HYDROGEN PRODUCTION BY ANAEROBIC MICROBIAL COMMUNITIES EXPOSED TO REPEATED HEAT TREATMENTS

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ABSTRACT

Biological hydrogen production by anaerobic mixed communities was studied in batch systems and in continuous-flow bioreactors using sucrose as the substrate. The systems were seeded with anaerobically digested municipal biosolids that had been heat treated at 100°C for 15 minutes. During operation, repeated heat treatments of the biomass in the reactors at 90°C for 20 minutes were performed. Results indicated that both initial heat treatment of the inoculum and repeated heat treatments of the biomass during operation promoted hydrogen production by eliminating non-spore forming hydrogen consuming microorganisms and by selecting for hydrogen producing spore forming bacteria. An operational pH of 5.5 was shown to be optimal for hydrogen production. The conversion efficiency and hydrogen yield were 0.0892 L-H_2/g-COD and 1.5291 mole of H_2/mole of sucrose, respectively. Terminal restriction fragment length polymorphism (T-RFLP) analysis showed that *Clostridium* and *Bacillus* species were dominant populations in the bioreactors. A positive correlation was observed between the total abundance of *Clostridium* species and hydrogen production during part of an operational run.

INTRODUCTION

Hydrogen production by anaerobic microbial communities (a process sometimes referred to as "dark fermentation") using organic waste as the substrate has drawn attention because of its ability to produce an environmentally friendly energy source, while simultaneously stabilizing waste. Several researchers have investigated the possibility of hydrogen production by continuously operated bioreactors (Fang and Liu, 2001, Lay *et al*., 2000, Lin *et al*., 1999, Ueno *et al.*, 1996, and Nakamura *et al.*, 1993), but sustainable hydrogen production remains a major challenge.

The theoretical maximum yield of hydrogen fermentation is reported to be four moles of hydrogen per mole of glucose (Thauer, 1977) or eight moles of hydrogen per mole of sucrose (Fang and Liu, 2001), if all of the substrate would be converted to acetic acid. These values correspond to a theoretical maximum yield of 0.467 L-H_2/g-COD. If all the substrate would be converted to butyric acid, these values are two and four moles of hydrogen per more of glucose and sucrose, respectively. In practice, a fraction of the substrate is used for biomass production and other metabolic products are also produced, resulting in a lower hydrogen yield. Hydrogen yields by pure or mixed cultures have been reported to range from 0.37 to 2.0 mole-H_2/mole-glucose (Kataoka *et al.*, 1997 and Kumar *et al.*, 1995). Considering the high theoretical yields, several researchers have begun exploring approaches to increase hydrogen production.

Several researcher have used physical methods to increase hydrogen yields by applying vacuum to the headspace of a bioreactor (Kataoka, *et al.*, 1997), by sparging the biogas with nitrogen gas (Mizuno *et al.*, 2000), by immobilizing cells (Kumar *et al.*, 1995), by vigorous stirring to allow the dissolved hydrogen to escape to the gas phase (Lamed *et al.*, 1988), or by using γ-Alumina, an activated alumina used as desiccant in chemical process industries, to adsorb volatile acids (Liang *et al.*, 2001).

One of the difficulties associated with hydrogen production using mixed communities in continuous flow systems is the coexistence of hydrogen consuming microorganisms, such as methanogens. Several studies have used heat treatment of the inoculum used to seed the reactors as a method to inactivate or eliminate these microorganisms. Lay (2000) and Okamoto (2000) used wet heat treatment (boiling for 15 minutes) of anaerobic digester sludge, whereas Van Ginkel *et al.* (2001) used dry heat treatment (baking at 104 °C for 2 hours) of compost and soils. The motivation for this heat treatment is to inactivate hydrogen consuming microorganisms and to select for hydrogen producing bacteria. This idea has merit since many of the hydrogen producing bacteria (e.g., *Clostridium* and *Bacillus* species) form endospores, which can be considered "survival structures" developed by these organisms when unfavorable environmental conditions are encountered (e.g., high temperature, desiccation, lack of carbon or nitrogen source, and chemical toxicity). When favorable conditions return, the spores germinate and become vegetative cells (Sylvia *et al.,* 1999 and Doyle, 1989). A similar idea (heat application at 80°C for 10 minutes) has been used to eliminate non-spore-forming bacteria during isolation procedures of spore-forming bacteria, such as *Clostridium* species (Doyle, 1989 and Alexander, 1977).

In addition to eliminating most vegetative cells (including hydrogen consuming microorganisms), heat treatment may also be beneficial to activate spore germination. Germination of a spore involves three steps—activation, germination, and outgrowth, and heat treatment (or heat activation) is one way to initiate spore germination. Several researchers have studied heat activation of *Clostridium botulinum* and *Clostridium perfringens*, two human pathogens (Doyle, 2002). For example, the optimal temperature/time combination for heat activation of spores of *C. perfringens* strains T-65 and S-45 were 80 °C/10min and 75 °C/120min, respectively (Hui *et al*., 1994 and Doyle, 1989). The spores of some heat-resistant strains of *C. perfringens* can be activated by heat treatment at 100 °C (Doyle, 2002). In most cases, heat activation at 75 to 80°C for 15-20 min is used to inactivate vegetative cells and activate germination of spores (Doyle, 1989). Similar studies have not been performed for other *Clostridium* species.

Germination of spores is considered a rapid process, which usually takes 60 to 90 min (Talaro and Talaro, 1996) and depends upon several parameters such as incubation temperature, pH, prior heat treatment, and reducing conditions (Doyle, 1989). One strain of *C. perfringens* germinated well at a pH of 5.5 and 7.0 but the germination rate was reduced by two-thirds when the pH was increased to 9.5. Lowering incubation temperature from 45 °C to 7 °C resulted in a decrease in the germination rate from 85% to 75% (at a pH of 6.0). Moreover, oxygen is known to inhibit germination. The addition of sodium bicarbonate and $CaCl_2$ enhanced germination of *C. perfringens* spores. Finally, the presence of lysozyme, a protein found in hen egg white, mucus, tears, and blood, helps germination of endospores (Doyle, 2002).

Spore formation or sporulation takes 6 to 8 hours in most spore forming species (Talaro and Talaro, 1996). In one strain of *C. perfringens*, free spores are released from cells within 7 to 8 hours. Depending on growth conditions, the maximum number of *C. perfringens* spores was obtained within 10 to 12 hours and 24 hours under anaerobic and aerobic conditions,

respectively (Hui *et al.*, 1994). During operation of continuously operated hydrogen fermentation processes, sporulation may lead to a decrease in hydrogen production (Minton and Clarke, 1989).

In this study, heat treatment of the inoculum was employed as a method to increase hydrogen production by inactivation of non-spore forming hydrogen consuming microorganisms and selection for hydrogen producing spore forming bacteria in both batch and continuous flow experiments. Repeated heat treatments were used to sustain hydrogen production in long term experiments.

METHODOLOGY

Inoculum

The inocula for batch and continuous flow studies were obtained from secondary anaerobic sludge digesters at a municipal wastewater treatment plant (Ames Water Pollution Control Facility, Ames, IA). The seed sludge was filtered through a screen (mesh size of 600 μm) and kept at 4°C prior to use.

Batch Experiments

Batch experiments were conducted by using 250-ml serum bottles with an active volume of 150 ml. After addition of the inoculum, phosphate buffer (1.97 g $Na_2HPO_4{\cdot}7H_20$ and 0.75 g K_2HPO_4, equivalent to 0.049 M $Na_2HPO_4{\cdot}7H_20$ and 0.029 M K_2HPO_4), 0.5 ml nutrient solution (prepared by adding 200 g NH_4HCO_3, 100 g KH_2PO_4, 10 g $MgSO_4{\cdot}7H_20$, 1 g NaCl, 1 g $Na_2MoO_4{\cdot}2H_20$, 1 g $CaCl_2{\cdot}2H_20$, 1.5 g $MnSO_4{\cdot}7H_20$, 0.278 g $FeCl_2$, 0.24 g $CoCl_2{\cdot}8H_20$, 0.12 g $NiCl_2{\cdot}6H_20$, and 0.06 g $ZnCl_2$ into 1 liter of distilled water), and 1.5 g sucrose were added to the serum bottles. The liquid volume was adjusted to 150 ml through the addition of distilled water, which resulted in a solution with a concentration of 10 g-sucrose/L or 11.23 g COD/L. The initial pH was adjusted to different values by adding 10-M HCl or 10-M NaOH. The bottles were flushed with nitrogen gas to provide anaerobic condition, capped, and mounted on a shaker operated with a horizontal rotational speed of 180 rpm. The batch experiments were conducted in the dark at 37 °C. Biogas production was measured by a plunger displacement method using appropriately sized wetted glass syringes, arranging from 5 to 50 ml (Owen *et al.,* 1979). Each condition (or batch) studied was duplicated or triplicated and average gas productions were reported.

To prevent any possible inhibitory condition (such as accumulated metabolic products) being carried over from the previous batches, a sludge washing procedure was conducted between runs. The washing procedure included a centrifugation step (2,000 x g for 20 minutes at 20 °C), followed by discarding of the supernatant (50 ml of content was retained), and addition of 600 ml of distilled water. This sequence was repeated once, and the centrifugation step followed by discarding of the supernatant, while retaining 50 ml of content, was repeated a third time. After this washing procedure, the serum bottles were exposed to the same conditions as those for the initial startup (initial pH, nutrient, buffer, and substrate concentration).

The inoculum was treated at 100 °C for 15 minutes. This treatment is referred to as “preheat treatment” in the rest of this paper. Repeated batch experiments were performed to test the capability of the seed sludge to continuously produce hydrogen. In addition to the washing procedure, the sludge in the batch experiments was reheated (at 100 °C for 15 minutes) (referred to as “repeated heat treatments”), unless stated otherwise.

Continuous Flow Experiments

Two completely-mixed anaerobic continuous flow reactors (New Brunswick Bioflo 2000, Edison, NJ), each equipped with a clarifier and a return sludge line, were used for the continuous flow experiments. One of the reactors (Reactor A) was equipped with an activation chamber, which exposed a fraction of the settled sludge to a temperature of 90°C for 20 minutes, while the other reactor (Reactor B) was not equipped with an activation chamber. Figure 1 shows a schematic of Reactor A.

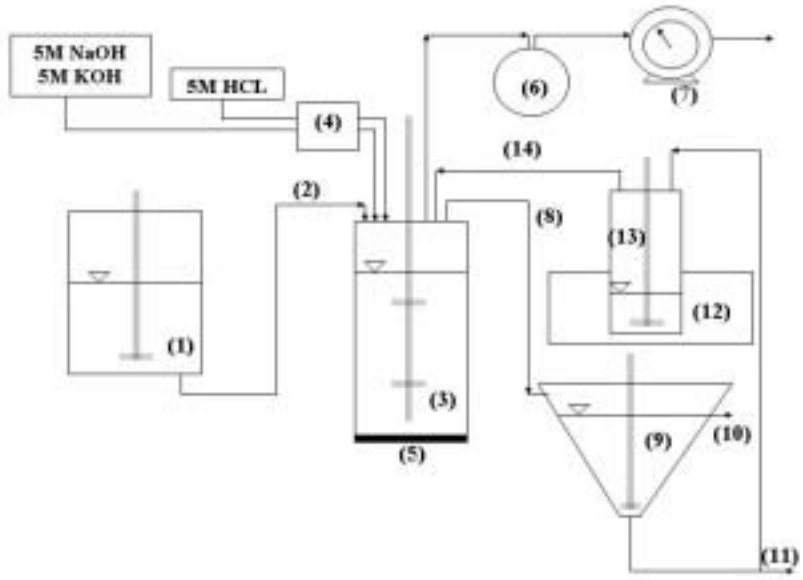

Figure 1: Schematic of the continuous flow reactor with activation chamber (Reactor A), (1) substrate reservoir, (2) influent, (3) 5-L completely-mixed anaerobic reactor, (4) pH controller, (5) heating mat, (6) balloon for pressure equalization, (7) gas meter, (8) reactor effluent, (9) clarifier, (10) system effluent, (11) sludge wastage, (12) water bath, (13) activation chamber, (14) return sludge line.

The temperature and mixing speed of both reactors were controlled at 37°C and 300 rpm, respectively. Sucrose was used as the substrate. Every 5 L of substrate solution was supplemented with 16.67 ml of nutrient solution as described in the batch experiment section above. The substrate was prepared daily and kept at 4°C. For the primary experiment described in this study, both reactors received a loading rate of 20 g COD/L/day at a hydraulic retention time (HRT) of 24 hours. The reactors were operated in semi-batch mode with feeding and decanting at regular intervals of 8 hours, three times daily except for the first day of operation when both reactors were operated in batch mode. For Reactor A, at the beginning of each cycle, 333 ml of return settled sludge (equivalent to 1,000 ml per day) was heated to 90°C for 20 minutes before being returned to the reactor. Total non-reaction time, which included pumping and settling of the sludge, was 1 hour. The pH of the reactor contents was controlled online at 5.5±0.1 through automatic addition of 5-M NaOH or 5-M KOH and 5-M HCl. Anaerobic conditions in the reactors were attained by flushing both liquid and head spaces with nitrogen gas immediately before startup. The seed sludge was treated at 100°C for 15 minutes. After

cooling to room temperature, the seed sludge was transferred to the reactors and the initial VSS concentration was adjusted to 7,000 – 8,000 mg/L.

Analytical Methods

The biogas composition was measured by two gas chromatographs (Gow Mac series 350) equipped with thermal conductivity detectors (TCD). Hydrogen was analyzed by one GC-TCD fitted with an 8' by 1" stainless steel column—SS 350A Molesieve 13X (80/100 mesh). Nitrogen was used as a carrier gas at a flow rate of 30 ml/min. The temperatures of the injection port, oven, and detector were 100, 50, and 100°C, respectively. Methane and carbon dioxide were analyzed by another GC-TCD fitted with a 3.3' stainless steel column packed with Porapak T (60/80 mesh). Helium was used as a carrier gas at a flow rate of 35 ml/min. The temperatures of the injection port, oven, and detector were at 150, 50, and 100°C, respectively. Individual VFAs and alcohols were analyzed by a gas chromatograph (Gow Mac series 580) equipped with a flame ionization detector (FID). The column used was a 6' by 8" stainless steel column—SS 580 FID, 10%SP-1200/1% H3PO4 (80/100 mesh). The temperatures of the injection port, oven, and detector were 140, 100, and 140°C, respectively, for individual VFA analysis, and 170, 70, and 170°C, respectively, for alcohol analysis. Helium was used as a carrier gas at a flow rate of 40 ml/min. Total VFA, volatile suspended solid (VSS), chemical oxygen demand (COD), and other measurements were made in accordance with the procedures listed in the Standard Methods (APHA, 1995). Carbohydrates were measured by phenol sulfuric acid method using glucose as a standard (Dubois, 1956). All gas production data reported were standardized to standard temperature (0 °C) and pressure (760 mm Hg) (STP).

Microbial Community Analysis

A fingerprinting method called terminal restriction fragment length polymorphism (T-RFLP) (Liu *et al.*, 1997) was used to identify the abundant populations in the bioreactors. DNA was extracted from biomass samples by the method described by Griffiths *et al.* (2000) with few modifications. Nucleic acid was precipitated at –20 °C overnight by adding 0.7 vol of isopropanol and 0.5 vol of 10-M ammonium acetate to the volume of aqueous sample recovered after the chloroform:isoamyl alcohol (24:1) extraction. Recovery of nucleic acid was accomplished by centrifuging 25 min at 16,000 x g at 4 °C. The pellet was washed once in 70% ethanol (ice cold) and resuspended in Tris-EDTA buffer (pH 7.4).

Polymerase chain reaction (PCR) amplification of the 16S ribosomal DNA (16S rDNA) gene was performed using the extracted DNA with a fluorescently (FAM) labeled forward primer 27f and an unlabeled reverse primer 1392r (Liu *et al.*, 1997). The PCR reaction mixture contained 1X PCR buffer, 2 mM $MgCl_2$, 0.2 mM each of deoxynucleoside triphosphate (dNTP), 0.2 µM each of forward and reverse primers, 20 ng of the DNA template and 2.5 U of *Taq* DNA polymerase (TaKaRa Biomedicals, Japan) in a final volume of 50 µl. The PCR was performed in a thermal cycler (PTC-200 DNA Engine, MJ Research Inc.). The amplification was done with a 5-min hot start followed by 30 cycles of 1 min denaturation at 94 °C, 1 min annealing at 55 °C and 1.5 min extension at 72 °C followed by a final extension of 10 min. Before purification of the PCR product (using purification kit, QIAGEN Inc, Valencia, CA), the PCR products were checked by running an agarose gel.

The purified fluorescently labeled PCR products were digested with restriction enzymes *HaeIII* (Gibco), *MspI* (Roche), and *RsaI* (Panvera) for 3 h at 37 °C. The fluorescently labeled terminal restriction fragments obtained in this manner were separated by gel electrophoresis at the

University of Illinois Biotechnology Center to determine the number and size of fragments obtained from each sample. Fragment analysis was conducted using GeneScan and the Ribosomal Database Project's online T-RFLP program (Marsh *et al.*, 2000) to identify the populations that were present in the original samples.

RESULTS AND DISCUSSION

Batch Experiments

To determine the specific hydrogen production (ml-H_2/g-VSS), specific hydrogen production rate (ml-H_2/hr/g-VSS), conversion efficiency (ml-H_2/g-COD), and lag phase duration (hours) in the batch experiments, the modified Gompertz equation (equation 1) was used to fit each cumulative hydrogen production curve obtained from the batch experiment (Lay *et al.,* 1999). This model has long been used for describing hydrogen, methane, or biogas production in batch experiment.

$$H(t) = P \cdot \exp\left\{-\exp\left[\frac{R_m \cdot e}{P}(\lambda - t) + 1\right]\right\} \quad (1)$$

where *H(t)* is cumulative hydrogen production (ml) during the incubation time, *t* (hours), *P* is the hydrogen production potential (ml), R_m is the maximum production rate (ml/hr), and λ is the lag phase duration (hours). The specific hydrogen production (ml-H_2/g-VSS) and specific hydrogen production rate (ml-H_2/hr/g-VSS) was acquired by dividing *P* and R_m, respectively, by gram VSS of sludge (g VSS). The conversion efficiency (ml-H_2/g-COD) was obtained by dividing *P* by grams of COD fed.

Three sets of batch experiments were conducted to validate the effectiveness and necessity of preheat treatment and repeated heat treatments for hydrogen production and to determine optimal values for certain parameters (e.g., pH) to be used in the continuous flow experiments. The first set of batch experiments consisted of three runs (Figure 2), with the washing procedure but no repeated heat treatments performed between each run. The seed sludge was untreated (8,500 mg-VSS/L). Even though the batches with initial pH values of 6.0, 6.5, and 7.0 exhibited larger amounts of hydrogen production compared to the experiments at the lower initial pHs, methane was detected and the methane levels increased after 251, 156, and 43 hours of incubation, respectively. This increase in methane production coincided with the depletion of hydrogen in the bottles, indicating that hydrogen consuming methanogens were active. The final pH values were 5.1, 5.5, and 6.0 for the batches with initial pH values of 6.0, 6.5, and 7.0, respectively. These three batches were not continued beyond run 1. The hydrogen production decreased substantially over the operating time for the batches operated at the lower initial pH values (4.5 to 5.5). For instance, at an initial pH of 5.5, the biogas production decreased from 482 ml in run 1 to 42 ml in run 2 and to 4 ml in run 3.

However, there was no methane detected in the biogas. Since the seed sludge was not preheated in this experiment, the hydrogen producing bacteria may have been outcompeted by other groups of fermenting bacteria as indicated by the decrease in pH values (final pH vales ranged from 3.1 to 3.5 for initial pH values of 4.5 to 5.5). This condition may have inhibited both hydrogen producing bacteria and methanogens and resulted in little or no hydrogen and methane production in subsequent runs. These observations suggested that significant changes in microbial community structure took place during repeated operation of batches.

Single batch experiments (without repeated batches) have been conducted using mixed cultures as the inoculum without heat treatment (Roychowdhury *et al.*, 1988) or without any other selection pressure (Ueno *et al.*, 1995) in order to study hydrogen fermentation and to obtain parameters, such as hydrogen yield and metabolic products profiles. However, these experimental parameters may not hold valid if the batches were to be repeated due to changes in microbial community structure as suggested by our work.

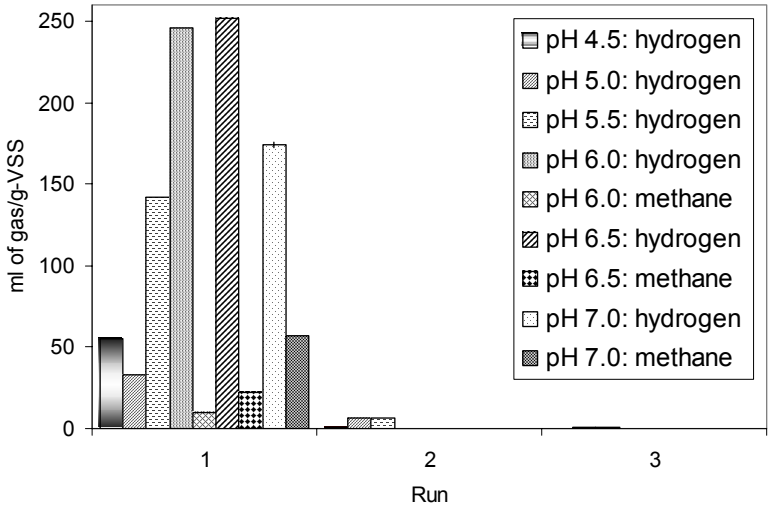

Figure 2: Hydrogen production potential and methane production in first set of batch experiments (without preheat treatment and without repeated heat treatments) at different initial pH values.

The second set of batch experiments (Figure 3) was conducted to find important parameters, especially pH, for hydrogen fermentation by using heat treatment (at 100°C for 15 minutes) of the seed sludge (7,100mg-VSS/L). Different initial pH values ranging from 4.5 to 6.5 were selected for this batch experiment. It was found that the batch operated at an initial pH value of 5.5 exhibited the highest specific hydrogen production (119.5 ml-H_2/g-VSS), hydrogen production rate (13.1 ml-H_2/hr/g-VSS), and conversion efficiency (75.5 ml-H_2/g-COD or 84.8 ml-H_2/g-sucrose). It was also found that the lag phase duration decreased as the initial pH increased. Lag phase duration at an initial pH of 5.5 was approximately 14 hours. The maximum decrease in pH during operation was one pH unit and occurred in the bottles with an initial pH of 6.5, indicating a strong buffering capacity. Van Ginkel *et al.* (2001) studied hydrogen production as a function of pH and substrate concentration, using heat treated compost, potato soil, and soy bean soil as inocula and found that a pH between 5.0 – 6.0 was suitable for hydrogen production, whereas the highest conversion efficiency occurred at a pH of 5.5. Lin and Chang (1999) reported significantly higher specific hydrogen production rates at pH 5.7 than that at pH 6.4 when using a mixed culture anaerobic reactor with an SRT of 0.25 – 0.5 day. However, Lee and co-workers (2002) found that the optimal initial pH was 9. This finding is unusual among the studies of hydrogen fermentation using mixed or pure cultures. In their study, they observed sharp decreases of the initial pH values of 6 to 9 to a final pH value of 4; therefore, the hydrogen production for the lower initial pH value experiments (lower than 9) may have been inhibited early on by these sharp decreases in pH values.

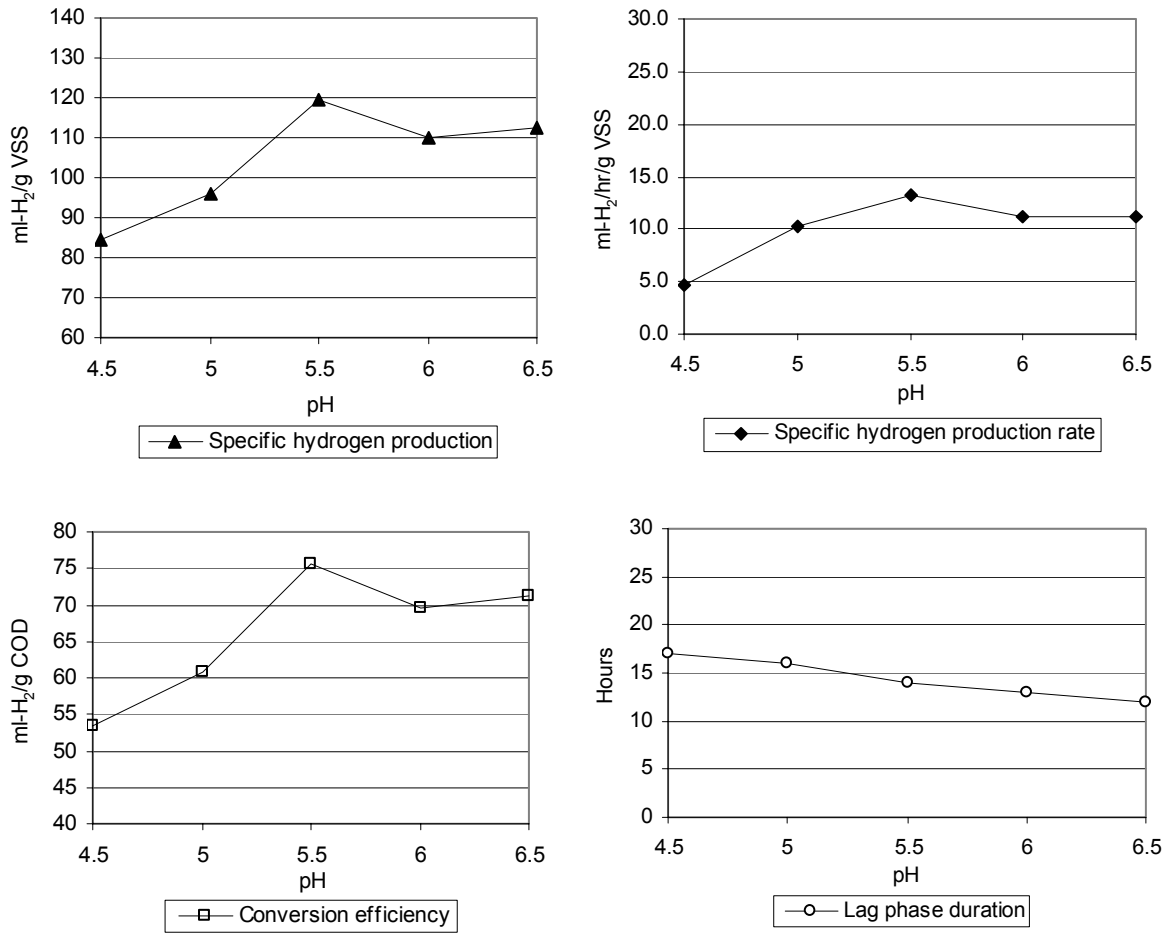

Figure 3: Effect of initial pH on hydrogen production and lag phase duration in second set of batch experiments (with preheat treatment).

The third set of batch experiments (Figure 4) was conducted to compare the effect of repeated heat treatments at 100°C for 15 minutes, after preheat treatment of the seed sludge (9,360mg-VSS/L) at 100°C for 15 minutes. The experiment was performed at an initial pH of 5.5 (optimal pH determined from the previous set). The batches in this experiment were repeated seven times (as indicated by runs 1 to 7 in Figure 4b) with washing between runs. The different experimental duration of each run was meant to be a simulation of continuous flow reactors with feeding and decanting. After run 7, it was found that the VSS concentration had decreased approximately 20 % compared to the initial concentration due to washing. The results indicated that the batches with repeated heat treatments produced consistently more hydrogen compared to the batches without repeated heat treatments. Methane was not detected in any batch in any run. Since heat treatment at 80°C for 10 minutes has been utilized for isolation of spore formers (Alexander, 1977), the heat treatment of 100°C for 15 minutes in this experiment should have had the same effect. Thus, it promoted the selection of spore-forming hydrogen producers and eliminated non-spore formers. This is important for future work when actual wastewater will be used as a substrate, since the microorganisms introduced through the wastewater should not be able to compete with the hydrogen producers. Apparently, repeated heat treatments will be able to accomplish this goal. Besides the benefit of heat treatment to eliminate non-spore

formers, such as hydrogen consumers, heat treatment can promote spore germination (Doyle *et al*., 1989 and Hui *et al.,* 1994). By eliminating non-spore formers and promoting germination of spores, the levels of active hydrogen producers in the bioreactor will be increased, leading to a greater potential hydrogen production

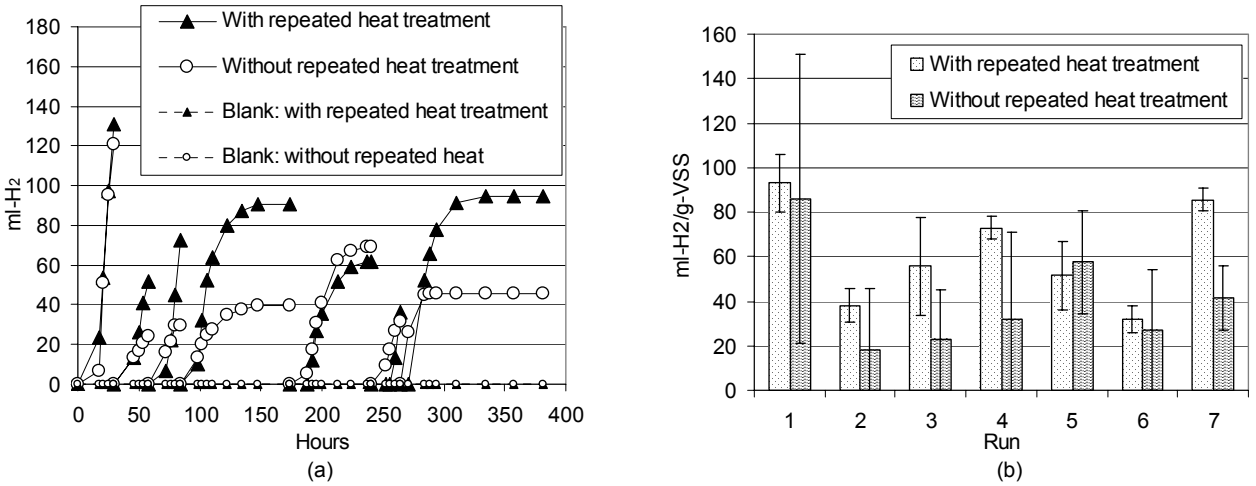

Figure 4: Effect of hydrogen production in third set of batch experiments with and without repeated heat treatments for seven runs at an initial pH of 5.5. Cumulative hydrogen production curves (a) and specific hydrogen production (b) (Error bars indicate ± standard deviation).

An interesting observation made during the third set of batch tests was the occurrence of a lag phase, a phase without any gas production (Figure 4a). A longer lag phase of about 11 hours was observed for run 7 of the batch with repeated heat treatments, while a significantly shorter lag time of about 5 hours was observed for those runs without repeated heat treatments. A lag phase duration of approximately 14 hours was also observed for the second set of batch experiments at an initial pH of 5.5. After heat treatment at 100°C for 15 minutes, presumably only spores survive. This lag phase may be explained by the fact that spores need time to germinate and vegetative cells need time to grow (after germination). Therefore, HRTs longer than the lag phase, i.e. 11 hours, would be needed to operate continuous-flow reactors.

Continuous Flow Experiments

The reactors were operated in semi batch mode, feeding and decanting every 8 hours. This approach was chosen because chemical or biological reactions will proceed faster in the desired direction if the products are withdrawn or if reactants are added. Specifically, for the acetic acid fermentation from sucrose (Equation 2), hydrogen formation should be favored if the concentration of sucrose is increased or if the concentrations of acetic acid and/or hydrogen are lowered. By decanting and then feeding, a higher concentration of sucrose and lower concentrations of VFAs and $H_{2(aq)}$ (due to dilution) will be achieved.

$$C_{12}H_{22}O_{11} + 5H_20 \rightarrow 4CH_3COOH + 8H_2 + 4CO_2 \qquad (2)$$

A preliminary experiment (operating parameters: HRT = 24 hours, semi-batch feeding three times a day, and pH controlled at 5.5) was performed at a volumetric loading rate of 6 g COD/L/day (Duangmanee *et al*., 2002). This experiment showed minimal hydrogen production

and production of methane. The activity of methanogens was somewhat surprising given that the seed sludge was preheated at 100°C for 15 minutes, that the return sludge underwent repeated heat treatments at 90°C for 20 minutes (using the activation chamber), and that the reactor pH was maintained at 5.5, which is below the optimal range for methanogens pH of 6.5-7.6 (Parkin *et al.*, 1986). However, since only approximately one third of the settle sludge underwent heat treatment in every cycle, it is likely that methanogens were able to grow and consume hydrogen despite the relatively low pH. Previous studies have shown that unionized volatile acid (UVA) concentrations in the range of 30 to 60 mg/L are inhibitory to methanogens (Parkin *et al.*, 1986). Even though the observed total VFA concentration of 2,000 mg/L as acetic acid at a pH of 5.5 and a temperature of 37 °C is equivalent to a UVA of approximately 316 mg/L, complete inhibition did not occur. In summary, a possible strategy could consist of operation at sufficiently high VFA concentrations to inhibit methanogenic activity (substrate inhibition), but at a sufficiently low VFA concentration to prevent inhibition of hydrogen producers.

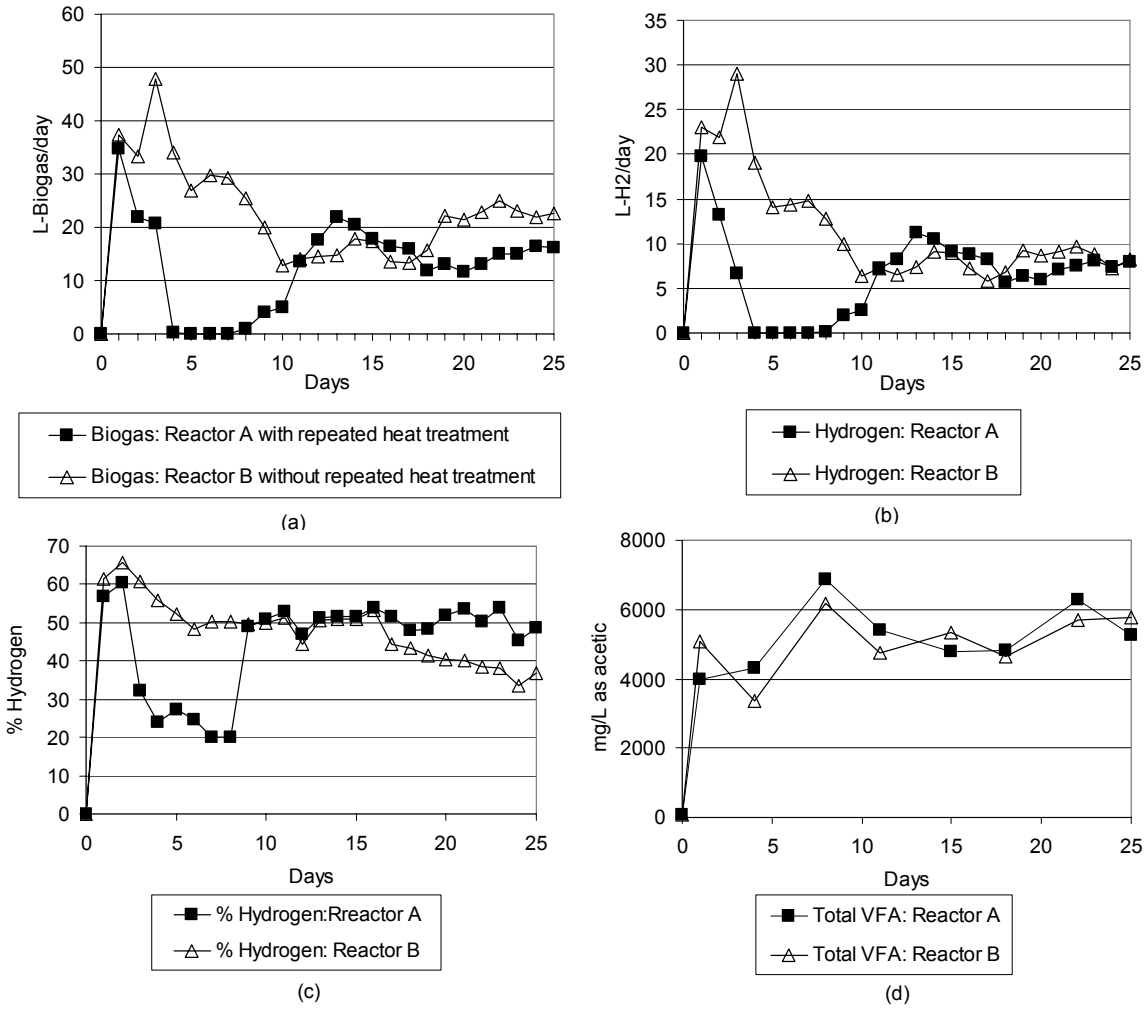

Figure 5: Total biogas (a) and hydrogen production (b) per day, percentage of hydrogen in biogas (c), and total VFA concentration (d) for continuous anaerobic reactor with (Reactor A) and without (Reactor B) repeated heat treatments of return settled sludge.

To maximize hydrogen production, the volumetric loading rate was increased to 20 g COD/L/day and two reactors were operated in parallel. Repeated heat treatments at 90°C for 20 minutes were applied to the return sludge from reactor A only. The inocula for both reactors were treated at 100°C for 15 minutes. The results indicated that the amount of hydrogen produced was similar in reactors A and B after 10 days of operation (Figure 5b); however, a higher percentage by volume of hydrogen was produced by reactor A (Figure 5c).

Methane was not detected in the biogas collected from either one of the reactors for the duration of the experiment. Total VFA concentrations as high as 6,000 mg/L (Figure 5d) as acetic acid were observed in both reactors. This value corresponds to approximately 950 mg/L of UVA, which is much higher than the reported range of 30 to 60 mg/L of UVA for inhibition of methanogens (Parkin *et al.*, 1986) and the value of 316 mg/L of UVA obtained for the preliminary experimental run at a loading rate of 6g COD/L/day. Thus, the high VFA concentrations in the reactors likely inhibited methanogenic activity. A decrease in hydrogen production coincided with a decrease in the VSS concentration from 7,750 mg/L (in both reactors) on day 0 to 2,000 and 2,800 mg/L in reactors A and B, respectively, on day 10, while VFAs were continually produced (Figure 5d). On average, 92 and 93% of sucrose was consumed by reactors A and B, respectively. After 10 days of operation, the hydrogen production, VSS concentration, and VFA concentration for both reactors stabilized (Table 1).

Table 1: Comparison of important parameters for Reactors A and B from day 11 to 25.

	Reactor A	Reactor B
Volumetric hydrogen production rate (L-H_2/L/day)	1.61 (2.25*)	1.61 (1.93*)
% of H_2/CO_2	50.72 / 46.02	42.65 / 54.01
Oxidation Reduction Potential (mV)	-320	-340
Reactor VSS (mg/L)	3,000	3,160
Effluent (system) VSS (mg/L)	1,780	1,400
Solids retention time, SRT (days)	1.75	2.26
Fraction of sucrose consumed	0.90	0.91
% of COD reduction (%)	19.46	21.92
Specific hydrogen production rate (L-H_2/g-VSS/day)	0.5353 (0.7533*)	0.5101 (0.6076*)
Conversion efficiency (L-H_2/g-COD)	0.0892 (0.1256*)	0.0886 (0.1055*)
Hydrogen yield (mole of H_2/mole of sucrose)	1.5291 (2.1530*)	1.5188 (1.8085*)
% of theoretical yield‡	19.11 (26.91*)	18.98 (22.61*)

*the maximum value obtained from operation from day 11 to 25
‡theoretical yield from acetic-producing hydrogen fermentation is 8 mole of H_2/mole of sucrose, 0.5240 L-H_2/g sucrose, or 0.4667 L-H_2/g COD

Microbial Populations

Samples were obtained on a regular basis from the two continuous flow reactors operated at a volumetric loading rate of 20 g COD/L/day and from the heat-treated inoculum used to seed the reactors. Samples collected were analyzed with T-RFLP. Results (Figure 6) indicate that two

major groups of *Clostridium* species were dominant in both reactors during the first 15 days of operation. The first dominant *Clostridium* group contained one or more of the following species: *C. beijerinckii, C. botulinum, C. putrificum,* and *C. sporogenes*. The second dominant population was identified to be *Clostridium butyricum*.

Clostridium tetanomorphum was found to be predominant in the inoculum after heat treatment, but was not present above the detection limit in either one of the reactors. There was a positive correlation between total abundance of the identified *Clostridium* species (Figure 6c) and hydrogen production (Figure 5b) during the first 15 days of operation in both reactors. In other words, a decrease in hydrogen production coincided with a decrease in the total levels of *Clostridium* species, and *vice versa*. But after day 18, the two identified *Clostridium* groups ceased to be dominant in reactor B although the hydrogen production remained stable. A *Bacillus* species, *Bacillus laveolaticus* (another spore former), became dominant after day 15 in reactor B and the same species became dominant in reactor A after day 22.

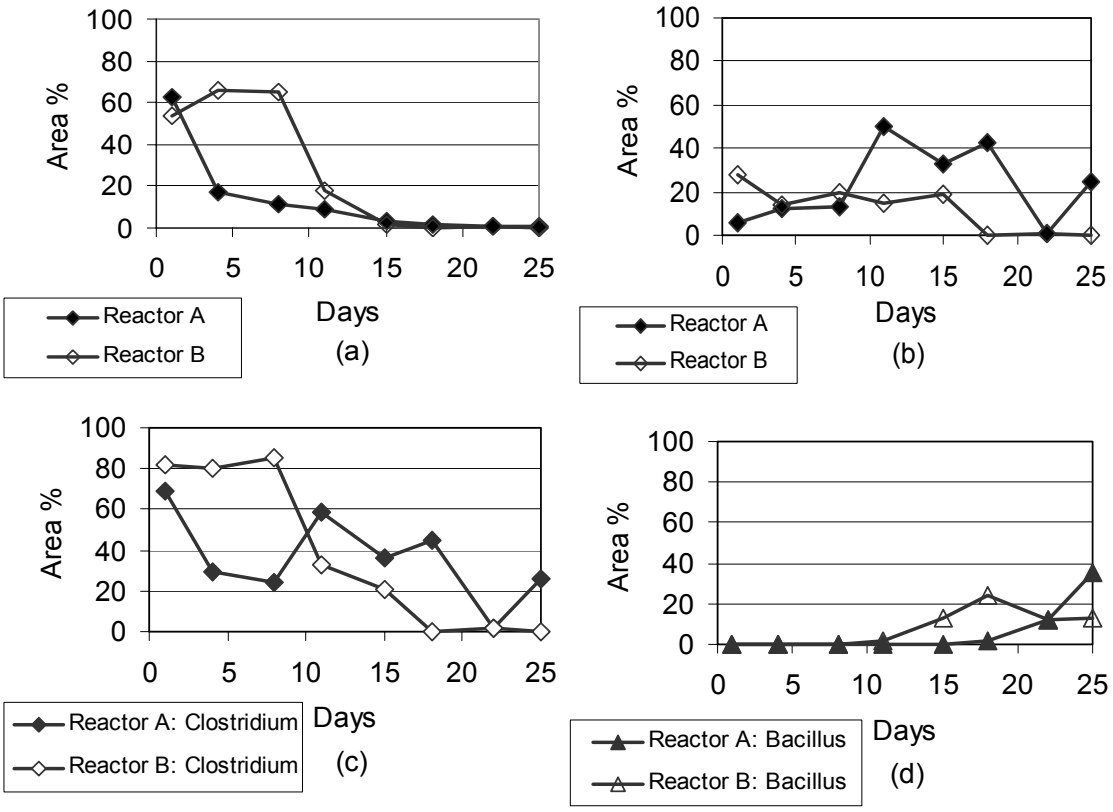

Figure 6: Relative levels of dominant *Clostridium* groups in Reactors A and B. *Clostridium* group 1 contained one or more of the following species *C. beijerinckii, C. botulinum, C. putrificum,* and *C. sporogenes* (a), *Clostridium* group 2 contained *C. butyricum* (b). Sum of relative abundance of both *Clostridium* groups identified (c), and total abundance of *Bacillus* species identified (d). Area % represents the relative population abundance.

Performance of Reactors

A summary of reactor performance data is reported in Table 1. Since the VSS in the effluent was higher for Reactor A than for Reactor B (1,780 and 1,400 mg-VSS/L, respectively), it is likely that heat treatments of sludge in Reactor A affected the settleability. Consequently, the SRT for Reactor A was lower than the SRT for Reactor B. On average, the percentage of hydrogen in the biogas of Reactor A was approximately 8% greater than for Reactor B since the specific hydrogen production and conversion efficiency were greater in Reactor A than in Reactor B.

Acetate, propionate, butyrate, ethanol, and butanol are the most common fermentation products produced by *Clostridium* species (Minton *et al.*, 1989). In this experiment, the combination of butyrate, acetate, and propionate accounted for 98% of VFAs determined in terms of COD, and butanol, ethanol, and propanol constituted additional major products (Table 2).

Table 2: Comparison of the percentage of metabolic products and unutilized carbohydrate (on a COD basis) of hydrogen production in Reactors A and B from day 11 to day 25.

Reactor	Soluble COD (%)	Butyrate (%)	Acetate (%)	Propionate (%)	Butanol (%)	Ethanol (%)	Propanol (%)	Unutilized Carbohydrate (%)
A	100	17.21	13.97	5.23	2.17	1.77	0.12	13.86
B	100	15.42	12.83	7.08	2.79	2.07	0.03	13.05

Figure 7 shows the distribution of the major metabolic products for the duration of the experiment. Similar amounts of metabolic products were obtained in both reactors after operation in batch mode on the first day of operation. From days 4 to 8, no biogas production (Figure 5a) was observed for Reactor A, but VFAs continued to be produced (Figure 5d). The amount of acetate was particularly high, up to 4,322 mg/L, and accounted for 70% of VFAs present. Homoacetogenic clostridia, such as *C. thermoaceticum*, *C. thermoautotrophicum*, and *C. magnum* (Minton *et al.*, 1989), have the ability to convert glucose or sucrose to acetate. They can also grow autotrophically using hydrogen and carbon dioxide to produce acetate. It is possible, that homoacetogenic clostridia were important during this time period, although T-RFLP data (see above) did not detect these bacteria. However, on day 11, hydrogen production resumed. In repeated experiment (data not shown), similar results were obtained. This observation indicates that repeated heat treatments during the early stage of operation are not necessary.

Since the difference in hydrogen production between Reactors A and B was minimal, we hypothesize that the amount of the return sludge heated in each cycle was too small (only one third of the return sludge was heated), that the heat treatment was performed too frequently (three times a day), and/or that a long lag phase after repeated heat treatments may have prevented good performance. Heat treatment at 90°C for 20 minutes kills most vegetative cells, leaving only spores. However, since only one third of the return sludge was heat treated, the two thirds of return sludge that were not treated may have contained sufficient amounts of non-hydrogen producing organisms to diminish hydrogen yields. The frequent heat treatments might have prevented all surviving spores to germinate and grow up to sufficient levels since germination of spores takes some time (60 to 90 min; Doyle *et al*., 1989) and heat treatment eliminates all vegetative cells, including those of hydrogen producing spore formers. A long lag

phase may also have been responsible for the poor performance since results from batch experiments indicated that the seed sludge that was subjected to heat treatment exhibited a lag period of about 14 hours at pH 5.5 (Figure 3). Another set of batch experiments (data not shown) conducted using an inoculum subjected to heat treatment at 100°C for 15 minutes and repeated heat treatments at 90°C for 20 minutes demonstrated a lag period of 18 hours. With a relatively long lag phase compared to the short SRT of 1.9 days in Reactor A, the limited improvement in performance by Reactor A, relative to the performance by Reactor B was not surprising.

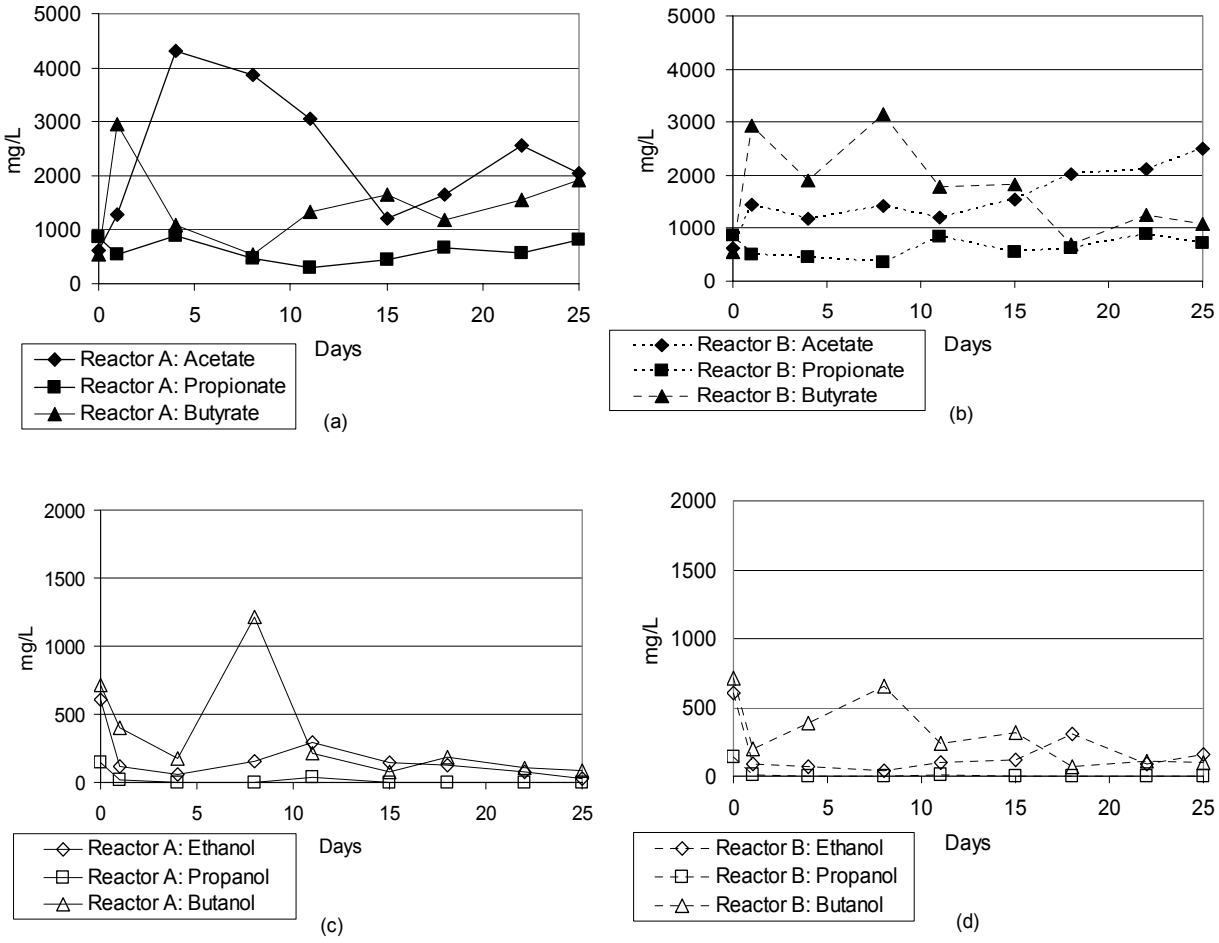

Figure 7: Major acids produced by Reactor A (a) and Reactor B (b), major alcohols produced by Reactor A (c) and Reactor B (d).

CONCLUSIONS

Sustainable hydrogen production was achieved by using a combination of pretreatment of the inoculum and repeated heat treatments of the biomass in anaerobic bioreactors. Operation with relatively high total VFA concentrations (around 6,000 mg/L as acetic acid) is preferred to ensure additional inhibition of hydrogen consuming microorganisms, such as methanogens. It was necessary to apply repeated heat treatments to the return sludge in continuous flow reactors to maintain hydrogen production. However, our results suggest that a larger portion of

the return sludge should be heated (larger than the one third fraction used in this study) and that less frequent heat applications may be beneficial (less frequent than once every 8 hours). Our future work will evaluate if such modifications will improve specific hydrogen production rates, conversion efficiencies, and hydrogen yields. We will also continue to use T-RFLP and introduce additional microbiological techniques to evaluate if our hypotheses with respect to selection of spore-forming hydrogen producing bacteria are valid.

ACKNOWLEDGEMENTS

This study was supported by a grant from the U.S. Department of Energy, contract number DE-FC36-00GO10530, through the Hydrogen Program.

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