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Biological Hydrogen Production Using Chloroform-treated Methanogenic Granules

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Abstract In fermentative hydrogen production, the low-hydrogen-producing bacteria retention rate limits the suspended growth reactor productivity because of the long hydraulic retention time (HRT) required to maintain adequate bacteria population. Traditional bacteria immobilization methods such as calcium alginate entrapment have many application limitations in hydrogen fermentation, including limited duration time, bacteria leakage, cost, and so on. The use of chloroform-treated anaerobic granular sludge as immobilized hydrogen-producing bacteria in an immobilized hydrogen culture may be able to overcome the limitations of traditional immobilization methods. This paper reports the findings on the performance of fed-batch cultures and continuous cultures inoculated with chloroform-treated granules. The chloroform-treated granules were able to be reused over four fed-batch cultures, with pH adjustment. The upflow reactor packed with chloroform-treated granules was studied, and the HRT of the upflow reactor was found to be as low as 4 h without any decrease in hydrogen production yield. Initial pH and glucose concentration of the culture medium significantly influenced the performance of the reactor. The optimum initial pH of the culture medium was neutral, and the optimum glucose concentration of the culture medium was below 20 g chemical oxygen demand/L at HRT 4 h. This study also investigated the possibility of integrating immobilized hydrogen fermentation using chloroform-treated granules with immobilized methane production using untreated granular sludge. The results showed that the integrated batch cultures produced 1.01 mol hydrogen and 2 mol methane per mol glucose. Treating the methanogenic granules with chloroform and then using the treated granules as immobilized hydrogen-producing sludge demonstrated advantages over other immobilization methods

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because the treated granules provide hydrogen-producing bacteria with a protective niche, a long duration of an active culture, and excellent settling velocity. This integrated two-stage design for immobilized hydrogen fermentation and methane production offers a promising approach for modifying current anaerobic wastewater treatment processes to harvest hydrogen from the existing systems.

Keywords Biological hydrogen production · Chloroform treatment · Granular · Immobilization · Integration with methane production

Introduction

Hydrogen gas is a clean energy; when it burns, it only produces water as the byproduct. Hydrogen also has the highest energy content per unit weight among any of the commonly known fuels. Although there are several ways to produce hydrogen—including electrolysis of water, thermo-catalytic reformation of organic compounds, and biological processes [1]—biological hydrogen production has attracted greater attention in recent years because of its capabilities as well as the growing environmental concerns regarding fossil fuel dependence. This process is particularly environmentally friendly because negative-valued waste materials such as cheese whey can be used as the raw material [2].

Biological hydrogen production using suspended-cell systems is normally inefficient and/or difficult to control in continuous operation. The low hydraulic retention rate of hydrogen-producing bacteria in a freely suspended-cell system limits the productivity of a reactor because of the long hydraulic retention time (HRT) required to maintain adequate bacteria population. Recycling biomass back to the reactor is one option for maintaining sufficient cell density, which is needed for high hydrogen production [3]. Recent studies show that immobilization of hydrogen-producing bacteria can also effectively enhance the bacteria population and increase hydrogen productivity [4, 5]. There are various challenges, however, for the polymer matrices to entrap and immobilize hydrogen-producing bacteria during the continuous operation. First, for the immobilized gel beads, as long as hydrogen gas is produced, the density of the gel beads decreases, which causes undesirable washout of the immobilized bacteria. Second, in most cases, the gel structure collapses after several batches because of damage resulting from several possible causes, including gel swelling, pH changes (many polymer matrices are pH sensitive), calcium loss, and so on. Third, bacteria leakage always decreases the biological stability of the immobilized gel beads. And finally, there are economic concerns, as entrapment of the bacteria into polymer matrices adds expense to the overall process [2, 5].

Anaerobic sludge granulation is a widely used self-immobilization method in anaerobic digestion. In an upflow anaerobic sludge bed (UASB) reactor, sludge agglutinates into granules, which results in an increase in biomass concentration and a reduction in sludge washout. The granules allow the organic loading rate of the UASB reactor to far exceed the typical loading rates applied in conventional activated sludge processes. Granules also have many advantages over other systems, which contribute to the success of the UASB design. First, the granules have faster settling velocity, which explains the reduction in sludge washout. Second, the granules provide a protective structure for microorganisms in a harsh environment, which ensures stable operation even if environmental shock occurs [6]. Third, in an anaerobic digestion system, granules are formed naturally and also have a porous structure, which is ideal for mass transfer of the nutrients required by the microorganisms and for the biogas being produced. And finally, it has been found that hydrogen-producing

biomass can develop into granules with high bioactivity [7]. Hydrogen-producing sludge has been shown to agglutinate into granules after 60 days of operation in continuous-flow tank reactors. Furthermore, granular sludge has been reported to be visible in the UASB systems after 120 days of reactor operation [8]. Rapid and efficient granular sludge formation has also been found in carrier-induced granular sludge bed (CIGSB) bioreactors with the addition of support carriers, especially activated carbon [9]. Overall, without the carrier inducement, direct granulation of hydrogen-producing bacteria is a time-consuming process, and there are many unknown factors that need to be investigated during the process.

One possible source for hydrogen producing granules is a conventional UASB reactor treating wastewaters. There are thousands of UASBs running worldwide, and once every 2 to 3 months, part of the methanogenic granules inside each of these reactors must be disposed of to maintain the reactor's efficiency. In previous research, chloroform treatment of granules was found to effectively eliminate methane production and to convert the culture into hydrogen production [6]. In this study, the use of chloroform-treated anaerobic granular sludge as immobilized hydrogen-producing bacteria in a hydrogen culture was investigated. In addition, the possibility of combining the immobilized hydrogen production with current anaerobic digestion was also examined.

Materials and Methods

Methanogenic Granules

The methanogenic granules were taken from a UASB that was treating starch wastewater. The average settling velocity of the granule was 29.5 m/h measure from 100 samples.

Glucose Culture Medium

The medium used for hydrogen (H_2) fermentation contained 20 g glucose chemical oxygen demand (COD)/L (i.e., 18.75 g glucose/L) as the carbon source and sufficient amounts of inorganic supplements [10] including: NH_4HCO_3 (5.24 g/L), $NaHCO_3$ (6.72 g/L), K_2HPO_4 (0.125 g/L), $MgCl_2$ $6H_2O$ (0.1 g/L), $MnSO_4$ $6H_2O$ (0.015 g/L), $FeSO_4$ $7H_2O$ (0.025 g/L), CuSO4 $5H_2O$ (0.005 g/L), and $CoCl_2$ $5H_2O$ (1.25×10^{-4} g/L).

Chloroform Pretreatment

To study the treatment level of the chloroform inhibition, 20 mL of culture medium was placed in a serum bottle. Chloroform was added to the culture medium for the pretreatment. Six levels of chloroform pretreatment were conducted, at 5, 2.5, 1, 0.5, 0.25, and 0.1%. Three milliliters (or another amount, if mentioned specifically) of sewage sludge granules were inoculated into the serum bottle for cultivation. The chloroform treatment level was chosen at 0.25% based on our previous studies [6]. In this case, the granules were cultured with 20 mL culture medium and 0.25% chloroform for more than 24 h. Then, the granules were filtered and washed to be used for inoculation.

Batch Culture

Twenty milliliters of culture medium was placed in a serum bottle. Three milliliters of pretreated granules from the pretreatment process was added to the serum bottle. Nitrogen

gas was then pumped into the serum bottle for 2 min before the fermentation to eliminate the oxygen inside the bottle. The culture temperature was maintained at 35 °C in an incubator (IC 600, Yamato), and the shaking speed was 100–150 rpm.

Fed-batch Culture

After the batch culture using chloroform-treated granules, 5 mL of fermentation broth was withdrawn for analysis. In addition, 5 mL of 80 g COD/L fresh culture medium with the same concentration of nutrients as the culture medium described above was added to the fermentation broth to start another batch. For the fed-batch culture without pH control, the pH was not adjusted at the beginning of each culture. For the fed-batch culture with pH adjustment, the pH was adjusted to about 7, after 5 mL of fresh culture medium was added into the serum bottles, and then a new batch was started.

Continuous Culture

An upflow reactor was set up with a volume of 450 mL and height of 18 cm. Fifty milliliters of methanogenic granules were treated with 0.1% chloroform following the method described previously. The chloroform-treated granules were packed in the upflow reactor as an expanded bed. Twenty grams of COD/L glucose culture medium was fed into the reactor from the bottom with the pump. Biogas and fermentation broth flowed out the top of the reactor then were separated in the separation bottles. Water replacement bottles were connected to the separation bottles to collect the biogas and measure the biogas volume. The reactor was maintained at a batch mode for 3 days, then at a continuous operation mode first with a HRT of 13 h. The HRT was then adjusted quickly to 5.3 h and kept in operation for 3 days until it ran constantly (here, "constantly" means that the pH of the effluent, the biogas and hydrogen production, the glucose conversion, and the volatile fatty acid [VFA] production were constant for more than 12 h). The HRT was gradually adjusted to 4 h and then held constant. The initial pH and the glucose concentration of the culture medium were then adjusted to measure their influence on the upflow reactor performance.

Scanning Electron Microscopy

The surface morphology of the granules was examined using a scanning electron microscope (Hitachi S-570). The freeze-dried granules were mounted on metal stubs, and the membranes were coated with gold for 6 min. The surfaces were then observed and photographed.

Transmission Electron Microscopy

The inside structure of the granules was examined using a transmission electron microscope (JEOL 1200 EX, equipped with digital camera and X-ray microanalysis system). The granules were fixed with 2.5% glutaraldehyde/2% paraformaldehyde in a Cacodylate buffer. They were then rinsed three times using a Cacodylate buffer, dehydrated with gradient ethanol and acetone, infiltrated and left overnight with acetone and SPURRs (1:1), infiltrated again with 100% SPURRs overnight, and embedded in the SPURRs and polymerized in the oven overnight. The resin was sectioned with Reichert-Jung ultra-microtomes at 70 nm thickness. Grids were stained with uranyl acetate for 10 min, rinsed

with water, then stained with lead citrate for 15 min and rinsed. Finally, the grids were observed and photographed.

Analysis

Total biogas production was measured at the end of the batch fermentation (Owen's method) [11]. The biogas was released into a U-tube with water. The biogas volume produced during the fermentation was measured through the water pressure out of the U-tube. The composition of biogas (H_2, CO_2, CH_4, and H_2S) in the headspace of the reactor was measured using a gas chromatograph (GC, CP-3800, Varian, Walnut Creek, CA) equipped with detectors, including a thermal conductivity detector for H_2 and CO_2, a flame ionization detector for CH_4, a Valco Instrument Pulsed Discharge Detector run in Helium Ionization Mode D2 for H_2S, an 18′×1/8″ HayeSep Q 80/100 Mesh Silcosteel column for CO_2, H_2, and CH_4 with nitrogen as the carrier gas, and a 50 m×0.53 mm×4 μm SilicaPLOT column for H_2S with helium as the carrier gas. VFAs were analyzed using a Dionex DX-500 system (Sunnyvale, CA) containing an AS11-HC (4 mm 10–32) column, a quaternary gradient pump (GP40), a CD20 conductivity detector, and an AS3500 auto-sampler [6].

Results and Discussions

Methanogenic Granules

The methanogenic granules were dark in color (probably because of the presence of sulfide produced by the sulfate-reducing bacteria in the digester) and were about 1 to 2 mm in size (Fig. 1a). The scanning electron microscopy (SEM) picture of the granules (Fig. 1b) shows the microorganisms packed together with extracellular polymers and with a porous structure, which facilitates the mass transfer, especially for the biogas to exit. There are several theories that explain the granulation process; however, the mechanism of the granules formation is not fully understood. Most researchers have concluded that filamentous *Methanosaeta concilii* is a key organism in granulation [12]. Filamentous species are clearly shown in the SEM picture (Fig. 1b), and the transmission electron microscopy (TEM) picture of the granules (Fig. 1c) also clearly shows the high activity of the *Methanosaeta* species. There is considerable consensus that the initial stage of granulation involves bacterial adhesion (a physical–chemical process), parallel to the early stages of biofilm formation. *Methanosaeta* aggregates to form nucleation centers, which in turn initiate granular development; then, acetogens adhere to the nuclei and form the second layer; finally, fermentative bacteria adhere to form the exterior layer of the granule in contact with the substrate. Methanogens can also be found in the exterior layer, which consume the free hydrogen produced by fermentative bacteria [13]. The SEM picture (Fig. 1b) confirmed that filamentous methanogens were distributed on the surface of the granules.

Chloroform Pretreatment

Chloroform pretreatment has been found to be an effective method for eliminating methanogenic activity and for switching a methane-producing system to a hydrogen-producing system [6]. It selectively inhibits methanogenic activity while allowing normal hydrogen production, so long as chloroform concentrations are low (Fig. 2). Chloroform,

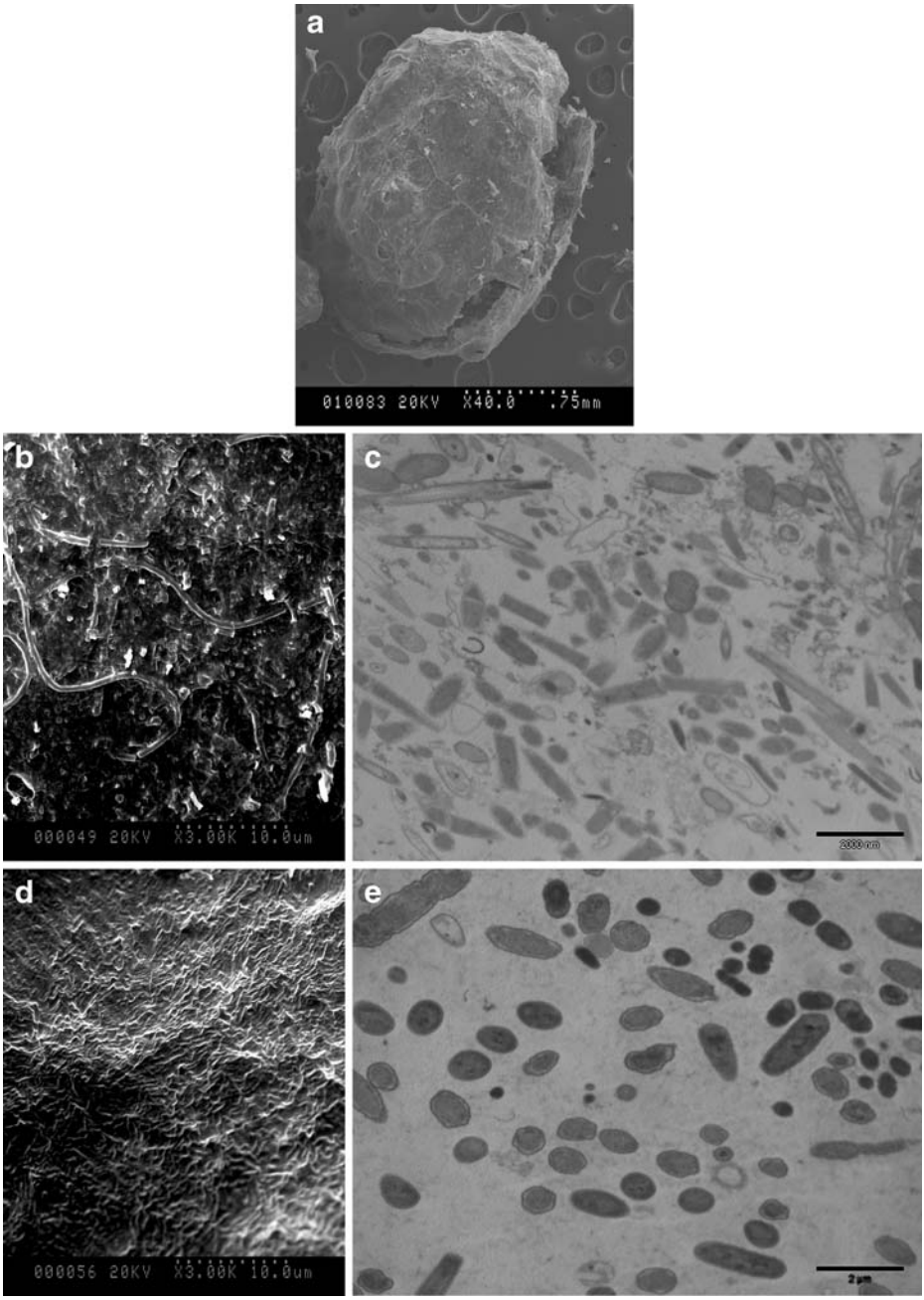

Fig. 1 **a** SEM picture of methanogenic granules. **b** SEM picture of methanogenic granules. **c** TEM picture of methanogenic granules. **d** SEM of chloroform treated granules (after 5 days culture). **e** TEM of chloroform treated granules (after 5 days culture)

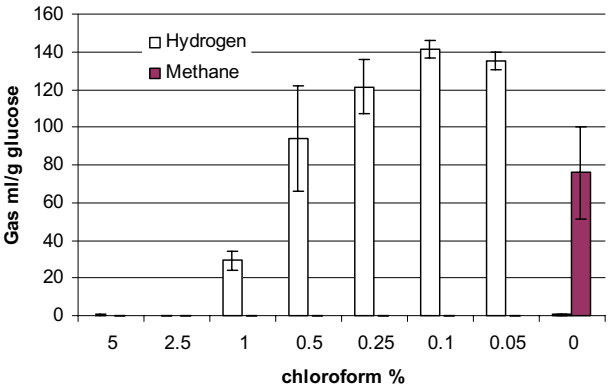

Fig. 2 Cumulative gas production for chloroform addition to granules sludge culture (Batch culture, 20 mL glucose culture medium, 3 days, inoculums 3 mL). Data are averages with *error bars* showing standard deviations (n=3)

however, causes damage to methanogens, and methanogenic activity is not easily recovered even after removal of the chloroform in subsequent cultures. In this study, the low amount of chloroform added to the culture medium in the pretreatment did not cause any damage to the structure of the granules. However, the chloroform pretreatment dramatically changed the microorganism distribution after several days of culture. Filamentous methanogens are clearly visible on the surface of the untreated granules (Fig. 1b), but there are no visible filamentous microorganisms on the chloroform-treated granules in the SEM picture (Fig. 1d). Similarly, in the TEM picture, the methanogens distributed in sections of the control methanogenic granules (Fig. 1c), while the TEM picture of the chloroform treated granules (Fig. 1e) is much clearer without the methanogens. The chloroform pretreatment showed good selectivity in inhibition of hydrogen-producing bacteria and methanogens, that is, permanently eliminating the methane production while allowing normal hydrogen production.

Fed-batch Culture

Figure 3a illustrates that for fed-batch cultures, the chloroform-treated granules can be stably reused four times with pH adjustment at the beginning of each fed-batch culture. After four fed-batches with pH adjustment, hydrogen production quickly decreased to zero, which showed strong final product inhibition. Without pH adjustment, hydrogen production was strongly inhibited after the second fed-batch, and it quickly decreased to zero. For the fed-batch cultures with pH adjustment (Fig. 3c), the metabolic pathway did not dramatically change after the first several batches because the pH of the initial culture medium was adjusted to neutral, and the VFA concentration increased with each batch. For the fed-batch cultures without pH adjustment (Fig. 3b), the metabolic pathway dramatically switched to lactic acid production, which does not produce hydrogen. VFAs can inhibit (or even be toxic to) the fermentative bacteria at high concentrations. The inhibition effect was studied by adding butyrate into the batch culture [14], and the addition of 8.36 to 12.54 g/L butyrate showed a moderate inhibitory effect. The butyrate concentration in this study reached 7.15±1.47 g/L at the third fed-batch with stable hydrogen production from cultures with pH adjustment. Then, the strong inhibition of VFA caused a drastic decrease in hydrogen production in subsequent fed-batch cultures. For the fed-batch cultures without

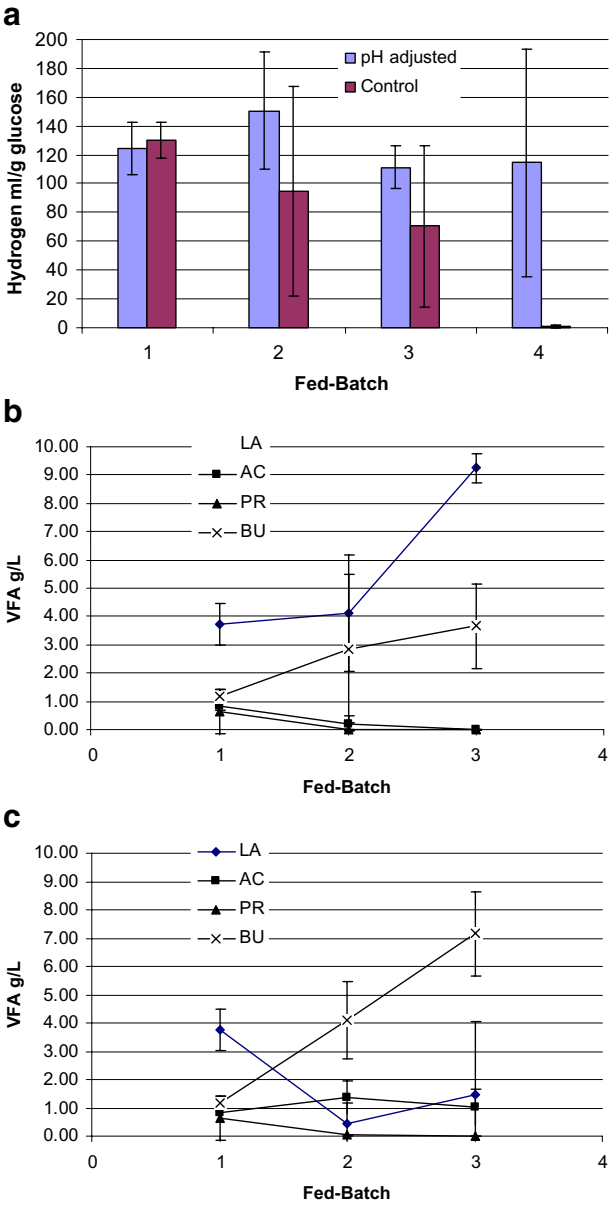

Fig. 3 **a** H_2 production from fed-batch culture of chloroform treated granules (granules were treated with 0.25% chloroform, 20 mL glucose culture medium, 3 days each batch, initial pH=8.0, inoculums 3 mL). Data are averages with *error bars* showing standard deviations (n=3). **b** VFA production from fed-batch culture of chloroform treated granules without pH adjustment (granules were treated with 0.25% chloroform, 20 mL glucose culture medium, 3 days each batch, initial pH=8.0, inoculums 3 mL). Data are averages with *error bars* showing standard deviations (n=3). *LA* Lactic acid, *AC* acetic acid, *PR* propionic acid, *BU* butyric acid. **c** VFA production from fed-batch culture of chloroform-treated granules with pH adjustment (granules were treated with 0.25% chloroform, 20 mL glucose culture medium, 3 days each batch, initial pH=8.0, inoculums 3 mL). Data are averages with *error bars* showing standard deviations (n=3). *LA* Lactic acid, *AC* acetic acid, *PR* propionic acid, *BU* butyric acid

pH adjustment, the pH value decreased batch by batch until the initial pH was too harsh for the growth of fermentative bacteria. After the second fed-batch culture, the pH of the culture medium had already been lowered to 3.8 and then increased to 4.5 with the addition of a new culture medium before the third fed-batch. The effects of VFAs on the fermentative hydrogen were associated with the pH of the solutions. At lower pH, the inhibition effects of VFAs were more likely to decrease the hydrogen fermentation activity [14]; our results clearly confirmed this finding.

VFAs, which are byproducts of the hydrogen fermentation process, can be feedstock for many other processes such as photo fermentation of hydrogen, microbial fuel cells, and methane production [15]. Higher levels of VFAs are always preferred because the separation and efficient utilization is difficult at lower concentrations. However, with the accumulation of VFAs, especially butyrate, in the fermentation broth, the switch from hydrogenesis to solventogenesis occurs. Before this "switch" to nonhydrogen production (solventogenesis), fed-batch cultures with pH adjustment seem to be an effective way to accumulate VFAs at higher concentrations.

Influence of HRT on the Continuous Fermentation with Granules

Our previous study has shown that the hydrogen productivity reached 11.6 L/L day at HRT 5.4 h [6], and remained stable even as the hydraulic resident time (HRT) decreased. In the present study, long-term continuous operation was continued to investigate the factors influencing the upflow reactor. As the HRT decreased, the hydrogen productivity gradually increased. However, the yield of hydrogen fermentation decreased dramatically when the HTR was shorter than 3 h because of the overloading of the upflow reactor (Fig. 4). For the UASB reactor, hydrogen productivity was stable at an HRT of 8 to 20 h and decreased dramatically at an HRT of 4 h [8]. A shorter HRT was reported for the CIGSB bioreactor with the addition of support carriers, where the hydrogen-producing biomass itself developed into granules [9, 10].

Influence of Culture Medium Initial pH on the Continuous Fermentation with Granules

For the continuous fermentation in the upflow reactor, the hydrogen productivity decreased when the initial pH of the culture medium was below 6.0 (Fig. 5). Our previous batch study

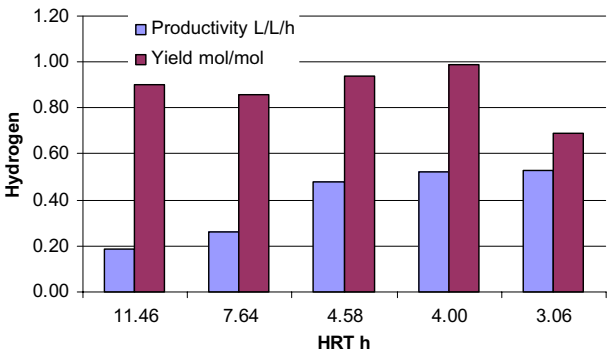

Fig. 4 Influence of HRT on the performance of the upflow hydrogen fermentation reactor (glucose culture medium 20 g COD/L, initial pH 8.0). Data were collected when they were stable for more than 12 h

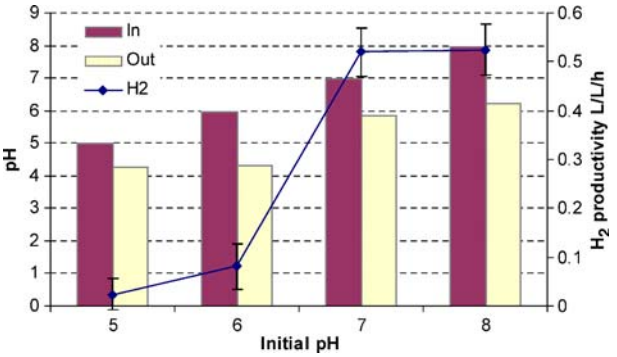

Fig. 5 Influence of initial pH of culture medium on the performance of the upflow hydrogen fermentation reactor (glucose culture medium 20 g COD/L, HRT 4 h). Data are averages with *error bars* showing standard deviations (n=3)

indicated that the culture conditions appeared to be too harsh for the hydrogen-producing bacteria to grow and nearly no hydrogen or VFA was produced when the initial pH was below 4.0 or above 9.0. The previous batch study also showed that the initial working pH for the batch culture medium ranged from 5.0 to 8.0 and that hydrogen production did not show a significant change within this initial pH range [2]. It seems that continuous culture in the upflow reactor required a narrower initial culture medium pH range. There was no significant difference for the hydrogen productivity between initial culture medium at pH 7.0 and 8.0, and the pH of the fermentation broth at these two conditions was around 6.0. When the initial pH was 5.0 or 6.0, the pH of the fermentation broth out of the reactor was about 4.2 (Fig. 5). In this case, the granules inside the reactor drifted up with the upflow and gradually washed out from the reactor, which drastically decreased the overall hydrogen productivity of the system. It is possible that this occurred because the fermentation broth was too acidic, and therefore, it might have been erosive to the extracellular polymer, which maintains the granular structure. Furthermore, because hydrogen-producing bacteria grow very slowly under acidic conditions, there might have been more bacteria detaching from the granules than bacteria reproducing and attaching to

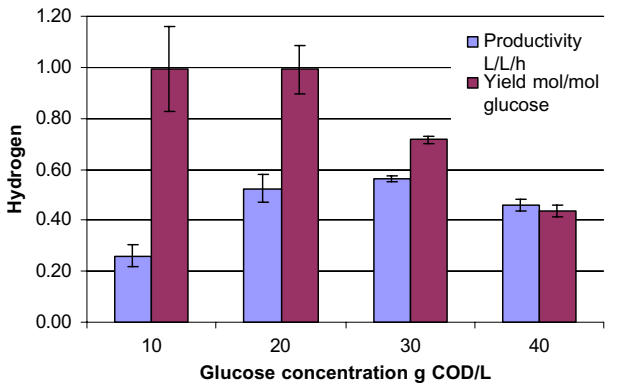

Fig. 6 Influence of glucose concentration of the culture medium on the performance of the upflow hydrogen fermentation reactor (glucose culture medium, Initial pH 8.0, HRT 4 h). Data are averages with *error bars* showing standard deviations (n=3)

the granules. The granules washing out of the reactor seems more like the floc without a granular structure, which confirms that the continuous granulized hydrogen production should remain at around neutral pH conditions because the granular structure collapsed under very acidic conditions. For many bioadsorption processes, the capacity of the adsorbent is pH sensitive, and consequently, the adsorption behavior becomes worse at very acidic conditions [16]. Most granulation theories suggest that granulation starts with bacteria adhesion to the inert nuclei [12], and our result confirms that bacterial adhesion inside the granule may be weakened when the conditions are too acidic.

Influence of Glucose Concentration of the Culture Medium on the Continuous Fermentation with Granules

Figure 6 clearly illustrates that substrate concentration influenced the continuous fermentation with granules. With an increase in glucose concentration and the associated COD loading of the upflow reactor, the hydrogen productivity reached a maximum and then decreased. There is no significant difference in hydrogen productivity between glucose concentration at 20 and at 30 g COD/L. However, when the glucose concentration was more than 20 g COD/L, the overall hydrogen yield decreased gradually because of overloading. Fermentation using high substrate concentrations is preferred because many raw materials (such as whey or manure) have a high COD content, and high concentrations of substrate are easy to heat and handle. Inhibition, however, has been found at higher feedstock concentrations [17].

Comparison of Different Immobilization Methods with Granular Treatment Method

Table 1 shows the maximum hydrogen (H_2) yield obtained in various types of H_2-producing reactors and processes. The hydrogen production yield is often influenced by several factors, such as initial glucose concentration, fermentation time, reactor type, and hydrogen partial pressure. Compared to other hydrogen-producing processes, the one investigated in this study achieved an average yield. Treating methanogenic granules and then using the treated granules as immobilized hydrogen-producing sludge, however,

Table 1 Comparison of the maximum H_2 yield obtained in various types of H_2-producing reactors.

Process	Organisms	Substrate	Maximum H_2 yield (mol H_2/mol glucose)	Reference
Batch (Blocking metabolites formation)	*Enterobacter aerogenes* HU-101 (mutant AY-2)	Glucose	1.17	[18]
Membrane reactor	Mixed culture	Glucose	1.0	[19]
N_2 sparging, continuous	Mixed culture (predominantly *Clostridium* sp.)	Glucose	1.43	[20]
UASB	Mixed culture with granular sludge	Sucrose	1.44±0.10	[21]
UASB	Mixed culture with granular sludge	Sucrose	0.92	[8]
Fluidized bed	Sewage sludge immobilized in silicone matrix	Sucrose	1.34	[22]
Batch	Chloroform treated methanogenic granules	Glucose	1.34±0.11	This study

demonstrated advantages over other immobilization methods because the granules provide the hydrogen-producing bacteria with a protective niche, a long duration, and excellent settling velocity. Different immobilization methods have been reported to enhance the biomass concentration for pure culture and mixed culture, such as calcium alginate entrapment [2]. When using a polymer matrix to trap hydrogen-producing bacteria, it is always difficult to maintain the polymer matrix because of erosion, degradation, destruction by the gas produced inside, and cell growth. In addition, the density of the matrix gel decreases as long as hydrogen and other biogases are produced, which cause the matrix gel to float and makes it more difficult to maintain the packing of the reactor. Granulation, which immobilizes the bacteria by self-flocculation, is easy to form and manipulate on an industrial scale. Fermentation using chloroform-treated granules provides a straightforward method for obtaining the hydrogen-producing granules, as methanogenic granules are available at wastewater treatment systems using a UASB.

VFA Production and Integration with Immobilized Methane Production

Table 2 shows that biohydrogen production, coupled with the subsequent step of methane production, can be an efficient process. After 3 days of biohydrogen fermentation using chloroform-treated granules, the fermentation broth was inoculated with methanogenic (untreated) granules for methane production. The initial pH of the culture medium was 8.0 for the hydrogen production, and the pH of the broth decreased to 6.5 because of VFA production before switching to the methane production reaction. Overall, the integration of the immobilized hydrogen production with immobilized methane production produced 1.01 mol hydrogen and 2 mol methane per mol glucose. It is well known that the formation of VFA during acidogenesis of organic matter precedes methanogenesis. The fermentation broth used in biohydrogen production can also be utilized with acetogensis and methanogensis to produce methane, which can alleviate the costs and environmental concerns associated with the biological hydrogen production process. The conversion of biohydrogen production follows the reaction as:

$$C_6H_{12}O_6 + 2H_2O \rightarrow 4H_2 + 2CH_3COOH + 2CO_2$$
$$C_6H_{12}O_6 \rightarrow 2H_2 + CH_3CH_2CH_2COOH + 2CO_2$$

If acetate was considered as the final product of biohydrogen production, the biochemical reaction of the integration of biohydrogen production and methane production can be illustrated as:

$$C_6H_{12}O_6 + 2H_2O \rightarrow 4H_2 + 2CH_4 + 4CO_2$$

It is evident that hydrogen production can still be improved. In stage 1, only 25% of hydrogen was produced (nearly 75% was missing). There might be multiple reasons for it:

Table 2 Integration of immobilized hydrogen production and immobilized methane production (20 g COD glucose culture medium, two sequential bioreactors, 35 °C).

	Time	H_2	CO_2	CH_4	Inoculums
Stag 1. H_2 production	3 days	47.1 mL	129.6 mL	0.0 mL	Chloroform treated granules
Stag 2. CH_4 production	6 days	0.0 mL	44.7 mL	135.2 mL	Methanogenic granules
Overall	9 days	47.2 mL	174.4 mL	93.3 mL	
Integration of H_2 and CH_4 production (mol/mol glucose)	1.01	3.74	1.99		

For the batch culture, with an increase in partial hydrogen pressure of the head space, the pathway switches to butyrate production instead of acetate, which gave theoretically only 2 mol hydrogen per mol glucose. If the pathway switched to another end product, such as solvent or propionate, no hydrogen would be generated at all. Ways to further increase hydrogen production is still a topic of investigation for many researchers, including the authors of this study.

Methane production reached 1.99 mol per mol glucose, nearly the same as the theoretical value in the integrated process. Because there are many other end products in stage 1, such as butyrate and lactate, the missing hydrogen production capability might be attributable to methane production with acetogensis, which might have produced hydrogen and acetate from butyrate, propionate, and solvent, providing the necessary raw material for methanogensis.

UASB reactors are widely used for anaerobically degrading various organic wastes for methane production. This study suggested a modification process to the current anaerobic digestion system using granules by adding a separate upflow reactor packed with chloroform-treated granules to harvest hydrogen before the waste stream feeds into the UASB. The integrated two-stage design for immobilized hydrogen fermentation and methane production offers a promising approach for modifying current anaerobic wastewater treatment processes to harvest hydrogen from these systems.

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