Electronic Journal of Biotechnology ISSN: 0717-3458
http://www.ejbiotechnology.info
DOI: 10.2225/vol14-issue5-fulltext-9 *RESEARCH ARTICLE*

Effect of temperature and initial pH on biohydrogen production from palm oil mill effluent: long-term evaluation and microbial community analysis

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Received December 21, 2010 / Accepted June 14, 2011
Published online: September 15, 2011

Abstract Anaerobic sludge from palm oil mill effluent (POME) treatment plant was used as a source of inocula for the conversion of POME into hydrogen. Optimization of temperature and initial pH for biohydrogen production from POME was investigated by response surface methodology. Temperature of 60ºC and initial pHof 5.5 was optimized for anaerobic microflora which gave a maximum hydrogen production of 4820 ml H_2/l-POME corresponding to hydrogen yield of 243 ml H_2/g-sugar. Total sugar consumption and chemical oxygen demand (COD) removal efficiency were 98.7% and 46%, respectively. Long-term hydrogen production in continuous reactor at HRT of 2 days, 1 day and 12 hrs were 4850 ± 90, 4660 ± 99 and 2590 ± 120 ml H_2/l-POME, respectively. Phylogenetic analysis of the mixed culture revealed that members involved hydrogen producers in both batch and continuous reactors were phylogenetically related to the *Thermoanaerobacterium thermosaccharolyticum*. Batch reactor showed more diversity of microorganisms than continuous reactor. Microbial community structure of batch reactor was comprised of *T. thermosaccharolyticum*, *T. bryantii, Thermoanaerobacterium* sp., *Clostridium thermopalmarium* and *Clostridium* NS5-4, while continuous reactor was comprised of *T. thermosaccharolyticum*, *T. bryantii* and *Thermoanaerobacterium* sp. POME is good substrate for biohydrogen production under thermophilic condition with *Thermoanaerobacterium* species play an important role in hydrogen fermentation.

Keywords: biohydrogen, long-term evaluation, palm oil mill effluent, response surface methodology, thermophilic condition

INTRODUCTION

Biohydrogen is a promising clean fuel as it is ultimately derived from renewable energy sources. It is also efficient and environmental friendly since its combustion converts to water, gives high energy yield with less energy intensive processes (Nielsen et al. 2001). One possible biological approach to producing hydrogen is to convert, often negative valued, organic wastes into hydrogen rich gas by anaerobic microbial flora (Montgomery, 2004). Disposal of agricultural and industrial wastes and residues are already an economic burden on communities and industries. Therefore, biohydrogen production by dark fermentation of wastes can both reduce waste disposal problem and decrease raw material cost (Zhang et al. 2003). Additionally, the major advantages of dark fermentative process are high hydrogen production capacities, operation without light sources and no oxygen limitation problems. These characteristics make it more competitive than other biological conversion of organic wastes into hydrogen gas (Hawkes et al. 2002). Dark fermentative hydrogen production gives relatively high theoretical values of hydrogen production. Theoretically, four moles of hydrogen are produced from glucose concomitantly with 2 moles of acetate and only 2 moles of hydrogen are produced when butyrate is the main fermentation product. Typically, 60-70% of the aqueous product during sugar

fermentation under mesophilic condition is butyrate (Liu et al. 2002). It is also observed that hydrogen production yield of 1-2 mol H_2/mol-hexose are obtained with mesophiles, while thermophiles display a yield higher than 2 mol H_2/mol-hexose (van Niel et al. 2002). Hydrogen yields can be improved by increasing hydrogen production through acetate end product reaction, and decreasing or preventing butyrate, ethanol and propionate product reaction. One way to accomplish this is through fermentation process with thermophiles or extreme thermophiles, operating at temperatures higher than 60ºC (Kadar et al. 2004; O-Thong et al. 2008). Thus, a higher temperature is more feasible for the conversion reaction toward hydrogen due to favourable thermodynamics conditions.

Thermophilic bacteria are considered as more promising microorganisms than mesophilic bacteria for hydrogen production. Thermophilic bacteria are able to utilize a wide range of organic wastes. Thermophilic mixed culture has been examined for their potential as hydrogen producers. High hydrogen production rate and less variety of fermentation end products were observed under thermophilic conditions compared to mesophilic ones (Ahn et al. 2005; O-Thong et al. 2008). These properties make application of thermophiles for hydrogen production economically and technical feasible. Among a large number of microbial species, strictly anaerobes, *Clostridium*, *Thermoanaerobacterium*, *Caldicellulosiruptor* and *Thermoanaerobacter*, are efficient hydrogen producers via fermentation process under thermophilic condition (Ueno et al. 2001; Shin and Youn, 2005; O-Thong et al. 2009). To date, the majority of research has been focused mainly on using organic wastes and wastewater from food industry to produce hydrogen with mixed culture. Advantages of using mixed culture over pure culture are lower cost (saving in sterilization cost), septic organic wastes can be used as substrate, and process using mixed culture gave stable yield of hydrogen production from non-sterile organic wastes (Noike and Mizuno, 2000). Anaerobic microorganisms from palm oil mill wastewater treatment plant have been utilized as inocula for hydrogen production from glucose in batch cultivation (Morimoto et al. 2004; Atif et al. 2005). Mixed culture was also used as inoculum for hydrogen production from POME under mesophilic condition and achieved both hydrogen production (0.42 L/g $COD_{destroyed}$) and COD reduction (40%) (Vijayarahavan and Ahmad, 2006). Our previously report studied on the statistical optimization of chemicals parameters such as C/N, C/P and iron concentration in cultural conditions for hydrogen production from POME by thermophilic mixed culture. Simultaneous hydrogen production (6.33 l H_2/l-POME) and COD removal (55%) were achieved (O-Thong et al. 2008). However, it cannot obtain long term stability of process operation via optimum nutrient. Only trace amount of hydrogen yield was obtained from previously reports because the fermentative hydrogen production is affected by many parameters such as pH, temperature and the nature of the microbial communities. The effect of pH is known to be crucial due to its effects on hydrogenase activity, metabolism pathways, and microbial communities (Fang and Liu, 2002). The microbial community is very important parameter for the success in biological hydrogen production process and is the key factor to bring sustainable biohydrogen production and industrial implementation (Iyer et al. 2004; Ren et al. 2006).

This work focused on the statistical optimization of physico-chemical parameters (pH, temperature) in cultural conditions for biohydrogen production from palm oil mill effluent (POME) in batch and continuous reactor, their interaction on hydrogen production, long-term evaluation of optimization condition and investigated the responsible microbial community using PCR–DGGE technique.

MATERIALS AND METHODS

Inoculum preparation and palm oil mill effluent

The seed microflora for hydrogen production was enriched from anaerobic sludge collecting from palm oil mill wastewater treatment plant. The sludge was settled and collected after decanting the supernatant. Seed microflora was prepared by load-shock pre-treatment to remove methanogenic bioactivity (O-Thong et al. 2009). Sludge was subsequently enriched in a synthetic medium consisting of sucrose (20 gCOD/l), NH_4HCO_3 5.2 g/l, K_2HPO_4 0.125 g/l, $MgCl_2{\cdot}7H_2O$ 0.1 g/l, $FeSO_4{\cdot}7H_2O$ 0.025 g/l, $MnSO_4{\cdot}6H_2O$ 0.015 g/l, $CuSO_4{\cdot}5H_2O$ 0.005 g/l, $CoCl_2{\cdot}5H_2O$ 0.0001 g/l, and $NaHCO_3$ 6.7 g/l and the initial pH value was adjusted to 5.5 (Fan et al. 2004). The enriched sludge having a volatile suspended solids (VSS) concentration of 2.0 g/l was acclimatized with 10%, 30% 60% and 100% of POME, respectively. The acclimatized sludge was operated in semi-continuous process by removing 50% of culture medium and adding 50% fresh POME into the reactor every 48 hrs. Raw POME was collected from the receiving tank of Trang Palm Oil Co, Ltd. in Southern Thailand. Raw POME has brown colour,

pH 4.2-4.5, a temperature of 70-80ºC. The chemical characteristics of the POME are given in Table 1. The POME was stored at a temperature range of 0-4ºC until used.

Table 1. Chemical characteristics of palm oil mill effluent used in this study.

Parameter	Concentration (g/l)
Biochemical oxygen demand (BOD_5)	22.0 - 54.3
Chemical oxygen demand be	75.2 - 96.3
Total sugar	16.3 - 20.2
Total nitrogen	0.83 - 0.92
Ammonium–nitrogen	0.02 - 0.03
Total phosphorus	0.09 - 0.13
Phosphate	0.01 - 0.02
Oil	8.3 - 10.6
Total solids	35.0 - 42.0
Suspended solids	8.5 - 12.0
Ash	4.2 - 4.5

Experimental design and data analysis

The experiment was performed in 1.0 l infusion bottles with working volume of 650 ml. 190 ml of inocula corresponding to 30% v/v, 50 ml nutrient solution and 410 ml of POME were added into infusion bottles. Each liter of nutrient solution contained 2.0 g NH_4HCO_3, 1.0 g of KH_2PO_4, 0.01 g $MgSO_4 \cdot 7H_2O$, 0.001 g NaCl, 0.001 g $Na_2MoO_4 \cdot 2H_2O$, 0.001 g $CaCl_2$ $x2H_2O$, 0.0015 g $MnSO_4 \cdot 7H_2O$, 0.00278 g $FeCl_2$ (Lay et al. 1999). The experiments were incubated at a temperature range of 35-75ºC and initial pH values range from 4.5-6.5. The initial pH values were adjusted using either 2 M NaOH or 2 M HCl. The evolved gas was collected with a gas collecting bag (Cali-5-bond, Calibrated Instruments, Inc.) and measured by water displacement method in a graduated cylinder filled with acidic water (pH ≤ 3) in order to prevent dissolution of the gas components (Owen et al. 1979). Factorial central composite experimental design (Box et al. 1978) was used to optimize the pH and temperature for hydrogen production from POME by thermophilic mixed culture. The cumulative hydrogen production curves were obtained over the time course of batch experiment. Central composite experimental design matrix with corresponding result under various pH and temperature are summarized in Table 2. The corresponding values of specific hydrogen production (P_s) and hydrogen production potential (P) were obtained by fitting with modified Gompertz equation (Equation 1) (Lay et al. 1999). R^2 for all parameters was larger than 0.95, indicating that the parameters were statistically significant.

$$H(t) = P.\exp\{-\exp[\frac{R_m.e}{P}(\lambda - t) + 1]\}$$

[Equation 1]

Where, *H(t)* (ml) = represents the cumulative volume of hydrogen production, P (ml) = the hydrogen production potential, R_m (ml/h) = the maximum production rate, and λ (h) = the lag time. The values of P, R_m and λ for each batch were determined by best fitting the hydrogen production data in the above equation using the Matlab 6.0 with optimization toolbox 2.1 (MathWorks, USA). The hydrogen yield (Y_{PS}) (ml H_2/g-sugar) was calculated by diving P with the initial quantity of total sugar in POME. The maximum specific hydrogen production rates (R_m) (ml H_2/g-VSS/h) was calculated by dividing R_m with the initial sludge VSS. Quadratic model was used to evaluate the optimization of environmental factors, including initial pH and temperature.

$$Y_i = \beta_0 + \beta_1 x_1 + \beta_2 x_2 + \beta_{12} x_1 x_2 + \beta_{11} x_1^2 + \beta_{22} x_2^2$$

[Equation 2]

Where Y_i = predicted response; x_1 and x_2 = parameters; β_0 = offset term; β_1 and β_2 = linear coefficients; β_{11} and β_{22} = squared coefficients; and β_{12} = interaction coefficients. Multiple regression with stepwise for Equation 2 was performed using Statistica program (Statsoft, USA).

Table 2. Central composite experimental design matrix with corresponding result under various pH and temperature.

Trials	Initial pH	Temperature (ºC)	Y_P (ml H_2/l-POME)	Y_{PS} (ml H_2/g-sugar)	Y_{COD} (%)	Y_{SC} (%)
1	5.5	55	4600 ± 125	230 ± 6	45 ± 1.5	95 ± 2.5
2	6.5	75	2050 ± 52	102 ± 2	22 ± 1.4	62 ± 1.3
3	5.5	35	1104 ± 60	55 ± 3	12 ± 0.6	62 ± 2
4	4.5	35	200 ± 5	2 ± 1	5 ± 1.5	23 ± 0.5
5	5.5	55	4564 ± 150	228 ± 7	43 ± 1.6	93 ± 1
6	5.5	55	4750 ± 130	237 ± 6	45 ± 0.5	96 ± 0.8
7	5.5	75	4100 ± 110	204 ± 5	40 ± 0.4	92 ± 2
8	5.5	55	4500 ± 105	229 ± 5	42 ± 0.7	95 ± 1.3
9	4.5	75	250 ± 10	3 ± 1	5 ± 1	25 ± 1
10	4.5	55	2584 ± 50	129 ± 2	20 ± 1.2	70 ± 1.2
11	5.5	55	4566 ± 110	227 ± 4	46 ± 0.5	97 ± 1.5
12	6.5	55	4300 ± 90	231 ± 5	41 ± 0.7	91 ± 2
13	5.5	55	4624 ± 60	230 ± 2	44 ± 0.9	94 ± 0.5
14	6.5	35	156 ± 11	42 ± 2	11 ± 0.5	33 ± 1

Continuous hydrogen production

Enriched microflora and optimum condition from batch tests and semi-continuous were applied to continuously hydrogen production from POME. Three identical 1.2 l glass continuous stirred tank (CSTR) reactors with 0.9 l working volume were used for continuous experiment. Experimental setup consists of feed bottle, feed pump, reactor, effluent bottle and gas meter. The temperature was controlled at 60ºC by circulating hot water inside the water jacket of the reactors. Mixing was provided by a magnetic stirrer located underneath the reactor. The initial anaerobic condition in the reactor was established by replacing the gaseous phase with nitrogen. The POME was continuously pumped into reactors six times a day at 4 hrs intervals, each time 122 ml POME for HRT 48 hrs, 225 ml POME for HRT 24 hrs and 450 ml POME for HRT 12 hrs. The amounts of evolved gas, soluble metabolites, and responsible microbial community were investigated. The reactors were operated until the system reached steady state. The steady-state condition was reached when hydrogen gas content, biogas volume and the volatile fatty acids (VFA) concentration in the effluent were stable (less than 10% variation) for a week.

Table 3. Quadratic model for the interaction effects of pH and temperature on hydrogen production potential (P), hydrogen yield (Y_{PS}), Total sugar consumption (Y_{SC}) and COD removal efficiency (Y_{COD}) of enriched microflora culture in batch testes while x_1 and x_2 represent pH and temperature, respectively.

Equation	R^2	Equation no.
$Y_P = - 57815 + 16093x_1 + 565x_2 - 1526x_1^2 - 6x_2^2 + 23x_1x_2$	0.96	3
$Y_{PS} = -2884 + 775x_1 + 31x_2 – 70x_1^2 - 0.3x_2^2 + 0.8x_1x_2$	0.95	4
$Y_{SC} = - 940 + 290x_1 + 7x_2 - 27x_1^2 - 0.1x_2^2 + 0.3x_1x_2$	0.97	5
$Y_{COD} = -544 + 156x_1 + 5x_2 -14x_1^2 - 0.1x_2^2 + 0.2x_1x_2$	0.93	6

Analytical methods

The biogas composition was measured by gas chromatography equipped with thermal conductivity detectors (GC-TCD). Hydrogen gas was analyzed by GC-TCD fitted with an 1.5 m stainless steel column SS350A packed with a molecular sieve (80/100 mesh). Nitrogen was used as a carrier gas at a

flow rate of 30 ml/min. The temperatures of the injection port, oven and detector were 100, 50 and 100ºC, respectively (Morimoto et al. 2004). Methane and carbon dioxide were analyzed by GC-TCD fitted with 3.3 ft stainless steel column packed with Porapak T (60/80 mesh). Helium was used as a carrier gas at a flow rate of 35 ml/min. The temperatures of the injection port, oven and detector were at 150, 50 and 100ºC, respectively (Chang and Liu, 2004). The gas sample of 100 µL was injected in duplicate. Volatile fatty acids (VFA) were analyzed by gas chromatography (Hewlett Packard, HP 6850 series) equipped with a flame ionization detector. A column capillary packed with nitroterephthalic acid-modified polyethleneglycol and with a length of 30 meter was used. The temperature of the injection port was 250ºC. The chromatography was performed using the following program: 100ºC for 5 min, 100-250ºC with a ramping of 10ºC/min, 250ºC for 12 min. The detector temperature was 300ºC. Chemical oxygen demand (COD), pH, suspended solid (SS), total suspended solid, oil concentration, total phosphorus and total Kjeldahl nitrogen were determined in accordance with the procedures described in the Standard Methods (Clescerl et al. 1998). Ammonium-nitrogen and phosphate concentrations were analyzed using commercial test kits from Spectroquant (Merck Ltd., Germany). The total sugar content was analyzed by the anthrone method (Morris, 1948).

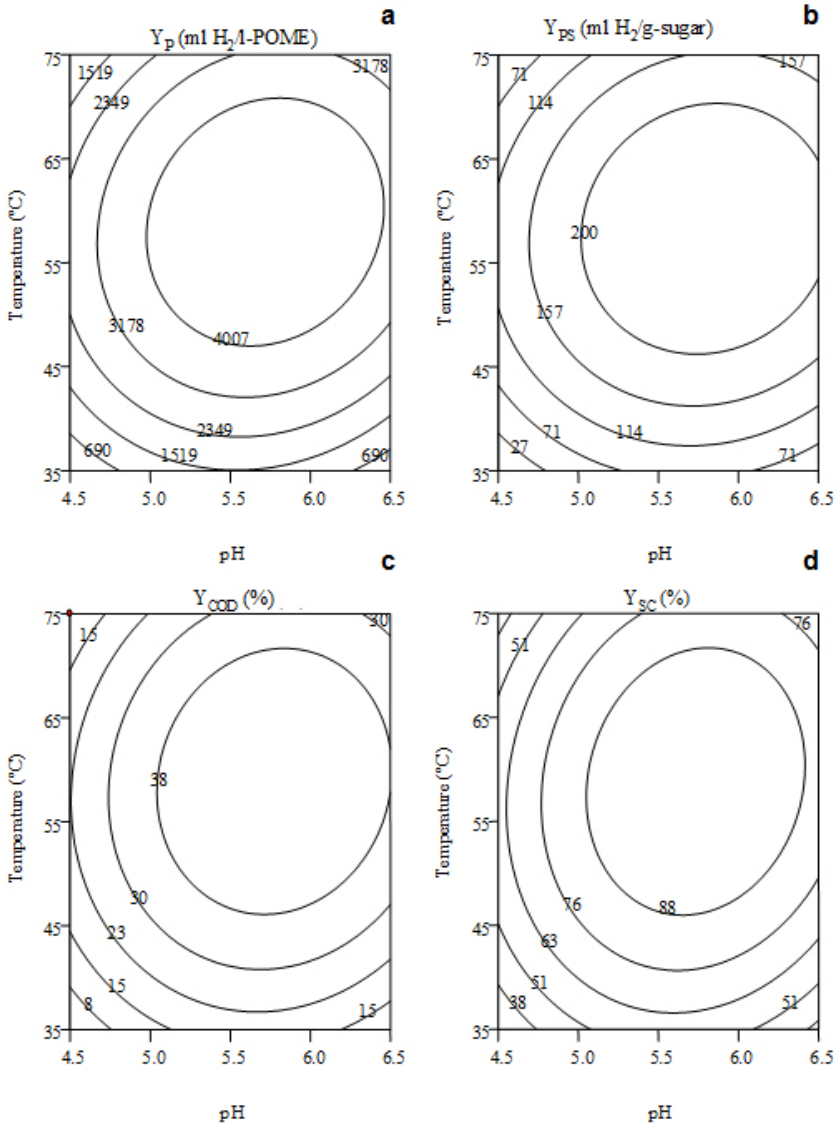

Fig. 1 Contour plot of Y_P (a), Y_{PS} (b), Y_{COD} (c) and Y_{SC} (d) from cultivation of enriched microflora on POME at varying of initial pH and temperature are estimated using Equation 3, Equation 4, Equation 5 and Equation 6, respectively.

Microbial community analysis

Total genomic deoxyribonucleic acid (DNA) was extracted from semi continuous experiment sludge samples using the Ultraclean Soil DNA Kit (MoBio Laboratory Inc., USA). The region of the 16S rRNA genes corresponding to position 340 to 518 in the 16S rRNA of *Escherichia coli* was PCR-amplified using the forward primer; L340f with a GC clamp at the 5' end (5'-CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGGCCTACGGGAGGCAGCAG-3') and the reverse primer; K517r (5'-ATTACCGCGGCTGCTGG-3') (Muyzer et al. 1993). PCR amplification was conducted in an automated thermal cycler using the following protocol; initial denaturation for 5 min at 94ºC, 30 cycles of denaturation for 1 min at 95ºC/annealing for 30 sec at 55ºC/extension for 1 min at 72ºC, followed by a final extension for 7 min at 72ºC. The DGGE analysis of the PCR products was performed by electrophoresis for 20 min at 20 V and 15 hrs at 70 V through a 7.5% polyacrylamide gel containing a linear gradient of denaturant (100% denaturant corresponds to 7 M urea and 40%(v/v) formamide deionised with AG501-X8 mixed bed resin) ranging from 30% to 60% in 0.5 x TAE buffer at a constant temperature of 60ºC (DGGE unit, V20-HCDC, Scie-Plas Limited, UK). The gel was stained with Sybr-Gold (1000x concentration) for 1 hr and visualized on a UV transilluminator. Most of the bands were excised from the gel and re-amplified with the forward primer without a GC clamp and the reverse primer. After re-amplification, PCR products were purified using E.Z.N.A cycle pure kit (Omega Bio-tek, USA) and sequenced using primer K517r and an ABI PRISM Big Terminator Cycle Sequencing Kit version 3.1 (Applied Biosystems, USA) in accordance with the manufacturer's instructions. Closest matches for partial 16S rRNA gene sequences were identified by database searches in GenBank using BLAST (Altschul et al. 1997).

RESULTS AND DISCUSSION

Effects of initial pH and temperature on enriched microflora

The effects of initial pH and temperature were studied using central composite design methodology with the aims of modelling and optimization on the conversion of POME to hydrogen by enriched microflora. The contour plots in Figure 1 were constructed using the equations listed in Table 3. The regression models of all equations were considered to represent the experimental data accurately as all values of R^2 are close to 1.0. The optimum conditions were found to be at the temperature of 60 ± 1ºC and initial pH at 5.5 ± 0.1. The pH and temperature both had a significant interaction on hydrogen production (Y_P), hydrogen yield (Y_{PS}), total sugar consumption (Y_{SC}) and COD removal efficiency (Y_{COD}). Maximum values of Y_P, Y_{PS}, Y_{SC} and Y_{COD} were 48120 ml H_2/l-POME, 243 ml H_2/g-sugar, 98.75%, 45%, respectively. The optimum point within the contour level of 4812 ml H_2/l-POME lies between 5.5-5.6 on pH axis and 60-61ºC on temperature axis (Figure 1a). To assess the amount of hydrogen gas of enriched microflora, equation 4 was used to construct the contour graph as shown in Figure 1b which provides the hydrogen yield of 243 ml H_2/g-sugar. The total sugar consumption was plotted in contour graph against pH and temperature (Figure 1d) created using Equation 5, giving the maximum value of 98.75% at pH 5.6 and temperature 61ºC, respectively. It is interesting to note that the optimum point of total sugar consumption was similar to that of hydrogen production and hydrogen yield. It may imply that evolved hydrogen come from total sugar degradation. The contour graph of COD removal efficiency derived from the quadratic model Equation 6 (Figure 1c) gave the highest value of 45%. The goodness of model was checked by R^2 values, it also indicate that only 4-7% of the total variable is not explained by the model Equation 3, Equation 4, Equation 5 and Equation 6. These hydrogen productions and COD removal efficiency agree with those obtained in previous reports (Atif et al. 2005; Fakhru'l-Razi et al. 2005; Vijayarahavan and Ahmad, 2006). Throughout this study, the hydrogen content in the biogas was in the range of 38.5-47% and no methane was found.

To validate the statistical experimental strategies and to gain a better understanding of hydrogen production efficiency, three batch reactors based on the optimal conditions (pH 5.5 and temperature 60ºC) were conducted. Confirmation experiments indicated that the obtained optimum conditions gave reproducible results; giving the value of Y_P, Y_{PS}, Y_{SC} and Y_{COD} of 4820 ± 120 ml H_2/l-POME, 228 ± 4.5 ml H_2/g-sugar, 94.3 ± 2.2% and 42 ± 3%, respectively, which were very close to the values, evaluated using the response surface methodology (Table 4). This agreement reveals that the Y_P, Y_{PS}, Y_{SC} and Y_{COD} of enriched cultures were reproducible. Validated experiment confirmed that high hydrogen conversion resulted from optimum pH and temperature. Khanal et al. (2004) suggested that pH is a pivotal parameter for biohydrogen production where the intermediate product (volatile fatty acids) drives the hydrogenase reaction. The enriched of microbial sludge from POME wastewater treatment

plant resulted in high hydrogen producing bacteria at thermophilic condition. It is interesting to known that what microorganisms play an important role in the thermophilic conversion of POME into hydrogen.

Table 4. Comparison between predicted and confirmation parameters of hydrogen production from the enriched microflora culture on POME under optimum values of pH at 5.5 and temperature at 60ºC.

Parameters	Maximum calculated value	Confirmation experiments
Y_{PS} (ml H_2/g-sugar)	243	228 ± 4.5
Y_P (ml H_2 /l-POME)	4812	4820 ± 120
Y_{SC} (%)	98.75	94.3 ± 2.2
Y_{COD} (%)	46	42 ± 3

Continuous hydrogen production

The comparison of hydrogen yields between batch and continuous cultivation are presented in Table 5. More total sugar in POME was decomposed at longer cultivation time (HRT 2 days). The highest hydrogen production yield of batch and continuous cultivation (HRT 2 days, 1 day and 1 hr) were 4745 ± 80, 4650 ± 90, 4550 ± 99 and 2259 ± 120 ml H_2/l-POME, respectively. Biogas from batch cultivation comprised of hydrogen (40%), carbon dioxide (38%), hydrogen sulphide (780 ppm) and no methane. Biogas of continuous cultivation comprised of hydrogen ranged between (38.5-47%), carbon dioxide (35-52%), hydrogen sulphide (620-440 ppm) and no methane gas. The total sugar consumption in batch and continuous cultivation (HRT 2 days, 1 day and 12 hrs) were 94%, 98%, 96% and 67%, respectively. The COD removal efficiency in batch and continuous cultivation (HRT 2 days, 1 day and 12 hrs) were 42%, 48%, 45% and 23%, respectively. Biogas production stopping was observed in continuous reactor cultivation after fed fresh medium for 6-12 hrs. The discontinued hydrogen production after adding fresh medium (during 0-6 hrs) may result from trace amount of dissolved oxygen contained in POME (Yokoi et al. 1995). This evidence resulted low hydrogen production yield at short hydraulic retention time (HRT 12 hrs) of continuous cultivation. Continuous cultivation operated at HRT 1 day yields less hydrogen than operated at HRT 2 days. These results seem to provide evidence that strictly anaerobic bacteria could play important role as hydrogen producers in the systems and are influenced by short hydraulic retention time or short cultivation time (Shin and Youn, 2005).

Table 5. Biogas composition, hydrogen production(Y_P) and hydrogen production rate (R_m) of batch and continuous cultivation for hydrogen production from POME by enriched microflora at temperature of 60ºC and pH of 5.5 exclude nitrogen gas that used for creating anaerobic condition (n = 6).

Parameters	Gacompositions				Y_P (ml H_2/l-POME)	R_m (ml H_2/l-POME/h)
	H_2 (%)	CO_2 (%)	H_2S (ppm)	CH_4 (%)		
Batch	40	38	780	0	4745 ± 80	98
Continuous at HRT 2-d	47	35	620	0	4850 ± 90	101
Continuous at HRT 1-d	46	36	510	0	4660 ± 99	194
Continuous at HRT 12-h	38.5	52	440	0	2590 ± 126	107

The change of hydrogen production and volatile fatty acids (VFA) was given in Figure 2. Acetic acid, butyric acid and formic acid were the main metabolites in batch and continuous processes, but butyric acid increased significantly in continuous cultivation and became dominated metabolites at HRT 1 day. The soluble metabolites of continuous cultivation were 6.8-7% formic acid, 30-40% acetic acid, 42-52% butyric acid, 0.7-0.8% propionic acid and small amount of undetermined volatile fatty acids (VFA) (8-10%). The hydrogen production was accompanied with the production of VFA. Butyric acid and acetic acid constituted more than 70% of total metabolites. Butyric acid and acetic acid are produced during thermophilic hydrogen fermentation by *Thermoanaerobacterium* (Ueno et al. 2001). In general, production of these two acids favours the production of hydrogen. The theoretical hydrogen yield from glucose with acetate formation is 450 ml H_2/g-sugar, which is twice as high as that of butyrate formation, 225 ml H_2/g sugar (O-Thong et al. 2008). However, biohydrogen yields were inhibited by self

produced acids (van Ginkel and Logan, 2005). From the result of volatile fatty acids production, it is obvious that at low HRT, more butyric acid was produced as compare to acetic acid. This might be due to nature of *T. Thermosaccharolyticum* as one of the butyric acid producers (O-Thong et al. 2009). The total sugar consumption in batch and continuous cultivation (HRT 2 days and 1 day) were 94%, 98% and 96%, respectively. Total sugar consumption showed that hydrogen production is limited by the bioavailability of the carbohydrates in the POME and therefore solubilisation of the undissolved carbohydrates could precede the whole process resulting at an even higher hydrogen recovery.

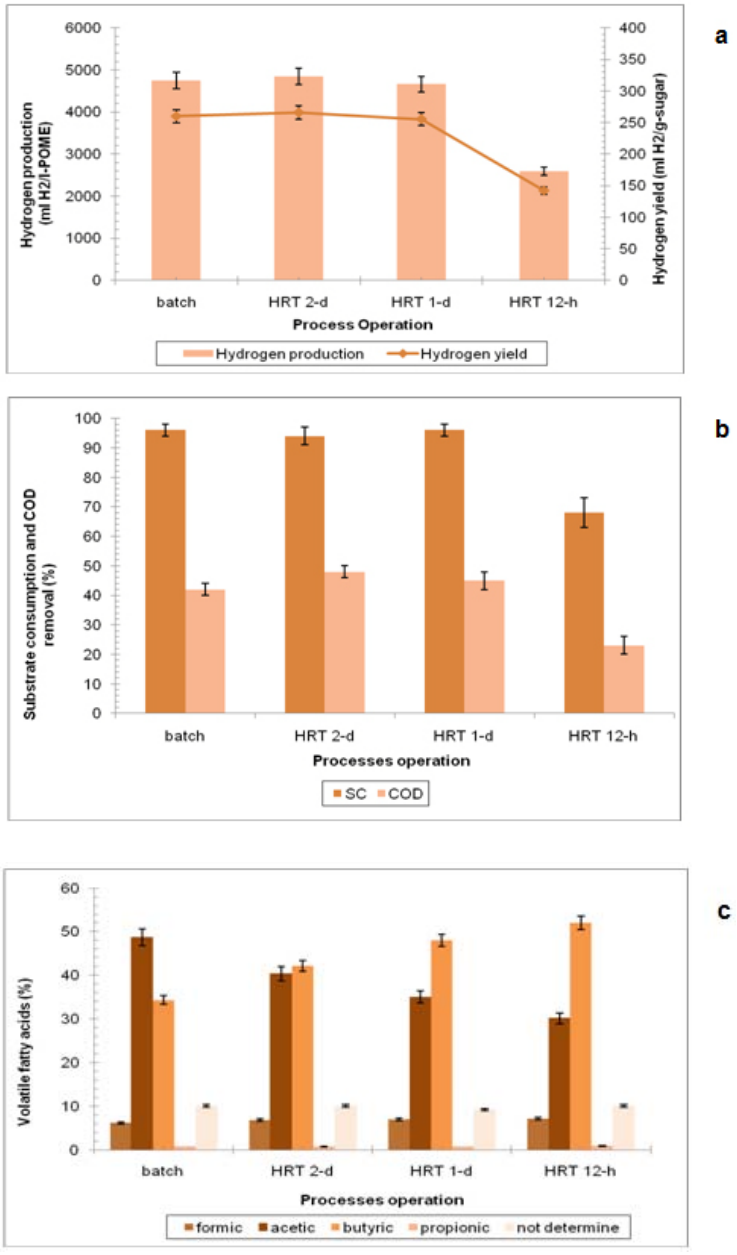

Fig. 2 Changes of hydrogen production (a) volatile fatty acids composition (b), substrate consumption and COD removal efficiency (c) during the cultivation of enriched cultures in palm oil mill effluent with batch and continuous operation.

Microbial community analysis

The diversity of microbial communities at different processes operation was analyzed and compared by DGGE technique. The DGGE profile of 16S rRNA gene fragments was shown in Figure 3. The DGGE profile clearly showed that different microbial population in batch and continuous processes operations. Batch cultivation showed more diversity of microorganisms than continuous cultivation. Microbial community structure of batch cultivation was comprised of *T. thermosaccharolyticum*, *T. bryantii, Thermoanaerobacterium* sp, uncultured bacterium (AY999014), *Clostridium thermopalmarium* and *Clostridium* NS5-4, while continuous cultivation comprised of *T. thermosaccharolyticum*, *T. bryantii* and *Thermoanaerobacterium* sp. *Clostridium thermopalmarium* and *Clostridium* NS5-4 gradually decreased in continuous cultivation and not present at HRT 1 day and 12 hrs. Each band of DGGE represents the specific species and the intensity of band relative to dominated species. All process operation was dominated by *T. thermosaccharolyticum*. Most of the DGGE related to *T. thermosaccharolyticum* which may proliferate under the thermophilic anaerobic conditions that are applied to the system. Soluble metabolites mainly consist of acetic acid and butyric acid also suggested that the strict anaerobic hydrogen producing bacteria (*T. thermosaccharolyticum*) play an important role in the anaerobic cultures enrichment. The major fermentation metabolites (acetic acid and butyric acid) depend on *Thermoanaerobacterium* species that are dominated in the anaerobic sludge (Zhang et al. 2003; Shin and Youn, 2005). Shin and Youn (2005) also reported that operated under thermophilic condition at pH 5.5, only *Thermoanaerobacterium* species was found. This can be inferred from the result that other microorganisms in original sludge were inactivated at the thermophilic and acidogenic operational condition, but it was a favourable environment for the growth of *Thermoanaerobacterium* species resulted in a predominant species in the system. *T. thermosaccharolyticum* is a thermophilic saccharolytic microorganisms involved in acetate and butyrate fermentation that leads to production of large amount of hydrogen from carbohydrate (Liu et al. 2003; Zhang et al. 2003; Ahn et al. 2005; Shin and Youn, 2005). The maximum growth of *T. thermosaccharolyticum* was obtained at the pH range of 5 to 6 and the optimum temperature for growth was 60ºC. The yields of hydrogen production from *T. thermosaccharolyticum* was 2.4 mol H_2/ mol glucose (270 ml H_2/g-sugar) that higher yields than *Clostridium* species and *Enterobacter* species (Ueno et al. 2001). Moreover, the microbial community changes in parallel with the decrease HRT from 1 day to 12 hrs. Previous studies also reported that the decrease in hydrogen yield of hydrogen was due to the hydraulic retention decrease in the reactor which was dominated by *Thermoanaerobacterium* species (Shin and Youn, 2005). *Clostridium* sp. and *Thermoanaerobacterium* sp. are strictly anaerobic bacteria and sensitive to oxygen from fresh POME at continuous operation which contributed to the decrease of hydrogen production (Liu and Shen, 2004). Oxygen in fresh medium was influenced to *Clostridium* sp. more than *Thermoanaerobacterium* sp., due to every low population of *Clostridium* sp. at continuous operation. Some oxygen contained in POME resulting to a non-strictly anaerobic condition subsequently leading to *Thermoanaerobactrium* species facing the long lag phase. The longer lag phase was likely due to the adaptation of the bacteria community by modifying their physiological state for the new environment (Zhang et al. 2003) after adding fresh POME. The hydraulic retention time or cultivation time could be an important factor to maintain a constant *Thermoanaerobacterium*-rich sludge.

CONCLUDING REMARKS

It has been proven that POME is good substrate for hydrogen production with approximately 4820 ml H_2/l-POME. Initial pH of 5.5 and temperature of 60ºC were the optimal condition for cultivation of the enriched microflora that is dominated by *Thermoanaerobacterium* as well as giving the high yields of hydrogen production from POME. These results confirm that environment factors such as pH, temperature and hydraulic retention time affect microbial community as well as hydrogen production yield. Batch cultivation showed more diversity of microorganisms than continuous cultivation. Microbial community structure of batch cultivation was comprised of *T. thermosaccharolyticum*, *T. bryantii, Thermoanaerobacterium* sp, uncultured bacterium (AY999014), *Clostridium thermopalmarium* and *Clostridium* NS5-4, while continuous cultivation comprised of *T. thermosaccharolyticum*, *T. bryantii* and *Thermoanaerobacterium* sp. *T. thermosaccharolyticum* is a hydrogen producing bacteria that is involved in butyric and acetic acid fermentation of carbohydrate in POME. The conversion of POME to hydrogen was strongly dependent on microorganisms, thus a suitable microbial community is an essential factor to obtain efficient and sustainable hydrogen production. Overall results suggested that enriched anaerobic cultures that are dominated by *Thermoanaerobacterium* were suitable for hydrogen production from POME and POME was found to be good substrate for hydrogen production under

thermophilic condition. Conclusively, the POME generated from processing of palm oil could be regarded as a useful by-product that can be used for the production of energy in the form of hydrogen.

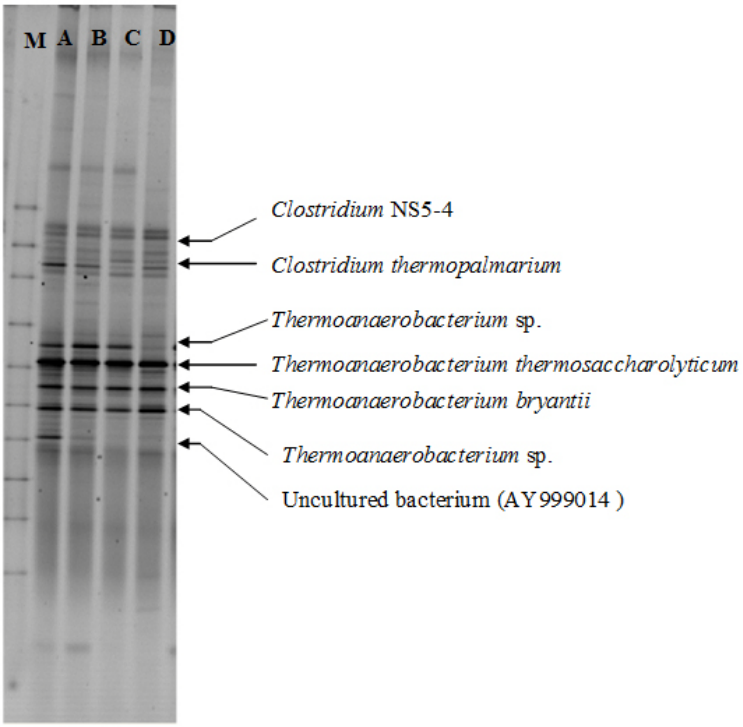

Fig. 3 DGGE profile of sludge from batch cultivation (A), continuous operating at HRT 2-d (B), continuous operating at HRT 1-d (C), continuous operating at HRT 12-h (D) under optimum condition of pH 5.5 and temperature 60ºC and D.

Financial support: This work was granted by the Thailand Research Fund (TRF), the Commission on Higher Education grant number MRG5280236, Research Group for Development of Hydrogen Production Process from Biomass by Microorganisms and the MEXT-ARDA.

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How to reference this article:

O-THONG, S.; MAMIMIN, C. and PRASERTSAN, P. (2011). Effect of temperature and initial pH on biohydrogen production from palm oil mill effluent: long-term evaluation and microbial community analysis. *Electronic Journal of Biotechnology*, vol. 14, no. 5. http://dx.doi.org/10.2225/vol14-issue5-fulltext-9