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Application of polyethylene glycol immobilized *Clostridium* sp. LS2 for continuous hydrogen production from palm oil mill effluent in upflow anaerobic sludge blanket reactor

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ABSTRACT

A novel polyethylene glycol (PEG) gel was fabricated and used as a carrier to immobilize *Clostridium* sp. LS2 for continuous hydrogen production in an upflow anaerobic sludge blanket (UASB) reactor. Palm oil mill effluent (POME) was used as the substrate carbon source. The optimal amount of PEG-immobilized cells for anaerobic hydrogen production was 12% (w/v) in the UASB reactor. 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 best volumetric hydrogen production rate of 336 mL H_2/L/h (or 15.0 mmol/L/h) with a hydrogen yield of 0.35 L H_2/g $COD_{removed}$ was obtained at a HRT of 12 h and an OLR of 5.0 g COD/L/h. The average hydrogen content of biogas and COD reduction were 52% and 62%, respectively. The major soluble metabolites during hydrogen fermentation were butyric acid followed by acetic acid. It is concluded that the PEG-immobilized cell system developed in this work has great potential for continuous hydrogen production from real wastewater (POME) using the UASB reactor.

1. Introduction

Fossil fuels are the life blood of modern civilization, but fossil fuel combustion causes global warming, health problems, and environmental pollution. 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 has been proposed as an alternative energy source because it is non-polluting and renewable [1]. Among the established hydrogen production technologies, biological hydrogen production has received considerable attention due to its potential as an inexhaustible, cost-effective, and carbon-neutral fuel. A wide variety of organic wastes or biomass could be used as substrates for biological hydrogen generation [2,3]. Biological hydrogen production can be achieved by anaerobic dark fermentation, in which anaerobic organisms convert carbohydrate-containing organic wastes into hydrogen [4]. Moreover, fermentative hydrogen production using organic waste and wastewater as substrates achieves both bioremediation and energy recovery.

In Malaysia, the estimated annual production of palm oil mill effluent (POME) is about 50 million tons. 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 [5,6]. The high nutrient content in POME makes it an ideal fermentation medium for anaerobic treatment processes [7]. Previous reports have utilized POME as a substrate for hydrogen production with defined microflora, or mixed cultures of POME sludge under continuous mode operation [8–12]. Badiei et al. [10] evaluated hydrogen production from POME in an anaerobic sequencing batch reactor using

Abbreviations: PEG, polyethylene glycol; POME, palm oil mill effluent; UASB, upflow anaerobic sludge blanket; COD, chemical oxygen demand; HRT, hydraulic retention times; OLR, organic loading rate; SEM, scanning electron microscope; TN, total nitrogen; VSS, volatile suspended solids; TS, total solid; GC, gas chromatograph; BOD, biochemical oxygen demand; TP, total phosphorus; TN, total nitrogen; VFAs, volatile fatty acids; HBu, butyric acid; HAc, acetic acid; BuOH, butanol; EtOH, ethanol; SMP, soluble microbial products; TVFA, total volatile fatty acid; HPr, propionic acid.

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enriched mixed microflora from POME sludge, and obtained maximum hydrogen production rate of 6.7 L H_2/L/d with a total COD removal of more than 37% at a HRT of 3 d, an OLR of 6.6 g COD/L/d, a pH of 6.8, and a temperature of 37 °C. Prasertsan et al. [11] obtained a maximum hydrogen production rate of 9.1 L H_2/L/d along with a COD removal of 57% at a controlled pH 5.5, a temperature of 60 °C, and optimum values of 48 h HRT and 60 g COD/L–POME/d OLR in the anaerobic sequencing batch reactor. Yusoff et al. [12] found that the maximum hydrogen production rate was 44 N mL/h/L–POME, and the maximum biohydrogen yield was 1054 mL/L–POME at a pH of 5.5, a temperature of 22–26 °C, a substrate concentration of 50–60 g/L COD–POME, and a HRT of 48 h in a 50 L continuously stirred tank reactor. Chong et al. [13] used a microorganism (*Clostridium butyricum* EB6) isolated from anaerobically digested POME sludge and raw POME as the substrate, with an initial concentration of 100 g COD/L–POME in a batch experiment at 37 °C and pH 6. Under these conditions, a maximum volumetric hydrogen production rate of 1034.7 mL/L/h was observed. However, these studies on biohydrogen production from POME only focused on suspended cell-systems, which are usually ineffective or difficult to handle in continuous operation because washout of bacteria with effluents may occur from the reactor at shorter HRTs [10] and result in a low biomass concentration in the bioreactor. Recycling of biomass is considered necessary to maintain a sufficient cell concentration in the reactor to maximize hydrogen production [14].

Another potential approach to enhance hydrogen production is to use an immobilized cell system [15]. 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 hydrogen production in a bioreactor [16–19]. Many different methods have been employed for biomass immobilization including adsorption to solid surfaces and entrapment in polymeric gel, biofilms, and granules [17,20–22]. The primary difficulty of the immobilized system is leak-out. However, it allows better biomass retention at low HRTs and creates a local anaerobic environment, which is well-suited to fermentative hydrogen production. In this work, a new cell immobilization system that employs polyethylene glycol (PEG) gel was employed to immobilize *Clostridium* sp. LS2 for continuous dark hydrogen production in a UASB reactor fed with POME. PEG was selected as the solid matrix for its ease of use, low toxicity, highly porous structure, and good mechanical properties [23]. The effect of HRT and OLR on the fermentative hydrogen producing capability of the UASB reactor containing immobilized cells was investigated. To the best of our knowledge, this is the first report of a PEG-immobilized *Clostridium* sp. LS2 system for continuous dark fermentative hydrogen production from real wastewater (POME).

2. Materials and methods

2.1. Fermentation medium

Raw POME was obtained from the final discharge point of a palm oil mill wastewater treatment plant, Lepar Hilir Pahang, Malaysia, and was used as the fermentation medium for hydrogen production. POME was preserved at 4 °C in a 25-L container to prevent biodegradation and acidification before use. Prior to being fed into the UASB reactor, the raw POME was diluted to a required COD concentration for fermentation. To assist the growth of hydrogen producing bacteria, the COD:N:P ratio was maintained at an average of 500:5:1 by adding peptone and $Na_2HPO_4{\cdot}2H_2O$ to the diluted POME. The chemical characteristics of the POME used in this study are shown in Table 1. Each sample was filtered by simple filtration to remove coarse particles from the sample.

Table 1
Characteristics of POME.

Parameter	Concentration (mg/L)
Biochemical oxygen demand (BOD)	23,100–55,200
Chemical oxygen demand (COD)	55,100–86,300
pH	4.0–5.0
Total carbohydrate	16,200–20,000
Total nitrogen (TN)	820–910
Ammonium–nitrogen	25–35
Total phosphorus (TP)	95–120
Phosphorus	14–20
Oil	2000–2500
Total solid (TS)	30,000–42,000
Volatile suspended solids (VSS)	8400–12,000

All values are in mg/L except pH.

2.2. Micro organisms

The hydrogen producing bacteria of *Clostridium* sp. LS2 was obtained from POME sludge after heat treatment for 30 min at 90 °C [24]. 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 °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*. The genomic DNA was extracted from cell pellets and the 16S rDNA gene was amplified by PCR as described [25]. The double-stranded PCR products were sequenced. The 16S rDNA sequence was aligned with others available in Gen Bank. 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. LS2. The isolated strain was stored in sterile 15% (v/v) glycerol solution at −30 °C before being subjected to immobilization.

2.3. Immobilization of cells in PEG

Clostridium sp. LS2 cells were immobilized by entrapment in a PEG prepolymer. First, the PEG prepolymer and the promoter N,N,N′,N′-tetramethylene-diamine were dissolved to achieve 16% (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 20 min to promote polymer formation. The immobilized-cell polymer was cut into 3-mm bead particles (density 1.40 g/cm^3). 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. The immobilization experiments were performed under anaerobic conditions at room temperature. 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 Fig. 1.

2.4. Mechanical bead testing

The mechanical stability of immobilized cells was measured according to the method described by Reys et al. [26] with small changes. 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 °C with shaking at 150 rpm. The number of intact

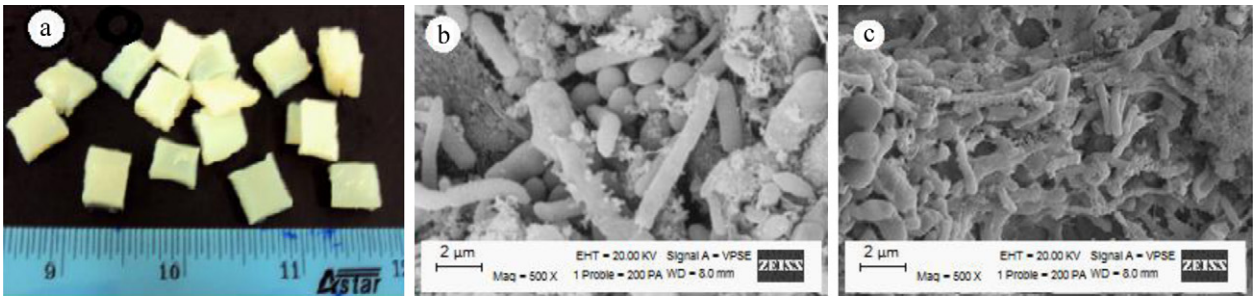

Fig. 1. SEM images of the PEG immobilized cell beads: (a) the shape and size of the immobilized beads; (b) immobilized *Clostridium* sp. LS2 in PEG at the starting phase of experiment. Scale bar: 2 μm; (c) immobilized *Clostridium* sp. LS2 in PEG at the end of experiment. Scale bar: 2 μm.

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, $f\,(\%) = [N/N_t] \times 100$, where N is the number of fractured beads and N_t is the total number of beads. Five independent experiments were carried out.

2.5. UASB reactor setup and operation for hydrogen production

A schematic of the UASB reactor is shown in Fig. 2. The UASB reactor was made of stain less steel with a total volume of 5126 cm^3 and a 5-L working volume. The temperature of the reactor during fermentation was maintained at 37 °C 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 loaded with an appropriate amount of PEG-immobilized beads to obtain a final solution of 6–15% (w/v). 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

Fig. 2. Schematic description of PEG immobilized cells containing UASB reactor for continuous hydrogen production. (1: Substrate feed tank; 2: feed pump; 3: manual valve; 4: immobilzed 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).

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 by changing the concentration of COD in POME. The steady-state condition was justified when hydrogen gas content, biogas volume, and volatile fatty acids (VFA) concentration were less than 10% variation. 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.

2.6. Assay methods

The biogas produced in the headspace of the UASB reactor was collected each day and measured by displacement of acidified water (pH 2–3). A gas chromatograph (GC 8500 PerkinElmer) 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 the biogas using nitrogen as a carrier gas at a flow rate of 25 mL/min. Methane and CO_2 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 100, 80, and 150 °C, respectively, for both columns. VFA and alcohol contents of filtered samples were determined with the same GC, using a flame ionization detector equipped with a Nukol capillary column. The temperature of the injector and detector were 240 and 300 °C, respectively. The oven temperature was set at 160 °C for 10 min, and there after increased to 200 °C. The system was calibrated with a mixture of standard volatile acids and alcohols from Supelco. COD, total nitrogen (TN), total solid (TS), volatile suspended solids (VSS), and pH were analyzed in accordance with the procedure described in the Standard Methods [27]. The dry weight of immobilized cells in bead was assessed by measuring the difference in dry weight between the biomass-associated bead and the bead alone [28].

2.7. Scanning electron microscopy and biochemical analysis

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 [29].

3. Results and discussion

3.1. Effect of immobilized cell concentrations on H_2 production

To determine the optimum amount of PEG-immobilized cell beads for hydrogen production, different amount of PEG-immobilized cell beads (6, 9, 12, and 15% (w/v)) were loaded in the UASB reactor. During the experiments, HRT and OLR were kept constant at 12 h and 3.3 g COD/L/h, respectively. The results are presented in Fig. 3. Hydrogen production rate and hydrogen yield were increased by increasing the amount of immobilized cells from 6% to 12% (w/v) (Fig. 3). At 12% (w/v), the hydrogen production rate and hydrogen yield reached maxima of 315 mL H_2/L–POME h and 0.33 L H_2/g $COD_{removed}$, respectively, which were higher compared to those attained at 6% and 9% (w/v). This suggested that increases in biomass loading increase the hydrogen production rate. The hydrogen content in biogas and COD removal remained stable at 58% and 56% when the reactor was loaded with 6% to 12% (w/v) of immobilized cells (Fig. 3). In contrast, the hydrogen fermentation

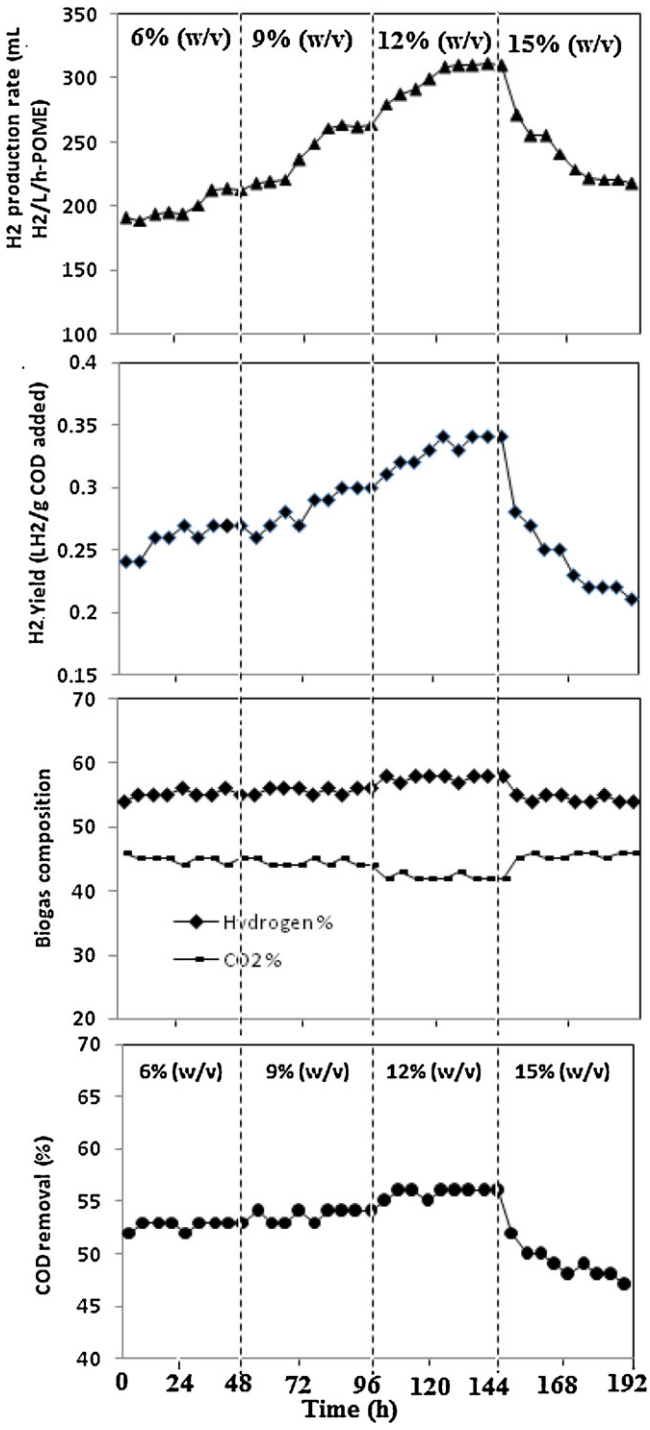

Fig. 3. Performance of hydrogen fermentation and COD removal under various loading amount of PEG immobilized cells in UASB reactor.

performance and COD removal efficiency were negatively impacted when the loading amount of immobilized-cell biomass increased from 12 to 15% (w/v) (Fig. 3). This poor hydrogen production performance with 15% (w/v) of immobilized cell could be due to the inefficient mass transfer arising from the improper immobilized cell loading amount. This result was similar to [30] research, which indicated that the internal mass transfer resistance increased when improper immobilized cell loaded in fermentative system. Moreover, excessive amount of bio-carrier in the bioreactor may reduce beads movement or contact between microflora and wastewater. Consequently, the overall performance of the anaerobic digester

was impaired [31,32]. This seems to suggest that the proper amount of the immobilized cells is requisite design parameter for a successful anaerobic hydrogen producing bioreactor. These results suggest that there is a critical amount of immobilized cells in the UASB reactor for successful anaerobic hydrogen production. On the basis of this result, subsequent experiments were performed with an immobilized cell amount of 12% (w/v).

3.2. 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 optimal loading amount of PEG-immobilized cells (12% w/v) (Fig. 4). 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 (Fig. 4). 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 L H_2/g $COD_{removed}$ to 0.3 L H_2/g $COD_{removed}$ as the HRT was shifted down from 24 to 12 h (Fig. 4b). 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 [10]. Further, there was a dramatic decrease in hydrogen yield from 0.3 L H_2/g $COD_{removed}$ to 0.14 L H_2/g $COD_{removed}$ when the HRT was shifted to 6 h (Fig. 4). 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 [33].

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 [34,35]. 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 [36]. 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 steady-state operation (Fig. 4). 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 2). 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.

3.3. 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 (Fig. 4).

Table 2
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[d] (g/L)	HPr[e] (g/L)	BuOH[f] (g/L)	EtOH[g] (g/L)	HBu[c]/HAc[d]	TVFA[i] (g/L)	SMP[h] (g/L)
24	3.3	219	0.17	3.9	2.0	1.2	1.8	1.0	1.9	7.1	9.9
18	3.3	246	0.22	5.3	1.8	0.9	1.6	0.4	2.9	8.0	10.0
12	3.3	317	0.30	6.4	2.1	0.6	1.1	0.1	3.0	9.1	10.2
6	3.3	314	0.14	5.8	2.3	0.5	0.9	0.3	2.5	8.6	9.8
12	1.6	292	0.24	4.8	2.2	1.3	1.9	0.7	2.1	8.3	10.9
12	3.3	318	0.29	5.8	2.0	0.5	1.3	0.2	2.9	8.3	9.8
12	5.0	336	0.35	7.0	2.2	0.1	0.4	0.09	3.1	9.3	9.7
12	6.6	221	0.14	5.1	2.4	0.6	1.2	0.6	2.1	8.1	9.9

[a] HPR = Hydrogen production rate.
[b] HY = Hydrogen yield.
[c] HBu = Butyric acid.
[d] HAc = Acetic acid.
[e] HPr = Propionic acid.
[f] BuOH = Butanol.
[g] EtOH = Ethanol.
[h] SMP = Soluble microbial products.
[i] TVFA = Total volatile fatty acid.

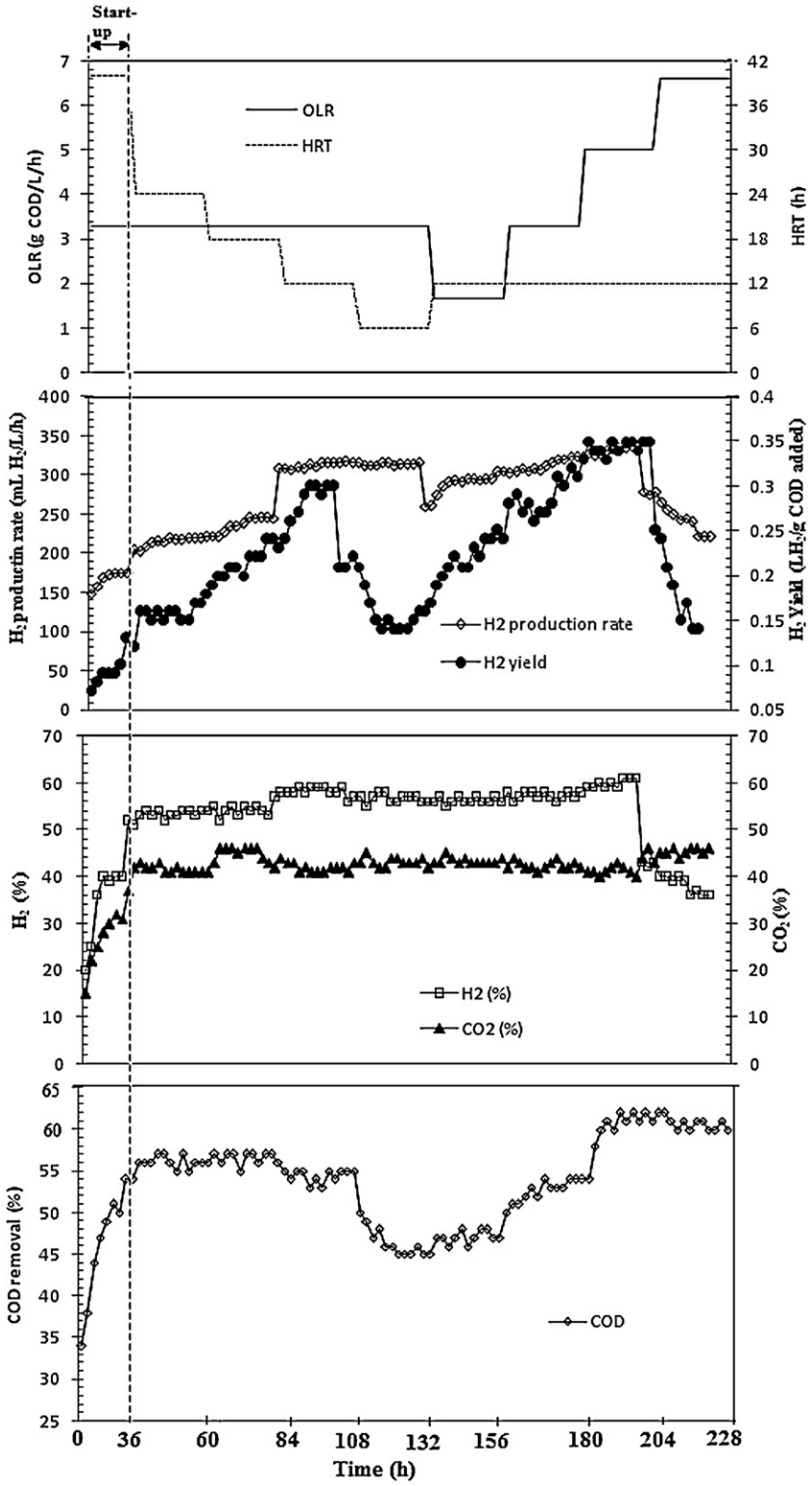

Fig. 4. Effect of HRT and OLR on the performance of the PEG immobilized cells containing UASB reactor with a constant OLR at 3.3 g COD/L/h when studying the effect of HRT and a constant HRT at 12 h when studying the effect of OLR.

The maximum hydrogen production rates of 336 mL H_2/L/h (or 15.0 mmol/L/h) and a hydrogen yield of 0.35 L H_2/g $COD_{removed}$ were obtained at 5.0 g COD/L/h OLR and a HRT of 12 h (Fig. 4). The results indicate that the hydrogen production rate increased from 261 mL H_2/L/h (or 11.6 mmol/L/h) to 336 mL H_2/L/h (or 15.0 mmol/L/h), and that the hydrogen yield increased from 0.16 L H_2/g $COD_{removed}$ to 0.35 L H_2/g $COD_{removed}$, when the OLR increased from 1.6 to 5.0 g COD/L/h (Fig. 4). However, the hydrogen production rate and yield dramatically decreased to 221 mL H_2/L/h (or 9.8 mmol/L/h) and 0.14 L H_2/g $COD_{removed}$, respectively, when the OLR was further increased up to 6.6 g COD/L/h (Fig. 4). The low hydrogen production at an OLR of 6.6 g COD/L/h could have been caused by the substrate inhibition of bacterial growth that occurs at high substrate feeding rates, or end-product inhibition, such as that

exerted by organic acids. 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 and recover hydrogen-producing microbial population, 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, whereas CO_2 production increased in response to a higher OLR (6.6 g COD/L/h) with a HRT of 12 h (Fig. 4). This suggests that some non-hydrogen producing bacteria started fermenting at a high carbon substrate (POME) loading rate and that this led to the conversion of carbon substrate to CO_2 without hydrogen production [37]. Although the obtained value of hydrogen yield (0.35 L H_2/g $COD_{removed}$) from the present study is still lower comparable to those reported by other researchers from pure carbohydrates (glucose and sucrose) [38,39]. 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 [40].

Fig. 4 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, 62% COD removal were higher than those previously reported [41,42]. However, the COD of the effluent was still high and subsequent treatment is needed, such as anaerobic treatment for hydrogen production in a combined dark and photo-fermentations 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 (Table 2). 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 (Fig. 1). 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. Indeed, the resulting PEG-immobilized cells displayed a very stable performance in continuous production of hydrogen from POME, allowing stable operation for over 5 weeks (data not shown). 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.

3.4. Soluble metabolites formation

Table 2 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 [43]. 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 2). In contrast, the production of ethanol, which is not beneficial for hydrogen production [44], 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 favored because the production of electron-consuming solvents (e.g., ethanol) was comparatively small [45]. However, butanol (BuOH) was the major species among the acidic solvents formed throughout fermentation in the immobilized-cell UASB reactor. This may be due to the fact that POME contains 750 mg/L of BuOH [10]. 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 [46]. 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 2). 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. LS2 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 [47,48]. 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 [37].

4. Conclusions

In this work, a novel polyethylene glycol prepolymer material for the immobilization of *Clostridium* sp. LS2 was investigated. 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 mL H_2/L/h (or 15.0 mmol/L/h) and a hydrogen yield of 0.35 L H_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. Additionally, the maximum COD removal efficiency in the reactor was 62%. 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. Microscopic examination clearly showed that the bacteria covered the surface of beads and were present in the core of the beads. The PEG-immobilized cells not only can enhance hydrogen production but can increase treatment efficiency of wastewater and thus hopefully immobilized hydrogen producing biocatalyst developed here can be combined with photo-fermentation for hydrogen production with high yield.

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