration of molasses (40 g L^{-1}) was introduced into the system. In addition, another parameter, i.e. the HRT, was adjusted from 24 h to 12 h. These steps were taken based on the understanding that hydrogen yield could be increased by changing the organic loading rate (OLR = feed concentration/hydraulic retention time) (Khemkhao et al., 2015; Luo et al., 2010). Lin, Lee, Tseng, & Shiao, (2006) reported a 32% increase in hydrogen yield when sucrose concentration was increased from 5 g COD L^{-1} to 40 g COD L^{-1} at 12 h HRT. On Day 14 of the present work, the hydrogen percentage was significantly increased from 5.69% to 76.96%. Additionally, the increment was observed to be 13 times higher when OLR was boosted from 20 g COD L^{-1} d^{-1} to 80 g COD L^{-1} d^{-1}. However, the hydrogen content was not sustained, and a sudden drop occurred from day 16 to day 17 when 60% raw POME was applied in the H_2-UASFF reactor, with the presence of methane gas in the reactor. The next step was to heat-treat the substrate, which was raw POME, at 80°C for an hour using a water bath on day 19 as a measure to kill methanogenic bacteria that might present in the substrate. The temperature was chosen based on the characteristics of raw POME from acidification pond that have a temperature between 80°C - 90°C.

On day 22, using 60% untreated raw POME, at 45°C, HRT was reduced to 8 h. MLSS and MLVSS in the H_2-UASFF reactor were 11.9 g L^{-1} and 8.85 g L^{-1}, respectively. On

day 26, using OLR of 80 g COD L^{-1} d^{-1}, HRT was reduced to 6 h to ascertain the effects of HRT on hydrogen percentage. Hydrogen percentage at first slightly increased to 10.55% and then it further increased to 22.85% on the following day. But on day 28, hydrogen percentage decreased back to 12.09% when 60% of untreated raw POME was reapplied into the reactor.

On days 29-30, the OLR was reduced to 40 g COD L^{-1} d^{-1} and temperature in the reactor was adjusted to 55°C. From Figure 5.2, the hydrogen percentage was 83.27% on day 29 before it decreased to 20.92% on day 30.

The conditions continued until day 32 where only 10 g COD L^{-1} of raw POME was mixed with 20 g COD L^{-1} of molasses in the reactor. The next day showed an increase in hydrogen percentage to 8.10% and 12.22%, which was day 34. On day 35, in order to study the optimum HRT for biohydrogen production, the HRT was increased from 6 h to 10 h. Here, the hydrogen percentage was increased to 78.76%. On day 36, when HRT and temperature were reduced back to 6 h and 50°C respectively, the hydrogen percentage significantly dropped to 2.65%. Effects of temperature were then tested by increasing it to 55°C on day 39 using the same HRT of 6 h. This condition lasted for four days and no significant increase in hydrogen percentage was recorded. Thanwised, Wirojanagud, & Reungsang, (2012) in their study with tapioca wastewater revealed that a shorter HRT (6 h, 32.3 ± 1.5°C) could increase the production of hydrogen to 883.19 ± 7.89 H_2 L^{-1} d^{-1} from 164.45 ± 4.14 H_2 L^{-1} d^{-1} (24 h HRT). However, at 3 h HRT, their hydrogen production rate (HPR) was decreased to 748.54 ± 13.84 mL H_2 L^{-1} d^{-1}. Kumar et al., (2016) reported a maximum hydrogen production rate of 25.9 L H_2 L^{-1} d^{-1} at a low HRT of 3 h, 35°C when feeding galactose with an OLR of 120 g L^{-1} d^{-1}.

The inability to sustain a high hydrogen percentage in the biogas from the H_2-UASFF reactor despite the different steps taken, including lowering the HRT and heat treating the substrate as described above, gave strong indications that the POME source might be a factor to be considered. As a final approach to improving the start-up of the H_2-UASFF, the source of raw POME was changed from the mill in Banting to the one in Dengkil, Selangor. The characteristics were measured based on Table 5.1. The differences in the characteristics of substrates used may attributed to the culture conditions. The culture conditions in each substrate completely dependent on the physical conditions and the ability to adapt to the conditions during cultures. By using 6 h HRT at 55°C, 60% of fresh raw POME (40% molasses) was fed into the reactor. pH in the reactor was recorded at 5.8 when the hydrogen percentage was 17.41% on day 44. pH in the reactor was then maintained at 5.5 on the next day and the hydrogen percentage was significantly increased to 41.82%. However, the hydrogen percentage fluctuated until day 53 when HRT and temperature were reduced to 4 h and 43°C, respectively.

Studies on the effects of temperature and HRT on biohydrogen production have been done by others (Chu et al., 2008; Dareioti & Kornaros, 2014; Irvan, Trisakti, Wongistani, & Tomiuchi, 2012; Karlsson, Vallin, & Ejlertsson, 2008; Trisakti, Manalu, Taslim, & Turmuzi, 2015). Karlsson et al., (2008) reported a temperature of 55°C being the most suitable condition for hydrogen production, supported by Chu et al., (2008) and Irvan et al., (2012). However, they reported a different HRT on the optimum hydrogen production studies (0.75 - 8 days). These references showed that under a thermophilic condition, the production of hydrogen could be enhanced under optimum HRT. Therefore, in this work Phase III is the final phase at which the hydrogen production under a thermophilic condition with a short HRT (43°C, 4 h) will be further investigated.

5.3.1.3 Phase III: 70-100% POME (54-59 days)

On day 54, with pH 5 in the reactor, hydrogen percentage was significantly increased to 68.29%. After more than three cycles in maintaining these conditions using 4 h HRT and 43°C (considered thermophilic), the raw POME portion in the feed substrate was gradually increased from 70% to 100% and the hydrogen percentage was observed to consistently fluctuate between the values of about 55% to 70%. The system is considered to have stabilized, which marked the end of the start-up experiment. In a continuous study using two-stage process, it shows that a thermophilic condition (55°C) could produce almost similar hydrogen yield and methane yield with another study by using digested sludge (Chu et al., 2008; Liu et al., 2006).

(a) ***Hydrogen Production Rate (HPR)***

HPR was calculated based on Equation 5.6. Figure 5.3 shows that in Phase I the maximum HPR of 6.86 L H_2 d^{-1} occurred with 100% molasses at HRT=1 day and T=37°C, but HPR showed a decreasing trend as raw POME percentage in the feedstock was increased to 50%. This was probably due to the presence of methanogenic bacteria in the mixed cultures, that caused the reduction in hydrogen production (O-thong et al., 2016).

After 60% of raw POME was used, the HPR started to increase and reached a maximum rate of 6.51 L H_2 d^{-1} by using 90% raw POME. There is no significant difference in the hydrogen production rate using 100% molasses and 90% raw POME. This indicated that POME is rich in BOD, COD, proteins, lipids and minerals (Krishnan et al., 2016) and can be a good source of substrate which is comparable to molasses. In general, it is a recommended approach to achieve both waste stabilization and energy recovery through the production of hydrogen using raw POME.

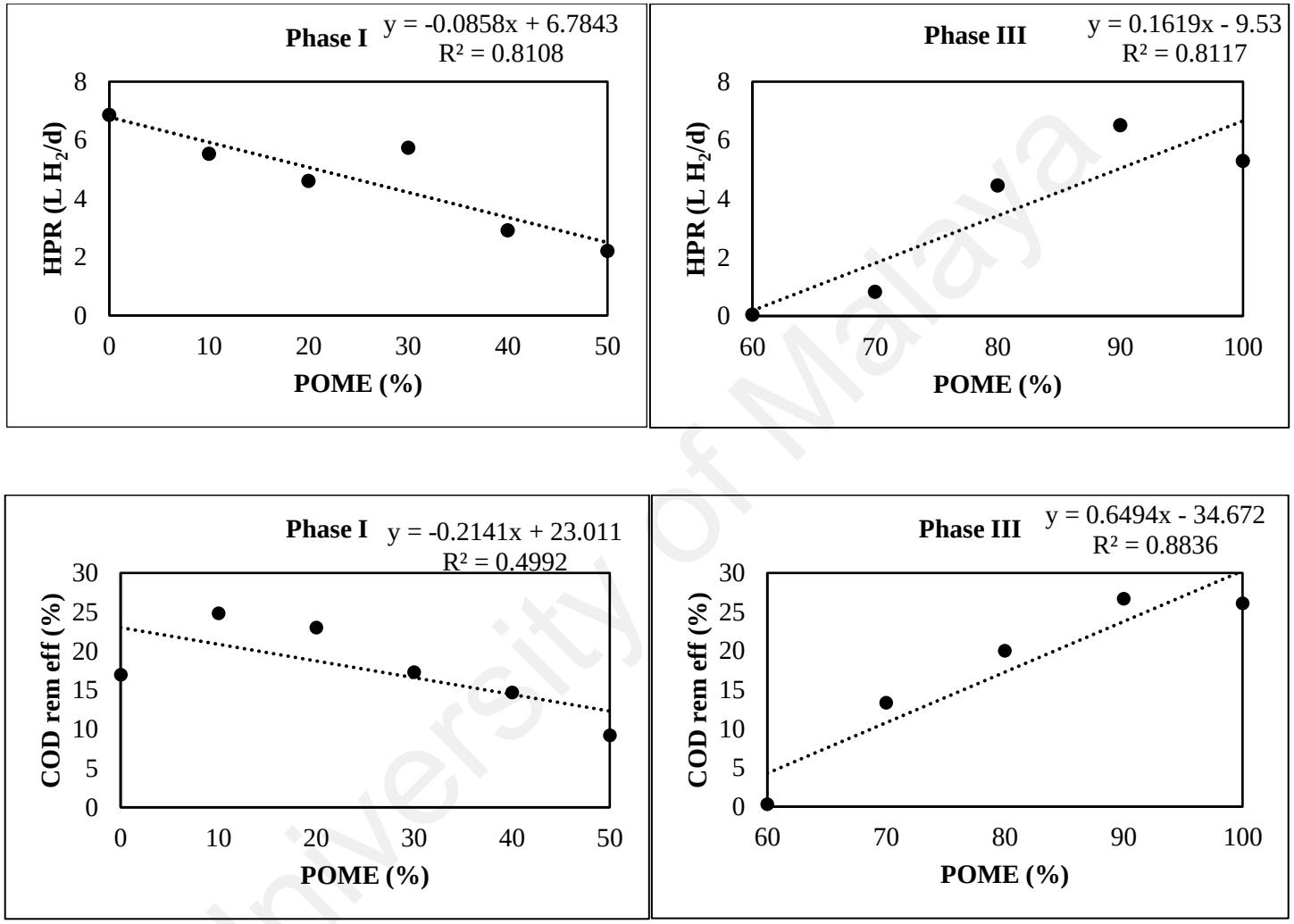


Figure 5.3: A Pattern of HPR and COD removal efficiency from Phase I and Phase III. (Phase II was neglected due to the low values reported as in Figure 5.2).

In Phase III (raw POME 70-100%), total biogas and hydrogen production rate increase as temperature increase to 43°C at 4 h HRT. This result is in agreement with other studies (Karlsson et al., 2008; Zhang et al., 2003). An optimal hydrogen production was reported when using thermophilic condition (55-65°C), as compared to at 37°C using starch as a substrate (Zhang, Kim, Lee, & Hwang, 2012). Thermophilic condition is better as compared to mesophilic because the former is known to enhance substrate degradation rates. With increasing temperature, the level of dissolved hydrogen will decrease. Thermodynamic equations done by several researchers on hydrogen-producing reactions found that higher temperature promoted hydrogen production (Lee & Zinder, 1988; Schink, 1997).

Wang, Mu, & Yu, (2005) in their study on the influence of temperature and substrate concentration using sucrose-rich wastewater found that the optimum temperature for biohydrogen production was 33.5°C when analyzed using RSM in a temperature range from 30 to 45°C. This is because acidogenesis was affected by temperature, pH and substrate concentration, thus the interaction between pH and temperature on hydrogen production was significant due to presence of volatile fatty acids (VFAs).

Raw POME is a complex compound; thus, it might be hard to be degraded by the mixed consortia present in the POME digested sludge. It also contains essential soluble minerals such as potassium (K), magnesium (Mg), calcium (Ca), zinc (Zn) and iron (Fe) (Table 1.1) that are readily available to microorganism. Lin & Lay (2005) reported that Mg was determined as the most important nutrient that will affect biohydrogen production. Fermentation process by UASFF bioreactor is suitable for high suspended solid waste with higher hydrolysis activity, which is the bottleneck for degradation of high suspended solids like POME. UASFF has been used for hydrogen and methane

production due to a promising anaerobic high-rate processes with short HRT (Mohammadi et al., 2017; Zinatizadeh & Mirghorayshi, 2017).

(b) *COD Removal Efficiency*

The same trend was also observed in the COD removal efficiency in the H_2-UASFF unit. During Phase I, the highest COD removal efficiency was at 10% raw POME used, i.e. 24.81%. After using 60% raw POME, COD removal efficiency was increased from 13.33% to 26.67% (90% raw POME, Phase III). At 100% raw POME application as a substrate, the COD removal efficiency was almost stable (26.10%). The low in COD removal efficiency in H_2-UASFF unit was because of the incomplete of mineralization of organic compounds under anaerobic conditions. The process is complete once methane gas was produced (Mamimin, Singkhala, & Kongjan, 2015).

In a study done by O-Thong, Mamimin, & Prasertsan (2011), low COD removal efficiency of POME were observed at pH 5.5, 35°C (12 ± 0.6 %), pH 4.5, 35°C (5 ± 1.5 %), and pH 4.5, 55°C (20 ± 1.2 %) for continuous hydrogen production. This is because pH and temperature have a significant interaction on hydrogen production. pH is a key for biohydrogen production where the VFAs drives the hydrogenase reaction (Khanal, Chen, Li, & Sung, 2004). The low COD reported might contributed to the rapid pH depletion that caused a metabolic alteration of the microorganisms involved in hydrogen production during the start-up. This cause the pathway of the intermediates production shifted and consequently decrease the COD removal efficiency and hydrogen production.

The correlation coefficient, R^2 of COD removal efficiency versus raw POME percentage is higher in Phase III (0.88) as compared to Phase I (0.50). This is because during Phase I, the fluctuations in pH, temperatures and HRT affected microbial community present in the mixed cultures, thus resulting in low hydrogen production rate.

This indicates that POME percentage plays a significant role in reducing the COD of the initial substrate in Phase III. COD in the POME was also reduced by conversion into hydrogen and CO_2 gas, as well as the microbial biomass during hydrolysis and acidogenesis (O-thong et al., 2016). During the third phase of the start-up period, methane percentage was only 12% observed at 100% raw POME (data not shown here), also indicating that less methanogenic activity occurred in the hydrogen reactor.

5.3.2 Stage II (CH_4-UASFF)

5.3.2.1 Methane Content (%), Methane Production Rate (MPR) and Gas Production Rate, COD removal efficiency

The CH_4-UASFF system was operated simultaneously using liquid effluent from the H_2-UASFF unit. Figure 5.4 shows that effluent from 0% POME feed (100% molasses used in H_2-UASFF unit), produced 68.95% methane with 31.05% CO_2. Methane content started to increase when the effluent from 20% POME was fed and became stable at 50% POME. The percentage of methane content decreased to 71.91% with CO_2 percentage of 28.09% with effluent from 60% POME as a result of the unstable conditions during second phase (Phase II) in the H_2-UASFF unit. Zahedi, Solera, Micolucci, Cavinato, & Bolzonella, (2016) reported 59 ± 9 % of methane in second stage methane-producing reactor during the fermentation process at the thermophilic condition. Figure 5.4 only shows the average value of methane content when the effluent from 60% raw POME was used. The percentage of methane is affected by methane production. The dropped in methane production at 60% POME used demonstrating that the effluent of hydrogen reactor was not readily usable for methanogenesis (Mamimin, Singkhala, & Kongjan, 2015). As MPR dropped at 60% POME applied due to decreased in HPR in H_2-UASFF unit, thus, methane content also dropped. This is because not all the suspended solids in H_2-UASFF reactor was decomposed by hydrolytic bacteria. A complete data was not

presented since the methane content percentage did not fluctuate significantly as methanogen reactor is easily stable for methane production. The CH_4-UASFF unit had already become stable under the anaerobic digestion process. In fact, the system was stable even when the effluent from 20% raw POME was used due to the acclimatization of the inoculum using glucose during the initial start-up. During Phase II of H_2-UASFF unit with 60% raw POME, extreme fluctuations in the output of that phase affected the overall methane production for 60% raw POME. Hence, for the CH_4-UASFF unit, the percentage of methane was stable and consistent with more than 90% of the methane produced. pH in CH_4-UASFF reactor was also observed and maintained between 7-8.

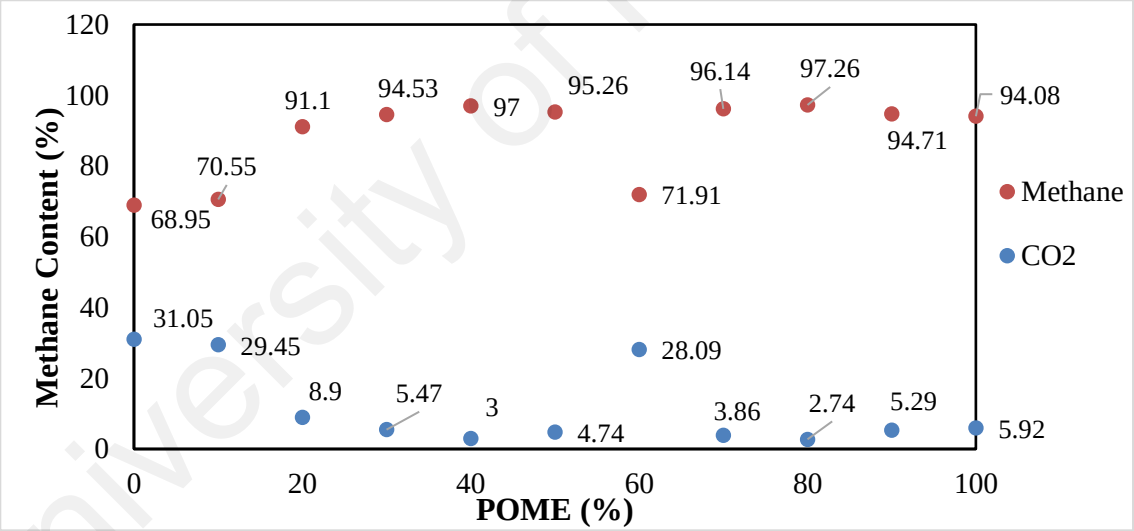


Figure 5.4: Methane and CO_2 percentage in CH_4-UASFF system.

The temperature applied in CH_4-UASFF unit was similar to H_2-UASFF unit as they shared the similar circulated water bath. It was observed that the increase in temperature from mesophilic (37°C) to thermophilic (43-55°C) did not significantly affect the methane percentage. The first two points in Figure 5.4 indicated the lag phase of the

methanogenic bacteria before they reached their exponential and stationary phase. The HRT of the system was maintained at 24 h with 1 m h^{-1} of V_{up}.

Figure 5.5 shows that as the percentage of POME increased, the gas production rate also increased (1.73 L d^{-1} to 6.84 L d^{-1}) from 0% POME effluent to 50% POME effluent applied. Methane production rate (MPR) was 1.19 L CH_4 d^{-1} during the initial feeding. At 60% of POME, the total gas production rate (GPR) (that also contains CO_2 gas) and MPR were dropped to 1.91 L d^{-1} and 1.37 L CH_4 d^{-1}, respectively. Changes in temperature and HRT in H_2-UASFF had a significant impact on the methane production rate in CH_4-UASFF bioreactor. After 60% of POME effluent was applied, methane production rate started to increase until it reached 9.60 L CH_4 d^{-1} with 100% POME effluent. It is found that there was no significant difference in the methane production rate when 100% POME was used instead of 100% molasses.

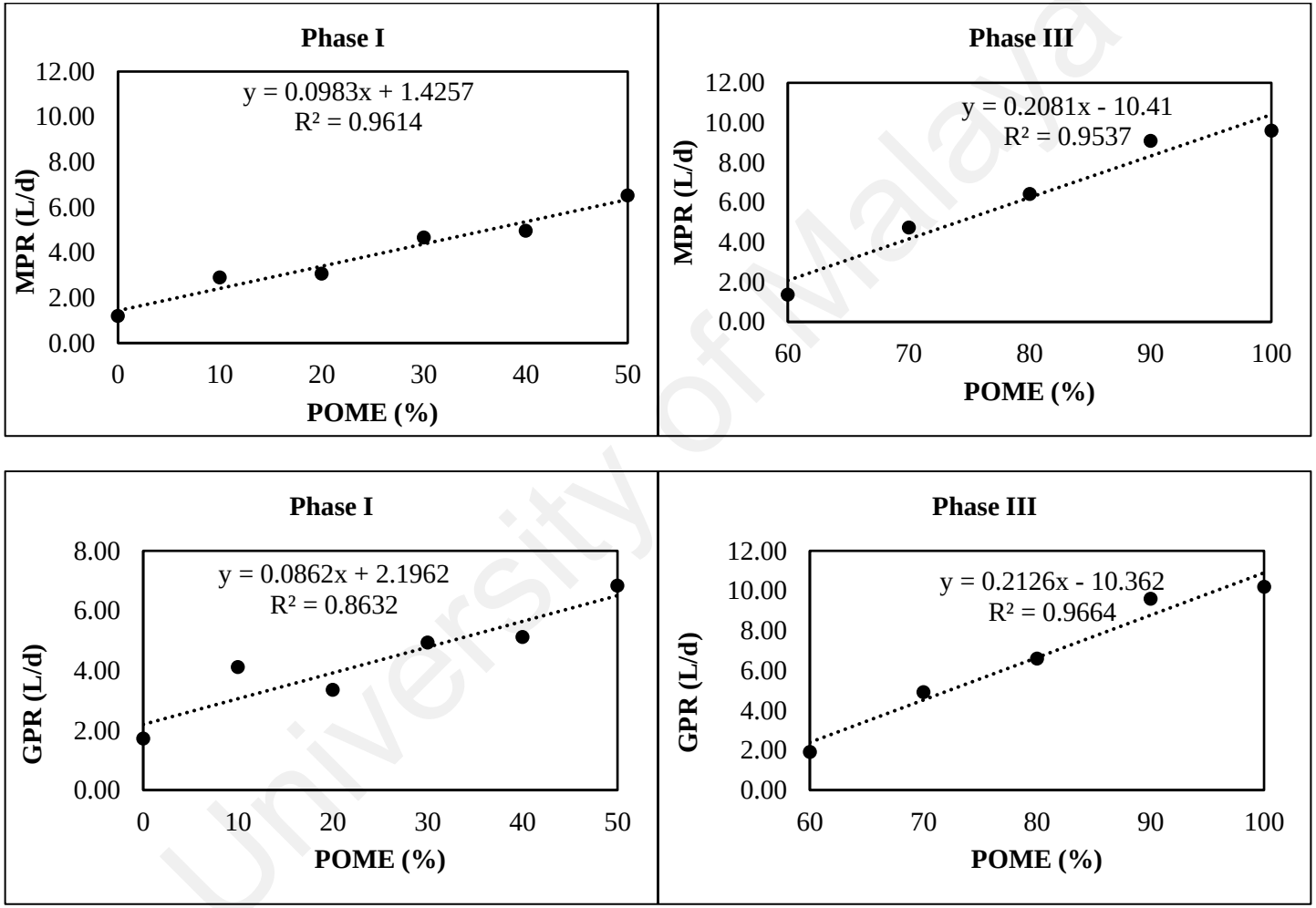


Figure 5.5: A pattern of methane production rate (MPR) and gas production rate (GPR) from Phase I and Phase III. Phase II was neglected due to the low values reported as in Figure 5.2 of the H_2-UASFF reactor.

Krishnan et al., (2016) reported a stable biogas production rate and maximum methane production rate of 2.93 L^{-1} d^{-1} and 3.2 L CH_4 L^{-1} d^{-1}, respectively using a two-stage thermophilic-mesophilic fermentation. Poh & Chong, (2009) in their study using two-stage anaerobic digestion treating POME found that methanogenic reactor quickly adapted with the feed from the acidogenic reactor, as well as able to tolerate its high OLR. They also reported a 90% COD reduction by using 30 kg COD m^3 d^{-1} with efficient methane conversion. Additionally, this finding has also shown that by using these two-stages of UASFF combined system, the stability of the second reactor was reached faster than a single-stage system.

Based on Figure 5.6, the COD removal efficiency in the CH_4-UASFF system decreased as the percentage of POME increased from 0% to 50%. However, as the H_2-UASFF system became stable after 60% POME, the COD removal efficiency started to increase by 19-fold, from 4.05% at 60% POME to 78.50% using 100% POME effluent from the H_2-UASFF system. The COD removal efficiency in the CH_4-UASFF system also observed to be stable after 70% POME effluent was applied. The same trend was also observed for the total COD removal efficiency for both systems with more than 70% of the COD could be removed using the two-combined UASFF systems.

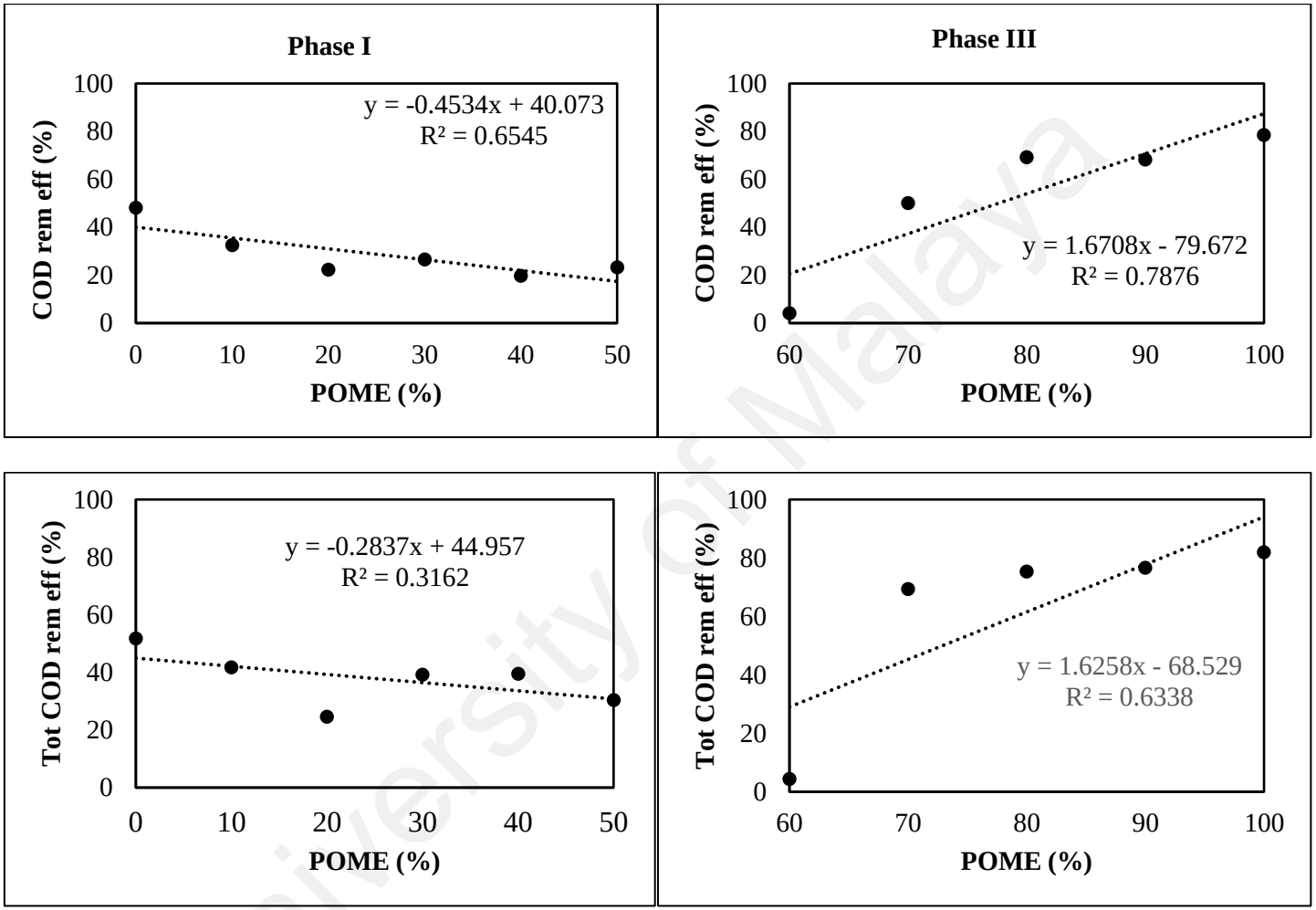


Figure 5.6: A pattern of COD removal efficiency (%) in CH_4-UASFF and Total COD removal efficiency (%) in two-stage UASFF bioreactor. Phase II was neglected due to the low values reported as in Figure 5.2.

5.3.2.2 The overall performance of UASFF bioreactor

The overall performance of a two-stage UASFF bioreactor was measured based on the stability of pH in the reactor, biogas production rate, total COD removal efficiency and biogas percentage. A low R^2 in Phase I (0.3162) shows that POME percentage used as a substrate for hydrogenic reactor was not significantly affected total COD removal efficiency in two-stage UASFF. This is because, total COD removal efficiency is depending on the initial POME % used for biohydrogen production (i.e. in H_2-UASFF unit) and its final treatment capacity in methanogenic bioreactor (CH_4-UASFF unit). The ability of treating POME in a second stage is depending on the volatile fatty acids present in H_2-UASFF effluent tank after hydrolysis/acidogenesis process.

A stable pH was observed at 70% raw POME to 100% raw POME (5.2 ± 0.1 and 7.5 ± 0.1 in H_2-UASFF and CH_4-UASFF, respectively). HPR was about to reach its stationary phase (Figure 5.3) starting from 80-100 % of raw POME while MPR achieved its stability (Figure 5.5) at 90-100% of H_2-UASFF effluent applied. A total COD removal efficiency of 83.75% was achieved using a two-stage UASFF bioreactor, as compared to a single reactor (26.67% in H_2-UASFF and 78.50% in a CH_4-UASFF reactor). Hydrogen percentage was reported to range between 50-70% at the end of the start-up using raw POME with methane gas composition was more than 90%.

The performance of the two-stage anaerobic fermentation system was also presented in Table 5.4, together with other studies using POME. The finding of this study was similar to studies done by Krishnan et al., (2016) and Mamimin et al., (2015) with total COD removal efficiency of 85% and 84%, respectively in a two-stage fermentation process treating POME. The optimal pH in the hydrogen reactor was maintained between

5-5.5 while in the methane reactor, the pH was between 7-7.5. Lowering the pH to 6.2 would reduce methane production (Oh et al., 2003). Under thermophilic condition, the optimum pH for methane production of 7.4 - 7.9 and 7.6 - 8.25, gave an average CH_4 content of 58.5% and 63%, respectively (Cavinato, Bolzonella, Fatone, Cecchi, & Pavan, 2011; Lee, Ebie, Xu, Li, & Inamori, 2010). While Krishnan et al. (2016) and Cavinato et al. (2011) operated their digesters at low HRT of 1.28 days and 3.3 days, respectively, thus, suggesting that the comparable performance is possible to be achieved in the CH_4-UASFF reactor with a shorter HRT of 1 day.

Table 5.4: Comparisons between studies integrating two-stages of biogas production using POME.

References	Krishnan et al. (2016)		Mamimin et al. (2015)		Suksong et al. (2015)		This study	
Parameters	**H_2**	**CH_4**	**H_2**	**CH_4**	**H_2**	**CH_4**	**H_2**	**CH_4**
Inoculum	Anaerobic seed sludge		Anaerobic seed sludge		Decanter cake		POME anaerobic sludge	
Substrate	POME		POME		POME		Raw POME	
Reactor Type	UASB	CSTR	ASBR	UASB	500 mL serum bottle		UASFF	UASFF
pH	5.5	uncontrolled	5.5	7.5	5.5	7.0	5.2	7.5
Temperature (°C)	55		55	35	60		43	
OLR	25-175 g COD $L^{-1}d^{-1}$	4-20 g COD $L^{-1}d^{-1}$	60 g COD L^{-1} d^{-1}	-	60-150 g VS L^{-1}		120 g COD L^{-1} d^{-1}	19.9-14.6 g COD L^{-1} d^{-1}
HRT (d)	0.375	12	2	15	1		1-0.17	1
Maximum COD removal efficiency in the 1st stage (%)	40		45		nil		26.67	

Table 5.4 continued.

Hydrogen Yield	49.22 mL g^{-1} COD applied	210 L kg^{-1} COD	16.6 mL g^{-1} VS	1021.74 mL g COD^{-1}
Hydrogen Production Rate (HPR) L H_2 L^{-1} d^{-1}	1.75	1.8	1.48	5.29
Methane Yield	155.87 mL g^{-1} COD applied	315 L kg^{-1} COD	240.65 mL g^{-1} VS	770 mL g^{-1} COD
Methane Production Rate (MPR)	325.13 mL g^{-1} MLVSS d^{-1}	2.6 L L^{-1} d^{-1}	51.59 L L^{-1} waste	9.60 L L^{-1} d^{-1}
Maximum COD removal efficiency in the 2^{nd} stage (%)	85	95	nil	78.50
Total COD removal efficiency (%)	85	84	62	83.70

5.4 Conclusion

The observations discovered in this study using an exploratory approach may provide a technical basis for the development of a larger-scale prototype design of UASFF, with regards to the production of H_2 and CH_4. However, increasing and maintaining H_2 and CH_4 production yields accompanied by a low BOD (< 20 mg L^{-1}) and COD concentration (< 100 mg L^{-1}) in the liquid effluent remains to be a challenging task, despite the ability of a two-stage UASFF bioreactor to produce biohydrogen and biomethane with POME as a substrate. Based on these observations, the application of UASFF bioreactor may still be limited for full-scale operation, nevertheless, efforts should be continued for further development of these systems in order to meet environmental compliance on POME discharge while maximizing the advantage of biogas as a renewable source of energy.

CHAPTER 6: OPTIMIZATION OF TEMPERATURE AND HYDRAULIC RETENTION TIME FOR BIOHYDROGEN PRODUCTION FROM POME IN H_2-UASFF BIOREACTOR USING RESPONSE SURFACE METHODOLOGY

6.1 Introduction

Malaysia is an equatorial country; therefore, anaerobic ponding systems are operated under mesophilic (30-40°C) temperature for 30-60 days of hydraulic retention time (HRT). During POME decomposition of organic matters, there are 60-70% of methane and 30-40% of CO_2 produced, with the rest consisting of a trace amount of H_2S (Loh et al., 2014). Current anaerobic digestion system implemented by all oil palm millers in Malaysia does not capture hydrogen. Hydrogen is an important intermediate product during the fermentation step and it has been an important clean renewable energy due to its favourable characteristics such as high energy yield (122 kJ/g) and efficiency, with various possible production (Aziz, Bagja, & Kurniawan, 2016; Singh & Wahid, 2015).

It was reported in the start-up study in Chapter 5 that using two-stage fermentation-anaerobic digestion process treating POME could shorten HRT and reduce more than 70% of total COD removal efficiency in a respective bioreactor with hydrogen and methane simultaneously produced under thermophilic temperature. When POME concentration is high, hydrogen yield and COD removal efficiency using fermentation system is low. Due to high lignin content and cellulosic-kind material, raw POME is hard to degrade by mixed cultures for hydrogen production under a short HRT. Therefore, a pre-treatment must be applied at the initial stage before it undergoes fermentation and digestion process. Different

pre-treatment methods have been proposed by Mohammadi et al., (2011). They reported that sludge heat pre-treatment is the simplest and useful method for enhancing hydrogen production and in increasing COD removal efficiency.

Raw POME is also a good substrate for POME digested sludge due to its high organic contents and high temperature (80-90°C). Cheong & Hansen, (2007) reported that hydrogen evaluation rate could be enhanced when treating wastewater at high temperatures. This is due to low liquid partial pressure in the liquid phase and propionate formation suppression. In a fermentative pathway, propionate fermentation will produce acetate, propionate and valerate. In this pathway, hydrogen will not involve. When running the experiment under thermophilic condition, they observed that propionate-producing bacteria were completely suppressed during the acid enrichment step. This is because the propionate-fermentative bacteria could not be involved with thermophilic hydrogen-producing potential. They also reported that when the inocula was heat-treated and pH was controlled at 5, the maximum hydrogen production potential was achieved when operated under thermophilic temperatures. The results indicated that hydrogen production was enhanced by thermophilic acidogenesis, which also consistent with the biochemical pathway of butyrate fermentation.

Fermentative production also can be carried out at ambient temperature (35-45°C) (Das, 2001). The high natural microbial diversity presence allows fermentation process to make use of variety of substrates. Therefore, the process will become easy by the mixed cultures. Several studies have documented the importance of microbiological aspects in POME (Chong, Abdul, et al., 2009; Chonticha Mamimin et al., 2012; Seiyaboh, Kigigha, Alagoa, & Izah, 2018). The diverse and mixed cultures communities are known to participate effectively

in bioremediation and biodegradation of POME. The study on microbiological characteristics in POME promotes a better understanding on the nature of microorganisms and consequently, their roles in hydrogen production (Bala, Lalung, & Ismail, 2014). Tan et al., (2015) also have summarized the microorganism involved in hydrolysis of fermentation process isolated from POME, as stated in Sub-section 2.5.1.

Meanwhile, several studies have been done by researchers using two-stage system for hydrogen and methane production treating POME wastewater in different bioreactor configurations under different operating conditions. They were using integrated system of up-flow anaerobic sludge blanket (UASB) – continuous stirred tank reactor (CSTR) and sequencing batch fermenters and anaerobic sequencing batch reactor (ASBR) - UASB (Krishnan et al., 2016; Mamimin et al., 2015; Mishra et al., 2016). Another studies also have been comprehensively reported in a single-stage fermentation process to produce hydrogen (Mohammadi et al., 2017) or methane (Zinatizadeh & Mirghorayshi, 2017) from POME using integrated up-flow anaerobic sludge blanket-fixed film (UASFF or UASB-FF) bioreactor. However, study on co-digestion of raw POME and POME digested sludge for hydrogen and/or methane in a two-stage of identical UASFF bioreactor is scarce.

Few researchers have documented hydrogen production potential from POME using different reaction temperatures and longer HRT (more than one day) in a two-stage anaerobic digestion system (Krishnan et al., 2016; Mamimin et al., 2015), but much is still unknown on the influences of shorter HRT on the conversion of POME to hydrogen using mixed cultures. Most of the studies examined different HRT between 36-96 h and 6 h (Badiei,

Jahim, Anuar, & Sheikh Abdullah, 2011; Krishnan et al., 2016; Singh et al., 2013) for biohydrogen production. However, studies on the effects of operating bioreactor at HRT less than those reported above utilizing POME is very limited. If the reactor is well controlled especially on the up-flow velocity, temperature, pH and organic loading rate (OLR), its performance could be enhanced even at lower HRT and biomass washout could be prevented.

On the other hand, different bioreactor configurations were used to treat POME using a two-stage system. Krishnan et al., (2016) were using up-flow anaerobic sludge blanket (UASB) – continuous stirred tank reactor (CSTR) for biohydrogen and biomethane production, Mamimin, Singkhala, & Kongjan, (2015) used integrated bioreactor of anaerobic sequencing batch reactor (ASBR) – UASB and Suksong et al., (2015) used 500 mL serum bottle – UASFF for hydrogen and methane production. They reported maximum COD removal efficiency of 40% and 45% under 9 h and 2 days of HRT, respectively, with hydrogen production rate (HPR) of 1.75 L H_2 L^{-1} day^{-1} and 1.8 L H_2 L^{-1} day^{-1}. To date, the optimization study of HRT and temperature using UASFF bioreactor for biohydrogen production in treating POME is hardly found.

Therefore, in the present work, the effects of temperature and low HRT were investigated to determine the optimum conditions for maximum biohydrogen production utilizing POME. This study focused on biohydrogen production in H_2-UASFF unit. Parameters at different conditions were analyzed, including hydrogen production rate and yield and also COD removal efficiency. A simple test was also done for microbial identification in POME digested sludge. Previous batch and start-up study operated under different operating, process

and environmental factors have provided some primary results and findings that supported the experimental set-up for this continuous study.

6.2 Materials and methods

This study is a continuity from the start-up study. Substrate used was from Sri Ulu Langat Palm Oil Mill in Dengkil, Selangor. The preparation was stated in Sub-section 3.2.1.1. The inoculum was remained in the bioreactor to keep the anaerobic condition and avoid any disturbances if the sludge is taken out, such as temperature shifts. Inoculum preparation was stated in Sub-section 5.2.1. The schematic diagram of two-stage UASFF bioreactor was stated in Chapter 5, Sub-section 5.2.3. This work will be focused solely on H_2-UASFF unit for biohydrogen production treating POME. Details of inoculum and substrate characteristics are shown in Table 6.1.

Table 6.1: Differences between raw POME and digested POME used in this study that acts as substrate and inoculum, respectively, for biohydrogen production.

Properties	Unit	POME	
Physicochemical		**Raw**	**Digested POME**
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}	n.a	50,000
Volatile Suspended solids (VSS)	mg L^{-1}	n.a	27,250
Soluble Chemical oxygen demand (SCOD)	mg L^{-1}	28,000	n.a

n.a = not analysed

6.2.1 UASFF set-up

The details of the reactor set-up were explained in Chapter 5, Sub-section 5.2.3. H_2-UASFF unit has a capacity of 2.5 L and pH in the bioreactor was maintained between 5-5.5.

Initial reactor conditions used were 2.51 L d^{-1} of feed flowrate (Q_F), 54.49 L d^{-1} of recycle flowrate (Q_R), and 1 m h^{-1} of up-flow velocity (V_{up}). Water bath was used to control the desired temperature in H_2-UASFF unit between 37°C - 70°C. Since there might be a heat loss when temperature is transferred from water bath to the bioreactor, therefore, several checking was done to identify the differences before applying the desired temperature. The calculations for HRT and V_{up} can be found in Chapter 5, Sub-section 5.2.5. To allow a dark fermentation process used in this study, the lowest section of UASB reactor was covered with aluminum foil in order to prevent the daily light that may promotes the growth of photosynthetic bacteria.

6.2.2 Experimental design

Based on the findings from UASFF start-up study, a model for H_2-UASFF unit was designed using central composite design (CCD) and analyzed using response surface methodology (RSM). The objective was to find the optimum temperature and HRT, therefore, two factors were chosen and assessed at low (37°C, 3 h), high (70°C, 9 h) and five central levels in order to estimate variability, design and corresponding results of the experiment. Temperature was chosen from 37°C to 70°C because based on the final finding obtained in Chapter 5 (i.e. 43°C during start-up), a preference of hydrogen producing bacteria (HPB) in the inoculum need to examine, either in an ambient, mesophilic or thermophilic condition. Meanwhile, HRT was chosen between 3-9 h because 4 h HRT is needed for production of biohydrogen based on the start-up study. Higher HRT is avoided to minimize the formation of methanogens in the UASFF bioreactor based on the previous experience during the start-up.

6.2.3 Analytical analysis

Analysis for chemical oxygen demand (COD) was mentioned in Sub-section 3.3.1.1 and calculations for COD removal efficiency and hydrogen yield was stated in Equation 5.5 and Equation 5.7, respectively. Biogas measurement and analysis was described in Sub-section 3.3.3.

6.2.4 Microscopic examination

The microorganism colony in POME sludge that involved in dark fermentation process for biohydrogen production was identified. Samples of untreated POME digested sludge and heat-treated POME sludge after dark fermentation process were sent to certified local laboratory for bacterial identification using microscopic examination. The details of the methods used is presented in Table 6.2. Three species of bacteria (*Clostridium sp., Bacillus sp*., and *E.coli*) were chosen based on studies done by Bala, Lalung, Al-Gheethi, Hossain, & Ismail, (2018), Chong, Abdul, Shirai, & Ali, (2009) and Seiyaboh, Kigigha, Alagoa, & Izah, (2018). They reported the presence of those species in POME, regardless of the plantation area.

Table 6.2: Analysis of bacteria identification of untreated and heat-treated POME digested sludge as inoculum.

Sample	Parameters	Methods Used (Method Reference)	Unit
Untreated POME	Heterotrophic plate count	APHA 9215B (*American Public Health Association (APHA). Standard Methods for the Examination of Water and Wastewater*, 1999)	CFU/mL

	Bacteria Identification	API 50 CHL (API Kit Manual)	-
Heat-treated POME	Anaerobic plate count	In-house No. M078 based on Merck Manual	CFU/mL
	Total coliform	APHA 9222B (*American Public Health Association (APHA). Standard Methods for the Examination of Water and Wastewater*, 1999)	CFU/mL
	Escherichia coli	APHA 9222G (*American Public Health Association (APHA). Standard Methods for the Examination of Water and Wastewater*, 1999)	CFU/mL
	Bacillus sp.	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	Microscopic Examination	-

6.3 Results and discussion

6.3.1 Effects of temperature on hydrogen production rate (HPR)

Table 6.3 demonstrates a reduced quadratic model for hydrogen production rate using ANOVA. Model indicates that there is a significant relation between HPR and temperature. At the point when temperature expanded from 37°C – 56.8°C, HPR was also increased

(Figure 6.2 (A)). Highest HPR of 13.07 L day^{-1} was obtained at 53.5°C, i.e. under thermophilic condition. Nonetheless, temperature between 56.8°C to 70°C demonstrates a diminished in HPR. This clearly showed that the difference in reaction time (HRT) used in this study did not gives a significant impact towards biohydrogen production using mixed cultures. In addition, this result suggests that different microbial communities are activated due to differences in fermentation temperatures.

Zhang et al., (2003) reported thermophilic temperature (55°C) could produce more hydrogen compared to mesophilic temperature when they used starch as a substrate. This is because at 55°C, thermophiles in the sludge may require a longer lag phase, even at a slower rate, to convert more substrate into hydrogen. Thermophiles are able to degrade various types of substrate such as cellulose, hemi-cellulose or pectin-containing biomass (Van De Werken et al., 2008). Another study also showed a higher hydrogen yield by thermophiles as compared to mesophiles (Groenestijn, Hazewinkel, Nienoord & Bussmann, 2002). They concluded that due to the entropy increased in the system, a thermodynamically higher temperature is favorable for hydrogen production, thus resulted in a more energetic process.

Table 6.3: Analysis of Variance (ANOVA) for Response Surface Reduced Quadratic Model for HPR and Response Surface Reduced Quadratic Model for COD removal Efficiency (%) and H_2 Yield.

Source	Sum of Squares	Mean Square	F Value	p-value Prob > F	
1. Hydrogen Production Rate (HPR)					
Model	18.87	9.43	48.92	<0.0001	significant
A-Temperature	4.87	4.87	25.24	0.0002	
A^2	14.00	14.00	72.60	<0.0001	
Residual	2.70	0.19			
Lack of Fit	1.30	0.22	1.24	0.3786	not significant
R-Squared	0.87				
Adj R-Squared	0.86				
2. COD removal Efficiency (%)					
Model	22.01	3.14	6.52	0.0060	significant
A-Temperature	0.84	0.84	1.74	0.2198	
B-HRT	6.32	6.32	13.11	0.0056	
AB	7.01	7.01	14.55	0.0041	
A^2	4.70	4.70	9.75	0.0123	
B^2	3.89	3.89	8.08	0.0193	
A^2B	7.28	7.28	15.11	0.0037	
A^2B^2	4.80	4.80	9.95	0.0117	
Residual	4.34	0.48			
Lack of Fit	1.47	1.47	4.08	0.0780	not significant
R-Squared	0.84				
Adj R-Squared	0.71				
3. H_2 Yield					
Model	27.71	5.54	12.10	0.0004	significant
A-Temperature	11.77	11.77	25.69	0.0004	
B-HRT	0.13	0.13	0.28	0.6046	
AB	2.55	2.55	5.56	0.0379	
A^2	7.79	7.79	17.00	0.0017	
B^2	0.33	0.33	0.71	0.4164	
Lack of Fit	2.86	0.95	3.50	0.0697	not significant
R-Squared	0.85				
Adj R-Squared	0.78				

6.3.2 Effects of hydraulic retention time (HRT) on COD removal efficiency (%)

In contrast with HPR, temperature did not have a significant effect towards COD removal efficiency, but hydraulic retention time (HRT). As shown in Figure 6.2 (B), the highest COD removal efficiency was detected at 70°C, 3 h HRT (42.14%). 3D plot showed that at 3 h HRT, COD removal efficiency increased as temperature increased. This showed that microbes in the inoculum degraded raw POME faster at lower HRT under thermophilic condition. In any case, when HRT increased from 3 to 9 h (at 37°C), COD removal efficiency decreased between 3 - 4 h before started to slowly increase back from 4 – 9 h. At 9^{th} h, COD removal increased (from 24.76% to 33.33%) between 37°C – 56.8°C, before decreased in productivity (from 33.33% to 14.29%) when temperature ascended to 70°C. In hydrolysis process, the enzymes involved may be very sensitive to temperature, thus resulting in a decrease of hydrolysis rate and affected the substrate degradation efficiency (Rizvi et al., 2015). Using a low HRT (6 h) could increase hydrogen yield, which corresponds to higher degradation efficiency. This is because the shorter HRT could reduce the microorganism diversity (with dominant species remains) which corresponds to propionate production suppression. Under short HRT, some of the microorganisms does not have enough time to consume the substrate. The production of propionate involves the consumption or both organic substrate and hydrogen (Zhang et al., 2006).

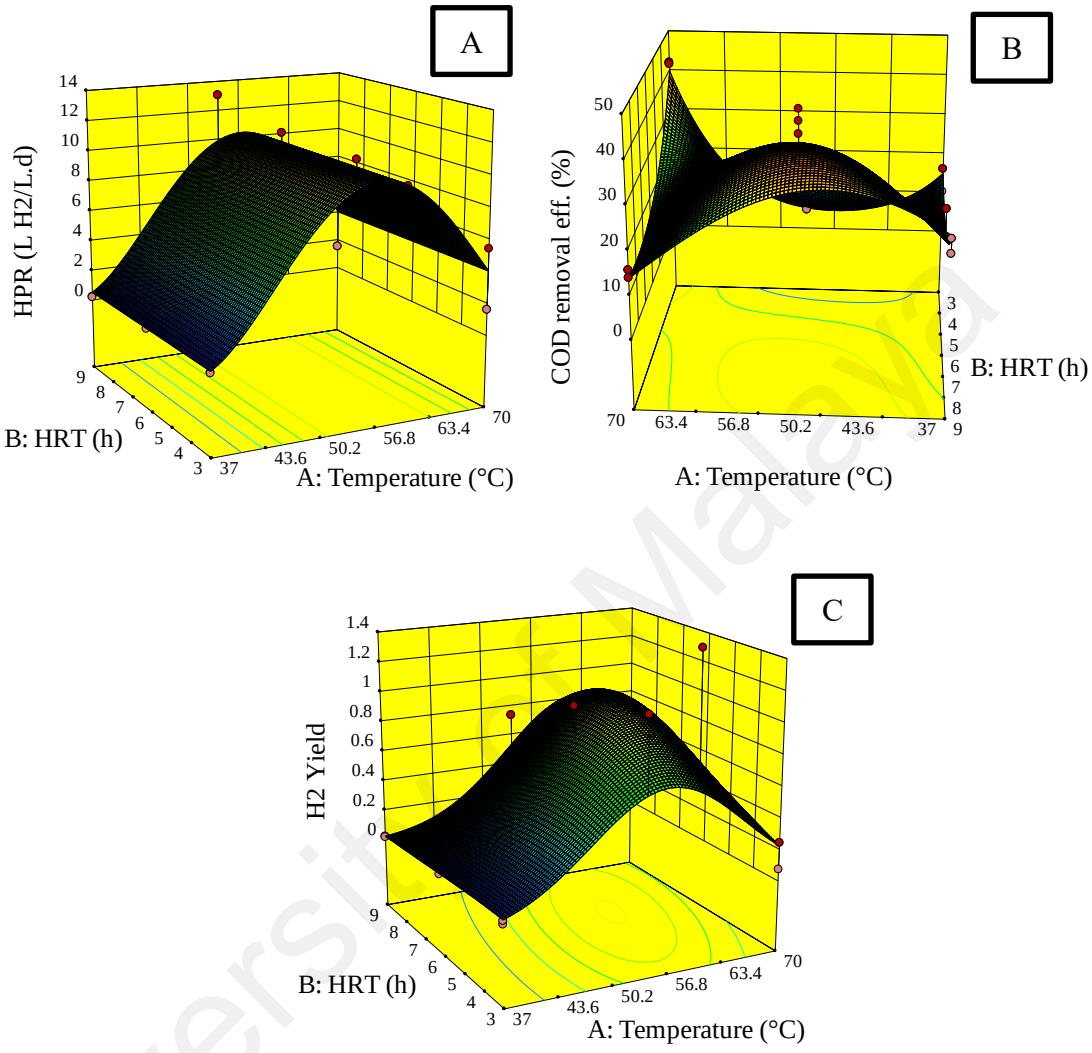


Figure 6.1: 3D surface of effects of temperature and HRT on three responses; A) hydrogen production rate (HPR); B) COD removal efficiency (%) and C) H_2 Yield.

6.3.3 Effects of temperature on hydrogen yield

The results of ANOVA show a significant relation between temperature and hydrogen yield using Response Surface Reduced Quadratic Model as shown in Table 6.3. In order to evaluate the results, regression analysis was used based on data in Table 6.2 using quadratic equation in Equation 6.1.

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

Equation 6.1

where A and B are coded value for temperature and HRT, respectively. Model shows a determination coefficient (R^2) of 0.85, indicating an 85% of variability in the response. Value for adjusting R^2 is 0.78, with Model F-value of 12.10 and values of Prob>F is 0.00036 (less than 0.05), which indicates the significance of the model and model terms, respectively. Meanwhile, 3D counter plot in Figure 6.2 shows an increase in hydrogen yield (0.25 – 0.61 L H_2 g^{-1} $COD_{removed}$) when HRT increased from 3 – 9 h at 70. Hydrogen yield is also increased (from 0.05 – 1.17 L H_2 g^{-1} $COD_{removed}$) when temperature increased from 37°C until temperature between 56.8 – 63.4°C at 3 h HRT, before the productivity started to decrease back when temperature hits 70°C (0.25 L H_2 g^{-1} $COD_{removed}$). This trend clearly represented that the mixed cultures in POME digested sludge is already adapted to thermophilic condition at lower HRT, based on the previous start-up operation of UASFF bioreactor. A similar result was also observed by Lin et al., (2008) where they obtained a gradual increased in hydrogen gas production at 50°C utilizing municipal sewage sludge as inoculum due to the mixed microflora adaptation at new HRT of 12 h with a strict enrichment of cultivation.

6.3.4 Optimization of T and HRT

Optimization was done to find the optimal conditions of T and HRT for highest output of HPR, H_2 yield and COD removal efficiency. The optimization results will be later used in the next study in Chapter 7 to find the interactions between the optimal conditions used in

H_2-UASFF unit with influence of two factors in CH_4-UASFF unit that producing biomethane (i.e. temperature and COD effluent from H_2-UASFF unit). In this study, 17 experimental data were pre-determined. By using numerical optimization, temperature and HRT were set in range while all three responses were in maximum goal. With this condition, only one solution was obtained with 78.90% desirability (Figure 6.3). Optimum conditions were found to be thermophilic (57°C) at 7 h HRT in order to achieve highest HPR, hydrogen yield and COD removal efficiency of 10.39 L H_2 d^{-1}, 0.95 L H_2 g^{-1} $COD_{removed}$ d^{-1} and 35.78%.

HPR was increased with increasing in temperature (37°C – 56.8°C) (Figure 6.3 (A)). The trend descends as temperature gets higher. Meanwhile, HPR is insignificantly affected by reaction time, HRT. Temperature plays a significant role towards biohydrogen production and microbial metabolism (Li & Fang, 2007; Mamimin, Chaikitkaew, et al., 2015). This is because, during dark fermentation, temperature affects the microbe's maximum specific growth rates and substrate conversion rate. In addition, the optimal temperature for biohydrogen production utilizing POME varies in different studies is because of the materials constituents and the communities of the microbes.

Study done by Mamimin, Chaikitkaew, et al., (2015) also reported that at 60°C, they obtained higher hydrogen production (2494 ± 196 mL H_2 L^{-1} POME) treating POME compared to when treated under mesophilic (15 ± 3 mL H_2 L^{-1} POME). Thermophilic was found to be favorable due to the mixed cultures that contained thermophilic microbial.

Distinctive temperature in biohydrogen production is associated with a change in microbial community and shift in the metabolic pathways (Balachandar et al., 2013).

Therefore, studies have proven that it is critical to comprehend temperature reliance of the microbial community, with the end goal to improve hydrogen production system.

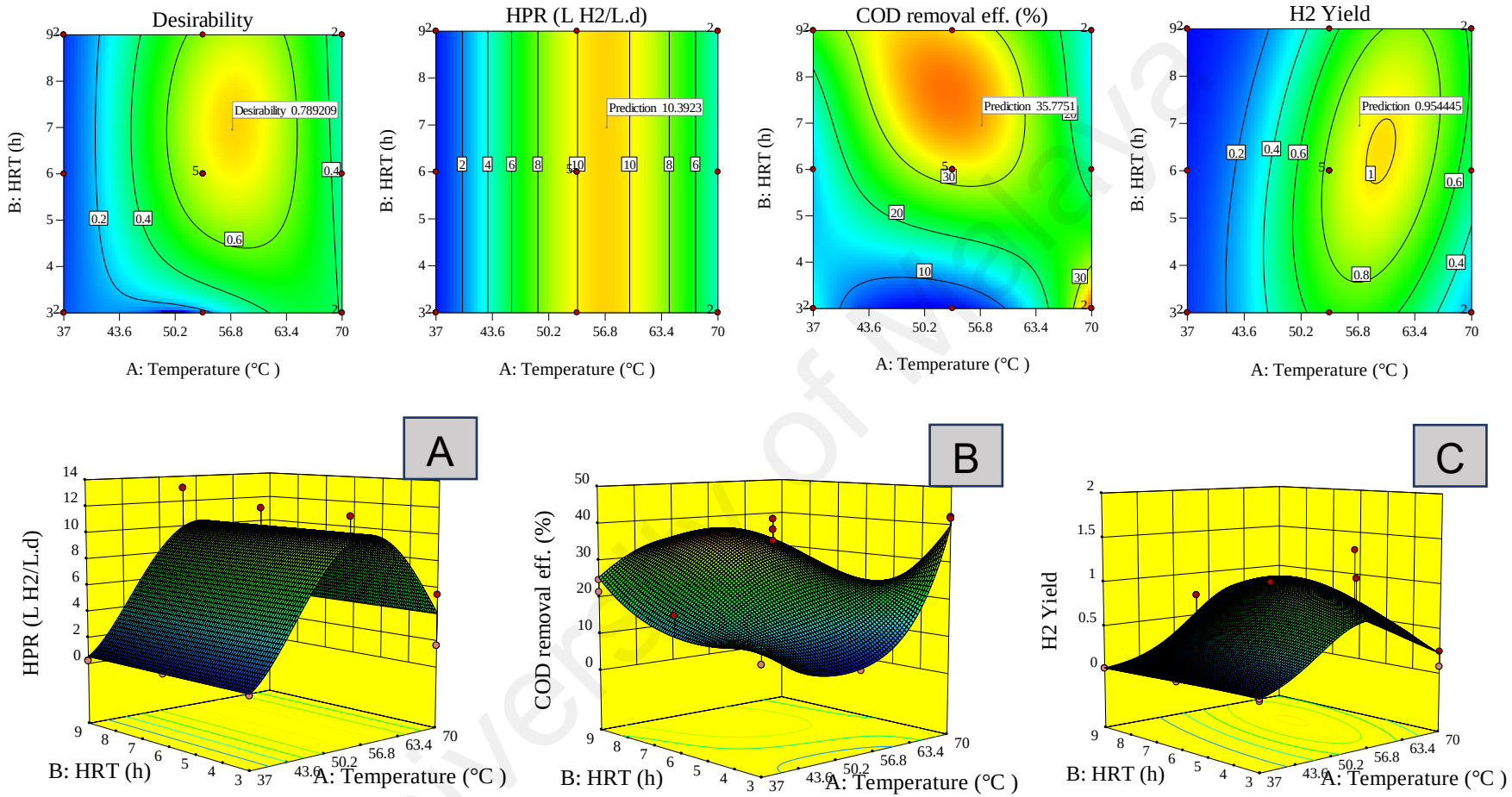


Figure 6.2: Counter and 3D plot diagram for A) hydrogen production rate (HPR); B) COD removal efficiency (%) and C) H_2 Yield with 78.92% desirability at optimum temperature and HRT of 57 and 7 h, respectively.

A counter plot for COD removal efficiency shows the highest percentage when temperature falls between 50.2°C to 56.8°C at 6 – 9 h HRT. The highest prediction of 0.95 L H_2 g^{-1} $COD_{removed}$ day^{-1} of hydrogen yield was observed between 56.8°C to 63.4°C at 6 – 7 h of HRT. Due to faster bacteria metabolic activity and limited growth of hydrogen consumers, thermophilic condition was found to yielded higher hydrogen yields. A similar experimental condition of inoculum was used by Karadag (2011) using mixed consortia from mesophilic sources for biohydrogen production. Zeidan & van Niel (2010) reported that hydrogen gas production is more effective when operated under thermophilic-anaerobic fermentation, compared to mesophilic fermentation. In their study utilizing glucose as substrate, the hydrogen yield achieved was as close as the theoretical yield (i.e. 4 mol H_2 mol^{-1} glucose). They also concluded that by controlling pH and nitrogen purging, the pressure in the bioreactor could be reduced, thus resulting in higher hydrogen yield.

6.3.5 Microscopic Analysis

A small amount of heat-treated POME sludge from H_2-UASFF bioreactor was examined for bacterial identification after the dark fermentation process. Based on the obtained results, the biggest colony found in heat-treated POME was gram negative rod-shape bacteria with 2.5 x 10^7 CFU/mL. POME degradation process includes hydrolysis of the long-chain carbon compounds, followed by acidogenesis and acetogenesis for hydrogen production (Tan et al., 2015). Table 6.4 shows the identified bacteria found in both untreated and treated POME sludge. The results from PERMULAB SDB BHD as an authorized and certified company analyzing both untreated and heat-treated POME sludge using microscopic examination was attached in Appendix.

Table 6.4: Bacterial identification and plate count for untreated and heat-treated POME sludge.

Sample	Parameters	Unit	Results	Bacteria Identification	Microscopic Examination
Untreated POME sludge	Heterotrophic plate count	CFU/mL	2.4×10^7	*Lactobacillus acidophilus*	Gram Positive Rod
Heat-treated POME sludge used in this study	Anaerobic plate count	CFU/mL	2.5×10^7	-	Gram Negative Rod
	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)	-	-

The bacteria in heat-treated POME was found to be rod in shape and gram negative. In a pilot-scale of biohydrogen production treating distillery effluent using defined bacterial culture, a rod shape negative bacteria was found in the study done by Vatsala, Raj, & Manimaran, (2008). Chaudhary, Thakur, Quraishi, & Jadhav, (2015) isolated and screened six different bacterial species for biohydrogen production. The effects of T, HRT and pH were studied. Results showed that the rod-shape gram negative bacteria have a significant impact on biohydrogen production due to its high amylase and fermentative ability. These results were similar to the characteristic of bacterial sp. found in this study.

6.4 Conclusions

The effects of temperature and HRT were investigated using H_2-UASFF bioreactor utilizing POME. This study shows that temperature plays a significant role in increasing hydrogen yield and hydrogen production rate whereas HRT has a significant impact on COD removal efficiency of POME. Therefore, at the optimum temperature and HRT of 57°C and 7 h,

maximum hydrogen production rate and yield of 10.39 L H_2 day^{-1} and 0.95 L H_2 g^{-1} $COD_{removed}$ with 35.78% of COD removal were achieved. Based on the optimization study using RSM, the obtained results confirmed that temperature and HRT significantly affected biohydrogen production rate, yield and COD removal efficiency, respectively by the ability of hydrogen-producing bacteria to degrade POME. Also, the Gram-negative rod bacteria which found dominant in the inoculum may attributed to the hydrogen production.

CHAPTER 7: EFFECTS OF TEMPERATURE AND DARK FERMENTATION EFFLUENT FROM H_2-UASFF ON BIOMETHANE PRODUCTION IN CH_4-UASFF

7.1 Introduction

Few technologies have been proposed for biomethane production during the last decades. Anaerobic digestion is one of the most promising processes and preferred by researchers. Anaerobic digestion can utilize a different type of organic wastes under different operational pressure and temperature while producing high biomethane production rate. This makes anaerobic digestion a favourite option for biomethane production. However, few factors will have an impact on either maximize the biomethane yield or process economy perspective such as the technology implemented, and type of organic wastes used.

Biogas production can be improved by optimizing the separate reactors as a step forward to the common anaerobic digestion process used (i.e. integrating hydrogen reactor and methane reactor). Therefore, in methane reactor, better quality substrate will be fed. Moreover, gases obtained can be used either separately or mixed together (also called biohythane), which has an average percentage composition of 10% H_2, 30% CO_2 and 60% CH_4 (Cavinato et al., 2011).

Production of methane using residual or effluent from the hydrogen reactor was reported by few researchers using different organic biomass residues with different operating factors such as organic loading rate (OLR), solid retention time (SRT) and different substrates (Xie et al., 2008; Xing, Dong-jie, Xiao-shuang, & You-cai, 2008). They concluded that: (i) energy

efficiency was improved when combining the hydrogen and methane production process and (ii) more than 80% of influent COD was removed at optimal conditions.

In two-stage anaerobic digestion process, fermentative hydrogen and methane fermentation is often used (Azbar & Speece, 2001; Demirel, Scherer, Yenigun, & Onay, 2010). In the second stage, a slow-growing acetogens and methanogens are present, which converting volatile fatty acids (VFAs) from the first stage to methane (CH_4) and carbon dioxide (CO_2). Regarding this two-stage process, the dark fermentation process that producing hydrogen does not significantly reduce the organic content of the substrate/feed.

In Chapter 6, results show that about 30% of chemical oxygen demand (COD) removal was achieved in H_2-UASFF unit. The undegraded COD can be transferred in a subsequent unit (i.e. CH_4-UASFF unit) with the organic content can be converted to methane. In two-stage anaerobic digestion, hydrogen (H_2) and CO_2 were produced in the first stage (i.e. H_2-UASFF unit) and the effluent of the first stage was transferred to the second stage (i.e. CH_4-UASFF unit) to be converted to CH_4.

Production of biohydrogen in H_2-UASFF unit was conducted to find the optimum temperature and HRT treating raw POME as a substrate while utilizing digested POME as an inoculum. Results showed that at 57°C with 7 h HRT, the highest hydrogen production rate (HPR) and hydrogen yield was achieved at 10.39 L H_2 d^{-1} and 0.95 mL H_2 g^{-1} $COD_{removed}$, respectively, with COD removal of 35.88%. In this two-stage UASFF bioreactor, a shared water bath was used. Therefore, effect of operating temperature was again studied and effect of effluent concentration towards biomethane production in the second stage (CH_4-UASFF

unit) and overall bioreactor performance was examined and analysed in order to find a significant relationship between the aforementioned parameters.

There is very limited study on determining the optimum temperature for biogas production treating POME (Choong, Chou, & Norli, 2018). Therefore, in this study, three different operating temperature (min. 37°C, max. 70°C) and effluent COD concentration from H_2-UASFF unit (lowest: 12,150 mg L^{-1}, highest: 19,967 mg L^{-1}) were designed using historical data in response surface methodology (RSM). Five responses were studied and analysed, *viz*. COD removal efficiency (%) and methane percentage (%) in CH_4-UASFF unit, methane production rate (MPR), methane yield, and total COD (in two-stage UASFF reactor). All responses were analysed using polynomial whereby quadratic was used in the designed model. At the end of the experiment, the optimum parameters were chosen based on the highest responses when effluent COD from H_2-UASFF unit and temperature were set in-ranged.

7.2 Materials and method

7.2.1 Inoculum and substrate preparation

Substrate and inoculum preparation was prepared as in Chapter 5, Section 5.2. Analytical analysis and all the calculations were as in Sub-Section 5.2.4 and 5.2.5 as well. Inoculum used was from the start-up study (Chapter 5). Meanwhile, the effluent concentration from H_2-UASFF unit (also called dark fermentation effluent) was prepared and used as a substrate. Effluent pH was detected at 4.7±0.1. This indicates that volatile fatty acid (VFA) was produced, thus lowering the pH value. Effluent pH was adjusted to 7-8 by using 6 N sodium hydroxide (NaOH) solution in order to maintain the pH in CH_4-UASFF bioreactor and

promotes methanogens for biomethane production. Pure nitrogen gas was then purged in a closed container containing the substrate at 10 mL min^{-1} for 10-15 min to maintain the anaerobic condition inside the bioreactor. The anaerobic digestion process starts when substrates were fed into the bioreactor.

7.2.2 Experimental set-up & design

A scale-up of integrated UASFF bioreactor mentioned in Sub-section 3.3.4. Circulating water bath was adjusted according to the desired temperature based on the study in H_2-UASFF unit for biohydrogen production (see Chapter 6, Subsection 6.2.2). CH_4-UASFF unit was operated at HRT of 24 h. Settling tank was used to collect any biomass washout and recycle them back in order to maintain the pH and biomass in the unit. The final effluent with a lower COD content will be discharged into the effluent tank.

To depict the intuitive impacts of temperature and substrate concentration on the responses, 17 continuous flow experiments were conducted with coded values of -1 and +1 for temperature (37°C, 70°C) and substrate concentration (12,150 mg L^{-1} and 19,967 mg L^{-1}). Temperature and substrate concentration were chosen as two independent factors in the experimental design. Meanwhile, COD and TCOD removal efficiency, CH_4 percentage in biogas, MPR and the yield are dependent outputs. The experimental design is shown in Table 7.1. RSM with Historical Data Design was used to optimize the key factors affecting the outputs. Historical Data is based on the data obtained from the effluent tank of H_2-UASFF unit for biohydrogen production.

7.2.3 Analytical methods

Methane volume and routine parameters such as COD and pH were measured as stated in Section 3.3. Biogas percentage was measured as stated in Sub-section 3.3.3. The details of the GC were reported in Chapter 4, Sub-Section 4.2.4.

7.3 Results and discussion

7.3.1 Statistical analysis

Table 7.1 displays the total of 17 runs from two responses used while Table 7.2 demonstrates the summarized results from analysis of variance (ANOVA). So as to fit the information, distinctive level of polynomial models was utilized. To quantify the curvature effects, experimental data were fitted to higher degree polynomial equations (e.g. quadratic, two-factor interaction (2FI), etc.) By using Design-Expert Software 10.0.06, data in responses were evaluated by default. The ANOVA results have shown some selected model terms were eliminated in terms of insignificant variables and their interactions. Despite the fact that there are no significant interaction terms (AB) in this investigation, notwithstanding, all models were huge dependent on the statistical analysis.

Table 7.1: Experimental conditions and responses using Historical Data for the design type in Response Surface Methodology (RSM).

Run	Factors		Responses				
	1. Temperature (°C)	2.(Substrate) CODin (mg L^{-1})	COD removal efficiency (%)	MPR (L CH_4 d^{-1})	Methane Yield (L CH_4 g^{-1} COD rem.d^{-1})	TCOD (%)	CH_4 (%)
1	53.5	19967	75.04	5.79	0.14	76.27	94.13
2	53.5	13530	54.55	12.59	0.63	70.71	92.04
3	53.5	15980	63.08	5.48	0.20	71.90	93.52
4	53.5	16300	61.96	9.09	0.33	70.48	94.66
5	53.5	12300	66.26	13.32	0.61	80.24	94.40
6	53.5	12893	69.79	18.98	0.78	81.45	94.13
7	53.5	14000	71.43	13.59	0.50	80.95	94.34
8	70	18000	64.91	9.25	0.29	69.92	91.74
9	70	17633	64.56	12.61	0.41	70.24	89.81
10	70	12233	43.97	12.12	0.83	67.36	90.16
11	70	12150	38.41	14.57	1.16	64.37	91.98
12	70	18633	62.97	9.45	0.30	67.14	87.50
13	37	18800	48.14	5.32	0.22	53.57	91.16
14	37	17560	43.05	6.53	0.32	52.38	90.65
15	37	17100	35.67	5.44	0.33	47.62	87.17
16	37	15800	45.89	12.42	0.63	59.29	86.23
17	37	16500	35.39	8.97	0.77	62.62	88.97

Table 7.2: ANOVA results of studied parameters, *viz.* A-temperature and B-substrate concentration (effluent from H_2-UASFF unit).

Responses	Modified equations with significant terms	R^2	Std Dev.	p-value Prob > *F*	Model Significance	Significant parameters
CH_4 (%)	94.12 + 1.13A + 0.94B - 2.37B - 5.11A^2	0.7926	1.43	0.0005	significant	Temperature (A)
MPR	10.44 + 0.66A - 5.29B + 3.03AB	0.7049	2.35	0.0009	significant	Influent COD (B)
COD rem. eff (%)	63.72 + 4.70A + 5.49B + 12.13AB - 16.71 A^2 + 8.35B^2	0.8678	5.81	0.0002	significant	-
Y_{CH4}	0.32 - 0.017A - 0.39B + 0.22A^2 + 0.10B^2	0.8106	0.14	0.0003	significant	Influent COD (B)
TCOD (%)	76.00 + 6.028A + 2.97AB - 14.02A^2	0.8209	4.67	<0.0001	significant	Temperature (A)

7.3.2 MPR, CH_4 yield and CH_4 content (%)

Table 7.1 shows different temperatures (37-70°C) and substrate concentrations (12,150 – 19,967 mg L^{-1}) used. Five responses were studied, *viz*. COD and TCOD removal efficiency, MPR, the yield, and finally methane content (%). Based on Figure 7.1, MPR increased as temperature rose from mesophilic to extreme thermophilic at the highest substrate concentration. The rate, however, declined when influent concentration increased under mesophilic condition (37°C).

The highest MPR of 17.88 L CH_4 day^{-1} was observed at 37°C using the lowest substrate concentration of 12,130 mg L^{-1}. Methane yield was decreasing as the substrate concentration increased from 12 g L^{-1} to almost 20 g L^{-1} at both 37°C and 70°C. The yield was decreased when thermophilic temperatures took place. The highest yield was reported to be 1.16 L CH_4 g^{-1} CODrem.d^{-1} at 70°C and 12,150 mg L^{-1} COD (Run 11). Meanwhile, the highest methane content was reported at 94.66% at 53.5°C and the substrate concentration of 16,300 mg L^{-1} COD. As shown in Figure 7.1, methane content was sharply increased when the temperature rose from mesophilic to thermophilic under lowest substrate concentration, before slowly decreased when temperature reaching 60°C.

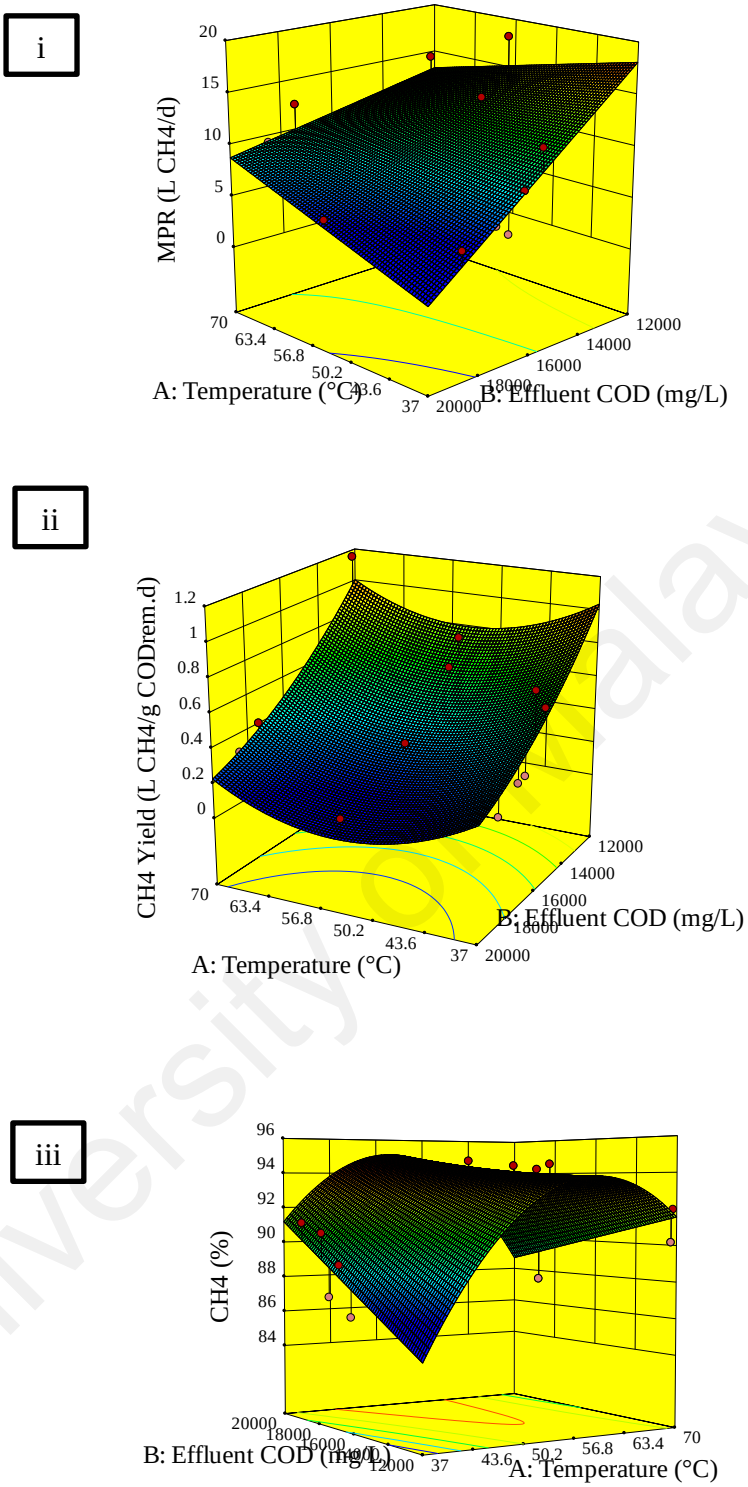


Figure 7.1: Effects of temperature and substrate concentration on (i) MPR; (ii) methane yield; (iii) methane content (%).

In the anaerobic digestion process, changes in temperature might lead to the changes in methane production rate as well. When POME was treated under different temperatures in other study, it revealed that thermophilic temperature gave a slightly higher in methane productivity when operated under thermophilic temperature (55°C) compared to mesophilic temperature (Choorit & Wisarnwan, 2007).

O-thong et al., (2016) utilized POME for biohydrogen and biomethane production using two-stage bioreactor. They obtained 9.8 L CH_4 L^{-1} POME within 15 days of HRT under mesophilic temperature (35°C). Meanwhile, Krishnan et al., (2017) achieved 3.2 L CH_4 L^{-1} day^{-1} with 5 days HRT using continuous stirred tank reactor (CSTR) under mesophilic condition. POME decomposition is a complex process in anaerobic digestion which involves the four biochemical processes. With each process, different specific microorganisms involved in CH_4-UASFF unit, especially during acetogenesis (hydrogenotrophic methanogens) and methanogenesis (acetoclastic methanogens) (Zabranska & Pokorna, 2017). An optimal HRT under anaerobic condition for organic content in hydrogenic effluent to be converted to methane by the role of methanogens was 15-20 days with pH of 7-8 (Mamimin et al., 2015). This proves that acetogenesis and methanogenesis took place, in which volatile fatty acids (VFAs) were converted to acetate and hydrogen in acetogenesis in H_2-UASFF unit before the conversion of acetate and CO_2 + H_2 to methane during methanogenesis. The referred studies were operated under more than a day of HRT for biomethane production. In this study, MPR was produced within a day with pH 7-8, indicating a good reactor performance as well. However, further study on finding the optimum HRT using the lower time for biomethane production in an integrated UASFF bioreactor is necessary to save the operation time while getting the highest substrate removal efficiency and yield as possible.

7.3.3 COD and TCOD removal efficiency (%)

A quadratic and reduced quadratic model was selected within the range of the factors to determine the response surface of COD removal efficiency and TCOD removal efficiency, respectively. The regression equations are presented as in Table 7.2, where A is temperature while B is substrate concentration. Figure 7.2 shows the simultaneous effects of those two factors towards COD and TCOD removal efficiency obtained from the equations in Table 7.2. The trend shows a significant decrease in COD removal efficiency in CH_4-UASFF unit solely with decreasing substrate concentration at a constant temperature (37°C). As shown in Table 7.1, the COD removal efficiency was decreased when low substrate concentration was used under extreme thermophilic condition. Changes in temperature in anaerobic digestion could affect the productivity of methanogens. Changes in environmental conditions must be carefully taken care of as this can lead to lowering the methane production rate, as methanogens are very vulnerable and prone to changes (Olvera & Alberto, 2015). A change in the source of the substrate and its concentration also could lead to this cause. This finding proved that effluent from dark fermentation in H_2-UASFF unit and temperature play a significant impact towards POME treatment efficiency in CH_4-UASFF unit under anaerobic digestion process.

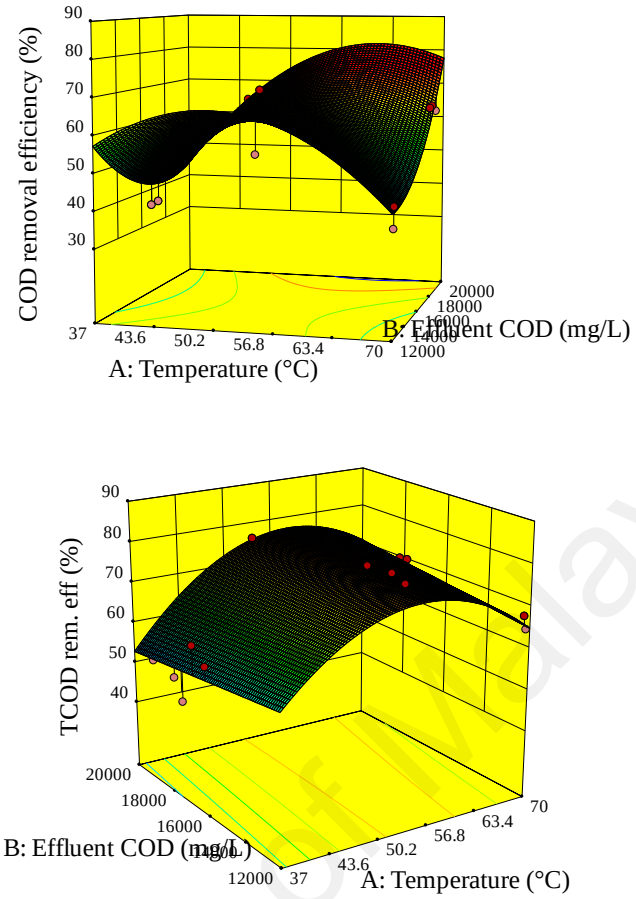


Figure 7.2: Effects of temperature and substrate concentration on COD removal efficiency and TCOD removal efficiency.

A significant increase in TCOD removal efficiency was observed when temperature increase from 37°C to between 50.2-56.8°C at a constant substrate concentration of 20 g L^{-1}. This is confirmed by its reduced quadratic model equation in Table 7.2 whereby temperature (A) had a greater effect on the response, compared to substrate concentration (B). The lowest efficiency of 47.62% was observed at mesophilic temperature (37°C) and highest substrate concentration. The efficiency was dropped between thermophilic to extreme thermophilic condition (56.8°C to 70°C) due to the biomass washout and foam formation observed in the gas collector. The foam formed due to rapid production of biogas in the reactor which might affected by the increase in

temperature (Xu, Li, Ge, Yang, & Li, 2018). The solubility between CH_4 and CO_2 is a big difference where methane was a bubble in the gas phase of the digestate, while the substantial amount of CO_2 was dissolved in the liquid phase as carbonic acid and HCO_3^- (Subramanian & Pagilla, 2014).

7.3.4 Process optimization using numerical and graphical to determine optimal conditions

In order to choose the optimum operating conditions, graphical optimization results were given in Figures 7.3 and 7.4. The shaded zones on the overlay plots in Figure 7.3 have met the proposed criteria. Based on the regression analysis from the historical data (data are based on the H_2-UASFF effluent concentration from Chapter 6), the optimum temperature and substrate concentration for maximum MPR, yield, CH_4 content (%), COD and TCOD removal efficiency were 54°C and 12 g L^{-1}. Under that optimum conditions, the maximum predicted MPR, yield, CH_4 content (%), COD and TCOD removal efficiency were 15.63 L CH_4 d^{-1}, 0.80 L CH_4 g^{-1} CODrem.d^{-1}, 93.31%, 66.30% and 76.10%, respectively with 77% desirability (Figure 7.4).

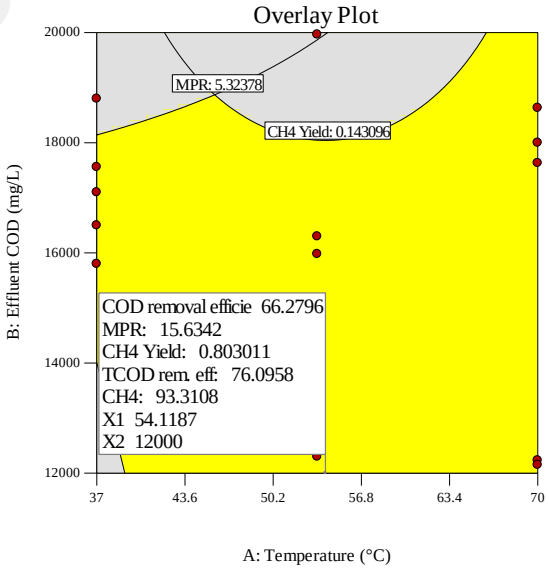


Figure 7.3: Optimization region (yellow) in an overlay plot utilizing POME wastewater in CH_4-UASFF unit.

From Figure 7.3, the intersection points show the conditions where the maximum temperature and substrate concentration (B) lied at 45°C and 18,863 mg COD L^{-1}, respectively. These results imply that with 24 h HRT, dark fermentation effluent from H_2-UASFF unit could be well treated under thermophilic condition. The thermophilic temperature used in this study might help in stabilizing the digestion process faster, besides promoting the growth of methanogenic bacteria and its efficiency. In addition, the thermophilic methanogens that were rapidly present in the mesophilic sludge/inoculum have become dominant under the new thermophilic conditions (Chachkhiani et al., 2004).

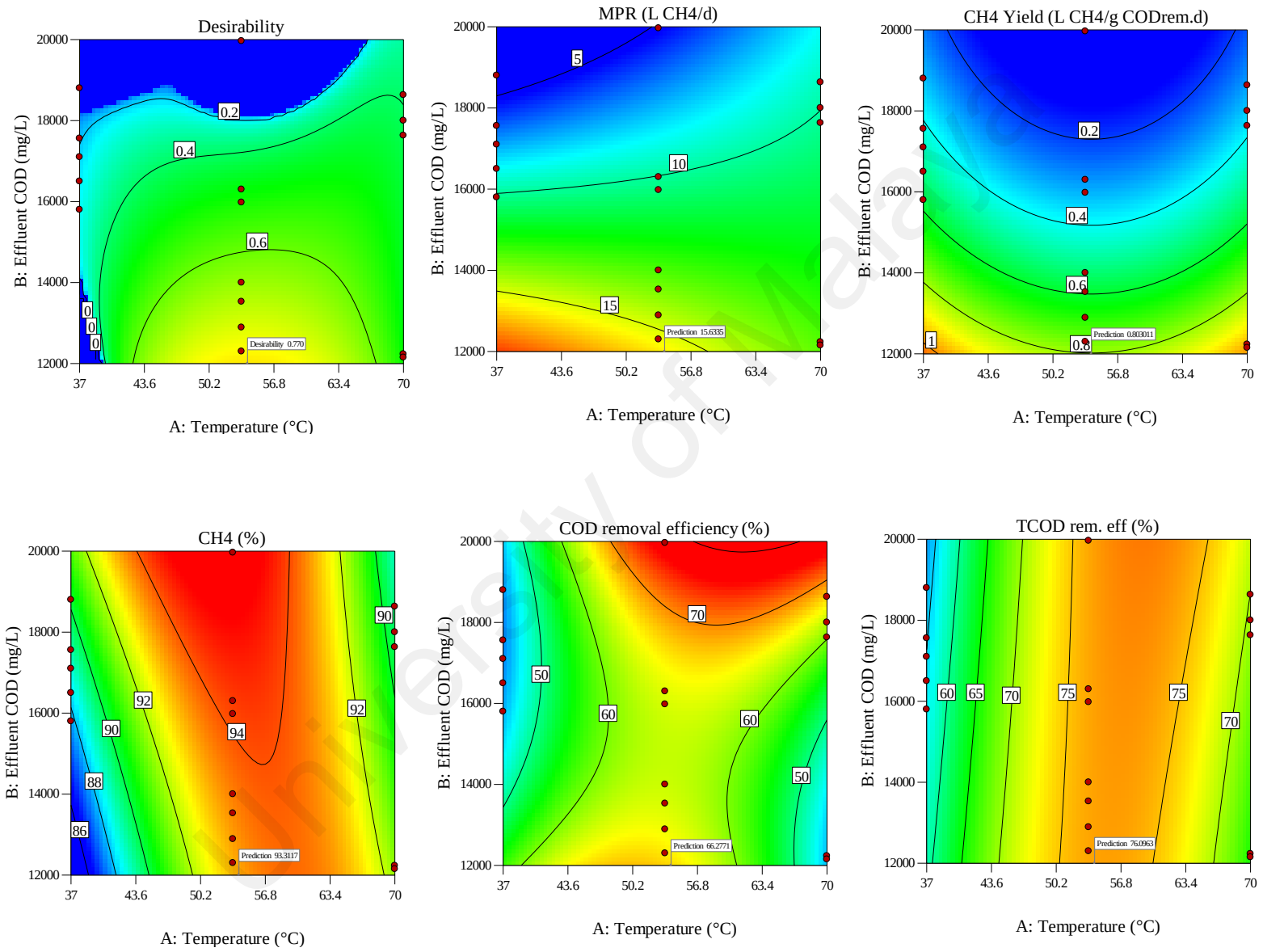


Figure 7.4: Counter plot for all responses with the highest desirability of 77% with optimum conditions of temperature (54°C) and influent COD (12 g L^{-1}).

7.4 Conclusion

Based on the experimental findings, 54°C was the optimum condition for biomethane production using two-stage UASFF bioreactor treating POME. With one day of HRT, anaerobic digestion process using UASFF bioreactor could achieve about 66% of COD removal efficiency in CH_4-UASFF unit alone. Also, utilizing dark fermentation effluent from H_2-UASFF bioreactor gave a significant increase in the methane production rate and yield of 15.63 L CH_4 d^{-1} and 0.80 L CH_4 g^{-1} $COD_{removed}.d^{-1}$, respectively. This concludes that hydrolysis and acidogenic processes were successfully operated in H_2-UASFF unit and thus, had significantly affect the performance of the CH_4-UASFF bioreactor. POME also could be treated by having 76% of total COD removal efficiency in two-stage UASFF bioreactor. However, further study on the kinetics of POME digestion reactions is necessary to ensure the consistency of reactor performance in a long-term operation.

CHAPTER 8: GENERAL CONCLUSION

8.1 Conclusion

POME is a good source as inoculum and substrate for biohydrogen and biomethane production in two-stage UASFF bioreactor. A two-stage UASFF bioreactor is good strategy in accelerating anaerobic granulation and achieving a high COD removal efficiency and H_2 yield in a relatively short period of time. The reactor was efficient in the fermentation of pre-settled POME at high OLR and short HRT. The bioreactor start-up time was less than two months after considering both the internal and external factors such as temperature, HRT, pH, substrate concentration, source of substrate, as well as minimizing the technical problems encountered during the process.

In batch study, the optimum inoculum to substrate ratio (I:S), temperature and HRT were 40:60, 50°C and 8 h, respectively with highest hydrogen yield was achieved at 28.47 mL H_2 g^{-1} $COD_{removed}$. When applying POME in two-stage UASFF bioreactor for a scale-up, several attempts had been applied during the start-up study to increase biohydrogen production. The results showed that source of substrate plays an important role for biohydrogen production, despite high temperature (i.e. 43°C) and short HRT (i.e. less than 24 h). To prove these conditions, an optimization study was conducted. Again, the results showed that in H_2-UASFF unit, temperature plays a significant role in hydrogen yield and hydrogen production rate whereas HRT has a significant impact on COD removal efficiency. The optimum temperature and HRT were 57°C and 7 h, respectively, with maximum hydrogen yield and hydrogen production rate of 0.95 L H_2 g^{-1} $COD_{removed}.d^{-1}$ and 10.39 L H_2 d^{-1}, respectively. The COD in POME was reduced up to 35%. Meanwhile, in a continuous operation, results in CH_4-UASFF unit showed that the

optimum temperature was 54°C with substrate concentration of 12 g L^{-1} in order to get highest methane yield, methane production rate, methane content an COD removal efficiency of 15.63 L CH_4 d^{-1}, 0.80 L CH_4 g^{-1} $COD_{removed}.d^{-1}$, 93.31% and 66.30%, respectively. The results concluded that thermophilic temperature significantly affected both hydrogen and methane production and increase overall COD removal efficiency in two-stage UASFF bioreactor by 76.10%.

Therefore, the significant and novelty of this study are:

1. By using high-rate anaerobic UASFF bioreactor, different wastewaters could be treated especially those contains high carbon source. For example, food and beverage (F&B) waste, sugar factory wastes (i.e. molasses), and municipal solid wastes.
2. Two-stage UASFF bioreactor treating POME for biohydrogen and methane production is the first study in Malaysia.

8.2 Recommendations for Future Research

For future research, these recommendations should be considered:

1. After optimizing operating factors (i.e. temperature, substrate concentration (I:S), HRT) in a two-stage UASFF bioreactor, efforts should be directed in the future for the application of kinetic and process data for scaling-up bioreactor to an industrial size with the economic evaluation.
2. For an industrial size of two-stage UASFF bioreactor, it is recommended to investigate the effect of mixing on biohydrogen and biomethane production from POME.

3. The feasibility of using different industrial wastewaters such as dairy, winery or food and beverages (F&B) for biohydrogen and biomethane production under the same operating condition should be compared and evaluated I the future using two-stage UASFF bioreactor.
4. A life cycle assessment (LCA) should be done for the estimation of energy used, energy recovery, carbon released and greenhouse gas (GHG) emission using two-stage UASFF bioreactor.
5. Microbial analysis using PCR-DGGE is recommended in the future to analyze the microbial community presence in the inoculum and substrate for their roles in biohydrogen and methane production.

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LIST OF PUBLICATIONS AND PAPERS PRESENTED

1. Effects of process, operational and environmental variables on biohydrogen production using palm oil mill effluent (POME). *International Journal of Hydrogen Energy*, 43 (2018): 10637-10644. **Published. Q1, IF 4.229**.
2. UASFF start-up for biohydrogen and biomethane production from treatment of Palm Oil Mill Effluent. *International Journal of Hydrogen Energy*, 44 (2018): 37 20725-20737. **Published. Q1, IF 4.229**.
3. Biohydrogen production from POME wastewater in UASFF bioreactor - Optimization of temperature and hydraulic retention time. *International Journal of Hydrogen Energy*. **Under Review. Q1, IF 4.229**.
4. Effects of temperature and effluent from H_2-UASFF on biomethane production in CH_4-UASFF bioreactor. *FUEL*. **Under Review. Q1, IF 4.908**.
5. A Review Study on Integrated System of Dark Fermentation Coupled with Anaerobic Digestion for Biohydrogen and Biomethane Production. *Polish Journal of Environmental Studies.* **Accepted. Q4, IF 1.120.**
6. Effects of process, operational and environmental variables on biohydrogen production using palm oil mill effluent (POME). 2nd International Hydrogen Technologies Congress – 2017, Cukurova University, March 15-18, Adana, Turkey. **ORAL PRESENTER.**
7. UASFF start-up for biohydrogen and biomethane production from treatment of Palm Oil Mill Effluent. McGill-Polytechnique Chemical Engineering Research Day, March 22nd, 2018, McGill University, Montreal, Canada. **ORAL PRESENTER.**

8. UASFF start-up for biohydrogen and biomethane production from treatment of Palm Oil Mill Effluent. IGEM 2018, Oct 17-20, 2018, Kuala Lumpur Convention Centre (KLCC). **POSTER PRESENTER.**
9. Part 1: Optimization of temperature and hydraulic retention time for biohydrogen production from POME in H_2-UASFF bioreactor using Response Surface Methodology. International Conference on Sustainable Energy and Green Technology (SEGT 2018), 11-14 December 2018, DoubleTree by Hilton, Kuala Lumpur, Malaysia. **ORAL PRESENTER.**
10. Part 2: Effects of temperature and effluent from H_2-UASFF on biomethane production in CH_4-UASFF bioreactor. 8th International Conference on Bioprocessing 2019 (IFIBiop 2019), 1st-5th May 2019, Miri, Sarawak, Malaysia. **ORAL PRESENTER.**
11. Biohythane from Agricultural Wastes. Climate Launch Pad (CLP 2018). Bootcamp from 25 July – 27 August 2018, MyMagiC, Cyberjaya. **PARTICIPANT.**
12. Treatment system for palm oil mill effluent (POME) wastewater and simultaneously producing biohydrogen and biomethane using two-stage up-flow anaerobic sludge fixed-film (UASFF) bioreactor. **PATENT APPLICATION SUBMITTED.**