BIOLOGICAL HYDROGEN PRODUCTION VIA SELF-IMMOBILIZED BACTERIA

By

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To the Faculty of Washington State University:

The members of the Committee appointed to examine the dissertation of BO HU find it satisfactory and recommend that it be accepted.

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BIOLOGICAL HYDROGEN PRODUCTION VIA SELF-IMMOBILIZED BACTERIA

Abstract

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This dissertation explored a creative bacteria immobilization method for hydrogen fermentation. The low growth rate of hydrogen producing bacteria limits the productivity of a suspended-growth reactor due to the requirement for long hydraulic resident time (HRT) to maintain adequate bacteria population. Microbial immobilization is an effective way for bacteria retention, however, traditional calcium alginate entrapment has many limitations for applications in hydrogen fermentation. Anaerobic granular sludge was proposed as the immobilized hydrogen producing bacteria to be used in hydrogen fermentation after methanogenic activity of the granules was eliminated in the pretreatment process. Chloroform treatment of methanogenic granules was compared against acid and heat treatments for the effectiveness to eliminate methanogenic activity. The results showed that chloroform treatment was the most effective among the three methods tested. Chloroform caused elimination of methanogenic activity while allowing normal hydrogen production. Chloroform treated anaerobic granular sludge was proposed as immobilized hydrogen producing bacteria to be used in the hydrogen fermentation. Chloroform treated granules could be re-used for over four fed-batch cultures with pH adjustment, and could be repeatedly cultured for eight times without noticeable damage. Continuous culture with chloroform treated granules showed that the granule structure

could be kept for over 15 days and new granules started to form after 10 days of operation. The hydrogen productivity reached 11.6 L/L/day at HRT of 5.3 hours. The optimum initial pH of the culture medium was neutral and the optimum glucose concentration was below 20 g COD/L.

This study also investigated the possibility of integrating both the immobilized hydrogen fermentation with chloroform treated granules and the immobilized methane production with untreated granular sludge. The results showed that the integrated batch cultures provided 1.01 mol hydrogen and 2 mol methane per mol glucose.

16s rRNA microbial analysis was used to investigate the community change during the chloroform treatment. Most of the *Methanogenic sp.* was eliminated by chloroform treatment, explaining the switching of methane production system to hydrogen production system. However, *Methanosaeta concilii*, the key organism in anaerobic granulation, was not eliminated from the hydrogen producing system, unexpectedly, which might help explain the granulation of hydrogen producing granules.

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Dedication

This dissertation is dedicated to my mother and father

who provided both emotional and financial support

CHAPTER ONE

INTRODUCTION

1. Anaerobic digestion

Anaerobic degradation occurs naturally in various anaerobic habitats. Anaerobic digestion is the industrial system that harnesses this natural process to treat waste, produce biogas that can be used as bioenergy. The digestive metabolism of anaerobic microorganisms is complicated and involves several intermediate steps. International Water Association's (IWA) Task Group has developed a generic anaerobic digestion model (Figure 1-1), Anaerobic Digestion Model No. 1 (ADM1) to address all the steps within the anaerobic digestion (Shang *et al.*, 2005). The steps include the flowing processes:

Different groups of microorganisms work together as a food chain to degrade the organic compounds to produce methane and carbon dioxide. Briefly, insoluble organic material is hydrolyzed to produce simple soluble organic materials including simple sugars, amino acids and long chain fatty acids. Acidogenic bacteria degrade simple organic soluble organics to produce volatile fatty acid (VFA) and hydrogen, which is called acidogenesis. Then, acetogenic bacteria produce acetate from VFA and solvents with acetogenesis. There is a group of acetogenic bacteria that can synthesis acetate from hydrogen and carbon dioxide, a process called homoacetogenesis. And finally methanogens use acetate or hydrogen to produce methane as the final product. There are also other bacterial groups involved with the anaerobic degradation, including sulfate reducing bacteria (Jeong *et al.*, 2005). Among these bacteria groups, bacteria involved with acitogenesis and acetogenesis were mostly identified as Clostridia species (Fang *et*

al., 2002). Clostridia are Gram-positive, spore-forming bacteria that can tolerate harsh environmental conditions by forming spores. Microorganisms involved with methanogenesis are mostly methanogen archaea species, and 1/3 of the methane is produced from hydrogen and carbon dioxide with the remaining methane from acetate.

Anaerobic sludge granulation is a widely used self-immobilization method in anaerobic digestion in which methane is produced. In an Upflow Anaerobic Sludge Bed (UASB) reactor, sludge agglutinates into granules, resulting in the increase of biomass concentration and the reduction of undesirable sludge 'washout'. The granules allow the loading rate of the UASB reactor far beyond the common loading rates applied so far in conventional activated sludge processes. Granules have many advantages over other systems, which contributes to the success of the UASB design. First, granules have superior settling velocity, which is the reason of the reduction of sludge "washout". Second, granules provide protective structure for the microorganisms in a harsh environment, which allows stable operation even in the case of an environmental shock (Hu and Chen, 2007). Finally, granules are naturally formed in the anaerobic digestion system, and they have a porous structure that is conductive for mass transfer of the nutrients microorganisms need and the biogas being produced.

2. Biological hydrogen production

Hydrogen gas is a clean energy as it produces only water when it burns and has the highest energy content per unit weight among any known fuels (Das and Veziroglu, 2001;Nath and Das, 2004b). Currently, hydrogen utilization amounts to 3% of world energy consumption and it is expected to grow significantly in the future (Nath and Das,

2004b). However, hydrogen is mainly produced from carbon-containing raw materials, primarily fossil in origin (Nath and Das, 2004b), which is not sustainable and not environmentally benign. There are different ways to produce hydrogen, including electrolysis of water, thermo-catalytic reformation of organic compounds, and biological processes (Levin *et al.*, 2004). Biohydrogen production methods that don't produce greenhouse gas include biophotolysis, photosynthesis /photo-fermentation, and fermentation.

In a biophotolysis process, H_2O is "split" into H_2 and O_2 by green algae or cyanobacteria through two photosystems, and high intensity light is required to drive this process. As the algal cells produce oxygen, this in turn strongly inhibits the biophotolysis process so that the overall hydrogen production efficiency is very low and an efficient oxygen removal procedure is needed. With photo-synthesis / photo-fermentation, photosynthetic bacteria use a short-chain organic acid (e.g., acetate) as substrate to produce H_2 through photosynthetic metabolism. Compared with biophotolysis, photofermentation is capable of using a wide spectrum of light, and it lacks oxygen-evolving activity, which would otherwise cause oxygen inactivation. In addition, photosynthetic bacteria can utilize organic substrates from wastewater and the conversion yield is relative high. Photo-fermentation, however, has its own limitations. Although oxygen inhibition is not as significant as in biophotolysis, it is still an inhibitor for the system. The process is also potentially limited by light availability. When bacterial cell density reaches a very high level, the mutual shading in the reactor will hinder the penetration of light into the reactor. As a result, photosynthetic bacteria cannot get sufficient light and the hydrogen evolution is limited.

Hydrogen can be produced within the anaerobic degradation as long as hydrogen consumers, in most cases methanogens, are eliminated from the system. In comparison with Clostridia species, *Methanogenic archaea* are vulnerable to harsh environmental conditions and grow slowly. Certain culture conditions can be chosen to limit the growth of the methanogens, such as low pH, short hydraulic retention times (HRT), and sludge retention times (SRT) in continuous cultures (Oh *et al.*, 2003). Heat-shock treatment was proved effective to enhance biohydrogen production because it can remove the non-spore forming microorganisms, for example, methanogens.

Dark fermentative hydrogen production from sugar has attracted more attention in recent years because of its numerous advantages over other forms of hydrogen production. The fermentative bacteria use a variety of carbon sources (e.g., glucose) as substrate to produce hydrogen and organic acids (e.g., acetate or butyrate) through anaerobic metabolic pathway, i.e.,

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

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

The most significant advantage of this process is that it requires no light. Additionally, it has no oxygen limitation problems because the process does not produce oxygen (Nath and Das, 2004a). Moreover, a variety of carbon sources can be used as substrates in the process, including negatively valued raw materials such as sugar-containing wastewaters (Nath and Das, 2004a). Hydrogen production via dark fermentation from numerous waste and wastewater sources has been reported, including

bean curd manufacturing waste (Zhu *et al.*, 2002), rice winery wastewater (Yu *et al.*, 2002), starch wastewater (Zhang *et al.*, 2003), livestock waste (Regan *et al.*, 2004), olive oil waste (Eroglu *et al.*, 2004), food processing, and domestic wastewater (Van Ginkel *et al.*, 2005). Moreover, mixed culture has been widely used requiring no sterilization in the process, for example acclimated sewage sludge has been cultured to generate hydrogen (Chen and Lin, 2001;Fang *et al.*, 2002;Van Ginkel *et al.*, 2001). Furthermore, dark fermentation also produces valuable by-products such as ethanol, acetate and butyrate. The concentration of the by-products in the current process is about 1%, which is too low to separate. There are several approaches to use these organic by-products without separation. First, volatile fatty acid (VFA) is the raw material for methane production via *Methanogenic archaea*. Second, VFA can be used to produce electricity via microbial-fuel cell (Liu *et al.*, 2005a). Thirdly, VFA can be used by photo-bacteria to produce hydrogen. Photosynthetic bacteria can use light to overcome the positive free-energy of the reaction from organic acid to hydrogen (Das and Veziroglu, 2001). The photosynthetic bacteria can use small-chain organic acids as electron donors for the production of hydrogen at the expense of light energy. Finally, VFA can be used for electrochemically assisted microbial production of hydrogen (Liu *et al.*, 2005b).

The major disadvantage of the dark fermentation is the low hydrogen yield. Fermentation for hydrogen production from hexoses, at most, produces 4 moles of H_2 per mole of hexose, with two moles of acetate. When butyric acid is produced as a major fermentation product, only 2 moles of hydrogen can be produced. Final product inhibition also contributes to the low yield of hydrogen. The partial hydrogen pressure is the most important parameter to influence the metabolic pathway of the fermentation. Four moles

of hydrogen can only be produced at zero hydrogen partial pressure and with sufficient fermentation time. With the increase of hydrogen partial pressure, the acetate pathway will be converted to butyrate pathway, or even solvent pathway without hydrogen production. Right now, several approaches are carried out to lower the hydrogen partial pressure, such as gas sparging (Mizuno *et al.*, 2000), vacuuming the headspace, and separating the biogas through silicone membrane (Nath and Das, 2004b), which all increase the hydrogen production yield. Carbon dioxide partial pressure also contributes to some metabolic pathway change away from the hydrogen production and removing carbon dioxide in the headspace increased the hydrogen production yield (Park *et al.*, 2005). Production of by-products such as acetic acid and butyric acid can drop the pH of the fermentation broth below 5, which inhibits the cell growth, and high concentration of acetate and butyrate (Van Ginkel and Logan, 2005a) decreases the hydrogen yield also. There is one report showing that high organic loading decreases the hydrogen production yield as well (Van Ginkel and Logan, 2005b).

Compared to other biological processes for hydrogen production, dark fermentation is closest to the industrial testing. One pilot test and one industrial case were reported in China where hydrogen was produced from wastewater via dark fermentation. From an engineering perspective, the low retention rate of hydrogen producing bacteria limits the productivity of a suspended-growth reactor due to the requirement for long hydraulic resident time (HRT) to maintain adequate bacteria population.

3. Bacteria immobilization and population enhancement

Continuous fermentation systems offer important economic advantages in comparison with batch culture systems for commercial applications. Most studies on microbial hydrogen production applied suspended-cell systems that are typically inefficient or difficult to handle in continuous operations. Recycling the biomass back to the reactor is a strategy used to maintain sufficient cell density for high hydrogen production. Recent studies show that different immobilization methods can be used with both pure culture and mixed culture, such as lignocellulosic materials supporting *Enterobacter cloacae* (Kumar and Das, 2001), and ethylene-vinyl acetate copolymer immobilized anaerobic sludge (Wu *et al.*, 2005). By using the active carbon as a support matrix to allow the retention of the H_2 producing bacteria within the reactor, fixed-bed bioreactors significantly enhanced the rate of hydrogen production (Chang *et al.*, 2002). Immobilized cells culture appears to be an effective way to increase hydrogen productivity.

It was found that the hydrogen producing biomass can develop into granules with high bioactivity (Fang *et al.*, 2002). Hydrogen producing sludge was demonstrated to agglutinate into granules after 60 days of operation in CSTR reactors. The granular sludge was reported visible in the UASB systems after 120 days of reactor operation (Chang and Lin, 2004). Rapid and efficient granular sludge formation was found with Carrier-Induced Granular Sludge Bed (CIGSB) bioreactor with the addition of support carriers, especially activated carbon (Lee *et al.*, 2004). Overall, without the carrier inducement, direct granulation of hydrogen producing bacteria is a time consuming process and there are many factors in this process that need to be investigated.

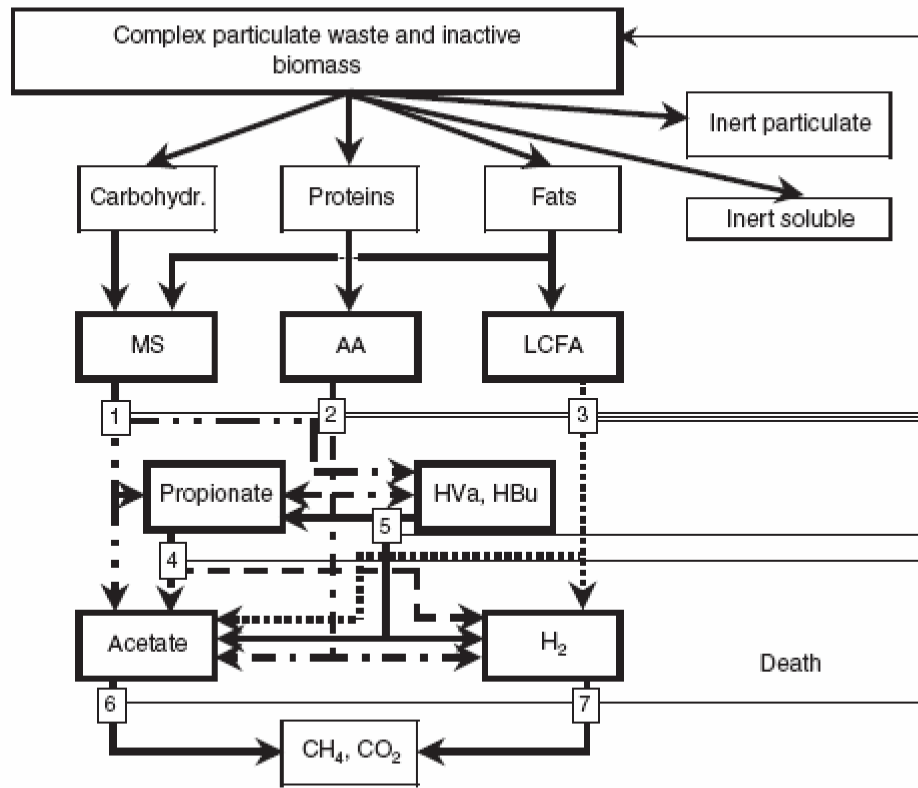

Figure 1-1 Processes involved in the anaerobic digestion: (1) acidogenesis from sugars, (2) acidogenesis from amino acids, (3) acetogenesis from LCFA, (4) acetogenesis from propionate, (5) acetogenesis from butyrate and valerate, (6) aceticlastic methanogenesis, and (7) hydrogenotrophic methanogenesis (Batstone *et al.*, 2002)

CHAPTER TWO

BIOLOGICAL HYDROGEN PRODUCTION VIA BACTERIA IMMOBILIZED IN CALCIUM ALGINATE GEL BEADS

1. Abstract

Hydrogen can be biologically produced by fermenting sugars in a mixed bacterial culture under anaerobic conditions. However, the slow growth rate of hydrogen-producing bacteria limits the productivity of a suspended-growth reactor due to the requirement for long hydraulic resident time in order to maintain adequate bacteria population. Calcium alginate gel entrapment was studied in this research as a possible method for enhancing biomass density through bacteria immobilization. Sewage sludge was used as the source for the hydrogen-producing bacteria, after pretreatment using acid to eliminate the *Methanogen archaea*. Experimental results indicated that these hydrogen- producing bacteria maintained high activity within a range of pH, i.e., from 5 to 8. Calcium alginate gel beads effectively entrapped the hydrogen- producing bacteria, resulting in increased hydrogen production rates. The immobilized hydrogen- producing bacteria with 30% inocula increased the hydrogen production over 50% when compared to the production with 15% inocula. Four repeated cultures were used to determine the effective life of the calcium alginate gel beads. The gel collapsed after 22 days, but during this time the hydrogen production held relatively constant. Cheese whey was used in this study as the nutrient for hydrogen production. The results showed that dilution was required for the suspended fermentation to obtain maximum hydrogen production yield. However, for the immobilized hydrogen fermentation, undiluted raw cheese whey could be directly used to produce hydrogen with maximum yield probably because substrate

inhibition was alleviated with the diffusion barrier provided by the immobilization matrix.

Keywords: biological hydrogen production; calcium alginate; immobilization; fermentation; cheese whey

2. Introduction

Hydrogen gas is a clean energy source because it produces only water upon combustion, and it has the highest energy content per unit weight among any known fuels (Das and Veziroglu, 2001;Nath and Das, 2004b). There are different ways to produce hydrogen, including electrolysis of water, thermo-catalytic reformation of hydrogen-rich organic compounds, and biological processes (Levin *et al.*, 2004). Currently, hydrogen consumed as energy is equal to three percent of the world energy consumption and this number is expected to grow significantly in the future. However, hydrogen is currently mostly produced from non-renewable fossil fuels, which poses a problem for future energy security (Nath and Das, 2004b).

Dark fermentative hydrogen production from sugar has attracted more and more attention in recent years because of its numerous advantages over other forms of hydrogen production. The most significant advantage of this process is that it requires no light energy. Additionally, it has no oxygen limitation problems because the process does not produce oxygen. Moreover, a variety of carbon sources can be used as substrates in the process, including negatively- valued raw materials such as sugar-containing wastewater (Nath and Das, 2004a). Such hydrogen production via dark fermentation from various waste and wastewater sources has been reported, including sources such as bean curd manufacturing waste (Zhu *et al.*, 2002), rice winery wastewater (Yu *et al.*, 2002), starch wastewater (Zhang *et al.*, 2003), livestock waste (Regan *et al.*, 2004), olive oil waste (Eroglu *et al.*, 2004), food processing, and domestic wastewater (Van Ginkel *et al.*, 2005). Moreover, mixed culture has been widely utilized therefore requiring no sterilization in the process. For example, acclimated sewage sludge was cultured to

generate hydrogen (Chen and Lin, 2001;Oh *et al.*, 2003). Most studies for microbial hydrogen production used suspended-cell systems that are normally inefficient or difficult to handle in continuous operations as a result of problems with recycling the biomass to maintain sufficient cell density in the reactor. Bacteria immobilization enhances the available bacteria population in the reactor, increases fermentation rates, shortens the fermentation period, and increases the productivity. Recent studies show that different immobilization methods can be used as effective means to increase the hydrogen productivity (Wu *et al.*, 2003) with both pure and mixed cultures. Examples include: agar gels-immobilizing *Rhodobacter sphaeroides* (Zhu *et al.*, 1999); lignocellulosic materials supported *Enterobacter cloacae* (Kumar and Das, 2001); and ethylene-vinyl acetate copolymer immobilizing anaerobic sludge (Wu *et al.*, 2005).

The immobilization techniques are based on the fact that bacteria can be entrapped within cross-linked polymers when a highly cross-linked network of polymers is formed in the presence of the bacteria. Such an immobilization method has a major advantage because it does not involve chemical modification of the bacteria. Alginate, a block copolymer of 1,4-linked β-D-mannuronic acid and α-L-guluronic acid, may be a suitable polymer candidate for this application (Tan *et al.*, 2003). It forms a calcium alginate gel when calcium ions are available. Entrapment by calcium alginate gel is widely used for immobilization of cells because it is a simple and reproducible technique using mild conditions (Shibasaki-Kitakawa *et al.*, 2001).

An effective way for hydrogen fermentation cost reduction is the use of waste as feedstock. Cheese whey, a byproduct of the dairy industry, is the liquid remaining after the precipitation and removal of milk casein during cheese making. It represents about

85-90% of the milk volume and 55% of milk nutrients (Siso, 1996). The large quantity of nutrients in whey, including lactose, soluble proteins, lipids, and mineral salts, is not fully utilized because of the low value and limited market for whey products. Because of its low concentration of milk constituent (for example, lactose content is only 4.5-5% (w/v)), whey has been commonly considered as a waste product. Whey has high organic matter content, exhibiting a BOD_5 of 30-50 g/L and COD of 60-80 g/L, with lactose being largely responsible for the high BOD and COD. Environmentally- friendly and economical disposal of these whey byproducts is a great challenge to the dairy industry (Liu *et al.*, 2006). However, bioconversion of cheese whey to single cell protein, ethanol, and methane has been reported recently, presenting possible solutions (Audic *et al.*, 2003;Demirel *et al.*, 2005;Kelleher *et al.*, 2002). Biological conversion of cheese whey to hydrogen, especially with immobilized fermentation of hydrogen, still needs to be investigated. In this study, hydrogen- producing bacteria were immobilized into calcium alginate gel beads, and the immobilized bacteria were used to treat cheese whey to produce hydrogen.

3. Materials and methods

H_2 producing sludge

Anaerobic sewage sludge, taken from the Wastewater Treatment Plant in Pullman, WA, was pretreated using two methods: acid pretreatment (Chen *et al.*, 2002) in which 100 ml of liquid sewage sludge was adjusted to pH 3.0 using 6 M HCl and shaken at 35°C for 24 hours; and heat pretreatment (Oh *et al.*, 2003) in which 100 g of solid sewage sludge was heated at 100°C in an oven for 2 hours. The acid- pretreated sewage

sludge was then cultured in a continuously- stirred tank reactor (CSTR) with glucose as the medium using the hydraulic retention time of 12-18 hours at room temperature (pH at around 5.5). The fermentation broth in the CSTR was used as hydrogen- producing seed stock in the batch cultures.

Calcium alginate immobilization of hydrogen producing bacteria

A 2% (w/v) solution (solution I) was prepared by dissolving sodium alginate in the seed broth from the CSTR. Solution I was then pressed through a nozzle into a beaker containing 2.5% (w/v) $CaCl_2$ solution (solution II) to form the beads. Calcium alginate beads were allowed to harden for 12 hours in solution II, then the beads were taken out, washed with water, and filtered (Tan *et al.*, 2003).

Culture medium

The glucose culture medium used for H_2 fermentation contained 20 g/L COD glucose (i.e., 18.75 g glucose /L. Here COD is only from glucose and one mole glucose equals 192 g COD) as the carbon source and sufficient amounts of inorganic supplements (Wu *et al.*, 2002), including: NH_4HCO_3 (5.24g/L), $NaHCO_3$ (6.72g/L), K_2HPO_4 (0.125g/L), $MgCl_2 \cdot 6H_2O$ (0.1g/L), $MnSO_4 \cdot 6H_2O$ (0.015g/L), $FeSO_4 \cdot 7H_2O$ (0.025g/L), CuSO4 $\cdot$ $5H_2O$ (0.005g/L), and $CoCl_2 \cdot 5H_2O$ (1.25×10^{-4}g/L). The initial pH of the culture medium was about 8, and was adjusted with 0.1M HCl and 0.1M NaOH during experiments to determine the influence of initial pH on the hydrogen production. The initial pH levels under investigation in this experiment range from 3 to 10. Cheese

whey, from Ferdinand's Ice Cream, Pullman, WA, was diluted with water, pH adjusted to 7, and then taken directly as cheese whey culture medium. The culture medium was stored at 4°C in the refrigerator for no more than one week. Whey used in this study had a COD of 70 g/L, pH 6.5, and Total Ammonia Nitrogen (TAN) of 109.7 mgN/L.

Batch culture

Twenty millimeters of culture medium were placed in a serum bottle. Three millimeters (15% inocula) or other amount if mentioned specifically of hydrogen-producing seeds (fermentation broth from the CSTR or alginate beads with the same amount of seed broth immobilized) was inoculated into the serum bottle for cultivation. Nitrogen gas was pumped into the serum bottle for 2 minutes before fermentation to eliminate the oxygen inside the bottle. The culture temperature was 35ºC and the shaking speed was 100-150 rpm (Incubator IC 600, Yamato).

Repeated culture

After one batch culture of the immobilized bacteria, the calcium alginate beads were taken out, washed with water three times, and filtered. These beads were then put into a serum bottle with the same amount of original culture medium to start another batch that was cultured under the same conditions as described above. This process was repeated intermittently for 4 batches.

Scanning Electron Microscopy

The surface morphology of the calcium alginate beads was examined using a scanning electron microscope (Hitachi S-570). Some of the freeze-dried beads were cut in half with a knife to inspect the structure inside. The gel beads, or their pieces, were mounted on metal stubs and their membranes were coated with gold for 6 minutes. Later, the surfaces were observed and photographed.

Analysis

Total biogas production was measured at the end of the batch fermentation (Owen's method) (Logan *et al.*, 2002). The biogas was released into a U-tube with water. The biogas volume produced during the fermentation was measured through the water pressured 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 50m × 0.53mm × 4µm SilicaPLOT column for H_2S with helium as the carrier gas. The time to analyze one sample was about 8 minutes. A high-performance anion-exchange chromatography apparatus was used to analyze several salts of VFA, such as lactate, acetate, butyrate and propionate. The salts of VFA are analyzed using a Dionex DX-500 system (Sunnyvale, CA, USA) including an AS11-HC (4mm 10-32) column, a

quaternary gradient pump (GP40), a CD20 conductivity detector, and an AS3500 auto-sampler. The effluent conditions are listed in Table 2-1 with the effluent flow rate: 1.5 ml/min; pressure limit: 2000~3000psi; SRS: 100mA.

4. Results and discussions

Treatment of the sewage sludge to obtain hydrogen- producing bacteria

As Figure 2-1 and 2-2 illustrates, no methane was produced even after 6 days of suspended fermentation with the acid- treated sewage sludge. Very little methane was produced with the heat- treated suspended sewage sludge, although the amount increased with fermentation time. In comparison with the untreated sewage sludge, these results suggested that *Methanogenic achaea*, which consumes hydrogen to produce methane, was eliminated completely via acid- treatment of the sewage sludge. Most hydrogen-producing bacteria are spore- forming bacteria, forming spores when their environment is stressed. Heat treatment, which is designed to kill most of the competing microorganisms and promote the activity of the spore- forming, hydrogen- producing bacteria, however, did not completely eliminate the methanogenic activity (Figure 2-1, 2-2), especially at long fermentation times. Although both acid- and heat- treatments have been reported to be effective ways to enrich hydrogen- producing bacteria (Chen *et al.*, 2002;Oh *et al.*, 2003), the results from this study indicated that the heat treatment used was not as effective as the acid treatment.

The influence of initial pH on hydrogen production

Figure 2-3 shows that the initial pH for the suspended culture medium was from 5.0 to 8.0. The hydrogen production did not show a significant change with the initial pH from 5 to 7, while the CO_2 / H_2 ratio increased dramatically and hydrogen production decreased slightly above pH 7.0. Initial pH of the culture medium has been reported to play a very important role in biological hydrogen production. Van Ginkel et al. (Van Ginkel *et al.*, 2001) determined that the best pH for the hydrogen production was 5.5, while Zhang mentioned that the maximum hydrogen yield from starch wastewater was found at a wastewater pH of 6.0, and the maximum specific hydrogen production rate was at a pH of 7.0 (Zhang *et al.*, 2003). There, the culture medium was glucose- limiting medium with a large quantity of bicarbonate salt. As long as the fermentation produced by-products such as volatile fatty acids, they reacted with bicarbonate and the pH of the broth decreased with the release of the carbon dioxide gas. The CO_2 / H_2 ratio increased in the head space with an increase of the initial culture pH because greater pH reduction during the fermentation caused the subsequent release of more CO_2. Additionally, it has been reported that high CO_2 partial pressure directs the pathway away from hydrogen production (Park *et al.*, 2005). Figure 4 shows that, at this metabolic shift at pH 8.0 and the higher CO_2 partial pressure, lactic acid production is seen to increase. Because lactic acid metabolism results in no hydrogen production, the hydrogen yield dropped slightly. In contrast to the effect of lactic acid, there was no significant influence on hydrogen production owing to the acetate, butyrate, and propionate production. Lastly, as the initial pH went below 4 or above 9, the culture conditions appeared to be too harsh for the bacteria to grow and so little to no hydrogen or volatile fatty acid was produced.

Bacteria immobilization in the calcium alginate gel beads

SEM clearly shows that rod-like bacteria (Figure 2-6) were entrapped within the 1.5 mm diameter calcium alginate gel beads (Figure 2-5). Due to the dehydrating effect of freeze-drying used to prepare the sample for SEM, distortion of the gel beads in the form of surface wrinkles can be easily observed. A cryo-scanning electron microscope (cryo-SEM) has been used as an alternative for the structure analysis of the calcium alginate as it does not produce structure distortion as was seen in the SEM (Serp *et al.*, 2002). Cryo-SEM studies have shown that polysaccharide chains inside the gel beads form in –homogenously-distributed aggregates, giving rise to a network with a pore size under 100 nm (Serp *et al.*, 2002). Nutrients diffuse into the gel beads and products spread out through these pores.

Hydrogen production from batch culture via immobilized bacteria

Hydrogen production via immobilized bacteria reached its maximum in 2 days' fermentation time while suspended culture needed 3 or more days (Figure 2-7). As long as inocula for both suspended and immobilized culture were the same, there was no significant difference ($P = 0.097$) in cumulative hydrogen production at the third day between suspended and immobilized culture, while hydrogen production from immobilized culture was higher than hydrogen production from suspended culture at the second day ($P = 0.004$). This suggests that hydrogen production rates significantly increased due to bacteria immobilization.

The influence of inocula size on hydrogen production

For the suspended culture, hydrogen production increased with the increase in inocula in the first day of the fermentation, while no significant difference was observed in the second day of fermentation (Figure 2-8 and 2-9). Inocula influenced the lag phase and production rate of the fermentation, but the cumulative hydrogen production ultimately was about the same. The suspended cell culture showed much higher hydrogen production at low levels of inocula (5%) compared to the production from immobilized cell culture at the second day of fermentation (Figure 2-8, 2-9). Hydrogen production increased with the increase in inocula in both the first and second day of the immobilized cell culture, and the immobilized fermentation showed much higher hydrogen production at high levels of inocula compared to the production from suspended cell culture (Figure 2-8, 2-9). Immobilization inhibits cell reproduction and the cell growth rate remains low, which causes the yield to increase (Doran, 1995). In addition, large inocula shorten the lag phase for the fermentation, and higher inocula also shorten the hydraulic retention time, which favors the hydrogen- producing bacteria. When the inocula size reached 6 ml, corresponding to 30% inocula for the culture, the hydrogen production increased over 50% when compared to the production from 15% inocula of suspended cells (Figure 2-8). Suspended-cell systems can hardly reach 15% inocula in a continuous production such as a CSTR system because “washout” may occur if the hydraulic retention time is too short. However, immobilized-cell systems can enhance the bacteria population, raising the inocula to as high as 30%, and increasing the productivity in the continuous operation.

Repeated culture via immobilized bacteria

The calcium alginate gel beads lasted for 22 days, after which they became transparent, soft, and partly broken, and were difficult to filter and could not be collected after filtration. Four repeat cultures were carried out intermittently during this period and the hydrogen production in these repeat batches stayed relatively constant (Figure 2-10). Calcium alginate gel beads have been reported to be pH sensitive (Tan *et al.*, 2003) and to undergo structure changes with the production of organic acids. Repeat acidic culture conditions lead to calcium loss and size erosion, which make the alginate beads fragile and finally collapse. Several approaches have been reported for enhancing the mechanical strength of calcium alginate beads, such as addition of activated carbon (Wu *et al.*, 2002).

Biological hydrogen production from cheese whey

As shown for the suspended culture (Figure 2-11), hydrogen production from batch cultures increased with increase of whey concentration if it was below 35g COD/L. Since the overall culture medium volume was a constant 20 ml in this study, the hydrogen production increased with the amount of cheese whey input. Hydrogen production remained constant or even slightly decreased when the whey concentration was passing over 35g COD/L to no dilution. This trend was clearer for the yield of hydrogen production, which reached a maximum 27 ml hydrogen /g COD at whey concentration 35g COD/L and decreased with higher whey concentration. The same effect of substrate concentration on hydrogen production was also reported with sucrose, and hydrogen yield decreased as substrate concentration increased to the extent that substrate overload occurred (Kim *et al.*, 2006;Kyazze *et al.*, 2006). For immobilized hydrogen fermentation (Figure 2-12), hydrogen production from batch cultures increased

with increased whey concentration while hydrogen yield remained constant. The entrapment of bacteria into the gel always creates mass transfer issues (Shibasaki-Kitakawa *et al.*, 2001), and this mass transfer barrier can explain the difference in performance of hydrogen fermentation between suspended and immobilized culture because the substrate needs to diffuse into the gel to reach the bacteria. Raw cheese whey could be directly used to produce hydrogen with maximum yield because substrate inhibition was alleviated with the diffusion barrier provided by the immobilization matrix.

5. Conclusion

Sewage sludge was used as the bacteria source for the hydrogen production. Acid pretreatment of sewage sludge was proved to eliminate the methanogenic activity and experimental results indicated that these hydrogen- producing bacteria maintained high activity within a pH range from 5 to 8. This study demonstrates an effective method to immobilize bacteria from sewage sludge, using calcium alginate entrapment, to produce hydrogen from glucose-limiting culture medium or cheese whey culture medium. Calcium alginate gel beads effectively entrapped the hydrogen- producing bacteria resulting in increased hydrogen production rates. When cheese whey was used as a nutrient to produce hydrogen using suspended fermentation, dilution was required to obtain maximum hydrogen production yield. However, in the immobilized hydrogen fermentation process, undiluted raw cheese whey could be directly used to produce hydrogen with maximum yield because substrate inhibition was alleviated due to the diffusion barrier provided by the immobilization matrix. The hydrogen production

reached 125 ml / g glucose or 23 ml / g whey with immobilized fermentation, and the calcium alginate gel bead immobilization matrix was shown to last for 22 days until it collapsed. Further study is needed to further increase the yield of hydrogen from whey, and to increase the effective life of the immobilization matrix.

Acknowledgments

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Table 2-1. Effluent conditions for VFA analysis

Time (min)	A (%)	B (%)	C (%)	Comments
Init	0.00	40.00	60.00	TTL1
0.00	0.00	40.00	60.00	
0.01	0.00	40.00	60.00	Inject
2.00	0.00	10.00	10.00	
18.00	0.00	99.00	1.00	
18.01	1.00	0.00	99.00	
28.00	30.00	0.00	70.00	
37.00	80.00	0.00	20.00	
38.50	80.00	0.00	20.00	
42.50	1.00	0.00	99.00	
42.51	0.00	99.00	60.00	
43.50	0.00	40.00	60.00	
50.00	0.00	40.00	60.00	

Effluent A: 100 mM NaOH; Effluent B: 1mM NaOH; Effluent C: water.

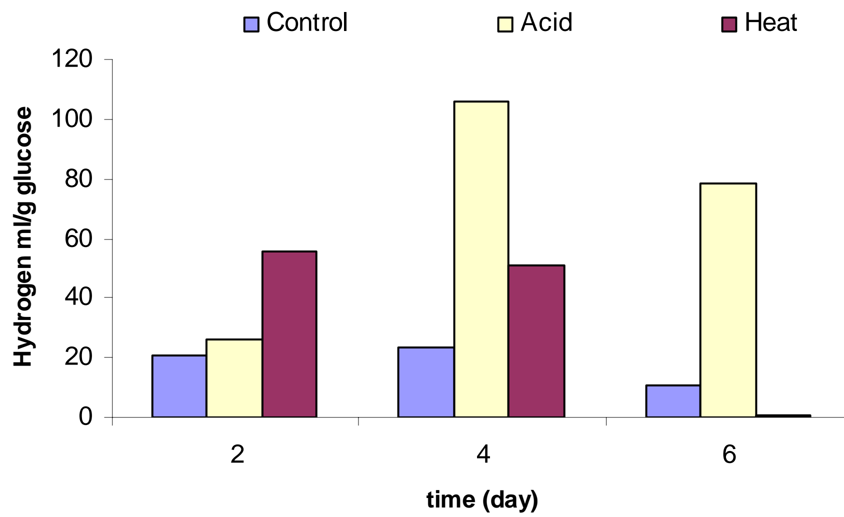

Figure 2-1. Hydrogen production for different pretreatments of sewage sludge (Batch culture, 50 ml glucose culture medium, initial pH=7.0). Data are the average of duplicate tests.

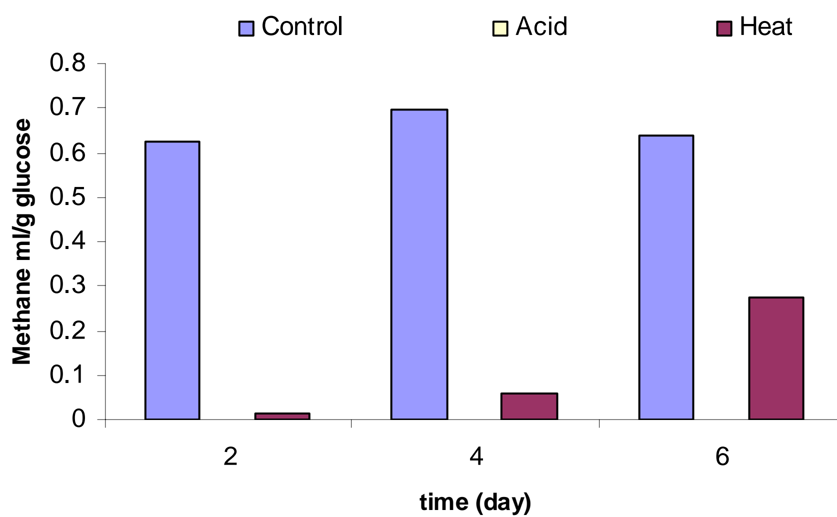

Figure 2-2. Methane production for different pretreatments of sewage sludge (Batch culture, 50 ml glucose culture medium, initial pH=7.0). Data are the average of duplicate tests.

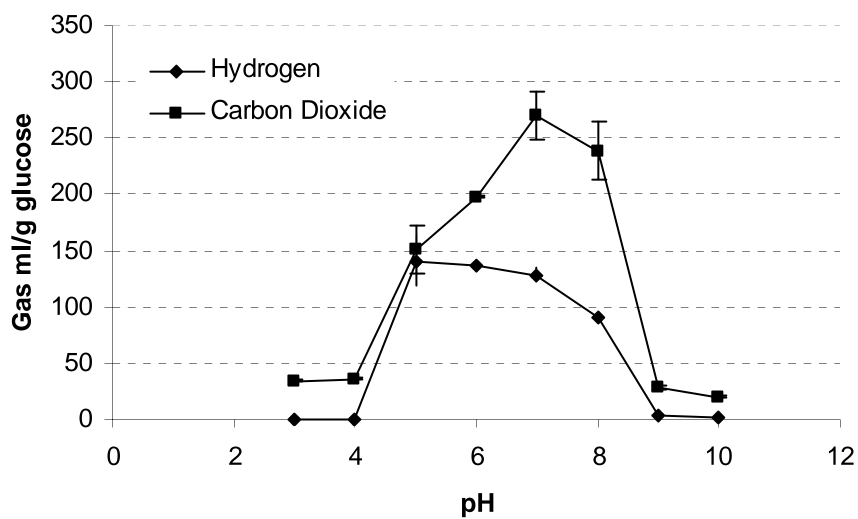

Figure 2-3. Biogas production from the culture with different initial pH (batch culture, 20 ml glucose culture medium, 3 days, inocula 3 ml). Data are averages with error bars showing standard deviations (n=3).

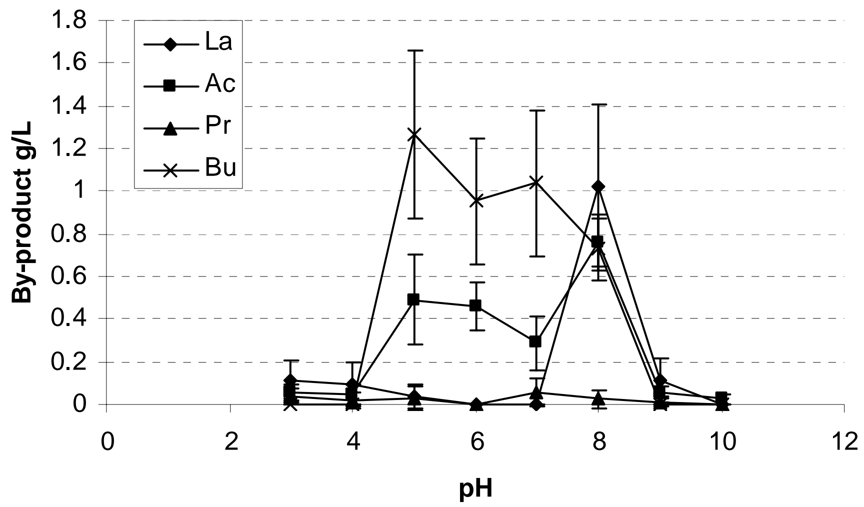

Figure 2-4. By-product production from the culture with different initial pH (batch culture, 20 ml glucose culture medium, 3 days). Data are averages with error bars showing standard deviations (n=3).

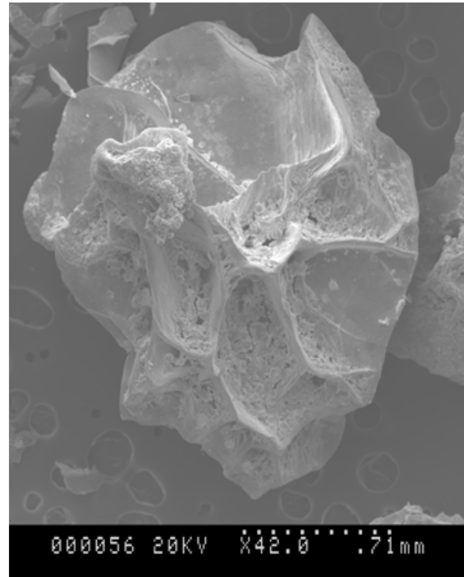

Figure 2-5. SEM picture of surface structure of the calcium alginate bead, immobilized H_2 producing bacteria.

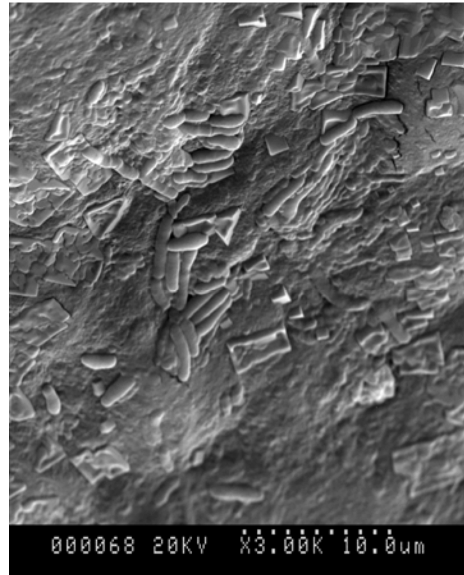

Figure 2-6. SEM picture of inside structure of the calcium alginate bead, immobilized H_2 producing bacteria.

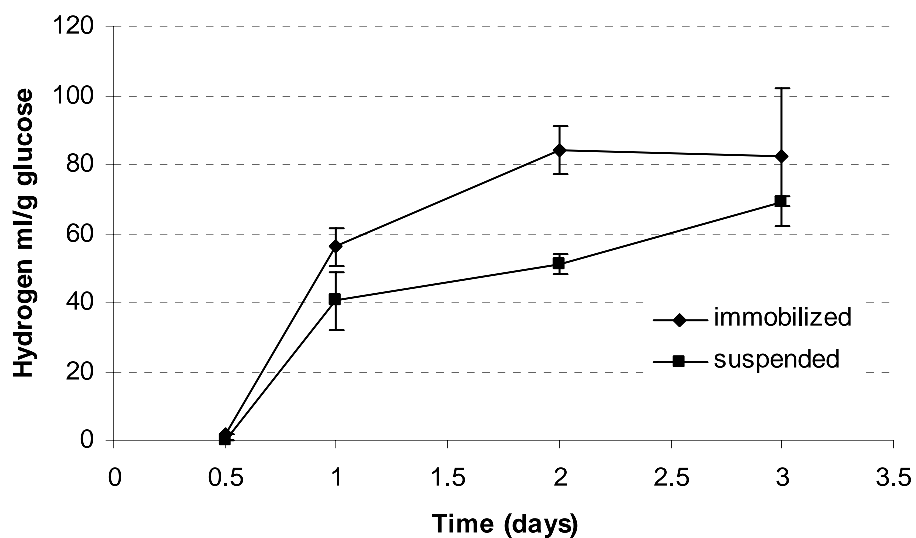

Figure 2-7. H_2 production via immobilized and suspended culture (batch culture, 20 ml glucose culture medium, initial pH=7.0, inocula 3 ml, initial pH=8.0). Data are averages with error bars showing standard deviations (n=3).

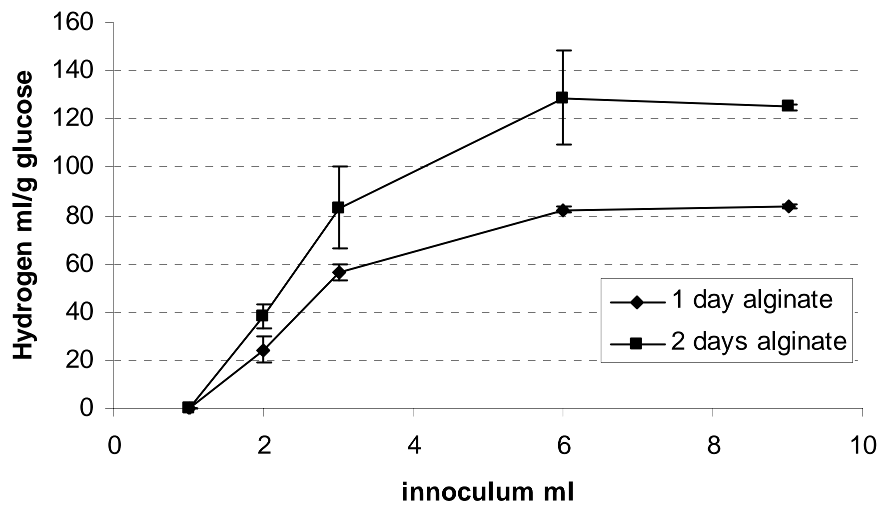

Figure 2-8. H_2 production from immobilized cultures with different inocula (batch culture, 20 ml glucose culture medium, initial pH=8.0). Data are averages with error bars showing standard deviations (n=3).

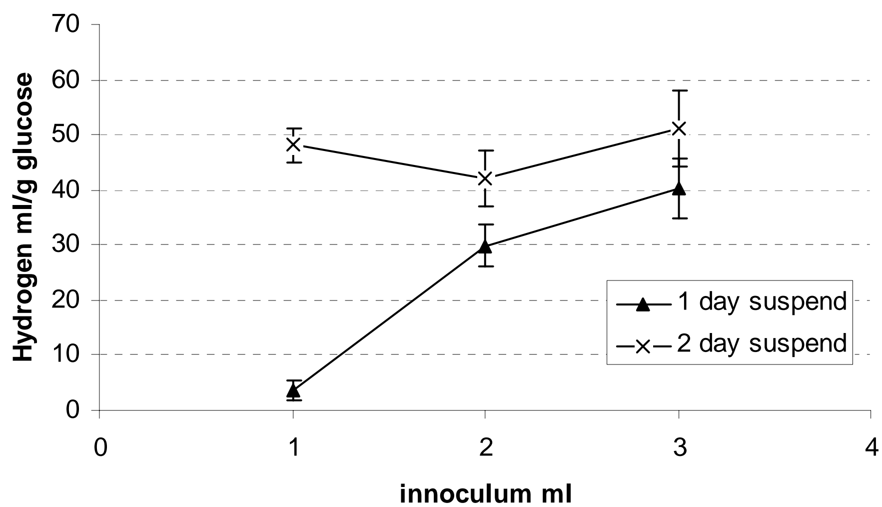

Figure 2-9. H_2 production from suspended cultures with different inocula (batch culture, 20 ml glucose culture medium, initial pH=8.0). Data are averages with error bars showing standard deviations (n=3).

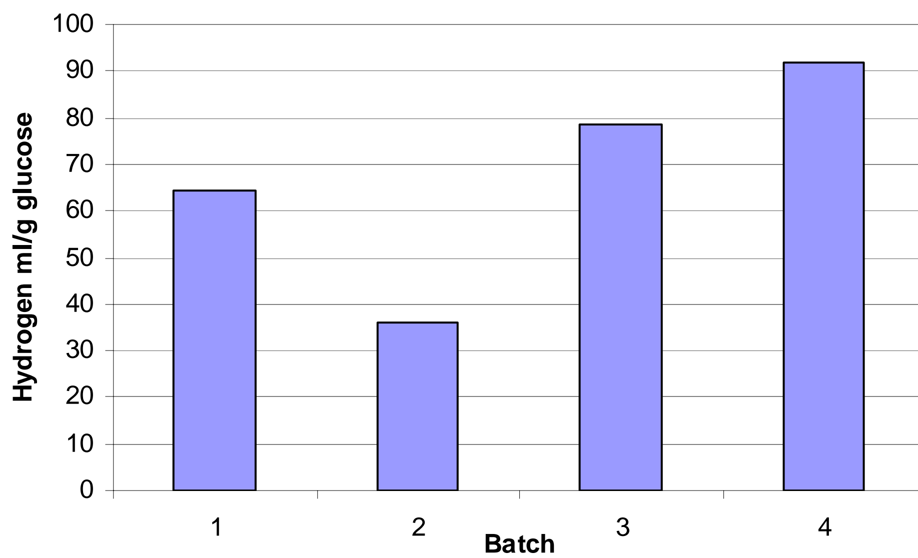

Figure 2-10. Repeat H_2 production from repeat culture via immobilized bacteria (20 glucose culture medium, 3 days each batch, initial pH=8.0, inocula 3 ml). Data are the average of duplicate tests.

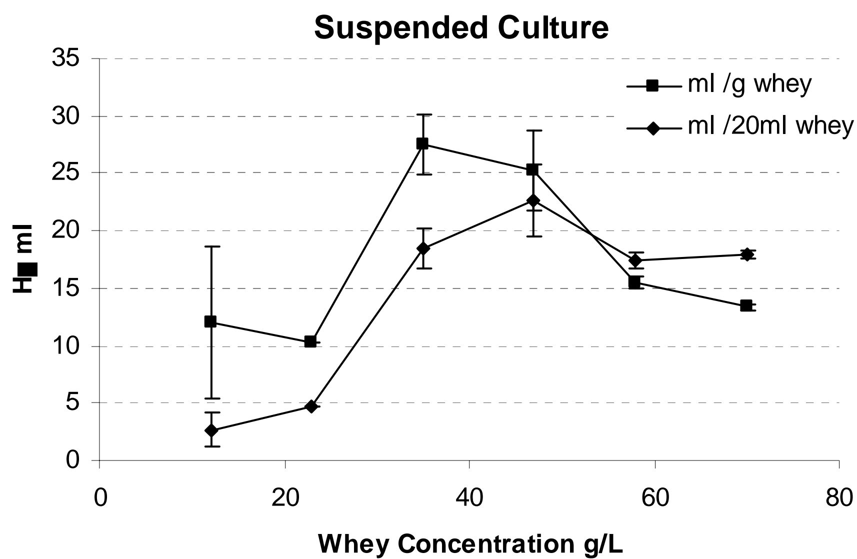

Figure 2-11. H_2 production from cheese whey via suspended culture (batch culture, whey culture medium, initial pH=7.0, inocula 3 ml, initial pH=8.0). Data are averages with error bars showing standard deviations (n=3).

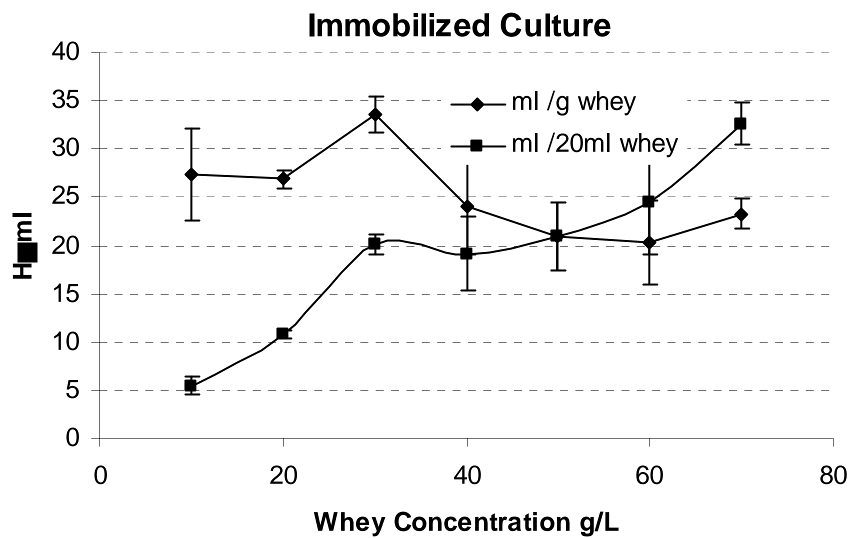

Figure 2-12. H_2 production from cheese whey via immobilized culture (batch culture, whey culture medium, initial pH=7.0, inocula 3 ml, initial pH=8.0). Data are averages with error bars showing standard deviations (n=3)

CHAPTER THREE

PRETREATMENT OF METHANOGENIC GRANULES FOR IMMOBILIZED HYDROGEN FERMENTATION

1. Abstract

Hydrogen can be produced through fermenting sugars in a mixed bacterial culture under anaerobic conditions. Anaerobic granular sludge was proposed as immobilized hydrogen producing bacteria to be used in hydrogen fermentation after methanogenic activity of the granules was eliminated in the pretreatment process. This paper reports an innovative treatment method to directly convert methanogenic granules to hydrogen producing granules using chloroform. Chloroform treatment was compared against acid and heat treatments of sewage sludge and methanogenic granules in terms of effectiveness to eliminate methanogenic activity. The results showed that chloroform treatment was the most effective among the three methods tested, whereas acid and heat treatments were less effective possibly due to the protection of bacteria within the granular structure. Methanogens were very sensitive to the chloroform and methane production was almost completely inhibited in both sewage sludge and granules with the treatment with only 0.05% chloroform. If the chloroform concentration was controlled at low levels, chloroform selectively inhibited methanogenic activity while not affecting hydrogen production. At high concentration range, chloroform also inhibited hydrogen production. Chloroform caused elimination of the methannogenic activity but hydrogen production recovered to normal after chloroform addition stopped. Chloroform showed desirable selectivity on inhibition of methanogens from hydrogen producing bacteria, eliminated the methane production, postponed the hydrogen consumption to acetic acid,

while allowing recovery for normal hydrogen production. Chloroform treated granules were repeatedly cultured for eight times without noticeable damage. Continuous culture with chloroform treated granules showed that the granule structure could be kept for over 15 days and new granules started to form after 10 days operation. The hydrogen productivity reached 11.6 L/L/day at HRT 5.3 hours. These experiment results all showed potential application of chloroform treatment of methanogenic granules in the immobilized hydrogen fermentation.

Key words: Biological hydrogen production, pretreatment, granules, chloroform.

2. Introduction

Fermentative hydrogen production from sugar has attracted increasing attention in recent years because of its ability to use biomass as well as addressing concerns about environment and fossil fuel limitations. The fermentative bacteria use glucose derived from a variety of sources as substrate to produce hydrogen and organic acids (e.g., acetate or butyrate) through anaerobic metabolic pathway, i.e.,

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

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

These reactions, accomplished by acitogenic bacteria, are part of the anaerobic digestion process that is widely used in environmental engineering. For the typical anaerobic methane production system, three groups of microorganisms coexist and work together to produce methane as follows: acitogens degrade various carbon sources to produce volatile fatty acids (VFA) and hydrogen; acetogens degrade some VFA such as propionate and butyrate, and other solvents to produce acetate and hydrogen; finally, acetate and hydrogen are used to produce methane by methanogens. Anaerobic fermentation has already been used for the production of methane and solvents such as acetone, butanol and ethanol (ABE fermentation). Anaerobic mixed fermentation can also be used to produce hydrogen, but the key to this process is blocking methanogenesis (Nath and Das, 2004b).

The major hydrogen producing microorganisms in anaerobic digestion are Clostridia, Gram-positive, spore-forming, rod-shaped bacteria. Clostridia are very resistant to heat or harmful chemicals, which provided important clues for treating the anaerobic sewage sludge to become hydrogen producing sludge. Many methods such as

acid treatment (Chen *et al.*, 2002), heat treatment (Iyer *et al.*, 2004;Zuo *et al.*, 2005), alkaline treatment(Cai *et al.*, 2004), etc. have been demonstrated for turning methane production system into hydrogen production system because these treatments eliminated most of the methanogens. Heat treatment has been reported as being more effective than pH control for enhancing biological hydrogen production; however, substantial hydrogen loss occurs during long-time batch culture due to homoacetogenic activity that consumes hydrogen to produce acetate (Oh *et al.*, 2003). Heat treatment seems unable to prevent acetogenesis, especially in most cases where some homoectogenic bacteria are also spore-forming bacteria. The continuous fermentation, short hydraulic retention time (HRT) method can be used to "wash out" the methanogens because Clostridia generally grow faster than methanogens (Lay *et al.*, 1999).

Chloroform reportedly inhibited the production of CH_4 from both H_2/CO_2 and acetate (Chidthaisong and Conrad, 2000). Very low concentrations (100μM) of $CHCl_3$ showed strong inhibition of methanogens. Furthermore, chloroform fumigation, which applies high $CHCl_3$ (vapor) concentrations, killed a large part of the soil microbial populations for subsequent extraction and determination of the microbial biomass carbon with preference to Gram-negative bacteria. These data indicate that chloroform treatment produces a shift towards a microbial community dominated by Gram-positive bacteria (Shibahara and Inubushi, 1995). $CHCl_3$ also inhibits acetate consumption by sulfate reducers. Furthermore, it was shown that the synthesis of acetate from CO_2 by cells of *Clostridium thermoaceticum* fermenting glucose was inhibited by $CHCl_3$ (R.K.Ghambeer, 1971).

Most studies on microbial hydrogen production have used suspended-cell systems, which are normally inefficient or difficult to control in continuous operations. Recycling the biomass back to the reactor must be used to maintain sufficient cell density for high hydrogen production. Different immobilization methods can also be used to enhance the biomass concentration for both pure culture and mixed culture, such as calcium alginate entrapment, lignocellulosic materials supporting *Enterobacter cloacae* (Kumar and Das, 2001), and ethylene-vinyl acetate copolymer immobilized anaerobic sludge (Wu *et al.*, 2005). By using active carbon as a support matrix to allow the retention of the H_2 producing bacteria within the reactor, fixed-bed bioreactors significant enhanced the rate of hydrogen (Chang *et al.*, 2002). Immobilized cell culture appears to be a more effective way to increase hydrogen productivity than suspended cell culture.

Granulation, which immobilizes the bacteria by self-flocculation, is simple to use and easy to control at an industrial scale. This immobilization technique can also be used in biological hydrogen production. It was found that hydrogen producing biomass can develop into granules with high bioactivity (Fang *et al.*, 2002). Hydrogen producing sludge was first demonstrated to agglutinate into granules in a well-mixed reactor treating a synthetic sucrose-containing wastewater at 26ºC, pH 5.5, with 6 hours of hydraulic retention. The granules became visible by day 15 and the size stabilized at around 1.6 mm diameter after day 60 (Fang *et al.*, 2002). UASB systems, where granule formation is an indicator of their successful operation, were also used for the hydrogen fermentation. The sludge granulation became visible after 120 days of reactor operation at HRT 8-20 hours and the average granular diameter peaked at day 173 at around 0.43 mm, which is smaller than general methane producing granular sludge (Chang and Lin, 2004). Rapid

and efficient granular sludge formation was found with Carrier-Induced Granular Sludge Bed (CIGSB) bioreactor with addition of support carriers, especially activated carbon. Packing a small quantity of carrier on the bottom of the upflow reactor significantly stimulated the granulation within 100 hours (Lee *et al.*, 2004). The mechanism of the granule formation however has not been fully understood yet. Most researchers concluded that *Methanosaeta concilii* is a key organism in methanogenic granulation (Pol *et al.*, 2004) and it is contradictory to recent reports (Chang and Lin, 2004;Fang *et al.*, 2002;Lee *et al.*, 2004) that showed granules formed directly by hydrogen producing system, from which *Methoanogen sp.* should be mostly eliminated. Possible explanation of these results is the Extracellular Polymer (ECP) excreted by bacteria during the granulation process. Although it has been found (Fang *et al.*, 2002) that there are slight differences in ECP composition between methanogenic granules and hydrogen producing granules, ECP plays the key role in agglutinating bacteria into granules and also in maintaining structural integrity of granules.

The straightforward way to obtain hydrogen producing granules is to treat the methanogenic granules by heat or chemicals, so that methanogens can be eliminated while hydrogen producing bacteria in the granular structure remain. The purpose of this study was to investigate the feasibility of treating methanogenic granules by different methods and to compare the treated granules for immobilized hydrogen production.

3. Materials and methods

Seed sewage sludge and methanogenic granules

The anaerobic sewage sludge was taken from the Wastewater Treatment Plant in Pullman, WA. The methanogenic granules were taken from a UASB treating starch wastewater, and the average settling velocity was 29.5 m/h (measured with 1000 ml graduated cylinder full of DI water, 100 samples).

Glucose culture medium

The medium used for H_2 fermentation contained 20 g glucose COD /L (i.e., 18.75 g glucose /L) as the carbon source and sufficient amounts of inorganic supplements (Fang *et al.*, 1995), including: NH_4HCO_3 (5.24g/L), $NaHCO_3$ (6.72g/L), K_2HPO_4 (0.125g/L), $MgCl_2 \cdot 6H_2O$ (0.1g/L), $MnSO_4 \cdot 6H_2O$ (0.015g/L), $FeSO_4 \cdot 7H_2O$ (0.025g/L), CuSO4 $\cdot$ $5H_2O$ (0.005g/L), and $CoCl_2 \cdot 5H_2O$ (1.25×10^{-4}g/L).

Acid pretreatment and heat pretreatment

The acidic pretreatment involved decreasing the pH of the sludge or granule solution to 3.0 using 0.1 N HCl solution for 24 h and a readjustment of pH back to 7.0 by 0.1 N NaOH solution. In the heat pretreatment, the sludge or granules was heated in a boiling water bath for a short period of time (10 to 30 minutes) first, then cooled down.

Batch culture

Twenty ml of culture medium was placed in a serum bottle with three ml pretreated sewage sludge or pretreated granules. Nitrogen gas was pumped into the serum bottle for 2 minutes before the fermentation to eliminate the oxygen inside the bottle. The culture temperature was 35ºC and the shaking speed was 100-150 rpm (Incubator IC 600, Yamato).

Chloroform pretreatment

Chloroform was added to the culture medium at the first batch culture for the chloroform pretreatments. Six levels of chloroform were used separately at 5%, 2.5%, 1%, 0.5%, 0.25% and 0.1%.

Repeat culture

After batch culture of granules with chloroform addition, another batch culture was performed without chloroform addition to see whether chloroform pretreatment caused irreversible damage to hydrogen producing bacteria and methanogen. The granules were taken out, washed with water and filtered. These granules were put into a serum bottle with the same amount of original culture medium to start another batch that was cultured with the same conditions as described above. Also, this procedure was repeated for several times to determine if the chloroform treated granules can be reused.

Continuous culture

An upflow reactor was set up with a volume of 450 ml and the height of the reactor was 18 cm. 50 ml methanogenic granules were treated with 0.1% chloroform

following the method mentioned previously. Chloroform treated granules were packed in the upflow reactor as an expanded bed. Glucose culture medium 20g COD/L was fed into the reactor at the bottom with the pump. Biogas and fermentation broth flowed out of the reactor at the top and were separated in a separation bottle. Water replacement bottles were connected with separation bottle to collect the biogas and measure the biogas volume. The reactor was maintained at batch mode for three days, and then began continuous mode at Hydraulic Retention Time (HRT) = 13 hours. The HRT was adjusted quickly to 5.3 hours and kept operational for three days until it ran constantly (constantly here means that the pH of the effluent, the biogas and hydrogen production, the glucose conversion and VFA production are constant for over 12 hours).

Analysis

Total biogas production was measured at the end of the batch fermentation (Owen's method) (Logan *et al.*, 2002). The biogas was released into a U-tube with water. The biogas volume produced during the fermentation was measured through the water pressured 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 50m × 0.53mm × 4μm SilicaPLOT column for H_2S with helium as the carrier gas. Volatile Fatty Acids (VFA) were analyzed using a Dionex DX-500 system (Sunnyvale, CA, USA)

containing an AS11-HC (4mm 10-32) column, a quaternary gradient pump (GP40), a CD20 conductivity detector, and an AS3500 auto-sampler. Settling velocity of granules was measured in water.

4. Results and discussions

Acid pretreatment

The methanogenic activity of granules was much higher than the sewage sludge (Table 3-1). The batch culture with granules as seeds (granule culture) produced 75.8 ml CH_4 / g glucose, while sewage culture only produced c.a. 0.74 ml CH_4 / g glucose. After acid pretreatment, the methane production for sewage culture decreased to close to zero, while the methane production for granule culture decreased slightly from 75.76 ml CH_4 / g glucose to 61.00 ml CH_4 / g glucose. Granules showed their advantage in methane production not only in terms of their higher methanogenic activity, but also by providing a protective niche for methanogens from a harsh environmental change, in this case the acid shot. Acid treatment has already been shown to be an effective way to eliminate methanogens from sewage sludge and turn sewage sludge to hydrogen producing sludge since most hydrogen producing bacteria in the sewage sludge are spore-forming Clostridia that can tolerate harsh environmental conditions. However, acid treatment of granules did not totally eliminate the methane production and could not convert methanogenic granules to be hydrogen producing granules because methanogens were, presumably, protected by the granular structure.

Heat pretreatment

Methanogenic activity was almost eliminated from sewage sludge culture after 10 minutes heat treatment of sewage sludge (Table 3-2). This method has already been proved to be effective and the results of this study confirm that. For granules on the other hand, methanogen was still very active with massive methane production after the granules were treated by heat for 10 minutes. Longer treatment time was needed to eliminate all methanogenic activity as shown in Table 3-2. After 30 minutes heat treatment, granule culture could produce hydrogen instead of methane. Presumably, as with acid treatment, the granular structure provided protection for methanogens against a harsh environment, leading to longer time required for heat treatment to eliminate methanogens.

Chloroform pretreatment

Chloroform showed strong inhibitory effects when added into both granules and sewage sludge culture systems (Table 3-3). More than 2.5% chloroform addition severely inhibited both hydrogen and methane production for both the granule culture and sewage sludge culture. However, the toxicity of chloroform was different between hydrogen producing bacteria and methanogens at lower chloroform concentration. Methanogens were so sensitive to the chloroform that only 0.05% addition into the culture medium almost completely inhibited methane production even for granulized methanogens. Chloroform has been used to inhibit methanogenic activity in different fields (Chidthaisong and Conrad, 2000) because chloroform can block the function of corrinoid enzymes and inhibit methyl-coenzyme M reductase. Lenly J. Weathers et al. (Weathers

and Parkin, 2000) found that the loss of methanogenic activity was strongly correlated with the mass of chloroform transformed, rather than with the time-integrated chloroform exposure that methanogens received.

With the decrease of chloroform addition from 2.5% to 0.05%, hydrogen production increased dramatically for both granule and sewage sludge culture. With only 0.1% chloroform added into the culture mediums, hydrogen production was even higher than in the sewage sludge control culture. The inhibition of chloroform to hydrogen production also showed strong correlation to the mass of chloroform added into the culture system (Figure 3-1). Hydrogen production was totally inhibited with the chloroform at a treatment level of above 2.5%, meanwhile, the system was transferred to methane production with no chloroform addition. The data of chloroform addition from 1% to 0.05% were fit to a linear equation

$$y = -117.11x + 148.75 \quad (1)$$

Where, y was the volume of hydrogen production from pretreatment batch culture per gram glucose and x was the % chloroform concentration added into the culture medium. Based on this model, it can be estimated that the hydrogen production would be totally inhibited if the chloroform addition is higher than 1.27% of the culture medium.

In addition to eliminating the methanogenic activity for hydrogen production system, chloroform can also inhibit the activity of hydrogen producing bacteria, mostly *Clostridia*, but if the concentration was controlled at low level, chloroform selectively inhibited methanogenic activity and did not affect the hydrogen production activity. It was also reported that chloroform can inhibit the activity of other hydrogen consuming bacteria such as homo-acetogens and acetate-consuming sulfate-reducing bacteria

(Scholten *et al.*, 2000). This might be part of the reason why hydrogen production at 0.1% chloroform addition for sewage sludge culture was even higher than in any other sewage sludge using other treatment methods.

Batch culture of chloroform pretreated granules

Figure 3-2 showed that all the chloroform treated granule cultures produced hydrogen and nearly no methane. For chloroform level below 2.5%, batch cultures produced nearly the same amount of hydrogen no matter whether pretreated chloroform concentration was different, which means that the hydrogen production ability was not affected by the chloroform inhibition in the pretreatment process. For the pretreatment level of 2.5%, the average hydrogen production of the batch culture reached 166.15 ml/g glucose, equivalent to a yield of 1.34 ± 0.11 mol H_2 / mol glucose calculated as ideal gas at room temperature. The yield of the hydrogen production can be influenced by several factors, such as initial glucose concentration, fermentation time, reactor type, hydrogen partial pressure, etc, and this yield is within the range reported fermentative hydrogen production by different organisms (Nath and Das, 2004b). To date, several approaches have been reported to increase the hydrogen production yield such as lowering the hydrogen partial pressure, or removing carbon dioxide in the headspace. Techniques for increasing the hydrogen yield will be a topic of research priority. For 5% chloroform treated granule culture, data showed large fluctuation of hydrogen production, which might be caused by the inhibition of the remaining chloroform from the previous pretreatment batch.

Table 3-4 showed the comparison of cumulative gas production for untreated and treated granule inoculums. For the batch culture inoculated with methanogenic granules, acetic acid was accumulated in the beginning of the fermentation and was converted completely to methane at 18 days of fermentation. Little hydrogen was accumulated in the beginning of the fermentation process, which indicates high homoacetogenic activity converting the hydrogen to acetate. For the batch culture inoculated with 0.1% chloroform treated granules, VFA was also accumulated because methane production was inhibited. However, after gas release at 3 days fermentation or 6 days fermentation, detectable methane was recorded at 18 days when the fermentation was continued. while acetic acid concentration of the broth at 18 days was significantly lower than at 3 days, indicating recovery of methanogenic activity and thus methanogens were not completely eliminated from the system. There is no significant difference of cumulative hydrogen production between gas release at day 3 and at day 6, however, for the batch culture without gas release in the beginning of the fermentation, little hydrogen was detected at day 18 and low amount of methane. CO_2 production was also decreased. Homoacetogenic activity showed significant effect at long fermentation time, which means that chloroform treatment postponed or increased the lag time of the homoacetogenic activity, thereby, avoiding rapid conversion of hydrogen and CO_2 to acetic acid.

Little or no methane was produced from the chloroform treated cultures, which means that chloroform caused irreversible damage to methanogens over period of time studies, and the methanogenic activity didn't recover even without continuous presence of chloroform. Meanwhile, hydrogen production recovered to normal level even though

chloroform partly inhibited hydrogen production at certain concentrations in the pretreatment. Chloroform showed good selectivity for inhibition of methanogens, eliminated the methane production, and postponed conversion of hydrogen to acetic acid, while allowing for recovery of hydrogen production to normal levels. Chloroform is required only in pretreatment process and the required concentration can be as low as 0.05%.

Comparison of acid treatment, heat treatment and chloroform treatment

Acid pretreatment and heat pretreatment have already been shown effective for obtaining hydrogen producing sludge from sewage sludge. Their effectiveness was also confirmed in this study. Chloroform addition as the first reported application for use in biological hydrogen production and this study showed similar effectiveness for treating sewage sludge, if the concentration was optimized. Compared to the acid treatment or heat treatment of sewage sludge, chloroform treatment is also a simple step that can be easily implemented. Chloroform is listed as a hazardous waste under Resource Conservation and Recovery Act (RCRA) and has been assigned EPA Hazardous Waste No. U044. It is approved for land disposal after treatment and only if the concentration of chloroform in the waste or treatment residual does not exceed 5.6 mg/kg (EPA, 2007). Chloroform treatment might cause some pollution, but the concentration required is low (0.05%) without need for repeated treatment.

From the experiment results, chloroform treatment showed many advantages over acid and heat treatments when dealing with granules presumably because granular structure forms a protective environment. Chloroform resulted in near complete

methanogens elimination. Furthermore, chloroform also postponed the activity of homoacetogens, another hydrogen consuming bacteria, whereas this latter phenomenon has not been reported for the acid treatment and heat treatment. Indeed, some homoacetogens are also spore-forming bacteria, which are not eliminated by the heat treatment method (Oh *et al.*, 2003).

Repeat culture of chloroform treated granules

There was no significant difference in hydrogen production for three repeated batch cultures with granules pretreated by 0.25% and 0.05% of chloroform. This result also confirmed that the chloroform treatment concentration can be lowered to 0.05%. Further repeated batch experiments only focused on 0.25% chloroform treated granules. Figure 3-2 showed that chloroform treated granules can last for at least 8 batches (24 days) with nearly constant hydrogen production yet without obvious morphology damage.

Continuous hydrogen production in the upflow reactor packed with chloroform treated granules

Figure 3-4 showed the performance of continuous hydrogen production in the upflow reactor packed with chloroform treated granules. The hydrogen productivity reached 11.6 L/L/day. After the HRT adjusted to below 4 hours, the overall yield decreased, so the initial test for the continuous operation stopped. The granules changed their color gradually from deep black to dark grey, which might be caused by the washing out of the sulfur reducing bacteria. The granular structure was mostly kept after 15 days

operation. New granules, which have shallow grey color, could be seen after ten days of operation.

5. Conclusion

Acid treatment and heat treatment were confirmed to be effective to sewage sludge while not completely effective to methanogenic microorganisms from granules presumably owing to granule's protective structure over harsh environment. Chloroform treatment can be an effective way for eliminating methanogenic activity. Hydrogen production also was inhibited by chloroform. If the concentration was controlled at low level, chloroform selectively inhibited methanogenic activity while it did not influence the hydrogen production activity. Chloroform caused irreversible damage to methanogens while hydrogen production ability recovered to normal after the pretreatment batch. Chloroform treated granules could be repeatedly cultured for eight time without obvious damage. The treated granules can keep their structure over 15 days and new granules started to form after 10 days operation.

Acknowledgments

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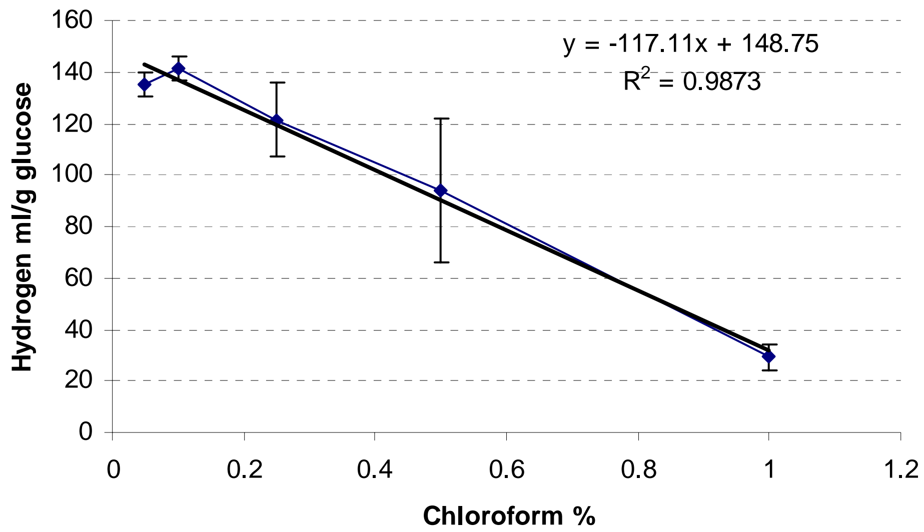


Figure 3-1. Chloroform inhibition on hydrogen production (20 ml glucose culture medium, 3 days each batch, initial pH=8.0, inoculums 3 ml). Data are averages with error bars showing standard deviations (n=3).

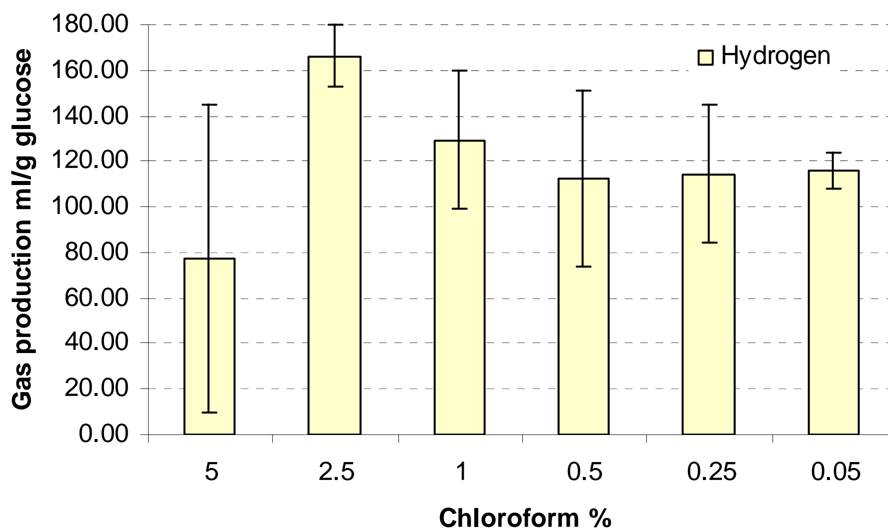


Figure 3-2. H_2 production from culture of chloroform treated granules (20 ml glucose culture medium, 3 days each batch, initial pH=8.0, inoculums 3 ml). Data are averages with error bars showing standard deviations (n=3).

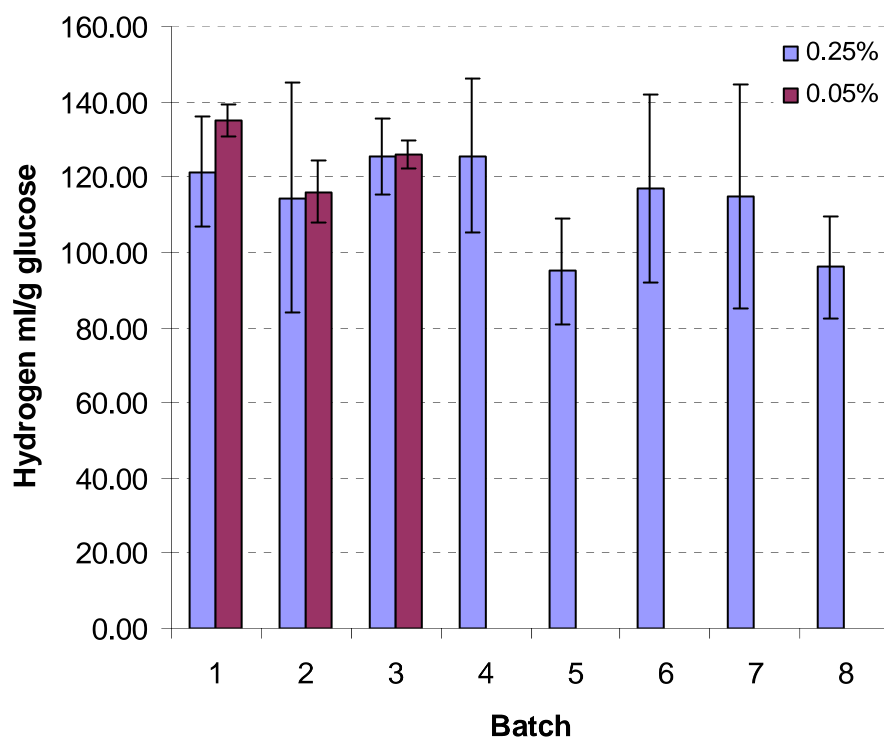


Figure 3-3. H_2 production from repeat culture of chloroform treated granules (20 ml glucose culture medium, 3 days each batch, initial pH=8.0, inoculums 3 ml). Data are averages with error bars showing standard deviations (n=3).

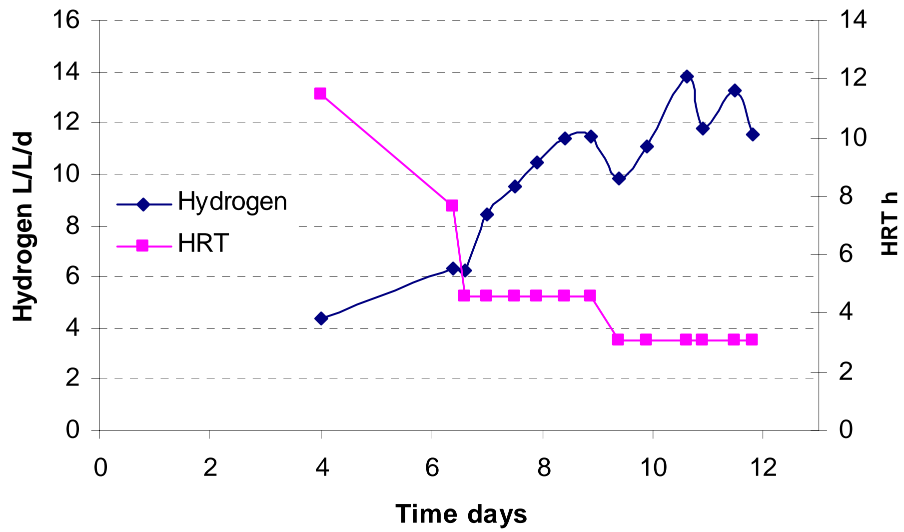


Figure 3-4. Continuous hydrogen production in the up-flow reactor packed with chloroform treated granules (20 g COD /L glucose culture medium, initial pH=8.0, 0.1% chloroform treatment).

Table 3-1. Gas production for acid treatment of granules and sewage sludge

Acid Pretreatment		H_2 ml/g glucose		CH_4 ml/g glucose	
		Average	Standard Dev	Average	Standard Dev
Granule	Control	0.4	0.5	75.8	24.8
	Acid	0.0	0.0	61.0	24.2
Sewage	Control	125.0	26.4	0.7	0.2
	Acid	89.0	14.5	0.3	0.1

(Batch culture, 20 ml glucose culture medium, 3 days, inoculums 3 ml). Data are averages with error bars showing standard deviations (n=3).

Table 3-2. Gas production for heat treatment of granules and sewage sludge

Heat pretreatment		H_2 ml/g glucose		CH_4 ml/g glucose	
		Average	Standard Dev	Average	Standard Dev
Granule	Control	0.4	0.5	75.8	24.8
	Heat(10 min)	0.3	0.5	60.0	12.5
	Heat(30 min)	118.3	0.0	0.1	0.0
Sewage	Control	125.0	26.4	0.7	0.2
	Heat(10 min)	121.3	25.7	0.0	0.0
	Heat(30 min)	134.1	5.8	0.0	0.0

(Batch culture, 20 ml glucose culture medium, 3 days, inoculums 3 ml). Data are averages with error bars showing standard deviations (n=3).

Table 3-3. Gas production for chloroform addition to granule and sewage sludge culture

Chloroform added into culture medium: %		H_2 ml/g glucose		CH_4 ml/g glucose	
		Average	Standard Dev	Average	Standard Dev
Granule	5	0.3	0.5	0.1	0.1
	2.5	0.0	0.0	0.0	0.0
	1	29.1	5.3	0.0	0.0
	0.5	94.1	27.9	0.0	0.0
	0.25	121.4	14.6	0.1	0.2
	0.05	135.1	4.4	0.0	0.0
	Control	0.4	0.5	75.8	24.8
Sewage	5	0.6	1.1	0.5	0.1
	2.5	0.0	0.0	0.6	0.1
	1	32.9	36.1	0.2	0.0
	0.5	45.6	17.5	0.1	0.0
	0.25	113.1	11.1	0.2	0.0
	0.1	145.3	27.9	0.1	0.0
	Control	125.0	26.4	0.7	0.0

(Batch culture, 20 ml glucose culture medium, 3 days, inoculums 3 ml). Data are averages with error bars showing standard deviations (n=3).

Table 3-4. Comparison of gas production for untreated and treated granule inoculums

	Time	LA	AC	PR	BU	H_2	CO_2	CH_4
	Days	g/L	g/L	g/L	g/L	ml/g glucose	ml/g glucose	ml/g glucose
Untreated Granule	3	0.00	3.13	1.65	0.00	7.8	292.7	80.9
	18	0.00	0.00	0.00	0.00	1.0	453.9	374.4
Treated Granule	3	1.91	1.82	0.00	2.65	109.9	290.1	0.0
	3(18)	0.00	0.79	0.35	1.58	180.6	317.9	1.4
	6(18)	0.00	0.67	0.71	2.71	176.7	364.5	1.5
	18	0.00	2.00	0.88	1.79	0.1	284.0	13.7

(Batch culture, 20 ml glucose culture medium, 3 days, inoculums 3 ml, chloroform treatment 0.1%). Data are averages with error bars showing standard deviations (n=3). LA: lactic acid; AC: acetic acid; PR: propionic acid; BU: butyric acid. Time 3(18) means that gas was released at day 3 and broth was measured at day 18, same as Time 6(18).

CHAPTER FOUR

BIOLOGICAL HYDROGEN PRODUCTION WITH CHLOROFORM TREATED METHANOGENIC GRANULES

1. Abstract

In fermentative hydrogen production, the low retention rate of hydrogen-producing bacteria limits the productivity of a suspended-growth reactor 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 for hydrogen fermentation such as limited duration time, bacteria leakage, cost and so on. Using 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 finding on the performance of fed-batch cultures and continuous cultures inoculated with chloroform treated granules. The chloroform treated granules could be re-used for 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 can be as low as 4 hours without decrease of 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 COD/L at HRT 4 hours. This study also investigated the possibility of integrating immobilized hydrogen fermentation with chloroform treated granules and immobilized methane production with untreated granular sludge. The results showed that the integrated batch cultures provided

1.01 mol hydrogen and 2 mol methane per mol glucose. Treating the methanogenic granules, and then using the treated granules as immobilized hydrogen producing sludge demonstrated advantages over other immobilization methods because the granules provide hydrogen-producing bacteria with a protective niche, a long duration, and excellent settling velocity. 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 the system.

Key words: Biological hydrogen production, chloroform treatment, granular, immobilization, integration with methane production

2. Introduction

Hydrogen gas is a clean energy; when it burns, it produces only water and it has the highest energy content per unit weight among any known fuels. Although there are several different ways to produce hydrogen—including electrolysis of water, thermo-catalytic reformation of organic compounds, and biological processes (Levin *et al.*, 2004),—biological hydrogen production has attracted more attention in recent years because of its hydrogen-producing capabilities and the growing environmental concerns regarding fossil fuel dependence. In addition, the process itself is particularly environmentally friendly because negative-valued waste materials (such as cheese whey) can be used in the process (Hu *et al.*, 2007).

Biological hydrogen production with suspended cell systems is normally inefficient or difficult to control in continuous operations. The low retention rate of hydrogen-producing bacteria limits the productivity of a suspended-growth reactor because of the long hydraulic retention time (HRT) required to maintain adequate bacterial population. Recycling biomass back to the reactor is one option for maintaining sufficient cell density, which is needed for high hydrogen production (Kraemer and Bagley, 2005). Recent studies show that immobilization of hydrogen-producing bacteria can also effectively enhance the bacterial population and increase hydrogen productivity (Kumar and Das, 2001;Wu *et al.*, 2005). There are various challenges, however, for the polymer matrices to entrap 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 washout of the immobilized bacteria. Second, in most cases, the gel structure collapses after several batches because of damage resulting

from several possible causes, such as gel swelling, pH changes (many polymer matrices are pH sensitive), calcium loss, and so on. Third, the loss of bacteria always decreases the biological stability of the immobilized gel beads. Finally, there are economic concerns because entrapment into polymer matrices adds additional costs to the overall process (Hu *et al.*, 2007;Wu *et al.*, 2005).

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 of sludge washout. The granules allow the loading rate of the UASB reactor to be far beyond the common loading rates applied to date in conventional activated sludge processes. Granules also have many advantages over other systems, which contribute to the success of the UASB design. First, granules formed inside the UASB reactor so that the granules have superior settling velocity, which explains the reduction of sludge washout. Second, the granules provide protective structure for the microorganisms in a harsh environment, which ensures stable operations even if environmental shock occurs (Hu and Chen, 2007). Finally, in an anaerobic digestion system, granules are formed naturally and have a porous structure, which is ideal for the mass transfer of nutrients required by the microorganisms and for the biogas being produced. In addition, it has been found that hydrogen-producing biomass can develop into granules with high bioactivity (Fang *et al.*, 2002). Hydrogen-producing sludge has been shown to agglutinate into granules after 60 days of operation in CSTR reactors. Furthermore, granular sludge has been reported to be visible in the UASB systems after 120 days of reactor operation (Chang and Lin, 2004). 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 (Lee *et al.*, 2004). 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.

There are thousands of UASBs running worldwide, and once every two to three months a portion of the methanogenic granules inside these reactors need to be disposed to maintain the reactor's efficiency. In previous research, chloroform treatment of granules was found to effectively eliminate methane production and convert the culture into hydrogen production (Hu and Chen, 2007). In this study, the use of chloroform treated anaerobic granular sludge as an immobilized hydrogen-producing bacteria in a hydrogen culture is investigated. In addition, the possibility of combining the immobilized hydrogen production with current anaerobic digestion is also examined.

3. Materials and methods

Methanogenic granules:

The methanogenic granules were taken from a UASB that was treating starch wastewater. The average settling velocity was 29.5 m/h (100 samples).

Glucose culture medium

The medium used for hydrogen (H_2) fermentation contained 20 g glucose COD /L (i.e., 18.75 g glucose /L) as the carbon source, and sufficient amounts of inorganic supplements (Fang *et al.*, 1995) including: NH_4HCO_3 (5.24g/L), $NaHCO_3$ (6.72g/L),

K_2HPO_4 (0.125g/L), $MgCl_2 \cdot 6H_2O$ (0.1g/L), $MnSO_4 \cdot 6H_2O$ (0.015g/L), $FeSO_4 \cdot 7H_2O$ (0.025g/L), CuSO4 $\cdot$ $5H_2O$ (0.005g/L), and $CoCl_2 \cdot 5H_2O$ (1.25×10^{-4}g/L).

Chloroform pretreatment

20 ml of culture medium was cultured with 0.25% chloroform addition for over one day, then, the granules were filtered and washed for inoculation.

Batch culture

Twenty ml of culture medium was placed in a serum bottle. Three ml pretreated granules from the pretreatment process was added. Nitrogen gas was pumped into the serum bottle for two minutes prior to the fermentation to eliminate the oxygen inside the bottle. The culture temperature was maintained at 35ºC in an incubator (IC 600, Yamato) and the shaking speed was 100-150 rpm.

Fed-batch culture

After the batch culture with chloroform treated granules, five ml of fermentation broth were taken out for analysis. In addition, five ml of 80 g COD/L fresh culture medium with the same concentration of nutrients as the culture medium mentioned above were added into the fermentation broth to start another batch. For the fed-batch culture without pH control, pH was not adjusted at the beginning of each culture. For the fed-batch culture with pH adjustment, pH was adjusted to about seven after five 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 centimeters. Fifty ml of methanogenic granules were treated with 0.1% chloroform following the method mentioned previously. Then 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 at the bottom with the pump. Biogas and fermentation broth flowed out of the top of the reactor and were separated in the separation bottles. Water replacement bottles were connected to separation bottles to collect the biogas and measure the biogas volume. The reactor was maintained at batch mode for three days, then at continuous mode, hydraulic retention time (HRT) for 13 hours. The HRT was adjusted quickly to 5.3 hours and kept in operation for three days until it ran constantly (here, constantly means that the pH of the effluent, the biogas and hydrogen production, the glucose conversion, and volatile fatty acid (VFA) production are constant for over 12 hours). The HRT was gradually adjusted to four hours and was kept constant. The initial pH and the glucose concentration of the culture medium were 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 granular was mounted on metal stubs and the membranes were coated with gold for six minutes. Later, the surfaces were 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 for 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 ultramicrotomes at 70 nanometers thickness. Grids were stained with uranyl acetate for 10 minutes, rinsed with water, then stained with lead citrate for 15 minutes 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) (Logan *et al.*, 2002). The biogas was released into a U-tube with water. The biogas volume produced during the fermentation was measured through the water pressured 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.

Volatile fatty acids (VFAs) were analyzed using a Dionex DX-500 system (Sunnyvale, CA, USA) containing an AS11-HC (4 mm 10-32) column, a quaternary gradient pump (GP40), a CD20 conductivity detector, and an AS3500 auto-sampler (Hu and Chen, 2007).

4. Results and discussions

Methanogenic granules

The methanogenic granules were dark in color (probably due to the presence of sulfide produced by the sulfate-reducing bacteria in the digester) and were about 1 to 2 mm in size (Figure 4-1). The average settling velocity of the granules in water was 29.5 m/h (100 samples). The SEM picture of the granules (Figure 4-2) shows the microorganisms packed together with extracellular polymers and it shows the porous structure of the granular, which facilitates the mass transfer, especially for biogas to exit. There are several theories that explain the granulation process; however, the mechanism granules' formation is not fully understood. Most researchers have concluded that filamentous *Methanosaeta concilii* is a key organism in granulation (Pol *et al.*, 2004). Filamentous species are clearly shown in the SEM picture (Figure 4-2), and the TEM picture of the granules (Figure 4-3) also clearly shows the high activity of the Methanogen species (predominance of cells with long-rod, sometimes filamentous, typical morphology are pointed out in Figure 4-3). There is considerable consensus that the initial stage of granulation is bacterial adhesion (a physical-chemical process), parallel to the early stage of biofilm formation. *Methanosaeta* aggregates to form nucleation centers that initiate granular development, then acetogens adhere to the nuclei

and form the second layer, and finally, fermentative bacteria adhere to form the exterior layer of the granules in contacting with the substrate. Methanogens are also found in the exterior layer where they consume the free hydrogen produced by fermentative bacteria (Macleod *et al.*, 1990). The SEM picture (Figure 4-2) confirmed that filamentous methanogens were distributed on the surface of the granules.

Chloroform pretreatment

Chloroform treatment has been found in our previous study (Hu and Chen, 2007) to be an effective method for eliminating methanogenic activity and for switching a methane producing system to hydrogen producing system. Chloroform selectively inhibits the methanogenic activity while allowing normal hydrogen production, as long as chloroform concentrations are low (Table 3-3) (Hu and Chen, 2007). Chloroform, however, causes damage to methanogens and the methanogenic activity is not easily recovered even upon removal of the chloroform in subsequent cultures. In this study, the low concentration of chloroform added in the culture medium in the pretreatment did not cause any damage to the structure of the granules. However, the chloroform treatment dramatically changed the microorganism distribution after several days of culture. Filamentous methaongens are clearly visible on the surface of the untreated granules (Figure 4-2), but there are no visible filamentous microorganisms on the chloroform treated granules in the SEM picture (Figure 4-4). Similarly, in the TEM picture, the methanogens are distributed and sectioned from the methanogenic granules (Figure 4-3), while the TEM picture of the chloroform treated granules (Figure 4-5) is much clearer without the methanogens. Chloroform showed good selectivity on inhibition of

hydrogen-producing bacteria and methanogens, eliminating the methane production while allowing normal hydrogen production.

Fed-batch culture

Figure 4-6 illustrates that for fed-batch cultures, the chloroform treated granules can be stably reused over four times with pH adjustment at the beginning of each fed-batch culture. After four fed-batches, 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 (Figure 4-8), 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 (Figure 4-7), the metabolic pathway dramatically switched to lactic acid production, which does not produce hydrogen. Volatile fatty acids (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 (Zheng and Yu, 2005) 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 appeared to a drastic decrease of hydrogen production in subsequent fed-batch cultures. For the fed-batch cultures without 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 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 hydrogen fermentation (Zheng and Yu, 2005); our results clearly confirmed this finding.

Volatile fatty acids (VFAs), which are byproducts of the hydrogen fermentation process, can be feedstock for many other processes such as photo fermentation of hydrogen, microbial fuel cell, and methane production (Nath and Das, 2004b). Higher levels of VFAs are always preferred because the separation and efficient utilization would be difficult at lower concentrations. However, with the accumulation of VFAs, especially butyrate, in the fermentation broth, the switch from hydrogenesis to solventogenesis always occurred. Before the pathway switched to non-hydrogen production, fed-batch cultures with pH adjustment seem to be an effective way to accumulate VFAs at higher concentrations.

Influence of HRT on the continuous granulized fermentation

Our previous study showed that hydrogen productivity reached 11.6 L/L/day at HRT 5.4 hours (Hu and Chen, 2007), 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 HRT decreased, hydrogen productivity gradually increased. The yield of hydrogen fermentation, however, decreased when the HTR was shorter than three hours, owing to the overloading of the upflow reactor (Fig 4-10). For the UASB reactor, hydrogen productivity was stable at an

HRT of 8 to 20 hours and decreased dramatically at an HRT of four hours (Chang and Lin, 2004). Shorter HRT was reported for the Carrier-Induced Granular Sludge Bed (CIGSB) bioreactor with the addition of support carriers, where hydrogen producing biomass itself developed into granules (Lee *et al.*, 2004).

Influence of initial pH of the culture medium on the continuous granulized fermentation

For the continuous fermentation in the upflow reactor, hydrogen production decreased when the initial pH of the culture medium was below 6.0 (Figure 4-10). In our previous batch study the culture conditions appeared to be too harsh for the hydrogen-producing bacteria to grow; nearly no hydrogen or volatile fatty acid (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 (Hu *et al.*, 2007). It seems that continuous culture in the upflow reactor required a narrower initial pH range of the culture medium. 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 (Figure 4-10). In this case, the granules inside the reactor were drifted up with the upflow and gradually washed out of the reactor, which drastically decreased overall hydrogen productivity of the system. It is possible that the fermentation broth was too acidic, and therefore, it might have been erosive to the extracellular polymer that maintains the

granular structure. Furthermore, because hydrogen-producing bacteria grow very slowly in acidic conditions, there might have been more bacteria detaching from the granules than bacteria reproducing and attaching to the granules. The granules washing out of the reactor appeared more like floc without granular structure, which confirmed that the continuous granulized hydrogen production should remain in around neutral pH conditions because the granular structure collapsed in very acidic conditions. For many bioadsorption processes, the capacity of the adsorbent is pH sensitive and consequently, the adsorption behavior decreased with increasingly acidic conditions (Tan *et al.*, 2004). Most granulation theories suggest that granulation starts with bacteria adhesion to the inert nuclei (Pol *et al.*, 2004), and our results confirm that bacterial adhesion inside the granules may be weakened when the conditions are too acidic.

Influence of glucose concentration of the culture medium on the continuous granulized fermentation

Figure 4-11 clearly illustrates that substrate concentration influenced the continuous granulized fermentation. With an increase in glucose concentration and the COD loading of the upflow reactor, hydrogen productivity reached maximum levels and then decreased. There was no significant difference in hydrogen productivity between glucose concentration at 20 g COD/L and at 30 g COD/L. When the glucose concentration was over 20 g COD/L, however, the overall yield decreased gradually. Fermentation with high substrate concentrations are preferred because many raw materials (such as whey or manure) have high COD content, and high concentrations of substrate are easy to heat and handle. Inhibition, however, has been found at higher

feedstock concentrations (Van Ginkel and Logan, 2005b). In this study, cultures with substrate concentration below 20 g COD/L have nearly the same yield; however, at higher substrate concentrations, overloading occurs, causing a decrease in hydrogen productivity and overall yield.

Comparison of different immobilization methods with granular treatment method

Table 4-1 shows the maximum hydrogen (H_2) yield obtained in various types of H_2 producing reactors and processes including initial glucose concentration, fermentation time, reactor type, hydrogen partial pressure etc. Compared to other hydrogen producing processes, the one used in this study has an average yield, although treating the methanogenic granules and then using treated granules as immobilized hydrogen producing sludge, has shown advantages over other immobilization methods because the granules provide the hydrogen-producing bacteria 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 (Hu *et al.*, 2007). By using polymer matrix to entrap hydrogen-producing bacteria, it is difficult to protect the polymer matrix from erosion, degradation, and destruction by the gas produced inside the system and by cell growth. In addition, the density of the matrix gel decreases as long as hydrogen and other biogas are produced, which cause the matrix gel to float and make it more difficult to maintain the packing of the reactor. Granulation, which immobilizes the bacteria by self-flocculation, is easy to induce and manipulate on an industrial scale. Furthermore, fermentation with chloroform treated granules provides a straightforward method for obtaining the hydrogen-producing

granules, because methanogenic granules are available as waste discharge at UASB wastewater treatment systems.

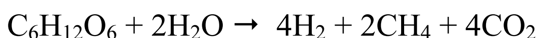
VFA production and integration with immobilized methane production

Table 4-2 shows that biohydrogen production, coupled with the subsequent step of methane production, can be very efficient. After three days of biohydrogen fermentation with 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 due to volatile fatty acid (VFA) production before converting to a methane production reactor. Overall, the integration of the immobilized hydrogen production with immobilized methane production provided 1.01 mol hydrogen and 2 mol methane per mol glucose. It is well known that formation of VFA during acidogenesis of organic matter precedes methanogenesis. The fermentation broth of biohydrogen production can therefore be used with acetogensis and methanogensis to produce methane, which can alleviate the costs and environmental concerns associated with the biological hydrogen production process. Production of methane from hydrogen fermentation broth brings extra value by producing methane, and the degradation of VFA also decreases the overall COD value of discharge from the 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 we take acetate as the final product of biohydrogen production, the theoretical 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 unaccounted). The reasons are multifarious. 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 gives theoretically only 2 mole hydrogen per mole glucose. If the pathway goes to another end product, such as solvent or propionate, no hydrogen will be generated. Ways to further increase hydrogen production is still a topic of investigation for many researches, including the authors of this study.

Methane production reached 1.99 mol per mol glucose, nearly the same as the theoretical value of the integrated process. Because there are many other end products in Stage 1, such as butyrate, lactate, and so on, the missing hydrogen generation capability appears to be directed to methane production with acetogensis, which produces hydrogen and acetate from butyrate, propionate and solvent, and provides the necessary raw material for methanogensis.

There are thousands of UASB reactors running worldwide that can anaerobically degrade various organic wastes for methane production. This study provides a modification process for current granulized anaerobic digestion system 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 the system.

Acknowledgments

Funding for this project was from the Washington State University Agriculture Research Center.

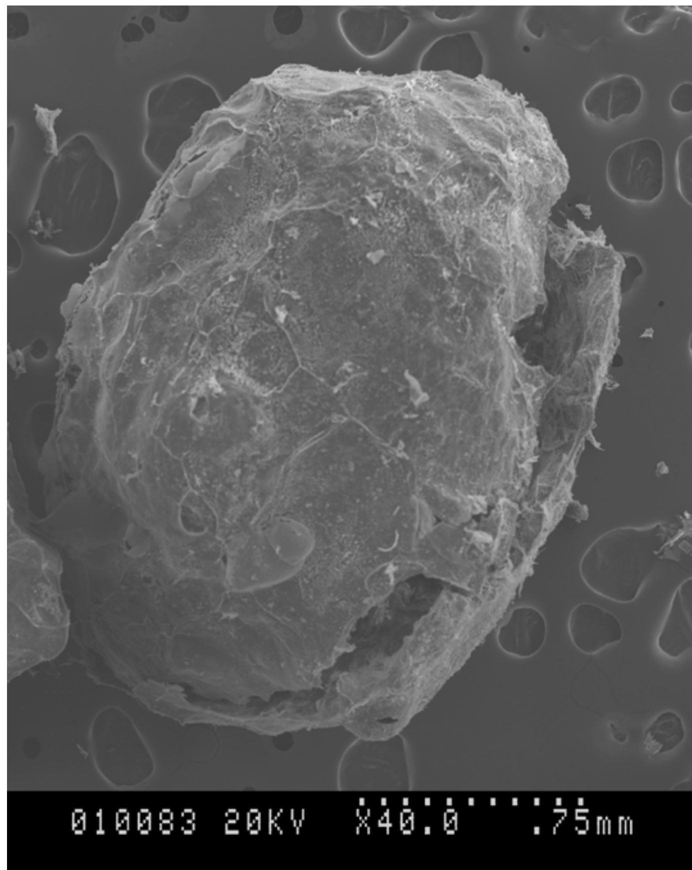


Figure 4-1. SEM picture of methanogenic granules.

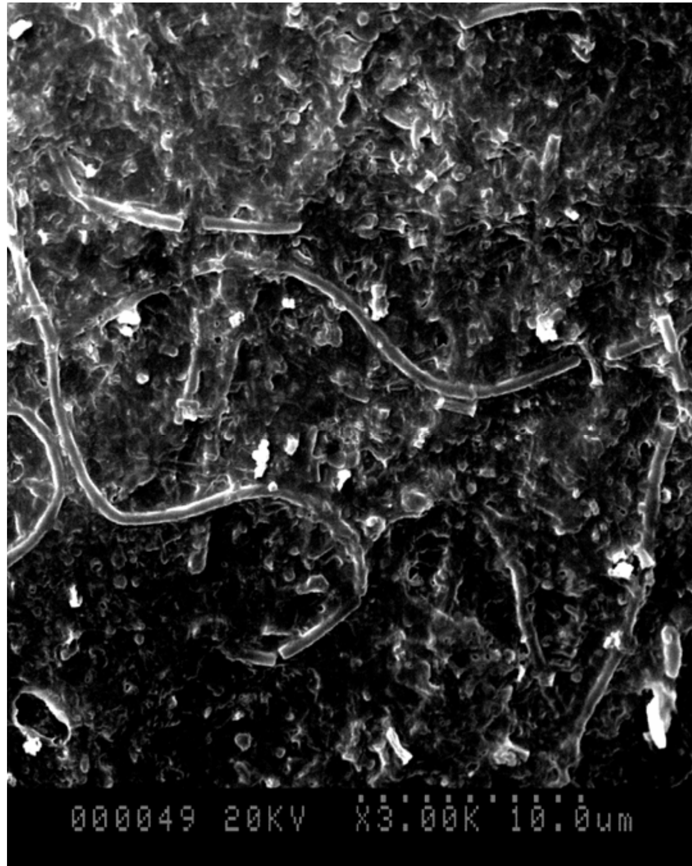


Figure 4-2. SEM picture of methanogenic granule surface.

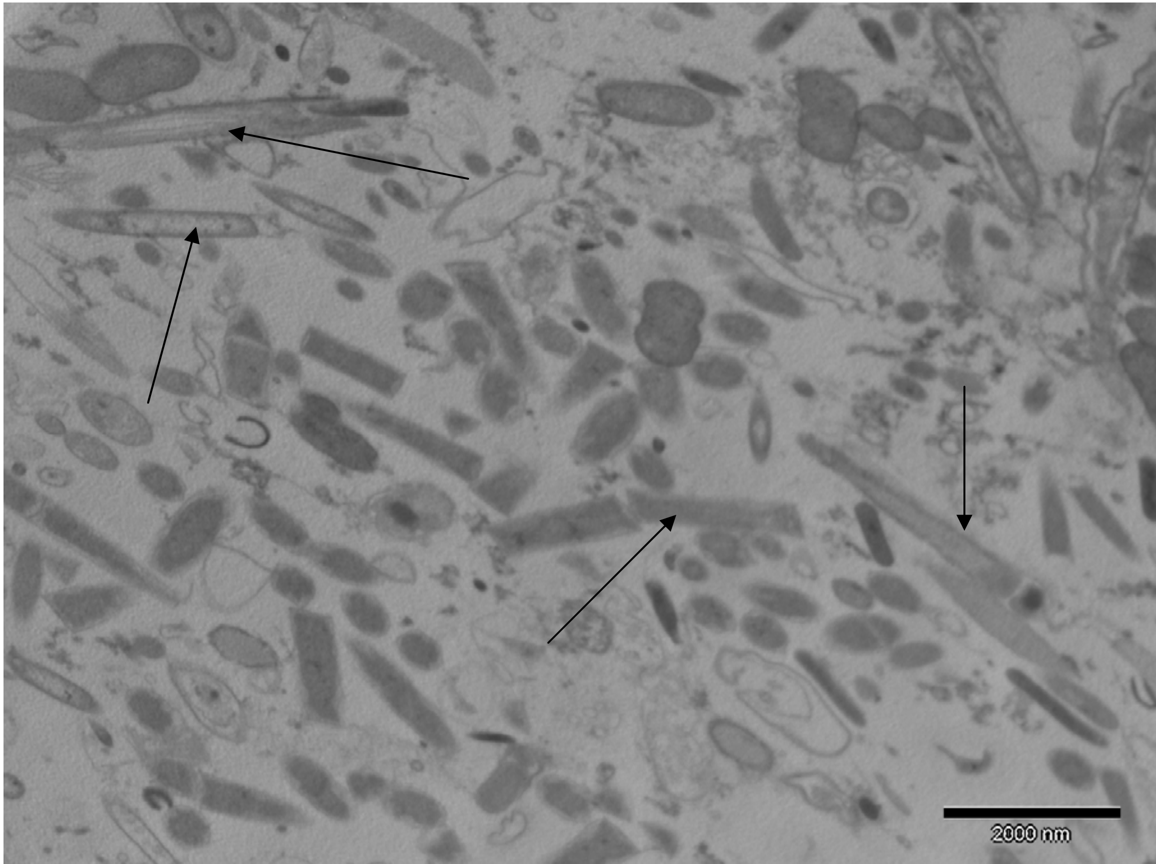


Figure 4-3. TEM picture of methanogenic granules. Arrows are pointing out the typical morphology of methanogens.

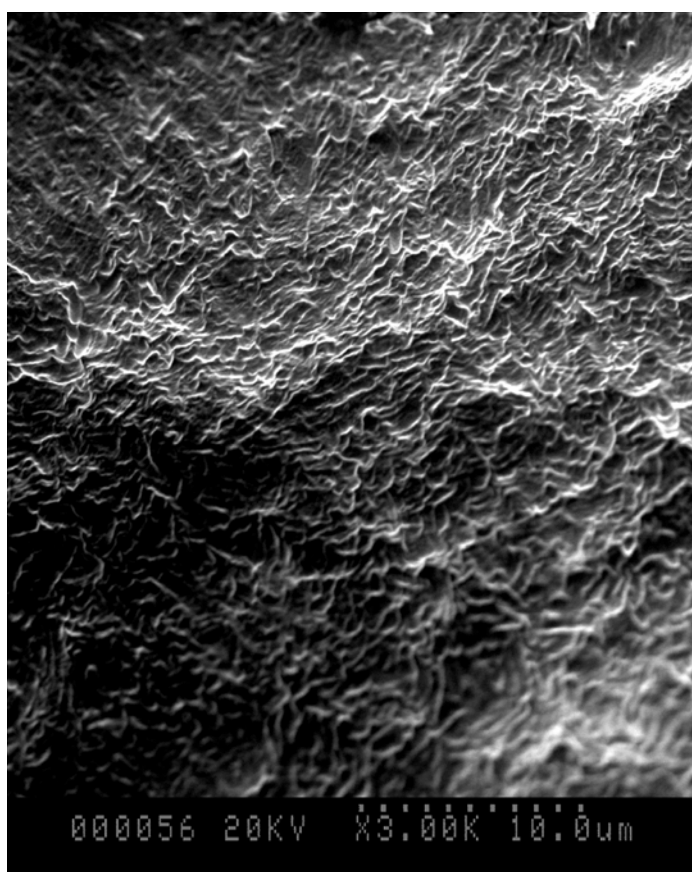


Figure 4-4. SEM of chloroform treated granules (after 5 days culture).

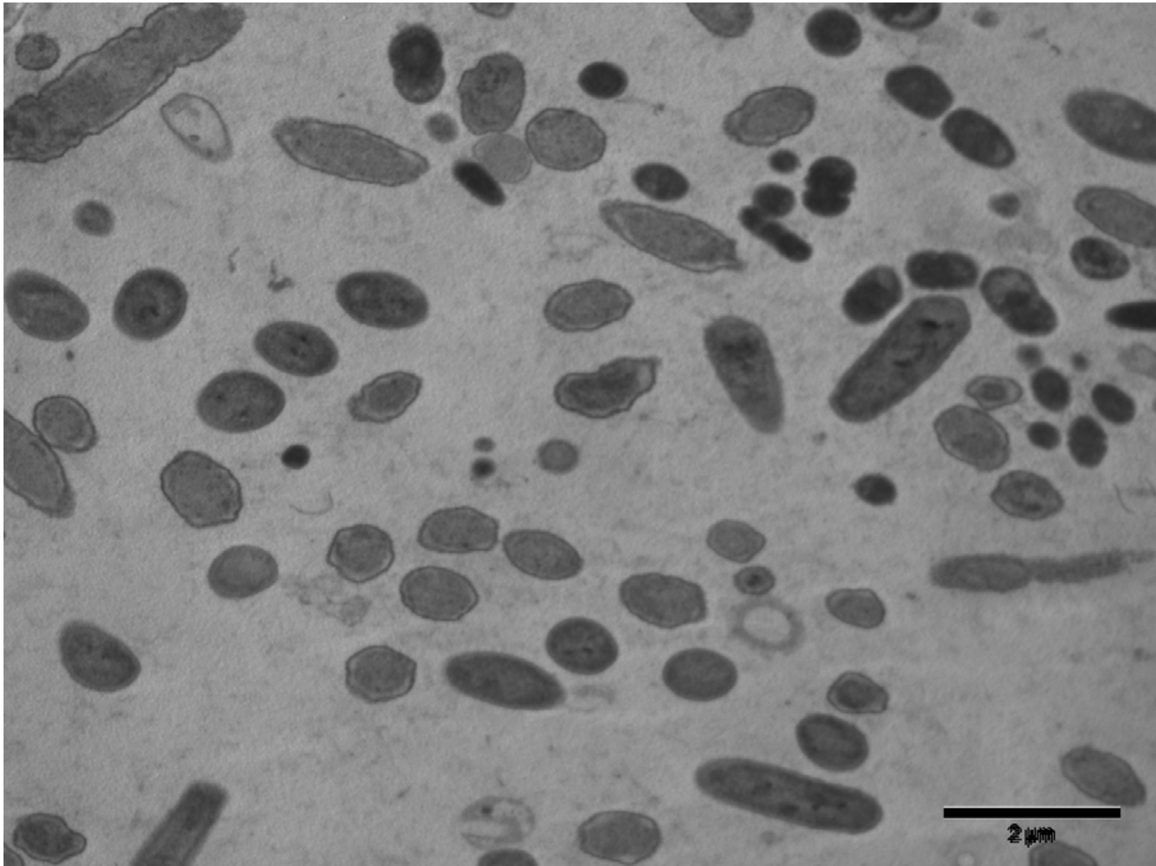


Figure 4-5. TEM of chloroform treated granules (after 5 days culture).

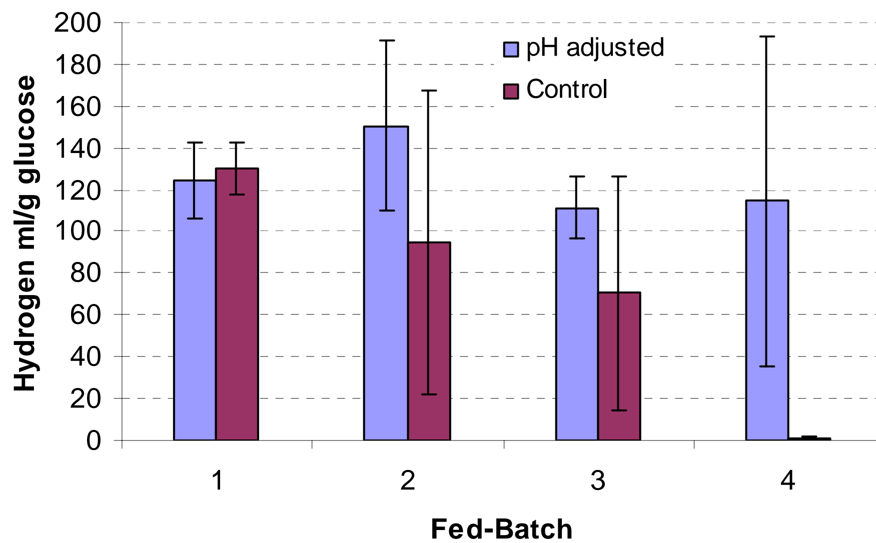


Figure 4-6. H_2 production from fed-batch culture of chloroform treated granules (granules were treated with 0.25% chloroform, 20 ml glucose culture medium, 3 days each batch, initial pH=8.0, inoculums 3 ml). Data are averages with error bars showing standard deviations (n=3).

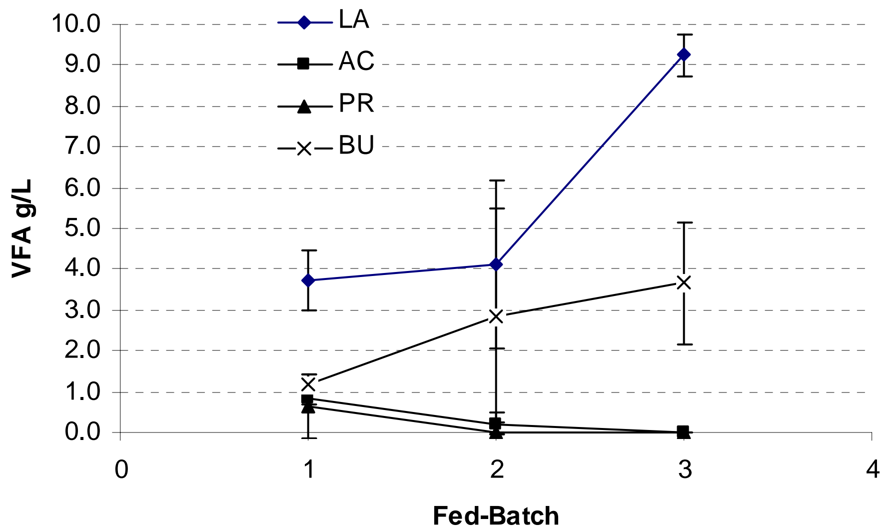


Figure 4-7. VFA production from fed-batch culture of chloroform treated granules without pH adjustment (granules were treated with 0.25% chloroform, 20 ml glucose culture medium, 3 days each batch, initial pH=8.0, inoculums 3ml). Data are averages with error bars showing standard deviations (n=3). LA: lactic acid; AC: acetic acid; PR: propionic acid; BU: butyric acid.

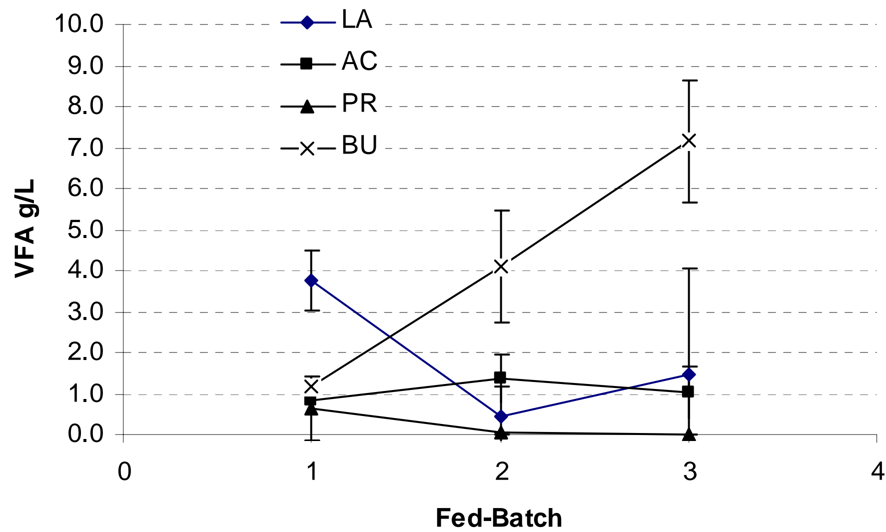


Figure 4-8. VFA production from fed-batch culture of chloroform treated granules with pH adjustment (granules were treated with 0.25% chloroform, 20 ml glucose culture medium, 3 days each batch, initial pH=8.0, inoculums 3 ml). Data are averages with error bars showing standard deviations (n=3).

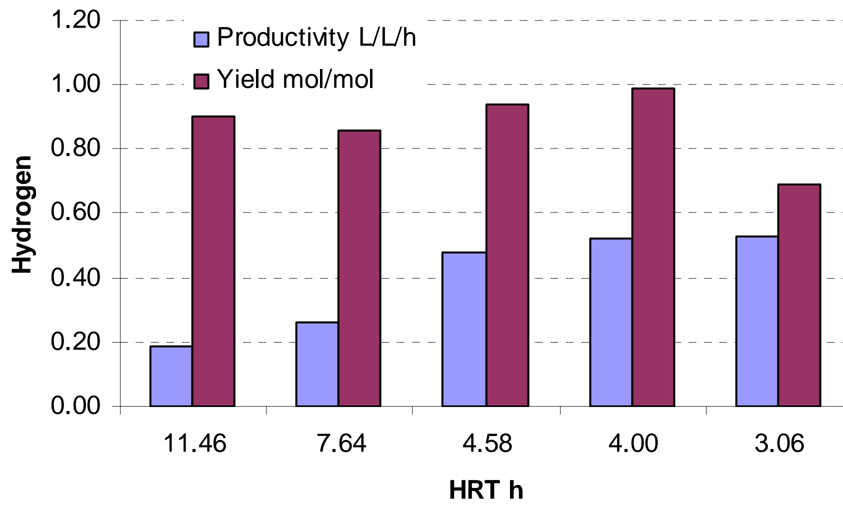


Figure 4-9. Influence of HRT on the performance of the upflow hydrogen fermentation reactor (glucose culture medium 20g COD/L, initial pH 8.0). Data were collected when they were stable for over 12 hours.

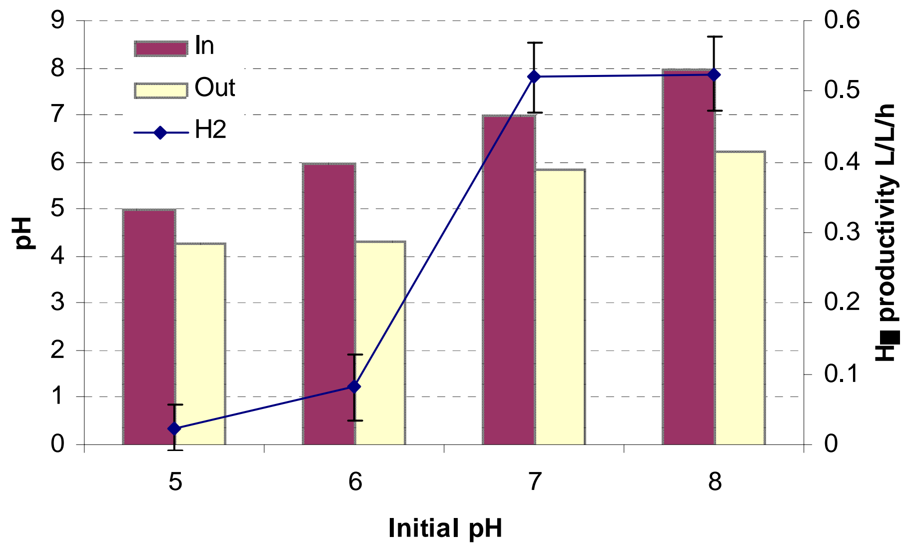


Figure 4-10. Influence of initial pH of culture medium on the performance of the upflow hydrogen fermentation reactor (glucose culture medium 20g COD/L, HRT 4 hours). Data are averages of steady state with error bars showing standard deviations (n=3).

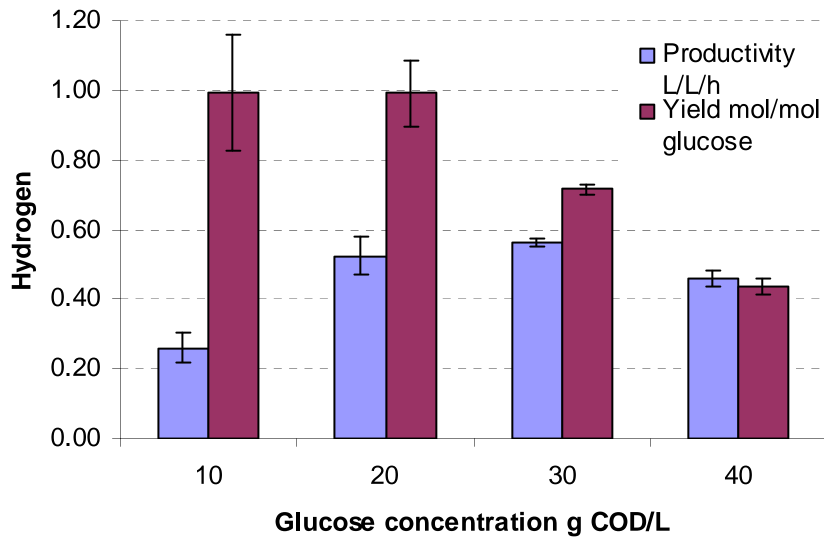


Figure 4-11. Influence of glucose concentration of the culture medium on the performance of the upflow hydrogen fermentation reactor (glucose culture medium, Initial pH 8.0, HRT 4h). Data are averages of steady state with error bars showing standard deviations (n=3).

Table 4-1. 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	(Rachman *et al.*, 1997)
Membrane reactor	Mixed culture	Glucose	1.0	(Oh *et al.*, 2004)
N_2 sparging, continuous	Mixed culture (Predominantly *Clostridium sp.*)	Glucose	1.43	(Mizuno *et al.*, 2000)
UASB	Mixed culture with granular sludge	Sucrose	1.44 ± 0.10	(Yu and Mu, 2006)
UASB	Mixed culture with granular sludge	Sucrose	0.92	(Chang and Lin, 2004)
Fluidized bed	Sewage sludge immobilized in silicone matrix	Sucrose	1.34	(Wu *et al.*, 2003)
Batch	Chloroform treated methanogenic granules	glucose	1.34 ± 0.11	This study

Table 4-2. Integration of immobilized hydrogen production and immobilized methane production

	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	

(20g COD glucose culture medium, two sequential bioreactors, 35°C).

CHAPTER FIVE

COMMUNITY CHANGE DURING CHLOROFORM TREATMENT OF METHANOGENIC GRANULES FOR IMMOBILIZED HYDROGEN FERMENTATION

1. Abstract

Hydrogen can be produced as an intermediate product during the anaerobic digestion. To produce hydrogen as a final product, hydrogen consumers, such as methanogens, need to be eliminated from the system. Chloroform treatment of anaerobic granular sludge was shown to be effective in eliminating the methane production and switching the methane producing system to a hydrogen producing system. Chloroform treated granules were tested for immobilized hydrogen production in the upflow continuous reactor and new hydrogen producing granules were formed after several days of operation and methane did not recover. Consequently, the purpose of this study was to determine how the microbial community structure changed following chloroform treatment and incubation in the reactor. Terminal restriction fragment length polymorphisms (T-RFLP) analysis demonstrated that there was a major community change during the chloroform treatment and that this community change was consistent between replicate experiments. Clone libraries of the 16s rRNA sequence were prepared from methanogenic granules, chloroformed treated granules, and continuous cultural samples from upflow reactor. Unexpectedly, *Methanosaeta concilii* was not eliminated from the hydrogen producing system, although this might help explain the successful formation of robust granules in this system; we surmise that the total population of *M. soehngnii* was probably very low given that no methane production was detected after chloroform treatment

Key words: Biological hydrogen production, granule, chloroform treatment, 16s rRNA microbial analysis, T-RFLP, clone library

2. Introduction

Anaerobic degradation occurs naturally in various anaerobic habitats. Different groups of microorganisms work together in a food chain to degrade the organic compounds resulting in production of methane and carbon dioxide as final byproducts. The digestive metabolism of anaerobic microorganisms is complex and involves several intermediate steps. Briefly, insoluble organic material is hydrolyzed to produce simple soluble materials such as simple sugar, amino acids, and long chain fatty acids. Acidogenic bacteria degrade simple soluble organics to produce volatile fatty acid (VFA) and hydrogen, a process called acidogenesis. Subsequently, acetogenic bacteria produce acetate from VFA and solvents via a process called acetogenesis. There is a group of acetogenic bacteria that can synthesize acetate from hydrogen and carbon dioxide, a process called homoacetogenesis. Finally, methanogens use acetate or hydrogen to produce methane as the final product. There are other bacteria groups involved with the anaerobic degradation, such as sulfate reducing bacteria (Jeong *et al.*, 2005).

The fermentative bacteria use a variety of carbon sources (e.g., glucose) as substrate to produce hydrogen and organic acids (e.g., acetate or butyrate) through anaerobic metabolic pathway, i.e.,

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

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

Chloroform inhibits the activity of methyl coenzyme M reductase, the key enzyme for methane synthesis, so it can eliminate methanogenic activity (Weathers and

Parkin, 2000). Very dilute chloroform treatment was reportedly eliminates methanogens from soil without changing the major microbial community structure (Chidthaisong and Conrad, 2000). Chloroform can also inhibit the activity of Carbon Monoxide Dehydrogenase (CODH) complex, the key enzyme system for the homoacetogenesis, which consumes hydrogen to produce acetate (Chidthaisong and Conrad, 2000). Chloroform treatment of granules has been found from our previous study to be an effective method for eliminating methanogenic activity and for switching a methane producing system to a hydrogen producing system (Hu and Chen, 2007). This selective inhibition of methanogenic activity allows normal hydrogen production, as long as chloroform concentrations are low. From our previous SEM and TEM analysis, there might be a microbial community change during the chloroform treatment: that is, methanogens should be eliminated, while hydrogen producing species such as *Clostridium* species should be accumulated. Further microbial analysis is needed to study the detailed community change during this process.

The microbial community changes inside the granules during the chloroform treatment and continuous culture were investigated in this study. Culture-independent method, the analysis of terminal restriction fragment length polymorphisms (TRFLP) of 16S ribosomal RNA (rRNA) was used first to analyze different samples in each condition. TRFLP offers a rapid overview of inter-individual differences in granular microbial communities (Liu *et al.*, 1997). When comparing the TRFLP data generated from different communities, variation can be found in the number and size of peaks and can be evaluated by adapting community parameters such as richness and evenness. These data provide quantitative information on the compositional differences of granular

microbial communities. As long as the community composition is consistent for each condition, and then only representative samples in each condition can be chosen to build the clone libraries for detailed description of community composition.

3. Materials and methods

Sample preparation

Condition 1 was the methanogenic granules, taken from a UASB treating starch wastewater. Condition 2 was the chloroform treated granules after 5 days culture and Condition 3 was the samples taken from the upflow reactor after 15 days culture of chloroform treated granules. Chloroform treatment and continuous culture were described and reported in Chapter 3 and Chapter 4 (Hu and Chen, 2007).

DNA extraction

Methanogenic granules were taken from a UASB treating starch wastewater. Chloroform treated granules were taken from the second batch culture (5 days) inoculated with the granules treated with 0.05% chloroform. Hydrogen producing granules were taken from the outlet of the continuous reactor with the fermentation broth. Genomic DNA was isolated with a commercial kit (QIAamp, Qiagen, Valencia, CA), and resultant DNA was stored at 4°C. The concentration of the genomic DNA was measured by both OD 260 and electrophoresis in 1.5 % agarose gel followed by staining with ethidium bromide and visualization under ultraviolet light.

PCR amplification

Internal fragments of 16s rRNA sequences were amplified from genomic DNA (gDNA) using universal bacterial primers 8f and 926r (based on *Escherichia coli* positions) (Zhou *et al.*, 2004), and universal archaea primers 112f and 934r separately (Baker *et al.*, 2003). Amplification was performed in 50 µl (total volume) reaction volumes that contained 20 ng (1 ul) of sample DNA, 1U of AmpliTaq DNA polymerase (Roche Molecular System, Inc., Branchburg, New, Jersey, USA), 1 x AmpliTaq reaction buffer, 1.5 mM $MgCl_2$, 100 mM dNTP, 5% DMSO and 0.05 mM each primer (Applied Biosystems, Middletown, CT). Initial DNA denaturation at 94°C in a PTC-100 Programmable Thermal Controller (MJ Research, Inc.) for 5 min followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 55°C (bacteria primers) or 60°C (archaea primers) for 1 min, and elongation at 72°C for 2 min, which was followed by a final extension at 72°C for 10 min. To confirm amplicon production, the mixture was analyzed by electrophoresis in 1.5 % agarose gel followed by staining with ethidium bromide and visualization under ultraviolet light.

Terminal restriction fragment length polymorphisms (T-RFLP) analysis

Fluorescent labeled primers (see above) were used to amplify gDNA to obtain a PCR product. C.a. 40 ng – 60 ng of each PCR product was digested at 37ºC for 3h with 10 U restriction enzymes HaeIII and MspI separately with enzyme-specific buffer. This mixture was then incubated for 20 min at 65°C to inactivate enzymes. This product (1µl) was then mixed with 0.5 µl of GeneScan-500 ROX size standard (Applied Biosystems) and 10 µl of formamide, then denatured at 95ºC for 5 min and immediately placed on ice

for at least 5 min. The T-RFLP analysis was performed on the ABI PRISM 3100 Genetic Analyzer (3100 PRISM Genetic Analyzer, Applied 150 Biosystems).

16s rRNA gene clone library

A clone library from the amplified DNA was generated with the TOPO TA Cloning kit. C.a., 20ng fresh 16s rRNA PCR product were cloned into PCR2.1-TOPO vector (20 ng) (Invitrogen Corporation, Carlsbad, NM, USA) using a vector/insert ratio of 1:1 and procedures recommended by the manufacturer. TOPO cloning reaction products (3 μl) were transformed into a vial of One Shot Chemical Competent *E.coli* (Invitrogen Corp) that were subsequently plated onto Luria-Bertani-Hogness agar plates containing 100 mg/ml kanamycin and 20 mg/ml x-gal to verify blue/white selection and incubated overnight at 37°C. Approximately 96 white, well-isolated white colonies were randomly selected from each library and grown in 1.5 ml of Luria-Bertani broth containing 100 mg/ml kanamycin in 96-well micro liter plates for 48 h. These cells were then harvested and the plasmid DNA was isolated using QIAprep96 Turbo Miniprep Kits (QIAGEN Inc) using a QIAGEN BioRobot 3000 workstation.

Sequencing and Sequence Analysis

Approximately 600 isolated plasmids with cloned inserts (approximately 920 bp length for all libraries) were sequenced with both bacteria 926r and archaea 934r primers. The insert sequences were determined using Big Dye ver. 3 cycle sequencing reactions (Applied Biosystems), and resolved using an automatic sequencer (3100 PRISM Genetic Analyzer, Applied 150 Biosystems). Sequences were trimmed to exclude the PCR primer

binding sites and manually corrected with Chromas 2 (Chromas Version 2. 22; http://www.technelysium.com.au/chromas.html). For identification of closest relatives, insert sequences were compared to those available in the Ribosomal Database Project [RDP] (Maidak et al., 2001) and GenBank [http://www.ncbi.nlm.nih.gov/] databases using standard nucleotide-nucleotide BLAST program [blastn]; [http://www.ncbi.nlm.nih.gov/].

4. Results and Discussion:

T-RFLP analysis

Optimization experiments demonstrated that 10 ng community gDNA worked best for PCR reactions (high concentration of gDNA had strong inhibited PCR amplification, especially for the archaea primers). The annealing temperature had to be increased to 60ºC to enhance specificity for archaea targets, although *Lactobacillus casei* was still amplified.

T-RFLP images showed similar peaks within each treatment, which indicates that there were limited differences in microbial communities within treatments, but we observed several differences between treatments. Bacteria T-RFLP image (Figure 5-1) showed many peaks from samples of methanogenic granules, and fewer peaks from the samples of chloroform treated granules. Some new peaks showed on the image for the chloroform treated granules and the relevant abundance is different from the methanogenic granules. These data confirm that there is a bacterial community change during the chloroform treatment. T-RFLP image of samples from the upflow reactor showed the fewest peaks, but these appear to have corresponding peaks in both

methanogenic and chloroform treated granule samples. This suggests that with the reactor, the community became less diverse relative to the other conditions tested herein.

Archaea T-RFLP image (Figure 5-2) showed many peaks from the sample of the methanogenic granules. Interestingly, samples of chloroform treated granules retained most of these peaks despite an absence of methanogenic activity. This might be explained by the presence of gDNA in metabolically inactive methanogens, or non-specific amplication of non-archaea targets. To examine this question further, we generated a 16s rRNA clone library.

16s rRNA libraries

Over 400 base of the sequence of each clone was compared to Genebank. We assigned presumptive genus names if there was ≥ 90% sequence similarity to an existing sequence. Clones with ≥ 97% sequence similarity were presumed to be of the same species, and clones with <90% sequence similarity were designated as novel phylotypes. For the bacteria clone library (Table 5-1), there were four major groups of bacteria in the methanogenic granule sample, including *Paenibacillus sp*. (13/86), *Enterococcus sp*. (7/86), *Clostridium sp.* (43/86) and *Bacteroides sp*. (16/86) (*Bacteroides sp*. and *Petrimonas sulfuriphila* are combined here owing to a close phylogenetic relationship). There were also four novel phylotypes in the community. After chloroform treatment, *Paenibacillus sp.* and *Bacteroides sp*. were reduced or eliminated from the granules, while hydrogen producing species *Eubacterium sp*. (22/95) and *Enterococcus sp.* (18/95) increased (Nath and Das, 2004b). For the sample from upflow reactor, both *Enterococcus sp*. and *Eubacterium sp*. disappeared after 15 days flushing. *Clostridium sp*., which

remained almost the same prevalence in the library following the chloroform treatment, dramatically increased inside the upflow reactor, this is consistent with a shift towards hydrogen production. The *Clostridium* species composition appeared to change with the chloroform treatment and continuous culture. *Clostridium butyricum*, a typical hydrogen producing species with butyrate pathway, became the dominant representative over time. With the increase of the hydrogen partial pressure, the pathway shifts to the butyricum pathway, and then a non-hydrogen producing pathway, such as formation of lactic acid. Thus, it was not surprising that we observed *Lactobacillus sp.* (23/94) in upflow reactor sample as this was consistent with the increasingly favored non-hydrogen pathway.

For the archaea clone library (Figure 5-2), most phylotypes were uncultured archaeon or euryarchaeote sequences. The uncultured euryarchaeote gene for 16S ribosomal RNA, partial sequence, clone:EA02; the uncultured archaeon TA05 16S ribosomal RNA gene, partial sequence and the uncultured archaeon TA01 16S ribosomal RNA gene, partial sequence were closely related to *Methanosaeta concilii* while the uncultured archaeon Arc No. 5 16S ribosomal RNA gene, partial sequence and others were close to other *Methanogen sp.* The archaea clone library for the sample from the upflow reactor included a number of clones of *Lactobacillus sp.* indicative of low selectivity from the archaea primers. This can also explain the difference of T-RFLP between chloroform treated granule sample and samples from upflow reactor. Overall, the archaea clone library for chloroform treated granules and sample from upflow reactor were similar, while there was a significant archaea community change during the chloroform treatment. Most of methanogens disappeared after chloroform treatment, while *Methanosaeta concilii (Methanothrix soehngnii)* clones remained constant in the

library. Most theories about granulation indicate that the acetotrophic methanogens *Methanosaeta concilii* (*Methanothrix soehngnii*) play a key role in the granulation (Pol *et al.*, 2004). Hydrogen producing system can form granules too. In our experiments, most of the *Methanogen sp*. was presumably eliminated during the chloroform treatment, consistent with the measured switch from methane producing system to hydrogen producing system. The presence of *Methanosaeta concilii* probably explains why granulation remained stable when the system produced mostly hydrogen (hydrogen producing bacteria are not known to produce granules). *Methanosaeta concilii*, which was renamed as *Methanosaeta concilii*, is a filamentous methanogen species that can only use acetate as its sole energy source and cannot use hydrogen as raw material. It is difficult to reconcile the presence of active methanogens in the absence of detectable methane, although if the total population remained very low methane production may have remained undetectable while PCR amplification of the target template might be sufficiently sensitive to amplify this genetic marker. Further research on the pathway of *Methanosaeta concilii* or its role in hydrogen producing granulation is needed.

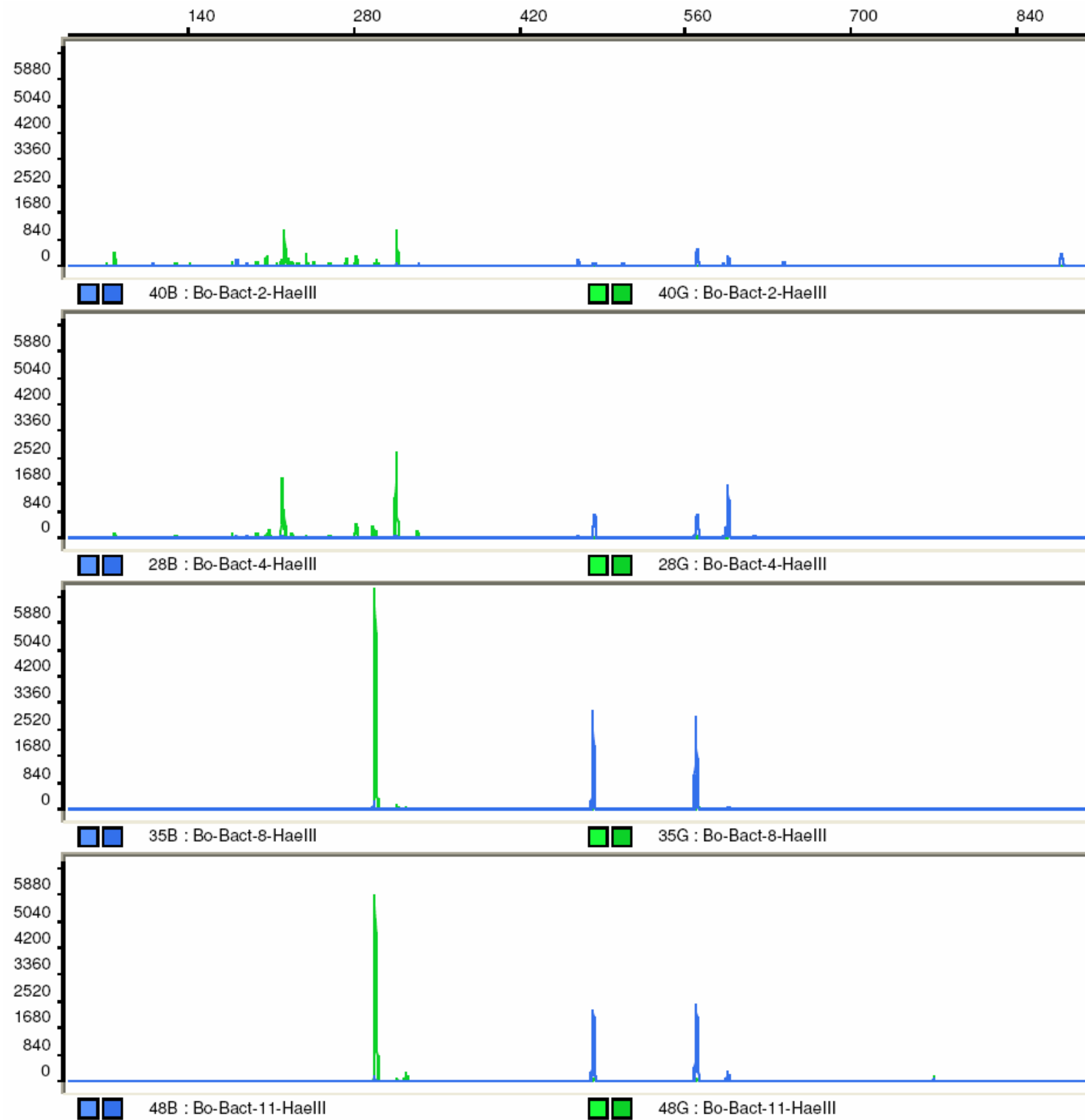


Figure 5-1. T-RFLP analysis of PCR products with bacteria primers. Bo-Bact-2-HaeIII represents methanogenic granule PCR products digested with HaeIII restriction enzyme. Bo-Bact-4-HaeIII represents chloroform treated granule PCR digested with HaeIII restriction enzyme. Bo-Bact-8-HaeIII and Bo-Bac-11-HaeIII represent samples from upflow reactor for 10 and 15 days culture.

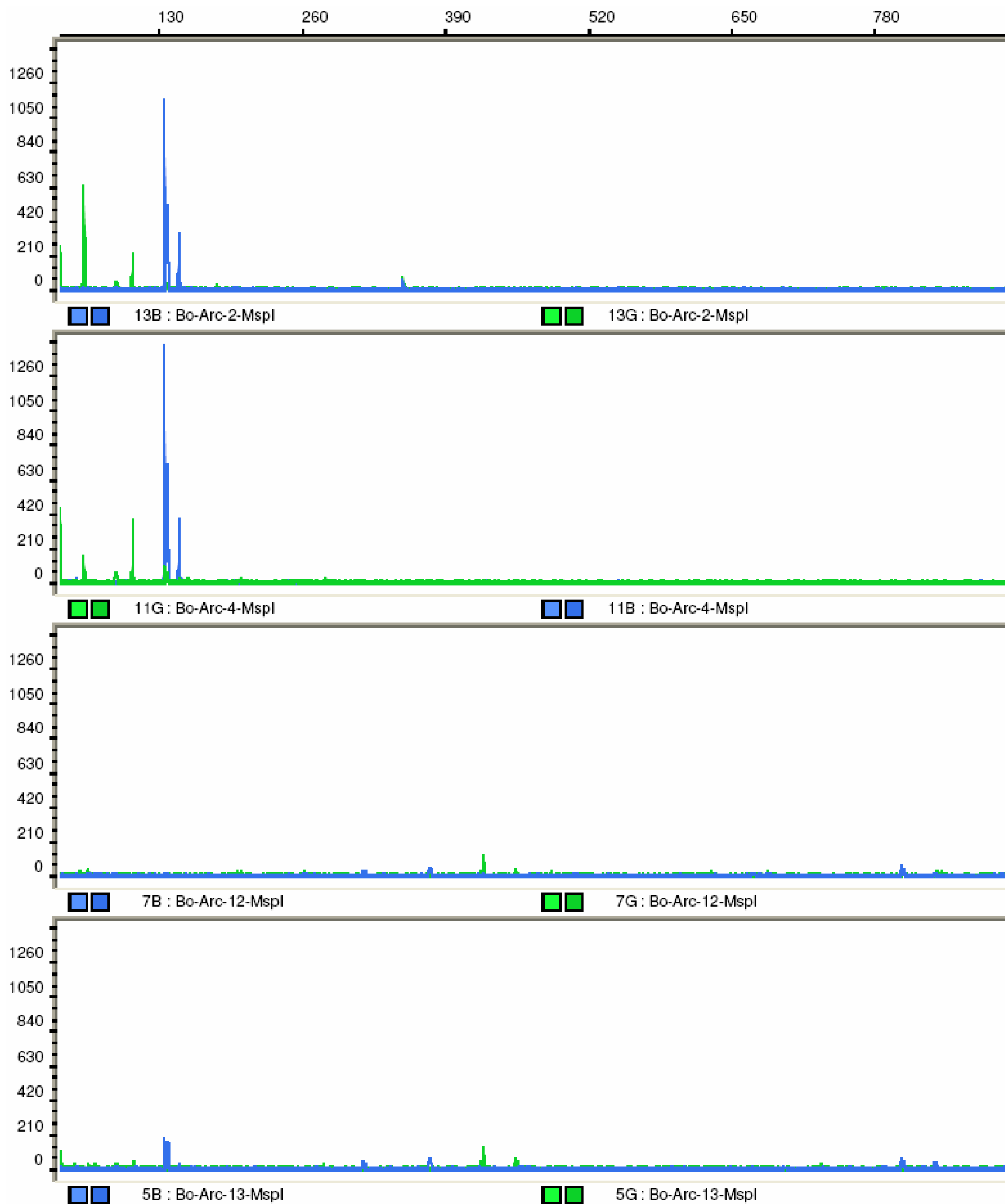


Figure 5-2. T-RFLP analysis of PCR products with archaea primers. Bo-Arc-2-MspI represents methanogenic granule PCR products digested with MspI restriction enzyme; Bo-Arc-4-MspI chloroform treated granules; Bo-Arc-8-MspI and Bo-Arc-11-MspI samples from the upflow reactor for 17 and 20 days.

Table 5-1. Bacteria phylotypes in 16S rRNA gene clone libraries

Phylotype*	B1 (n= 86)	B2 (n=95)	B3 (n=94)	
AY382190 Paenibacillus sp. R-7204.	12	0	0	
AY382190 Paenibacillus sp.	1	0	0	*
AF135452 Eubacterium sp. 1275b	0	19	0	
DQ411811 Enterococcus avium; ATCC 14025	7	17	0	
DQ411811 Enterococcus sp.	0	6	0	*
DQ019166 Exiguobacterium aurantiacum; DSM 6208	0	2	0	
X76746 Clostridium neopropionicum	13	0	0	*
AY781385 Clostridium intestinale	3	0	0	
Y18175 Clostridium sartagoforme (T); DSM 1292	9	12	0	
Y18175 Clostridium sp	0	1	0	*
AF005092 Clostridium sp. SP3	0	2	0	*
AB032556 Clostridium paraputrificum; M-21.	9	0	0	
AY949856 Clostridium sp.	3	0	0	*
X95274 Clostridium sp.; LMG 16094.	1	0	0	
DQ196622 Clostridium sp. BL-30.	1	0	0	
AY548781 Clostridium sp. PPf35E1.	2	1	0	*
AF227826 Clostridium sp. 75064	0	1	0	*
U59278 Clostridium sp	1	0	0	*
U51843 Clostridium chauvoei (T); ATCC 10092T.		2	0	
U51843 Clostridium sp	0	17	0	*
AF005092 Clostridium sp. SP3.	0	2 (*)	4	
AY862517 Clostridium sp. HPB-55.	0	1	0	*
AF316590 Clostridium butyricum	0	0	55	
AJ305238 Clostridium sp.	0	0	2	
Clostridium	42	39	61	***
Y18189 Anaerobacter sp	0	1	0	*
AB003390 Bacteroides sp.; sp14	15	0	0	
AJ319867 Dysgonomonas sp.	1	0	0	*
AB243672 anaerobic filamentous bacterium sp.	0	1	0	*
DQ071273 Thermovirga sp	0	2	0	
X82874 Syntrophobacter sp.	1	1	0	
X82875 Syntrophobacter sp.	1	2	0	*
AJ133796 Syntrophus sp.	0	1	0	*
AY960767 bacterium Ellin504 sp.	0	1	0	*
CP000423 Lactobacillus casei ATCC 334.	0	0	22	
DQ523490 Lactococcus lactis; LA35.	0	0	1	
DQ223887 Enterococcus casseliflavus; P10.	0	0	1	
DQ358738 Klebsiella sp. JT42.	0	0	4	
AJ871856 Klebsiella oxytoca; SB71.	0	0	2	
AB180662 Stenotrophomonas sp. Toyama-1.	0	0	1	
Novel phylotype	4	0	0	

B1 represents bacteria clone library for the sample from condition 1, the methanogenic granules; B2 as condition 2, chloroform treated granules; and B3 as condition 3, sample from upflow reactor.

* Phylogenetically related clones which on average had ≥ 90% sequence similarity to a reference strain were presumed to be of the same genus, and clones which on average had ≥ 97% sequence similarity were designed with the corresponding epithet. Number of clones with ≤90% and >97% sequence similarity were parenthesized. Over 400 bp was compared for each clone. n, number of clones analyzed.

*** Summary of *Clostridium sp.*

Table 5-2. Archaea phylotypes in 16S rRNA gene clone libraries

Phylotype	A1 (n= 95)	A2 (n=96)	A3 (n=60)
Methanothrix soehngenii gene for 16S ribosomal RNA	52	82	48
Methanosaeta harundinacea	28	3	0
Uncultured archaeon 16S rRNA gene, clone GZK31	8	2	8
Uncultured archaeon clone H11T20L165 16S ribosomal RNA gene, partial sequence	0	0	1 *
Uncultured Methanosaeta sp. clone M1 16S ribosomal RNA gene, partial sequence	0	0	1 *
Uncultured archaeon partial 16S rRNA gene, clone PM25		2 *	0
Uncultured archaeon 2C300X 16S ribosomal RNA gene, partial sequence	1 *	2 *	0
Unidentified archaeon 16S rRNA gene, clone 321	1 *	1 *	0
Uncultured archaeon clone CBd-366G 16S ribosomal RNA gene, partial sequence	1	0	0
Uncultured Methanomethylovorans sp. Clone KB-1 2 16S ribosomal RNA gene, partial sequence	1	0	0
Uncultured euryarchaeote gene for 16S ribosomal RNA, partial sequence, clone:BA03	1	0	0
Uncultured archaeon partial 16S rRNA gene, clone OuO-24		1	0
Uncultured archaeon partial 16S rRNA gene, clone OuI-11		1	0
Unidentified archaeon clone vadinDC06 16S ribosomal RNA gene, partial sequence	2	1	0
Methanobacterium sp. C5/51 16S rRNA gene, strain C5/51	1		0
Uncultured archaeon clone D10T20L254 16S ribosomal RNA gene, partial sequence	1	1	0
Novel Phylotype			1
Lactobacillus			28

A1 represents archaea clone library for the sample from condition 1, the methanogenic granules; A2 as condition 2, chloroform treated granules; and A3 as condition 3, sample from upflow reactor.

* Phylogenetically related clones which on average had ≥ 90% sequence similarity to a reference strain were presumed to be of the same genus, and clones which on average had ≥ 97% sequence similarity were designed with the corresponding epithet. Number of

clones with ≤90% and >97% sequence similarity were parenthesized. Over 400 bp was compared for each clone. n, number of clones analyzed.

A1 to A3 represent samples amplified with archaea primers. n, Number of clones analyzed.

CHAPTER SIX

CONCLUSIONS AND RECOMMENDATIONS FOR FUTURE WORK

A novel immobilization method was developed for biological hydrogen production in this study that included three efforts: chloroform treatment to switch methane production system to the hydrogen production system; taking chloroform treated granules as immobilized hydrogen producing sludge for immobilized hydrogen fermentation; and integrating the immobilized hydrogen fermentation into current anaerobic digestion system. The main conclusions and recommendations for future work obtained from this research are summarized below:

1. Chloroform treatment to eliminate methanogenic activity

Chloroform treatment was the most effective method, compared to acid and heat treatments, for directly converting methanogenic granules to hydrogen producing granules. Low dose chloroform treatment eliminated methanogenic activity while allowing normal hydrogen production.

2. Biological hydrogen production with chloroform treated granules

Chloroform treated anaerobic granular sludge could be used in the immobilized hydrogen culture. Furthermore, the chloroform treated granules could be re-used for over four fed-batch cultures with pH adjustment, and could be repeatedly cultured 8 times without noticeable damage. Continuous culture with chloroform treated granules showed that the granule structure was stable for over 15 days and new granules started to form after 10 day's operation. The hydrogen productivity reached 11.6 L/L/day at HRT of 5.3

hours. The optimum initial pH of the culture medium was neutral and the optimum glucose concentration of the culture medium was below 20 g COD/L. This level of production has an average yield as reported, but the treatment of methanogenic granules and then using the treated granules as immobilized hydrogen producing sludge demonstrated advantages over other immobilization methods in that the granules provide hydrogen producing bacteria with a protective niche, long duration and excellent settling velocity.

3. Integration of immobilized hydrogen production with current anaerobic digestion

The integrated batch cultures of immobilized hydrogen fermentation with chloroform treated granules and methane production with untreated granules provided 1.01 mol hydrogen and 2 mol methane per mol glucose. The hydrogen yield is similar as those reported in the literature and the methane yield is close to theoretical value. 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 the system.

4. Community change during chloroform treatment

Chloroform treatment, which eliminated the methanogenic activity, caused a major microbial community change inside the granules. Most of *Methanogen sp*. was eliminated by chloroform treatment, explaining the switching of methane production system to hydrogen production system. Unexpectedly, however, *Methanosaeta concilii*

was not eliminated from the hydrogen producing system, although this might help to explain the successful formation of robust granules in this system.

5. Future work

Integration of immobilized hydrogen fermentation with chloroform treated granules into current UASB anaerobic digestion system needs investigation, especially with respect to scaling the process and determining if the two-stage process is stable under long-term reactor conditions. In addition, quantitative PCR could be used to better understand how *Methanosaeta concilii* is being maintained in this system. Further research on the pathway of *Methanosaeta concilii* or its role in hydrogen producing granulation is also needed.

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