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Performance characteristics of single-stage biohythane production by immobilized anaerobic bacteria

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Biohythane produced via dark fermentation is much greener than hythane that is generated using natural gas. Biohythane production using a single-stage system has potential to increase the economic viability since it requires fewer controls than a two-stage system that has individual acidogenic and methanogenic reactors. This single-stage system is an innovative method in producing biohythane. The present work investigated the performance of a mesophilic single-stage system with a batch mode operation to generate biohythane. The reactor was seeded with hydrogenic and methanogenic bacteria (HB and MB), which were entrapped in κ-carrageenan/gelatin beads (2%/2% w/w) using the dripping method. The energy yield of 0.41 to 1.48 kJ g^{-1} glucose and the hydrogen content in biohythane (H2/(H2 + CH4)) of 0.35 to 0.69 were obtained. These results indicate that different biohythane compositions would be obtained by regulating the HB/MB bacteria concentration ratio, substrate concentration and cultivation pH. Moreover, a comparison of two-stages and single-stage systems as well as the challenges were also elucidated.

Keywords: anaerobic digestion, biohythane, cell immobilization, κ-carrageenan, gelatin

INTRODUCTION

The global warming limitation of 2°C for the 2000–2050 period was adopted by over a hundred countries to mitigate the risks, impacts and damages of climate change. In the result, the usage of fossil fuels should be restricted to keep the cumulative carbon emissions to not exceed 1000 Gt of CO_2 in the 21st century [1]. Furthermore, the global fossil fuel source was calculated to be depleted in about 27, 99 and 29 years for oil, coal and natural gas, respectively, which soon results in a critical issue for humanity energy supply [2]. The generation of bioenergy hence offers a promising alternative to fossil fuels as their properties of low carbon emission [3] and greater energy security [4].

Hythane, trademarked by Eden Energy, has attracted growing attention due to its versatile advantages as its low-carbon emission, environment-friendly and alternative to the fossil fuels [5]. It was considered a perfect combination of the inflammable hydrogen (H_2) and the slow burning, hard igniting methane (CH_4) [6, 7]. However, the recent production of hythane using natural gas (blending clean H_2 to CH_4) is

greatly limited since H_2 is mainly produced by physical/chemical methods, which heavily relied on fossil based energy. Thus, biohythane produced via dark fermentation can be a greener approach to produce hythane. The most successful method for biohythane production appears to be two-stage dark fermentation since the H_2/CH_4 ratio can be easily regulated by adjusting the microbial fermentation conditions of each stage [8]. Nevertheless, operation of two reactors demands more energy consumption, manpower and infrastructure, which lowers the economic viability. Therefore, a single-stage system could be another approach for biohythane production.

Recently, single-stage fermentation for biohythane production has been proposed to reduce the drawbacks of operating a two-stage system [9]. This bioreactor was filled with coir pith as a fixed bed material to support the growth of anaerobic consortium. Other than the fixed bed, there are many methods that could be applied to improve the bacterial growth such as cell immobilization. This immobilized-cell system has shown many reactor performance advantages over suspended culture such as help avoiding cell-washout [10], has easy product recovery [11], could enlarge cell density [12] and give cost effective since the cells could be used several times without losing significant activities [13]. Moreover, the polymer matrix gel can protect the cells from harsh conditions and prevent them from nutrient competition and overgrowth [14]. Furthermore, many immobilized-cell studies have shown the improved performance in either H_2 or CH_4 production, when compared with suspended cells [15–21]. Thus, cell immobilization is considered able to enhance the biohythane production in a single-stage system.

Based on the above considerations, this work was aimed to investigate the biohythane production performance of a single-stage bioreactor containing immobilized hydrogenic (HB, H_2-producing consortium) and methanogenic bacteria (MB, CH_4-producing mixed culture). The performance indicators were biohydrogen yield (HY), methane yield (MY), energy yield (EY) and H_2 content in a biohythane gas (HCH, calculated by volume H_2(mL)/[volume-H_2(mL) + volumeCH_4(mL)]) under the effects of bacteria concentration ratio (HB/MB), substrate concentration and initial cultivation pH. The dripping method was applied for the cell immobilization as of its simplicity and popularity at lab-scale [22]. κ-carrageenan was chosen as a carrier for its properties of non-toxic, easy-preparation and gelation under mild conditions. Gelatin was added as an additive to enhance gas diffusion inside the beads [23].

METHODOLOGY

Microorganisms

The seeds HB and MB were collected from the effluents of biohydrogen- and methane-producing bioreactors that fed on textile desizing and beverage wastewaters, respectively, in our laboratory. Their volatile suspended solids concentration (VSS g L^{-1}, to express the seed biomass), H_2 production rate (HPR, mL L^{-1} d^{-1}), CH_4 production rate (MPR, mL L^{-1} d^{-1}), HY (mL H_2 g^{-1} glucose) and MY (mL CH_4 g^{-1} glucose) are shown in Table 1.

Table 1. **Characteristics of HB and MB**

Seed inoculum	VSS (g L^{-1})	Maximum HPR/MPR (mL L^{-1} day^{-1})	Maximum HY/MY (mL g^{-1})
HB	4.01	10,110/0	506/0
MB	7.51	293/44	29/140

Three seed types (Seed I, II and III) with different concentration ratios of HB/MB (Table 2) were used for investigating the influence of bacteria concentration ratio. Prior to immobilization, these collected seeds were individually centrifuged (Universal 320) at 9000 rpm for 10 minutes and washed three times using reverse-osmosis (RO) water and then re-suspended in RO water.

Table 2. **Characteristics of the seeds before and after immobilization**

Seed	Before immobilization		After immobilization
	HB/MB volume (mL : mL)	HB/MB VSS (g : g)	HB/MB VSS concentration (g L^{-1} : g L^{-1})
I	100 : 100	0.4 : 0.75	8.4 : 15.7
II	0 : 200	0 : 1.5	0 : 34.1
III	0 : 300	0 : 2.25	0 : 47.1

Substrate and nutrient

Glucose was used as the substrate. The nutrient composition was (mg L^{-1}) [24]: $(NH_4)_2HPO_4$ 700, NH_4Cl 850, $MgSO_4 \cdot 7H_2O$ 250, KCl 750, $MgCl_2 \cdot 6H_2O$ 810, $FeCl_3 \cdot 6H_2O$ 420, $NaHCO_3 \cdot 6720$, $CoCl_2 \cdot 6H_2O$ 18.

Cell immobilization

After being centrifuged, the temperatures of the seeds were elevated to 50°C before κ-carrageenan and gelatin were gently added to obtain mixtures of κ-carrageenan, gelatin (2%/2% each polymer) and anaerobic microorganism. To form the beads, these mixtures were dropped (using a syringe) into 3 separated 0.3 M KCl solutions (stirred at 120 rpm by a magnetic stirrer) [23]. Note that the droplets should avoid the central vortex. The beads were then immersed in a KCl solution for 2 h prior to use. The VSS concentrations of these immobilized seeds are shown in Table 2.

Single-stage biohythane production experiments

The biohythane production experiments were conducted at mesophilic temperature (37°C) in a batch reactor with total and working volume of 2 and 1.2 L, respectively. According to our previous study, there was almost no biogas production in 24 h after substrate loading [25]. Thus, in the biohythane production experiments the substrate was fed for every 24 h. $NaHCO_3$ (0.1 M) and glacial acetic acid were used to adjust the cultivation pH before the reactor was flushed with argon gas to create an anaerobic environment. This whole process (substrate addition, initial cultivation pH adjustment and then anaerobic environment establishment) was defined as "a cycle". Biogas production and composition were monitored at designated time intervals. Biogas was measured frequently at the first 12 h (4th, 8th and 12th h) of each cycle since most of the biogas was produced during this time range. At 23rd hour, gas was measured again before starting another cycle.

Table 3 summarizes the experimental conditions for investigating the influences of bacteria concentration ratio, substrate concentration and cultivation pH on biohythane production. The effects of bacteria concentration ratio were conducted up to 3 cycles for each HB/MB concentration (Seed I, II and III (immobilized-cell)), depending on the mechanical stability of the beads structure. The substrate concentration effect experiments were carried out using 4 cycles, which had glucose concentrations of 10, 6, 3, 1.5 g L^{-1} at the 1st, 2nd, 3rd and 4th cycle, respectively. The pH effects were examined by 8 cycles, in which the first 4 cycles were done at an initial cultivation pH of 7.5. At the 5th cycle, the initial cultivation pH was 5.5 to examine how abrupt pH change affecting the biohythane production. At the 6th, 7th and 8th cycle, the initial cultivation pH values were 6.0, 6.5 and 7.0, respectively, to study the change of biohythane production when pH went from an acidic to a neutral value.

Table 3. **Experimental conditions for investigating the influences of bacteria concentration ratio, substrate concentration and cultivation pH on biohythane production**

Influence of	Seed used	Substrate concentration (g glucose L^{-1})	Initial pH
Bacteria concentration ratio	I, II, III	6	7
Substrate concentration	II	10, 6, 3, 1.5	7
pH	II	3.0	5.5, 6, 6.5, 7, 7.5

Analytical methods

Gas composition was analysed by a gas chromatography (China Chromatography 8700T) with a thermal conductivity detector. The column was made of a stainless steel pipe (1/8 mm ID*4 m). Carrier gas was argon. Injector, detector and column temperatures were 40, 40 and 28°C, respectively. Water quality analysis was according to APHA for volatile suspended solid (VSS) [26]. Biogas production was measured by a gas bag and a syringe. Since the energy contents of H_2 and CH_4 are 12.8 kJ L^{-1} and 35.8 kJ L^{-1}, respectively [27], the energy yield (EY) was calculated as follows:

$$EY\ (kJ\ g^{-1}) = \frac{(\%H_2 \times 12.8 + \%CH_4 \times 35.8) \times Volume_{biogas}(L)}{100\% \times Mass_{glucose}(g)}$$

RESULTS AND DISCUSSION

Influence of bacteria concentration ratio on cell immobilization efficiency and biohythane production

The beads of Seeds I and II both had an average diameter of 3 mm (Fig. 1). After 24 h operation, the beads' colour changed from white-grey (Fig. 1a) to black (Fig. 1b), which might be due to the growth of MB inside the beads. Such MB growth on the consumed gelatin was reported [23]. In contrast, the Seed III beads (Fig. 1c) were degraded within 12 h in the reactor, which might resulted from the beads' egg shape since its mechanical resistance is reduced compared to spherical shape beads [28].

The slope down of HY after cycle 1 correlated to the increase of MB activities, which resulted in the rise of MY in cycles 2 and 3 (Fig. 2a, b). This was the results of an accumulation of volatile fatty acids (VFAs) that were produced by HB in glucose digestion [29]. The HY and MY values at each cycle obtained by Seed I were 51, 44 and 36 ml H_2 g^{-1} glucose and 0.6, 5, 25 ml CH_4 g^{-1} glucose at cycles 1, 2 and 3, respectively. These values were greater than that of Seed II (34, 31, 10 ml H_2 g^{-1} glucose and 0.1, 9, 18vml CH_4 g^{-1} glucose at cycles 1, 2, 3, respectively). This fact shows the enhancement of HB activities when more HB was added to the seeds. These results show the possibility of regulating biohythane composition by adjusting HB/MB ratio. Recently, a different biohythane composition at various HB/MB ratio was also reported [30]. The highest EY of 1.34 and 0.77 kJ g^{-1} glucose, which corresponded to the HCH of 0.58 and 0.35 were obtained by Seed I and Seed II, respectively (Fig. 2c).

Influence of substrate concentration on biohythane production

Figure 3a shows the affection on cultivation pH drop (ΔpH, the cultivation pH difference between initial and final values during fermentation) of glucose concentration during fermentation. The ΔpH values were 0.2, 0.5, 1.0 and 2.0 at the glucose concentrations of 1.5, 3, 6 and 10 g L^{-1}, respectively. It was reported that a low cultivation pH environment (pH 4.5–5.5) favoured H_2 production and inhibited CH_4 production while a neutral cultivation pH (6–7) yields a reverse result [31, 32]. Thus, as displayed in Fig. 3b, HY rose while MY dropped down as glucose concentration increased at an initial cultivation pH of 7 (final cultivation pH was 6.8, 6.5, 6 and 5, which yielded 5, 45, 67, 148 mL H_2 g^{-1} glucose and 10, 14, 5, 1 mL CH_4 g^{-1} glucose at glucose concentrations of 1.5, 3, 6, 10 g L^{-1}, respectively). At a glucose concentration of 1.5 g L^{-1}, the MY was lower than MY at 3 g L^{-1}, which correlated to the malfunction of HB activities (HY = 5 mL H_2 g^{-1} glucose) since HB provides VFAs for MB [33].

It was reported that in a batch biohydrogen production system, HY (ranged from 99–124 mL H_2 g^{-1} glucose) did not change significantly at glucose concentrations of 0.86–10 g L^{-1} [34]. Other reports show HY values reached 145

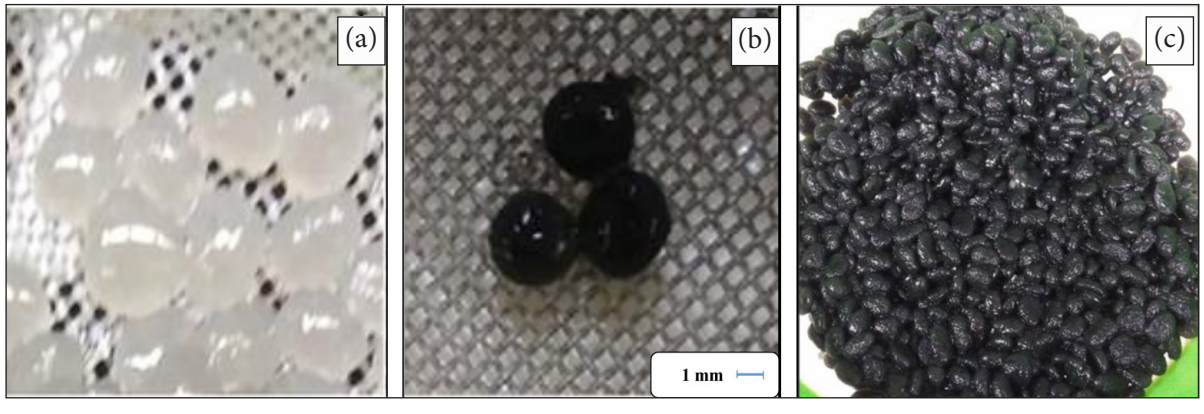

Fig. 1. Colour change of Seeds I and II from white-gray (a) to black (b) after 24 h operation and appearance of Seed III after 12 h operation (c)

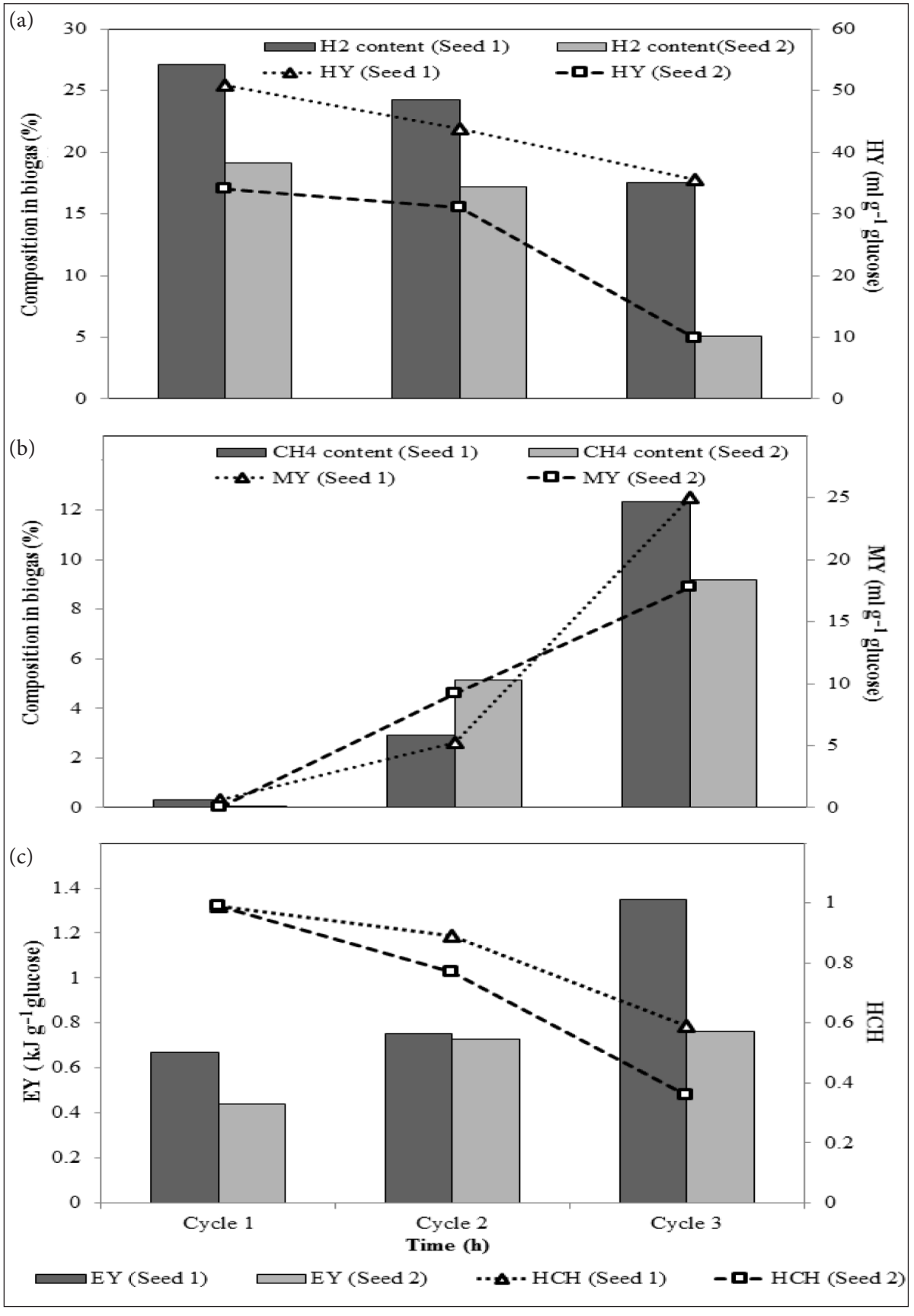

Fig. 2. Relationships between H_2 composition, HY (a); CH_4 composition, MY (b); EY, HCH (c) and operation cycle of Seeds I and II

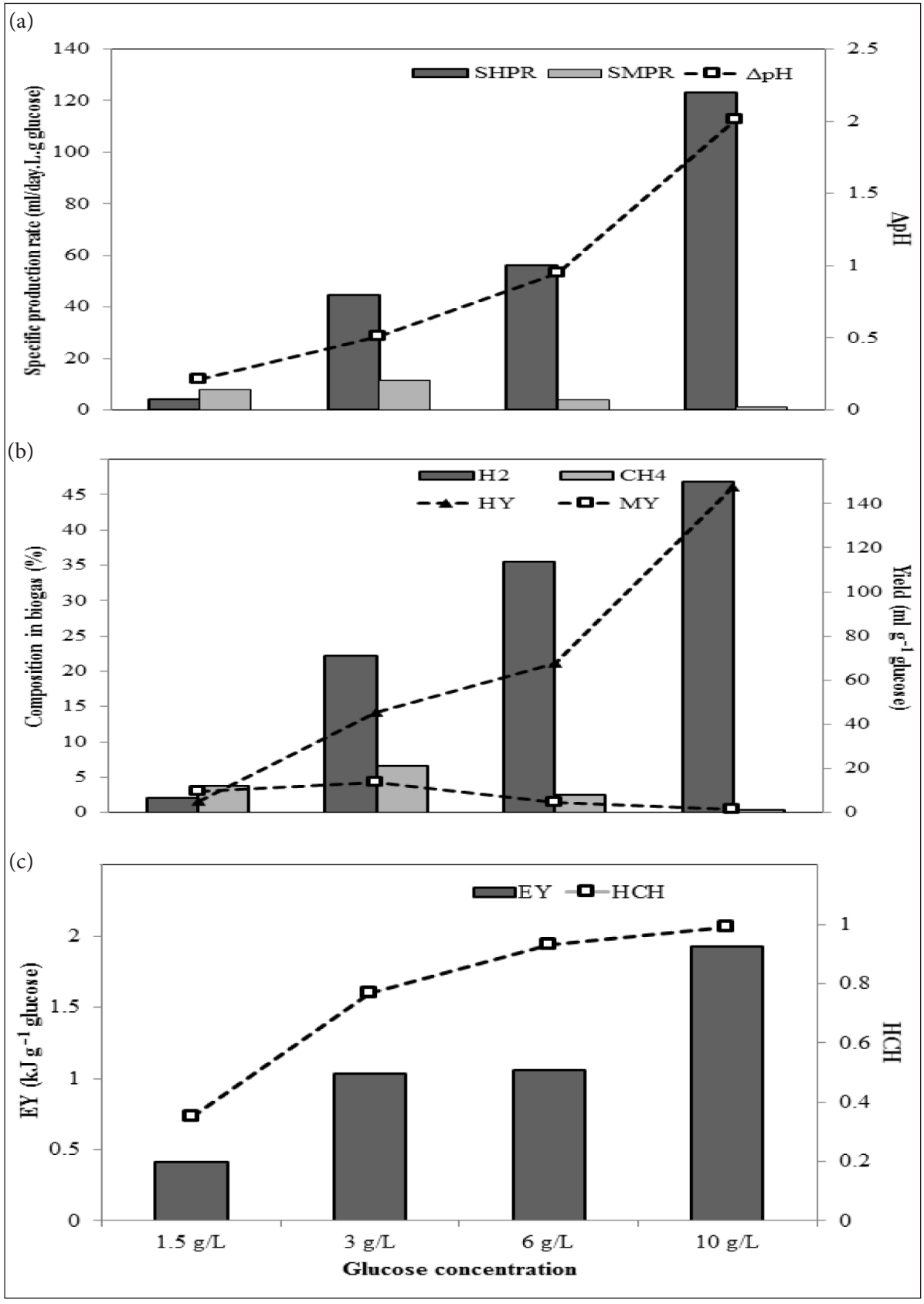

Fig. 3. Influences of substrate concentration on SHPR (specific HPR – HPR per g glucose), SMPR (specific MPR – MPR per g glucose) and ΔpH (a); H_2 and CH_4 compositions, HY and MY (b); EY and HCH (c)

and 217 H_2 g^{-1} glucose at glucose concentrations of 3.76 and 10 g L^{-1}, respectively [35, 36]. These results show that except glucose concentration 10 g L^{-1} (this study), HB activities were weakened when HB was mixed with MB. This phenomenon was also reported when recycling the methane reactor effluent to the hydrogen reactor in a two-stage system, which greatly reduced the hydrogen yield from 171 to 22 mL H_2 g^{-1} glucose (87% reduction) [8]. The EY of 1.93, 1.03, 1.06 and 0.41 kJ g^{-1} glucose, which corresponded to the HCH of 0.99, 0.93, 0.77 and 0.35 were obtained at glucose concentration of 10, 6, 3 and 1.5 g L^{-1}, respectively (Fig. 3c), which shows the decrease in the energy recovery at lower substrate concentration.

Influence of cultivation pH on biohythane production

Figure 4 displays the rapid decline of HY at an initial cultivation pH of 7.5 (32, 25, 1.4 and 0.05 mL H_2 g^{-1} glucose at cycle 1, 2, 3 and 4, respectively). It was reported that at cultivation pH 7.7, a batch sole biohydrogen production yielded 140 mL H_2 g^{-1} glucose at glucose concentration 3 g L^{-1} [37]. Therefore, the low HY obtained in this study should be correlated to the MB activities, which were increasingly enhanced over cycles (MY was 2, 7, 8 and 37 mL CH_4 g^{-1} glucose at cycle 1, 2, 3 and 4, respectively).

Figure 5a illustrates the recovery of HY to 13 mL H_2 g^{-1} glucose (from 0.05 mL H_2 g^{-1} glucose at initial cultivation pH of 7.5) at an initial cultivation pH of 5.5 due to low pH. At this pH, MB still can produce CH_4, which was reviewed in [8] as the activities of the hydrogenotrophic methanogens (hydrogen-consuming MB) under acidic condition. Nevertheless, as the inhibition effect at low cultivation pH reported in [32], MY at initial cultivation pH of 5.5 (14 mL CH_4 g^{-1} glucose) was significantly sloped down compared to MY at initial cultivation pH of 7.5 (37 mL CH_4 g^{-1} glucose). At initial cultivation pH values of 6 and 6.5, HY continued to increase (21 and 58 mL H_2 g^{-1} glucose, respectively), though at cultivation pH 7, it dropped down to 52 mL H_2 g^{-1} glucose, as the effect of neutral pH. The increase of initial cultivation pH was also accompanied by the gradual rise in MY (12, 20, 23 mL CH_4 g^{-1} glucose at cultivation pH 6, 6.5 and 7, respectively) which was also reported in [31]. The EY of 0.66, 0.71, 1.46, 1.48 and 1.33 kJ g^{-1} glucose, which corresponded to the HCH of 0.47, 0.63, 0.74, 0.69 and 0.001 obtained at initial cultivation pH values of 5.5, 6, 6.5, 7, 7.5, respectively (Fig. 5b), indicates the initial cultivation pH range of 6.5–7 was the most suitable for single-stage biohythane production.

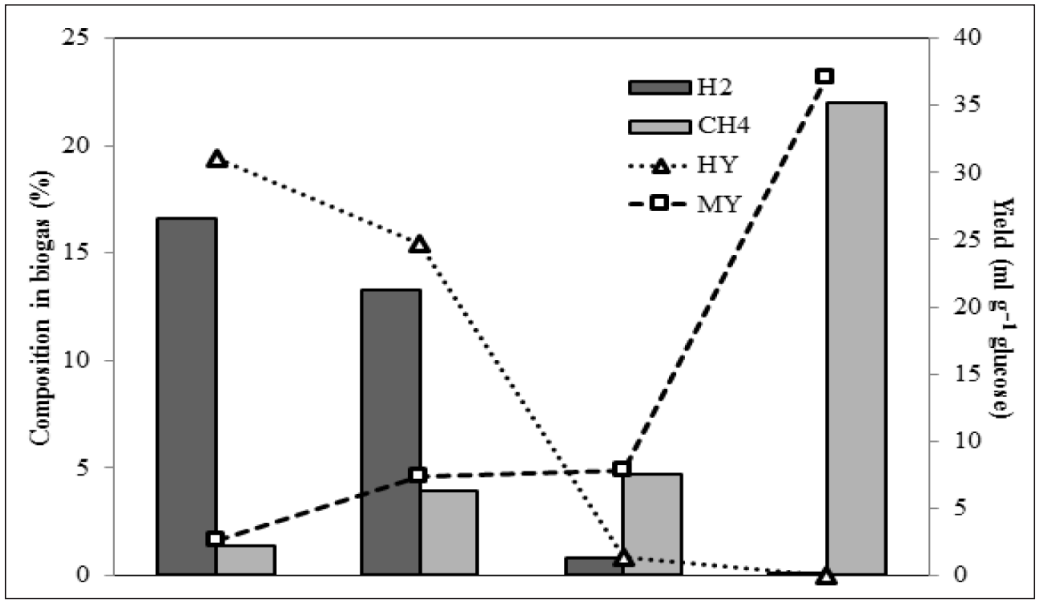

Fig. 4. Relationship between biohythane composition and yield and operation cycle at initial cultivation pH of 7.5

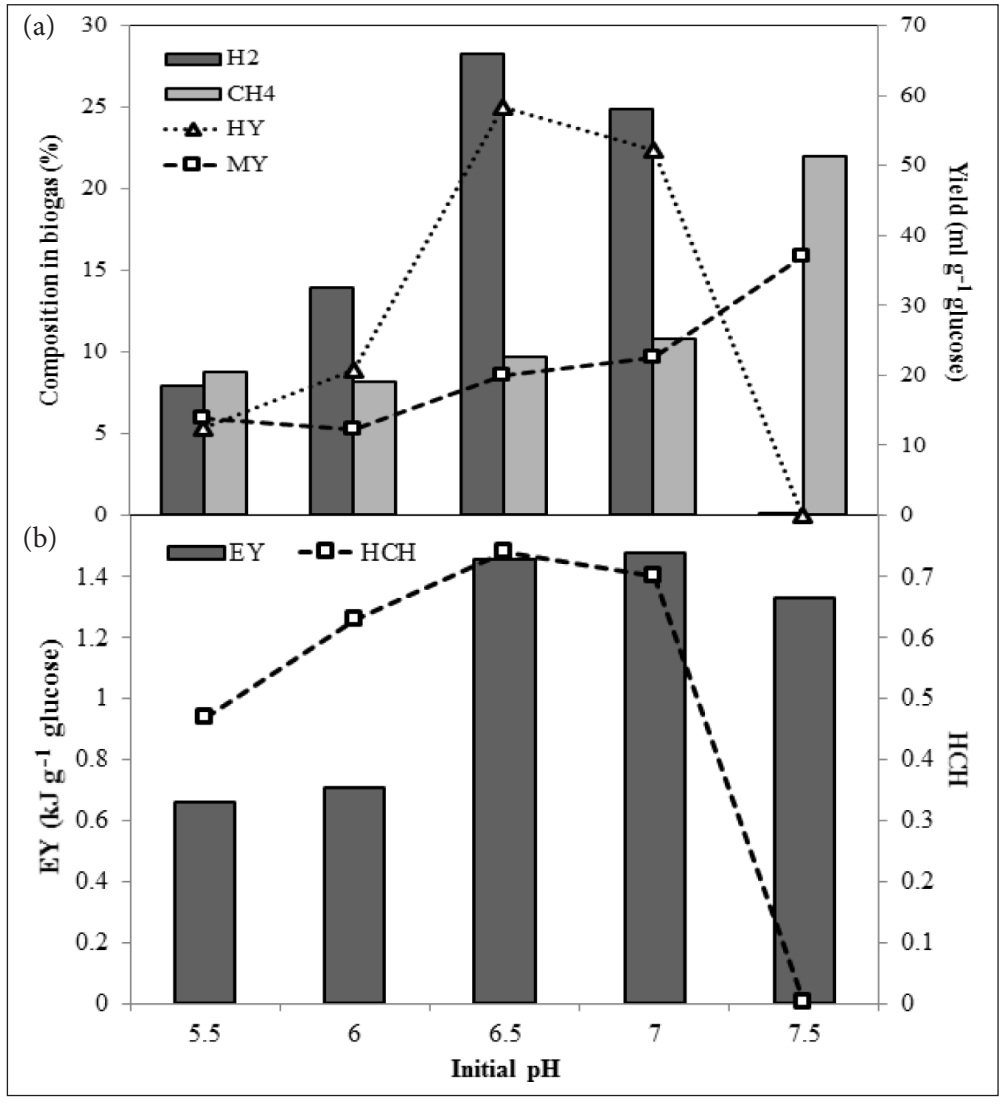

Fig. 5. Variations of biohythane composition and yield (a); EY and HCH (b) time at different initial cultivation pH value

Challenges for single-stage biohythane production

Biohythane production in a single-stage system is still a novel method. Up to now, there are very few published studies for this approach (Table 4).

When substrate was glucose, HY and MY of a single-stage biohythane system were shown malfunctioned compared to a two-stage biohythane system as reviewed in Table 5. It has been reported that a H_2 content of 7% by energy

Table 4. **Recent studies on single-stage biohythane production**

Substrate	Operation	EY	HCH	Reference
Distillery spent-wash	Batch, fixed bed	15.64 kJ g^{-1} COD	0.24	[9]
Glucose	Batch	1.03 kJ g^{-1} glucose	0.23	[30]
Glucose	Batch, cell-immobilized	3.16 kJ g^{-1} glucose	0.08	[25]
Glucose	Batch, cell-immobilized	0.77 kJ g^{-1} glucose	0.35	This study
Glucose	Batch, cell-immobilized	1.45 g^{-1} glucose	0.69	This study

in hythane is optimum for the reduction of NO_x (about 50%) in vehicle combustion. This value is equivalent to 20% by volume (HCH = 0.2) [38]. The results of this study have shown the difficulties in balancing the EY and HCH in single-stage biohythane production, in which high EY was obtained with a very low HCH (HCH = 0.001, EY = 1.33 kJ g^{-1} glucose) or very high HCH (HCH = 0.99, EY = 1.93 kJ g^{-1} glucose); while HCH closing to 0.2 was attained with low EY (HCH = 0.36, EY = 0.77 kJ g^{-1} glucose; HCH = 0.35, EY = 0.41 kJ g^{-1} glucose). Similar phenomenon was also found in [25] (HCH = 0.08, EY = 3.1 kJ g^{-1} glucose) and [30] (HCH = 0.23, EY = 1.03 kJ g^{-1} glucose).

It has been suggested that thermophilic temperature should be applied to restrain hydrogenotrophic methanogens for obtaining a higher HY [8]. However, it was reported that mesophilic temperature is more efficient for MB activities due to the prevention of ammonia inhibition [39]. This difference in optimal temperature leads to a challenge for single-stage biohythane production. Results in [9] were so far the best performance for single-stage biohythane production with EY comparable to two-stage system (15.64 kJ g^{-1} COD) with good HCH (0.24). Though, these results were only achieved for 48 h in total 240 h of operation time, which was incomparable to results from two-stage systems, such as [40] (10 days) and [41] (5 days). This left another challenge for system stability for single-stage biohythane production.

CONCLUSIONS

The potentials of cell immobilization and single-stage reactor for biohythane production were demonstrated. Immobilized anaerobic bacteria by κ-carrageenan/gelatin (2%/2% w/w) beads had successfully operated in a batch biohythane production reactor. The biosystem was able to attain 52–58 mL H_2 and 20–23 mL CH_4 per g glucose, with energy yield of 1.46–1.48 kJ g^{-1} glucose and HCH of 0.7–0.75 were achieved at the operating conditions of MB concentration 31.4 g VSS L^{-1}, glucose concentration 3 g L^{-1} and initial cultivation pH range 6.5–7. Although there are still many challenges, this single-stage system approach could help reduce the cost of secondary bioreactor and facilitates of a two-stage system, which holds promise for both production enhancement and economy viability of biohythane.

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Table 5. **Performance comparison of single-stage and two-stage biohythane production using glucose as substrate**

System (Stage)	Operation	EY (kJ g^{-1} glucose)	HCH	Reference
Single	Batch, cell-immobilized	0.77	0.35	This study
Single	Batch, cell-immobilized	1.45	0.69	This study
Single	Batch	1.03	0.23	[30]
Single	Batch, cell-immobilized	3.16	0.08	[25]
Two	CSTR*	1.07	0.74	[42]
Two	CSTR	13.81	0.56	[43]
Two	CSTR	7.24	0.75	[44]
Two	SBBR*	17.07	0.41	[45]

* CSTR: Continuous stirred-tank reactor; SBBR: Sequencing batch biofilm reactor

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VIENPAKOPĖS BIOHITANO GAMYBOS ĮMOBILIZUOTOMIS ANAEROBINĖMIS BAKTERIJOMIS PROCESO CHARAKTERIZAVIMAS

Santrauka

Biohitanas, pagamintas tamsios fermentacijos būdu, yra ekologiškesnis nei hitanas, gaminamas iš gamtinių dujų. Biohitano gamyba naudojant vienpakopę sistemą gali padidinti ekonominį rentabilumą, kadangi reikalinga mažesnė kontrolė nei dviejų pakopų sistemoje, turinčioje atskirus rūgštinius ir metanogeninius reaktorius. Ši vienpakopė sistema yra inovatyvus biohitano gamybos būdas. Straipsnyje pateiktas mezofilinės vienpakopės sistemos veikimas su vienos įkrovos režimu biohitanui generuoti. Reaktoriuje inokuliuotos vandenilinės ir metanogeninės bakterijos (HB ir MB), kurios lašinimo metodu įterpiamos k-karagenino / želatinos granulėse (2 % / 2 % m / m). Gliukozės energetinė išeiga 0,41–1,48 kJ/g ir vandenilio išeiga biohitane (H2 / (H2 + CH4)) 0,35–0,69. Šie rezultatai rodo, kad skirtingos biohitano kompozicijos būtų gaunamos reguliuojant HB / MB bakterijų koncentracijos santykį, substrato koncentraciją ir substrato pH. Palygintos dviejų pakopų ir vienpakopės sistemos, taip pat išaiškinti iššūkiai.

Raktažodžiai: anaerobinis įsisavinimas, biohitanas, ląstelių imobilizavimas, k-karageninas, želatina