Development of a continuous hydrogen fermentation process

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Development of a continuous hydrogen fermentation process

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ABSTRACT

For global environmental considerations, production of hydrogen by biological reactions from renewable organic waste sources represents an important area of bioenergy production. Many investigations have been conducted using pure cultures of hydrogen-producing bacteria. Some of the studies showed promising results. However, since there is bacterial contamination in wastewater, it is difficult to maintain the purity of a wastewater treatment reactor. Therefore the treatment of wastewater has to be a mixed culture process. Although some researchers tried using mixed culture, none of their experiments attained stable hydrogen production – the hydrogen production was stopped by either microbial shift or metabolic alteration. So the present study was aimed at developing a feasible anaerobic hydrogen fermentation process, in which the consistency of the enriched mixed culture can be maintained. Environmental conditions such as pH, feeding substrate and intermediates production were also evaluated to maximize hydrogen production.

In this study, both continuous flow experiments and batch tests were employed. Two New Brunswick Bio-flow 2000 fermentors with different hydraulic retention times were used for the continuous flow study and batch tests were conducted in 250 mL serum bottles. Non-sterile sucrose/starch solution was used as substrate for the experimental study. Through a 5-stage operation of the continuous flow reactors, it was found that with the addition of a sludge activation and return process, consistent hydrogen production could be attained. The maximum hydrogen production obtained for the continuous flow reactors was 6.5 L/day for the reactor with an organic loading rate of 10.8 g COD/L-day and 3.5L/day for the reactor with an organic loading rate of 7.1 g COD/L-day. A high loading was found to be more favorable for hydrogen production.

Under same operation conditions, the hydrogen conversion potential from sucrose was higher than that from starch. pH had a significant effect on both hydrogen production potential and hydrogen production rate. The optimum pH range for hydrogen production obtained from batch tests was between 5 and 5.5. Major intermediates such as propionate, acetate and butyrate detected in the process could also affect hydrogen production.

CHAPTER 1. GENERAL INTRODUCTION

Introduction

As a sustainable energy source with minimal or zero use of hydrocarbons, hydrogen is a promising alternative to fossil fuels. It is a clean and environmentally friendly fuel, which produces water instead of greenhouse gases when combusted. In addition, hydrogen can be used in fuel cells to produce electricity without any combustion. Last but not the least, there is also a need to eliminate waste generated by humans in an environmentally friendly manner. Much of this waste is carried in water and is the product of food processing (e.g., waste from corn, soybean, rice and meat processing plants). Conventionally, these waste streams are high in energy content measured as chemical oxygen demand (COD), are treated in aerobic wastewater treatment plants. Aerobic treatment methods could be used to treat these wastes but the energy content cannot be recovered and a significant amount of energy would have to be added to supply aeration. The dual needs of energy production and waste reduction has brought forth anaerobic digestion process as a viable process to produce energy in the form of hydrogen or methane and stabilize wastes. By using a similar hardware, the economics of hydrogen fermentation could be more favorable than industrial methane fermentation due to its faster reaction rate. Besides, hydrogen has a heating value of 61,100 Btu/lb while methane has a heating value of 23,879 Btu/lb, which is only about one third that of hydrogen.

Compared to biohydrogen fermentation from wastes, some of the conventionally used methods of hydrogen production can only generate energy but not accomplish waste reduction, e.g. electrolysis, which involves electrically splitting water into hydrogen and oxygen, or chemical methods such as cracking of hydrocarbons into hydrogen.

Furthermore, these methods require large inputs of electricity derived from fossil fuel combustion. Because they use conventional polluting energy sources, they do not qualify as alternative renewable energy sources. For global environmental considerations, microbial hydrogen production or production of hydrogen by biological reactions from renewable organic waste sources represents an important area of bioenergy production.

Many scientific publications have reported on the groundwork for creating renewable hydrogen production systems through either "biophotolysis" - hydrogen production through photo-synthetically splitting water – or "dark" fermentation of low-cost substrates or wastes. Photo processes usually require high activation energy for the reaction, and naturally occurring organisms examined so far show rather low rates of hydrogen production; the consequence is thus low solar energy conversion efficiencies. Hydrogen production by the dark process is much simpler than photo process on technical grounds, and the dark process can generate hydrogen from a large range of carbohydrates frequently obtained as refuse or waste products.

Anaerobic bacteria use organic substances as the sole source of electrons and energy, converting them into hydrogen. The reactions involved in hydrogen production [Equations 1 and 2] are rapid and these processes do not require solar radiation, making them useful for treating large quantities of wastewater by using a large fermentor.

$$\text{Glucose} + 2H_2O \rightarrow 2\ \text{Acetate} + 2CO_2 + 4H_2 \qquad \Delta G = -184.2\ \text{kJ} \qquad (1)$$

$$\text{Glucose} \rightarrow \text{Butyrate} + 2CO_2 + 2H_2 \qquad \Delta G = -257.1\ \text{kJ} \qquad (2)$$

Since they cannot utilize light energy and the free energy of the above equations is only –46 kJ/mol hydrogen reaching bottom of the system, the decomposition of organic substrates is incomplete. Further decomposition of the remaining organic substances

(acetic acid) has to be completed in other biological processes. Nevertheless, these reactions are still suitable for pre-treating wastewater, and they could be followed by methane production for maximum energy conversion. Another option with great potential is combining H_2 fermentation with production of high value co-products such as acetic acid and other organic acids from organic wastes. Biohydrogen production from wastes requires comparatively less time than methane fermentation, which translates into a smaller capacity of the bioreactor and lower capital costs for construction. Where a local market for H_2 exists, or if small-scale (< 1 M Watt) fuel cell technology becomes competitive, H_2 waste fermentation could be favored over methane fermentation. Thus, dark H_2 fermentation of wastes could be competitive with fossil fuel-derived H_2, providing a promising approach to practical biohydrogen production.

However, most of the research on biohydrogen production from organic substances over the past two decades has focused on the use of photosynthetic bacteria (Sasikala *et al.*, 1993) or pure culture of fermentative bacteria. A successful process of H_2 fermentation using mixed culture from industrial waste has not yet been demonstrated, the challenges faced here are: 1. minimizing contamination from the wastewater and secure the consistency of enriched culture for hydrogen production; 2. maximizing hydrogen production by controlling environmental conditions such as pH, temperature, substrate and organic loading rate etc.. This is because theoretical maximum fermentative H_2 yields are 4 moles of H_2 per mole of glucose substrate as shown in equation (1) and (2), but in practice, only a maximum of 10-20% of the energy content in glucose is recoverable as H_2 (Benemann, 1998) due to the limited metabolic energy derived from H_2 fermentation (Schink, 1997). Nevertheless, by controlling the environmental conditions, microbial growth can be arrested and the thermodynamics of the reaction could be shifted

in favor of H_2 production (Heijen, 1995). Metabolic engineering could also be used to redirect bacterial metabolism towards H_2 production (Keasling *et al.*, 1998).

Clearly, more research and development is required. The objectives of the present study are: 1. to develop a consistent continuous flow biohydrogen fermentation process by imposing selection pressures; 2. to investigate the effects of environmental conditions on hydrogen production in order to provide valuable information for future hydrogen production optimization.

Thesis Organization

Separate manuscripts were prepared addressing each of the two objectives of this study for submission to a peer-reviewed journal. The first paper evaluates the performance of two continuous flow reactors at different operating conditions. The second paper describes the effects of pH, intermediate production etc. on hydrogen production based on batch tests. The two papers constitute the major part of the thesis. A chapter for general conclusions and recommendations is also prepared. Literature review is included under general introduction. References for all chapters are listed at the end of the thesis.

Literature Review

In anaerobic mineralization, the organic matter is converted to methane and carbon dioxide via a series of interrelated microbial metabolism, including hydrolysis/fermentation, acidogenesis, and methanogenesis. Fermentative bacteria hydrolyze and ferment carbohydrates, proteins, and lipids to volatile fatty acids, which are further converted to acetate, carbon dioxide, and hydrogen by acidogenic bacteria.

These products are finally converted to methane by methanogenic bacteria. For biohydrogen production, we will limit our focus to the fermentation phase. The schematic diagram of substrate metabolism in hydrogen fermentation is shown in Figure 1 as below.

In regard to the anaerobic microbial food chain, if the bioactivity of hydrogen-consumers is inhibited, the anaerobic fermentation process will possess significant capacities for transforming organic matter, such as carbohydrates, into hydrogen gas. Most studies on the production of hydrogen gas involve the inhibition of hydrogen consumers - especially methanogens (Lay *et al.*, 1999a; Roychowdhury *et al.*, 1988; Sparling *et al.*, 1998; and Ueno *et al.*, 1995). To date, acetylene, 2-bromoethanesulfonate (BES), as well as aeration have been used to inhibit methanogens. According to Sparling *et al.* (1998), the inhibition of methanogens is essential for efficient hydrogen production.

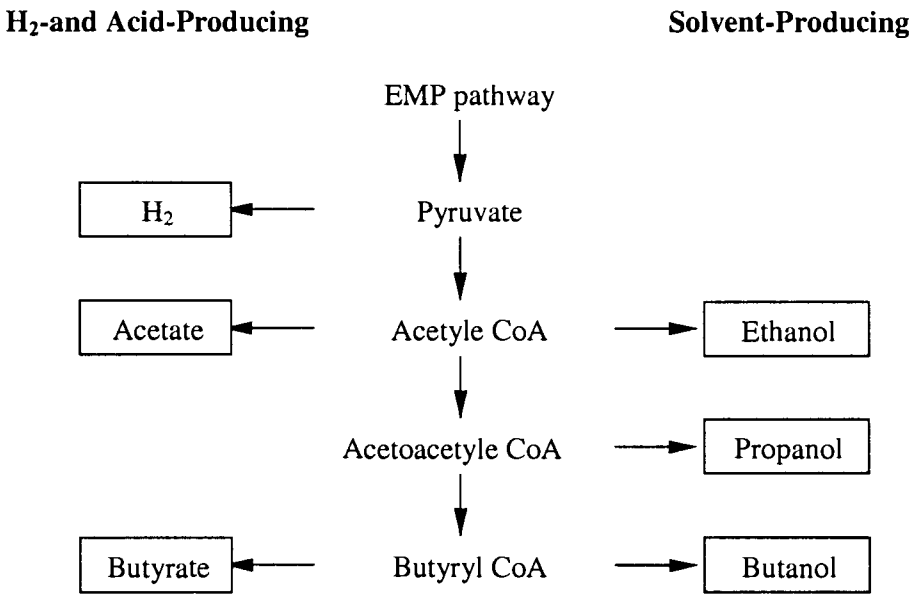

Figure 1. Schematic diagram of substrate metabolism in hydrogen fermentation. Adapted from Yan et al. (1988).

Acetylene can be used as an inhibitor but it has been known to inhibit the hydrogenase enzyme of other anaerobes. It has been mentioned in their study that a 5% acetylene concentration inhibits 50% of the hydrogenase activity in some cultures. BES could be used but there are mutants resistant to this chemical. Another method of inhibition is heat shock. If a liquid medium containing bacteria is subjected to high temperatures, many of the bacteria will not survive. Those that do survive are likely to be sporeformers. Incubation for ten minutes at temperatures between 70°C and 90°C is sufficient to eliminate thermophilic nonsporeformers (Sparling *et al.*, 1998).. Once the liquid medium is subjected to natural bacterial growing conditions, these spores can germinate producing viable, growing cells.

A group of acidogens of the genus *Clostridium* has been well studied for its ability to form spores and for its potential to generate hydrogen gas (Brosseau and Zajic, 1982; Lay *et al.*, 1999b; Ueno *et al.*, 1995). There are many types of *Clostridium* including saccharolytic and proteolytic forms. Saccharolytic acidgoens ferment carbohydrate and proteolytic acidogens degrade protein or amino acids. Saccharolytic clostridia are spore formers. Their fermentation pathway is exemplified by the *Clostridium* type strain-*C. butyricum*, which produces butyric acid as the major fermentation product together with CO_2, acetate and H_2, the reactions are shown above as Equation (1) and (2). This pathway is found in approximately 50% of all clostridial species that have been isolated to date. There are totally about 22 species that can produce acids plus molecular H_2. *Clostridium butyricum* has a high hydrogen – producing capability, corresponding to 2.4 mol/mol-hexose. Other fermentation reactions by sacchrolytic clostridia are those leading to the production of propionate by *C. arcticum* (Jodan and McNicall, 1979), succinate by *C. ccoides* (Kaneuchi *et al.*, 1976 a) and lactate by *C. barkeri* (Stadtman *et al.*, 1972).

As mentioned, a heat shock treatment could be used to select an enriched culture of *Clostridia* and other spore-formers such as *Bacillus*. However, if spore-forming bacteria are selected as hydrogen producers, sporulation triggered by the onset of other unfavorable conditions, such as the depletion of an essential nutrient and presence of radiation, and toxic chemicals etc. should also be manipulated to help the hydrogen production. According to Kim *et al.* (1967), many Bacillus and Clostridium species sporulate satisfactorily at their optimal growth temperatures. Shih *et al.* (1996) stated that sporulation-promoting ability was present in vegetative and sporulating CSFs (concentrated culture supernatant fluid) of both enterotoxin-positive (Ent+) and Ent- strains. Waldburger *et al.* (1993) also reported that the CSF from Bacillius subtilis 168 stimulating sporulation of B.subtilis BR 151. The sporulation-promoting substances released by bacterial cells was termed sporulation factor. It was described before as sporogen (Srinivasan, 1966) for Bacillus or extracellular differentiation factor (Grossman, 1988). It was found that the sporulation-promoting ability of C. perfringens CSF was concentration dependent. Some organic acids were thought as inducers of sporulation, and the acidic environment may serve to stimulate sporulation (Shih, 1995), though in his later study (Shih and Labbe,1996), it was found that the organic acids such as acidic acid is unlikely the responsible agent in the CSF.

The clostridial genus is an obligate anaerobic heterotroph that does not contain a cytochrome system (Nandi and Sengupta, 1998). This genus produces hydrogen using the activities of pyruvate-ferredoxin-oxidoreductase and hydrogenase enzymes and has a desired pH range of 6.5 to 7.0, which is similar to that of methanogens (Grady *et al.*1999; Minton and Clarke, 1989). The activity of hydrogenase, an iron-containing enzyme inhibited by low pH, was reported to be one of the most important factors in the overall hydrogen fermentation (Holt *et al.*, 1988; Afschar et al., 1986; Dabrock et al., 1992; Ueno

et al., 1996). In certain cases, the hydrogen evolution activity of hydrogenase may be suppressed in the presence of high levels of hydrogen (Kondratieva, 1983). A decrease in hydrogen concentration will favor hydrogen formation and permit the bacteria to metabolize acetyl-CoA through the energy-efficient path leading to acetate and ATP production. Alternatively, increased hydrogen concentration inhibits the formation of hydrogen via hydrogenase (Lamed *et al.*, 1988).

In typical anaerobic processes, hydrogen is produced in a step called anaerobic oxidation. Hydrogen is produced during the exponential growth phase of *Clostridia*. When the population reaches the stationary phase of growth, solvents or alcohols are produced. This shift occurs when the pH drops to 4.5 (Byung and Zeikus, 1985). Apparently, the build up of volatile fatty acids and hydrogen during the exponential growth phase inhibits this activity. While other researchers (Gottwald and Gottschalk, 1985) found that the shift occurred at a pH level above 5.7, due to enzyme synthesis or enzyme activation, which is necessary for solvent production. Accordingly, it is important to remove excess hydrogen from the system and control the pH to maintain hydrogen production.

According to Harper and Pohland (1985), hydrogen must be continually removed from the system to assure the continued production of acetic acid, which is the main precursor to methane. If H_2 builds up in the system, higher molecular weight acids such as butyric and propionic acids are produced instead of acetic acid (Grady *et al.*, 1999). Lay et al. (1999a) determined that most of the hydrogen production coincides with the production of butyric acid and acetic acid. Van Andel *et al.* (1985) reported that more efficient fermentation of glucose increased over a broad range in parallel with the growth rate at very high glucose input concentrations and in parallel with decreasing partial pressure of hydrogen. The increasing $q_{acetate}/q_{butyrate}$ ratio at higher growth rates is

accompanied by an increased production of H_2. However, in hydrogen production, with the inhibition of methanogenesis, the conversion of acetic acid into carbon dioxide and methane is blocked, so acetic acid builds up in the system potentially lowering the pH to inhibitory levels.

Little information is available on the preferential acid uptake by the microorganisms. If methane formers are inhibited, hydrogen and acids must be removed by other means to control the pH for continuous hydrogen production. One way to achieve this goal is by using a continuously stirred reactor to help volatize the hydrogen gas and flush out the acids to ensure the continuous production of hydrogen. In this way, hydrogen is removed and some COD conversion can be accomplished, although this COD reduction is quite small compared to methanogenesis.

Much research has been done using pure cultures of bacteria to produce hydrogen (Brosseau and Zajic, 1982a, Kalia *et al.*, 1994; Karube *et al.*, 1976). Some of the work focused on immobilized pure cultures (Kumar *et al.*, 1995; Xu et al., 1995) while other studies used a completely mixed regime - chemostat. It is mentioned here that the comparatively high conversion rates from the immobilized cultures were probably due to the high biomass concentration supported by this method. However, Brosseau and Zajic (1982b) claimed that chemostats have a higher hydrogen production potential because immobilized cultures can become diffusion limited and volatilization of hydrogen gas may become a problem in an immobilized system.

Whether a chemostat or immobilized bacteria is used, the purity of any wastewater treatment reactor cannot be maintained due to the bacterial contamination in wastewater, therefore the treatment of wastewater has to be a mixed culture process. Akashah *et al.* (1996) showed the possibility of hydrogen production using a mixed microbial population from acclimated sludge taken from a local treatment plant.

Although conditions were favorable for the production of hydrogen gas in the acidogenesis phase and a high percentage of hydrogen was observed in the biogas, a stable continuous operation state could not be obtained. Substrate inhibition seemed to be a controlling factor.

In anaerobic waste treatment processes, separation of main reaction steps (methanogenesis and acidogenesis phases) has proven advantages and its controlled optimum conditions enable a stable production of methane gas and sludge metabolic characteristics of different bacterial groups (Lawrence and McCarty, 1970; Ghosh and Pohland, 1974). The role of hydrogen gas as a controlling tool in anaerobic digestion processes has been suggested and studied (Mosey, 1983, Mosey and Fernandez 1989, Harper, 1985). However, very few studies have been done to specifically exploit the acidogenic bacteria to produce only volatile acids and hydrogen gas. If acidogenic hydrogen production can be achieved before the acids are directed into the subsequent methanogenesis phase, the maximum benefit can be attained from anaerobic waste treatment.

In conclusion, optimization of biological hydrogen production must be based on further research on the effects of operating conditions such as pH, temperature, mixing, substrate loading, etc. to achieve a successful metabolic pathway shift.

CHAPTER 2. CONTINUOUS FLOW HYDROGEN FERMENTATION FROM SYNTHETIC WASTEWATERS

A paper to be submitted to Water Science and Technology

Ling Li and Shihwu Sung

Abstract

In this study, biological hydrogen production was from synthetic wastewater achieved at mesophilic temperatures using anaerobic, mixed cultures with natural inocula-compost material. The suitability and performance of continuous flow reactors for biohydrogen production was evaluated under different operating conditions such as pH, organic loading rates and different substrates. Batch tests were also conducted in parallel to evaluate the hydrogen conversion efficiency from different substrates. A heat shock method was applied as a selective pressure to inhibit nonspore-forming hydrogen-consuming bacteria. Non-sterile mineral salts-starch/sucrose medium was used as the synthetic substrate. The experimental results showed that an external sludge activation chamber could retain predominance of H_2 producers in reactor and ensure stability of the operation. Batch feeding of higher organic loading rates favored hydrogen production. The hydrogen conversion potential of starch was lower than sucrose, and a better conversion efficiency from starch could be attained at a lower pH level. However, addition of sucrose into starch could improve hydrogen production potential and hydrogen production rate from starch. Major intermediate products detected were volatile fatty acids such as acetate, butyrate and propionate. Although the COD conversion efficiency of hydrogen fermentation process was

limited, it could still be a good pretreatment method for industrial wastewater to maximize energy conversion from organic waste and reduce pollutants release into environment.

Key words:

Continuous flow hydrogen fermentation, sludge activation, sucrose, starch, organic loading rate, spore-forming bacteria, hydrogen production potential.

Introduction

The waste streams from food processing plants such as corn, soybean, and meat processing plants pose a major burden on the environment. Aerobic wastewater treatment conventionally used for the treatment of these wastes requires energy input to provide aeration whereas anaerobic digestion process can achieve dual benefits of energy production in the form of hydrogen or methane and waste stabilization. By using hardware that is similar to that used in industrial methane fermentation, the economics of hydrogen fermentation could be favorable due to its faster reaction rate. Additionally, hydrogen has a heating value of 61,100 Btu/lb while methane has a heating value of 23,879 Btu/lb, nearly one third that of hydrogen. Furthermore, the combustion product of hydrogen is a nonpollutant, water thus reducing greenhouse gas emission. However, most of the research on biohydrogen production from organic wastes over the past two decades has focused on the use of photosynthetic bacteria (Sasikala, *et al.*, 1993). Benemann (1996) concluded that the dark fermentation of wastes is a more promising approach to biohydrogen production than photosynthetic bacteria.

Last two decades have seen much research being carried out using pure cultures of bacteria for hydrogen production (Brosseau and Zajic, 1982a, Kalia *et al.*, 1994; Karube *et*

al., 1976). Some of the work focused on immobilized pure cultures (Kumar *et al.*, 1995; Xu *et al.*, 1995) while other studies used a completely mixed regime - chemostat. Whether a chemostat or immobilized bacteria is used, bacteria contaminate wastewater affecting the purity of any wastewater treatment reactor and therefore the treatment of wastewater has to be a mixed culture process. Some researchers (Akashah *et al.*, 1996; Uneo *et al.*, 1996; Mizuno *et al.*, 1997) showed the possibility of hydrogen production using mixed microbial population from acclimated sludge. However, satisfactory stability and molar yields have not been obtained in continuous flow studies with mixed culture due to either substrate inhibition or microbial competition.

In regard to the anaerobic microbial metabolic chain, if the bioactivity of hydrogen-consumers is inhibited, the anaerobic fermentation process will possess significant capacities for transforming organic matter, such as carbohydrates, into hydrogen gas. Most of the studies on the production of hydrogen gas involve the inhibition of hydrogen consumers - especially methanogens (Lay *et al.*, 1999a; Roychowdhury *et al.*, 1988; Sparling *et al.*,1998; and Ueno *et al.*,1995). To date, acetylene, 2-bromoethanesulfonate (BES), as well as aeration have been used to inhibit methanogens (Sparling *et al.*, 1998).

The genus, *Clostridium*, has been well studied for its ability to form spores and generate hydrogen gas (Brosseau and Zajic, 1982; Lay *et al.*, 1999a; Ueno *et al.*, 1995). According to Minton and Clarke (1989), over 50% of clostridia were found to produce butyric acid as the major fermentation product together with CO_2, acetate and H_2. Previous experimental study (Van Ginkel *et al.*, 2000a, 2000b) showed that using heat shock as a selection pressure, high hydrogen production could be achieved in batch experiments with sucrose as a substrate. The present study was intended to develop a continuous flow

hydrogen production process using same mixed culture obtained from natural inocula – compost material. A sludge activation and return process was included to retain enriched hydrogen-producing culture in the reactor. Other operation parameters such as pH, organic loading rate and mixing were also controlled to help maintain and optimize hydrogen production. Hydrogen conversion efficiency from different substrates was evaluated by continuous flow experiment supplemented with batch tests.

Methodology

Experimental Setup for Continuous Flow Experiments

Two Bioflo-2000 Modular Benchtop Fermentors (New Brunswick CO.), each with a working volume of 5 L (Figure 1), were used to carry out the experiments. One reactor was operated at a HRT of 20 hours; the other was operated at a HRT of 30 hours. Both of the reactors were operated at 37°C and the mixing speed was set at 250 rpm. Identical pH levels were maintained in both reactors. pH was monitored by a pH probe (Ingold Co.) and controlled by the addition of 4 N base solution (2N NaOH plus 2N KOH) regulated by a pH Control Module (New Brunswick pH-2000).

Compost material obtained from Iowa State University composting pile was used as the seed source. It was baked in the oven at 105°C for two hours and then cooled to room temperature in a desiccator. The baked compost material was ground and sieved through a No. 30 sieve. The sieved fines were mixed with distilled water, settled sands were removed and the slurry was added into the reactors. The reactors were started with sucrose (50 g) and mineral salts stock solution. Each liter of mineral salts stock solution contained 200 g of

NH_4HCO_3, 100g of KH_2PO_4, 10g $MgSO_4 \cdot 7H_2O$, 1.0 g of NaCl, 1.0 g of $Na_2MoO_4 \cdot 2H_2O$, 1.0 g of $CaCl_2 \cdot 2H_2O$, 1.5g of $MnSO_4 \cdot 7H_2O$ and 0.278g of $FeCl_2$, which was slightly modified from Lay *et al.* (1999a). 8 g K_2HPO4 and 21 Na_2PO4 were added in as buffer. The initial pH was adjusted to 6.5 by adding HCL acid. They were operated in batch mode (without feeding and decanting) for 24 hours until continuous biogas production was observed. Subseqently, the substrate of 8 g /L sucrose amended with mineral salts was fed to the reactors in sequencing batch mode and pH was maintained at 4.8 in both reactors. The reactors were fed 4 times per HRT; it took about 12 minutes for decanting and 20 minutes for feeding. Effluent was pumped out by a Masterflex peristaltic pump controlled by a timer (ChronTrol) and influent was fed by both external Masterflex peristaltic pump and feed pump controlled by Addition Module (New Brunswick FP 2000). A foam/level probe connected to the modular sensed the water level in the reactors. Feed was kept refrigerated at 4°C until feeding. In stage 4 and 5, soluble starch (Acs Co.) solution of 8g/L was used as the substrate.

With the introduction of sludge activation and return process, a settling tank and activation chamber were added to the process. The schematic flow chart of the system is shown in Figure 2. A portion of sludge separated from the effluent of the reactors was directed into a activation chamber to be heated at 90°C for about 15 minutes until boiling for 2 more minutes. Then the activated sludge was returned into the reactors.

Batch Tests on Hydrogen Conversion Efficiency

For batch tests on the hydrogen conversion potential of different substrates, 250 ml serum bottles were used. The experimental design is shown in Table 1. Seed sludge for

batch tests was collected from the same source. Equal amounts of substrate (on COD basis) were added into each bottle. The substrates used in the study were: starch solution only, sucrose only and mixture of starch solution and sucrose (1:1). Nutrient stock solution and pH buffers were also added into each bottle evenly and the bottles were filled up to 150 mL level using deionized water allowing 100 mL headspace. pH was adjusted to designed initial levels by addition of NaOH or HCL solution. pH was measured using a calibrated pH probe. The bottles were flushed with N_2 and capped tightly before being put on an orbital shaker table running at 180 rpm in a 37°C constant temperature room. Biogas production was measured by plunger displacement method with appropriately sized wetted glass syringes (Owen *et al.* 1979). The percentage of hydrogen gas in the headspace was determined by a GC-TCD. Liquid samples were collected using a 2 mL plastic syringe for pH measurement. A modified Gompertz model was used to fit the cumulative hydrogen production curves for each bottle to obtain the hydrogen production potential P, the maximum hydrogen production rate R and lag phase λ.

$$H = P * exp\{-exp[(R_m * e / P)(\lambda - t) + 1\ ]\} + \varepsilon \qquad (1)$$

Where H = cumulative hydrogen production (mL),

λ = lag-phase time (hr),

P = hydrogen production potential (mL),

R_m = maximum hydrogen production rate (mL/hr),

t = incubation time (hr) and

e = $exp(1)$

and ε is a random error, which represents the difference between a measurement H and a point on the line of model.

The parameters of Equation (1) were estimated using the function of "solver" in Microsoft Excel version 5.0 (Microsoft, Inc.). This program uses a Newton algorithm. Up to a hundred iterations were used to converge the ratio sum of square error (SSE) to correlation coefficient (R^2) between the experiment and the estimate to a minimum. Starting parameter values were estimated using a built – in visual procedure based on a limited fit algorithm (Lay *et al.*, 1998). The specific hydrogen production potential P_s (mL/g COD) was obtained by dividing P by amount of substrate COD applied; the specific hydrogen production rate R_s (mL/hr-g VSS) was obtained by dividing R_m by the amount of volatile suspended solids added. Hydrogen conversion efficiency for different substrates was compared based on P_s and R_s.

Analysis

Gas production was recorded by a wet-gas meter (Schlumberger Co.) connected to the reactor. Biogas composition was analyzed daily using two gas chromatographs (Gow Mac series 350) equipped with a thermal conductivity detector (TCD). Hydrogen gas was analyzed using a GC-TCD connected with an 8' by 1" stainless column - SS 350A Molesieve 13X (80/100 mesh). Nitrogen was used as the carrier gas at a flow rate of 30 mL/min. The operational temperatures of the injection port, the oven and the detector were 100, 50, and 100°C, respectively. Methane and carbon dioxide in biogas were measured using another GC-TCD connected with a 3.3' stainless column packed with Porapak T (60/80 mesh). The operational temperatures of the injection port, the oven and the detector were maintained at

100, 50, and 100 ^{0}C, respectively. Helium was used as the carrier gas at a flow rate of 35 mL/min. Individual volatile fatty acids (VFA) and solvents were analyzed using a gas chromatograph (Gow Mac series 580) equipped with a flame-ionized detector (FID). The column used was a 6'by 8" stainless column – SS 580 FID, 10%SP-1200/1% H_3PO_4 (80/100 mesh). The operational temperatures of the injection port, the oven and detector were maintained at 140, 100, and 140°C respectively for volatile acids analysis, and 170, 70, 170°C respectively for solvent analysis. Helium was used as the carrier gas at a flow rate of 40 mL/min.

Total VFA as acetate, chemical oxygen demand (COD), suspended solids (SS), volatile suspended solids (VSS) were measured according to Standard Methods for the Examination of Water and Wastewater (APHA, 1995). Carbohydrate content was measured by the phenol-sulfuric method using glucose as a standard (Oi *et al.*, 1982). Daily composite samples of the effluent were collected and the influent was sampled directly from the feed container. COD, VFA, solvent, SS, VSS and carbohydrate were measured every alternate day.

Results and Discussion

Effect of Activation Chamber on Reactors' Performance

The system was operated continuously but with some changes of the operation conditions. There were 5 stages involved in the experiment as listed in Table 1. The first stage was start up of the system using 8g/L sucrose as substrate plus mineral salts; the pH was set at 4.8 for both reactors. The biogas production was very low at 1.0 L/day for the

reactor with HRT = 20 hours (R1) and 0.6 L/day for the reactor with HRT = 30 hours. The H_2 content in the biogas was 12.6% and 20.6% for R1 and R2, respectively. It seemed that there was a continuous loss of biosolids from the system. So in the second stage, a portion of settled sludge collected from the effluent was reactivated and returned into both reactors daily. It was seen that the reactivation of sludge improved the system performance dramatically as shown in Figure 3. The average daily biogas production increased to 4.3 L for R1 and 1.6 L for R2 with H_2 contents of 37.8% for R1, and 19.0% for R2. R1 showed a better performance than R2.

Freshness of Substrate

Although activation of sludge improved the performance of the reactors, it was observed that biogas production fluctuated with feed age – a better performance was observed when freshly prepared substrate was fed to the reactor, and a drop in reactor performance was noted with longer feed age. Influent samples were collected for VFA measurement and it was found that on the third day the substrate had a total VFA content of 53.7 mg/L as acetic acid. It was suspected that the volatile organic acids in the feed could inhibit hydrogen production by triggering the sporulation of hydrogen producers. Further more, volatile fatty acids, being one of the major products of the reaction, might hinder the progress of the reaction in the favorable direction.

$$C_6H_{12}O_6+2H_2O \rightarrow 2CH_3COOH+2CO_2+4H_2\uparrow \quad (2)$$

$$C_6H_{12}O_6 \rightarrow CH_3CH_2CH_2COOH+2CO_2+2H_2\uparrow \quad (3)$$

Based on this hypothesis, the substrate was prepared on a daily basis to ensure its freshness from the third stage. From Figure 4 and Table 2, it can be observed that the performance of the reactors started peaking after the initial 3 days operation with a fairly constant daily biogas production of about 13.2 L/day for R1 and 8.5 L/day for R2, and an overall average daily biogas production of 12.1 L for R1 and 7.7 L for R2. The hydrogen contents of the biogas were approximately 48.8% for R1 and 41.3% for R2.

Continuous Hydrogen Fermentation from Starch

Wastewaters from most food processing units such as corn, soybean, and meat etc., contain high concentration of carbohydrates in forms other than sugar. At the fourth stage of experiment, the substrate used was changed to starch at a concentration of 8 g/L. The organic loading rate (ORL) was maintained at 10.8 g COD/L-day for R1 and 7.1g COD/L-day for R2. According to van Ginkel *et al.* (2000), pH level around 5.5 might be more suitable for hydrogenesis than solventogenesis of microbes involved, which had been tested with sucrose in a batch experiment. Thus, a pH level of 5.2 was tried at stage 4. It was found that the reactors' performance dropped sharply with an average daily biogas production of 3.9 L for R1 and 1.7 L for R2, the hydrogen content also dropped to 35.7% and 21.5% respectively as shown in Figure 5 and Table 2. Carbohydrate tests showed that there was a slightly higher content of undegraded carbohydrates in the effluent of stage 4 (2.0%) than in the effluent from stage 3 (0.8%). However this is not sufficient evidence to explain the much lower hydrogen production in stage 4.

Hydrogen Conversion Efficiency

To further investigate above observed phenomenon, batch serum bottle assays were conducted to test the hydrogen conversion potential of different substrates. Three different types of substrates were used as described in the Test Procedure Section, i.e. starch, sucrose and mixture of starch and sucrose (1:1 on COD basis). Inside each bottle, aliquot of substrate was added on COD basis. The initial pH was adjusted at different levels from 4.5 to 5.5 for different samples. The bottles were duplicated for each sample. The test results are shown in Figures 6 through 8. It can been seen that for bottles with starch as substrate, the hydrogen production potential was consistently lower than that with sucrose as substrate at all pH levels, while the bottles with starch plus sucrose mixture as substrate had hydrogen conversion potentials better than starch but lower than sucrose at corresponding pH levels. The highest hydrogen production for starch occurred at an initial pH of 4.5, while for sucrose it occurred at 5.5. When starch was used as substrate, lag phases saying from 18 to 20 hours were observed; while for sucrose, they were at least 3 hours shorter for corresponding bottles. The starch plus sucrose mixture had similar results as sucrose with highest hydrogen production at critical pH = 5.5 but had the shortest lag phase of 13 hrs. It was interesting to note that for starch, although the bottles with initial pH 4.5 had lower hydrogen production rate (about 7mL/hr-g VSS) compared with the others (over 14 mL/hr-g VSS), they had the maximum hydrogen production potential. This could be by the gradual release of hydrolyzed starch to microbes at lower pH levels and consequently longer-lasting hydrogen production process. The specific hydrogen production potential P_s at different pH levels for the three substrates were calculated and summarized in Figure 9. It was clear that hydrogen conversion potentials for starch were lower than those for the mixture and sucrose. The

lower the pH, higher the hydrogen conversion potential for starch. But for sucrose and the mixture, the optimum pH seemed to be close to 5.5. This suggested that the addition of sucrose to starch might help in the conversion of starch to hydrogen, not only by increasing the total amount of hydrogen produced, but also increasing the hydrogen production rate which occurred around pH = 5.0 ~ 5.5.

pH Effect on Continuously Flow Hydrogen Fermentation of Starch

Based on the serum bottle tests, at the fifth stage of the continuous system operation, starch was still used as the substrate but the pH levels in the two reactors were set at 4.8. The reason to use 4.8 rather than a lower value is that from previous trials conducted by the authors with a continuous flow reactor, a prolonged lower pH (<4.6) would trigger the microbial shift from hydrogenesis to solventogensis. Other researchers (Byung and Zeikus, 1985) have also reported pH = 4.5 as the threshold for production of the solvents. As Figure 5 and data listed in Table 2 show, although hydrogen production was still relatively low, the performance of the rectors improved in terms of biogas production and hydrogen content in the biogas compared with the performance at the previous stage. The hydrogen production increased hundred times that stage 4.

Effect of Organic Loading Rate and Feeding Mode

Figure 5, 10, 11 and Table 2 also show that at all stages, HRT of 20 hours reactor (R1) performed better than HRT of 30 hours reactor (R2) in terms of hydrogen production. In a previous trial conducted by the authors with a 3L working volume reactor (data are not shown here), when the reactor was operated in continuously fed mode, continuous hydrogen

production could hardly be observed, however, when it was operated at same HRT in a sequencing batch fed mode, a fairly consistent hydrogen production of 0.4~0.5L/day could be achieved. Both these results suggested that higher loading rates and famine-feast mode might be favorable for spore forming bacteria to re-germinate and produce hydrogen. In addition, sequencing batch mode might be helpful in flushing out some of the potential toxicants excreted by most spore forming bacteria (Shih and Labbe, 1996; Srinivasan, 1966).

Mass Balance for System Evaluation

To make a quantitative evaluation of performance of the reactors, mass balances were conducted for stages 3, 4 and 5 for the two reactors on COD basis. The COD distributions by products detected are tabulated in Table 3-a and 3-b. The unknown soluble COD fraction was considered to be other metabolic products of carbohydrates including solvents. It was determined by subtracting carbohydrate remaining and VFA from the total soluble COD of the effluent. 1.42 g COD per gram VSS was assumed for biosolids COD calculation. The maximum COD conversion in hydrogen production was 8.1%, compared to the theoretical value of 33.3 %. This result is similar to the values reported by other researchers (Akashah *et al.*, 1997). About 48% to 65% percent of soluble COD in the effluent was constituted by volatile fatty acids, the remaining fraction being solvents and other intermediate products. These intermediate products are all easily biodegradable substances, which could be subsequently removed by an anaerobic methane production processes. Therefore, hydrogen fermentation from wastewater is best suited for pretreatment of industrial wastewater for maximum energy conversion.

Conclusion

The results presented here showed that external activation chamber can retain a predominance of H_2 producers in the reactor and ensure stability of operation. Freshness of substrate was important for hydrogen production; existence of volatile fatty acids was inhibitory to hydrogenic activity. Batch feeding was better suited to hydrogen production since it could flush out substances promoting sporulation and enhances hydrogen production. Batch feeding with a shorter HRT, in other words, higher organic loading rate was found to be favorable for hydrogen production. The hydrogen conversion potential of starch was lower than that of sucrose; and lower the pH, better the conversion of starch to hydrogen. However, addition of the sucrose into starch could improve hydrogen production rate and hydrogen production potential of starch. Although the COD conversion efficiency in hydrogen fermentation process was limited, it still could be a good pretreatment process for industrial wastewaters to maximize energy conversion from negative value organic wastes and reduce pollutants release into environment.Further research should focus on the optimization of hydrogen production by changing operational parameters such as pH and organic loading rate.

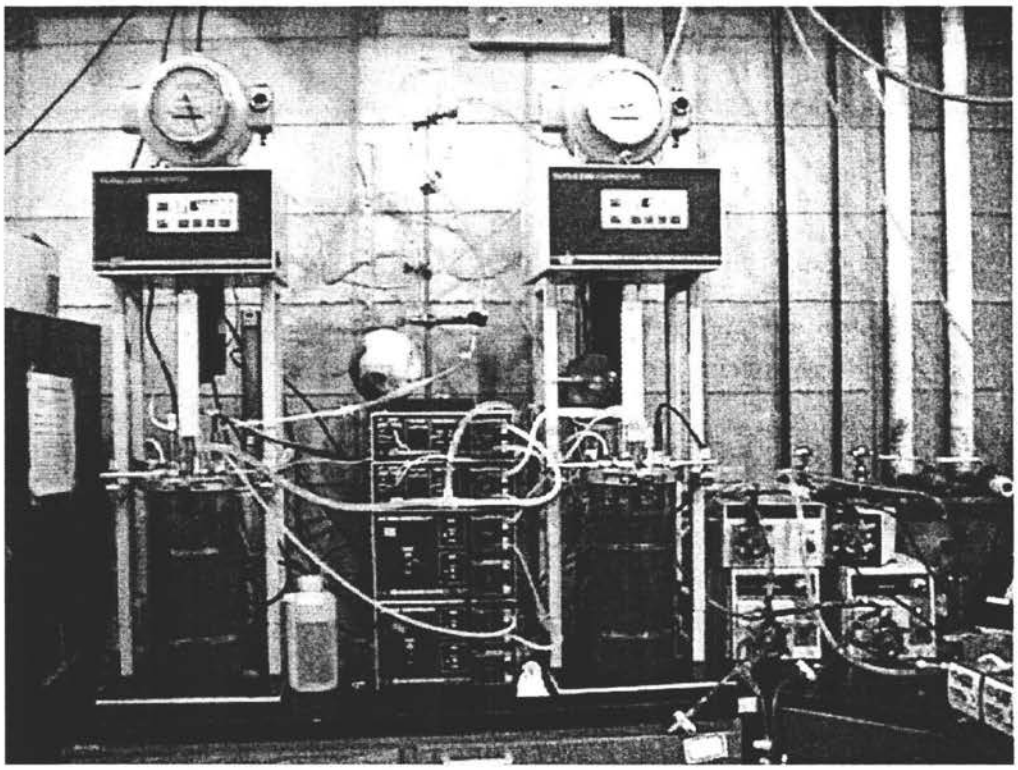

Figure 1. Photo of continuous-flow biohydrogen fermentors.

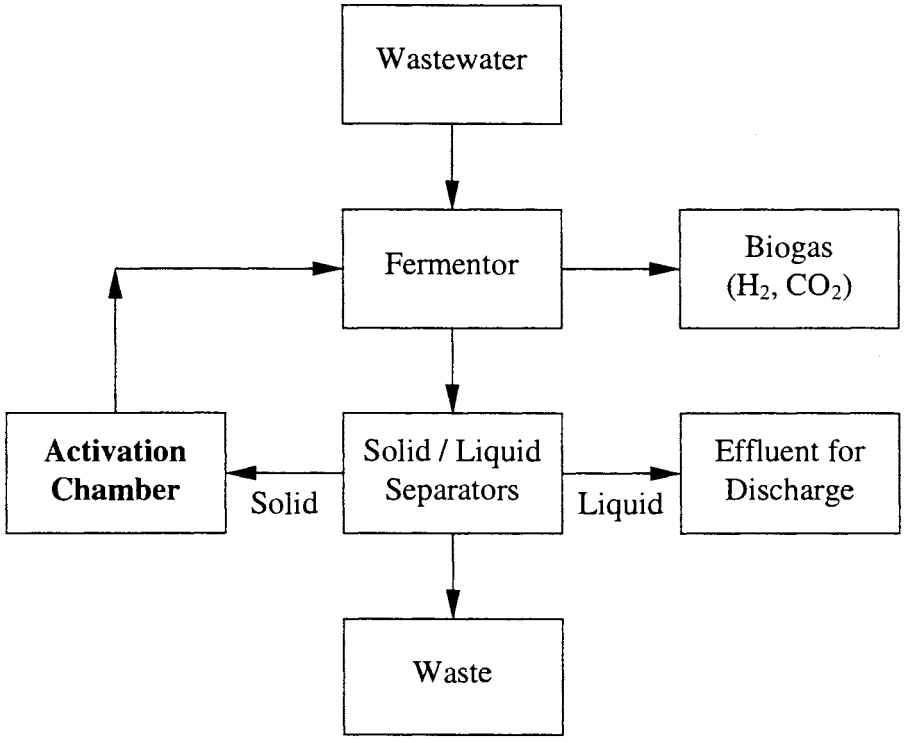

Figure 2. Block diagram of the combination of an activation chamber to the hydrogen fermentation process.

Table 1. Design of batch test for hydrogen conversion potential

		Samples (duplicated)				
pH		4.5	5.0	5.5	6.0	6.5
Substrate	Starch (g)	1.5	1.5	1.5	1.5	1.5
	Sucrose (g)	1.5		1.5		1.5
	Starch (g) +Sucrose (g)	0.75 +0.75		0.75 +0.75		0.75 +0.75

Table 2. Experimental stages and the performance of the reactors

Stage	Duration (Days)			Ave. Biogas (L/d)		Ave. H_2 (%)	
	From	To	Total	R1	R2	R1	R2
1st	7-Mar	15-Mar	9	1.0±0.9	0.6±0.8	12.6±19.8	20.6±10.8
2nd	16-Mar	30-Mar	15	4.3±2.7	1.6±1.1	37.8±19.3	19.0±9.5
3rd	31-Mar	10-Apr	11	12.1±1.9	7.7±1.6	48.8±1.5	41.3±3.9
4th	11-Apr	24-Apr	14	3.9±0.6	1.7±0.4	35. 7±3.9	21.5±2.6
5th	25-Apr	7-May	13	5.2±0.7	2.4±0.6	43.4±4.8	30.4±4.9

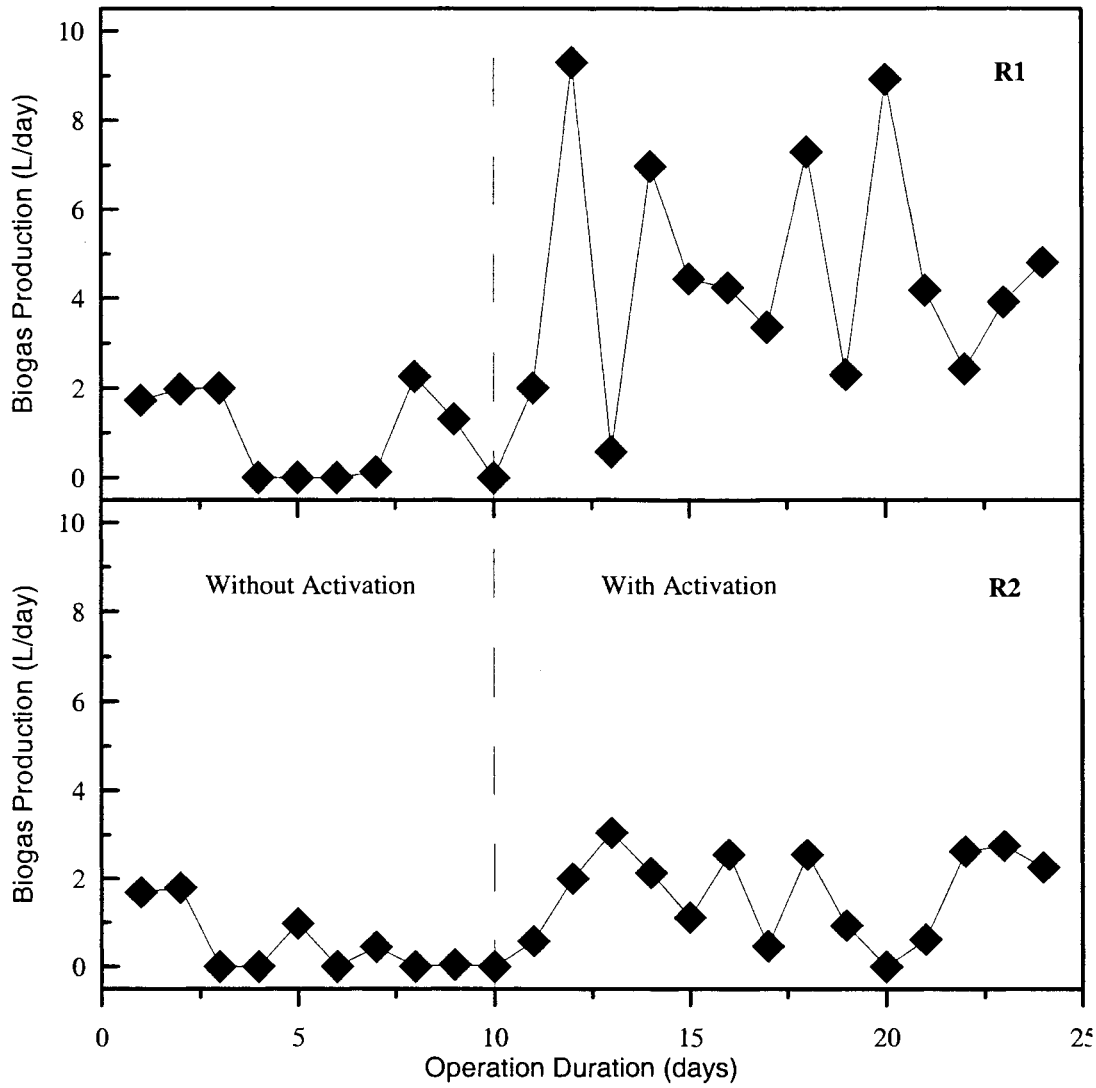

Figure 3. Activation effect on the performance of the reactors.

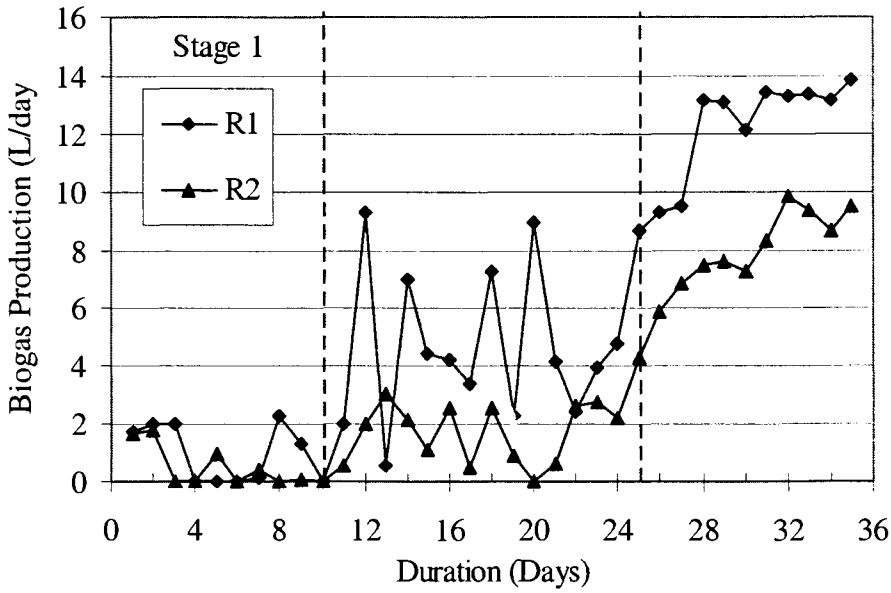

Figure 4. Sludge activation and substrate freshness effect on the performance of the reactors.

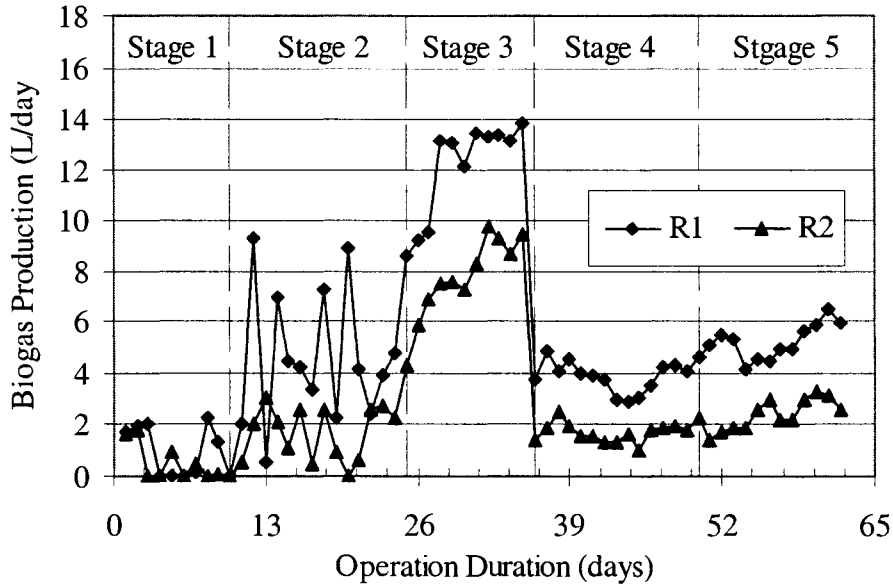

Figure 5. Performance of two reactors at five different stages.

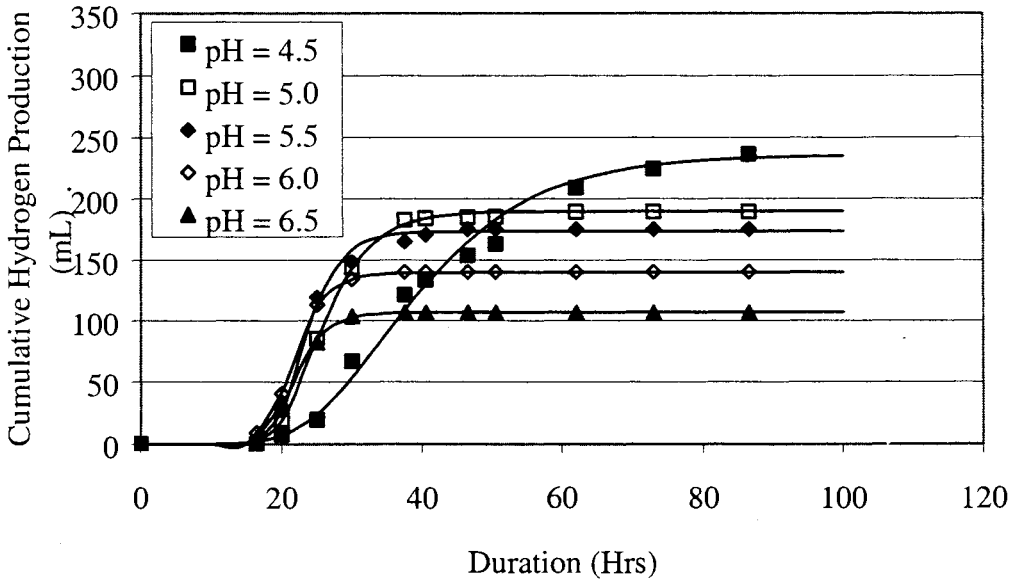

Figure 6. Cumulative hydrogen production from starch with different initial pH.

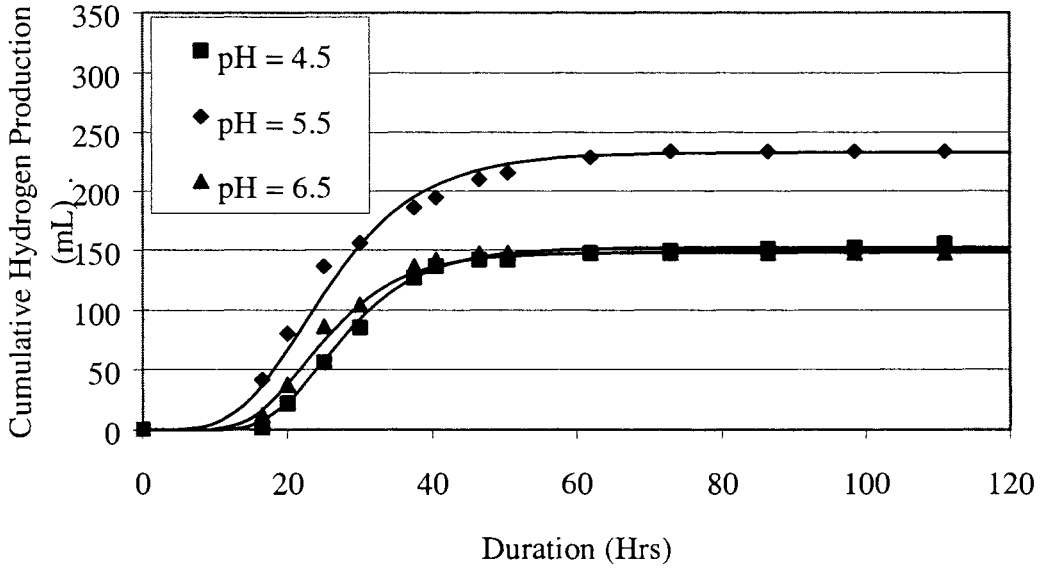

Figure 7. Cumulative hydrogen production from mixture of starch and sucrose with different initial pH.

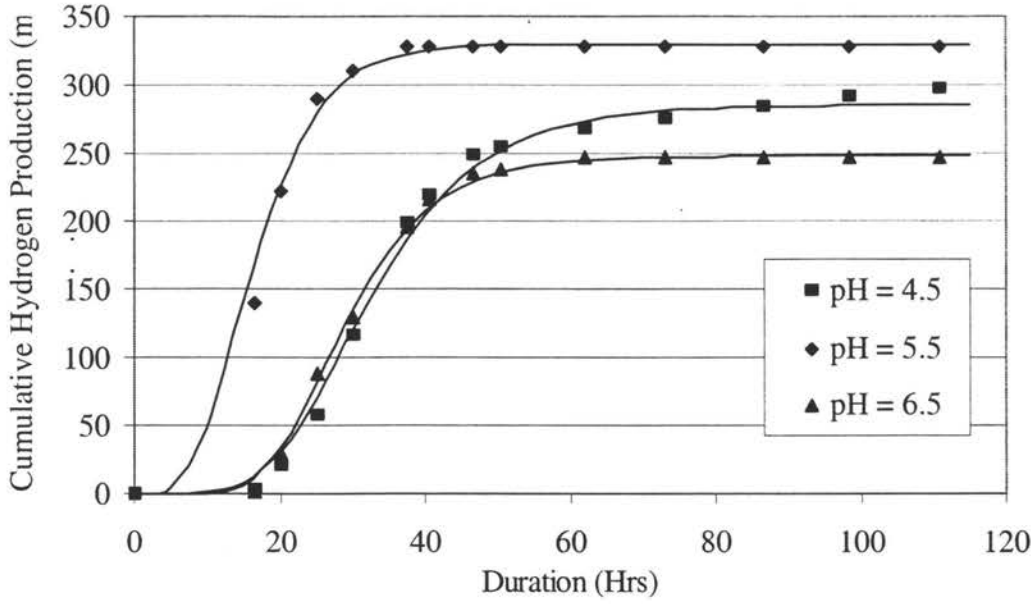

Figure 8. Cumulative hydrogen production from sucrose.

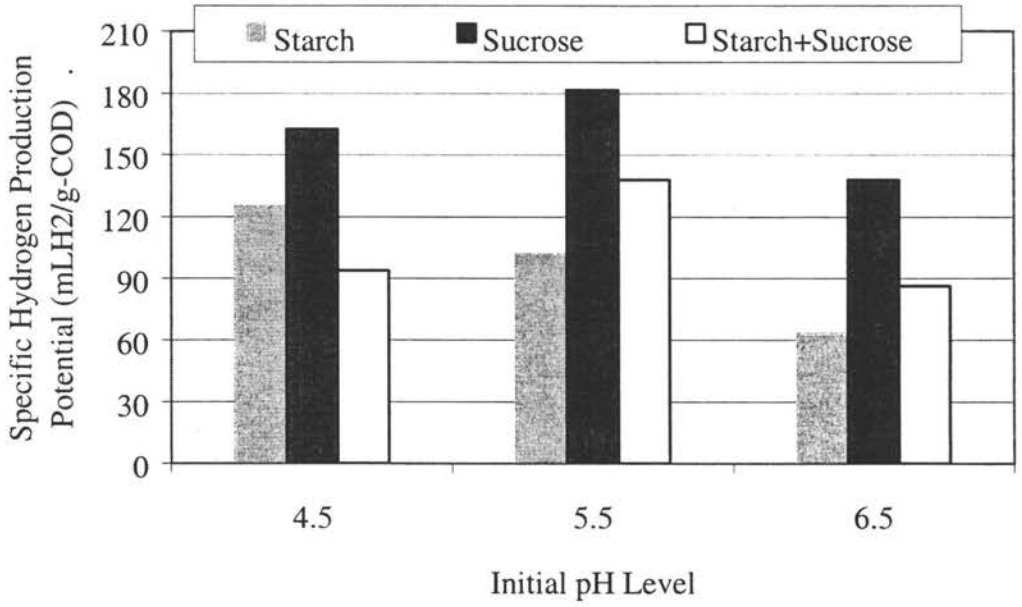

Figure 9. Comparison of hydrogen conversion potential from different substrate.

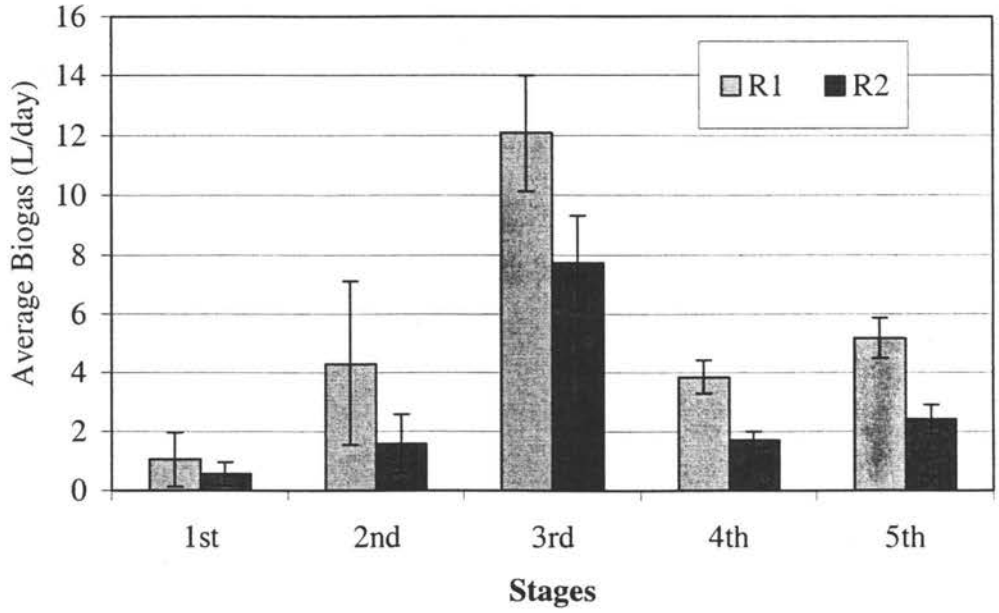

Figure 10. Comparison of averaged biogas production at different stages.

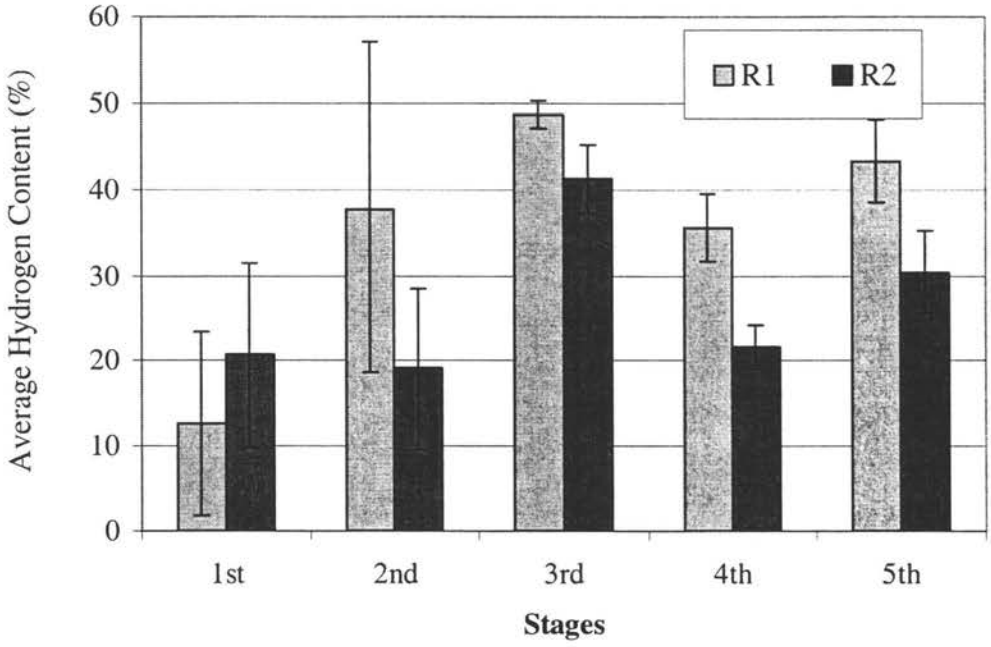

Figure 11. Comparison of averaged hydrogen content in biogas at different stages.

* Error bar shows the standard deviation

Table 3a. System COD Balance (%) of Reactor 1 (R1).

Stage	Soluble Products					Gas		Biomass	Total COD
	Remained Carbohydrate	VFA			Unknown Products	Hydrogen	Methane	Effluent VSS	
		Acetate	Propionate	Butyrate					
3rd	1.1±0.3	15.4±1.1	22.2±3.4	3.3±1.3	26.1±4.2	8.1±1.2	ND*	23.1±6.8	99.3
4th	3.0±0.5	17.2±2.3	26.9±3.7	5.5±0.2	23.8±8.2	1.8±0.3	ND	20.3±1.7	98.5
5th	1.2±0.2	13.1±1.9	25.1±1.8	5.8±1.2	29.9±2.1	3.5±0.6	ND	23.0±3.1	101.6

* Not detected within the detect limit

Table 3b. System COD Balance (%) of Reactor 2 (R2).

Stage	Soluble Products					Gas		Biomass	Total COD
	Remained Carbohydrate	VFA			Unknown Products	Hydrogen	Methane	Effluent VSS	
		Acetate	Propionate	Butyrate					
3rd	1.0±0.3	11.2±2.3	16.7±2.5	10.3±0.5	39.9±8.3	6.8±1.8	1.9±0.4	15.9±4.5	103.7
4th	2.5±0.4	17.9±3.5	20.3±2.7	13.6±1.9	25.6±2.8	0.7±0.2	4.9±1.3	13.9±1.1	99.4
5th	1.5±0.3	15.2±3.4	17.9±2.1	11.3±2.7	34.2±4.6	2.8±0.8	2.3±0.7	15.8±2.9	101.0

* All data in the table are reported in % COD of the feed substrate.

CHAPTER 3. THE EFFECTS OF PH AND INTERMEDIATE PRODUCTION ON HYDROGEN FERMENTATION

A paper to be submitted to Water Environment Research

Ling Li and Shihwu Sung

Abstract

To optimize the hydrogen production in continuous biohydrogen fermentation processes, batch tests were conducted to investigate the effects of pH and intermediates production on hydrogen fermentation. Tests were run in serum bottles to determine the optimal operation conditions, which may prove beneficial in maximizing hydrogen production. Sucrose and starch were used as substrates for the study. Apart from hydrogen, variations in pH, volatile fatty acid, and solvent concentrations were also monitored. Initial pH was found to have an effect on both hydrogen production potential and hydrogen production rate. The lowest initial pH = 4.5 gave the highest specific hydrogen production potential of 214 mL H_2/g COD and 125 mL H_2/g COD for sucrose and starch respectively, but the lowest specific maximum hydrogen production rate. At higher initial pH values, although hydrogen production started earlier with a high production rate, the duration of the production was shorter. So the total hydrogen produced was less. The rapid pH depletion could have caused a metabolic alteration of the microorganisms involved resulting in a change in the intermediates production pattern (variation of the acetate/butyrate ratio) and a consequent decease in hydrogen production. The hydrogen production rate was highest at the optimal pH range of 5.0 to 5.5. A mixed culture of microorganisms was involved in the fermentation process with propionate, acetate, butyrate, CO_2, and H_2 as the major products.

Key words:

pH effect, hydrogen production potential, hydrogen production rate, volatile fatty acids, solvents, acetate/butyrate ratio, mixed culture

Introduction

Acidogens of genus, *Clostridia*, has been well studied for its ability to form spores and for its potential to generate hydrogen gas (Brosseau and Zajic, 1982; Lay *et al.*, 1999a; Ueno *et al.*, 1995). They are the presumed hydrogen producers in the present study. There are many types of *Clostridia* including saccharolytic and proteolytic forms. Saccharolytic acidogoens, which are the spore formers, ferment carbohydrate and proteolytic acidogens degrade proteins or amino acids. The fermentation pathway of saccharolytic clostridia is illustrated by the *Clostridia* type strain-*C. butyricum* (Minton and Clarke, 1989), which produces butyric acid as the major fermentation product together with CO_2, acetate and H_2. The reactions are shown as Equations (1) and (2).

$$C_6H_{12}O_6+2H_2O \rightarrow 2CH_3COOH+2CO_2+4H_2\uparrow \quad (1)$$

$$C_6H_{12}O_6 \rightarrow CH_3CH_2CH_2COOH+2CO_2+2H_2\uparrow \quad (2)$$

This pathway is found in approximately 50% of all clostridial species that have been isolated to date. Other fermentation pathways found in sacchrolytic clostridia are those leading to the production of propionate by *C. arcticum* (Jodan and McNicall, 1979), succinate by *C. ccoides* (Kaneuchi *et al.*, 1976) and lactate by *C. barkeri* (Stadtman *et al.*, 1972). The clostridial genus is an obligate anaerobic heterotroph that does not contain a cytochrome system (Nandi and Sengupta, 1998). This genus produces hydrogen using the activities of pyruvate-ferredoxin-oxidoreductase and hydrogenase enzymes. This

genus has a desired pH range of 6.5 to 7.0, which is similar to that of methanogens (Grady *et al.*1999; Minton and Clarke, 1989). The activity of hydrogenase, an iron-containing enzyme inhibited by low pH, was reported to be one of the most important factors in the overall hydrogen fermentation (Holt *et al.*, 1988; Afschar *et al.*, 1986; Dabrock *et al.*, 1992; Ueno *et al.*, 1996). In certain cases, the hydrogen evolution activity of hydrogenase may be suppressed in the presence of high levels of hydrogen (Kondratieva, 1983). A decrease in hydrogen concentration will favor hydrogen formation and permit bacteria to metabolize acetyl-CoA through the energy-efficient path leading to acetate and ATP production.

In a typical anaerobic process, hydrogen is produced during the exponential growth phase of *Clostridia*. When the population reaches the stationary phase of growth, solvents or alcohols are produced. This shift occurs when the pH drops to 4.5 (Byung and Zeikus, 1985). Apparently, the build up of volatile fatty acids and hydrogen during the exponential growth phase would potentially induce this shift. While other researchers (Gottwald and Gottschalk, 1985) found that the shift occurred at pH levels above 5.7, due to enzyme synthesis or enzyme activation, which is necessary for solvent production. Accordingly, it is important to remove excess hydrogen from the system and control pH to maintain hydrogen producition. If H_2 builds up in the system, higher molecular weight acids such as butyric and propionic acids are produced instead of acetic acid (Grady *et al.*, 1999). On the other hand, if the pH cannot be maintained in a desired range, it could be inhibitory to hydrogen production or cause a microbial population shift resulting in a cessation of hydrogen production. So it is important to monitor and maintain the pH at the optimum level in the hydrogen production process.

Theoretically, in the fermentation displayed by *Clostridium butyricum*, ATP generation by *C. butyricum* can vary from 3 (mol ATP)/(mol glucose fermented), when

glucose is fermented completely to butyrate, CO_2 and H_2, to 4 (mol ATP)/(mol glucose fermented). In practice, during the fermentation process both butyrate and acetate are formed, but the ratio may vary with growth conditions within thermodynamically determined limits (Thauer *et al.*1977). Van Andel *et al.* (1985) found that decreasing the partial pressure of H_2 results in an increase in the $q_{acetate}/q_{butyrate}$ ratio accompanied by an increased production of H_2. Therefore, if we know the actual metabolic pattern, it would be possible for us to drive the pathway towards a higher $q_{acetate}/q_{butyrate}$ ratio and parallel higher H_2 production by controlling environmental conditions such as pH, mixing intensity, HRT, organic loading rate and nutrient.

The objective of this study was to investigate the effects of environmental factors such as initial pH, partial pressure of H_2 (here measured as volume percentage of H_2 in the biogas), and intermediate products on hydrogen production and to determine the optimum operational pH range for hydrogen production, which is evaluated based on specific hydrogen production rate. For these purposes, the initial pH values of the serum bottles were adjusted to different levels by adding sufficient buffer. Biogas was released from the bottle frequently (3 to 4 hours interval) and biogas composition was analyzed. Liquid samples were taken for pH monitoring and intermediate products (VFAs, solvents) analysis. Two types of substrates – sucrose and soluble starch (Acs Co.) were used to validate the results from each other.

Methodology

The experiment was run in 250 ml serum bottles. Compost material obtained from Iowa State University composting pile was used as seed source. It was baked in the oven at 105°C for two hours and then cooled to room temperature in a desiccator. The baked compost material was ground and sieved through a No. 30 sieve. The sieved fines

was mixed with nano-pure water to make a 2 L slurry solution. It was settled for 30 seconds to remove sands and the volume was adjusted to 2 L again with nano-pure water. 8g K_2HPO_4 and 21 g Na_2HPO_4 were added as a buffer. The slurry was mixed completely and an aliquot of the slurry was added into each serum bottle. To each bottle, 1.5 g sucrose or 10 mL concentrated starch solution (150g/L) and 0.5 mL of nutrient stock solution were added. Each liter of nutrient stock solution contained 200 g of NH_4HCO_3, 100g of KH_2PO_4, 10g $MgSO_4 \cdot 7H_2O$, 1.0 g of NaCl, 1.0 g of $Na_2MoO_4 \cdot 2H_2O$, 1.0 g of $CaCl_2 \cdot 2H_2O$, 1.5 g of $MnSO_4 \cdot 7H_2O$ and 0.278g of $FeCl_2$, which was slightly modified from Lay *et al.* (1999a, 1999b). The bottles were filled to the 150 mL level mark using nano-pure water. pH was adjusted by concentrated HCL or NaOH solution to desired initial levels: 4.5, 5.0, 5.5, 6.0, and 6.5. The bottles were flushed with N_2 and capped tightly before being put on an orbital shaker table running at 180 rpm in a 37°C constant temperature room. Each sample was duplicated and two control bottles (blank) were also prepared without addition of substrate.

Biogas produced was measured by plunger displacement method with appropriately sized wetted glass syringes (Owen *et al.* 1979) and gas composition in the headspace was determined using two gas chromatographs (Gow Mac series 350) equipped with thermal conductivity detectors (TCD). Hydrogen was analyzed using one GC-TCD connected with an 8' by 1" stainless column - SS 350A Molesieve 13X (80/100 mesh). Nitrogen was used as the carrier gas at a flow rate of 30 mL/min. The operational temperatures of the injection port, the oven and the detector were 100°C, 50°C, and 100°C, respectively. Methane and carbon dioxide in biogas were measured using another GC-TCD connected with a 3.3' stainless column packed with Porapak T (60/80 mesh). The operational temperatures of the injection port, the oven and the detector were

maintained at 100°C, 50°C, and 100°C, respectively. Helium was used as the carrier gas at a flow rate of 35 mL/min. A modified Gompertz model was used to fit the cumulative hydrogen production curves for each bottle to obtain the hydrogen production potential P, the hydrogen production rate R and lag phase λ.

$$H = P * exp\{-exp[(R_m * e / P)(\lambda - t) + 1\ ]\} + \varepsilon \qquad (1)$$

Where H = cumulative hydrogen production (mL),

λ = lag-phase time (hr),

P = hydrogen production potential (mL),

R_m = maximum hydrogen production rate (mL/hr),

t = incubation time (hr) and

e = $exp(1)$

and ε is a random error, which represents the difference between a measurement H and a point on the line of model.

The parameters of Equation (1) were estimated using the function of "solver" in Microsoft Excel version 5.0 (Microsoft, Inc.). This program uses a Newton algorithm. Up to a hundred iterations were used to converge the ratio sum of square error (SSE) to correlation coefficient (R^2) between the experiment and the estimate to a minimum. Starting parameter values were estimated using a built – in visual procedure based on a limited fit algorithm (Lay et al., 1998). Then specific hydrogen production potential P_s (mL/g COD) was obtained by dividing P by the amount of substrate COD applied; the specific hydrogen production rate R_s (mL/hr-g VSS) was obtained by dividing R_m by the

amount of volatile suspended solids added. Hydrogen conversion potential for different substrates was compared based on P_s and R_s.

Liquid samples were collected 6 to 7 times during the process using a 10 mL plastic syringe. 2 mL sample was taken each time and placed into a 15 mL centrifuge tube. pH was measured using a calibrated pH probe. The liquid was centrifuged and the supernatant was transferred to a 2 mL centrifuge tube with the addition of a half-drop of concentrated sulfuric acid. The sample was mixed and capped before being kept into the freezer. The samples were thawed and centrifuged again before analysis. Individual volatile fatty acids (VFA) and solvents were analyzed using a gas chromatograph (Gow Mac series 580) equipped with a flame ionization detector (FID). The column used was a 6'by 8" stainless column – SS 580 FID, 10%SP-1200/1% H3PO4 (80/100 mesh). The operational temperatures of the injection port, the oven and detector were maintained at 140°C, 100°C, and 140°C respectively for volatile acids analysis, and 170°C, 70°C, 170°C respectively for solvent analysis. Helium was used as the carrier gas at a flow rate of 40 mL/min.

Chemical oxygen demand (COD), suspended solids (SS), volatile suspended solids (VSS) were measured according to Standard Methods for the Examination of Water and Wastewater (APHA, 1995).

Results and Discussion

Effects of Initial pH

In the previous study conducted by Van Ginkel (2000), it was concluded that initial pH value did not have as much of an effect on hydrogen production rate as it did on specific hydrogen production potential. To confirm these results, batch tests were

conducted with different substrates – starch and sucrose at same concentration levels (on COD basis), but adjusting the initial pH to different levels in each bottle. The cumulative hydrogen production from sucrose and starch plotted in Figure 1-a and Figure 1-b showed that the initial pH did have an effect on both hydrogen production potential and hydrogen production rate – higher the initial pH, lower the total hydrogen production potential; although there seemed not much difference in the hydrogen production rates when the initial pH was above 5 for both starch and sucrose by visual observation, paired T-test still showed a significant difference between samples with a p-value of 0.05, especially the bottles with initial pH = 4.5 showed the lowest hydrogen production rate. The initial pH effect was more pronounced for starch than for sucrose. This might be because of the easier degradability of sucrose compared to starch, making the inhibitory effect less evident during the reaction.

To have a better picture of the pH effect on hydrogen production, the specific hydrogen production potential Ps and λ obtained from Gompertz model were plotted versus corresponding initial pH values as shown in Figures 2-a, 2-b, 3-a and 3-b. From Figures 2-a and b, it can be observed that sucrose and starch batches had similar trends for specific hydrogen production potential. The slightly more curvature shown by the trend line for starch suggests that hydrogen conversion potential from starch depends more on pH. As to the lag phase, again Figures 3-a and b show a similar trend for sucrose and starch. The bottles with starch as substrate had a 3~4 hours longer lag phase than the corresponding ones with sucrose as substrate. However, for both starch and sucrose, the bottles with initial pH = 4.5 had the longest lag phase, while the others had similar lag phase with 14±1 and 18±1 hours for sucrose and starch batch respectively.

To further elucidate the observed results, the pH profile during the course of hydrogen production was plotted for each bottle as shown in Figure 4-a and Figure 4-b.

From these two figures it can be observed that at a higher initial pH, greater was the pH drop during hydrogen production and the duration of the hydrogen production was shorter. This suggested that higher initial pH levels were more favorable to trigger hydrogen production at a higher production rate. However, rapid hydrogen production was accompanied with acids accumulation, which in turn depleted the pH very quickly to the inhibitory levels. The hydrogen producers could not adapt to the fast change in environment, thereby halting the reaction. On the other hand, at a lower initial pH level (especially pH = 4.5), the starting environment might not be suitable for hydrogen producers, however, with their adaptation and limited self-adjustment of environmental conditions such as pH (Gottwald and Gottschalk, 1985), they started to produce hydrogen gradually at a moderate production rate. The amount of acids produced was insufficient to cause a dramatic depletion of pH. The active hydrogen producers could last longer in a relatively consistent environment.

Van Ginkel *et al.* (2000a) reported that maximum hydrogen production rate occurred before any inhibitory effects were observed. On the basis of this observation, he might undermine the effect of initial pH value on hydrogen production rate. In the authors' point of view, without monitoring the pH profile during the course of the reaction it would be difficult to determine the role of pH in hydrogen fermentation process. Moreover, since Van Ginkel varied both the initial pH and substrate concentration in his tests, it would not have been possible to study the sole effect of either of these. Nevertheless, it is interesting to note that with the consideration of lag phase, which was about 14 hrs for most bottles of sucrose batch and 18 hrs for most bottles of starch batch, hydrogen production in each bottle started around pH = 5.5. This is in agreement with the earlier work (Lay *et al.*, 1999a, Lay, 2000, and Van Ginkel *et al.*, 2000a) where pH = 5.5 deemed the optimum initial pH for hydrogen production.

Optimum Operational pH

With the pH profile during hydrogen production, it is possible for us to determine the actual pH values when hydrogen production rate was the maximum. This could be applied as the optimum operational pH for the continuous flow system. In a continuous process for hydrogen production, it is possible to maintain a constant high rate of microbial growth by controlling the operational parameters such as pH, sludge retention time (SRT) etc. (Wang *et al.* 1979; Brosseau and Zajic 1982b), and consequently maximum hydrogen production rate and productivity could be obtained. With this in mind, a combined plot was developed superimposing the cumulative hydrogen production curve fit by Gompertz model and pH profile plot for the individual bottles. The pH values corresponding to maximum hydrogen production rate were determined from the combined plot. Specific hydrogen production rate was then calculated. The results are plotted in Figures 5-a and 5-b. It can be noted that for sucrose, the maximum rate occurred at a pH level slightly higher than 5, while for starch, the maximum rate occurred in the pH range of 5 to 5.5. In general, the optimum operational pH range was determined to be between 5 and 5.5.

Intermediate Production and Its Effects

Hydrogen production is usually accompanied by volatile acids (VFAs) and solvents etc. production. The production of these intermediates could reflect changes in the metabolic pattern of the microorganisms involved, a better knowledge of which would help us learn more about hydrogen production and the environmental conditions favorable for hydrogen production. So during the course of hydrogen production, liquid samples were collected and analyzed for volatile fatty acids and solvents by a GC-FID.

The major VFAs detected in the process were propionate, acetate, and butyrate. The typical VFAs production profile is shown in Figures 6-a and 6-b for sucrose and starch respectively. The results shown here were obtained from bottles with initial pH = 6.0. It can be observed that there was a gradual production of acids before the hydrogen production that caused a pH change towards 5.5. This was followed by rapid production of hydrogen with an increase in acids production till the stationary phase of hydrogen production. The hydrogen content in the biogas peaked before the maximum hydrogen gas production was attained. In the stationary phase, the production of acetate decreased slightly but an increase in butyrate production was noticed. Nevertheless the amounts of acetate and butyrate produced were independent of pH in the range studied. This is in agreement with the results obtained by Van Andel et al. (1985) based on a study using pure culture of *C. butyricum*.

To have a better look of the change of metabolic pattern, the concentration ratio of acetate and butyrate was calculated and plotted together with the cumulative hydrogen production curve for different bottles in the two batches as shown in Figure 7-a and Figure 7-b. The acetate/butyrate ratio during hydrogen production showed a similar pattern for all the bottles - the highest ratio occurred during the exponential phase, especially for sucrose batch, it occurred close to the time when the hydrogen production rates were maximum; before this time, the ratio kept increasing, afterwards, it became decreasing and this followed by the stationary phase of hydrogen production. Although the total amounts of acetate and butyrate produced were independent of the initial pH, the acetate/butyrate ratio seemed to show larger variation for lower initial pH; the maximum acetate/butyrate ratio value was also higher at lower initial pH. This coincided with the trend of total hydrogen production for different bottles. According to the presumed major metabolic pathway involved in hydrogen production as shown in Equation 1 and 2, it

might be interpreted that although both the reactions occurred during the process, a lower initial pH could have driven the reaction towards acetate production resulting in higher production of hydrogen. The changes in acetate/butyrate ratio implied a metabolic alteration due to environmental changes such as pH, partial pressure of hydrogen (hydrogen content in the biogas) and the accumulated amounts of intermediate products in the bottles. A satisfactory repetition of this finding can be observed from the duplicated samples in the tests (data are not shown here). In another batch of experiments with half the quantity of sucrose added into each bottle, the results (data are not shown here) showed a similar trend but the variation among bottles were less. The extent of variation being different for these two batches, the quantitative change of ratio could not be concluded.

It was interesting to note that the amounts of propionate detected in the present study were consistently higher than acetate and butyrate produced. This is different from the results obtained by other researchers (Lay, 1990, Noike *et al.*, 2000 and Akashah *et al.*, 1997). In most of their experiments, butyrate was the predominant product. This suggests the presence of not only *C. butyricum* but a different species of saccharolytic *Clostridia,* possibly *C. arcticum*, which produces propionate (Jodan and McNicall, 1979) or other *Clostridia* species, such as *C. novyi* (Holdemann et al., 1977) and *C. propionicum.*(Cardon and Barker, 1947). This fact strengthened the view that a mixed culture was involved in the hydrogen production process. This adds significant meaning to the engineering prospect since a mixed culture is more viable than a pure one in wastewater treatment practice.

During hydrogen production, the concentration of solvents produced was less than 500 mg/L for all the bottles. This implied that microbial shift to solventogenesis did not occur in the pH range (4.5 ~ 6.5) studied. In his earlier work, Lay (2000) stated that

microbial shift from hydrogenesis to solventogenesis would occur at a pH level as low as 4.1. Above this level, metabolic alternation but not hydrogenase activity is the most likely cause for decrease in hydrogen production rate. In a previous trial of continuous flow experiments conducted by the authors, it was found that under prolonged conditions of pH lower than 4.6, shift to solventogensis could be observed based on the results from analysis of intermediate products (VFAs and solvents) by a GC-FID.

Conclusion

By detailed monitoring of pH change and intermediates production during hydrogen production using sucrose or starch as substrate, it was found that initial pH level did have an effect on both hydrogen production potential and hydrogen production rate. The lower the initial pH, higher the total hydrogen production potential, but lower the hydrogen production rate. This effect was more obvious for hydrogen conversion from starch. The maximum specific hydrogen production potential was 214 mL H_2/ g COD and 125 mL H_2/g COD at initial pH = 4.5 for sucrose and starch respectively. An initial pH as low as 4.5 could defer hydrogen production, but hydrogen production could last longer compared to a higher initial pH, thus giving a higher hydrogen production.

For the samples with higher initial pH, the changes in environmental conditions caused by the rapid depletion of pH might have resulted in a metabolic alteration, and consequent inhibition of hydrogen production. The changes in the intermediates production pattern, especially the concentration ratio of acetate/butyrate, support this hypothesis. The stationary phase of hydrogen production for all bottles occurred after the decrease of acetate/butyrate ratio although the extent of ratio variation differed with the initial pH. Hydrogen partial pressure in the serum bottles might be another environmental factor affecting the metabolic alteration.

Based on the evaluation of maximum hydrogen production rate, the optimum operational pH range was determined as 5~5.5. This result could be applied in future continuous flow processes to maintain a high rate of hydrogen production.

In addition to acetate and butyrate, high concentrations of propionate detected during the process suggested the presence of more than one species of microbes. It is beyond doubt that a mixed culture process be more feasible for future industrial waste treatment.

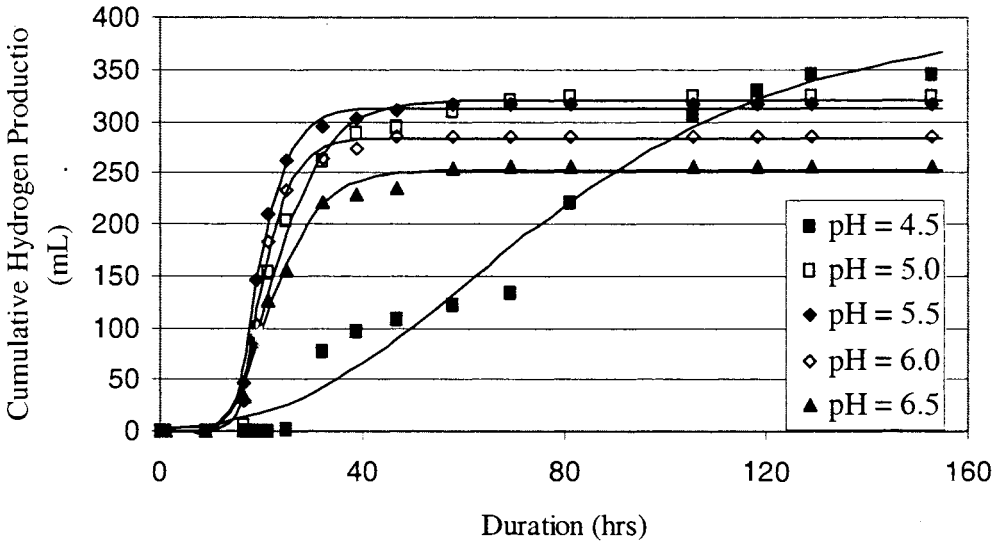

Figure 1-a. Cumulative hydrogen production from sucrose.

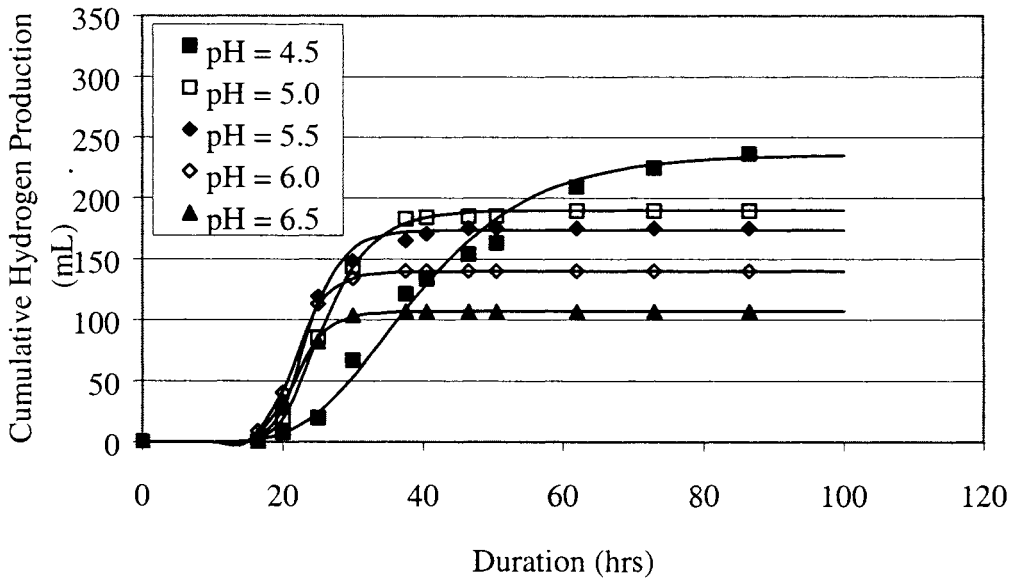

Figure 1-b. Cumulative hydrogen production from starch.

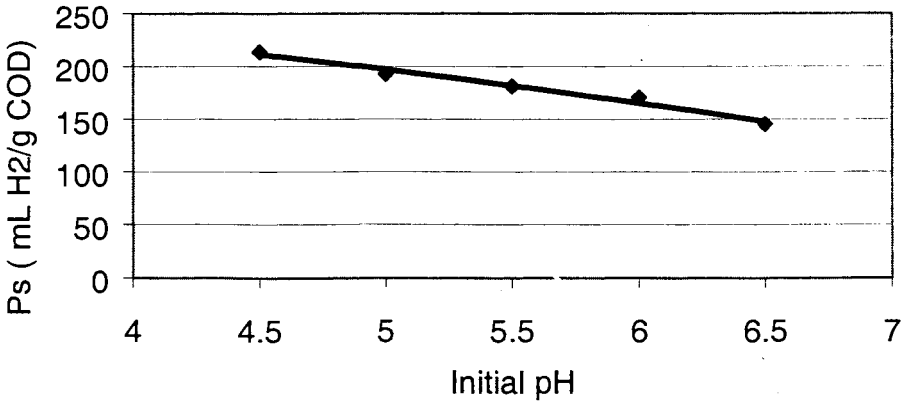

Figure 2-a. Specific hydrogen production potential vs. initial pH for sucrose batch.

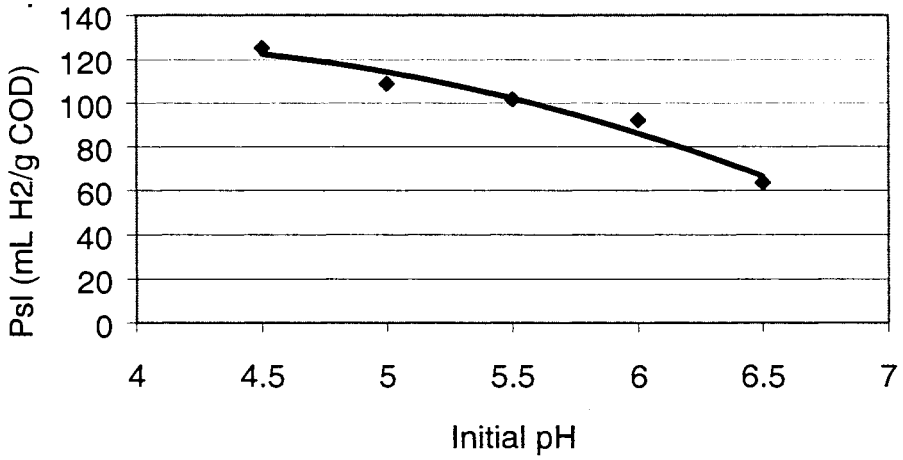

Figure 2-b. Specific hydrogen production potential vs. initial pH for starch batch.

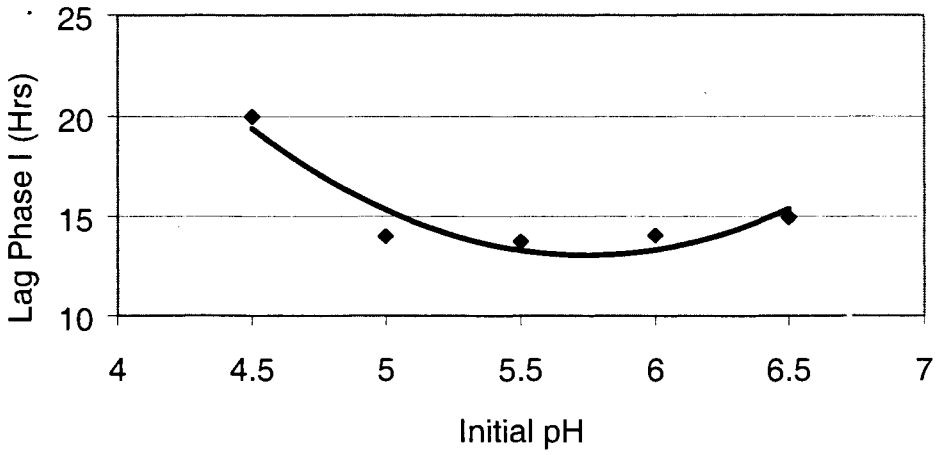

Figure 3-a. Lag phase vs. initial pH for sucrose batch.

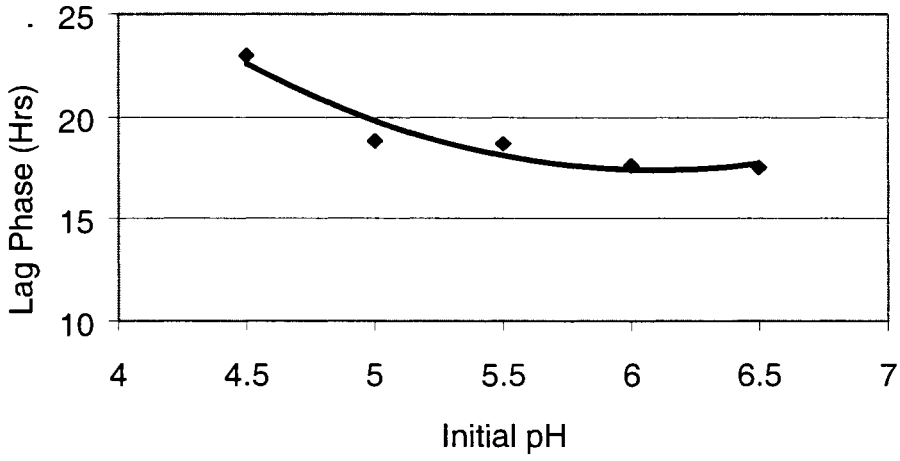

Figure 3-b. Lag phase vs. initial pH for starch batch.

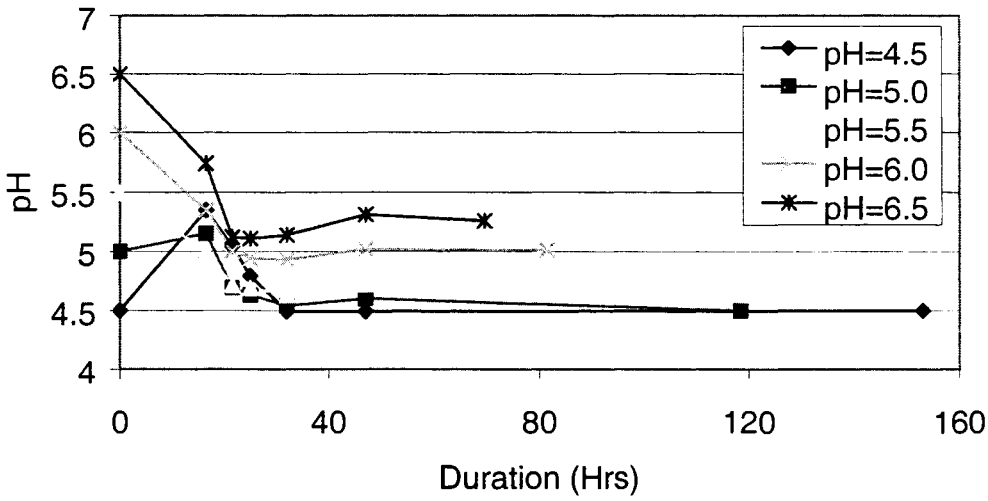

Figure 4-a. pH change profile during hydrogen production for sucrose batch.

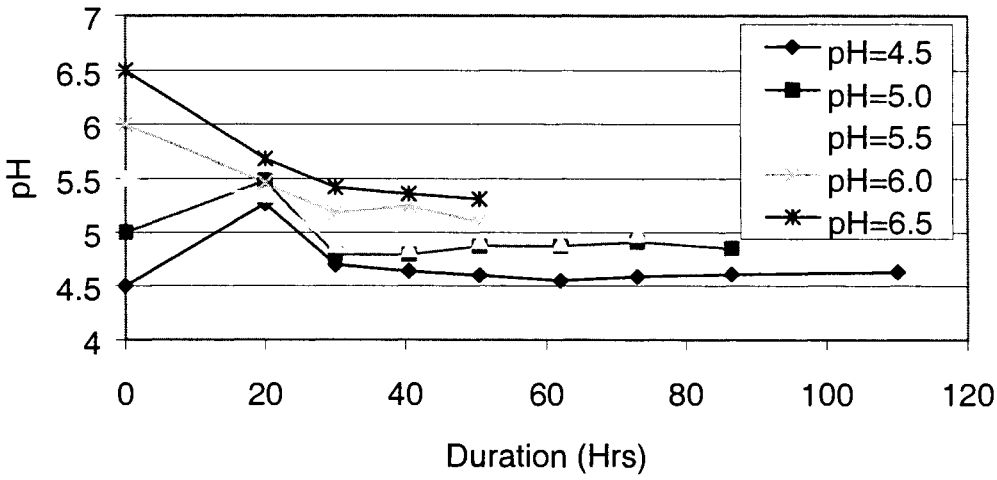

Figure 4-b. pH change profile during hydrogen production for starch batch.

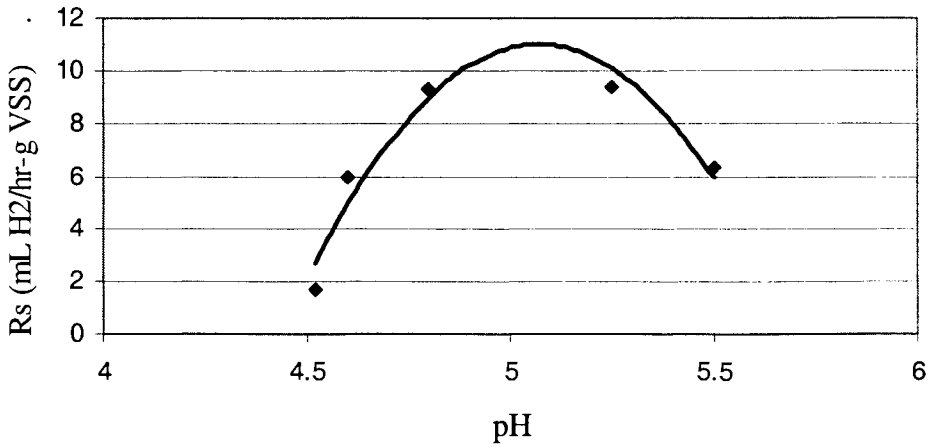

Figure 5-a. Maximum specific hydrogen production rate Rs vs. pH for sucrose batch.

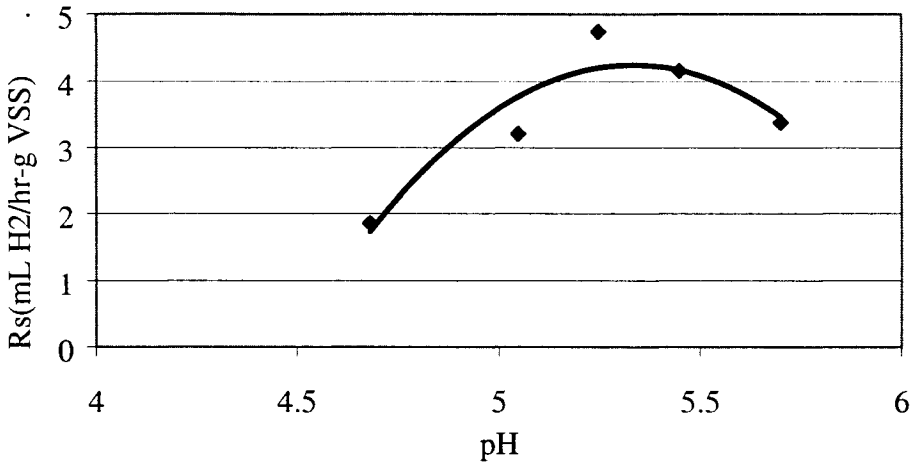

Figure 5-b. Maximum specific hydrogen production rate Rs vs. pH for starch batch.

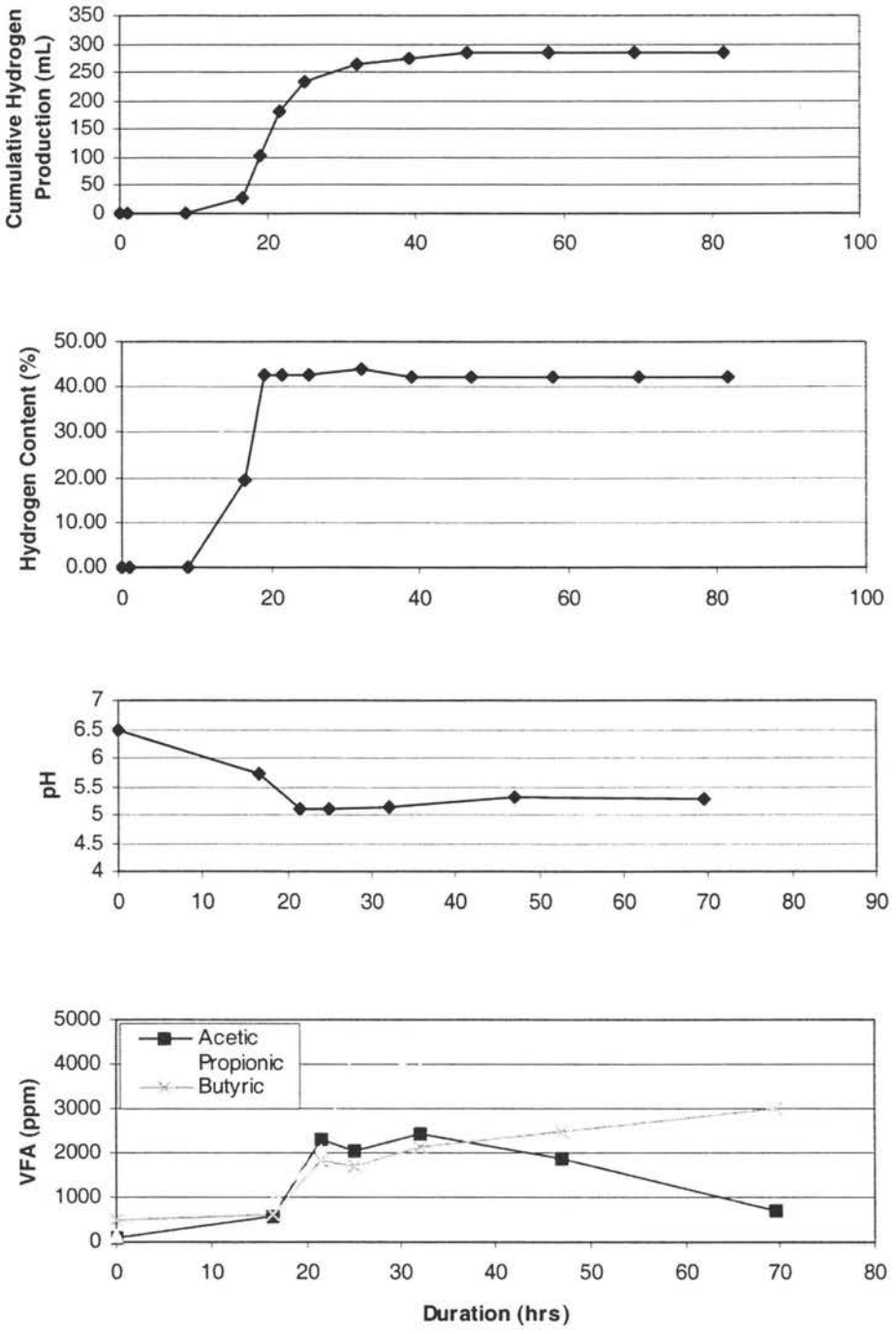

Figure 6-a. Hydrogen production, hydrogen content, pH and volatile acids production profiles for sucrose batch (initial pH = 6.0).

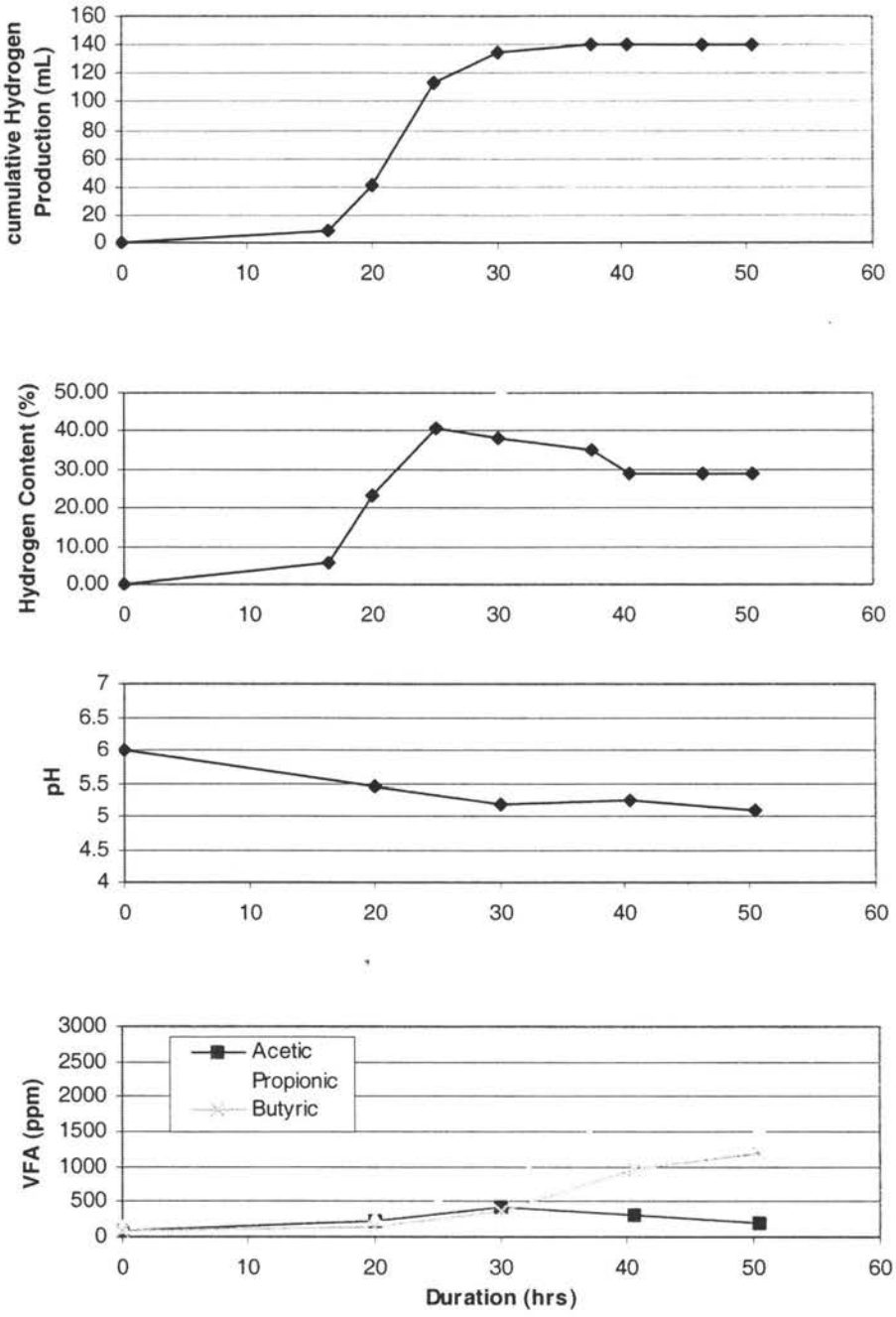

Figure 6-b. Hydrogen production, hydrogen content, pH and volatile acids production profiles for starch batch (initial pH = 6.0).

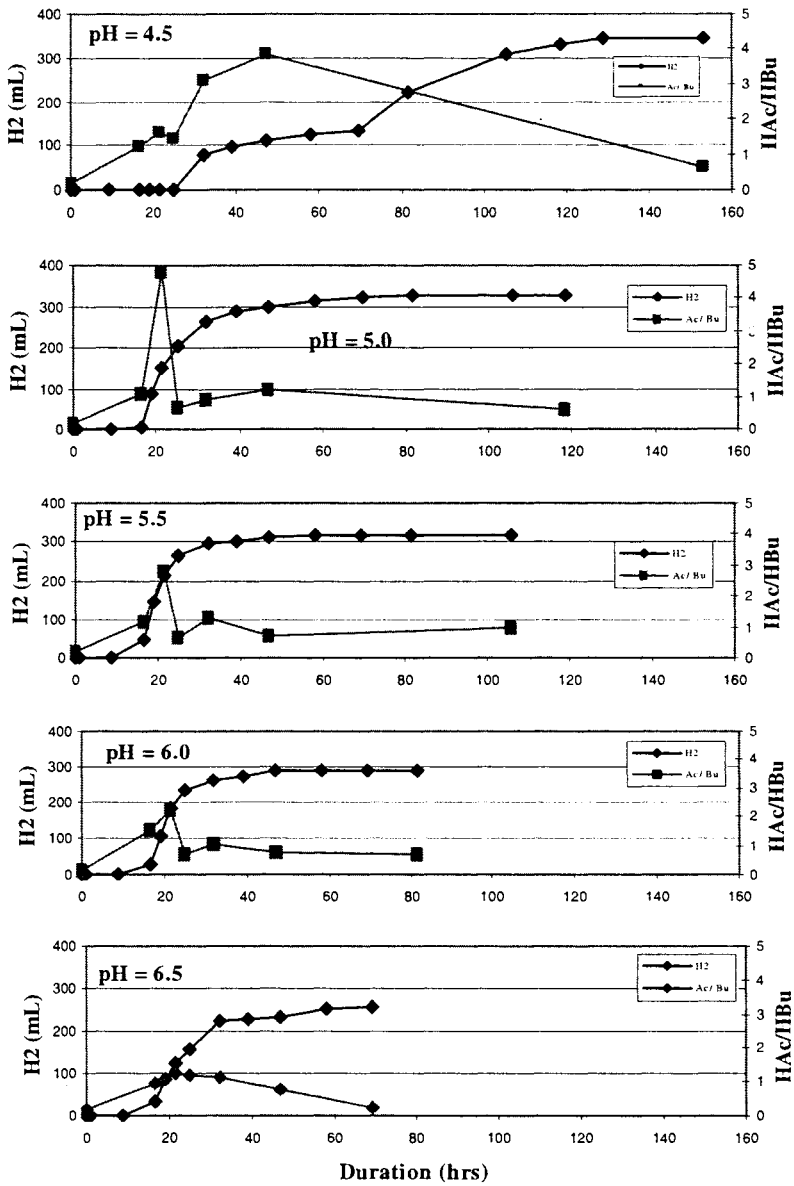

Figure 7-a. The profile of acetate/butyrate ratio and cumulative hydrogen production for sucrose batch.

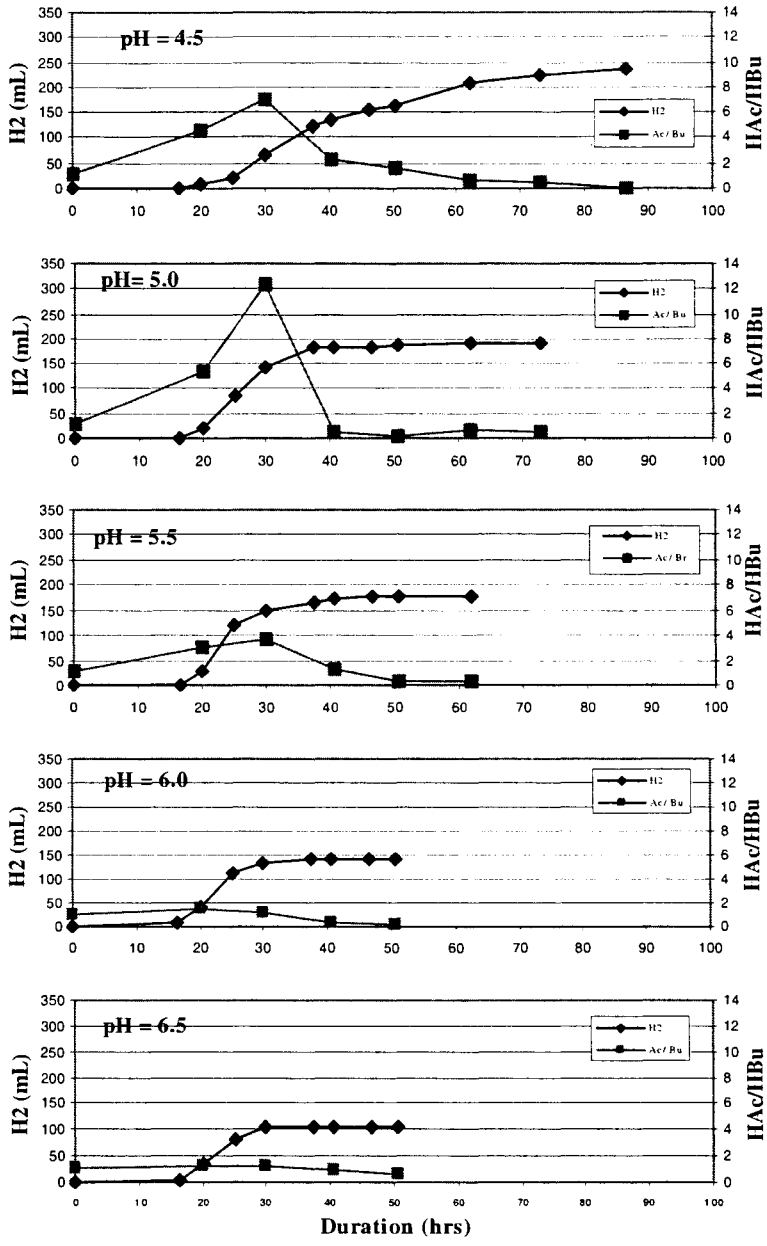

Figure 7-b. The profile of acetate/butyrate ratio and cumulative hydrogen production for starch batch.

CHAPTER 4. GENERAL CONCLUSIONS

General Discussion

Based on the experimental results, it is possible to maintain a consistent continuous flow biohydrogen fermentation process by introducing an external activation chamber. The activation process could eliminate other competitive microorganisms, while retaining hydrogen producers in the spore form. Under proper environmental conditions including sufficient substrate and nutrients, the spores in the reactivated sludge can germinate rapidly and recover the capability to produce hydrogen. This activation process is easy to implement in practice, making the hydrogen fermentation process promising and technically feasible.

Batch feeding is better suited for hydrogen production. The famine-feast mode could also impose a selection pressure on the microorganisms. During low substrate conditions, hydrogen producers could sustain in its spore form, while a shock loading of substrate could promote germination of the spores. Batch feeding could also provide a better flush out of intermediate products such as volatile fatty acids produced during the process and other substances excreted by hydrogen producers themselves that promote sporulation.

The hydrogen producers operate efficiently at shorter HRT. Therefore, easily degradable sucrose has higher hydrogen conversion efficiency than starch at the same HRT. However, the addition of sucrose to starch could enhance the hydrolysis of starch thus increasing the substrate availability to hydrogen producers, consequently improving the hydrogen production potential and hydrogen production rate. This approach may be applied in hydrogen conversion from other substrates as well.

Hydrogen conversion from starch seems more dependent on pH. Continuous flow experiments showed that an operation pH of 4.8 could give a better reactor performance than pH = 5.2 based on total amount of hydrogen produced. However, batch tests showed that the optimum pH for hydrogen production would be 5 ~ 5.5 based on hydrogen production rate. Though two results look contradictory, it should be noted that the environmental conditions and microbial physiology in batch tests are different from those existing in a continuous flow reactor. In addition to operation pH, other environmental conditions should also be optimized to obtain maximum hydrogen production rate. Even though batch test study is an easy, fast way to gain valuable information for the development of a continuous hydrogen fermentation process, the feasibility of the process ought to be verified in a continuous flow experiment. A consistent microbial physiology could be maintained in a continuous flow process and it is better suited for hydrogen production.

Although the COD conversion efficiency was low in the present continuous flow study, it could be improved by further optimization since conversion efficiencies as high as 21% (Van Ginkel, 2000a) have been achieved from batch tests and the theoretical highest COD conversion efficiency is 33%. The majority of intermediate products formed during the process are volatile fatty acids, which can be easily mineralized in subsequent biological processes such as an anaerobic methane production process. Biological hydrogen fermentation process could be a good pretreatment for industrial wastewater to maximize energy conversion from negative value organic wastes and reduce pollutant release into the environment.

Recommendations for Future Research

From the present study, a shorter HRT with higher organic loading rate provided a better reactor performance. It would be worthwhile to study the effect of shorter HRTs. However, a very short HRT could pose the potential danger of sludge washout (completely mixed reactors are used here). The influent substrate concentration can also be varied to get a higher organic loading rate. The microbial growth rate was not tested in the present study, but it would be valuable information to help optimize the reactor performance. Operation at other pH conditions should also be tested in the continuous flow study to verify the results obtained from batch tests. Mixing intensity is another environmental factor to be studied for the control of hydrogen partial pressure. Effects of oxidation-reduction potential could also be evaluated to attain optimal environmental conditions.

Batch test results showed that the pattern of the intermediate production could affect hydrogen production, within the scope of engineering, it could be interesting to study the factors that could drive the metabolic reaction towards hydrogen production. Microbial morphology and physiology study should be conducted to achieve this goal, as well as to validate the obtained results.

Other than synthetic wastewater, feasibility of hydrogen production from real wastewaters should be tested. For the near-term, readily degradable liquid wastes appear to be the most likely candidates for H_2 fermentations.

Clearly, more research and development is required. According to Benemann (1996), if developed, H_2 fermentation from negative value organic wastes would be economically more attractive than methane fermentation (in the range of $4-8/MBTU). He further concluded that the "dark fermentation of wastes is a more promising approach to biohydrogen production than other biological hydrogen production approaches."

REFERENCES

Afschar, A. S., Schaller, K., Schugerl, K.,1986. Continuous Production of Acetone and Butanol with Shear-activated *Clostridium acetobutylicum. Appl. Microbiol. Biotechnol.* **23**: 315-322.

Akashah, M., Ogiso, N., Nakamura, M., and Mastsumoto, J., 1996. Study on Preparation of Seed Sludge for Hydrogen Production in Anaerobic Acidogenesis Phase, *Proc. of The 51st Annual Con. Of the JSCE*, Nagoya, 19-21 September.

Akashah M., Yoshida M., Watanabe M., Nakamura M., and Matsumoto J., 1997. Hydrogen Gas Production from Glucose and its Microbial Kinetics in Anaerobic Systems. *Wat. Sci. Tech.* **36** (6-7): 279-286.

APHA, 1995. Standard Methods for the Examination of Water and Wastewater, 19th edn. American Public Health Association.

Benemann, J.R., 1996. Hydrogen Biotechnology: Progress and Prospects. *Nature Biotechnology*, **14**.

Biebl, H., 1999. Clostridium acetobutylicum. In : Robinson, R., Batt, C. and Patel, P., editors. Encyclopedia of Food Microbiology, 445-451, Academic Press.

Brosseau, J. D. and Zajic, J.E., 1982a. Hydrogen Gas Production with *Citrobacter intermedius* and *Clostridium pasteurianum, J. Chem. Tech. Biotechnol.*, **32**: 496.

Brosseau, J.D. and Zajic, J.E., 1982b. Continuous Microbial Production of Hydrogen gas. *Int. J. Hydrogen Energy*, **7**: 623 – 628.

Byung, H.K. and Zeikus, J.G., 1985. Importance of Hydrogen Metabolism in Regulation of Solventogenesis by *Clostridium acetobutylicum* Continuous Culture System of Hydrogen-Producing Anaerobic Bacteria. *Proc. 8th International Conf. On Anaerobic Digestion*, **2**: 383-390.

Cardon, B. P., and Barker, H. A., 1947. Amino Acid Fermentations by *Clostridium propionicum* and *Diplococcus glycinophilus*, *Arch. Biochem.* **12**: 165-180.

Dabrock, B., Bahl, H., Gottschalk, G. 1992. Parameters Affecting Solvent Production by *Clostridium pasteurianum. Appl. Environ. Microbiol.*, **58**:1233-1239.

Endo, G., Noike, T. and Matsumoto, J., 1986. Characteristics of Solid Cellulose and Soluble Glucose Degradation in Acidogenesis Phase of Anaerobic Digestion, *The Technology Reports of the Tohoku University*, **51**:103-117.

Garcia-Alvarado, J. S., M. A. Rodriguez, and R. G. Labbe. 1991. Starch hydrolysis by enterotoxin-positive and enterotoxin-negative strains of Clostridium perfringens type A at elevated termperatures. Appl. Environ. Microbiol. **58**: 326-330.

Ghosh, S. and Pohland, F. G., 1974. Anaerobic Acidogenesis of Wastwater Sludge, *Journal WPCF.*, **47**: 30-45.

Gottschalk, G. (1979). Bacterial fermentations. In: Bacterial metabolism. Springer Verlag, New York, pp 277-327.

Gottwald, M. and Gottschalk, G., 1985. The Internal pH of *Clostridium acetobutylicum* and its Effect on the Shift from Acid to Solvent Formation, *Arch Microbiol.*, **143**: 42-46.

Grady, C. P. Leslie, Jr., Daigger, G. T. and Lim, H. C., 1999. Biological Wastewater Treatment. Second Edition. Chapter **13.9**.

Grossman, A., and R. E. Levin. 1992. Extracellular control of spore formation in *Bacillus subtilis. Proc. Natl. Acad. Sci.* USA **85**, 4369-4373.

Hanaki, K., Matsuo, T., Nagase, M. and Tabata, Y., 1986. Evaluation of Effectiveness of Two-Phase Anaerobic Digestion Process Degrading Complex Substrate. *Wat. Sci. Technology*, **19**, 311-322.

Harper, S. R. and Pohland, F. G., 1985. Recent Developments in Hydrogen Management During Anaerobic Biological Wastewater Treatment, Biotechnology Report, *Biotechnol. Bioeng.*, **XXVIII**: 585-602.

Heijnen, S. J., 1995. Thermodynamics of Microbial Growth and its Implication for Process Design. *Trends in Biotechnology*, **12**: 483-492.

Holt, R. A., Cairns, A. J., Morris, G. J. 1988. Production of Butanol by *Clostridium puniceum* in Batch and Chemostat Culture. *Appl. Microbiol. Biotechnol.* **27**: 319-324.

Holdeman, L. V., Cato, E. P., and Moore, W. E. C., 1977. Anaerobe Laboratory Manual, 4th. Ed., Anaerobe Laboratory, Virginia Polytechnic Institute and State University, Blacksburg, 1-156.

Jordan, D. C., and McNicoll, P. J., 1979. A New Nitrogen-Fixing *Clostridium* Species from a High Arctic Ecosystem, *Can. J. Microbiol.* **25**: 947-948.

Jungermann, K., Thauer, R. K., Leimenstoll, G., and Decker, K., 1973. Function of Reduced Pyridine Nucleotide-Ferredoxin Oxidoreductase in Saccharolytic *Clostridia. Biochem. Biophys. Acta.* **305**: 268-280.

Kalia, V.C., Jain, S. R., Kumar, A., and Joshi, A. P., 1994. Fermentation of Biowaste to H_2 by *Bacillus licheniformis*, *World J. Microbiol. Biotechnol.*, **10**: 224-232.

Kaneuchi, C., Benno, Y., and Mitsuoka, T., 1976a, Clostridium coccoides, a new species from the feces of mice, *Int. J. Syst. Bacteriol.* **26**:482-486.

Karube, I., Matsunaga, T., Tsuru, S., and Suzuki, S., 1976. Continuous Hydrogen Production by Immobilized Whole Cells of *Clostridium butyricum*, *Biochem. Biophys. Acta*, **44**: 338-344.

Keasling, J. D., Benemann J., Pramanik, J., Carrier, T. A., Jones, K. L, and Van Dien, S. J., 1998. A Toolkit for Metabolic Engineering of Bacteria: Application to Hydrogen Production. In: BioHydrogen, Plenum Press. 87-98.

Kim, C. H. R. Cheney, and M. Woodburn. 1967. Sporulation of *Clostridium perfringens* in a modified medium and selected foods. *Appl. Microbiol*, **15**: 871-876.

Kondratieva, E. N., 1983. Production of Molecular Hydrogen in Microorganisms. *Adv. Biochem. Eng.*, **28**: 139-191.

Kumar, A., Jain, S. R., Sharma, C. B., Joshi, A. P., and Kalia, V. C., 1995. Increased Hydrogen Production by Immobilized Microorganisms. *World J. Microbiol. Biotechnol.*, **11**: 156-159.

Labbe, R. G., and C. L. Duncan. 1974. Sporulation and enterotoxin production by *Clostridium perfringens* type A under conditions of controlled pH and temperature. *Can. J. Microbiol.*, **20**: 1493-1501.

Lamed, R. J., Lobos, J. H. and Su, T. M., 1988. Effects of Stirring and Hydrogen on Fermentation Products of *Clostridium thermocellum. Appl. Environ. Microbiol.*, **54**(5): 1216-1221.

Lawrence, A. W., and McCarty, P. L., 1970. A Unified Basis for Biological Treatment Design and Operation, *J. Sanit. Eng. Div. ASCE.*, **96**: 757-778.

Lay, J. J., Li, Y., Y., Noike, T. 1998. Mathematical Model for Methane Production from Landfill Bioreactor. *J. Environ. Eng. ASCE*, **124**: 730-736.

Lay, J.J., Lee Y.J. and Noike T., 1999a. Feasibility of Biological Hydrogen Production from Organic Fraction of Municipal Solid Waste, *Wat. Res.*, **33**: 2576–2586.

Lay, J. J., Noike, T., 1999b. Biological Hydrogen Production with Variables of Cellulose Concentration and Digested Sludge Density, Submitted for Publication.

Lay, J. J., 2000. Modeling and Optimization of Anaerobic Digested Sludge Converting Starch to Hydrogen, *Biotechnol. Bioeng.*, **68**(3): 269-278.

Microsoft Excel User Guide, version 5.0, 1994. Microsoft Corporation.

Minton, N.P., Clarke D.J., 1989. Biotechnology Handbooks **3**, Clostridia. Plenum Press.

Mizuno, O., Ohara, T. and Noike, T, 1997. Hydrogen Production from Food Processing Waste by Anaerobic Bacteria. *JSCE*, **II-5**(573): 111-118.

Mosey, F. E. and Fernandez, X. A., 1989. Patterns of Hydrogen in Biogas from the Anaerobic Digestion of Milk-sugar. *Wat. Sci. Tech.*, **21**: 187-196.

Mosey, F. E., 1983. Mathematical Modeling of the Anaerobic Digestion Process Regulatory Mechanisms for the Formation of Short-chain Volatile Acids from Glucose. *Wat. Sci. Tech.*, **15**: 209-232.

Nandi, R. and Sengupta, S., 1998. Microbial Production of Hydrogen: an Overview. *Crit. Rev. Microbiol.*, **24**: 61-84.

Noike, T. and Mizuno, O., 2000. Hydrogen Fermentation of Organic Municipal Wastes. *Wat. Sci. Tech.*, **42** (12): 155-162.

Okamoto, M., Miyahara, T., Mizuno, O., and Noike, T., 2000. Biological Hydrogen Potential Material Characteristic of the Organic Fraction of Municipal Solid Wastes. *Wat. Sci. Tech.*, **41** (3): 25-32.

Owen, W. F., Stuckey, D. C., Healy Jr., J B., Young, L. Y., McCarty, P. L., 1979. Bioassay for Monitoring Biochemical Methane Potential and Anaerobic Toxicity. *Wat. Res.*, **13**: 485 – 493.

Roychowdhury, S., Cox, D., Levandowsky, M., 1988. Production of Hydrogen by Microbial Fermentation, International Association of Hydrogen Energy.

Sasikala, K., Ramana, C. V., Rao, P. R. and Kovacs, K. L., 1993. Anoxygenic Phototrophic Bacteria: Physiology and Advances in Hydrogen Technology, *Adv. Appl. Microbio.*, **38**: 211-295.

Schink, B., 1997. Energetics of Syntrophic Cooperation in Methanogenic Degradation. *Microbial. and Molecular Biol. Reviews*, **61**: 262-280.

Shih, N. J., 1995. Purification, Characterization and Regulation of Anylase from *Clostridium perferingens*. Ph.D. Dissertation. University of Massachusetts, Amherst.

Shih, N. J. and Labbe, R. G., 1996. Sporulation-Promoting Ability of *Clostridium perfringens* Culture Fluids. *Appl. and Environ.l Microbiol.*, **62** (4): 1441-1443.

Sparling R., Risbey D., and Poggi-Varaldo, H.M., 1998. Hydrogen Production from Inhibited Anaerobic Composters, *Int. J. Hydrogen Energy.*, **22**: 563-566.

Srinivasan, V. R. 1966. Sporogen – an inducer for bacterial cell differentiation. *Nature* (London), **209**: 537-540.

Stadtman, E. R., Stadtman, T. C., Pastan, I., and Smith, L. D. S., 1972. *Clostridium Barkeri* sp. n., *J. Bacteriol.*, **110**: 758-760.

Taguchi, F., Chang, J. D., Takiguchi, S., Morimoto, M., 1992. Efficient Hydrogen Production from Starch by a Bacterium Isolated from Termites. *J. Ferment. Bioeng.*, **73**: 244-245.

Taguchi, F., Yamada, K., Hasegawa, K., Taki-Saito, T., and Hara, K., 1996. Continuous Hydrogen Production by *Clostridium* sp. Strain no. 2 from Cellulose Hydrolysate in an Aqueous Two-Phase System, *J. Ferment. Bioeng.*, **82**: 80-87.

Thauer, R. K., Jungermann, K., Decker, K., 1977. Energy Conservation in Chemotrophic Anaerobic Bacteria. *Bacterial Rev.*, **1**:100-180.

Ueno, Y., Kawai, T., Sato, S., Otsuka, S., and Morimoto, M., 1995. Biological Production of Hydrogen from Cellulose by Natural Anaerobic Microflora. *J. Ferment. and Bioeng.*, **79**: 395–397.

Ueno, Y., Otsuka, S., Morimoto, M., 1996. Hydrogen Production from Industrial Wastewater by Anaerobic Microflora in Chemostat Culture. *J. Ferment. Technol.*, **82**: 194-197.

Van Andel, J.G., Zoutberg, G. R., Crabbendam, P. M. and Breure, A. M.(1985). Glucose Fermentation by *Clostridium butyricum* Grown under a Self Generated Gas Atmosphere in Chemostat Culture Solvent Formation. *Applied Microbiology Biotechnology*, **23**:21-26.

Van Ginkel, S., Sung, S. and Lay, J. J., 2000 a. The Effect of Substrate Concentration on Biohydrogen Production. Accepted by *Wat. Sci. and Tech.*

Van Ginkel, S., Sung, S. Li, L., and Lay, J. J., 2000b. The Role of Initial Sucrose and pH Level on Natural, Hydrogen – Producing, Anaerobe Germination. Submitted to *Wat. Sci. and Tech.*

Waldburger, C. D. Gonzalez, and G. H. Chambliss. 1993. Characterization of a new sporulation factor in *Bacillus subtilis*. *J. Bacteriol.*, **175**: 6321-6327.

Wang, D. I. C., Cooney, C. L., Demain, A. L., Dunnill, D., Humphrey, A. E., and Lilly, M. D., 1979. Fermentation and Enzyme Technology, John Wiley, Toronto.

Xu, X., Yu, X., and Zheng, P., 1995. Studies on Kinetics of Substrate Utilization of Hydrogen Production from Wastewater with Immobilized Cells of Photosynthetic Bacteria. *Chinese Journal of Biotechnology.*, **11**: 1-11.

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