BIOHYDROGEN PRODUCTION FROM PALM OIL MILL EFFLUENT USING POLYETHYLENE GLYCOL IMMOBILIZED CELLS IN UPFLOW ANAEROBIC SLUDGE BLANKET REACTOR

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STATEMENT OF AWARD FOR DEGREE

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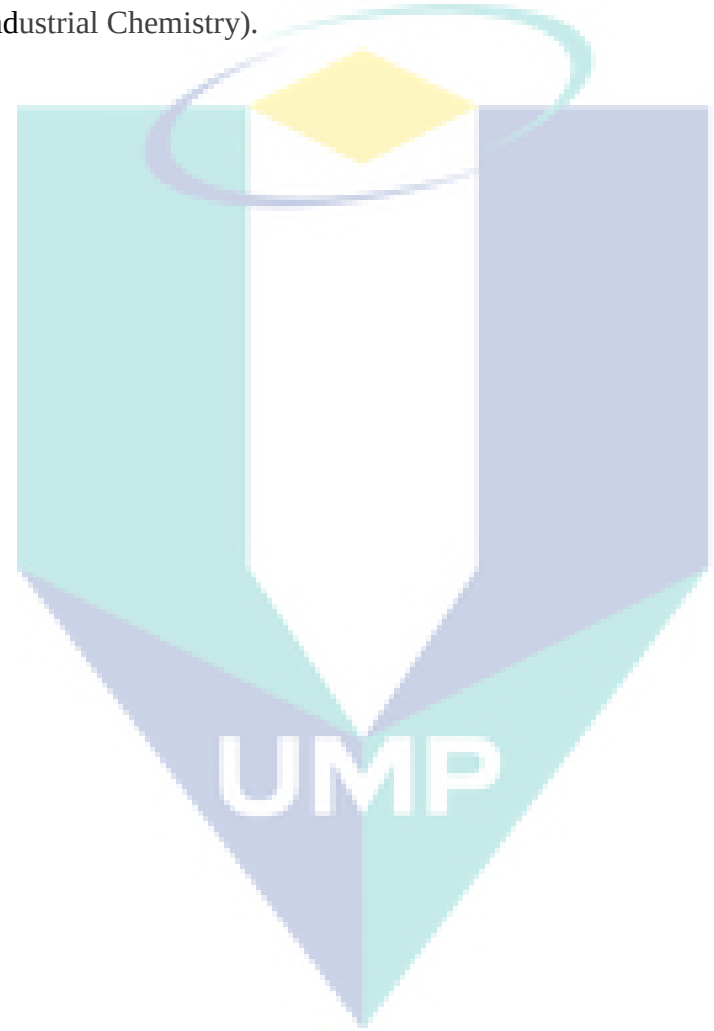

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Specially dedicated to my parents who have supported me all the way since the beginning of my studies. Also, this thesis is dedicated to my dearest fiancé who has been a great source of motivation and inspiration.

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ABSTRACT

The demand for improvement of the hydrogen production by dark hydrogen fermentation is increasing. Recently, a number of cell-immobilization systems were used to improve dark hydrogen production. The main objective of this research was to examine the polyethylene glycol immobilized cells system in enhancing hydrogen production and treatment of palm oil mill effluent (POME). During this research five experiments were performed. In the first experiment, PEG gel was fabricated and used as a carrier to immobilize *Clostridium sp.* for biohydrogen production using POME. POME was diluted and used as a substrate. The resulting PEG-immobilized cells were found to yield 5.35 L H_2/L-POME, and the maximum hydrogen production rate was 0.5 L H_2/L-POME/h (22.7 mmol/L h). The Monod-type kinetic model was used to describe the effect of substrate (POME) concentration on the hydrogen production rate. Furthermore, PEG- immobilized cell was examined for H_2 production in comparison to suspended cell reactor. The suspended-cell containing reactor was able to produce hydrogen at an optimal rate of 0.348 L H_2/L-POME/h at HRT 6 h. However, the immobilized-cell containing reactor exhibited better hydrogen production rate of 0.589 LH_2/ L-POME/h which occurred at HRT 2 h. When the immobilized-cell containing reactor was scaled up to 5 L, the hydrogen production rate was 0.553–0.589 L H_2/ L-POME/h. Another study addressed the application of a PEG-immobilized upflow anaerobic sludge blanket (UASB) reactor using *Clostridium sp.* for enhancing continuous hydrogen production from POME. The UASB reactor containing immobilized cells was operated at varying hydraulic retention times (HRT) that ranged from 24 to 6 h at 3.3 g chemical oxygen demand (COD)/L/h organic loading rate (OLR), or at OLRs that ranged from 1.6 to 6.6 at 12 h HRT. The maximum volumetric hydrogen production rate of 0.336 LH_2/L/h (15.0 mmol/L/h) with a hydrogen yield of 0.35 LH_2/g $COD_{removed}$ was obtained at a HRT of 12 h and an OLR of 5.0 g COD/L/h. The effect of immobilized cell packing ratio, HRT and POME concentration on continuous hydrogen production and treatment efficiency of palm oil mill effluent was studied. The UASB reactor with a PEG-immobilized cell packing ratio of 10% weight to volume ratio (w/v) was optimal for dark hydrogen production. The highest volumetric hydrogen production rate of 0.365 L H_2/L/h (16.2 mmol/L/h) with a hydrogen yield of 0.38 LH_2/g $COD_{removed}$ was obtained at POME concentration of 30 g COD/L and HRT of 16 h. The average hydrogen content of biogas and COD reduction were 68% and 66%, respectively. In the final study, optimization of the hydrogen production capability of the immobilized cells, including PEG concentration, cell loading, curing times as well as effects of temperature and different inorganic components concentrations on hydrogen production rate were studied. Result showed that with an optimal PEG concentration (10 % w/v), cell loading (2.4 g dry wt.), curing time (80 min) and inorganic components **(**$NiCl_2$ 1 mg/L, $FeCl_2$ 300 mg/L and $MgSO_4$ 100 mg/L), attaining an excellent hydrogen production rate of 7.3 L/L-POME/d and a hydrogen yield of 0.31 L H_2/g COD in continuous operation.

ABSTRAK

Permintaan untuk peningkatan pengeluaran hidrogen oleh penapaian hidrogen gelap semakin meningkat. Banyak kajian mengenai sistem pergerakan sel telah dijalankan untuk meningkatkan pengeluaran hidrogen secara anaerobik. Objektif utama kajian ini adalah untuk mengenalpasti PEG yang tidak bergerak dalam sel-sel sistem bagi meningkatkan pengeluaran biohidrogen serta rawatan efluen kilang minyak sawit (POME). Lima eksperiman telah dijalankan dalam menjayakan kajian ini. Pada eksperiman yang pertama, gel novel PEG telah difabrikasi digunakan sebagai bahan bagi mengangkut *Clostridium* sp bagi tujuan penghasilan biohidrogen daripadaPOME. POME telah dicairkan dan digunakan sebagai substrat. PEG-sel tidak bergerak yang terhasil didapati menghasilkan 5.35 L H_2/L-POME, dan kadar pengeluaran hidrogen maksimum adalah 0.5 L H_2/L-POME/ h (22.7 mmol/L h). Model Monod kinetik telah digunakan untuk menggambarkan kesan kepekatan substrat (POME) terhadap kadar pengeluaran hidrogen. Selain itu, sel PEG-tidak bergerak telah diperiksa dalam penghasilan hidrogen berbanding sel reaktor-terampai. Sel terampai terkandung dalam reaktor mampu menghasilkan hidrogen pada kadar yang optimum iaitu 0.348 L H_2/L-POME/h pada HRT 6 h. Walau bagaimanapun, reaktor mengandungi sel tidak bergerak menunjukkan kadar pengeluaran hidrogen yang lebih baik 0.589 L H_2/ L-POME/h, yang berlaku pada HRT 2 h. Apabila sel tidak bergerak digunakan dalam reaktor yang berskala 5 L, kadar pengeluaran hidrogen adalah 0.553-0.589 L H_2/L-POME/h. Kajian lain pula menggariskan penggunaan PEG-bergerak aliran ke atas selimut enapcemar anaerobik (UASB) reaktor yang menggunakan *Clostridium* sp. dalam meningkatkan pengeluaran hidrogen daripada POME secara proses berterusan. Reaktor UASB yang mengandungi sel tidak bergerak telah dioperasikan pada pelbagai masa tahanan hidraulik (HRT) antara 24 ke 26 jam pada 3.3 g oksigen dengan kandungan kimia (COD) /L/h kadar daya organik (OLR), atau pada OLRs yang berjulat antara 1.6 ke 6.6 pada 12 h HRT. Isipadu maksimum pengeluaran hidrogen adalah pada kadar 0.336 LH_2/L/h (atau 15.0 mmol/L/h) dengan hasil hidrogen iaitu 0.35 LH_2/g $COD_{removed}$ telah diperolehi pada HRT 12 h dan OLR sebanyak 5.0 g COD/L/h. Kesan daripada nisbah pembungkusan sel bergerak, HRT dan kepekatan POME ke atas penghasilan hidrogen secara berterusan dan keberkesanan rawatan ke atas efluen kilang kelapa sawit telah dikaji. Nisbah pembungkusan sel bagi reaktor UASB dengan sel PEG-tidak bergerak menunjukkan 10% daripada berat kepada nisbah isipadu adalah ratio yang optimum bagi pengeluaran hidrogen dalam keadaan gelap. Kadar pengeluaran hidrogen tertinggi dengan isipadu 0.365 LH_2/L/h (atau 16.2 mmol/L/h) dengan penghasilan hidrogen sebanyak 0.38 L H_2/g $COD_{removed}$ telah diperolehi pada POME yang berkepekatan 30 g COD/L dan HRT pada jam ke-16. Purata kandungan hidrogen dan pengurangan COD adalah sebanyak 68% dan 66%. Pada pengakhiran kajian, pengoptimuman keupayaan penghasilan hidrogen bagi sel-sel yang tidak bergerak termasuk kepekatan PEG, daya sel, kaedah pengawetan serta kesan suhu dan bahan kimia inorganik pada kepekatan berlainan terhadap kadar pengeluaran hidrogen turut dikaji. Keputusan menunjukkan bahawa dengan penumpuan PEG optimum (10% w / v), beban sel (2.4 g kering berat.), mengubati masa (80 min) dan komponen-komponen organik ($NiCl_2$ 1 mg / L, $FeCl_2$ 300 mg / L dan $MgSO_4$ 100 mg / L), mencapai kadar pengeluaran hidrogen yang sangat baik sebanyak 7.3 L / L-POME / d dan hasil hidrogen 0.31 L H_2 / g COD dalam operasi yang berterusan.

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LIST OF ABBREVIATIONS/SYMBOLS

ASBRs	Anaerobic sequencing batch reactors
AHB	Annular hybrid bioreactor
BOD	Biochemical oxygen demand
BuOH	Butanol
CPO	Crude palm oil
COD	Chemical oxygen demand
CO_2	Carbon dioxide
CH_4	Methane
CSTR	Continuous stirrer tank reactor
CA	Calcium alginate
CH	Chitosan
CDW	Cell dry weight
DOE	Department of Environment
DNA	Deoxyribonucleic acid
K_2HPO_4	Dipotassium phosphate
EtOH	Ethanol
EQA	Environmental Quality Act
EFB	Empty fruit bunches
EVA	Ethylene-vinyl acetate
FFB	Fresh fruit bunches
F/M	Food to microorganism ratio
$FeCl_2$	Ferrous chloride

GHG	Green house gases
GC	Gas chromatograph
GAC	Granular activated carbon
HRT	Hydraulic retention time
HBu	Butyric acid
HAc	Acetic acid
HPr	Propionic acid
HPR	Hydrogen production rate
IBR	Immobilized bioreactor
KOH	Potassium hydroxide
MT	Metric Tons
MPOB	Malaysian Palm Oil Board
MBA	N,N'-methylenebisacrylamide
$MgSO_4$	Magnesium sulphate
NaOH	Sodium Hydroxide
NaCl	Sodium chloride
$NiCl_2$	Nickel chloride
OLR	Organic loading rate
POME	Palm oil mill effluent
PEG	Polyethylene glycol
PVA	Polyvinyl alcohol
PAM	Polyacrylamide
PDMS	Polydimethylsiloxane

PMMA	Polymethyl methacrylate
PO_4^{3-}	Phosphate
$K_2S_2O_8$	Potassium persulfate
SCB	Sugarcane bagasse
SEM	Scanning electron microscope
SMP	Soluble microbial products
SS	Suspended solids
TN	Total nitrogen
TKN	Total Kjeldahl nitrogen
TS	Total solid
TP	Total phosphorus
TVFA	Total volatile fatty acid
UASB	Upflow anaerobic sludge blanket
UFR	Up-flow reactor
VSS	Volatile suspended solids
VFAs	Volatile fatty acids
w/v	weight to volume
^oC	Degree Celsius
h	Hour
m^3	Meter cube
min	Minute
MW	Megawatt
g/L	Gram/Liter

CHAPTER 1

INTRODUCTION

1.1 INTRODUCTION

This chapter describes a brief description of palm oil industry in Malaysia, palm oil mill effluent (POME), cell immobilization, upflow anaerobic sludge blanket (UASB) reactor and hydrogen production. The first and second part of chapter explains about palm oil industry in Malaysia, hazard of POME wastewater and reuse of POME as fermentation media. Third part explain about cell immobilization system. The fourth part discusses production of biohydrogen. Finally, in the last part of this chapter, problem statement, objectives and scope of study are described.

1.2 BACKGROUND

1.2.1 Palm oil industry in Malaysia

Oil Palm was first introduced to Malaysia in year 1875 as an ornamental plant (DOE, 1999). Malaysia has the most ideal climate conditions for growing oil palm. The growth of the palm oil industry in Malaysia has been phenomenal over the last 4 decades. The Malaysian palm oil industry has grown rapidly over the past few years to become the world's second largest producer of palm oil, accounted for 10.3% of the world's oils and fats production in year 2007 (Lam and Lee, 2011). The palm oil industry served as an important backbone to the country economy and has significantly increased the standard of

living among Malaysians (Yusoff and Hansen, 2007). Morever, the palm oil industry provides a source of livelihood to rural families in govermental land schemes and private small holders, as well as employment opportunities to, agricultural workers in estates (Wu et al., 2010). For example, in 2003, more than 3.79 million hectares of land were under oil palm cultivation, occupying more than one-third of the total cultivated area in Malaysia and 11% of the total land area. In year 2008, the total export of palm oil and derived products raked RM 64,808 million (USD 20,268 million), or 9.8% of the total national revenue (Yusof, and Yew, 2009). As of 2009, there were 416 palm oil mills operating in Malaysia, 249 mills from Peninsular Malaysia and 167 from Sabah and Sarawak. There were 120 mills with total capacity 29,893, 200 tonnes FFB per year located in Sabah alone. Total of 17,564,937 Metric Tons (MT) crude palm oil (CPO) produced in year 2009 and 31.03% of total CPO was produced in Sabah (MPOB, 2009). The oil palm planted area in 2011 increased 3% y/y to 5 mn hectares due to increase in planted area in Sabah and Sarawak. CPO production in 2011 increased 11.3% y/y to reach a record-high of 18.9 mn. It was claimed that further expansion of oil palm industry will cause severe negative impacts toward environment such as destruction of orangutan habitat, deforestation and green house gases (GHG) emission due to over exploration of peat land for oil palm plantation (Laurance et al., 2010, and Yule, 2010). Nevertheless, criticisms are not only limited to plantation sector, but also include palm oil mills.

1.2.2 The palm oil mill effluent

The production of palm oil is increasing every year due to its application for biodiesel production after the announcement of Fifth Fuel Policy under Eighth Malaysia Plan (2001–2005) (Lim and Teong, 2010). This leads to the increasing amount of palm oil mill effluent (POME); a by-products from the oil-palm extracting process (Poh and Chong, 2009). In Malaysia, the estimated annual production of palm oil mill effluent (POME) is about 50 million tons. POME is a viscous brown liquid cosisiting of 92-94% water, 6-7% total solids, 2-4% suspended solids (SS) and 0.7-0.8% oil. Discharge of untreated POME directly into the water streams, its is certain to cause considerable environmental problems due to highy value of chemical oxygen demands (COD) and biochemical oxygen demands

(BOD) that it generates (Lam and Lee, 2011). The palm oil industry in malaysia has thus been identified as that which discharges the largest pollution load into the water bodies and the environment throught the country (Wu et al., 2010). This adverse environmental effect from the palm oil industry cannot be ignored. Thus, there is an urgent need to find an efficient and practical approach to preserve the environment while maintaining the economy in good condition.

1.2.3 POME as fermentation media

The high compositions and concentrations of carbohydrate, protein, nitrogenous compound, lipid, mineral and nutrient content in POME makes it an ideal fermentation medium for biotechnological means (Hwang et al., 1978 and Habib et al., 1997). POME and its derivatives have been exploited as fermentation media to produce various products/metabolites such as bioinsecticides, antibiotics, polyhydroxyalkanoates, solvents, organic acids as well as enzymes to varying degrees of success (Wu, T.Y. 2007). Since POME contains high level of organic matters and thus, adoption of anaerobic digestion in the first stage of the treatment process is a necessity to convert the bulk of the wastes to biogas (biomethane). The treated effluent is further subjected to an aerobic treatment in order to meet the required discharge standards. These treatment steps have been applied either as an open pond or open digesting tank systems in Malaysian palm oil mills. However, due to the lack of infrastructure and low demand of renewable energy (biogas) in the county, biomethane is not captured but escapes directly to the atmosphere and thus caused serious air pollution. Methane has been categorized as one of the GHG with its global warming potential 21 times more potent than CO_2. On other hand, potential of using raw POME as the main substrate to produce biohydrogen has been intensively studied (Ismail et al., 2010) since the generated hydrogen and its combustion production do not count as green house gases (Koroneos et al., 2004). Naturally, POME contains lignocelluloses and hemicelluloses material (complex carbohydrate polymers) which resulted to high COD value (15,000–100,000 mg/L) (Chong et al., 2009a). Due to this reason, POME can become a suitable substrate for biohydrogen production and act as a

wastewater treatment process simultaneously. To date, production of biohydrogen from POME in commercial scale is not ready based on current production technology.

1.2.4 Cell immobilization

An immobilized cell is defined as a microbe that prevented from moving independently of its neighbors to all parts of the aqueous phase of the system by natural or artificial means. Cells immobilization is a versatile tool that serves to increase the stability of a microbial system, allowing its application under extreme environmental conditions, its reuse and the development of continuous bioprocesses (Anisha and Prema, 2008). Immobilization of living cells can improve the process economy by increasing reusability and/or feasibility (Salter and Kell, 1991). This technology provides an innovative procedure for the immobilization of bacteria that can be used to improve the performance and stability of biological treatment systems. The use of immobilized cell systems is well documented for the production of valuable product like enzymes, antibiotics, organic acid and alcohols. Several methods can be applied to immobilization microorganism on the carriers by using artificial way, these methods including: covalent bonding, cross-linking of microorganism, adsorption and encapsulation into a polymer-gel and entrapment in a matrix, and so on (Cassidy et al., 1996). The cell entrapment is one of the most widely applied methods for cell immobilization, in which microorganisms are enclosed in a polymeric matrix which is porous enough to allow the diffusion of substrates to the cells and of product away from the cells. Entrapment of bacteria provides both an appropriate growth environment for the organism (Zhu et al., 1999) and structural, thermal and chemical stability to the entrapped cells (Dickson et al., 2009). Thus, immobilization-cell systems are also adapted with a feature of creating a local anaerobic environment, which is well suited to oxygen perceptive fermentative hydrogen production.

The application of immobilized cells to hydrogen production has been reported such as ethylene vinyl acetate copolymer (Wu et al., 2005b), polyvinyl alcohol (Tian et al., 2009), lignocellulosic materials (Kumar and Das, 2001), sodium alginate (Ishikawa et al., 2006), calcium alginate (Hu et al., 2007). Nearly all their work showed that immobilized

fermentative bacteria can enhance and stabilize hydrogen production process. However, Natural polymers (agar, agarose, alginate, kappa-carragenan) possess poor mechanical strength and durability, although they are not toxic to microorganisms. Conversely, synthetic polymers have strong mechanical strength and durability but are often toxic to microorganisms (Kuu and Polack, 1983). Polyethylene glycol (PEG) is a promising type of synthetic polymer, which is cheap, non-toxic to microorganisms, good mechanical properties and highly porous structure that helps to sustain immobilized cell viability (Leenen et al., 1996). PEG is the only immobilization matrix used in large-scale wastewater–treatment plant in Japan and more than five years of gel durability has been demonstrated (Takeshima et al., 1993).

1.2.5 Up-flow anaerobic sludge blanket (UASB) reactor

In spite of their early introduction, the interest on anaerobic systems as the main biological step in wastewater treatment was scarce until the development of the upflow anaerobic sludge blanket (UASB) reactor in the early 70s (Lettinga et al., 1980). The success of the UASB concept depends on the establishment of a dense sludge bed in the bottom of the reactor, in which the biological processes take place (Lettinga, 1995). The formation of sludge bed takes place by the accumulation of the incoming suspended solids and the growth of bacteria. UASB reactor essentially consists of gas-liquid-solids separator (to retain the anaerobic sludge within the reactor), an influent distribution system and effluent draw off facilities. UASB reactor is a high effective, energy saving and less construction area technology. UASB process is a combination of physical and biological processes. The main feature of physical process is separation of solids and gases from the liquid and that of biological process is degradation of decomposable organic matter under anaerobic conditions. Wastewater enters at the bottom of the reactor. At the top, biogas is collected and the effluent of treated water leaves (Figure 1.1). At the upper part of the reactor, above the sludge bed, a blanket zone is formed where some particles of biomass are suspended. This zone acts as a separation zone between the water flowing up and the suspended biomass. Performance depends on the mean cell residence time and reactor volume depends on the hydraulic residence time, therefore, UASB reactor can efficiently

convert wastewater organic compounds into biogas. Among thousands of anaerobic full scale tretment facilities worldwide, approximately 60% are based on the UASB design concept, treating a various range of industrial wastewaters (Jung et al., 2012 and Jantsch et al., 2002).

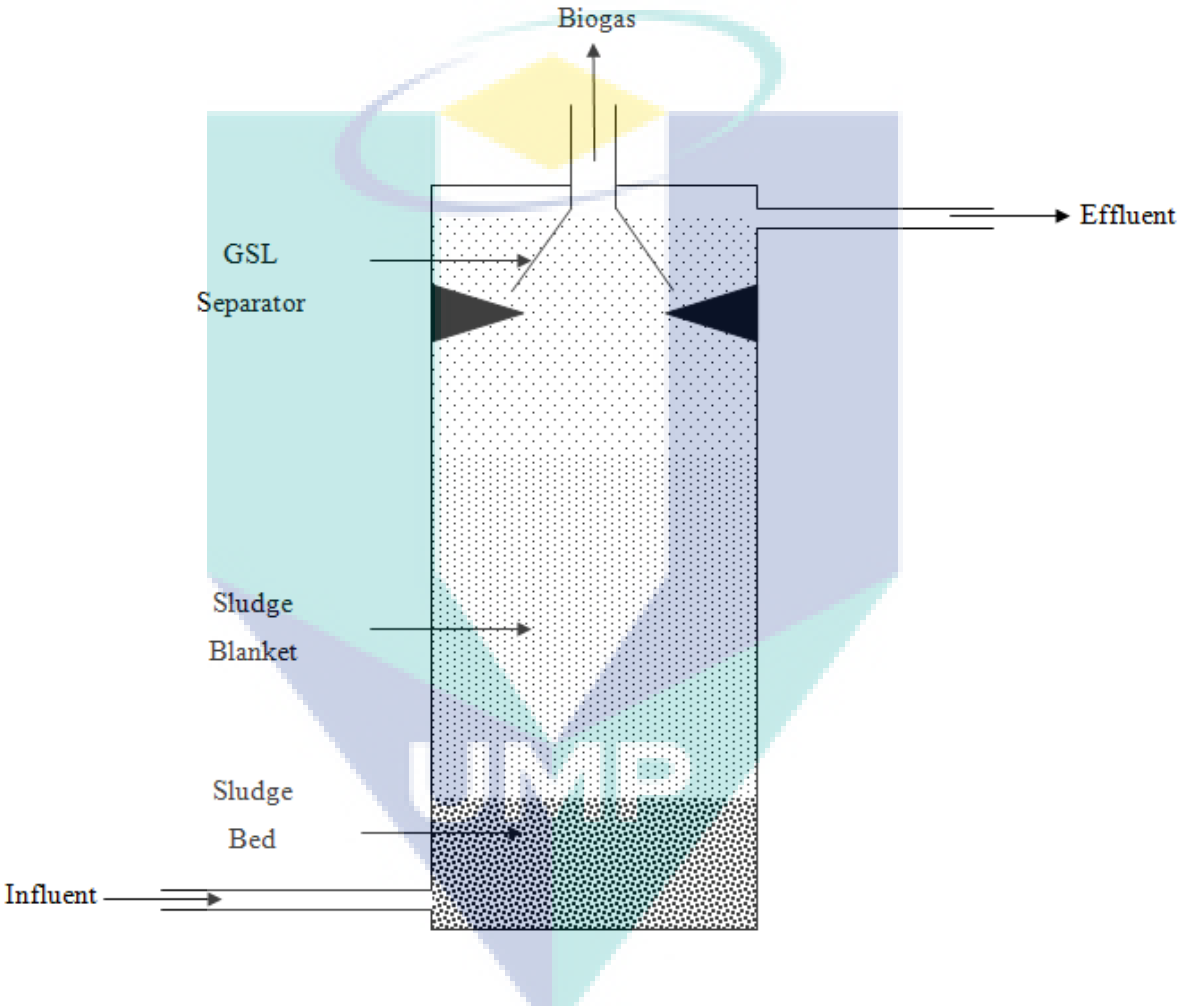

Figure 1.1: Upflow anaerobic sludge blanket reactor

1.2.6 Biohydrogen production

The joint challenges of environmental crises and dwindling fossil fuel supplies are driving intensive research focus in alternative energy production. Hydrogen is widely regarded as one of the most potential future energy vectors, capable of assisting in issues of

environmental emissions, energy security and versatility as fuel (Pakarinen et al., 2008). When hydrogen is used as a fuel, its main combustion product is water which can be recycled again to produce more hydrogen. In recent years widely uses of hydrogen have been demonstrated *viz.* hydrogenfueled transit buses, ships and submarines etc., including chemical and petrochemical applications. Despite the "green" nature of hydrogen as a fuel, it is still usually produced via steam reforming of natural gas, petroleum hydrocarbon, nonrenewable materials and other hydrogenation reactions, which makes hydrogen production environmentally unfriendly (Ginkel and Sung, 2001). Biological process for hydrogen production is one of the alternative methods, can be operated at ambient temperatures, pressures, less energy intensive and more eco-friendly compared to conventional chemical method (Wang and Wan, 2009). This process is not only eco-friendly, but also escort to open new path for the exploitation of renewable energy resources which are unlimited. Biohydrogen production can be achieved by biophotolysis, photosynthesis /photo-fermentation, and dark fermentation. In a biophotolysis process, H_2O is "split" into H_2 and O_2 by green algae or cyanobacteria through two photosystems, and high intensity light is required to drive this process. With photo-synthesis/photo-fermentation, photosynthetic bacteria use a short-chain organic acid (e.g., acetate) as substrate to produce hydrogen through photosynthetic metabolism. Compared with biophotolysis, photo-fermentation is capable of using a wide spectrum of light, and it lacks oxygen evolving activity, which would otherwise cause oxygen inactivation. In addition, photosynthetic bacteria can utilize organic substrates from wastewater and the conversion yield is relative high. Photo-fermentation, however, has its own limitations (Kapdan and Kargi, 2006). Although oxygen inhibition is not as significant as in biophotolysis, it is still an inhibitor for the system. The process is also potentially limited by light availability.

Dark fermentation of sugar and organic waste materials presented a promising route of biohydrogen production because of its numerous advantages over other forms of hydrogen production. The major advantages of dark fermentative process are high rate of cell growth, operation without light source and no oxygen limitation problems (Levin et al., 2004). Various attempts have been made to generate fermentative hydrogen from biomass and wastewater like sugarcane bagasse (SCB) (Pattre et al., 2008), wheat straw (Chu, Y.

2011), cheese whey wastewater (Azbar et al., 2009) and dairy waste (Mohan et al., 2009). However, low hydrogen yields, low production rates and reactor instability for continuous high volume hydrogen production remain the major problems of the anaerobic method for hydrogen production at commercial levels. Another potential approach to enhance hydrogen production in anaerobic hydrogen production is to use an immobilized cell system (Hu and Chen, 2007). Immobilized cells offer distinct advantages over suspended cells, because they are resistant to cell wash-out during continuous operation and can maintain a higher cell density that increases hydrogen production. Immobilized cells have been successfully used for continuous biohydrogen production in a bioreactor (Chu, C.Y. 2011; Peixoto et al., 2011 and Keskin et al., 2011).

1.3 PROBLEM STATEMENT

Palm oil mill wastewater treatment systems are one of the major sources of green house gases in Malaysia due to their biogas emission (36% CH_4 with a flow rate of 5.4 l/min.m^2) from open digester tanks (Yacob et al., 2005). Also, the treated POME using ponding system sometimes could not meet the required discharge standards.

However anaerobic treatment of POME by UASB process is the most promising and useful technology due to the positive energy balance, inexpensive and high rate treatment system along with the production of usable biogas (Latif et al., 2011). Methane production through anaerobic digestion of POME is already broadly applied. Besides, methane has a relative lower calorific value (36.3 kJ/g), and CO_2 (22 g) is released during its combustion. In contrast, hydrogen gas has higher calorific value (118.2 kJ/g), and its reaction with oxygen does not produce green house gases such as CO_2. For example, to produce 1 kWh of eletricity need to burn ~ 30.5 g hydrogen where as to produce same amount of eletricity around ~ 90 g of methane (three times) must be burned. From this perspective, anaerobic bioconversion or organic wastes to hydrogen gas is an attractive option that achives both the goals.

Recently, the potential of using POME as the main substrate to produce biohydrogen has been revealed by severals group of researchers (Chong et al., 2009a; Chong et al., 2009b; Ismail et al., 2010 and Prasertsan et al., 2009). Up to now, biohydrogen production from POME in industrial scale is not ready based on current production technology. Apart from storage and safety problems of biohydrogen, problems associated with reactor design, long retention time and washout of bacteria with effluents may occur from the reactor at shorter HRTs of continuous dark fermentation have been identified as the major constrains to biohydrogen production. Another potential approach to conceptualize commercial scale of biohydrogen production is through cell immobilization. Bacteria immobilization enhances the available bacteria population in the reactor, increases fermentation rates, shortens the fermentation period, and increases the productivity. Recent studies show that different immobilization methods can be used as effective means to increase the hydrogen productivity with both pure and mixed cultures without washout of bacteria in the reactors at shorter HRTs (Chu, C.Y. 2011; Plangklang et al., 2012, and Zhao et al., 2012). This special feature clearly suggests that using immobilized cells might reduce the operational cost by gaining a comparable hydrogen producing capacity at a higher organic load rate (or lower HRTs). Polyethylene glycol (PEG) with additional merits was selected in this work for entrapment due to its simple immobilization procedure, low toxicity, good mechanical properties and highly porous structure that helps to sustain immobilized cell viability (Leenen et al., 1996). To the best of our knowledge, this is the first report of state of the art PEG-immobilized cell system for dark fermentative hydrogen production from POME.

1.4 RESEARCH OBJECTIVES

a) Main objective

The main objective of this study was to examine the enhanced biohydrogen production from POME using PEG-immobilized cells in a UASB reactor.

b) Specific Objectives

i. To determine the feasibility of PEG as a carrier to immobilize *Clostridium sp.* for hydrogen production from POME in batch test.

ii. To determine the hydrogen production performance of PEG-immobilized cell containing reactor in comparison to suspended cell reactor.

iii. To examine the application of a PEG-immobilized cell for continuous hydrogen production from POME in UASB reactor.

iv. To determine the effect of immobilized cell packing ratio, hydraulic time retention time and POME concentration on hydrogen production and treatment efficiency of POME.

v. To determine the optimized condition for hydrogen production capability of the immobilized cell, the conditions for cell immobilization including PEG concentration, cell loading, curing times as well as effects of temperature and inorganic components on hydrogen production.

1.5 SCOPE OF STUDY

To accomplish the above objectives, the following tasks were undertaken:

1. Raw POME was obtained from the final discharge point of a palm oil mill wastewater treatment plant, Lepar Hilir Pahang, Malaysia.
2. *Clostridium sp.* was obtained from Institute for Medical Research, Kuala Lumpur, Malaysia and used as an inoculum. Bacteria was acclimatized and stored in sterile 15% (v/v) glycerol solution at −10 ^{o}C before being subjected to immobilization.
3. Polyethylene glycol (PEG) gel was fabricated and used as a solid carrier to immobilize *Clostridium sp.*
4. Scanning electron microscopy (SEM) was used in order to examine the entrapped cells inside the PEG-immobilized bead.
5. The mechanical stability of the immobilized beads was expressed by the fracture frequency of the beads, f (%) = [N/Nt] × 100, where N is the number of fractured beads and Nt is the total number of beads.

6. Batch tests were carried out to study the effect of time 4 to 24 h on cell leakage from the bead into medium suspension and consequent effect on hydrogen production. Monod type kinetics model was used to study the effect of acclimated immobilized cells and unacclimated immobilized cell on hydrogen production.
7. The UASB reactor was designed by using stain less steel with a total volume of 5126 cm^3 and a 5-L working volume and used for continuous hydrogen production in this study.
8. Comparison of hydrogen production performance of immobilized cell reactor and suspended cell reactor at different HRT 2-12 h was also carried out.
9. The effect of packing ratio of immobilized cell, HRT, POME concentration on continuous hydrogen production and COD removal was carried out in UASB reactor.
10. Exploration and optimization of the hydrogen production capability of the immobilized cells as well as effect of temperature and addition of inorganic components on biohydrogen production was carried out.

1.6 THESIS ORGANIZATION

This thesis contained 5 chapters. In Chapter 1 (Introduction), a brief introduction of palm oil industry in Malaysia, palm oil mill effluent, Cell immobilization, UASB reactor, Biohydrogen production is described. Then a problem statement was given with some basis and rationale to find the directions and gaps in the study. Furthermore, research objectives of the present study are elaborated in detail together with the scope of the study to be covered. Additionally, the organization of thesis is also given in this chapter.

Chapter 2 (Literature Review) provide more detailed explanation of what has been summarized in Chapter 1. It also present reviews of published literatures, covering topics related to this study. Strength and shortcoming of some methods have also been discussed.

Chapter 3 (Materials and Methods) describes the materials and methods used in this research. A detail of experimental set-up is elaborated in this chapter. The last part of the chapter describes some important process control parameter calculations and analytical methods which have been used for experiment.

Chapter 4 (Results and Discussion) presents the findings obtained from the experiments carried out as in Chapter 3. A detailed discussion pertaining to the results obtained in this study and that from other studies will be provided.

Chapter 5 (Conclusions and Recommendations) concludes the findings from the current studies and recommendations for future studies in the related field made from the understanding and information generated in the present study. These recommendations are given due to their significance and importance to be further investigated and explored by future research work in this area.

CHAPTER 2

LITERATURE REVIEW

2.1 INTRODUCTION

This chapter provides a brief review of POME, palm oil mill flow description along with the hydrogen production as a clean source of renewable energy from POME. Anaerobic digestion also been discussed in this chapter. Review on the rationale of whole cell immobilization and its application in biotechnology is discussed. This chapter also focuses on the UASB reactor for biogas production and wastewater treatment potential along with its advantages and disadvantages. Moreover, this chapter also focuses on the biohydrogen production methods are discussed. Besides that, role of different microorganism participate in the biological hydrogen generation systems are discussed.

2.2 PALM OIL MILL EFFLUENT

Oil palm production is one of the major agricultural industries in tropical countries (Zinatizadeh et al., 2007). In Malaysia, palm oil industry is one of the most important contributors to boost Malaysia economy. The total oil palm planted area in the country has increased by 4.3% to 4.48 million hectares, and fresh fruit bunch production has reached 90.5 million tons in 2008 (MPOB, 2009). In year 2009, 43.8 million m^3 (11,600 million gallon) of POME was generated from Malaysian palm oil mills base on the production of 17.56 million tons of total crude palm oil (MPOB, 2010). However, sustainability of palm

oil production has always been questioned, especially by non-governmental organizations (NGOs). Moreover, POME is a non-toxic colloidal suspension brownish liquid with unpleasant smell; consisting of 92–94% water, 6–7% total solids (TS) and pH ranges from 4-5 with average COD and BOD values of 50,000-85000 and 25,000-45,000 mgL^{-1}, respectively. Its COD and BOD values are very high enough to cause serious pollution and environmental problem to the rivers and community (Wu, T.Y. 2007). The measured physico-chemical characteristics of the POME given by different researchers are shown in Table 2.1.

Table 2.1: Measured physico-chemical characteristics of POME

Parameter (g/L)	(Ma and Ong, 1985)	(Oswal et al., 2002)	(Ahmad et al., 2003)	(Najafpour Et al., 2006)	Choorit and Visarnwan, 2007)	(Yusoff et al., 2010)	(Zhang et al., 2008)
Temp.	80-90	NA	55	56	NA	NA	NA
pH	4.5	5	4.7	3.8-4.4	4.4	4.5	4.8
BOD	25	11	25.0	23.0-26.0	65.7	20-35	NA
COD	55	24.6	50.0	42.5-55.7	100.3	60-80	79.7
TS	NA	NA	40.5	NA	72.5	20-30	67.2
TVS	NA	NA	NA	NA	NA	NA	49.3
SS	19	NA	18.0[a]	16.5-19.5[a]	46.2	25.8	NA
TKN	NA	NA	0.750	0.50-0.70[b]	13.8	0.2-0.4	6.7
Oil & Grease	80	NA	4.0	4.9-5.7	9.5	1-1.5	17.4

All parameters are in g/L except pH

NA: Not available

The physico-chemical characteristics of POME change widely throughout the year because of seasonal cropping and mill operations (Yacob et al., 2005). To date, 85% of the palm oil mills using facultative, algae and anaerobic ponding system to treat POME, which is followed by an open-tank digester coupled with extended aeration (Siang, 2006). However, ponding treatment systems are getting less attractive because methane gas is released to the atmosphere and it is believed that those systems have contributed to the atmospheric methane concentration increment. Henson (2009) reported a net carbon emission from POME is approximately 1.4×10^6 tons per years while Yacob et al. (2005) reported 5.5 kg of CH_4 (or approximately 36% of biogas) is emitted from open digesting tanks. Existing treatment in a series of open lagoons at high ambient temperatures, results in the uncontrolled production of methane and carbon dioxide, which are both Green House Gases (GHGs). It was reported that the atmospheric methane concentrations has significantly increased by 30% in the last 25 years (IPCC, 2006). With the increased worldwide concern on environmentally friendly production processes particularly the emission of methane, it is important to develop an alternative concept for POME treatment. Anaerobic digestion is considered to be an effective treatment process for POME. There have been growing interests in anaerobic fermentative hydrogen production from POME treatment as a clean non-polluting and renewable fuel (Morimoto et al., 2004). With increasing demand for energy and environmental crises anaerobic fermentative hydrogen production has become the focus of worldwide. Moreover, fermentative hydrogen production using organic waste and wastewater as substrates achieves both bioremediation and energy recovery.

2.2.1 Environmental regulations of POME discharge

The main agenda of Environmental Quality Act (EQA) was to set adequate standards for the emission and discharge of pollutants into the environment rather than prevention, with an exemption given to the necessities on environmental impact measurements. The environmental restrictions in palm oil industry were decided to be a necessary licensed approach that would permit close control of individual factories. On the basis of prevailing environmental circumstances, environmental restrictions also provide a

mechanism for permitting variable effluent standards. The environmental quality regulations for the crude palm oil industry were the first set of regulations promulgated under the EQA, 1977 for control of industrial pollution source (Thani et al. 1999), enforced by the Department of Environment, (DOE). Therefore, palm oil mill owners have to obtain the license for factories operation that includes ensuring acceptable condition of effluent discharge, proper waste disposal and air emission control throughout the operation (EQA 1974, 2005).

The proper treatment, management, and disposal of POME must be ensured and existing facilities must be upgraded for the acceptable discharge to rivers and streams. As per the provision, the Department of Environment (DOE) has been assigned to observe the implementation of these rules, and the Malaysian Palm Oil Board (MPOB) will be required to submit annual reports regarding the status of POME in their areas to the DOE. These rules are applicable to MPOB in Malaysia, which is responsible for POME management. In addition, there are EQA by different industries such as the FELDA. These EQA also deal with environmental pollution caused by improper disposal of POME.

The Malaysian Government proposed and legalized standards for POME discharge into water courses. Since then, palm oil mills are required to treat their POME prior to discharging it into rivers. The effluent discharge standards ordinarily applicable to crude palm oil mills are presented in Table 2.2.

Table 2.2: Parameter limits for watercourse discharge for POME

Parameter*	**Parameter limits**
pH	5-9
Temperature	45
BOD	1
COD	NA
TS	NA
SS	0.10
Oil and Grease	0.01
Total Nitrogen	0.2

* All parameters in g/L except pH and temperature (°C)

NA: Not available

(Adopted from Environmental Quality Act 1974, 2005)

2.2.2 Cleaner production as a sustainable strategy for POME management

Recently, end-of-pipe standards imposed through "command and control regulations" are the basis of environmental provision. Nevertheless, in worldwide trend promoting pollution prevention through cleaner production, which is based on the 5 R plan (Figure 2.1); namely reduction, replacement, reuse, recovery and recycling, is emerging. Within this framework, it is proposed herewith that a wastewater management based on the promotion of cleaner production and environmentally sound biotechnologies could be integrated as a part of the POME management in Malaysia in order to attain a sustainable development. Such a strategy could take benefit of the current international interest in promoting cleaner production as the driving force of a new and sustainable industrial development technique.

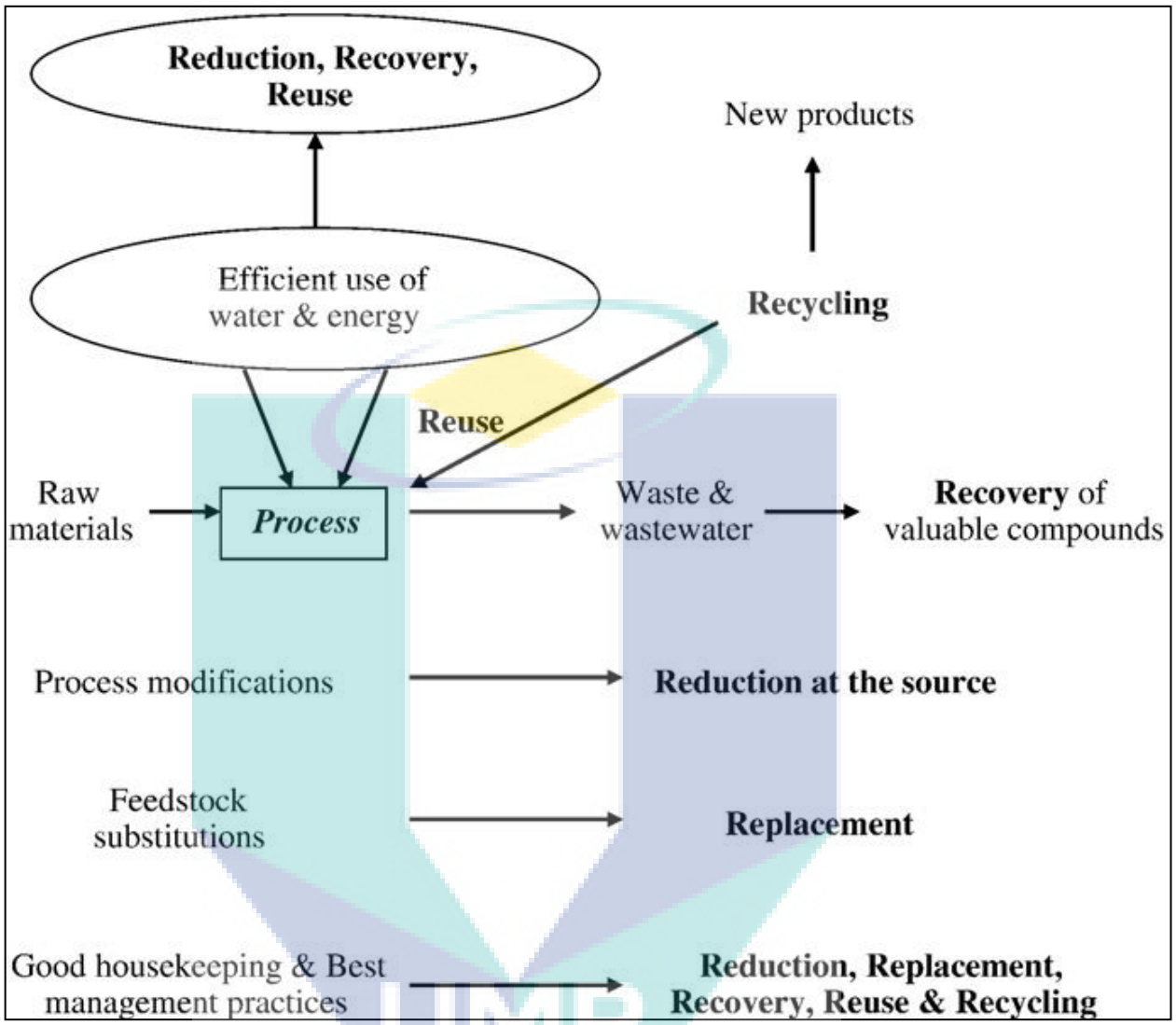

Figure 2.1: The 5 R policy

(Drawn after Olguín et al., 2004).

2.2.3 Bioenergies production from POME

POME is an important renewable biomass energy source that can be harmful to the environment if untreated POME is discharged directly to the surroundings, due to high values of biochemical oxygen demand (BOD) and chemical oxygen demand (COD) that it

generates (Wu et al., 2009). With increasing demand for energy and cost effective environmental protection, anaerobic treatment in wastewater has become the focus of worldwide attention. The key advantage of implementing this approach is reduction in wastewater treatment cost by producing green energies as by-products that is also very useful towards environmental protection. Meanwhile, since POME contain high level of organic matter and nutrient, adoption of anaerobic digestion in the first stage of treatment process is important to convert a bulk of wastes to biogas (methane) before it is further subjected to an aerobic treatment in order to meet the required discharge standard. In Malaysian palm oil mills, the current practice of treating POME by either using open digesting tank and/ or ponding systems (Ma, 1999). However, due to low demand of renewable energy (biogas) and the lack of infrastructure in the county, biomethane is not captured but emitted to the atmosphere and thus caused serious air pollution. Methane gas is one of the most crucial GHGs among other gases because its global warming potential is 21 times more than carbon dioxide (Ishigaki et al., 2005). A life cycle assessment study on Malaysian palm oil mill processes revealed that the non-recovered biomethane emission from POME contributed the highest impact towards the environment (climate change category) and therefore makes the overall processes not environmentally friendly (Subramaniam et al., 2008).

On other hand, biohydrogen production from renewable source, also known as "green technology" in accordance with sustainable development and waste minimization issue. Naturally, POME contains lignocelluloses and hemicelluloses material (complex carbohydrate polymers) which resulted to high COD value (15,000–100,000 mg/L) (Chong et al., 2009a). Due to this reason, POME can become a suitable substrate for biohydrogen production and act as a wastewater treatment process simultaneously. The potential of using POME as a fermentation medium to produce biohydrogen gas has been recently researched (Badiei et al., 2011; Chong et al., 2009a; Chong et al., 2009b; Ismail et al., 2010; Jamil et al., 2009; Prasertsan et al., 2009, and Yusoff et al., 2010) since the generated hydrogen and its combustion products do not count as green house gases (Koroneos et al., 2004). The hydrogen production yield from POME lies in the range of 1.1 and 6.9 L H_2/L POME depending on the types of microorganisms and fermentation method (Table 2.3).

Table 2.3: Biohydrogen production yield from POME

Microorganism	Temperature (^{o}C)	pH	Biohydrogen yield (L H_2/L POME)	Reference
Dark fermentation				
Mixed culture	60	5.5	2.3	(Atif et al., 2005)
Mixed culture	55	5.5	2.6	(Ismail et al., 2010)
Mixed culture	23-25	5.5	1.2	(Yusoff, et al., 2009)
Clostridium butyricum	37	55	3.4	(Chong et al., 2009a)
Clostridium butyricum	36	5.7	6.9	(Chong et al., 2009b)
Thermoanaerobacterium	60	5.5	6.3	(O-Thong et al., 2007)
Thermoanaerobacterium	60	5.5	6.5	(O-Thong et al., 2007)
Photofermentation				
Rhodopseudomonas palustris	NA	6.0	1.1	(Jamil et al., 2009)

NA: Not available

Based on 43,800 million L of POME generated in 2009, it was estimated that 197,100 million L of biohydrogen can be produced. If this amount of biohydrogen is successfully captured and utilized as energy, the energy attained is equivalent to 2.51 PJ in which contributes to 6.5% of the projected renewable energy demand (375 MW) in Malaysia in year 2010 (Oh et al., 2010). Apart from that, the potential of biohydrogen

derived from POME is further strengthened by referring to (Figure 2.2) that shows the yield of biohydrogen from POME is the highest in comparison with other wastes and wastewaters.

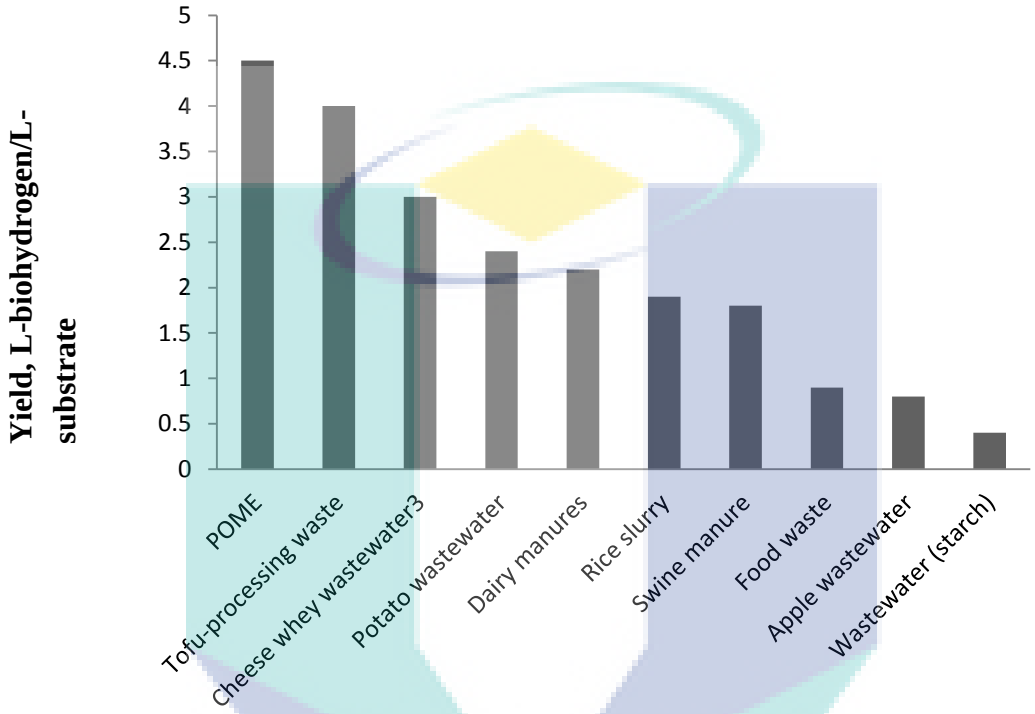

Figure 2.2: Biohydrogen production yield by using different feedstock

(Modified from Lam and Lee, 2011)

Up to now, biohydrogen production from POME in industrial scale is not ready based on current production methods. Apart from storage and safety problems of biohydrogen, problems associated with reactor design and long retention time of dark fermentation have been identified as the major constrains to up-scale biohydrogen production. A study was conducted previously in a pilot plant using 50 L of CSTR reactor to produce biohydrogen from POME (Yusoff, et al., 2009). The result significantly indicated that lab-scale biohydrogen production efficiency did not correlate well when up-scaled to pilot plant. The biohydrogen production attained in pilot plant and lab scale was

33 mL/h/L-POME (25% biohydrogen content) and 74 mL/h/L-POME (70% biohydrogen content), respectively. These two distinguish observations were mainly due to difficulty in pH and temperature control during pilot plant operation. Thus, further investigation and research works should be done with special focus on up-scaling processes, optimization of process conditions, selection of suitable microorganisms, improvement on reactor configuration and parameters control.

2.2.4 Palm oil mill flow description

The wet process of palm oil milling is the most common and typical way of extracting palm oil from fresh fruit bunches (FFB), especially in Malaysia (Wu et al., 2010). In this palm oil milling process, large amounts of steam and hot water are used (Zinatizadeh et al., 2006), which in turn generate a large volume of wastewater. This large quantity of polluted wastewater is commonly referred to as palm oil mill effluent (POME). This section involves the description of some important stages of palm oil processing as shown in Figure 2.3.

2.2.5. Sterilization of fresh fruit bunches

Sterilization of the FFB is carried out by sterilizing the fresh fruit bunches in steam sterilizers for 50 min at about 140 ^{o}C and a pressure of 3×10^{5} Pa (Wu et al. 2010) in order to neutralize the fat splitting enzymes that are naturally present in the palm fruit mesocarp thereby stabilizing the quality of the palm oil and its levels of Free Fatty Acids (FFA). However, it is important to ensure that air is evacuated from sterilizer in order to increase the efficiency of heat transfer and to avoid oil oxidation by air (Poku, 2002). When high-pressure steam is used for sterilization, the heat causes the moisture in the nuts to expand. From the foregoing, it is obvious that sterilization (cooking) is one of the most important operations in oil processing, ensuring the success of several other phases. The condensate coming out from sterilizer constitutes as one of the major sources of POME.

2.2.6 Stripping, digestion and pressing of fruits

After sterilization, the FFBs are fed to a rotary drum-stripper where the fruits are stripped from fruit bunches. This step generates the empty fruit bunches (EFB). Digestion is the process of releasing the palm oil in the fruit through the rupture or breaking down of the oil-bearing cells. Separated fresh fruits are put into a digester, where they are mashed under steam heated conditions by the rotating arms. Through the action of rotation at high temperature (80–90 °C) (Wu et al. 2010), the fruits' outer covering (mesocarp) are loosened from the nuts and subsequently being channeled to a mechanical press machine to squeeze out the crude palm oil. Twin screw presses are generally used to press out the oil from digested mash of fruit under high pressure.

2.2.7 Clarification

The main point of clarification is to separate the oil from its entrained impurities. The fluid coming out of the press is a mixture of palm oil, water, cell debris, fibrous material and 'non-oily solids'. Because of the non-oily solids the mixture is very thick (viscous). Hot water is therefore added to the press output mixture to thin it. The dilution (addition of water) provides a barrier causing the heavy solids to fall to the bottom of the container while the lighter oil droplets flow through the watery mixture to the top when heat is applied to break the emulsion. Consequently, the bottom phase from the clarifier is drained off as sludge or POME for further purification before being discharged. Water is added in a ratio of 3:1.

2.2.8 Kernel oil recovery

The residue (press cake) from the pressing of digested fruit consists of a mixture of moisture, oily fiber and nuts, and the cakes are conveyed to a depericarper for nuts and fiber separation. After the separation of fiber from nuts by strong air current induced by a suction fan, the fiber is usually sent to boiler house as boiler fuel while nuts are sent to a rotating drum where any remaining fiber is removed before they are sent to a nut cracker

and finally to hydrocyclone. Hydrocyclone is commonly used to separate the kernels from empty shells after cracking the nuts (Wu et al., 2010). The discharge from this process constitutes the last source of POME.

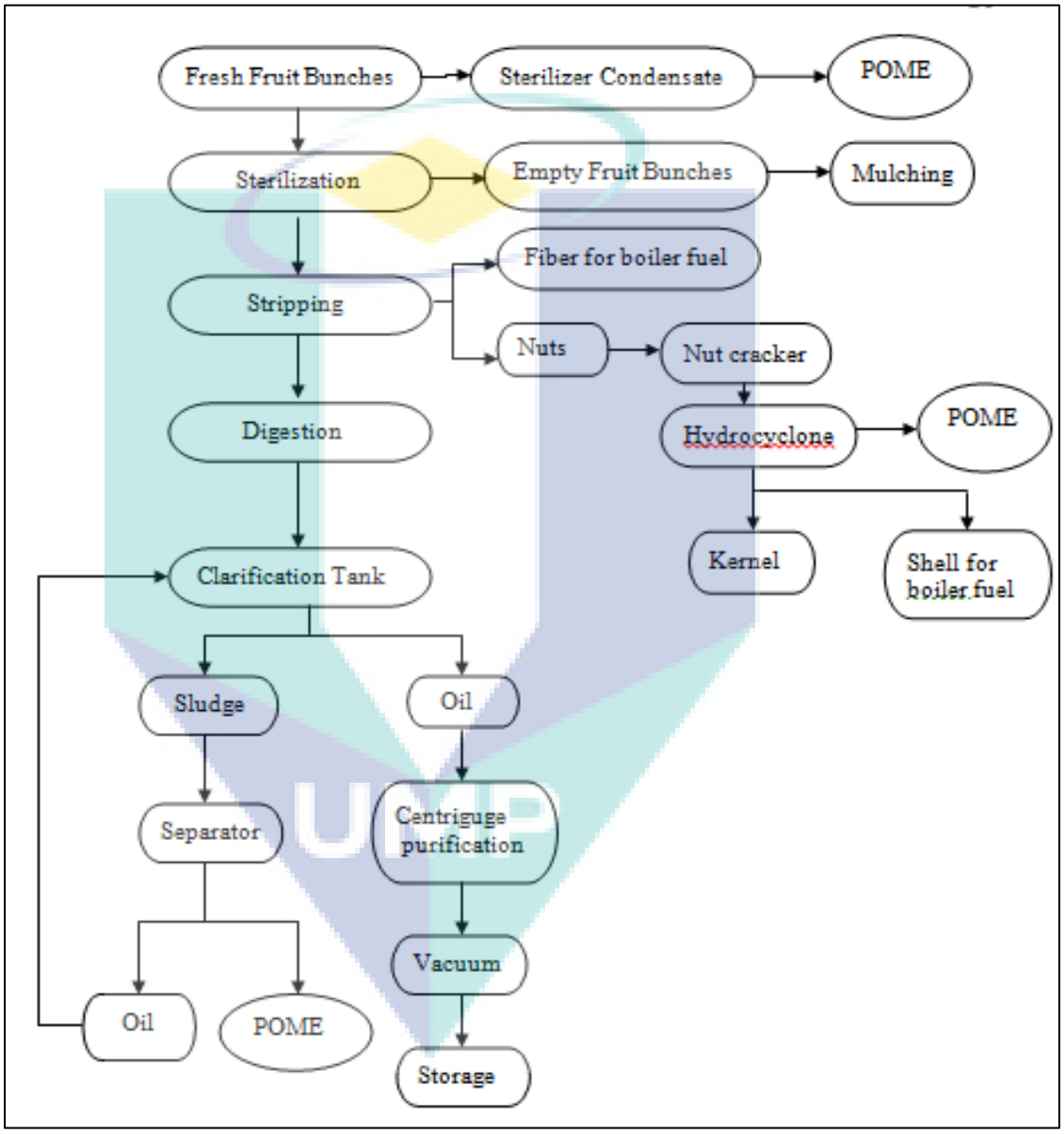

Figure 2.3: A typical flow diagram of a palm oil mill

(Adapted from Lam and Lee, 2011)

2.3 ANAEROBIC DIGESTION

Anaerobic digestion is a process by which almost any complex organic matters can be biologically transformed into another form in the absence of oxygen. Anaerobic digestion is the industrial system that harnesses this natural process to treat wide range of material including agriculture, municipal and industrial wastes, produce biogas that can be used as bioenergy (Chen et al., 2008, and Jingura and Matengaifa, 2009). This process has some advantages over aerobic process due to a low energy requirement for operation and a low biomass production (Kim, D.H. 2006). Moreover, the anaerobic digestion of organic waste is also an environmentally useful technology.

This process is time consuming as bacterial consortia responsible for the degradation process requires time to adapt to the new environment before they start to consume on organic matter to grow. The digestive metabolism of anaerobic microorganisms is complicated and involves several intermediate steps. Generally, there are four basic steps which involve in an anaerobic digestion of organic matter namely hydrolysis, fermentation (acidogenesis and acetogenesis) and methanogenesis (Charles et al., 2009). These four steps are further illustrated schematically in Figure 2.4. Different group of microorganisms work together as a food chain to degrade the organic matter to produce biogas. Briefly, the digestion process begins with bacterial hydrolysis of insoluble organic material to produce simple soluble organic materials including simple sugars, amino acids and long chain fatty acids and make them available for bacteria. Acidogenic bacteria degrade simple organic soluble organics to produce volatile fatty acid (VFA) and hydrogen, which is called acidogenesis. Then, acetogenic bacteria produce acetate from VFA and solvents with acetogenesis. There is a group of acetogenic bacteria that can synthesis acetate from hydrogen and carbon dioxide, a process called homoacetogenesis. Methanogens bacteria finally are able to use acetate or hydrogen to produce methane and carbon dioxide as the final product (Hartmann and Ahring, 2006). Among these bacteria groups, bacteria involved with acitogenesis and acetogenesis were mostly identified as Clostridia species (Fang et al., 2002). *Clostridia* are Gram-positive, spore-forming bacteria that can tolerate harsh environmental conditions by forming spores.

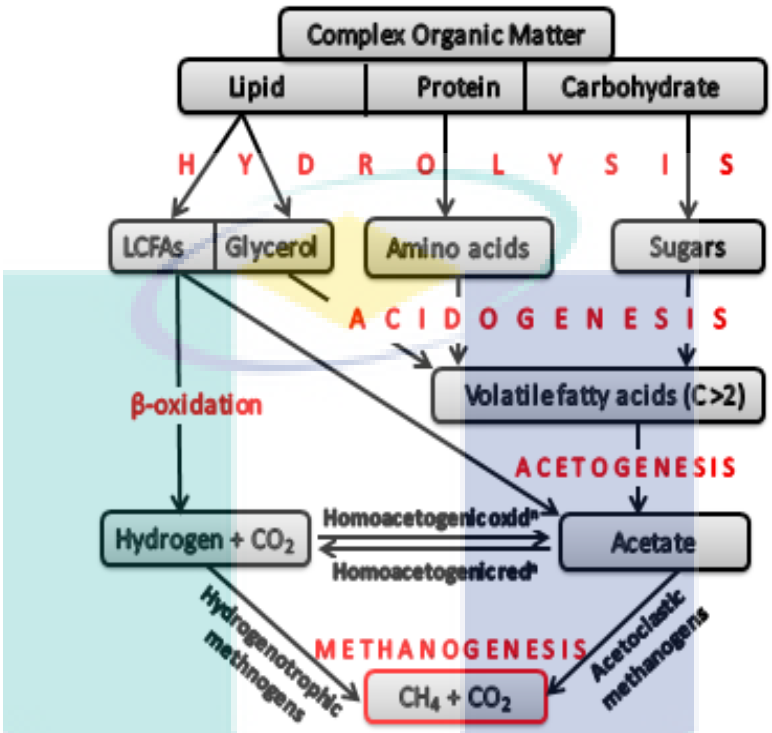

Figure 2.4: Anaerobic degradation process of wastewater treatment depicting both acetogenesis/acidogenic and methanogenic process during conversion of complex organic materials

(Drawn after Mohan, 2008).

2.4 IMMOBILIZATION CELL TECHNOLOGY

An upsurge use of cell immobilization began in the early 19th century (Chitbata and Tosa, 1981). Since that time, immobilization of bacterial cells has been developed and utilized in a wide range of fields such as, enzyme immobilization, biomedical, alcohol production and wastewater treatment. This is mainly due to the several advantages that cell immobilization offers; it permits higher cell densities in bioreactors, improve stability, enhanced fermentation productivity and lower costs of recovery and recycling and

downstream processing (Margaritis and Merchant, 1984, and Stewart and Russel, 1986). An immobilized microorganism is defined as ‘‘the physical confinement or localization of intact cells to a certain region of space with preservation of some desired catalytic activity’’ (Karel et al., 1985). Numerous techniques can be applied to immobilization microorganism on the carriers by using artificial way, these techniques including: adsorption or attachment on solid carrier surfaces, covalent bonding, cross-linking, encapsulation into a polymer-gel and entrapment within a porous matrix (Figure. 2.5). In adsorption, the binding of the cells to the surface of the carriers by (Vander-Waals forces) or electrostatic forces and sometime bacterial exopolymers are involved in the process as well. The advantages of adsorption are that it is simple to carry out and has little influence on conformation of the biocatalyst. However, a major disadvantage of this technique is the peeling off adsorbed microorganisms during the operation (Krekeler et al., 1991). The immobilization of cells/enzymes on solid supports by covalent coupling and metal coordination is one of the most widely applied methods which immobilize the microorganism with bonding reaction of reactive groups (e.g. –COOH or - NH_2 groups). Normally, it involves two steps, first, activation of the support and second coupling of enzyme to the activated support. After the coupling immobilization, the stability of the microorganism will increase significantly, but the bioactivity of microorganism will decrease quickly during the post-operation process (Shriver-Lake et al., 2002). Cross-linking technique was frequently used to link the bio-macromolecular each other with covalent bonds by using multifunctional reagents, such as bisdiazobenzidine, glutaraldehyde, and hexamethylene diisocyanate. This mentioned method is very simply, but the procedure is difficult to control properly (Ramakrishna and Prakasham, 1999).

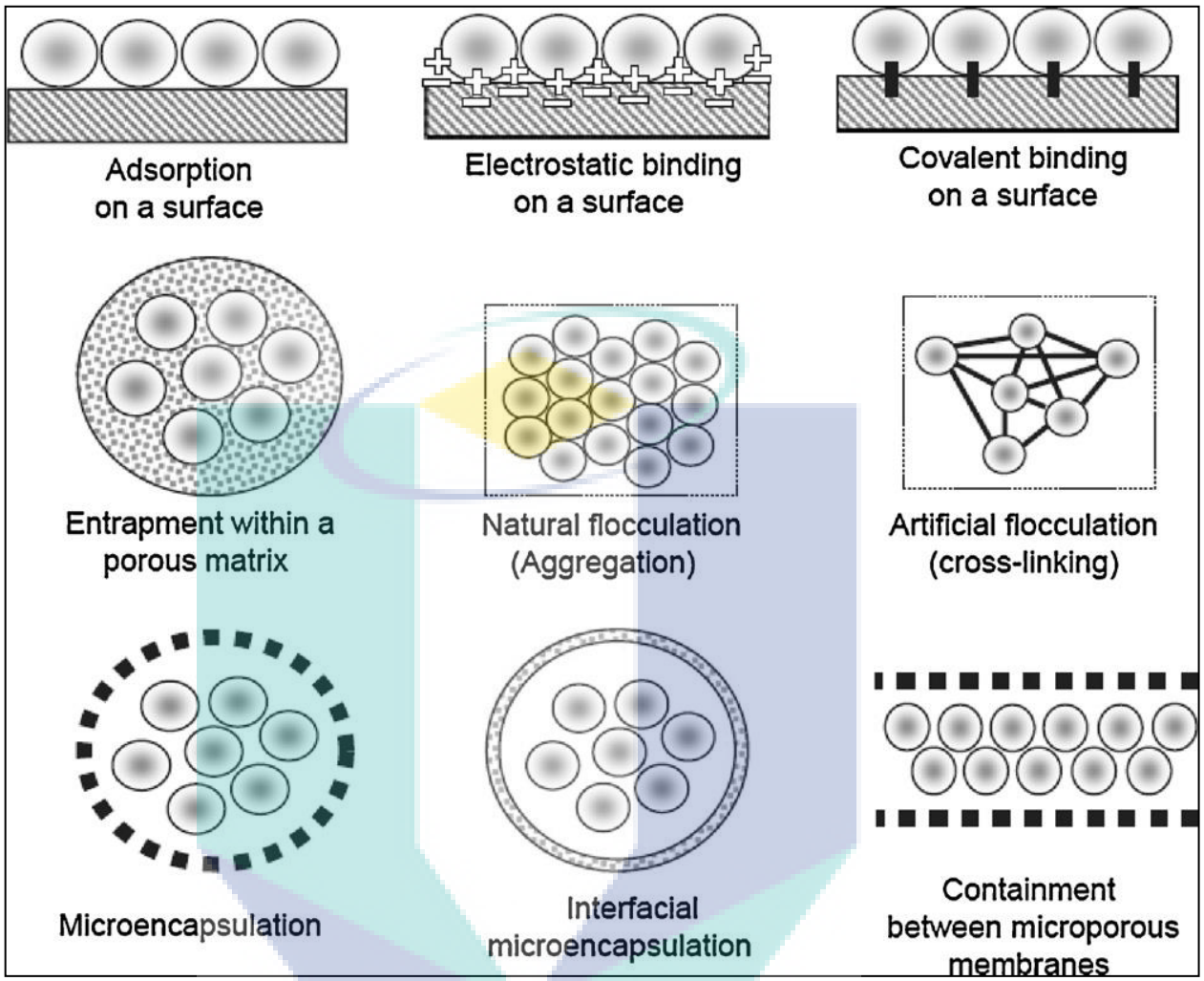

Figure 2.5: Basic methods of biocatalyst immobilization

(Adapted from Kourkoutas et al., 2004)

Immobilization by encapsulation has been widely used for immobilization of whole cells, compared to the other immobilization methods (Orive et al., 2002). In this process, cells are immobilized with the use of many synthetic polymers such as polyvinyl alcohol (PVA), polyacrylamide (PAM), etc., and a lot of natural polymers, for example agarose, carrageenan, alginate, agar, cellulose, etc. are often used as encapsulated carriers (Jen et al., 1996). Compared with other immobilization methods, the major limitation of this technique is diffusion limitation and slow leakage of cells during continuous long-term operation.

Entrapment of the microorganisms in porous polymer carrier was extremely used to capture the microorganisms from suspended solution and then obtain the immobilized microorganisms. The polymer matrix used in this method confining microorganisms has porous structure, and thus various metabolic products could easily diffuse through into the matrix. In this method, a lot of porous polymers can entrap microorganisms under ambient conditions. What's more, the achievement of higher active biomass concentration, shock loads, enhanced tolerance to toxic compounds and greater plasmid stability of genetic engineered microorganisms offer a lot of virtues, and make this method as excellent options for cell immobilization (Verma et al., 2006). The synthesis and design of novel immobilized carries should be a research hot topic in the area of immobilized microorganism because these works would also play very important role in immobilized cell system.

2.4.1 Immobilization whole cell systems for hydrogen production

Immobilized whole cell systems for fermentative hydrogen production are a rapidly expanding research area, although applications of this technology at industrial scale are limited. Continuous fermentation systems offer important economic advantages in comparison with batch culture systems for commercial applications. Most studies on microbial hydrogen production applied suspended-cell systems that are typically inefficient or difficult to handle in continuous operations (Hu and Chen, 2007). Recycling the biomass has to be used to maintain sufficient cell density for high hydrogen production. Cell immobilization technology, applied to hydrogen fermentation, has been shown several advantages as compared to suspended cell systems, which are mentioned in Table 2.4. Moreover, immobilization-cell systems are also adapted with a feature of creating a local anaerobic environment, which is well suited to oxygen perceptive fermentative hydrogen production.

Table 2.4: Advantages of immobilized cells over suspended cell

Advantages
1. Prolonged activity and stability of the biocatalyst.
2. The immobilization support may act as a protective agent against stressful environmental conditions.
3. Higher cell densities per unit bioreactor volume, which leads to high volumetric productivity, elimination of non-productive cell growth phases and shorter fermentation times.
4. Increased substrate uptake and yield improvement.
5. Effective in enhancing biomass retention and can operate at higher dilution rates without biomass washout from the reactor during continuous operation.
6. Increased tolerance to high substrate concentration and reduced end product inhibition.
7. Regeneration and reuse of the biocatalyst for extended periods in batch operations, without removing it from the bioreactor.
8. Ability to use smaller bioreactors with simplified process designs and therefore lower capital costs.
9. Reduction of risk of microbial contamination due to high cell densities and fermentation activity.

Support materials for cell immobilization can be synthetic polymers, such as alginate and polyvinyl alcohol, or naturally available, such as lignocellulosic materials from agricultural residues (Plangklang et al., 2012). Some researchers have concluded that, the immobilized cells culture systems appears to be more effective way to increase hydrogen production. The number of immobilized hydrogen studies in the literature are quite limited (Table 2.5). Ismail et al. (2011) studied hydrogen production from palm oil mill effluent (POME) using polydimethylsiloxane (PDMS) as the immobilization ion matrix and reported the hydrogen production rate of 2.1 LH_2/ L-POME with immobilized mixed cultures, and they showed efficient soluble carbohydrate consumption and volatile

suspended solids removal of 88% and 62% under continuous operation for hydrogen production. Patel et al. (2010) used mixed and pure cultures immobilized by a lignocellulosic matrix for enhancement of continuous hydrogen production and obtained a higher hydrogen production rate by immobilized cells than that found with free cells. The hydrogen-producing anaerobe, *Clostridium tyrobutyricum* JM1, was isolated from a food waste treatment process and immobilized in a packed-bed reactor using polyurethane foam as the support medium to improve the efficiency of substrate utilization and continuous hydrogen production (Jo et al., 2008). The maximum hydrogen yield and hydrogen production rate were 223 mL/g hexose and 7.2 L H_2/L d with an influent glucose concentration of 5 g/L. Therefore, the immobilized reactor using *Clostridium tyrobutyricum* JM1 was an effective and stable system for continuous hydrogen-production efficient substrate utilization. Immobilization of cells in polymeric materials e.g. ethylene-vinyl acetate copolymer (EVA) and polymethyl methacrylate (PMMA) have been used successfully (Wu et al., 2005b, and Wu, K.J. 2007) for hydrogen production from simple sugars in both batch and continuous modes. These materials have been used widely because of low cost, high mechanical strength and durability with ease of handling (Park and Chang, 2000). Acclimated sludge entrapped in composite polymer matrix (PMMA/collagen/activated carbon) drastically increased the hydrogen production rate from 1.21 L H_2/ L h (in suspended system) to 1.80 L H_2/ L h (in immobilized system). Cells immobilized by a calcium alginate matrix supplemented with chitosan and titanium oxide carriers (CA+CH+TiO_2) were found by (Wu et al., 2006) to be more effective for hydrogen production and provided a maximum production rate of 21.3 mmol/L h (at 35 °C and 20 g COD/L sucrose), which was 3-fold greater than that obtained with suspended cells (6.8 mmol /L h). The results of this work suggested the potential use of immobilized-cell systems for hydrogen production. Zhao et al. (2012) were investigated the performance of hydrogen production in continuous processes by *Clostridium sp*. T2 immobilized on mycelia pellets and compared with free cell or traditional carrier sodium alginate. It was obtained that the maximum hydrogen production immobilized on mycelia pellets reached 2.76 mmol H_2/L h at the HRT of 10 h, which was considerably improved compared with carrier-free process of 1.13 mmol H_2/L h, but slightly lower than the one immobilized in sodium alginate. Liu et al. (2011) reported the use of an immobilization technique for photo

fermentation bacteria for hydrogen production. However, they reported that the immobilized photo fermentation bacteria could not only enhance hydrogen production but could increase acid-tolerance capacity. Hydrogen was produced even at a pH of 5.0, and so immobilized photo fermentation bacteria can hopefully be applied in combined of dark and photo fermentation for improved the hydrogen production capacity. Hydrogen production from tofu wastewater by the anoxygenic phototrophic bacterium immobilized in agar gel was discussed by Zhu et al. (1999), who demonstrated that this provided an effective method for simultaneous hydrogen production and wastewater treatment. The maximum rate of hydrogen production from the wastewater was 2.1 $L/h/m^2$ gel which were even slightly higher than that from glucose medium (as control). Yokoi et al. (1997) compared seven different immobilization carries and polysaccharide gels on *Enterobacter aerogenes* HO-39 and found that immobilized system with agar and porous glass showed higher performance in hydrogen production rate and hydrogen yield than k-carrageenan, alginate, fabric, chitosan, agar, nonwoven porous glass and cellulose foam.

Thus, hydrogen production from immobilized cells is a more promising system than the production from suspension cultures. It is of scientific and engineering significance to evaluate the hydrogen production performance with immobilized biomass, and to analyze the microbial population and activities immobilized. Better understanding and characterization on these aspects are highly desired. In addition, these studies were mostly performed on synthetic substrates (glucose), particular real wastewater as substrates are recommended for hydrogen production.

Table 2.5: Cell immobilization technologies for fermentative hydrogen production

Material used as solid support matrices for immobilization	Reactor	Inoculums	Substrate	Hydrogen production with suspended-cell system	Hydrogen production with immobilized-cell system	Ref.
Polydimethyl-siloxane	CSTR	Immobilised PDMS-mixed cultures cubes	Palm oil mill effluent	NA	2.1 LH_2/ L POME	(Ismail et al., 2011)
Agar gel	Batch	*Rhodopseudomonas fae- calis* strain RLD-53,	Acetate	NA	3.15 mol H_2/mol acetate	(Liu et al., 2011)
PMMA collagen, and activated carbon	Continuous, conditions	Anaerobic sludge	Sucrose based synthetic water	1.21 L H_2/ L h	1.80 L H_2/ L h H_2/mol sucrose	(Wu, K.J. 2007)
Ligno-cellulosic wastes-banana leaves and coconut coir	Batch	Mixed microbial cultures (MMCs)	Glucose	40-60 mL H_2/day	300-330 mL H_2/day	(Patel et al., 2010)
Calcium alginate matrix (CA+CH+TiO_2)	Batch	Anaerobic sludge	Sucrose	6.8 mmol/L h	21.3 mmol/L h	(Wu et al., 2006)
Biological mycelia pellets	CSTR	*Clostridium sp*. T2	Xylose	1.13 mmol H_2/L h	2.76 mmol H_2/L h	(Zhao et al., 2012)
Lignocellulosic agroresidues	Bioreactor	*Enterobacte cloacae* IIT-BT 08	Lignocellulosic	37 mmol/L h	62 mmol/L h	(Kumar and Das, 2001)
Polyurethane form	IBR	*Clostridium tyrobutyricum* JM1	Glucose	NA	7.2 L H_2/L d	(Jo et al., 2008)
Granular activated carbon	AFBR	Activated sludge and digested sludge	Glucose synthetic wastewater	NA	2.36 L H_2/ L h	(Zhang et al., 2007)

EVA (ethylene vinyl actate copolymer)	Batch	Sewage sludge	Sucrose	NA	488 ml H_2/g VSS	(Wu et al., 2005b)
Ager gel	Batch	*Rhodobacter sphaeroides*	Tofu wastewater	1.9 l/h/m^2 gel	2.1 L/h/m^2 gel	(Zhu et al.,1999)
Ager gel or on porous glass beads	Column reactor	*Enterobacter uerogenes* strain HO-39	Glucose	0.54 mol Hz/mol glucose	0.73 mol Hz/mol glucose	(Yokoi et al., 1997)
Coir	Up-flow (UFR) reactor	Modified *Escherichia coli* BL-21	Glucose	NA	1.45 L-H_2/h/L	(Chittibabu et al., 2006)
Aliginate and ALSC	UFR	Domestic sewage	Sucrose	NA	0.93 L-H_2/h/L	(Wu et al., 2003)
Loofah sponge, expanded clay, Activated carbon (AC)	UFR	Domestic sewage	Sucrose	NA	1.32 L-H_2/h/L	(Chang et al., 2002)
AC	UFR	Domestic sewage	Sucrose	NA	7.30 L-H_2/h/L	(Lee et al., 2004)

NA: Not available

2.5 UP-FLOW ANAEROBIC SLUDGE BLANKET (UASB) REACTOR

Recently, modern anaerobic processes used for high rate reactors have been widely applied to the treatment of a wide variety of industrial wastewaters with a high soluble COD content (Tchobanoglous et al., 2003), including paper-pulp liquors (Elliott and Mahmood, 2007), POME (Borja and Banks, 1994, and Chaisri et al., 2007) and those wastewater from the food industry (Stabnikova et al., 2008). Among the high rate reactors, the UASB is the most commonly used process, with more than 500 installations in the world (Tchobanoglous et al., 2003). The concept of the UASB reactor was developed in the 1970s for CH_4 production, and it has prevailed successfully for anaerobic treatment of various types of wastewater to produce methane, because of its high treatment efficiency and excellent process stability (Lettinga et al., 1980, and Wang, Y. 2007). The underlying principle of the UASB operation is to have an anaerobic sludge which exhibits good settling properties (Lettinga, 1995). This reactor has a longitudinal structure with a gas/liquid/solid separator at the top, where microbial granules with high settling velocity are formed, resulting in a thick biomass blanket zone at the bottom (Jung et al., 2010). Performance depends on the mean cell residence time and reactor volume depends on the hydraulic residence time, therefore, UASB reactor can efficiently convert wastewater organic compound into biogas. Numerous works have dealt with hydrogen producing UASB reactor, since hydrogen-producing granule (HPG) formation was first reported by (Fang et al., 2002), and as mentioned above, this reactor generally shows high and stable performance. However, for most studies in this field, synthetic wastewater is generally applied as a substrate. It was found that hydrogen yield was HRT-dependent and stabilized at 1.5 mol H_2/mol sucrose at HRT of 8–20 h (Chang and Lin, 2004). The yields decreased drastically at a HRT of 4 or 24 h. At a HRT of 8 h, the maximum hydrogen production and specific hydrogen production rates were recorded at 0.25 L/ h/L and 53.5 mmol H_2/g VSS/d, respectively. Biomass retention reached the maximum level of 7.2 g VSS/L at a HRT of 24 h, but decreased to 5.0 g/L at the optimum HRT of 8 h. Mu et al. (2006) attempted to optimize the operational pH condition from sucrose using an UASB reactor seeded with sludge taken from an anaerobic reactor treating citrate-producing wastewater. A maximum HY of 1.68 mol H_2/mol hexose and a HPR of 0.145 L H_2/L/h were obtained at

pH 4.2. Wang, X.J. (2007) reported the highest HY of 1.33 mol H_2/mol hexose and a HPR of 0.1 L H_2/L/h using sucrose as a substrate; however, a start-up period of 150 d was needed to establish stable operation. Wu et al. (2005a) designed a reactor containing silicone immobilized and self-flocculated sludge and obtained the highest HPR (15 L H_2/L/h) documented to date. A high concentration of biomass up to 35.4 g VSS/L was maintained even at 0.5 h of HRT. However, application of the UASB reactor is obstructed by the major drawback of a long start-up period, where a few months are generally required for HPG formation, as mentioned above. Thus far, the application of actual wastewater for dark fermentative hydrogen production using an UASB reactor has been limited, although it has high potential for significant enhancement of economic viability. Four studies on dark fermentative hydrogen production from actual wastewater have been conducted thus far (Yu et al., 2002; Huang et al., 2004, and Jung et al., 2010, 2011a, b). Rice winery wastewater was applied as a substrate for dark fermentative hydrogen production in order to optimize HRT, substrate concentration, and operational pH conditions (Yu et al., 2002). A maximum HY of 2.14 mol H_2/mol hexose and a HPR of 0.16 L H_2/L/h were reported; however, no information on the HPG was provided. Jung et al. (2011a, b) reported that maximum HY and HPR average values of 1.78 mol H_2/mol hexose and 2.76 L H_2/L/h, respectively, were obtained from CDMW.

Recently, UASB process are being increasingly used for the treatment of various high-strength industrial wastewaters, many problems are encountered, for instance granulation may not occur and the seeded sludge may get wash out at hydraulic stresses, high and very low up-flow velocities (Revanuru and Mishra, 2008). The advantages and disadvantages of UASB reactor have been summarized in Table 2.6. Under properly controlled conditions, hydrogen-producing biomass may be granulated, similar to the methanogenic biomass in UASB reactor. Granulation results in substantial increase of biomass concentration in the reactor and thus improves hydrogen production efficiency (Fang et al, 2002, and Chang and Lin, 2004). However, unlike the methanogenic granules, hydrogen-producing granules seem to lack the mechanical integrity and result in poor settling. This was partly attributed to the lower proteinaceous constituent in the extracellular polymers of the hydrogen producing granules (Liu and Fang, 2002), which

were easily disintegrated and thus washed out from the reactor. These shortcomings may be improved by immobilization of the hydrogen-producing biomass in bioreactors (Difu, 2007). Recently, immobilized cell systems have been successfully applied for biohydrogen production in UASB reactor (Chang and Lin, 2004, and Yu and Mu, 2006).

Table 2.6: Advantages and disadvantages of UASB reactor

Advantages

1. Good removal efficiency can be achived in the system, even at high loading rates and low temperatures.
2. Anaerobic treatment can easily be applied on either a very large or a very small scale.
3. When high loading rates are accommodated, the area needed for the recator is small thus reducing the capital cost.
4. Reduction of CO_2 emissions due to low demand for foreign (fossil) energy and surplus energy production.
5. Can handle organic shock load effectively.
6. Wide range of wastewater concentration.
7. Low nutrients and chemical requirement especially in the case of sewage, an adequate and stable pH can be maintained without the addition of chemicals.

Disadvantages

1. Foaming and sludge floatation at high OLRs.
2. Seeded sludge may get wash out at hydraulic stresses, high and very low up-flow velocities
3. Low sludge yield of microorganisms, therefore a long start-up time is required to reach the desired concentration of biomass in the reactor, if not fed with granulated seed sludge during start-up.
4. Proper temperature control (15-35 ^{o}C) required for colder climates.
5. Requirement of post-treatment of anaerobic effluent to meet water discharge standards for organic matter, nutrients and pathogens.

2.6 HYDROGEN BIOTECHNOLOGY

2.6.1 General use and production of hydrogen

Worldwide, the extensive use of fossil fuels for power plants, automobiles, and rapid industrialization are increasing day by day, resulting in not only environmental pollution, but also economic and diplomatic problems owing to their limited reserves and uneven distributions (Jung et al., 2010). Furthermore, the burning of fossil fuels (coal, natural gas, oil, petroleum) in different sectors contributes to the emission of greenhouse gases as shown in Figure 2.6.

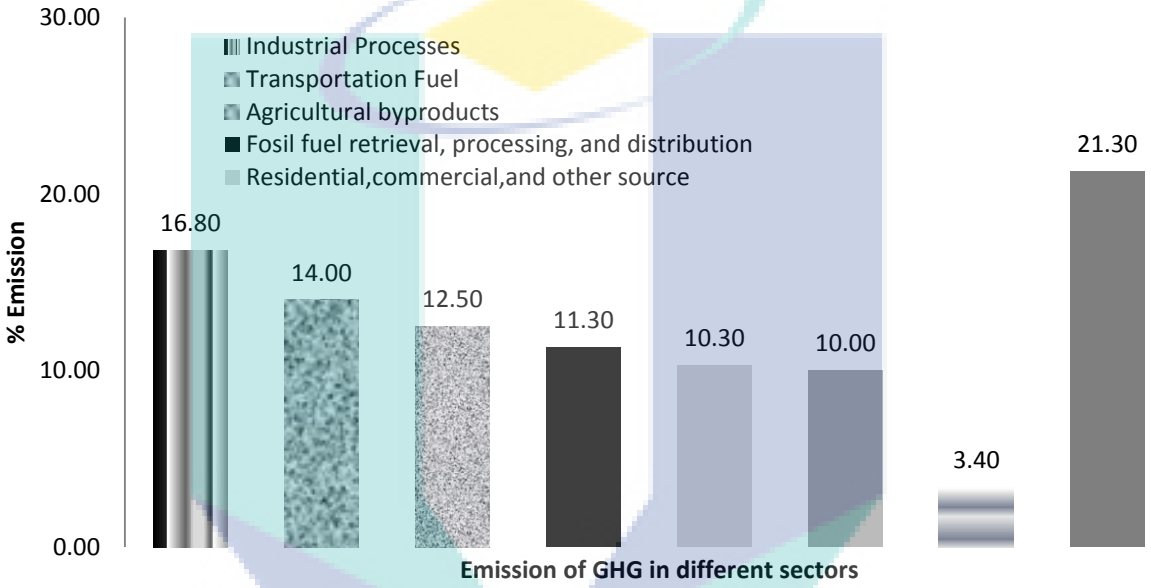

Figure 2.6: Annual GHG Emissions by different Sectors

(Source: Global anthropogenic green house gas emissions in 2004)

Exploration of new sustainable energy alternatives to fossil fuels has been a major challenge during this century to overcome the impending energy crisis and avoid problems arising from global climate change. Hydrogen is widely regarded as one of the most potential future energy vector, capable of assisting in issues of environmental emissions, energy security and versatility as fuel (Pakarinen et al., 2008)**.** This yield is 2.75 times higher than energy yields of hydrocarbon fuel. When hydrogen is used as a fuel, its main combustion product is water which can be recycled again to produce more hydrogen (Figure 2.7).

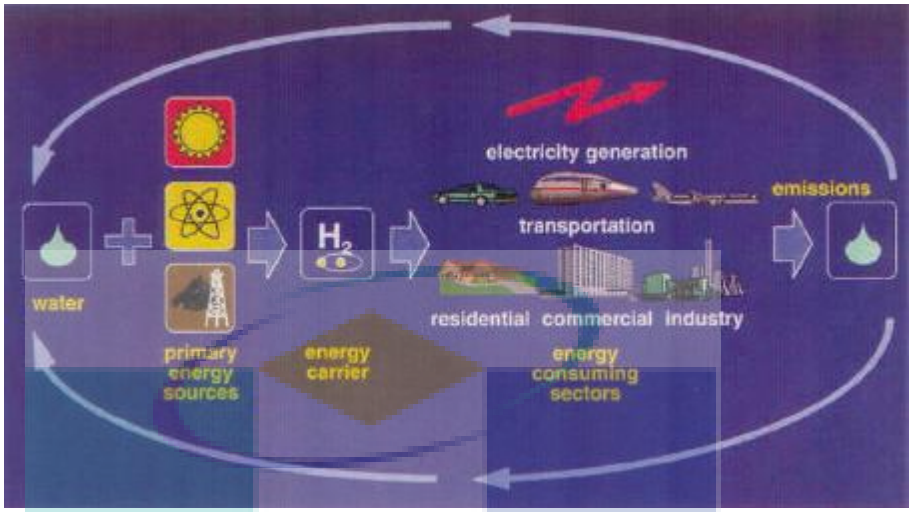

Figure 2.7: Hydrogen energy system

(Source: International Association of Hydrogen Energy, USA)

Apart from its use as a clean fuel, hydrogen can be used for various other reasons in chemical process industries. It is used as a reactant in hydrogenation process to produce lower molecular weight compounds. It can also be used to saturate compounds, crack hydrocarbons or remove nitrogen and sulphur compounds. It is a good oxygen scavenger and can therefore be used to remove traces of oxygen to prevent oxidative corrosion in the presence of a catalyst. In the manufacturing of synthesis gas, methanol and ammonia, the use of hydrogen is well known.

In recent years, a significant use of hydrogen has been demonstrated for hydrogen-fueled transit buses, ships and submarines, where it will help reduce pollution. As of today, all these areas of hydrogen utilization are equivalent to 3% of the energy consumption, but it is expected to grow significantly in the years to come (Nath and Das, 2003). Currently, about 98% of hydrogen comes from fossil fuel (Kalinci et al., 2009). Worldwide, 48% production of hydrogen from natural gas or steam reforming of hydrocarbon, 30% from oil, 18% from coal, and the remaining 4% via water electrolysis. However, these processes

involved with electricity and which comes from fossil fuel combustion so that they are energy exhaustive, expensive and not always environmental friendly (Ginkel and Sung, 2001). Given the economic uncertainties and environmental hazards of fossil fuels, working out the technical and economic feasibility of hydrogen production is becoming a major priority in the 21st century. Biological process for hydrogen production, in that aspect, is uniquely poised to make a significant contribution to that effort. This process is not only eco-friendly, but also escort to open new path for the exploitation of renewable energy resources which are unlimited.

2.6.2 Biohydrogen production

Hydrogen produced from renewable sources (organic wastes, water, and biomass) either biologically or photobiologically is called "biohydrogen". All biohydrogen production technologies depend on either nitrogenase or hydrogenase for hydrogen evolution and derive energy by the use of two main types microorganisms, photosynthetic (photoautotrophic and photoheterotrophic) and fermentative. Various researches have been reported on microbial hydrogen production (Zajic et al., 1978; Roychowdhury et al., 1988, and Sasikala et al., 1993). Biological hydrogen production methods fundamentally rely on the presence of a hydrogen metabolizing enzyme, namely nitrogenase or hydrogenase. Nitrogenases release hydrogen as a byproduct during nitrogen fixation. Hydrogenases catalyze what is arguably the simplest chemical reaction: $2H^+ + 2e^- \leftrightarrow H_2$. The reaction is reversible, and its direction depends on the redox potential of the components able to interact with the enzyme. In the presence of hydrogen and an electron acceptor, it will act as a hydrogen uptake enzyme; in the presence of an electron donor of low potential, it may use the protons from water as electron acceptors and release hydrogen. At present three enzymes carrying out hydrogen metabolism are known: Nitrogenase, Fe Hydrogenase, Ni-Fe Hydrogenase (Sinha and Pandey, 2011). Sinha and Pandey (2011) have described the methods related to biohydrogen production and have pointed out the advantages, as well as the disadvantages of such processes (Figure 2.8).

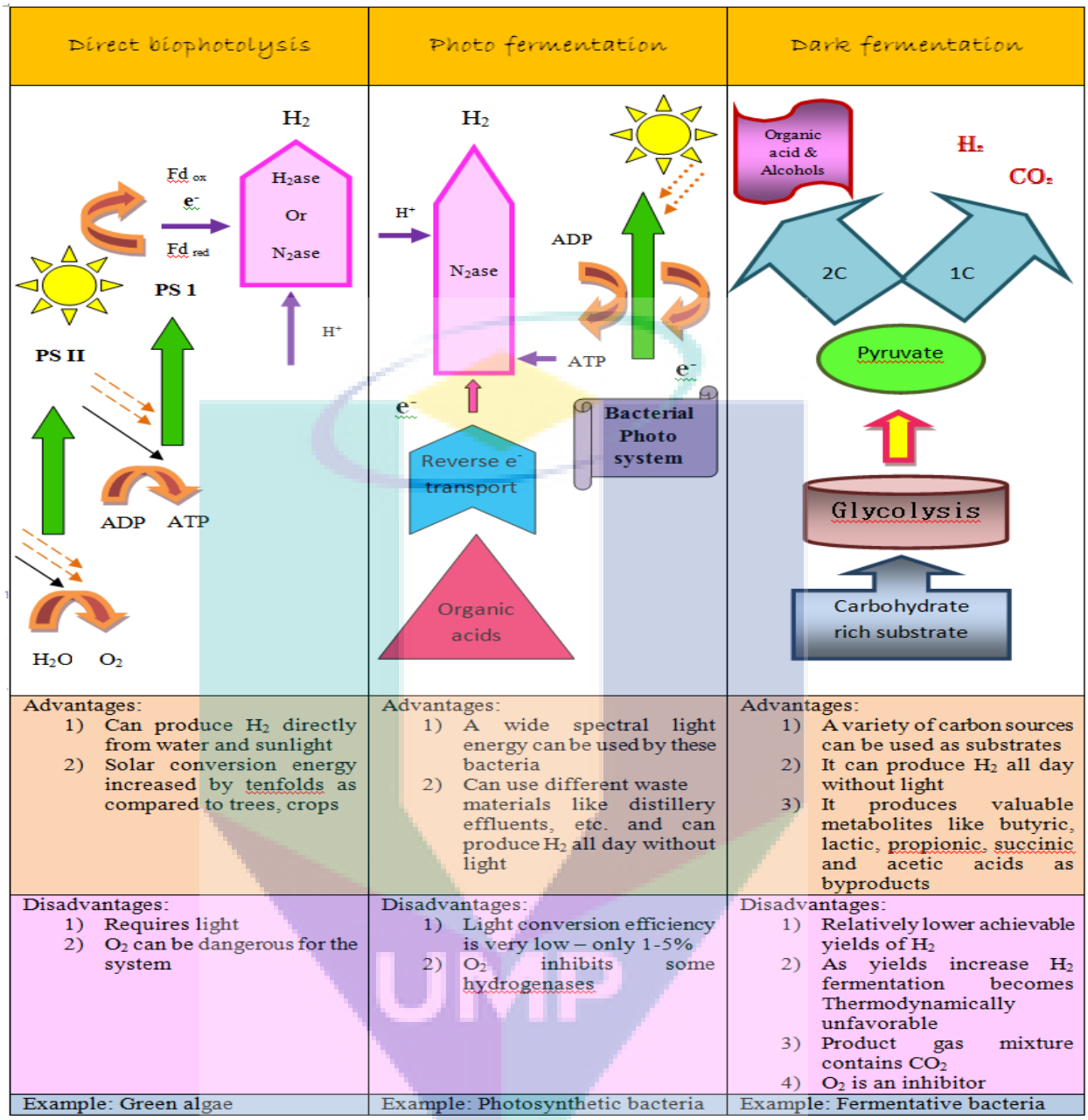

Figure 2.8: Comparison of three biological hydrogen production processes

(Modified from Sinha and Pandey, 2011)

Fermentative production of hydrogen via dark fermentation has some advantages of rapid hydrogen production rate and simple operation over photosynthetic hydrogen production. In addition, fermentative hydrogen production using organic waste and

wastewater as substrates achieves both bioremediation and energy recovery. Thus fermentative route is more feasible and widely used for hydrogen production and has a higher feasibility for industrialization. Fermentative hydrogen can be produced by anaerobic bacteria such as facultative anaerobes (enteric bacteria, e.g. *Citrobacter, Escherichia coli,* and *Citrobacter*) and strict anaerobes (clostridia, rumen bacteria methanogenesis, methylotrophic, and rumen bacteria), is one of those organisms capable of converting various organic waste to hydrogen (Das and Veziroglu, 2001, and Liu and Wang, 2012). Enteric bacteria are rod-shaped, gram-negative facultative anaerobes, less sensitive to oxygen and are able to recover following air exposure (Nath and Das, 2004) the presence of oxygen, however, causes degradation of formate – a major precursor for hydrogen production, without hydrogen formation. *Clostridia* are major hydrogen producing microorganism in anaerobic fermentation and are gram-positive, spore-forming, rod-shaped bacteria. *Clostridia* are very resistant to high temperature, harmful chemicals, carbon or nitrogen deficiency. *Clostridia* species produce hydrogen gas during the exponential growth phase. The dominant culture of *Clostridia* can be easily obtained by heat treatment of biological sludge. The organisms belonging to genus *Clostridium* has been widely studied for hydrogen production. *Clostridia* species are capable of using different organic substrates such as proteins (Thabet et al., 2004), sucrose (Chen et al., 2005) cellulose (Levin et al., 2006), POME (Chong et al., 2009a) and sugarcane juice (Plangklang et al., 2012). The hydrogen production pathway of clostridia using glucose as a model during dark fermentation is presented in Figure 2.9. Glucose is metabolized to pyruvate through glycolysis. The pathway of pyruvate decomposition through acetyl-CoA with the formation of ferredoxin (red) using pyruvate ferredoxin oxidoreductase. The reduction of a proton by ferredoxin (red) produces hydrogen through hydrogenase activity. The acetyl-CoA gives variety of end products (acetate or ethanol, viz). Hydrogen production occurs mainly during the acid (acetate and butyrate) production phase. The fermentation pathway is highly dependent on pH. A maximum of 4 mol H_2/glucose can be obtained by acetate-type fermentation, whereas up to 2 mol H_2/glucose can be produced in butyrate-type fermentation.

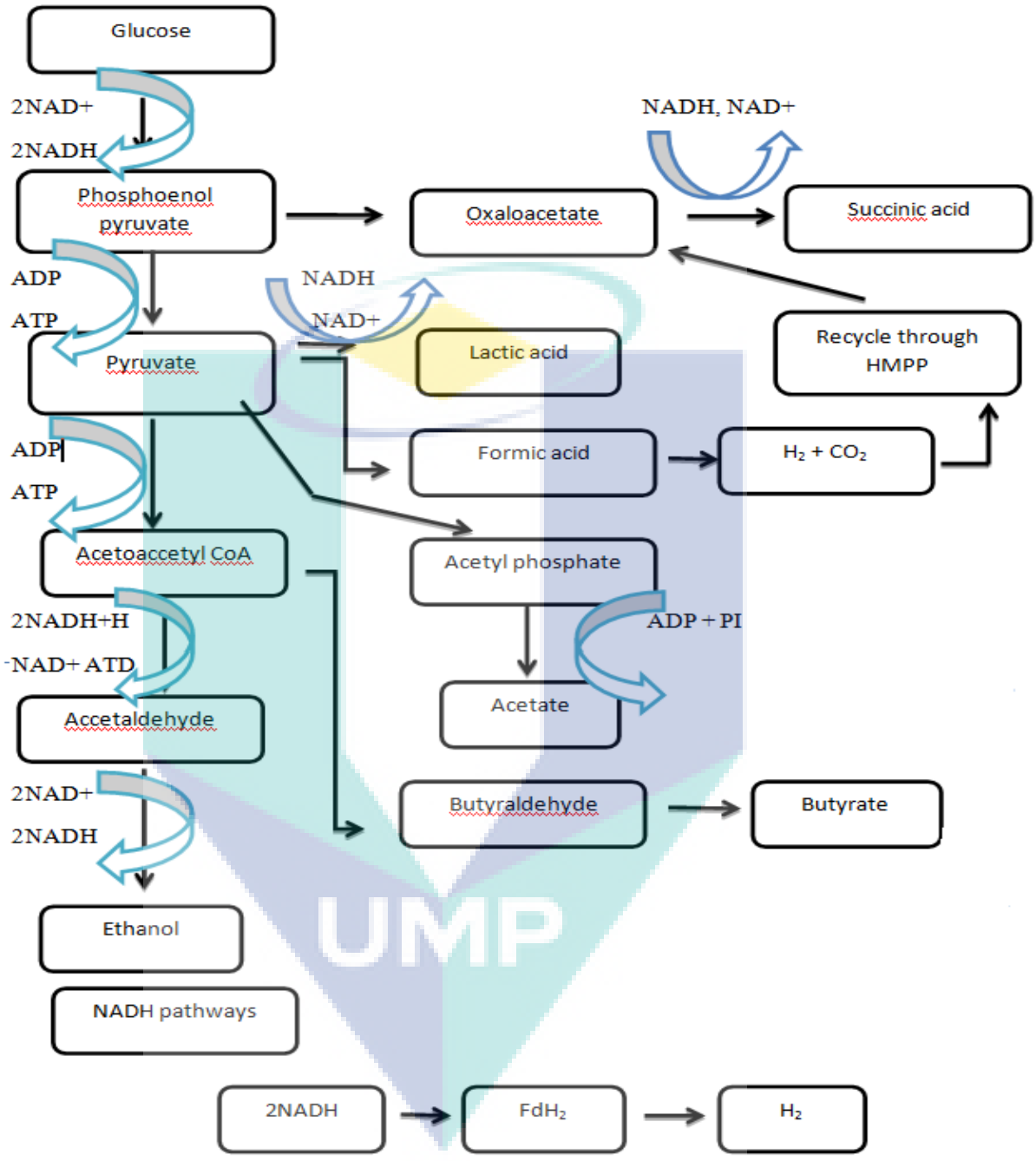

Figure 2.9: Hydrogen production pathway of clostridia during dark fermentation

(Modified after Nath and Das, 2004).

Much research has been conducted on the generation of hydrogen via dark fermentation from waste and wastewater sources, including food waste (Kim, S.H. 2008), waste ground wheat (Sagnak et al., 2011), cassava starch processing wastewater (O-Thong et al., 2011), coffee drink manufacturing wastewater (Jung et al., 2010) and POME (Badiei et al., 2012). Furthermore, dark fermentation also produces valuable by-products such as acetate, butyrate and ethanol. The major disadvantage of the dark fermentation is the low hydrogen yield. Most of the study on dark hydrogen production form waste and wastewater conducted on suspended-cell system. These systems are usually ineffective or difficult to handle in continuous operation, and the recycling of biomass is considered necessary to maintain sufficient cell concentration in the reactor to maximize hydrogen production (Hu and Chen, 2007). Compared to other biological processes for hydrogen production, dark fermentation is closest to the industrial testing. One pilot test and one industrial case were reported in China where hydrogen was produced from wastewater via dark fermentation. From an engineering perspective, the low retention rate of hydrogen producing bacteria limits the productivity of a suspended-growth reactor due to the requirement for long hydraulic resident time (HRT) to maintain adequate bacteria population. Right now, Cell-immobilization technology provides an alternative approach to suspended-cell systems in continuous operations to improve hydrogen production.

CHAPTER 3

MATERIALS AND METHODS

3.1 INTRODUCTION

This chapter includes materials and methods used for this study. This includes collection and preservation POME and bacteria samples. PEG gel was fabricated and further investigated as a carrier for immobilization of cells. Scanning electron microscopy (SEM) was performed to determine the entrapped cells inside the PEG-immobilized beads. The application of PEG-immobilized cells was tested for batch hydrogen production from POME. Monod type kinetics model was used to study the effect of acclimated immobilized cells and unacclimated immobilized cell on hydrogen production. PEG-immobilized cells were further investigated for hydrogen production performance in comparison to suspended cell reactor. Furthermore, to examine the application of PEG-immobilized cells in UASB reactor for enhanced continuous biohydrogen production and treatment efficiency of POME were studied. Finally, to optimization the condition for hydrogen production capability of the immobilized cell, the conditions for cell immobilization including PEG concentration, cell loading, curing times as well as effects of temperature and inorganic components on hydrogen production was investigated. The last part of chapter consists, analytical methods and some important process control parameter calculations which have been used for experiment. The flow chart of different phases of experimental study has been shown in Figure3.1.

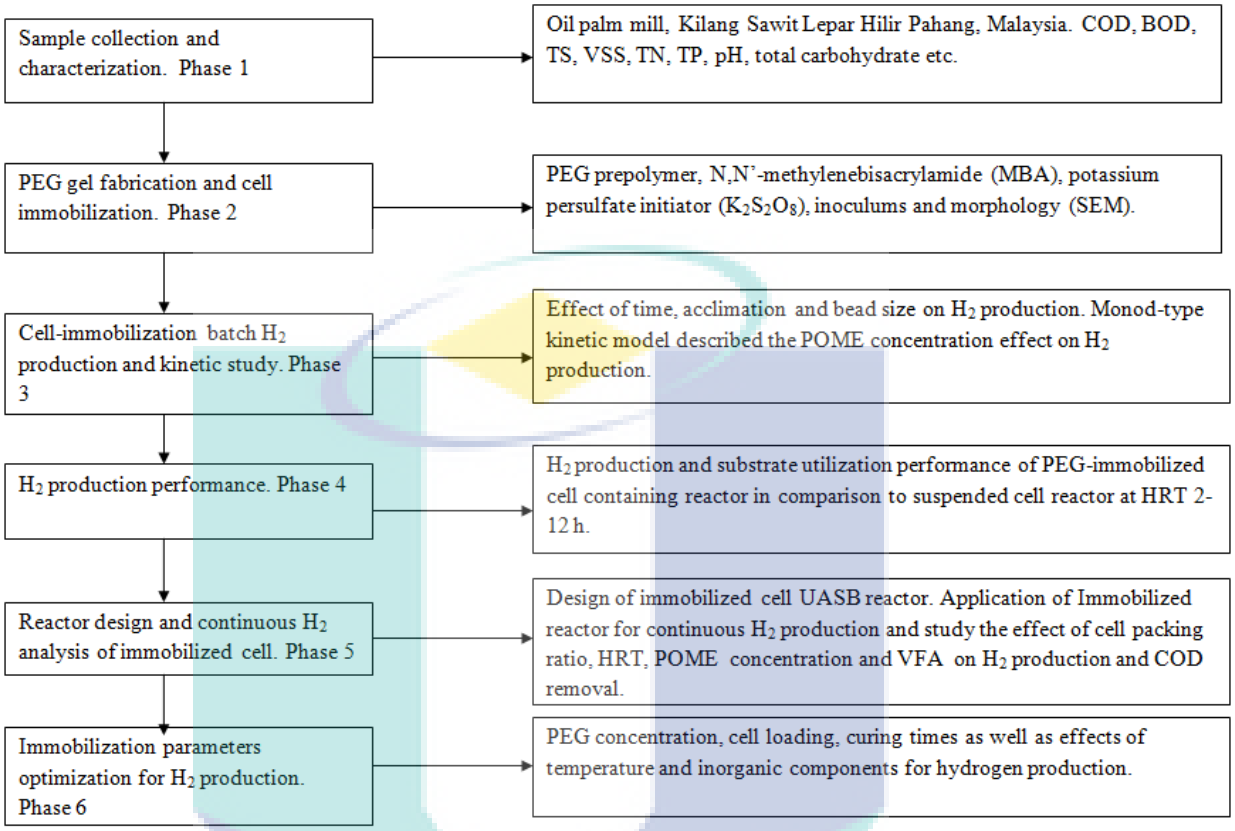

Figure 3.1: Schematic diagram of experimental overview of this study

3.2 BIOHYDROGEN PRODUCTION FROM PALM OIL MILL EFFLUENT USING IMMOBILIZED *CLOSTRIDIUM SP.* IN POLYETHYLENE GLYCOL

3.2.1 Materials

The materials used in this study were nutrient broth for cell culture. PEG (MERCK, K36428809-805) was used to entrap the bacteria (*Clostridium sp.).* PEG was selected in this research for entrapment of cells due to its simple immobilization procedure, low toxicity, good mechanical properties and highly porous structure that helps to sustain immobilized cell viability. N,N'-methylenebisacrylamide (MBA) (SIGMA, 101072856)

and potassium persulfate ($K_2S_2O_8$) (ACROS, 202012500) were used as a cross linker and initiator for hard immobilization beads formation. 2–L glass bioreactor was used for batch hydrogen production. Reciprocal shaker was used to perform batch test. A stainless steel laboratory-scale UASB reactor was used in this study for continuous hydrogen production due to simple to construct and operate and is able to tolerate high organic and hydraulic loading rates. Anaerobic conditions were created in the bioreactor by nitrogen gas sparging for 10 min before use.

3.2.2 POME sample collection and characterization

POME was collected from the tank of the palm oil mill at Kilang Sawit Lepar Hilir Pahang and was used as the substrate (carbon source) for hydrogen fermentation. The POME was preserved at 4 ^{o}C to prevent self-biodegradation and acidification. Each sample was sieved through mesh size of 600 µm to remove the coarse particles from the sample. Figures 3.2 showed the collected samples of POME. POME samples were diluted using tap water according to required COD concentrations. The characteristics of POME used in this study are summarized in Table 3.1.

Figures 3.2: POME samples collected from KSLH-Palm Oil Mill

Table 3.1: Characteristics of palm oil mill effluent

Parameter	Concentration (mg/L)
Biochemical oxygen demand (BOD)	30,100-55,200
Chemical oxygen demand (COD)	55,100-86,300
pH	4.0-5.0
Total carbohydrate	16,200-20,000
Total nitrogen	870-910
Ammonium -nitrogen	20-35
Total phosphorus	100-120
Phosphorus	15-20
Oil and grease	2000-2500
Total solid (TS)	30,000-40,000
Volatile suspended solids (VSS)	9000-12,000
Alkalinity	100-130

All values are in mg/L except pH

3.2.3 Inoculum

Clostridium sp. was obtained from Institute for Medical Research, Kuala Lumpur, Malaysia and used as inoculum. The culture medium (per liter) of strain consisted of 10 g of meat extract, 0.5 g of starch, 4 g of glucose, 4 g of peptone, 3 g of yeast extract, 0.5 g of agar, 0.5 g of l-cysteine·HCl·H_2O, 5 g of NaCl and 2 g of CH_3COONa. The initial pH was adjusted to 6.5 with 0.1M NaOH. The bacterium was stored in 10% glycerol at −10 ^oC before being subjected to immobilization.

3.2.4 Cell-immobilization

Cell immobilization *Clostridium sp.* cells were immobilized by entrapment into PEG prepolymer. PEG was selected as the solid matrix for its ease of use, low toxicity, highly porous structure, and good mechanical properties. First, 10% (w/v) PEG and 0.6% (w/v) N,N-methylenebisacrylamide (MBA) cross linker were dissolved in a water. The resulting mixture and 20 ml of inoculum (in exponential growth phase) was quickly mixed in a beaker. The dry weight of cells in 20 ml of inoculum was approximately 2.2 g. To start polymerization, 0.25% (w/v) potassium persulfate initiator ($K_2S_2O_8$) was mixed in and the mixture allowed to stand for about 30 min to promote bead formation. The resulting immobilized cell bead was cut into 3-mm beads. The biomass content of the immobilized bead was ~10 mg cell/g bead. The dry weight of immobilized cell in immobilized beads was assessed by measuring the difference in dry weight between the biomass-associated beads and the beads alone (Owen et al., 1978). The immobilized cells were repeatedly acclimatized with POME in a 2 L bioreactor for 2-6 h.

3.2.5 Mechanical bead testing

The mechanical stability of immobilized cells was measured according to a method described by (Reyes et al., 2006), with some modifications. Eighty PEG immobilized beads were incubated for 15 h in a shaking flask containing 100 mL 0.80% NaCl with six glass beads at 37 ^{o}C with shaking at 150 rpm. The number of intact immobilized beads in the flask at the end of the experiment was examined under a microscope. The mechanical stability of the immobilized beads was expressed by the fracture frequency of the beads, f (%) = [N/Nt] × 100, where N is the number of fractured beads and Nt is the total number of beads. Five independent experiments were carried out.

3.2.6 Batch operation for hydrogen production

A total of 300 g of immobilized beads was inoculated in a 2-L bioreactor with 1L POME as a substrate. Anaerobic conditions were created in the bioreactor by nitrogen gas

sparging for 10 min. The batch tests were performed to determine the effects of different sizes of immobilized beads on hydrogen production. The bioreactor was maintained at a constant temperature of 37 ^{o}C and a pH of 5.5, which were found to be favorable for hydrogen production (Chong et al., 2009a). The bioreactor was capped with a stopper and placed in a reciprocal shaker (150 rpm). At the end of each experiment, the beads collected from the spent medium were washed with distilled water. As a control, the test was also conducted with free cells (without immobilization) to examine the hydrogen production. All tests were conducted in triplicate.

3.2.7 Repeated batch experiments

After the initial batch test, repeated batch tests were instigated by reserved the immobilized beads and replacing the liquid medium with fresh medium (1 L). Similar procedures were repeated 15–20 times to the extent that the mechanical strength of the immobilized beads allowed. The composition of the gas and the concentrations of soluble products were measured throughout the experiments.

3.3 BIOHYDROGEN PRODUCTION PERFORMANCE IN IMMOBLILIZED CELL REACTOR VERSUS SUSPENDED CELL REACTOR.

3.3.1 Materials

The materials used in this study are the same as described in section 3.2.1.

3.3.2 POME sample collection and characterization

POME sample and characteristics have been previously described in section 3.2.2.

3.3.3 Inoculum

The seed was obtained from a same palm oil mill wastewater treatment plant used

as inoculum. The collected sludge was sieved using 500 mm mesh. Hydrogen productivity of the sludge was increased by heat treatment at 80 ^{o}C for 50 min (Chang and Lin, 2004). The pH was restored to 6.0 by 0.1 N NaOH and the 2 L volume of filtrate was transferred to the fermentor. The initial volatile VSS and TS concentration of the sludge were 8.0 and 11.4 g/L, respectively. Before subjected to immobilization, the heat treated sludge was acclimated with synthetic wastewater in a UASB reactor at 37 ^{o}C.

3.3.4 Preparation of PEG-immobilized cells

Ten gram of PEG, 1 g MBA cross linker and 50 mL of distilled water was carefully heated to 40 ^{o}C to completely dissolve the PEG. The solution than was cooled down to below 30 ^{o}C. One portion of centrifuge H_2-producing POME activated sludge at 2000 rpm for 15 min (100 ml ~ 100 g) and one portion of PEG solution (100 ml) mentioned above thoroughly mixed. To start polymerization, 0.5 g $K_2S_2O_8$ was mixed in and the mixture allowed to stand for about 30 min to promote bead formation. The resulting immobilized sludge bead was cut into 3 mm beads shown in Figure 3.3. The biomass content of the immobilized beads was ~ 10 mg VSS/g bead.

Figures 3.3: PEG-immobilized sludge pallets

3.3.5 Set-up and operations of UASB reactor for hydrogen production

UASB reactors with a working volume of 500 mL or 5 L were used in this study. The reactors were flushed with oxygen-free nitrogen gas for 20 min to established anaerobic condition (Chong et al., 2009). The pH of the medium was controlled at 5.5 with 1 M NaOH and 1 M HCl, constantly mixing and temperature maintained at 37 ^{o}C through water jacket. Immobilized sludge beads (100 g or 200 g) were placed in a UASB reactor (500 mL or 5 L) containing aforementioned medium. The feed was pumped into the reactor using a peristaltic pump (Masterflex L/S, Cole Palmer Instrument, USA). During the reaction phase, the reactor was intermittently mixed with liquid recirculation to provide better distribution of biomass and improve contact of microflora with wastewater and also prevented inhibitory effect of oxygen from influent on strictly anaerobic mixed culture in reactor (O-Thong et al., 2007). The gas line was connected to the gas holder to measure the daily biogas production by water displacement method. The composition of biogas and soluble metabolites produced during hydrogen fermentation was determined at designated time intervals. The reactor was operated at HRT of 1–12 h by adjusting the volumetric flow rate of the feed.

3.4 APPLICATION OF POLYETHYLENE GLYCOL IMMOBILIZED *CLOSTRIDIUM SP* FOR CONTINUOUS HYDROGEN PRODUCTION FROM PALM OIL MILL EFFLUENT IN UPFLOW ANAEROBIC SLUDGE BLANKET REACTOR.

3.4.1 POME sample collection and characterization

POME sample and characteristics have been previously described in section 3.2.2. Prior to being fed into the UASB reactor, the raw POME was diluted to a required COD concentration for fermentation.

3.4.2 Inoculum

The hydrogen producing bacteria of *Clostridium sp.* was obtained from POME sludge after heat treatment for 30 min at 90 ^{o}C (Kim et al., 2006). The sludge was taken from an anaerobic digester in the palm oil mill of Lepar Hilir Pahang, Malaysia. The heat-treated sludge was diluted with distilled water, and then cultured in medium under anaerobic conditions using an anaerobic jar at 36 ^{o}C. The composition of the culture medium was (per L): 10 g of meat extract, 0.5 g of starch, 4 g of glucose, 2.5 g of peptone, 3 g of yeast extract, 0.5 g of agar, 0.5 g of l-cysteine·HCl·H_2O, 5 g of NaCl, 2 g of CH_3COONa, pH 6.5 (adjusted with 0.1 M NaOH). Biochemical identification was carried out by the rapid ANA II microtest system indicated that the strain belonged to the genus *Clostridium*. For biochemical identification the strain was sent to First-Base, Malaysia. By aligning with the 16S rDNA gene sequences from GenBank releases, the strain LS2 exhibited 99% sequence identity with genus Clostridium. So the hydrogen producing strain was considered to belong to *Clostridium sp*. The isolated strain was stored in sterile 15% (v/v) glycerol solution at −30 ^{o}C before being subjected to immobilization.

3.4.3 Immobilization of cells in PEG

Clostridium sp. cells were immobilized by entrapment in a PEG prepolymer. First, the PEG prepolymer and the promoter N,N,N,N-tetramethylenediamine were dissolved to achieve 12% (w/v) and 0.6% (w/v) solutions, respectively. The resulting mixture and 24 ml of inoculum (approximately 2.8 g of cells, dry wt.) was quickly mixed in a beaker. To start polymerization, an initiator ($K_2S_2O_8$) at 0.25% (w/v) was added and the mixture was allowed to stand for approximately 30 min to promote polymer formation. Prior to use, the immobilized cells were stored in physiological saline solution for 2 h and then washed thoroughly with distilled water. The biomass content of the beads was ~ 18 mg cells/g bead. Scanning electron microscopy (SEM) images of the immobilized-cell beads showed that rod-like bacteria covered the surface and penetrated to the core of the beads, as shown in Figure. 3.4.

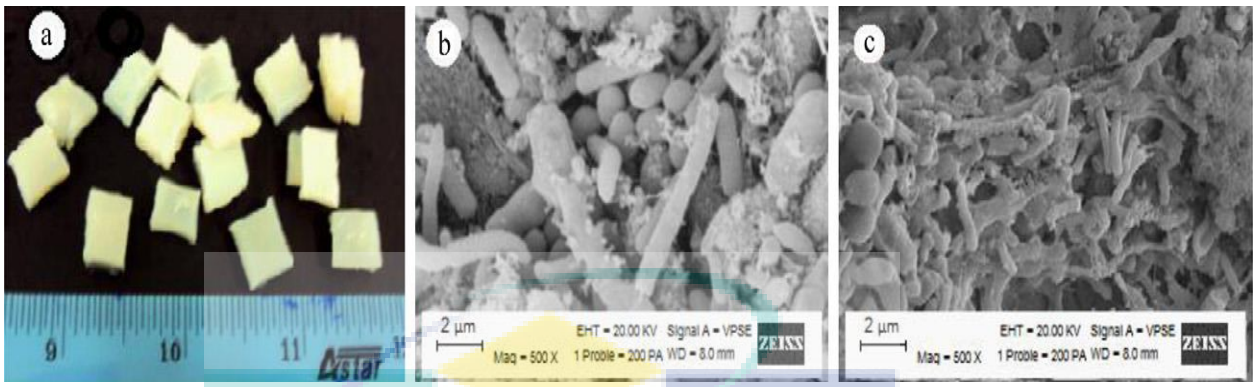

Figure 3.4: SEM images of the PEG immobilized cell beads: (a) the shape and size of the immobilized beads; (b) immobilized *Clostridium sp.* in PEG at the starting phase of experiment. Scale bar: 2 µm; (c) immobilized *Clostridium sp.* in PEG at the end of experiment. Scale bar: 2 µm.

3.4.4 UASB reactor setup and operation for hydrogen production

A schematic of the UASB reactor is shown in Figure. 3.5. The reactor was fabricated in university workshop. The UASB reactor was made of stain less steel with a total volume of 5126 cm^3 and a 5-L working volume. Two sampling ports were installed at 15, 65 cm from bottom of the reactor. These sampling ports were made by 6 mm of pneumatic fitting which were connected to manual valves in order to prevent leakage. An effluent post, an overflow pipe and baffles were installed 5 cm, 10 cm and 20 cm below the top of reactor respectively. The baffles angle of inclination was 45 degree with inner diameter 40% lesser than the actual reactor diameter. The temperature of the reactor during fermentation was maintained at 37 ^oC by hot water circulation through the water jacket. The pH was maintained at 5.5 by adding 1 M NaOH or 1 M HCl and by using pH sensors. POME was fed from the bottom of the reactor together with PEG-immobilized cells by using a peristaltic pump. Intermittent mixing was applied to avoid settling of PEG-immobilized beads in the reactor and to provide better contact between the immobilized cells and the wastewater. Two sampling points were introduced at appropriate heights in

the UASB reactor. A gas–liquid separator was introduced at the top of the reactor for biogas collection. The reactor was purged with nitrogen gas for 10 min to promote anaerobic conditions. The UASB reactor was operated at 40 h HRT during the first 36 h start-up period. When the system reached steady-state the effects of different HRT and OLR on hydrogen production and COD removal were studied. The HRT was decreased stepwise from 24 to 6 h at a constant OLR of 3.3 g COD/L/h. A suitable HRT was selected to study the effect of OLR at 1.6, 3.3, 5.0, and 6.6 g COD/L/h. The steady-state condition was justified when hydrogen gas content, biogas volume, and volatile fatty acids (VFA) concentration were less than 10% variation (Prasertsan et al., 2009). Biogas production primarily consisted of hydrogen and carbon dioxide (CO_2), COD removal efficiency (%), volatile fatty acids (VFAs), pH, and temperature were monitored at designated time intervals.

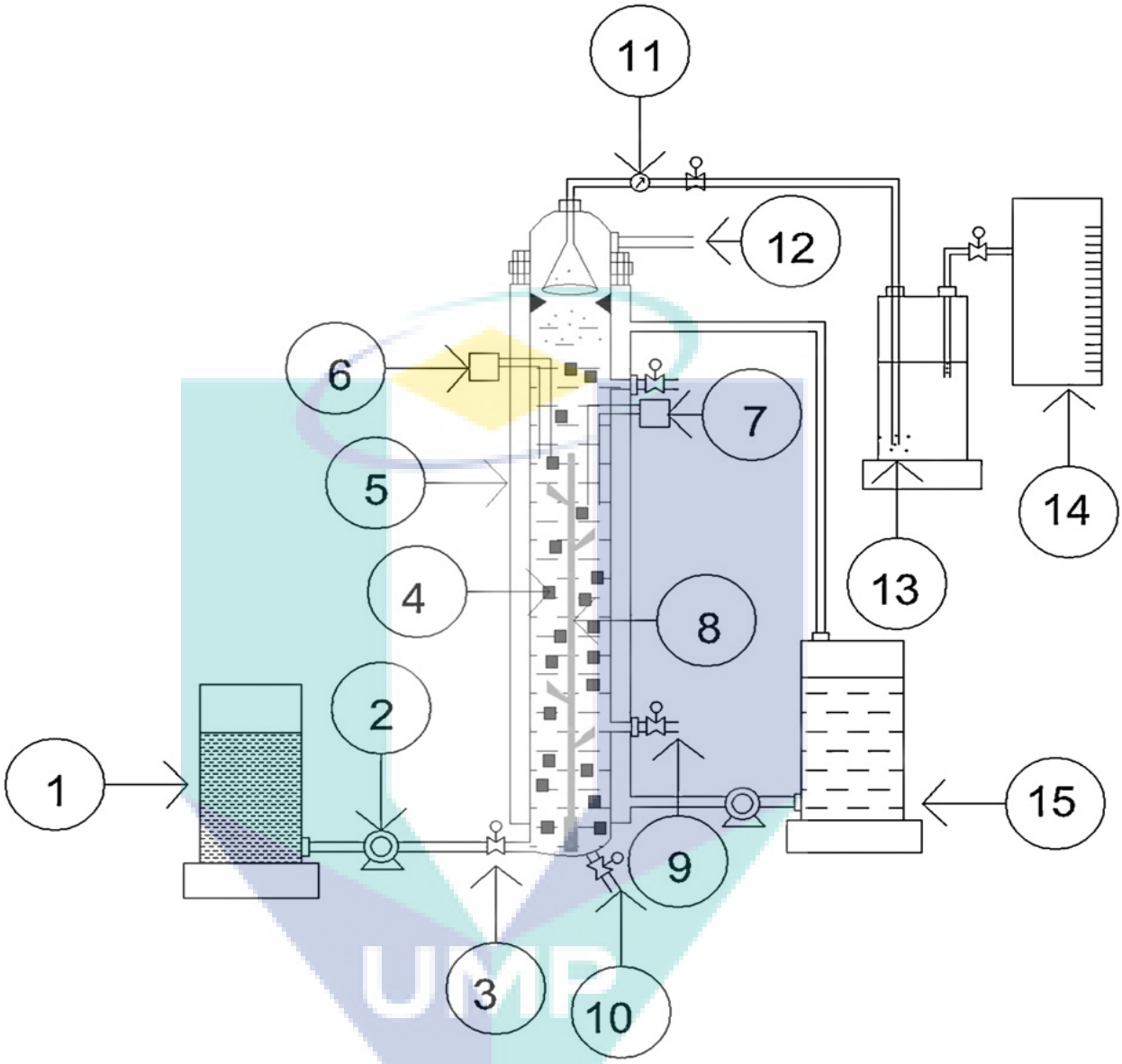

Figure 3.5: Schematic description of PEG immobilized cells containing UASB reactor for continuous hydrogen production. (1: Substrate feed tank; 2: feed pump; 3: manual valve; 4: immobilized cell beads; 5: water jacket; 6: temperature indicator; 7: pH indicator; 8: stirred blade; 9: sampling point; 10: drain; 11: gas flow meter; 12: effluent outlet line; 13: biogas collection system; 14: hydrogen gas holder; 15: hot water tank)

3.5 EFFECT OF CELL PACKING RATIO, HRT AND POME CONCENTATION ON CONTINNOUES BIOHYDROGEN PRODUCTION AND TREATMENT EFFICIENCY OF PALM OIL MILL EFFLUENT IN IMMOBILIZED CELL UASB REACTOR

3.5.1 POME sample collection and characterization

POME sample and characteristics have been already described in section 3.2.2. Prior to being fed into the reactor, the POME was diluted to a required COD concentration for fermentation.

3.5.2 Inoculum

Microorganisms used in this study are the same as described in section 3.4.2.

3.5.3 Cell immobilization

Cell immobilization method used in this study is the same as described in section 3.4.3.

3.5.4 Reactor operation and monitoring

A stainless steel laboratory-scale UASB reactor (5126 cm^3) with 5 L working volume was used in this study Figure 3.5. The UASB reactor was operated at temperature 37 ^oC and temperature was maintained by hot water circulation through the water jacket. Throughout the experiments, the pH of the reaction medium in the reactor was adjusted around 5.5 by adding 1 M NaOH or 1 M HCl solutions. The medium was fed into the UASB reactor together with PEG-immobilized cells. Sampling points were introduced at appropriate heights in the reactor. A gas-liquid separator was introduced at the top of the reactor, where the biogas and soluble microbial product (SMP) collected separately. The

reactor was purged with nitrogen gas for 10 min to create anaerobic conditions. The UASB reactor was loaded with an appropriate amount of PEG-immobilized cells to obtain a final solution of 4-16% (w/v). The UASB reactor was operated on batch mode for 6 h before being switched to continuous mode at a HRT of 32-8 h and a POME concentration of 10-40 g COD/L. The quantity and composition of the biogas, COD removal efficiency (%), volatile fatty acids (VFAs), pH, and temperature were monitored at designated time intervals.

3.6 EXPLORATION AND OPTIMIZATION OF HYDROGEN PRODUCTION CAPBILITY OF THE IMMOBILIZED CELLS AS WELL AS EFFECT OF TEMPERATURE AND INORGANIC COMPONENTS ON BIOHYDROGEN PRODUCTION

3.6.1 POME sample collection and characterization

POME sample and characteristics have been already described in section 3.2.2.

3.6.2 Inoculum

Microorganisms used in this study are the same as described in section 3.4.2.

3.6.3 Immobilization

Clostridium sp. cells were immobilized by entrapment into PEG prepolymer. The cells of anaerobic bacteria were harvested by centrifugation at 8000 rpm for 12 min. A culture sample was heated at 80 °C for 3 min prior to use for immobilization. First, 12% (w/v) PEG and 0.6% (w/v) MBA cross linker were dissolved in water. The resulting mixture and 40 mL of inoculum (in exponential growth phase) was quickly mixed in a beaker. The dry weight of cells in 30 mL of inoculum was approximately 3.6 g. To start polymerization, 0.25% (w/v) $K_2S_2O_8$ was mixed in and the mixture allowed to stand for

about 60 min to promote bead formation. The resulting immobilized cell bead was cut into 3-mm beads. The biomass content of the immobilized bead was ~10 mg cell/g bead.

3.6.4 Optimization of immobilization parameters

As immobilization matrices, hydrogels should possess high strength, stability, porosity, and permeability of gel beads. To meet these requirements, conditions for hydrogel bead preparation need to be optimized. Thus, selecting a suitable initiator ($K_2S_2O_8$), as well as initial biomass that can be loaded in the bead and PEG prepolymer concentrations, is important for the synthesis of a hydrogel immobilization matrix that has high strength and elasticity. For this purpose, varying PEG prepolymer concentrations (2-12% w/v) and different volumes of inoculum were used for immobilization, such as 10, 20, 30 and 40. The dry weight of cells in 10 mL of inoculum was approximately 1.2 g. The beads were cured for varying periods (20–120 min) at 4 °C to check the time requirement for proper curing of the beads.

3.6.5 Batch hydrogen production using immobilized cell beads

The immobilized cells obtained as above were used to inoculate 250 mL serum bottles containing 150 mL of prepared POME (20 g COD/L) as a fermentation medium. The gas space of the vial was vacuumed and flushed with argon (99%) for 15 min to create anaerobic conditions. Vials were capped with a stopper and placed in a reciprocal shaker (150 rpm, 37 ^{o}C and pH 5.5). During the course of experiments, gas samples were withdrawn at designated time intervals and checked for composition of gas products (mainly, H_2 and CO_2). Liquid sample were also taken regularly and monitored under microscope for cell leakage. Hydrogen production rate was measured while varying, PEG concentration (2-12%), cell loading concentration in beads (10-40 mL), curing time (20-120 min) and temperature (25-45 ^{o}C). The effect of inorganic components concentration such as $NiCl_2$ (0.5-2.0 mg/L), $FeCl_2$ (100-400 mg/L) and $MgSO_4$ (50-200 mg/L) on the hydrogen production capability of the immobilized cells was also investigated. Unless stated otherwise, the standard conditions were defined as follows: PEG concentration in the

solution used for immobilization, 10% (w/v); cell concentration in the suspension used for immobilization, 30 ml (3.6 g dry wt.); temperature, 37 ^{o}C; curing time, 80 min; pH, 5.5.

3.6.6 Continuous experiments using PEG-immobilized cells

Optimized conditions of immobilization were selected for effective cell encapsulation in continuous operation. Continuous experiments were conducted in 5126 mL reactor with working volume of 5 L. The reactor was flushed with argon for 15 min to create anaerobic conditions. The reactor was operated at 37 ^{o}C using a temperature controller and a stirring speed of 100 rpm. The pH of the medium was maintained at pH 5.5 with 1 M NaOH and 1 M HCl.

3.7 ANALYTICAL METHODS

The COD, BOD, TN, TKN, TP, TS, TSS, and VSS were measured by the Standard Methods (APHA, 1998).

3.7.1 Chemical Oxygen Demand (COD) determination method

The chemical oxygen demand was measured by direct digestion method, using HACH apparatus LR (3-150 mg/L COD); HR (20-1500 mg/L COD) and HR plus (20-15000 mg/L COD and above). The COD measurement was always carried out by diluting the original sample to meet the vial results. The vials (blank and sample) after adding the sample were placed in COD digester reactor for 2 hours at 150 ^{o}C which were kept remained in reactor until the reactor temperature was lower down to 120 ^{o}C. After moving vials from reactor, these were allowed to cool down further at room temperature for accurate results. The program 435 HR COD was used accordingly for results.

3.7.2 Biochemical Oxygen Demand (BOD) determination method

In the standard BOD test, a small sample of the wastewater to be tested was placed in a 300 mL BOD bottle. The bottle was then filled with dilution water saturated in oxygen and containing the nutrients required for biological growth. To ensure that meaningful results are obtained, the sample must be suitably diluted with specially prepared dilution water so that adequate nutrients and oxygen will be available during the incubation period.

Before the bottle was stoppered, the oxygen concentration in the bottle was measured. After the bottle was incubated for 5 days at 20 °C ± 1 °C, the dissolve oxygen (DO) concentration was measured again. The difference in dissolved oxygen before and after incubation was calculated for BOD measurement. The dissolved oxygen was measured through DO meter (YSI 5100). The BOD of the sample is the difference in the dissolve oxygen concentration values, expressed in milligrams per liter, divided by the decimal fraction of sampled used. BOD concentration and BOD removal efficiency can be calculated by the following formula.

$$\mathbf{BOD} = \frac{\mathbf{D_1} - \boldsymbol{D_2}}{\mathbf{P}}$$

Where,

BOD = Biochemical Oxygen Demand (mgL^{-1})

D1 = DO of diluted sample immediately after preparation (mgL^{-1})

D2 = DO of diluted sample after 3-days incubation at 27 °C (mgL^{-1})

P = fraction of wastewater sample volume to total combined volume

3.7.3 Total Nitrogen (TN) determination method

Total nitrogen was measured by persulfate digestion method using HACH program 350N, LR (0.5-25 mg/L) TNT. The nitrogen hydroxide reagent vials with added total nitrogen persulfate pillows and 2 mL sample (blank and sample) were kept in COD reactor for 30 minutes at 105 ^{o}C. After removing and cooling both the vials to room temperature,

total nitrogen reagent pillows A, B and C were added by giving them the reaction time of 3, 2 and 5 minutes respectively in HACH apparatus. The blank was also run with same manner as used for original sample for apparatus calibration. The obtained results were obtained by multiplying the original results with the dilution factor.

3.7.4 Total Kjeldahl Nitrogen (TKN) Determination Method

Total Kjeldahl nitrogen (TKN) was measured by Nessler method where digestion of sample is carried out by means of digestion apparatus. After digestion 10 mL of sample was taken in a 25 mL graduated cylinder and same amount of deionized water was taken for blank. Then one drop of TKN indicator was added to each cylinder. After that drops of 8.0 N KOH were added until a flash of blue color is appeared. For taking permanent blue color, a drop of 1.0 N KOH was added and mixed by gentle shaking of cylinder then other drop was added and mixed until permanent blue color appeared. The cylinders were then filled up 20 mL mark with de-ionized water and three drops of mineral stabilizer were added to each cylinder. The three drops of polyvinyl alcohol dispersing agent were also added and mixed in each cylinder and filled with de-ionized water up to 25 mL mark. 1.0 mL of Nessler reagent was pipette in each cylinder and cylinders were inverted repeatedly. The cylinders were kept consecutively in HACH apparatus pre-selected program 399 nitrogen, TKN and two minute reaction time was given to each of the cylinder. After that the 10 mL volume from each of the cylinder was poured in separate square sample cell. The blank square sample cell was placed in apparatus for zero and then original sample was placed for results. The measured amount was multiplied with a factor 75 and then divided with volume of digester for analysis and volume taken in square sample cell.

3.7.5 Total Phosphorus (TP) Determination Method

Total phosphorus was measured by molybdovanadate method with acid persulfate digestion. Two total phosphorus test ‘N tube vials used and each vial was added with 5 mL of de-ionized water and sample solution respectively and potassium persulfate powder pillows were added to each vial. The de-ionized water was used for blank. The vials were

then placed in reactor preheated at 150 ^{o}C for 30 minutes. After digestion the vials were placed in vial holder and when these vials cooled down at room temperature, 2 mL of 1.54 N sodium hydroxide solution was added to each vial. Then 0.5 mL of molybdovanadate reagent was added to each vial. The vials were then placed in HACH apparatus for seven minutes reaction period. The total phosphorus was calculated by selecting a 542 HR P total program. The results obtained were in mg/L PO_4^{3-} and results for only phosphorous (P) were obtained by multiplying a factor of 0.3261 to the original results. The obtained results were obtained by multiplying the original results with the dilution factor.

3.7.6 Total Solids (TS) Determination Method

All organic matter contains some water. Total Solids (TS) is a measure of the actual solid content of a substance. Only portions of the solid material are actually bio-converted. TS is determined by weighing a sample, oven-drying it to remove all moisture (103 to 105 ^{o}C), and then re-weighing the dried sample. TS% is determined by dividing the "dry" weight by the "wet" weight.

3.7.7 Total Suspended Solids (TSS) determination method

TSS is solid materials, including organic and inorganic, that are suspended in the water. Total suspended solids (TSS) include all particles suspended in water which will not pass through a filter. The total suspended solids were measure by centrifuge method and the centrifuge was run at 1400-2000 rpm for 5 minutes and the process was repeated again for better solids settling in the centrifuge tube. The centrifuged contents were transfered in porcelain dish and placed in oven for 2-4 hours at 104 ^{o}C. The weight difference was then divided by the sample volume and multiplied with the unit conversion factor to obtain the results in mgL^{-1}. It can be calculated by the following formula:

$$\mathbf{TSS} = \frac{\mathbf{(A - B) \times 1000}}{\mathbf{Sample\ volume, mL}}$$

Where,

TSS = total suspended solids (mgL^{-1})

A = weight of crucible + paper + solids (mg)

B = weight of crucible + paper (mg)

3.7.8 Volatile Suspended Solids (VSS) determination method

Volatile solids are those solids lost on ignition (heating to 550 ^{o}C). They are useful to the treatment plant operator because they give a rough approximation of the amount of organic matter present in the solid fraction of wastewater, activated sludge and industrial wastes. The TSS residue obtained after oven drying was then processed for ignition at 550 ^{o}C to obtain volatile suspended solids concentration. The difference in weight loosed in ignition and before ignition was measured and multiplied by the unit conversion factor to obtain the results in mgL^{-1}. It can be calculated by the expression given below:

$$\mathbf{VSS} = \frac{\mathbf{(A-B)\times 1000}}{\mathbf{Sample\ volume, mL}}$$

Where,

VSS = volatile suspended solids (mgL^{-1})

A = weight of dish + paper + solids (mg) (before ignition)

B = weight of dish + paper (mg) (After ignition).

3.7.9 Mixed Liquor Volatile Suspended Solids (MLVSS) (Standard Method 2540-B and E)

The well-mixed sample was filtered through a weighed standard glass-fibre filter, and the residue retained on the filter was dried to a constant weight in a Protect Oven at 103-105 ^{o}C, and then cooled in desiccator to balance temperature, and weighed. Now the dried solids ignited to constant weight at 550±50 ^{o}C in a Carbolite furnace. The difference between the weights of ash and dish is the weight of mixed liquor suspended solids. The MLVSS can be calculated by the formula given below:

$$\mathbf{MLVSS} = \frac{\mathbf{(A - B) \times 1000}}{\mathbf{Sample\ volume, mL}}$$

Where,

MLVSS = mixed liquor volatile suspended solids (mgL^{-1})

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

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

3.7.10 pH and Alkalinity determination method

The pH was measured using HACH pH meter. Alkalinity was measured by the direct titration method. 50 ml sample and 0.1 N standard H_2SO_4 solutions were prepared. Now 50 mL sample was then titrated with 0.1 N standard H_2SO_4 solutions using a calibrated pH meter to an end point of pH 4.5. After each addition of acid, the sample was mixed thoroughly and gently until a constant reading was obtained. While reaching near the end point, the titration rate was slow down and to ensure that pH equilibrium was reached before adding more titrant. The alkalinity value of the sample was calculated according to the equation given below:

$$\mathbf{Total\ Alkalinity\ (mg/L)} = \frac{\mathbf{S \times N \times 1000}}{\mathbf{Sample\ volume, mL}}$$

Where,

S = mL of titrant to reach end point

N = equivalents H_2SO_4 per liter titrant

3.7.11 Oil and Grease determination method

Oil and grease were determined according to standard methods (Partition-Gravimetric method). The samples were collected in 1 liter glass bottle which pre-rinsed with n-hexane. The sample was acidified to pH 2 with 1:1 hydrochloric acid (HCl) and transferred into a 500 mL separatory funnel. By using 100 mL n-hexane, the oil was extracted from 100 mL of sample in the separator funnel. The sample was shaken vigorously for two minutes. After that, the two layers started separating in the funnel. The aqueous layer and small amount of organic layer was drained into original sample container, while the solvent layer was drained through a funnel containing solvent moisture of Advatec 150 mm filter paper to a distilling flask. The extraction was repeated two times and the extracts were combined in the same distilling flask. Hexane was distilled in water bath at 70 ^{o}C until all of the solvent was evaporated. The sample was then dried in a Protect Oven at 103-105 ^{o}C, cooled and weighed. The oil and grease content of the sample was calculated using equation given below:

$$\textbf{Oil and Grease (mg/L)} = \frac{\textbf{(A - B)} \times \textbf{1000}}{\textbf{Sample volume, mL}}$$

Where,

A = weight of oil extracted + dried distilling flask (mg)

B = weight of blank distilling flask (mg)

3.7.12 Volatile Fatty Acids (VFA) Determination (HACH-Esterification Method 8196)

Volatile fatty acids (VFAs) were measured by esterification method using HACH apparatus. A 25 mL sample was centrifuged and supernatant was collected in to separate sampling bottle. The 0.5 mL of this supernatant was taken into another 25 mL sampling bottle. Ethylene glycol 1.5 mL was added to the sample and swirled for mixing. The 0.2 mL of 19.2 N sulfuric acid standard solution was added and mixed prior to putting the

sample bottle into water bath for boiling. The HACH program 770 volatile acids was selected and boiled sample was placed in it thus giving a reaction time of three minutes. After beep the sample bottle made cool down until 25 ^{o}C and 0.5 mL of hydroxylamine hydrochloride solution was added and mixed. Then 2 mL of 4.5 N sodium hydroxide solution was pipette into the sample and 10 mL of ferric chloride solution as also added and mixed. 10 mL of deionized water was added to the sample and well mixed. The 10 mL of sample was taken into clean dry square sample cell. Similarly whole procedure was also adapted in order to prepare the blank sample. This square sample cell was placed in the apparatus for giving a 3 minute reaction time. The volatile acids were noted in mg-acetic acid/L by placing the sample cell into apparatus.

3.7.13 Gas production measurement (Water Displacement Method)

Gas production was measured by liquid displacement method (Figure 3.5). An inverted burette was connected to one end of a flexible tube and the other end of flexible tube was then jointed with another burette and this full set of U-tube was filled with water up to a graduated level. The gas was entered from one side of the burette and gas volume was measure by the liquid displacement to other side caused by the exerted pressure of entering gas. The change in volume in burette was noted for total gas volume produces at that time. Hydrogen gas was calculated by the same method but the gas was first passed through soda lime in order to remove carbon dioxide and remaining hydrogen gas was then subjected to pass through U-tube water displacement set for measurement. The hydrogen gas contents were measured according to design time interval during experiment. The dry weight of immobilized cell in immobilized beads was assessed by measuring the difference in dry weight between the biomass-associated beads and the beads alone (Owen et al., 1978).

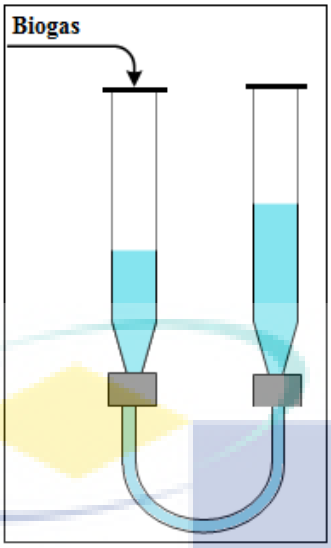

Figure 3.7: Water displacement method for biogas measurement

3.7.14 Biogas Composition by Gas Chromatographic method

A gas chromatograph (GC 8500 Perkin Elmer) equipped with a thermal conductivity detector and a 2-m stainless-steel SS350A column packed with a molecular sieve (80/100 mesh) was used to determine the fraction of hydrogen in biogas using nitrogen as a carrier gas at a flow rate of 25 mL/min. The operating temperatures of the injection port, oven and detector were 100 ^{o}C, 85 ^{o}C and 100 ^{o}C, respectively. Methane and carbon dioxide were analyzed using the same model GC with a 2-m stainless-steel column packed with Porapak T (60/80 mesh) using helium as a carrier gas at a flow rate of 30 mL/min. The operating temperatures of the injection port, oven and detector were 150 ^{o}C, 80 ^{o}C and 100 ^{o}C, respectively. The gas sample of 50 µl for methane and 250 µl for hydrogen was injected in duplicate. For alcohol analysis, 1 cm^3 of sample acidified with 0.003 cm^3 25% H_2SO_4, was analyzed using GC–FID and capillary column of the same model and type, respectively.

3.7.15 Scanning Electron Microscopy (SEM) and biochemical analysis

The morphology of the immobilized bacteria before and after the operation was observed by SEM. The immobilized cells were washed with distilled water and treated with glutaraldehyde for 2 h. The samples were dehydrated by graded ethanol solutions (50–90%) for 20 min in each solution. The dehydrated immobilized cells were transferred into a freeze dryer. The dried samples were covered with a layer of gold under vacuum prior to being subjected to SEM (Zeiss EVO50, Germany). The rapid ANA II microtests (Remel) for anaerobic bacteria were utilized for biochemical identification (Lo et al., 2009).

3.8 IMPORTANT CALCULATION METHOD APPLICABLE DURING EXPERIMENT

3.8.1 The F/M Ratio

The food to microorganism ratio (F/M) is the key factor controlling anaerobic digestion. Microorganisms will most efficiently break down the organic matter in water if they are present in the right proportion. At a given temperature, the bacterial consortia can only consume a limited amount of food each day. In order to consume the required number of gm of waste, the proper number of gm of bacteria must be supplied. The ratio of the gm of waste supplied to the gm of bacteria available to consume the waste is the F/M ratio. This ratio is the controlling factor in all biological treatment processes. A lower the F/M ratio will result in a greater percentage of the waste being converted to gas. Thus, F/M ratio can be used as a parameter for testing UASB performance and COD reduction. Unfortunately, the bacterial mass is difficult to measure since it is difficult to differentiate the bacterial mass from the influent waste. The task would be easier if all of the influent waste were converted to biomass or gas. In that case, the F/M ratio would simply be the digester loading (g-COD) divided by the concentration of biomass (g-VSS) in the digester (e.g. g COD/gVSS day). For any given loading, the efficiency can be improved by lowering the F/M ratio by increasing the concentration of biomass in the digester. Also for any given

biomass concentration within the digester, the efficiency can be improved by decreasing the loading.

$$\frac{\mathbf{F}}{\mathbf{M}} = \frac{\textbf{Organic loading rate}}{\textbf{Volatile solid}}$$

Where,

Organic loading rate = COD of the influent stream (g-CODL^{-1}.day)

Volatile solids = Volatile suspended solids concentration in the reactor (g-VSSL^{-1})

F/M = kg-COD/kg-VSS. day

Thus, F/M ratio generally represents the amount of foods available to the number of microorganisms present in the system. High COD reduction can be achieved at proper F/M ratio which could be in the range of 0.2 to 0.7 gCOD/gVSS.d (Rumana, 2012).

3.8.2 The Hydraulic Retention Time

Hydraulic Retention Time (HRT) calculate before proceeding experiments is also an important process control parameter. HRT measures the length of time that liquid remains in the system. The HRT is the ratio of the reactor volume to the flow rate of the influent substrate. Therefore, it is the time that substrate spends in the bioreactor in contact with the biomass. The HRT plays an important role while anaerobic digestion of which the liquid has to stay within the digester until degradation. The time required to achieve a given degree of treatment depends on the rate of microbial metabolism and subsequently on the type and composition of input organic material. The HRT equals the volume of the reactor divided by the influent flow rate.

Followed by the F/M ratio, the hydraulic retention time (HRT) can be calculated as,

$$\mathbf{HRT} = \frac{\mathbf{CODin}}{\mathbf{OLR}}$$

Where,

HRT = Hydraulic retention time (days)

OLR = Organic loading rate (g-CODL^{-1}.d^{-1})

CODin = Influent COD (g-COD L^{-1})

3.8.3 The Flow Rate

A fluid power variable that describes how much fluid is being moved and how much work is being performed within a given or standard period. Flow rate is usually controlled by means of a peristaltic pump with corresponding tube hosing of different diameter. The lower will be the diameter of tube hose; less will be the flow rate of the influent stream and vice versa. Flow rate can be calculated by the formula as given below:

$$Q = \frac{Vw}{HRT}$$

Where,

Q = Influent flow rate (Ld^{-1})

V_w = Working volume of the reactor (L)

HRT = Hydraulic retention time (days)

3.8.4 The Upflow Velocity

For effective solids-liquid separation in reactor it is necessary that the rate of rise of the liquid (the upflow velocity) should be significantly less than the natural settling velocity of the solids. If this is not the case there will be carryover of solids with the final effluent discharge. Upflow velocity is calculated by dividing the total flow through the reactor by the surface area of the reactor.

$$\mathbf{Vup} = \frac{\mathbf{Q}}{\mathbf{A}}$$

Where,

V_{up} = Upflow velocity (m/h)

Q = Flow rate of influent stream (m^3/h)

A = Cross-sectional area of the reactor (m^2)

The upflow velocity can also be calculated by the following expression.

$$\mathbf{Vup} = \frac{\mathbf{H}}{\mathbf{HRT}}$$

Where,

V_{up} = Upflow velocity (m/h)

H = Height of the reactor up to working volume (m)

HRT = Hydraulic retention time (hours).

CHAPTER 4

RESULTS AND DISCUSSION

4.1 INRODTUCTION

This chapter describes the experimental results and discussion. This chapter is divided into five parts. The first part, describes results and discussion on feasibility of PEG-immobilized cell for batch hydrogen production from POME. In the second part, the results of hydrogen production performance of PEG-immobilized mixed culture in comparison to suspended cell reactor are described. The third part describes the results of PEG-immobilized cell for continuous hydrogen production from POME in UASB reactor. In the fourth part, results regarding the effect of immobilized-cell packing ratio, HRT, and POME concentration on continuous hydrogen production and treatment efficiency of POME. The last part of this chapter describes the results and discussion on optimization of cell immobilization parameters including PEG concentration, cell loading, curing times as well as effects of temperature, pH and inorganic components on hydrogen production.

4.2 BIOHYDROGEN PRODUCTION FROM PALM OIL MILL EFFLUENT USING IMMOBILIZED *CLOSTRIDIUM SP.* IN POLYETHYLENE GLYCOL

4.2.1 Performance of immobilized-cells compared to that of free cell cultures

A comparison of a hydrogen production rate by free and immobilized cells of *Clostridium sp.* was done by performing batch fermentations test. In this study, hydrogen production rate values obtained from the PEG entrapment technique were higher than those obtained from the free cells experiments (Table 4.1). The results suggested that the immobilization technique could improve the hydrogen production efficiency of *Clostridium sp.* from POME. This could be due to the fact that the cell wall membrane system interacts strongly with the gel matrix network. Since this network can present different structural arrangements, unconventional three-dimensional adjustments between the cell wall and the matrix molecules are possible. Therefore, these altered spatial organizations at discrete molecular levels induce the cells at determined specific conditions to enhance the biosynthesis of metabolites and increase the permeability of the cells inside the bead to the substrate (Karel et al., 1985), hence enhancing the rate of hydrogen production.

Table 4.1: Comparative study on the efficiency of hydrogen fermentation processes

Fermentation process	**Substrate**	**Culture type**	**H_2 production rate**	**Ref.**
Batch	Sugarcane juice	IMC	5.78 mmol/L h	(Plangklang et al., 2012)
Continuous	Glucose	IMC	2.76 mmol /L h	(Zhao et al., 2012)
Batch	POME	IMC	22.7 mmol/L h	Present work
Batch	POME	SPC	12.3 mmol/L h	Present work
Continuous	POME	SPC	16.9 mmol/L h	(Prasertsan et al., 2009)
Feed Batch	POME	SPC	19.4 mmol/L h	(Atif et al., 2005)
Continuous	POME	SPC	12.4 mmol/L h	(Badiei et al., 2011)
Batch	Food wastewater	SPC	24.5 mmol /L h	(Wu and Lin, 2004)

IMC: Immobilized cells system, SPC: Suspended cells system.

Figure 4.1 shows the effect of time on the cell leakage from the beads in the medium suspension and consequent effect on hydrogen production. The prolongation of experiment time from 4 h to 24 h, the cell leakage in the suspension medium is negligible, which resulted in slightly increase in hydrogen production rate. The values of standard deviation and error for hydrogen production rate and cell leakage in the medium suspension have been shown in Table A1.

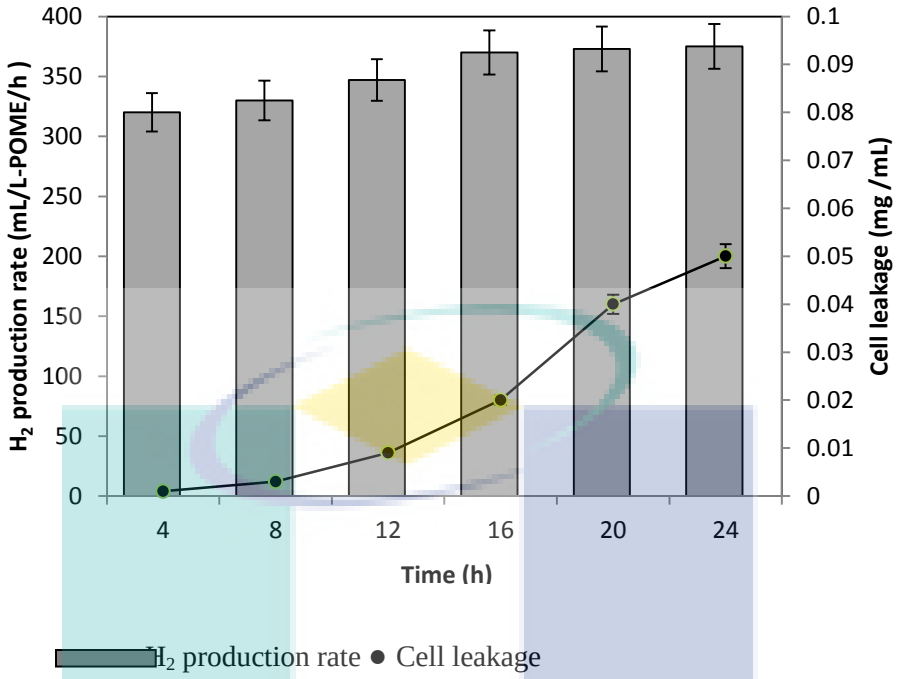

Figure 4.1: The effect of time on the cell leakage from the beads in the medium suspension and consequent effect on hydrogen production

The SEM micrographs show that structure of PEG beads had lot of pores inside the gels, which was a suitable for the entrapment of microbial cells and provided an anaerobic environment for the growth of the microorganisms (Figure. 4.2). Table 4.1 lists the hydrogen production rates in the literature with free and immobilized cells from different substrate for comparison. Although the hydrogen production obtained from the present study is still lower than that obtained from food wastewater using free cells culture (Wu and Lin, 2004). The possible reasons for higher hydrogen production from food wastewater could be due to the different substrate, their COD concentration ranges and reaction conditions.

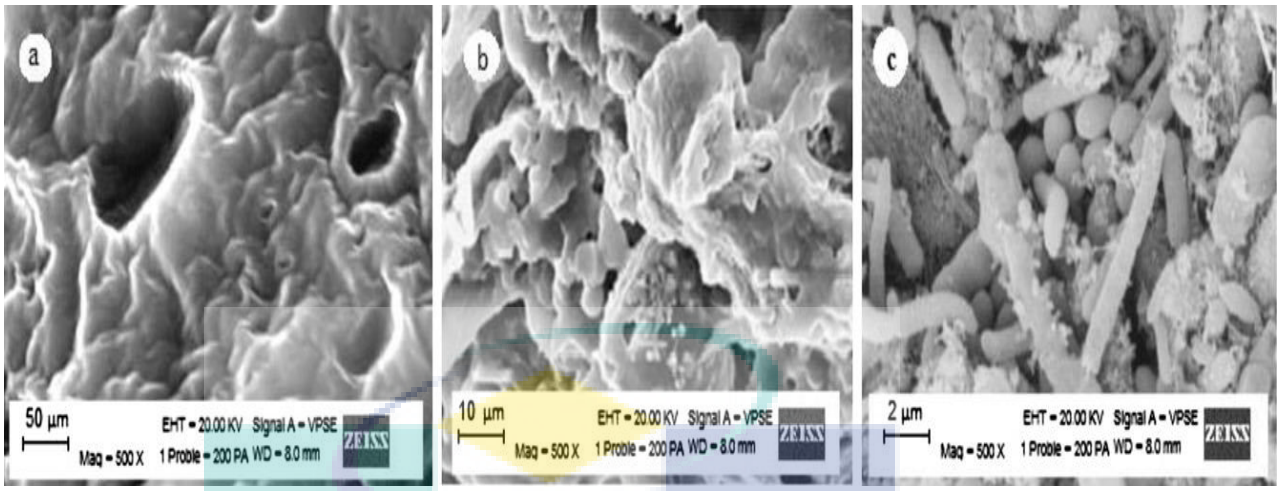

Figure 4.2: SEM images of the PEG-immobilized cells. (a) Immobilized beads without cells. Scale bar: 50 µm. (b) Immobilized beads with cells. Scale bar: 10 µm (c) Cross-sectional image of the immobilized beads with cells. Scale bar: 2 µm.

4.2.2 Effect of acclimated immobilized cells on hydrogen production

In the current study, immobilized cells tended to have enhanced hydrogen production rates when they were repeatedly adapted with POME medium for a period of time. Therefore, to increase hydrogen production rates, the immobilized cells were repeatedly cultivated in POME until an optimal H_2 production rate was obtained. Significant improvement in hydrogen production rate was observed with acclimated immobilized cells (see Figure. 4.3) compared with unacclimated immobilized cells. As shown in Table 4.2, there were significant differences in hydrogen production rates (R_{H_2}) and yields between the immobilized culture with and without acclimation. Acclimated immobilized culture gave a 1.5–5.0-fold improvement in R_{H_2} and 1.5–2-fold increases in yield compared with unacclimated immobilized cells. Enhanced hydrogen production activity of immobilized cells with acclimation could be due to the exposure of cells to the substrate may trigger the production of the necessary enzymes involved in the utilization of the substrate more efficiently for hydrogen production.

A maximum hydrogen production rate of 510 mL H_2/L-POME h was attained when the acclimated immobilized cells were cultivated at an initial substrate concentration of 60,000 mg COD/L-POME. In response to changes in initial substrate concentration (20,000-60,000 mg COD/L-POME), the hydrogen yield was slightly increased from 5.11 to 5.35 LH_2/L-POME (Table 4.2). It is likely that the change in organic loading of 20,000–60,000 mg COD/L-POME did not affect the microbial community structure in the immobilized cells. This work revealed that acclimation plays a crucial role in speeding up hydrogen production from real wastewater (POME).

Table 4.2: Effect of different POME concentrations on H_2 production performance with PEG immobilized cells

	Substrate concentration (mg COD/L-POME)	**H_2 production rate (mL H_2/L-POME h)**	**H_2 yield (LH_2/L-POME)**	**H_2 content (%)**
Without acclimation	20,000	92	3.14	50.1
	30,000	144	3.55	50.3
	40,000	268	3.51	51.5
	50,000	310	3.81	51.2
	60,000	349	3.91	52.1
With acclimation	20,000	168	5.18	57.1
	30,000	315	5.22	60.0
	40,000	481	5.35	58.8
	50,000	461	5.11	60.0
	60,000	510	5.19	59.9

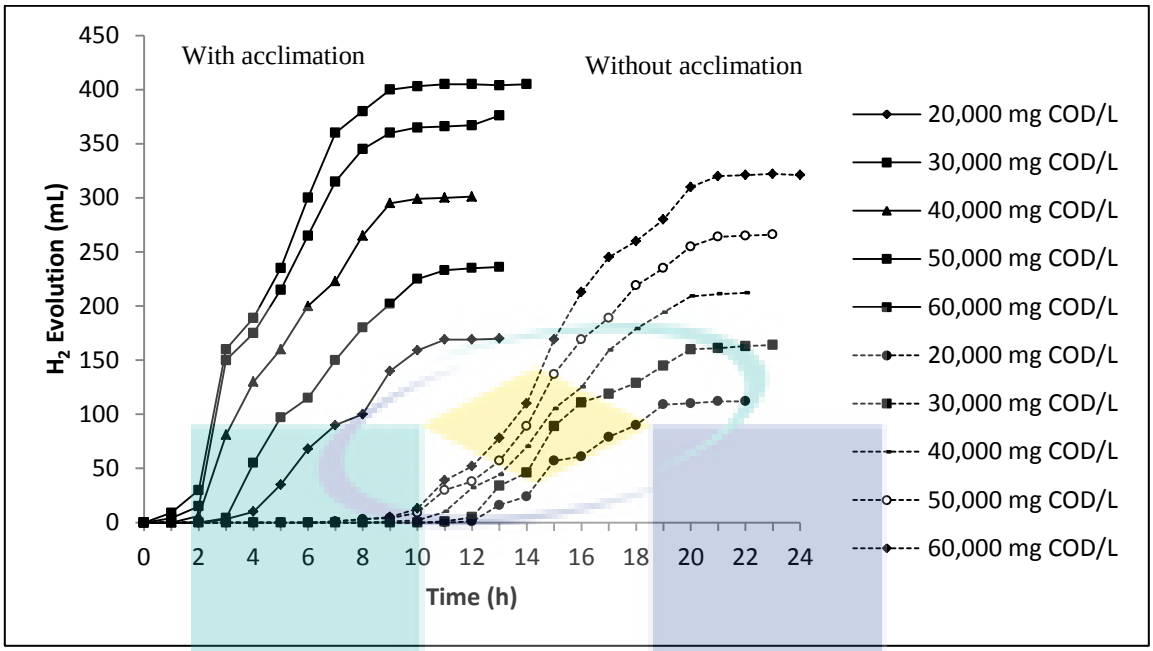

Figure 4.3: The effect of acclimation on H_2 production performance for PEG-immobilized cells; the initial POME concentration = 40,000 mg COD/L; T = 37 ^{o}C; pH = 5.5; Biomass loading = 30 g of cell/L; (black line: result from immobilized acclimated cells; dotted line: result from without acclimation)

4.2.3 Kinetics of hydrogen production with immobilized cultures using POME substrate

The dependence of the R_{H_2} of immobilized cells (with and without acclimation) on different POME concentrations is shown in Figure 4.4. The R_{H_2} increased initially with increase in POME concentration and then leveled off at a maximum value as POME concentration exceeded 30,000–50,000 mg COD/L-POME. The relation between the medium concentration (POME) and R_{H_2} can be explained by Monod-type kinetics:

$$R_{H_2} = \frac{R_{\max,H_2} S_{POME}}{K_P + S_{POME}} \qquad (1)$$

where K_P is the half-saturation constant (mg COD/L), $R_{\max,H_2}$ is the maximum specific hydrogen production rate (mL/g of cell/h) and S_{POME} is the POME concentration (mg COD/L). The correlation r^2 value obtained by the nonlinear regression analysis of Eq. (1)

was greater than 0.92 for immobilized cells. The values of K_P and R_{max,H_2} were estimated from the experimental data by numerical simulation. For acclimated immobilized cells, the K_P and R_{max,H_2} values were 20,000 mg COD/L and 490 mL/g of cell/h, respectively, whereas for unacclimated cells, the values were 30,000 mg COD/L and 315 mL/g of cell/h, respectively. Figure 4.4 shows that acclimated immobilized cells had significantly higher R_{max,H_2} values than those obtained from unacclimated cells. R_{max,H_2} value in the Monod model is considered a liner function of total active biocatalyst concentration. Therefore, the higher R_{max,H_2} value for acclimated immobilized cells represents higher concentration of active biocatalyst than that of unacclimated immobilized cells.

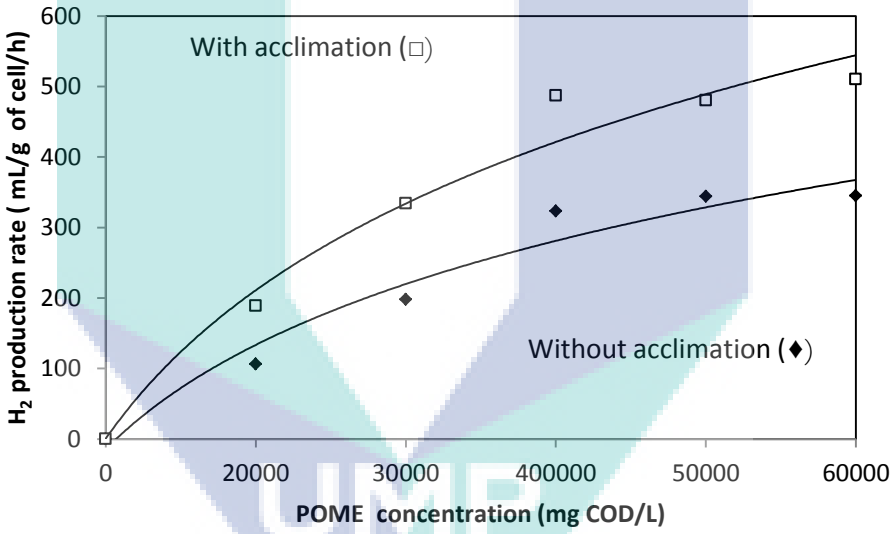

Figure 4.4: The dependence of H_2 production rate on different POME concentration for PEG-immobilized cells. The initial biomass loading was 30 g of cell/L; T = 37 ^oC; pH = 5.5. (Lines: represent simulation predictions with Monod model; symbols: represent the experimental data)

4.2.4 Repeated batch operations

To determine the stability and durability of PEG-immobilized cells in hydrogen production, the acclimated immobilized cells was subjected to repeated batch mode

experiments. The results are presented in Figure 4.5. The PEG-immobilized cells maintained stable and efficient H_2 production for 20 repeated cycles. Meanwhile, the PEG-immobilized cells remained intact with good settling efficiency and mechanical and biological stability during prolonged operation. Several types of natural and synthetic polymer immobilization matrices have previously been used for hydrogen production (Zho et al., 2012, and Wu et al., 2003), but most of them exhibited lower stability and less favorable mechanical properties compared with the PEG-immobilized cells systems developed in our present work. This indicates the potential of using the PEG-immobilized for practical applications in continuous hydrogen production operations.

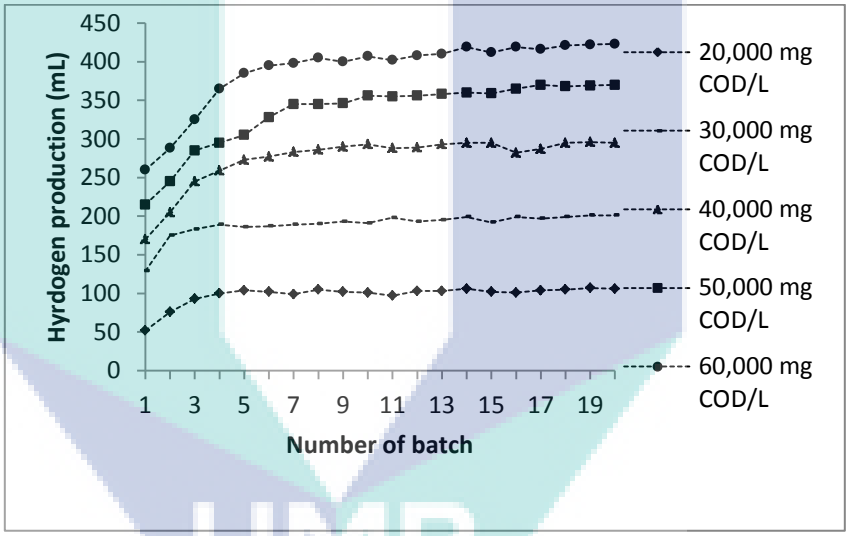

Figure 4.5: Hydrogen evolved during repeated batch tests with PEG- immobilized cells; operating condition for each batch test was; initial POME concentration = 40,000 mg COD/L; T = 37 ^{o}C; pH = 5.5

4.2.5 Effects of immobilized bead size on hydrogen production

The effect of three different PEG bead sizes (3, 4 and 5 mm) on the hydrogen production rate was assessed while keeping the PEG bead dosage (300 g) constant. As shown in Figure 4.6, the maximum rate of 427 mL H_2/L-POME h was observed with bead size of 3 mm. However, the lower hydrogen production rate was observed when the bead

size was at 4 and 5 mm. The differences in hydrogen production performance may result from the cells immobilized in PEG with different bead size. In previous work has confirmed that fluidization quality affected by particle size (Mastellone and Arena, 1999). At the same velocity, different particle size groups show different flow patterns. The particle size is the factor that differentiates the particle motion inside the reactor (Papadikis et al., 2010). In this study, the bead size (3 mm) was observed to be fluidized and evenly distributed in the medium during the stirrer operation at 150 rpm, so the cells immobilized in the carrier to provide better distribution of biomass and more active exchange of cells with the POME. Therefore, more H_2 production was attained by bacteria immobilized in PEG with bead size of 3 mm. However, the beads movement or contact between cells and wastewater may be reduced by the carrier of 5 mm, due to larger bead size. Consequently, less hydrogen was produced by bacteria immobilization in carrier of 5 mm. From the above finding it may be concluded that the beads of 3 mm offered lesser diffusion resistance compared to the larger beads. The values of standard deviation and error for hydrogen production rate have been shown in Table A2.

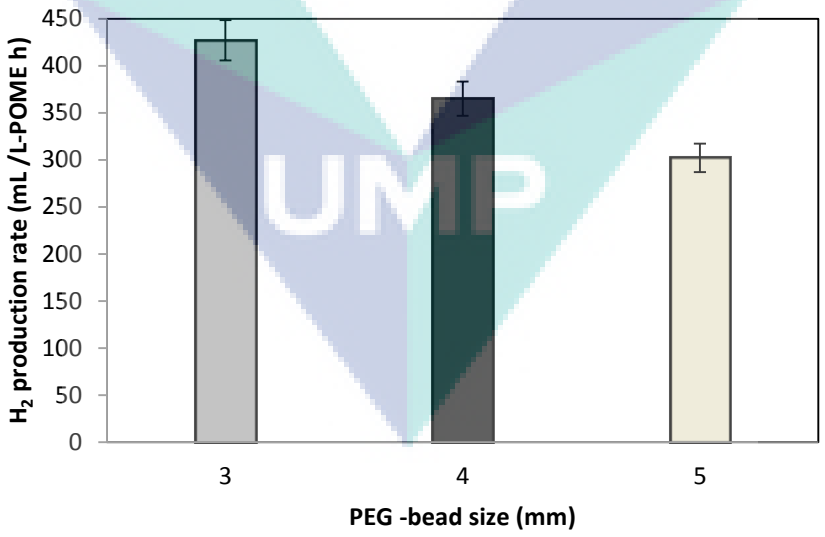

Figure 4.6: Effect of PEG beads size on H_2 production rate by immobilized cells

4.3 BIOHYDROGEN PRODUCTION PERFORMANCE IN IMMOBLILIZED CELL REACTOR VERSUS SUSPENDED CELL REACTOR

4.3.1 Continuous hydrogen production

UASB reactors with a working volume of 500 mL were operated continuously to produce hydrogen from POME wastewater using suspended and immobilized-cells. The effects of HRTs on the biogas production rate, composition of biogas and soluble metabolites, and COD removal efficiency were examined during the course of hydrogen production. The data for suspended and immobilized-cell reactors are showed in Figure 4.7 and 4.8, respectively. The correlations between HRTs and hydrogen generation efficiency as well as COD removal efficiency were summarized in Table 4.3. In all cases, the biogas primarily consisted of CO_2 and H_2. Typical soluble metabolites included butyric acid (HBu), acetic acid (HAc), propionic acid (HPr), and ethanol (EtOH).

4.3.2 Hydrogen production performance with suspended-cell system

Decrease in HRT from 12 to 6 h: Figure 4.7 shows that the suspended-cell reactor was started at HRT 12 h, where the steady-state hydrogen generation rate was 0.275 L H_2/(L POME h). As the HRT was decreased to 6 h, hydrogen production rate increased to 0.348 L H_2/(L POME h) and then slightly declined. Initially, production of EtOH dominated the soluble metabolites, when hydrogen generation was not significant, it then sharply decreased as production rate exceeded 0.2 L H_2/(L POME h) and VFA became major soluble products. Solvent formation is unfavorable to anaerobic production of hydrogen gas due to loss of electrons protons to the formation of more reduced product (Yan et al., 1988).

Decrease in HRT from 6 to 1 h: As the HRT was decreased from 6 h to 2 and then to 1 h, the hydrogen production rate did not enhance, while CO_2 (and total biogas) production increased significantly in response to higher organic loading rate for lower HRTs (Figure 4.7). The hydrogen content in biogas reduced to less than 15% at HRT 1 h,

in contrast to 39% of H_2 at HRT 6 h (Table 4.3). The hydrogen generation rate decreased to as low as 0.192 L H_2/(L POME h). Meanwhile, formation of HPr increased dramatically when HRT shifted down from 6 to 1 h, while composition of HBu and HAc did not vary considerably during this period (Figure 4.7). Analysis of the variations in composition of biogas and soluble metabolites suggests that operation at a high carbon substrate (POME) loading rate (or a low HRT) led to conversion of POME to CO_2 by bacterial populations that were inefficient in hydrogen production, but somehow dominated substrate utilization to produce CO_2 and HPr as the major gas and soluble products. Therefore, HPr was most likely produced by bacterial populations other than the primary hydrogen producer in the culture. Thus, observation of an overwhelming HPr production may be considered as a signal of inefficient hydrogen fermentation with the sludge culture. In addition, when HRT was adjusted from 2 to 1 h, significant washout of cells occurred. Thus, operation at HRT=1 h appeared to be unstable for the suspended-cell reactor.

Increase in HRT from 1 to 6 h: Since operation at HRT 1 h resulted in low hydrogen generation efficiency and a considerable washout of the cells, the HRT was readjusted to 6 h. It was found that production of hydrogen increased to 0.214 L H_2/(L POME h) (Figure 4.7). The hydrogen content also increased as hydrogen accounted for 23% of the biogas during this period (Table 4.3). In the meantime, the production of HBu and HAc increased considerably, accompanied by decreases in HPr and EtOH, indicating that the hydrogen generating population revived. Interestingly, HPr production decreased when HRT increased from 1 to 2 h, implying that the activity of HPr producing bacteria in the suspended-cell containing reactor were stimulated only when substrate feeding rate exceeded a high level.

Decrease in HRT from 6 to 2 h: After the reactor reached steady state for a prolonged period at HRT = 2 h, the HRT was subsequently lowered to 1 h to examine how the reactor would respond to an increased organic loading rate at this time. Again, production of total biogas and CO_2 increased due to faster supply of substrate, but the hydrogen concentration in biogas became lower (18%), in contrast to 23% at HRT 6 h (Table 4.3). Consequently, the hydrogen generation rate at HRT 2 and 6 h was quite same.

Overall assessment of the data in Figure 4.7 and Table 4.3 shows that optimal HRT was 6 h for suspended sludge containing UASB reactors in continuous hydrogen production. When the reactor was operated at HRT 6 h, the best hydrogen production rate was 0.348 L H_2/(L POME h) (Figure 4.7).

Table 4.3: Performance of hydrogen fermentation in suspended and immobilized-cell reactor at different hydraulic retention times (HRTs)

Reactor system	**HRT (h)**	**[a]Hydrogen content in biogas[a] (%)**	**Hydrogen production rate[a] (L H_2/L POME/h)**	**COD removal efficiency[a,b] (%)**
Suspended-cell system	12	39.0	0.255	54
	6	36.5	0.348	58
	2	22.5	0.244	52
	1[c]	12.1	0.091	50
	6[c]	23.1	0.312	56
	2[c]	18.2	0.302	53
Immobilized-cell system	6	40.1	0.257	62
	2	37.1	0.589	63
	1	32.2	0.102	57
	2[c]	28.4	0.419	63
	1[c]	34.1	0.532	62
Immobilized scale-up reactor	10	32.3	0.553	62
	6	29.2	0.587	63
	2	26.4	0.011	54
	8	28.1	0.119	57
	2[c]	34.2	0.632	64

[a]Steady-state values or mean values.

[b]COD removal efficiency= $(COD_{in} - C_{eff.})/COD_{feed}100\%$, where, COD_{in} and C_{eff} represent COD influent and effluent in (g COD/L).

[c]Repeated HRT (the same HRT was used earlier in this run).

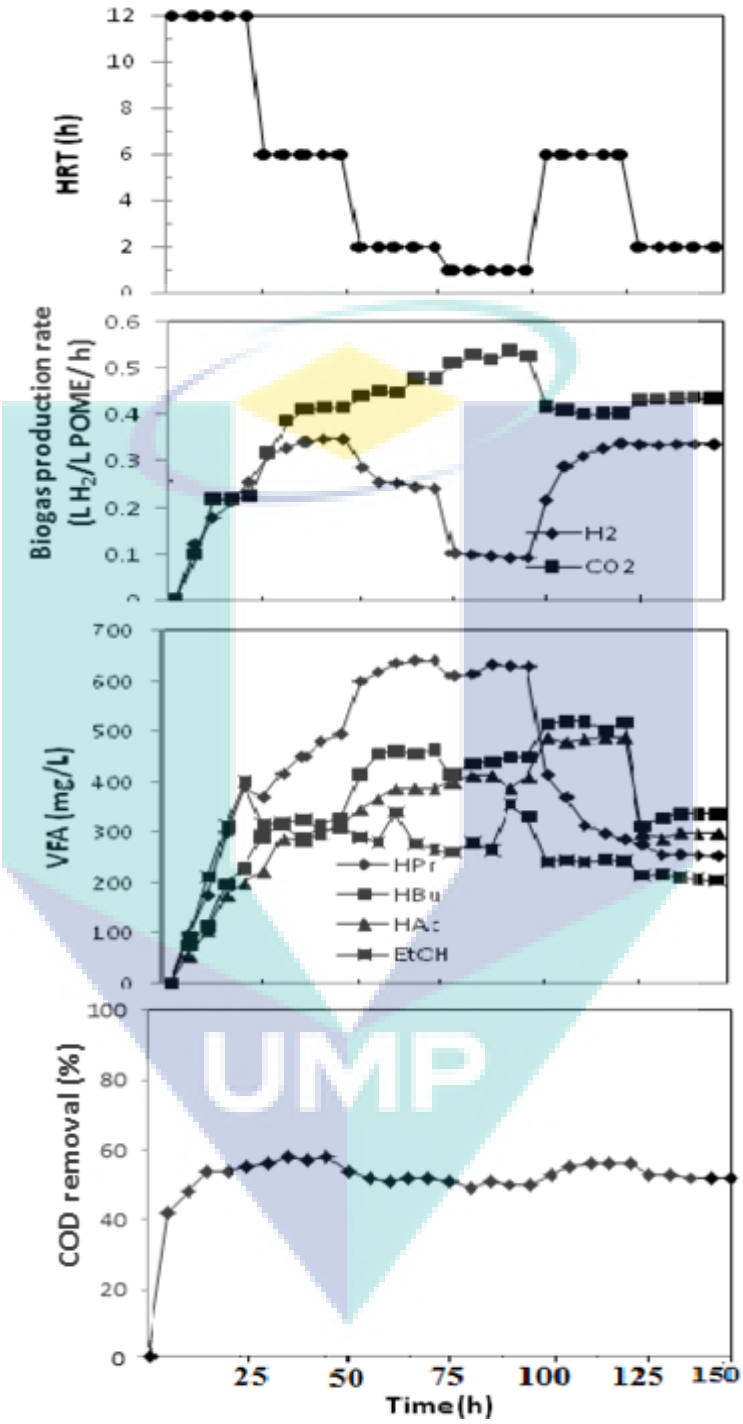

Figure 4.7: HRT-dependent profiles of biogas production rates, volatile fatty acid (VFA) production, and COD removal in the effluent with suspended-cell containing reactor

4.3.3 Performance of immobilized-cell UASB reactor

Figure 4.8 shows that the steady-state hydrogen production rate of PEG immobilized-cell reactor increased from 0.291 to 0.589 L H_2/(L POME h) when HRT was shifted down from 6 to 2 h. During this stage, the hydrogen content in the biogas was around 37–40% (see Table 4.3) and the major VFA product was clearly HBu. As the HRT was reduced further to 1 h, a slight increase in CO_2 production was observed. However, the hydrogen production was not enhanced but rather decreased slightly to lower hydrogen content (33%). This trend is similar to what was observed in suspended cell containing UASB reactor when HRT was decreased to 1 h (see Figure 4.7), except that the increase of CO_2 production in immobilized reactor corresponding to a HRT drop was much less significant than that occurred in suspended cell containing reactor. Moreover, HPr was not the dominant VFA product as it was in suspended-cell containing reactor. Therefore, the immobilized reactor was relatively stable when it was operated a low HRT (1 h). Accidental power failure occurred at 75 h when the immobilized reactor was operated at HRT 1 h (see Figure 4.8). The feeding stream was stopped for 15 h, and the reactor was then restarted at HRT 2 h. Evolution of hydrogen recovered immediately after the feeding was restored and hydrogen production rate reached a steady-state value of 0.249 L H_2/(L POME h) (see Table 4.3).

The HRT was then switched to 1 h again and production rate increased further to a peak value of 0.532 L H_2/(L POME h), and then started to decrease as the operation at HRT of 1 h continued. During the periods of efficient hydrogen production (25 to 60 h and 100 to 125 h), HBu was the most abundant acid product, followed by HAc and HPr, while production of EtOH was insignificant. In contrast to hydrogen production rate, the COD removal did not vary significantly with change in HRT, but still reached highest level of 63% when the reactor was operated at HRT of 6 h and 2 h, respectively, which were higher than suspended-cell process. Immobilized-cell UASB reactor showed a better performance on hydrogen production in comparison to suspended-cell reactor (Table 4.3). Despite operation at a HRT of 2 h, immobilized cell UASB reactor attained a maximum hydrogen

production rate of 0.589 L H_2/(L POME h), which was 55 % higher than suspended-cell reactor in this study. The hydrogen content in biogas and COD removal values were also higher then those obtained from the suspended-cells reactor (see Table 4.3).

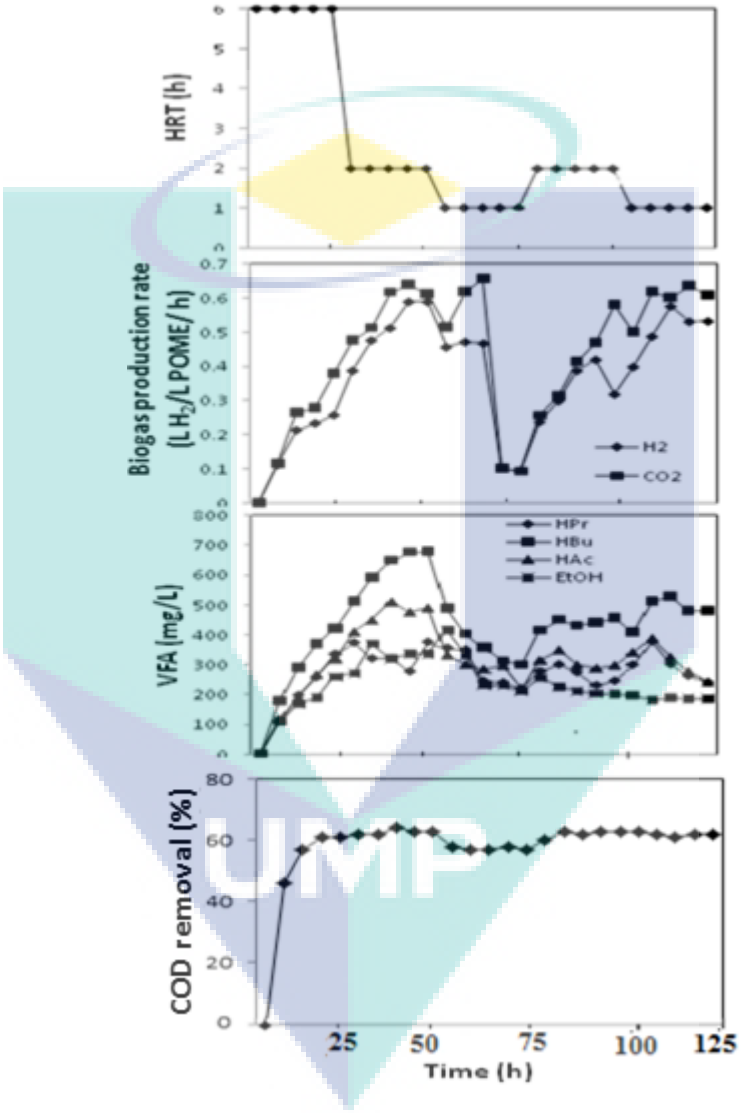

Figure 4.8: HRT-dependent profiles of biogas production rates, volatile fatty acid (VFA) production, and COD removal in the effluent with immobilized-cell containing reactor

4.3.4 Scale-up of immobilized-cell containing reactor

To assess the feasibility of using PEG-immobilized cells containing reactor for large scale hydrogen production, the reactor was scaled up to 5 L. The scale up experiment was carried out under identical condition to those used for the smaller reactors. As indicated in Figure 4.9 and Table 4.3 with the 5 L reactor, the hydrogen production rate nearly constant at 0.589 L H_2/(L POME h) when the HRT was 6 to 10 h. Whereas further decrease in HRT to 2 h resulted in a significant decrease in hydrogen production rate and hydrogen content (Table 4.3). This may be due to an overload of medium causing a substrate inhibition effect on hydrogen producers. As CO_2 did not decrease along with the decrease in hydrogen, it is likely that some non-hydrogen producers started to dominate the culture at high loading rates (i.e., low HRTs) and converted the substrate to CO_2 without hydrogen production. This idea was supported by the restoration of hydrogen production performance after the culture was subject to a heat treatment to inactivate the non-hydrogen-producing populations (Figure 4.9).

To prove that the decline of hydrogen production rate at HRT of 2 h (Figure 4.9) was due to domination of non-hydrogen producers in the culture, a thermal treatment was conducted after operation at HRT of 2 h for 1 h. Thermal treatment was found to be effective in acclimation of hydrogen-producing populations (Chu, Y. 2011), because hydrogen producers in anaerobic cultures are often sporeforming bacteria, such as *Clostridium* sp. (Das and Veziroglu, 2001), which are resistant to high temperatures. The heat treatment was expected to be capable of selecting for the hydrogen-producing bacteria by eliminating the other bacterial populations at high temperatures. As shown in Fig. 4.9 after the heat treatment (employed between 122 and 123 h), the reactor was shortly revived at HRT 8 h and was then adjusted back to HRT 2 h. During this stage, hydrogen production rate reach to high value of 0.632 L H_2/(L POME h) and hydrogen content in biogas also improved to 34%. The concentration of soluble metabolites produced during conversion of POME to hydrogen in the 5 L immobilized reactor essentially followed the order of HBu>HAc>HPr>HAc>EtOH, comparable to what was obtained in the 500 mL immobilized reactor. The total quantity of soluble metabolites decreased considerably as

the HRT was shortened (Figure 4.9), probably due to the increase in the dilution rate. This result clearly suggested that using PEG-immobilized cells UASB reactor might reduce the operational cost by gaining a comparable hydrogen producing capacity at a low HRT (or a wide range of OLR).

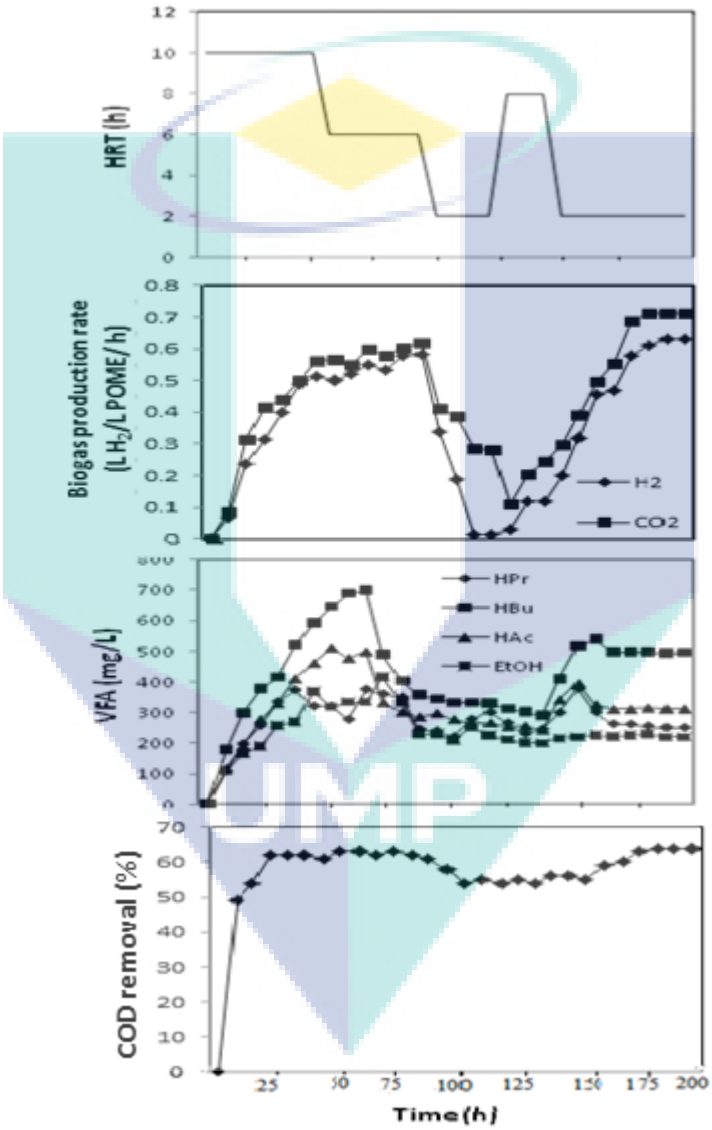

Figure 4.9: Performance of hydrogen fermentation during scale-up operations of immobilized-cell containing UASB reactor (working volume = 5 L)

4.4 APPLICATION OF POLYETHYLENE GLYCOL IMMOBILIZED *CLOSTRIDIUM SP.* FOR CONTINUOUS HYDROGEN PRODUCTION FROM PALM OIL MILL EFFLUENT IN UPFLOW ANAEROBIC SLUDGE BLANKET REACTOR.

4.4.1 Effect of HRT on hydrogen production

To evaluate the operational stability of the immobilized-cell UASB reactor for the production of hydrogen, continuous operation was carried at different HRTs (24-6 h) but at a constant OLR of 3.3 g COD/L/h, a pH of 5.5, a temperature of 37 °C, and an loading amount of PEG-immobilized cells (12% w/v) (Figure 4.10). The immobilized-cell UASB reactor was operated at a HRT of 40 h as a start-up period for the first 36 h, and then the HRT was decreased stepwise to 24, 18, 12, and 6 h (Figure 4.10). The hydrogen production rate increased from 220 mL H_2/L/h (or 9.8 mmol/L/h) to 317 mL H_2/L/h (or 14.1 mmol/L/h). Hydrogen yield increased from 0.17 LH_2/g $COD_{removed}$ to 0.3 LH_2/g $COD_{removed}$ as the HRT was shifted down from 24 to 12 h (Figure 4.10). Furthermore, the hydrogen production rate was essentially constant when the HRT decreased from 12 to 6 h. These results suggest that the UASB reactor containing immobilized cells could maintain a high cell concentration even at the lower HRT of 6 h. This indicates that the immobilized-cell UASB reactor was protected from cell washout and could be operated at a lower HRT during operation for continuous hydrogen production. By contrast, previous reports showed a decrease in hydrogen production rate in suspended-cell systems caused by cell washout at low HRT (Badiei et al., 2011). Further, there was a dramatic decrease in hydrogen yield from 0.3 LH_2/g $COD_{removed}$ to 0.14 LH_2/g $COD_{removed}$ when the HRT was shifted to 6 h (Figure 4.10). This may be due to vigorous hydrogen production at a short HRT (6 h), which causes a sudden increase in hydrogen partial pressure, leading to a lower hydrogen yield (Ginkel et al., 2001).

At a longer HRT of 24 h, both hydrogen production rate and yield were lower. One explanation for poor hydrogen production at HRT 24 h might be due to the lower substrate loading rate caused by decrease medium replacement ratio which could bring the

fermentation system to a substrate deficient state and lower the specific bacterial activity (Plangklang et al., 2012, and Kapdan et al., 2009). Other possible reason is generation of inhibitive by-products such as organic acids which was insufficiently washed out due to longer HRT. Accumulation of these types of products can cause imbalance in the reactor and prevent the culture from effectively utilizing the substrate (Stamatelatou et al., 2003). The hydrogen content in the biogas and the COD removal percentage were maintained in the range of 52-58% and 54-60% at all HRTs of 24-6 h during study-state operation (Figure 4.10). At all ranges of HRT, the biogas produced consisted of hydrogen and CO_2, but CH_4 was not detected. The soluble microbial products (SMP) increased with decreasing HRT and mainly included butyric acid (HBu) and acetic acid (HAc). Within the range of HRT (24-6 h), HBu and HAc increased from 39-62% and 18-23% respectively, of the total SMP (Table 4.4). The nearly stable hydrogen content and COD removal performance suggest that the immobilized hydrogen producing cells had high operation stability regardless of changes HRT. Thus the immobilized-cell UASB reactor may be suitable where substrate costs are not very important and where a stable productivity is essential.

4.4.2 Effect of OLR on hydrogen production

After finding a suitable HRT (12 h), the hydrogen production capability of the immobilized-cell UASB reactor was evaluated by increasing stepwise OLR from 1.6 to 6.6 g COD/L/h (Figure 4.10). The maximum hydrogen production rates of 336 mLH_2/L/h (or 15.0 mmol/L/h) and a hydrogen yield of 0.35 LH_2/g $COD_{removed}$ were obtained at 5.0 g COD/L/h OLR and a HRT of 12 h (Figure 4.10). The results indicate that the hydrogen production rate increased from 261 mLH_2/L/h (or 11.6 mmol/L/h) to 336 mLH_2/L/h (or 15.0 mmol/L/h), and that the hydrogen yield increased from 0.16 LH_2/g $COD_{removed}$ to 0.35 LH_2/g $COD_{removed}$, when the OLR increased from 1.6 to 5.0 g COD/L/h (Figure 4.10). However, the hydrogen production rate and yield dramatically decreased to 221 mLH_2/L/h (or 9.8 mmol/L/h) and 0.14 LH_2/g $COD_{removed}$, respectively, when the OLR was further increased up to 6.6 g COD/L/h (Figure 4.10). The reason for low hydrogen production rate and yield is likely to be end-product inhibition by over-accumulation hydrogen gas in the liquid at high organic loading rates. Lower hydrogen production rate at high OLR agreed

with previous study (Tawfik and Salem, 2012, and Prasertsan et al., 2009). Higher hydrogen production ability at OLR of 5.0 g COD/L/h with an HRT of 12 h possibly correlates to existing appropriate condition to activate spore formed bacteria, which can utilize the POME more efficiently for hydrogen production. The hydrogen content in biogas decreased from 61 to 31% with an increase of the OLR from 5.0 to 6.6 g COD/L/h (Figure 4.10). Although the obtained value of hydrogen yield (0.35 LH_2/g $COD_{removed}$) from the present study is still lower comparable to those reported by other researchers from pure carbohydrates (glucose and sucrose) (Logan et al., 2002, and Van Ginkel et al., 2001). This major cause for lower yield could be due the POME contains very high particulates or VSS which require long hydrolysis time. Hydrolysis is known to be rate limiting step for carbohydrate conversion (Miron et al., 2000).

Figure 4.10 also shows that the COD removal efficiency gradually increased with stepwise increases in OLR from 1.6 to 6.6 g COD/L/h, indicating that the removal percentage of COD becomes higher with increasing OLR. Under the optimum conditions in this study, 63% COD removal were higher than those previously reported (Vijayaraghavan and Ahmad, 2006, and Ren et al., 2006). However, the COD of the effluent was still high and subsequent treatment is needed, such as integrated fermentation-methanogenic process before being discharge to the environment. HBu and HAc were the most abundant products, with contents in the range of 44-71% and 20-24% of total SMP, respectively (see Table 4.4 in section of 4.4.3). SEM analysis showed that rod-shaped bacteria were firmly attached to the surface and interior part of the beads at the initial and final stage of experiments (see Figure 3.4). The PEG-immobilized beads also had a porous microstructure that facilitated the transfer of nutrients and substrates, thereby ensuring the growth of microorganisms for hydrogen production. We conclude that the UASB reactor containing PEG-immobilized cells is very efficient for anaerobic hydrogen production and treatment of high-strength wastewater (POME) at high OLR and short HRT values. In addition, the PEG-immobilized cells were able to achieve a stable and high hydrogen production at a relatively high dilution rate (low HRT) without cell washout. This special feature clearly suggests that using PEG cells might reduce the operational cost by gaining a

comparable hydrogen producing capacity at a low HRT (or a wide range of OLR) while compromising the cost for production of PEG beads.

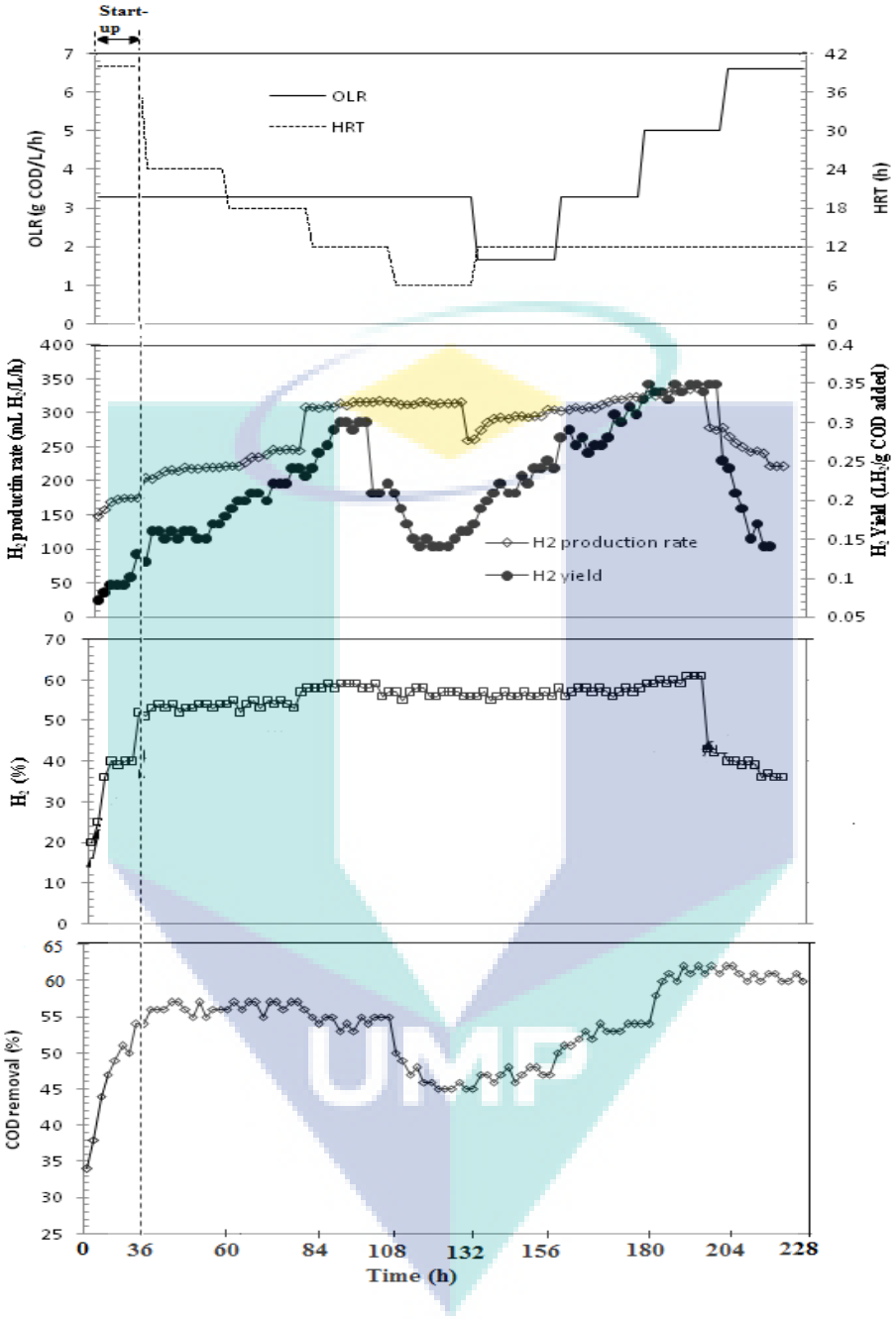

Figure 4.10: Effect of HRT and OLR on the performance of the PEG immobilized cells containing UASB reactor with a constant OLR at 3.3 g COD/L/h when studying the effect of HRT and a constant HRT at 12 h when studying the effect of OLR

4.4.3 Soluble metabolites formation

Table 4.4 presents the composition and distribution of soluble microbial products (SMP) that are produced, including total volatile fatty acids (TVFAs) and alcohols at various HRT and OLR values. The concentration of TVFAs and their relative proportions have been successfully used as indicators of hydrogen production in anaerobic processes (Chu, Y. 2011). In this research, HBu and HAc were the main soluble metabolites and constituted more than 70-85% of total SMP, whereas propionic acid (HPr) was produced at a lower amount (Table 4.4). In contrast, the production of ethanol, which is not beneficial for hydrogen production (Yan et al., 1988), was relatively insignificant (less than 7% of SMP) throughout the tested HRT and OLR ranges. The low amount of ethanol in the solvent suggests that hydrogen production was favoured because the production of electron-consuming solvents (e.g., ethanol) was comparatively small (Ueno et al., 1996). The HBu/HAc ratio ranges from 2.0 to 3.1 and has been used as an indicator for hydrogen production in dark fermentation systems (Oh et al., 2004). The HBu/HAc ratios in the present study varied from 1.9 to 3.1 at various HRT (24-6 h) and OLR (1.6-6.6 g COD/L/h) values in the immobilized-cell UASB reactor (Table 4.4). The highest hydrogen production performance occurred at 12 h HRT and an OLR of 5.0 g COD/L/h, resulting in a HBu/HAc ratio of 3.1 (Table 2). The high HBu/SMP ratio and the abundance of TVFAs in total SMP suggest that hydrogen production with the immobilized *Clostridium sp.* was directed by acidogenic pathways and was essentially butyrate-type fermentation. The high butyrate concentrations are likely to have been generated by *Clostridium* species, because these bacteria engage in butyrate-type fermentation (Chen et al., 2005, and Dinopolou et al., 1988). The observed HPr level was low and did not vary markedly over different HRT (24-6 h) and OLR (6.6-1.66 g COD/L/h) ranges during fermentation. It is known that HPr interferes with hydrogen production (Chang et al., 2002).

Table 4.4: Soluble metabolites production during anaerobic hydrogen fermentation in the immobilized UASB reactor at different HRT and OLR value

HRT (h)	OLR (g COD/L/h)	HPR[a] (mL H_2/L/h	HY[b] (L H_2/g $COD_{removed}$)	HBu[c] (g/L)	HAc[c] (g/L)	HPr[c] (g/L)	BuOH[c] (g/L)	EtOH[c] (g/L)	HBu/HAc	TVFA (g/L)	SMP (g/L)
24	3.3	219±0.02	0.17±0.05	3.9±0.01	2.0±0.06	1.2±0.03	1.8±0.02	1.0±0.01	1.9±0.03	7.1±0.02	9.9±0.04
18	3.3	246±0.05	0.22±0.01	5.3±0.02	1.8±0.02	0.9±0.01	1.6±0.03	0.4±0.06	2.9±0.02	8.0±0.05	10.0±0.04
12	3.3	317±0.02	0.30±0.04	6.3±0.04	2.1±0.05	0.6±0.06	1.1±0.01	0.1±0.03	3.0±0.04	9.1±0.01	10.2±0.01
6	3.3	314±0.07	0.14±0.06	5.8±0.01	2.3±0.01	0.5±0.02	0.9±0.05	0.3±0.01	2.5±0.05	8.6±0.04	9.8±0.03
12	1.6	292±0.01	0.24±0.05	4.8±0.05	2.2±0.02	1.3±0.04	1.9±0.06	0.7±0.02	2.1±0.01	8.3±0.01	10.3±0.02
12	3.3	318±0.04	0.29±0.02	5.8±0.04	2.0±0.03	0.5±0.01	1.3±0.03	0.2±0.04	2.9±0.02	8.3±0.03	9.8±0.02
12	5.0	336±0.03	0.35±0.01	7.0±0.03	2.2±0.01	0.1±0.01	0.4±0.05	0.09±0.1	3.1±0.06	9.3±0.03	9.7±0.04
12	6.6	221±0.02	0.14±0.04	5.1±0.05	2.1±0.04	0.9±0.02	1.2±0.02	0.6±0.01	2.1±0.01	8.1±0.02	9.9±0.06

[a] HPR= Hydrogen production rate

[b] HY= Hydrogen yield

[c] HBu= butyric acid; HAc=Actic acid; HPr; = propionic acid; BuOH=butanol; EtOH;=ethanol

SMP = soluble microbial products

TVFA = total volatile fatty acid

TVFA = HAc + HPr + HBu

SMP = TVFA + ethanol+butanol

4.5 EFFECT OF CELL PACKING RATIO, HRT AND POME CONCENTATION ON CONTINNOUES BIOHYDROGEN PRODUCTION AND TREATMENT EFFICIENCY OF PALM OIL MILL EFFLUENT IN IMMOBILIZED CELL UASB REACTOR

4.5.1 Effect of packing ratio of the PEG-immobilized cell concentrations on hydrogen production

To determine the biomass loading for higher hydrogen production and stabilize the operations in the UASB reactor, a suitable amount of the PEG-immobilized cells were added in the reactor for hydrogen production. To determine the superior packing ratio of the PEG-immobilized cells in UASB reactor, a packing ratio of 4, 8, 12, and 16% (w/v). During the experiments, reactor was fed under a constant POME concentration of 20 g COD/l and HRT of 8, 16, 24 and 32 h. The results are presented in Table 4.5. Hydrogen production rate was increased with a decrease in HRT from 32 to 8 h, when packing ratio of immobilized cells 4% and 12% (w/v) was used. On other hand, hydrogen yield showed an opposite trend as it decreased when HRT was shortened. The hydrogen production rate trend is due primarily to the increase the organic loading rate at a shorter HRT, thereby allowing an increase in production rate. The latter trend could be a result of an abrupt increase in hydrogen partial pressure due to vigorous hydrogen production at a short HRT, leading to a lower hydrogen yield. At a packing ratio of 12%, the hydrogen production rate and hydrogen yield reached maximum of 345 mL H_2/L-POME h and 0.35 LH_2/g $COD_{removed}$, respectively, which were higher compared to those attained at 4% and 8%. This suggested that increases in biomass loading increases the hydrogen production rate. However, the hydrogen production rate and yield decreased, when the packing ratio of immobilized-cell beads in the reactor increased from 12 to 16%. The lower hydrogen production performance at packing ratio of 16% might have been caused by the use of a large amount of substrate for growth, thus directing substrate utilization away from hydrogen fermentation. The hydrogen content in biogas and COD removal remained stable at 68% and 64% when the reactor was loaded with 4% to 12% of immobilized cells. In contrast, the hydrogen percentage and COD removal efficiency were negatively impacted

when the loading amount of immobilized-cell biomass increased from 12 to 16%. This could have been caused by reduced bead movement or contact between microflora and substrates at high immobilized-cell biomass levels. Consequently, the overall performance of the anaerobic digester was impaired (Karim et al., 2005, and Latif et al., 2011). These results suggest that a critical amount of immobilized cells in the UASB reactor for successful anaerobic hydrogen production is required.

Table 4.5: Effect of various packing ratio of PEG-immobilized cells in UASB reactor on hydrogen production

HRT	Packing ratio of PEG-immobilized cells (w/v)%	HPR[a] (mL H_2/L/h)	HY[b] (L H_2/g $COD_{removed}$)	H_2 content in biogas (%)	COD removal (%)
32	4	292	0.275	65	59
24		305	0.260	66	60
16		310	0.259	64	61
8		317	0.251	66	60
32	8	302	0.313	65	58
24		310	0.299	66	60
16		323	0.283	67	61
8		331	0.272	66	60
32	12	325	0.350	67	62
24		337	0.341	68	61
16		342	0.327	68	64
8		345	0.312	67	64
32	16	294	0.319	62	58
24		299	0.283	60	57
16		276	0.246	61	57
8		268	0.235	59	56

a HPR= Hydrogen production rate
b HY= Hydrogen yield

4.5.2 Effect of HRT

The effect of HRT on hydrogen production was evaluated as the reactor was operated at different HRTs (32-8 h) but at a constant pH of 5.5, a temperature of 37 °C, and POME concentration of 20 g COD/L. Based on the foregoing results, the loading of PEG-immobilized cells (i.e., a packing ratio of 12% (w/v)) was used in UASB reactor operation. After 6 h of batch operation, the continuous operation was started at a stepwise decreasing HRT from 32 h to 8 h (Figure 4.11). The hydrogen production rate increased from 215 mL H_2/L/h (or 9.5 mmol/L/h) to 350 mL H_2/L/h (or 15.6 mmol/L/h). Hydrogen yield increased from 0.16 LH_2/g $COD_{removed}$ to 0.36 LH_2/g $COD_{removed}$ as the HRT was decreasing from 32 to 16 h. Furthermore, the hydrogen production rate was essentially constant when the HRT decreased from 16 to 8 h. These results suggest that the UASB reactor containing immobilized cells could maintain a high cell concentration even at the lower HRT of 8 h. This indicates that the immobilized-cell UASB reactor was protected from cell washout and could be operated at a lower HRT during operation for continuous hydrogen production. By contrast, previous reports showed a decrease in hydrogen production rate in suspended-cell systems caused by cell washout at low HRT (Badiei et al., 2011). The hydrogen yield in general decreased as the HRT decreased. The hydrogen yield values were within the range of, but the hydrogen yield significantly decreases from 0.36 LH_2/g $COD_{removed}$ to 0.15 LH_2/g $COD_{removed}$ when the HRT was shorter to 8 h. The hydrogen content in the biogas and the COD removal percentage were maintained in the range of 66-68% and 64-66% at all HRTs of 32-8 h during study-state operation. The nearly stable hydrogen content and COD removal performance suggest that the immobilized hydrogen producing cells had high operation stability regardless of changes HRT.

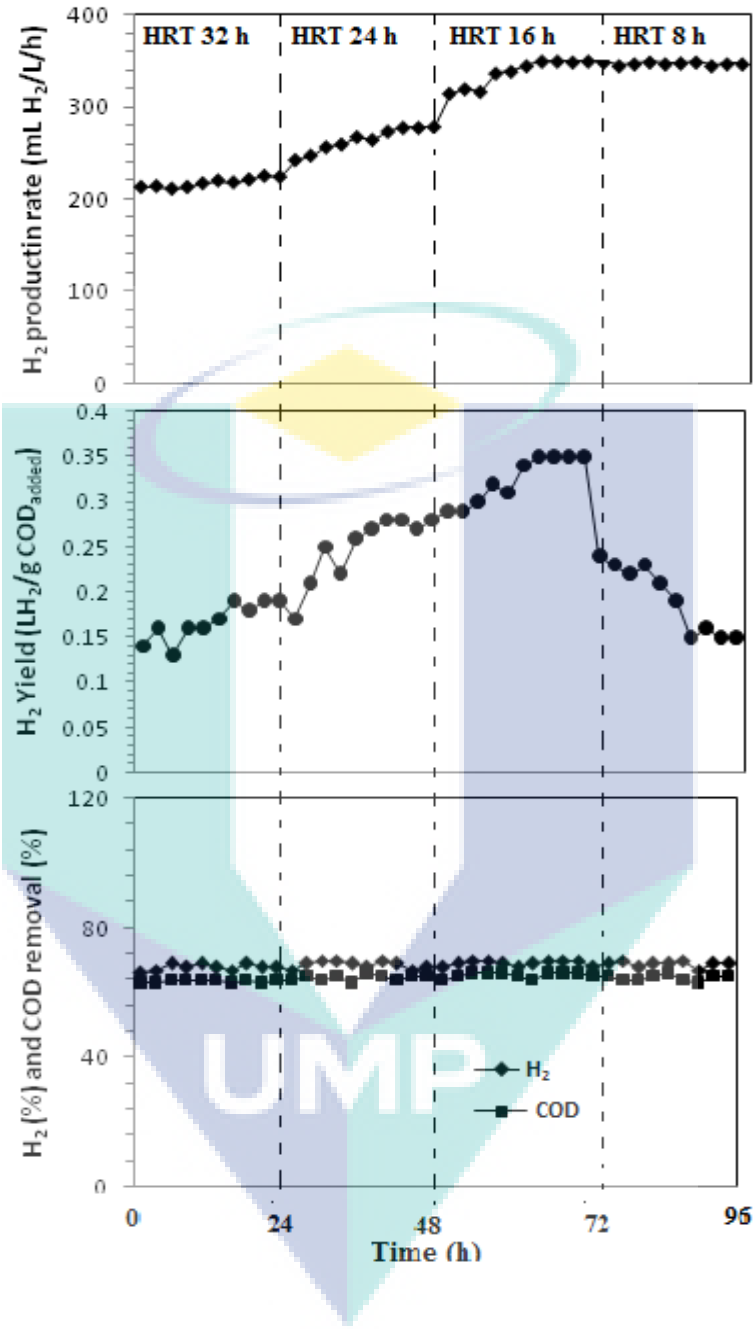

Figure 4.11: Effect of different HRTs on hydrogen production rate, hydrogen yield, hydrogen content and COD removal

4.5.3 Effect of influent POME concentration

To evaluate the effect of influent POME concentration on anaerobic hydrogen production, a test was conducted at 16 h HRT and varying POME concentration, from 10 to 40 g COD/L (Figure 4.12). The result clearly showed that a POME concentration of 30 g COD/L demonstrated the maximum hydrogen production rates of 365 mL H_2/L/h (or 16.2 mmol/L/h) and hydrogen yield of 0.38 LH_2/g $COD_{removed}$. The hydrogen production rate and hydrogen yield both increased with feed concentration increase from 10 to 30 g COD/l, while they decreased significantly when the feed concentration was elevated further to 40 g COD/L. Hydrogen composition decreased from 66 to 43% with an increase of the feed concentration from 30 to 40 g COD/L. A decrease in hydrogen production at higher POME concentration 40 g COD/L might be due to the competing reactions in hydrogen fermentation pathway in which the substrate as used to produce the other products such as volatile fatty acids and alcohols. End product inhibition as a result of overloading has been reported by several other researchers studying fermentative hydrogen production using waste materials such as cheese whey (Davila-Vazquez et al., 2009), and simpler substrates such as sucrose (Kim, S.H. 2006) and glucose (Van Ginkel and Logan, 2005). The COD removal efficiency gradually increased with stepwise increases of feed concentration from 10 to 40 g COD/L, indicating that the removal percentage of COD becomes higher with increasing substrate concentration. We conclude that the UASB reactor containing PEG-immobilized cells is very efficient for anaerobic hydrogen production and treatment of high-strength wastewater at short HRT and high substrates concentration, which could result in significant economic benefits.

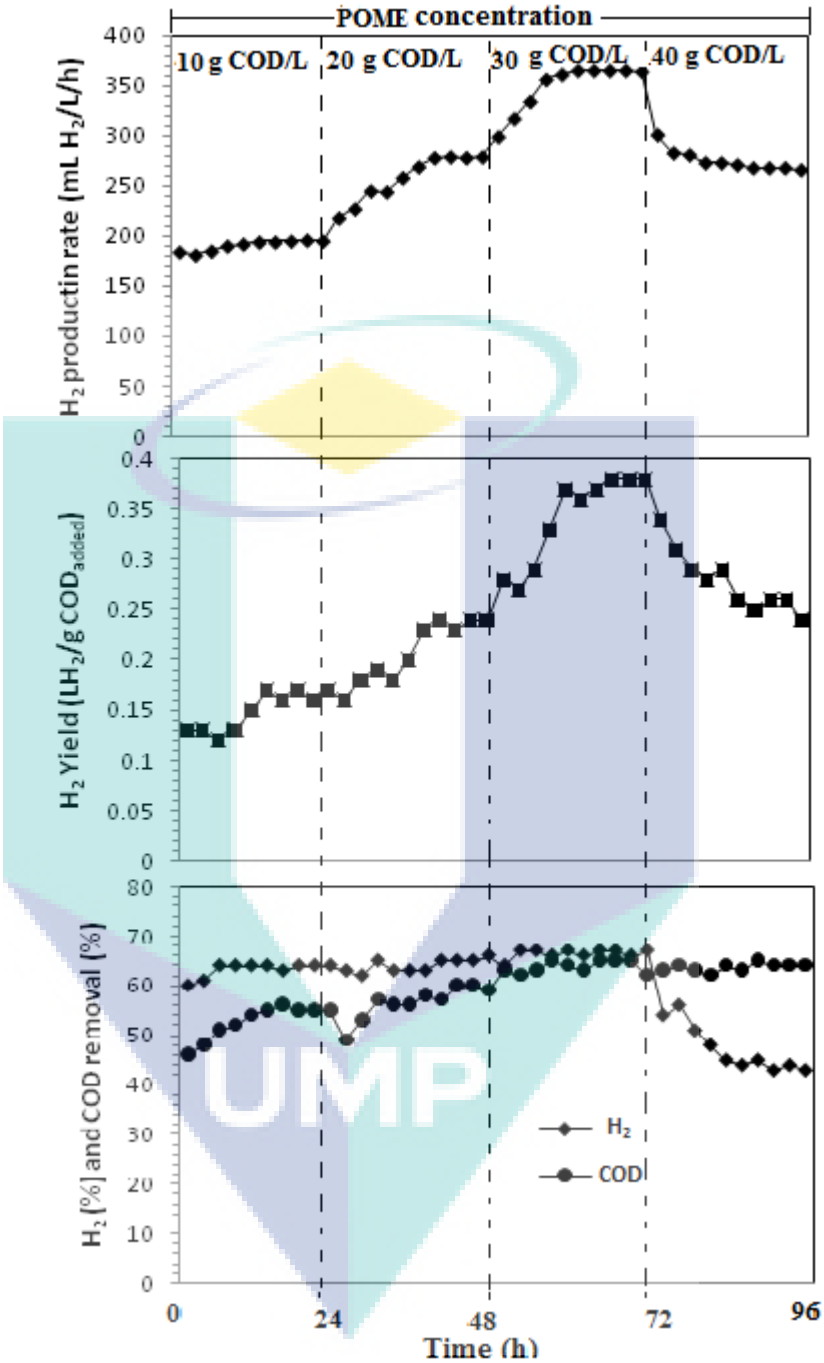

Figure 4.12: Effect of POME concentration on hydrogen production rate, hydrogen yield, hydrogen content and COD removal

4.5.4 Variation of VFA concentration during hydrogen production

Result of total volatile fatty acids (TVFAs) including soluble microbial products (SMP) that are produced, and alcohols at various HRT and feed concentration presented in Table 4.6. The concentration of TVFAs and their relative proportions have been successfully used as indicators of anaerobic hydrogen production (Chu, Y. 2011). In this study, HBu and HAc were the major soluble metabolites and accounting more than 75-85% of total SMP, the cultures appeared to carried out metabolic pathways in favor of hydrogen production. In contrast, the production of propionic acid (HPr) and ethanol, which are not unfavorable for hydrogen production, was relatively insignificant (less than 17% of SMP) throughout the operations. The HBu/HAc ratio ranges from 2.0 to 3.2 and has been used as an indicator for dark fermentative hydrogen production. The HBu/HAc ratios in the present study varied from 1.9 to 3.1 at various HRT (32-8 h) and feed concentration (10-40 g COD/L) values in the immobilized-cell UASB reactor (Table 4.6). The high HBu/SMP ratio and the abundance of TVFAs in total SMP suggest that hydrogen production with the immobilized *Clostridium sp.* was directed by acidogenic pathways and was essentially butyrate-type fermentation (Dinopolou et al., 1988).

Table 4.6: Soluble product composition in immobilized UASB reactor at different HRT and POME concentration

HRT (h)	POME concentration (g COD/L)	HPR[a] (mL H_2/L/h	HY[b] (L H_2/g $COD_{removed}$)	HBu (%)	HAc (%)	HPr (%)	BuOH (%)	EtOH (%)	HBu/HAc	TVFA (g/L)	SMP (g /L)
32		225	0.19	36	15	15	22	12	2.4	2.2±0.02	2.9±0.04
24		278	0.28	53	20	12	11	7	2.6	2.6±0.05	3.3±0.04
16	20	350	0.35	55	19	10	14	2	2.9	3.2±0.01	3.4±0.01
8		347	0.14	63	21	7	7	2	2.5	2.8±0.04	3.2±0.03
	10	185	0.17	32	17	13	16	7	1.9	1.7±0.01	2.5±0.02
	20	299	0.24	66	23	15	20	4	2.8	2.4±0.03	3.1±0.02
16	30	365	0.38	65	20	6	8	0.9	3.1	2.9±0.03	3.2±0.04
	40	268	0.26	51	25	10	12	2	2.1	2.2±0.02	3.4±0.06

a HPR= Hydrogen production rate

b HY= Hydrogen yield

HBu= butyric acid; HAc=Actic acid; HPr; = propionic acid; BuOH=butanol; EtOH;=ethanol

TVFA = HAc + HPr + HBu

SMP = TVFA + ethanol+butanol

4.6 EXPLORATION AND OPTIMIZATION OF HYDROGEN PRODUCTION CAPABILITY OF THE IMMOBILIZED CELLS AS WELL AS EFFECT OF TEMPERATURE AND INORGANIC COMPONENTS ON BIOHYDROGEN PRODUCTION

4.6.1 PEG concentration

The concentration of PEG determines the permeability, rigidity, and porosity of gel beads. The effect of PEG concentrations on hydrogen production by *Clostridium sp.* and cell leakage is shown in Figure 4.13. The different PEG concentrations in the range of 2-12% (w/v) were used in this study. The maximum hydrogen production rate of 327 mL/L-POME/h was obtained with beads prepared using 10% (w/v) PEG. At PEG concentration of 12% (w/v) the pellet rigidity was improved but the hydrogen production rate decreased due to diffusional limitations that limited the transfer of substrate and products through the bead (Ellaiah et al., 2004). On the other hand, when PEG concentration was 8% (w/v), especially when less than 6% (w/v), beads breakage was significant and cell leakage into the medium was high. This indicates that, at PEG concentrations of 10% (w/v), substrate and products are easily transferred through the bead. Therefore, PEG concentration of 10% (w/v) was employed for further studies. The values of standard deviation and error for hydrogen production rate and cell leakage of different PEG concentration have been shown in Table A3.

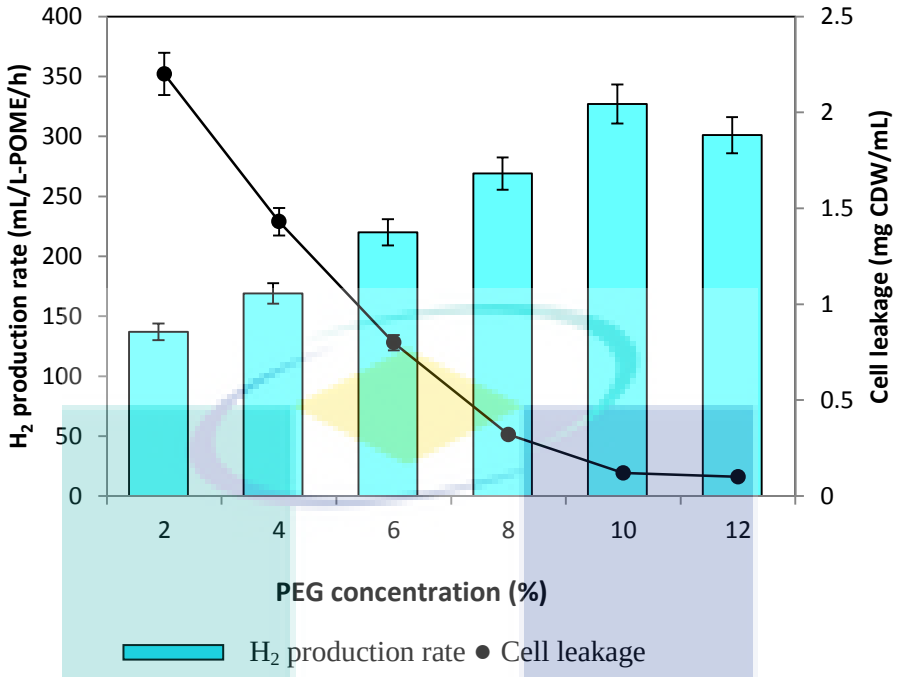

Figure 4.13: Effect of PEG concentration on hydrogen production and cell leakage

4.6.2 Cell load and curing time

The effect of initial biomass that can be loaded in immobilized bead on hydrogen production rate was studied in the range between 1.2 to 6.0 g dry wt. in gel (Figure 4.14). As the cell concentration in the beads was increased, the cell leakage into the fermentation medium also increased. Hydrogen production rate increased when the initial cell concentration in the beads was decreased from 3.6 to 2.4 g dry wt. in gel. Though immobilized cell systems are reported to enable the maintenance of high cell densities resulting in higher overall reaction rates and higher product yields, which is not possible with suspension cultures (Zhao et al., 2012, and Wu, K.J. 2007). In the present study the initial biomass of 3.6 g was too high to support highest hydrogen production. The decreased hydrogen production activity with high amount of biomass in bead might be due to substrate into the bead was constant and excessive biomass were employed in the fermentation system, which can lead to the consumption of a lot of substrate to maintain the energy require for their growth, thus reducing the use of substrate for hydrogen production

and immobilized cells activity was prevented. In addition, the beads prepared with high cell load above 2.4 g dry wt. were mechanically unstable during hydrogen production and their breakage was observed. For the following studies, 2.4 g was employed for bead preparation. The values of standard deviation and error for hydrogen production rate and cell leakage of different initial cell concentration have been shown in Table A4.

Figure 4.15 (Table A5) shows the effect of curing time on the stability of gel beads. The prolongation of hardening time from 20 to 80 min led to enhanced stability of bead, which resulted in decreased cell leakage. Further extension of curing time resulted in only slight increase in hydrogen production rate.

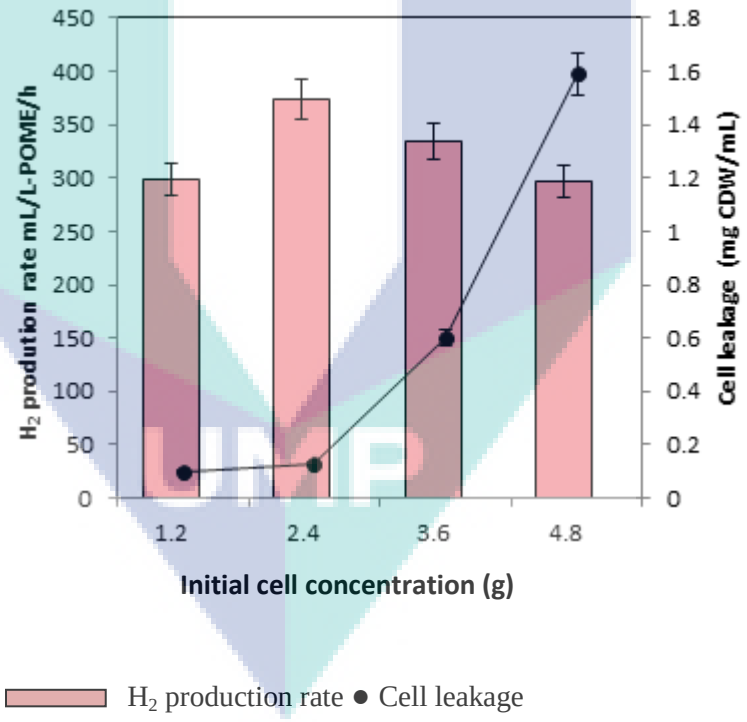

Figure 4.14: Effect of initial cell concentration on hydrogen production and cell leakage

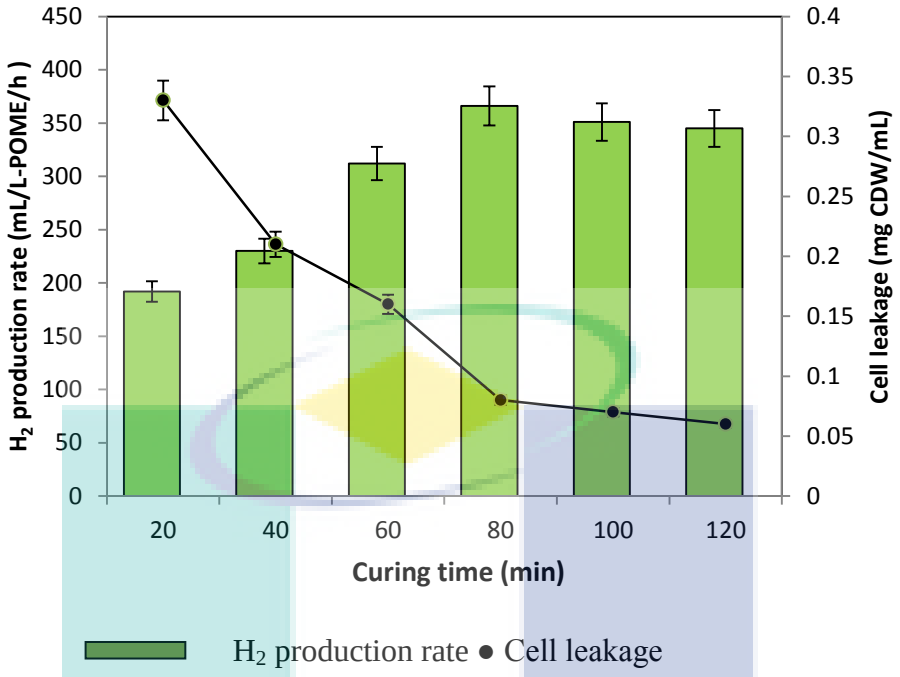

Figure 4.15: Effect of curing time on hydrogen production and cell leakage

4.6.3 Effect of temperature

Temperature is one of the important operating factors affecting the activity of microorganisms and fermentative biohydrogen production (Mu et al., 2007). The bacterial activity is inhibited when the environmental temperature is not suitable for their growth. Figure 4.16 demonstrates the performance of hydrogen production rate between a temperature of 25-45 °C. The values of standard deviation and error for hydrogen production of different temperature have been shown in Table A6. With an increase in temperature from 25-37 °C, the hydrogen production rate increases and reach the maximum value of 363 mL/L-POME/h at 37 °C. The hydrogen production rate significantly decreases when the culture temperature is further increased to 40 °C, suggesting that the hydrogen–producing population in beads belong to mesophilic group. Similar observation was reported by Seol et al. (2011) for immobilized E. coli SH5. On the other hand, lower hydrogen production rate at lower temperature is expected to slow down the bacterial activity. For further studies, temperature 37 °C was selected.

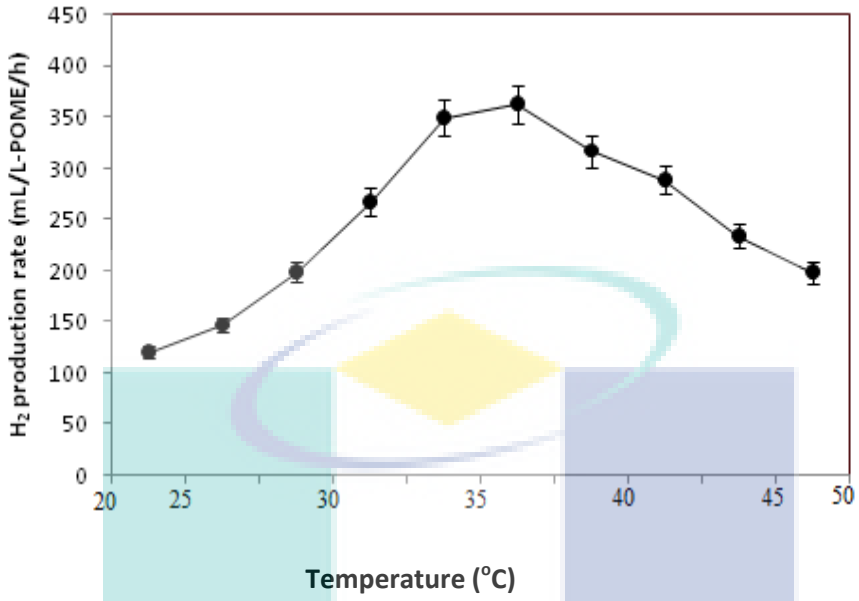

Figure 4.16: Effect of temperature on hydrogen production rate by immobilized cells of *Clostridium sp.*

4.6.4 Effect of inorganic components on hydrogen production

In previous study, the effect of inorganic components such as $NiCl_2$, $FeCl_2$ and $MgSO_4$ on hydrogen production using immobilized cell was not well studied. Figure 4.17 illustrates the effect of different $NiCl_2$, $FeCl_2$ and $MgSO_4$ concentrations on hydrogen production rate. Hydrogen production rate rose from 356 to 425 mL/L-POME/h when $NiCl_2$ increased from 0.5 to 1 mg/L. However, hydrogen production declined to 278 mL/L-POME/h when $NiCl_2$ increased from 1 to 2 mg/L. This indicated that Ni^{2+} could stimulate or inhibit hydrogen production rate depending on their concentration. Reason may be due to that the insertion of Ni^{2+} active site of [Ni–Fe] hydrogenases influence the fermentative hydrogen production by influencing the activity of [Ni–Fe] hydrogenases (Li and Fang, 2007) and thus may play an important role in fermentative hydrogen production. On the contrary, at much higher Ni^{2+} concentration inhibits the hydrogen production (Wang and Wan, 2008). The hydrogen production rate rose from 289 to the maximum value of 498 when $FeCl_2$ increased from 100 to 300 mg/L in the culture medium, because Fe^{2+}

participates in the ferredoxin synthesis in *Clostridium butyricum*, which mediate electron transfer of reducing power en route to hydrogenase-catalyzed hydrogen production (Lee et al., 2001). On the other hand, hydrogen production rate decreased to 498 mL/L-POME/h when $FeCl_2$ concentration further increased to 372 mg/L, because excessive Fe^{2+} inhibits hydrogen production. The hydrogen production rate in batch test increased from 301 to 404 mL/L-POME/h with increasing Mg^{2+} concentration form 50 to 100 mg/L, because enolase and phosphorylase need Mg^{2+} for the growth of microorganisms (Wang, X.J. 2007). However, HPR trended to decrease from 404 to 201 mL/L-POME/h when further increasing Mg^{2+} concentration from 100 to 200 mg/L, because excessive Mg^{2+} restricts hydrogen production. This result suggests that in an appropriate range of $MgSO_4$, $NiCl_2$ and $FeCl_2$ concentration, biomass waste could be used as a source for efficient hydrogen production.

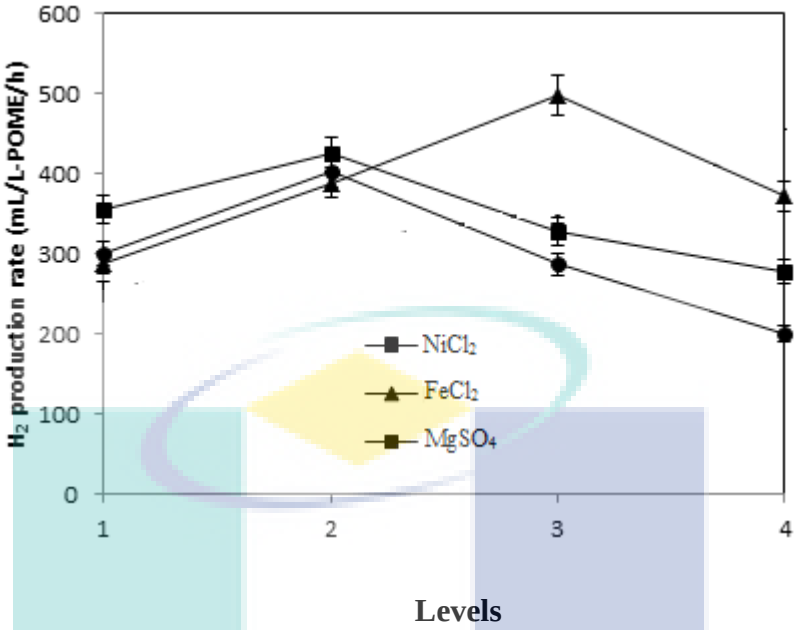

Figure 4.17: Effects of inorganic components on hydrogen production rate. $NiCl_2$ levels: (1) 0.5 mg/L, (2) 1.0 mg/L, (3) 1.5 mg/L, (4) 2.0 mg/L. $FeCl_2$ levels: (1) 100 mg/L, (2) 200 mg/L, (3) 300 mg/L, (4) 400 mg/L. $MgSO_4$ levels: (1) 50 mg/L, (2) 100 mg/L, (3) 150 mg/L, (4) 200 mg/L

4.6.5 Stability of hydrogen production during continuous operation

The fermentation of POME was carried out according to the optimal conditions obtained in batch test to further examine the operational stability of the immobilized biocatalyst and suitability for continuous dark hydrogen production process. The reactor was scaled up to 5000 mL working volume and operated at 36 h HRT. As shown in the Figure 4.18, the feeding started in the second day. After short term variations, COD removal efficiency and hydrogen production reached steady-state. Hydrogen production rate of 7.3 L/L-POME/d (13.5 mmol/L/h) and yield of 0.31 L H_2/g COD with about 66% COD removal. The hydrogen content in biogas was also fairly stable at 72%, demonstrating that the immobilized-cell culture consisted of a stable bacterial population, which converted the substrate via a steady metabolic flux. The scanning electron microscopic studies of the

PEG beads with immobilized cells showed that PEG beads had lot of pores inside the gels, which was a suitable for the entrapment of cells and provided a suitable microenvironment for the growth of the microorganisms (Figure 4.19). The hydrogen production value was higher than those of other researchers using various bioreactors, in which biomass are either immobilized cell system or suspended cell system (Table 4.7). It is worth mentioned that (Prasertsan et al., 2009) obtained a slight increase in the hydrogen production rate at thermophilic temperature using suspended cells in an anaerobic sequencing batch reactor. Their research resulted in hydrogen production rate of 9.1 L/L-POME/d at OLR of 60 g COD/L/d and HRT 2-d. High productivity could be attributed to thermophilic temperature and higher influent concentration used.

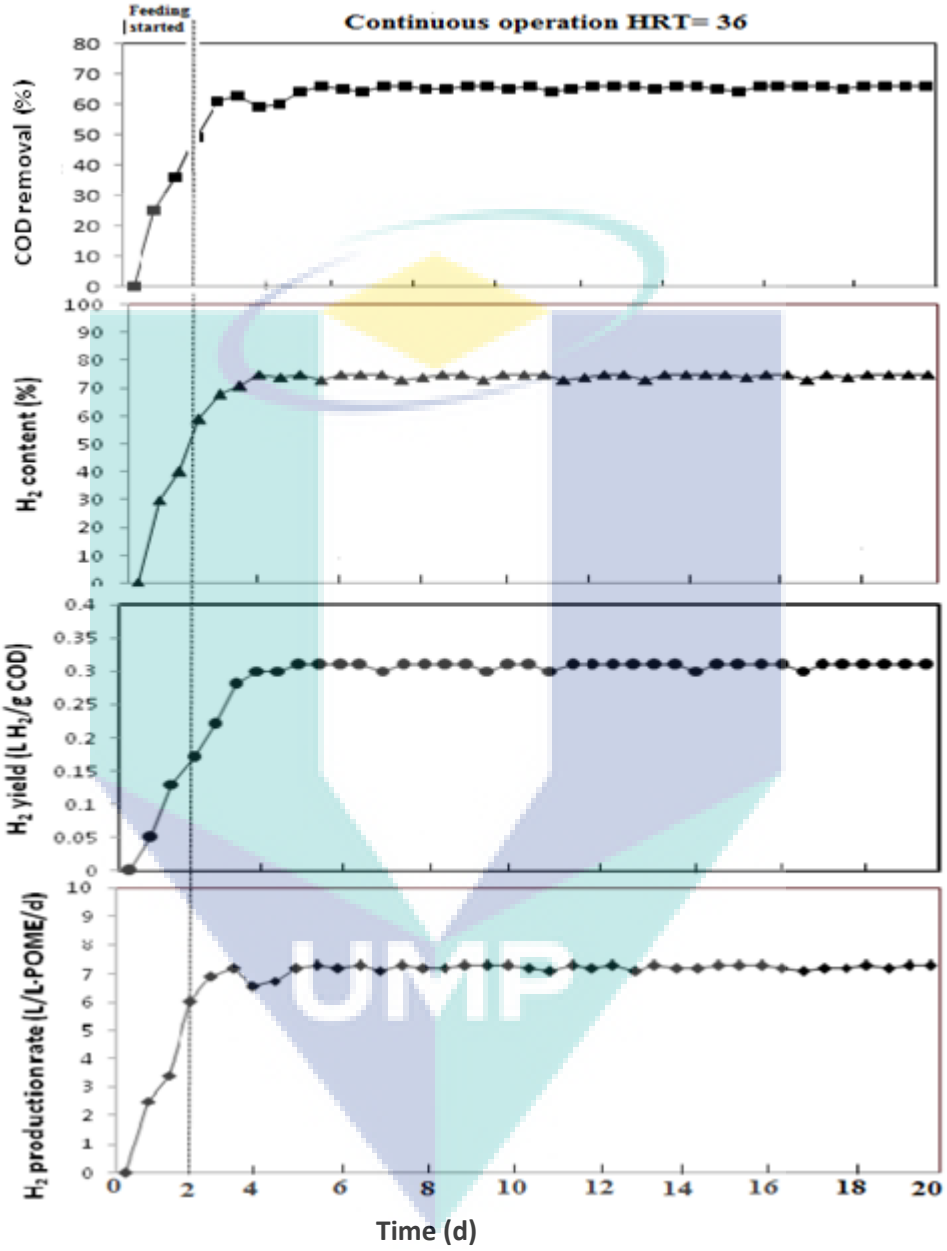

Figure 4.18: Continuous hydrogen production by *Clostridium sp.* immobilized in PEG: (a) COD removal (%), (b) H_2 content in biogas, (c) H_2 yield, (d) H_2 production rate (PEG concentration, 10% w/v; cell concentration, 2.2 g dry weight; curing time, 80 min; temperature, 37 ^{o}C; inorganic components, $NiCl_2$ 1 mg/L, $FeCl_2$ 300 mg/L and $MgSO_4$ 100 mg/L

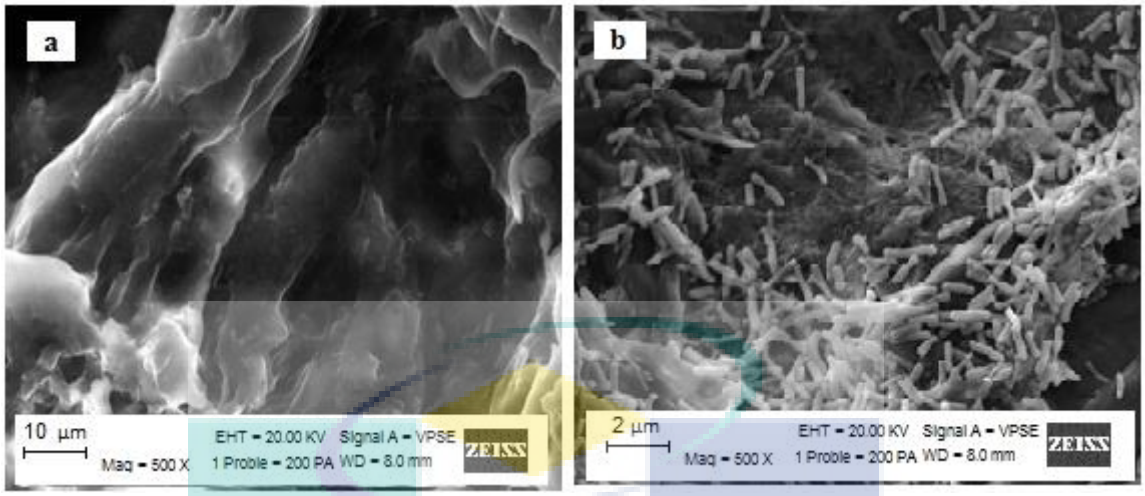

Figure 4.19: Scanning electron microscopy images of the PEG- bead: (a) before immobilization and (b) after immobilization

Table 4.7: Comparison of hydrogen production rate from various substrates by free cells and immobilized cells.

Bacterial growth mode/support matrix	**Substrate**	**Microorganism**	**H_2 production rate (L H_2/L/d)**	**Ref.**
Suspension/none	POME	Mixed/anaerobic	9.1	(Prasertsan et al., 2009)
Suspension/none	Sucrose	*C. butyricum*	3.50	(Chen et al., 2005)
Suspension/none	Corn starch	Mixed/anaerobic	4.12	(Farhan et al., 2008)
Immobilized cell/sugarcane bagasse	Sugarcane juice (sucrose)	*C. butyricum* TISTR 1032	3.50	(Plangklang et al., 2012)
Immobilized cell/polyurethane foam	Glucose	*C. tyrobutyricum* JM1	7.2	(Jo et al., 2008)
Immobilized cell /ceramic ball	sucrose	Anaerobic sludge	2.7	(Keskin et al., 2012)
Immobilized cell/PEG	POME	*Clostridium sp.*	7.3	This study

CHAPTER 5

CONCLUSIONS AND RECOMMENDATIONS

5.1 CONCLUSIONS

In this study, the feasibility and performance of fermentative hydrogen production from POME using immobilized cell in UASB reactor under various operational conditions were investigated. The main conclusions obtained from this research are summarized below.

1. **Feasibility of PEG as a carrier to immobilize *Clostridium sp*. for fermentative hydrogen production from POME.**

   - The maximum rate of hydrogen production with acclimation was 510 mL H_2/L-POME h at an organic loading of 60,000 mg COD/L-POME, compared with 349 mL H_2/L-POME for unacclimated immobilized cells.

   - The effects of medium concentration (POME) on the hydrogen production rate were well explained by Monod-type kinetics. K_P and $R_{\max, H_2}$ values for acclimated cells were 20,000 mg COD/L and 490 mL/g of cell/h, respectively. However, for unacclimated immobilized cultures the values were 30,000 mg COD/L and 315 mL/g of cell/h, respectively.

- It was found that the bead size should be of 3.0 mm to obtain the optimum hydrogen gas production.

- SEM micrographs illustrated that the PEG beads were porous structure, which facilitated the transport of substrate and product, ensuring the growth of the cells.

- This work has shown that PEG prepolymer served as a good matrix for the entrapment of *Clostridium sp.* for hydrogen production from POME.

II. Hydrogen production performance of PEG-immobilized cell containing reactor in comparison to suspended cell reactor.

- Hydrogen production from POME wastewater was attained using immobilized and suspended-cell culture in UASB reactors with optimal hydrogen generation rate of 0.589 L H_2/L POME h (HRT 2 h) and 0.348 L H_2/L POME h (HRT 6 h), respectively.

- The PEG-immobilized biomass loaded reactor seems to be a better choice of the two for continuous hydrogen fermentation since it exhibited higher hydrogen production rate, and also showed more stability when it was operated at low hydraulic retention time (HRTs).

- Over the HRT range 1–12 h, the hydrogen production rate did not always increase along with decreases in HRT, while the optimal HRTs for immobilized and suspended-cell reactors were 2 and 6 h, respectively.

III. Continuous hydrogen production from POME in immobilized cell UASB reactor.

- The results showed that PEG-immobilized cells were efficient for continuous hydrogen production and the treatment of POME in a UASB reactor under different HRT and OLR conditions.

- The UASB reactor loaded with PEG-immobilized cells generated an optimal hydrogen production rate of 336 mLH_2/L/h (15.0 mmol/L/h) and a hydrogen yield of 0.35 LH_2/g $COD_{removed}$, with an effluent containing mainly butyric acid and acetic acid when operated at a HRT of 12 h and a COD/L/h OLR of 5.0 g.

- The satisfactory hydrogen production and efficient performance over low HRT and high OLR ranges in the UASB reactor can be attributed to the maintenance of a high and stable concentration of bacteria by the PEG-immobilization technique.

IV. Effect of immobilized cell packing ratio, HRT and POME concentration on hydrogen production and treatment efficiency of POME.

- The UASB reactor with a PEG-immobilized cell packing ratio of 10% weight to volume ratio (w/v) was optimal for dark hydrogen production.

- The hydrogen production rate tended to increase as the HRT was shortened at constant substrate concentration of 20 g COD/L. On other hand, the hydrogen yield did not have a common trend against HRT and POME concentration, where it usually decreased at high POME concentration and lower HRT.

- The UASB reactor loaded with optimal PEG-immobilized cells generated an highest hydrogen production rate of 365 mL H_2/L/h (or 16.2 mmol/L/h) and a hydrogen yield of 0.38 LH_2/g $COD_{removed}$, with an effluent containing mainly butyric acid and acetic acid when operated at a HRT of 12 h and a POME concentration of 30 g COD/L.

- The average hydrogen content of biogas and COD reduction were 68% and 66%, respectively.

V. Optimization of immobilization parameters and effect of inorganic components on biohydrogen production.

- Through optimization of bead preparation method, high hydrogen production could be obtained.

- Additionally, adding proper concentration of $NiCl_2$, $FeCl_2$ and $MgSO_4$ ions in fermentation medium with immobilized cells could be effective in enhancing hydrogen production.

- With an optimal PEG concentration (10 % w/v), cell loading (2.4 g dry wt.), curing time (80 min) and inorganic components (**(**$NiCl_2$ 1 mg/L, $FeCl_2$ 300 mg/L and $MgSO_4$ 100 mg/L), attaining an excellent hydrogen production rate of 7.3 L/L-POME/d and a hydrogen yield of 0.31 L H_2/g COD in continuous operation.

- Therefore, the immobilized-cell system used in this study seems to have the potential to be practically applied for industrial scale hydrogen production from POME.

5.2 RECOMMENDATIONS

A polyethylene glycol (PEG) gel was fabricated and successfully used as a carrier to immobilize dark-fermentative bacteria for biohydrogen production and treatment of palm oil mill effluent. However, further work should take the following directions:

- Hydrogen production from POME using PEG-immobilized cell in UASB anaerobic digestion system needs investigation, especially with respect to scaling the process and determining if the two-stage process is stable under long-term reactor conditions.

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- The kinetics study of hydrogen production from palm oil mill effluent with immobilized cell in UASB reactor should be performed for higher biogas production.

- The COD of the effluent was still high and subsequent treatment is needed, such as integrated fermentation-methanogenic process before being discharge to the environment.

- Feasibility of PEG-immobilized cells for hydrogen production from palm oil mill at pilot scale UASB reactor should be carried out.

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APPENDIX A1

EFFECT OF TIME ON THE CELL LEAKAGE FROM THE BEADS IN THE MEDIUM SUSPENSION AND CONSEQUENT EFFECT ON HYDROGEN PRODUCTION

Time (h)	HPR(mL/L-POME/h)			Cell leakage (mg /mL)		
	AV	±SD	±SE	AV	±SD	±SE
4	320.3	1.91	0.90	0.001	0.03	0.001
8	330.2	1.17	0.67	0.003	0.05	0.01
12	347.3	1.56	0.85	0.009	0.06	0.02
16	370.4	1.34	0.77	0.02	0.10	0.01
20	373.6	1.10	0.63	0.04	0.11	0.09
24	375.2	1.23	0.71	0.05	0.13	0.10

(Values in mean ± SD, n=3)

APPENDIX A2

VARIATIONS OF HYDROGEN PRODUCTION RATE WITH BEAD SIZE

Bead size (mm)	Av. HPR(ml/L-POME/h)	±SD	±SE
3	427.3	**2.13**	**1.23**
4	365.2	**1.43**	**0.82**
5	302.7	**1.16**	**0.67**

(Values in mean ± SD, n=3)

APPENDIX A3

VARIATIONS IN HYDROGEN PRODUCTION RATE AND CELL LEAKAGE AS A FUNCTION OF PEG CONCENTRATION

PEG Concentration	HPR(mL/L-POME/h)			Cell leakage (mg CDW/mL)		
	AV	±SD	±SE	AV	±SD	±SE
2	137.3	0.95	0.54	2.2	0.34	0.19
4	169.2	1.17	0.67	1.43	0.26	0.15
6	220.3	1.56	0.90	0.8	0.27	0.15
8	269.4	1.34	0.77	0.32	0.17	0.09
10	327.6	1.10	0.63	0.12	0.15	0.08
12	301.2	1.23	0.71	0.1	0.19	0.10

(Values in mean ± SD, n=3)

APPENDIX A4

EFFECT OF CELL CONCENTRATION ON HYDROGEN PRODUCTION RATE AND CELL LEAKAGE

Cell concentration (g)	HPR(mL/L-POME/h)			Cell leakage (mg CDW/mL)		
	AV	±SD	±SE	AV	±SD	±SE
1.2	298.2	1.98	1.14	0.1	0.13	0.07
2.4	374.1	2.03	1.17	0.13	0.16	0.09
3.6	335.6	1.68	0.97	0.6	0.20	0.11
4.8	297.3	1.16	0.67	1.59	0.06	0.03
6	210.5	1.33	0.76	2.37	0.17	0.09

(Values in mean ± SD, n=3)

APPENDIX A5

EFFECT OF CURING TIME ON HYDROGEN PRODUCTION RATE AND CELL LEAKAGE

Curing time (min)	HPR(mL/L-POME/h)			Cell leakage (mg CDW/mL)		
	AV	±SD	±SE	AV	±SD	±SE
20	192.5	2.11	1.21	0.33	0.12	0.06
40	230.1	1.51	0.87	0.21	0.09	0.05
60	312.8	1.42	0.82	0.16	0.14	0.08
80	366.4	1.89	1.09	0.08	0.03	0.01
100	351.2	1.10	0.63	0.07	0.09	0.05
120	345.2	1.23	0.71	0.06	0.02	0.01

(Values in mean ± SD, n=3)

APPENDIX A6

VARIATIONS OF HYDROGEN PRODUCTION RATE WITH TEMPERATUER

Temperature (^{O}C)	Av. HPR(mL/L-POME/h)	±SD	±SE
25	147.3	**1.14**	**0.65**
30	267.3	**1.76**	**1.01**
35	363.6	**1.02**	**0.58**
40	289.1	**0.99**	**0.57**
45	198.8	**1.18**	**0.68**

(Values in mean ± SD, n=3)

APPENDIX A6

LIST OF PUBLICATIONS

Published Papers:

Lakhveer Singh, Zularisam A. Wahid, Muhammad Faisal Siddiqui, Anwer Ahmad, Mohd Hasbi Ab. Rahim, Mimi Sakinah**,** Application of immobilized upflow anaerobic sludge blanket reactor using *Clostridium* LS2 for enhanced biohydrogen production and treatment efficiency of palm oil mill effluent. **International Journal of hydrogen energy** 38 (2013) 2221-2229. **(Elsevier)**

Lakhveer Singh, Zularisam A. Wahid, Muhammad Faisal Siddiqui, Anwer Ahmad, Mohd Hasbi Ab. Rahim, Mimi Sakinah. Biohydrogen production from palm oil mill effluent using immobilized *Clostridium butyricum* EB6 in polyethylene glycol**. Process Biochemistry** 48 (2013) 294-298. **(Elsevier)**

Lakhveer Singh, Muhammad Faisal Siddiqui, Anwer Ahmad, Mohd Hasbi Ab. Rahim, Mimi Sakinah, Zularisam A. Wahid[*]**,** "Application of polyethylene glycol immobilized *Clostridium sp.* LS2 for continuous hydrogen production from palm oil mill effluent in upflow anaerobic sludge blanket reactor." **Biochemical Engineering Journal** 70 (2013) 158-165. **(Elsevier)**

Lakhveer Singh, Muhammad Faisal Siddiqui, Anwer Ahmad, Mohd Hasbi Ab. Rahim, Mimi Sakinah, Zularisam A. Wahid[*]**,** "Biohydrogen production from palm oil mill effluent using immobilized mixed culture**. Journal of Industrial and Engineering Chemistry 1**9, Issue (2013) 659-664. **(Elsevier)**

Papers Under-review:

Lakhveer Singh, Zularisam A. Wahid, Muhammad Faisal Siddiqui, Anwer Ahmad, Mohd Hasbi Ab. Rahim, Mimi Sakinah. Hydrogen production from biological processes: **Under Review in Journal of Environment technology.**

Lakhveer Singh, Zularisam A. Wahid, Muhammad Faisal Siddiqui, Anwer Ahmad, Mohd Hasbi Ab. Rahim, Mimi Sakinah. Enhanced bio-hydrogen production from palm oil mill effluent using cell immobilization technique. **Under Review in Journal of Biomass and Bioenergy.**

Lakhveer Singh, Zularisam A. Wahid, Muhammad Faisal Siddiqui, Anwer Ahmad, Mohd Hasbi Ab. Rahim, Mimi Sakinah. Optimization of biohydrogen production from palm oil mill effluent using immobilized biomass. **Under Review in Journal of Industrial and Engineering Chemistry.**

Conference and Proceeding:

Lakhveer Singh, Anwar Ahmad. Hydrogen production from POME wastewater by using UASB reactor: Poster presentation in post graduate poster competition 2011,be held in: University Malaysia Pahang on 7th April 2011.

Lakhveer Singh, Anwar Ahmad, Zularisam Abd. Wahid. Bio-hydrogen production from POME waste water by immobilized sludge process. Paper presented in, Vth World Aqua congress, be held in India Habitat Centre, New Delhi, India, on November 16-18, 2011.

Lakhveer Singh, Zularisam Abd. Wahid. Biohydrogen production from palm oil mill effluent using immobilized biomass UASB reactor. Paper presented in, NCON conference, be held in Universiti Malaysia Pahang (UMP), on September 10-11, 2012.

Lakhveer Singh, Zularisam Abd . Optimization of biohydrogen production from palm oil mill effluent using immobilized biomass. Paper presented in, 1st international conference in chemical, environment and energy, be held in Kualalampur Malaysia on September 21-23, 2012.

Lakhveer Singh, Zularisam Abd. Novel biocatalyst for enhanced hydrogen production and treatment efficiency of palm oil mill effluent. Poster presented in international conference in post graduation education, be held in Dewan Sultan Iskandar (DSI) UTM Johar Baharu Malaysia on 18-19, Dec, 2012.