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International Journal of Hydrogen Energy 32 (2007) 4761–4771
International Journal of HYDROGEN ENERGY
www.elsevier.com/locate/ijhydene

Biohydrogen production from cheese processing wastewater by anaerobic fermentation using mixed microbial communities

Peilin Yang[a,*], Ruihong Zhang[b], Jeffery A. McGarvey[c], John R. Benemann[d]

[a]*Department of Agricultural and Biological Engineering, Mississippi State University, Mississippi State, MS 39762, USA*
[b]*Department of Biological and Agricultural Engineering, University of California at Davis, Davis, CA 95616, USA*
[c]*U.S. Department of Agriculture, Agricultural Research Service, Foodborne Contaminants Research Unit, Albany, CA 94710, USA*
[d]*Benemann Associates, Walnut Creek, CA 94595, USA*

Received 15 October 2006; received in revised form 2 June 2007; accepted 16 July 2007
Available online 4 September 2007

Abstract

Hydrogen (H_2) production from simulated cheese processing wastewater via anaerobic fermentation was conducted using mixed microbial communities under mesophilic conditions. In batch H_2 fermentation experiments H_2 yields of 8 and 10 mM/g COD fed were achieved at food-to-microorganism (F/M) ratios of 1.0 and 1.5, respectively. Butyric, acetic, propionic, and valeric acids were the major volatile fatty acids (VFA) produced in the fermentation process. Continuous H_2 fermentation experiments were also performed using a completely mixed reactor (CSTR). The pH of the bioreactor was controlled in a range of 4.0–5.0 by addition of carbonate in the feed material. Maximum H_2 yields were between 1.8 and 2.3 mM/g COD fed for the loading rates (LRs) tested with a hydraulic retention time (HRT) of 24 h. Occasionally CH_4 was produced in the biogas with concurrent reductions in H_2 production; however, continuous H_2 production was achieved for over 3 weeks at each LR. The 16S rDNA analysis of DNA extracted from the bioreactors during periods of high H_2 production revealed that more than 50% of the bacteria present were members of the genus *Lactobacillus* and about 5% were *Clostridia*. When H_2 production in the bioreactors decreased concurrent reductions in the genus *Lactobacillus* were also observed. Therefore, the microbial populations in the bioreactors were closely related to the conditions and performance of the bioreactors.

Keywords: Biohydrogen; Anaerobic fermentation; Cheese whey wastewater; pH control; *Lactobacillus*; VFA

1. Introduction

There is increased interest in the production and use of hydrogen (H_2) as a clean fuel for various applications. H_2 can be used by itself or blended with another fuel, such as methane (CH_4). Combustion of H_2 and CH_4 mixtures results in lower NO_x emissions from internal combustion engines [1,2], which is highly desirable for air quality. Furthermore, H_2 use in fuel cells is superior to CH_4 and alcohol combustion as it results in higher energy efficiency [3].

There are two major biological processes for the production of H_2 by microorganisms. One utilizes photosynthetic organisms, such as *Oscillatoria* sp. and *Rhodopseudomonas*, *capsulate* and the other involves fermentative, H_2-producing organisms such as *Clostridia butyricum* and *Escherichia coli* [4]. Based on current technologies it is not economically feasible to grow photosynthetic bacteria in large photo-bioreactors using synthetic culture media as described previously [5,6]. Production of H_2 by anaerobic fermentation of organic substrates may be more practical than by photo-biological conversion if a low/no cost feed material can be obtained because anaerobic fermentation does not require the expensive and large surface area photo-bioreactors necessary to utilize solar energy efficiently [6].

There have been many studies regarding anaerobic fermentations for H_2 production, with research accelerating in recent years. The substrates for these fermentation reactions are typically simple sugars or starches, which are not economically feasible to use due to their high cost. Waste/wastewater

* Corresponding author. Tel.: +1 662 325 3365; fax: +1 662 325 3853.
E-mail address: py27@msstate.edu (P. Yang).

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doi:10.1016/j.ijhydene.2007.07.038

containing high concentrations of carbohydrates generated from agricultural processes (such as animal wastes and agricultural residues) and food industries (such as dairy processing and vinery wastewaters) is preferred for economic reasons. A few researchers have studied the production of H_2 via the anaerobic fermentation of wastes, such as municipal solid waste and wastewaters [7–10], sugar manufacturer wastewater [11], synthetic wastewater [12], dining hall food waste [13], alcohol manufacturer wastewater [14], starch manufacturer waste [15], and rice slurry [16].

The objective of this study was to investigate batch and continuous anaerobic fermentation processes for H_2 production from cheese whey wastewater using mixed microbial cultures and to develop a stable anaerobic fermentation process for continuous H_2 production. California is the largest dairy producing State in the United States, and thus also produces the greatest amount of whey byproduct. Developing innovative technologies for the utilization of this byproduct as a valuable resource, such as H_2, is important from both an economical and environmental standpoint.

2. Methods and materials

2.1. Experimental design

Batch experiments were performed to study the degradation rate and potential H_2 yield of cheese whey permeate. Four feed-to-microorganisms (F/M) ratios (0.5, 1.0, 1.5, and 2.0) were tested in duplicate. The feed concentration (F) was based on the chemical oxygen demand (COD) of cheese whey permeate and the microorganism concentration (M) was estimated by the volatile suspended solid (VSS) concentration of the anaerobic digester sludge used as the inoculum. The F/M ratios were calculated using a feed concentration of 5.0 g COD/L and different VSS concentrations of the anaerobic sludge. The initial and final pH levels of the fermentation solution were measured. Total biogas production after 48 h was measured and converted to the volume at 25 °C. Hourly biogas production from one of the batch bioreactors was also measured to determine the rate of the biogas production and to estimate the retention time needed to run continuous fermentation bioreactors. The H_2 yield was calculated from the volume of biogas produced and H_2 content of the biogas.

Continuous experiments were conducted to study process variables, such as hydraulic retention time (HRT), organic loading rate (LR), and pH, on H_2 production. Three bioreactor systems (denoted as Systems 1, 2, and 3) were operated. System 1 was designed to determine the effect of different LRs (10, 12, and 14 g COD/L/d) at a selected HRT (24 h) and was started at an LR of 5.0 g COD/L/d. System 2 was designed to study the effect of different HRTs (12, 18, and 24 h) corresponding to different LRs (10, 6.7, and 5 g COD/L/d). System 3 was designed to allow visual observation of the inside of the bioreactor for biofilms build-up and was operated at two LRs (10 and 12 g COD/L/d) at a HRT of 24 h. The microbial communities in the continuous bioreactors were analyzed to identify the types of bacteria present when high and low H_2 production levels were reached in order to identify the types of bacteria involved in H_2 production.

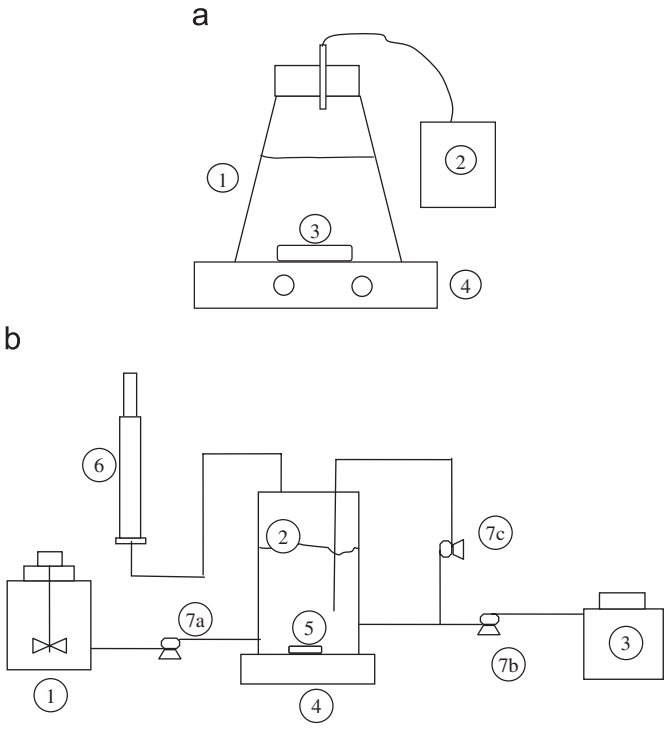

Fig. 1. Schematic of H_2 fermentation systems. (a) Batch bioreactor set-up: (1) bioreactor, (2) gas collector, (3) magnetic stirrer, and (4) magnetic plate. (b) Continuous bioreactor system set-up: (1) feed tank, (2) bioreactor, (3) effluent tank, (4) magnetic stir plate, (5) magnetic stirrer, (6) gas meter, and (7) pumps: (a) feed influent, (b) bioreactor effluent, and (c) recirculation.

2.2. Experimental set-up of batch and continuous bioreactor systems

The set-up of the batch bioreactors is shown in Fig. 1a. The 1-L Erlenmeyer flasks (Fisher Scientific, Pittsburgh, PA) were sealed with a gas-impermeable rubber septum, mixed with a magnetic stirring bar, and placed in an incubator maintained at 35–38 °C. The biogas produced in each bioreactor was collected in a 3-L Tedlar bag (SKC, Eighty Four, PA) and its volume measured using a water displacement technique and converted to the volume at 25 °C for reporting.

The set-up of continuous fermentation bioreactor systems is shown in Fig. 1b. The general set-up was the same for all three systems. Systems 1 and 2 were identical, using bioreactors with a working volume of 1.5 L. In order to visually observe the inside of the bioreactor, System 3 used an 1-L Erlenmeyer flask. Each of three bioreactors was housed in an incubator maintained at 35–38 °C. The feed material storage tank was maintained at 4 °C in a refrigerator. The total biogas production for each system was measured using a water displacement meter. Each bioreactor was fed and decanted using peristaltic pumps (Cole Parmer Instrument Company, Vernon Hills, IL) that were controlled automatically. The bioreactors in Systems 1 and 2 were mixed using liquid recirculation and magnetic stirrers,

Table 1
Characteristics of the seeding inocula (unit: mg/L)

TKN	NO_3–N	$P_{(Total)}$	$K_{(Total)}$	$S_{(Total)}$	$Ca_{(Total)}$	$Mg_{(Total)}$	$B_{(Total)}$	$Zn_{(Total)}$	$Mn_{(Total)}$	$Fe_{(Total)}$	$Cu_{(Total)}$	TS	VS	$C_{(Total)}$
2050	0.245	613	107	601	1790	456	2.95	44	10	711.5	34.3	44,300	25,700	11,500

Table 2
Characteristics of the cheese whey permeate powder

Moisture (%)	4.8
Lactose (%)	83
Nitrogen (mg/g)	5.49
COD (mg/g)	1034.3
Calcium (mg/g)	8.7
Magnesium (mg/g)	1.3
Phosphorus (mg/g)	7.2
Potassium (mg/g)	23.5
Sodium (mg/g)	5.7

while the bioreactor in System 3 was mixed with a magnetic stirrer only.

2.3. Seed inocula

The seed sludge used to inoculate the bioreactors was collected from a two-stage mesophilic anaerobic digester with a 24-d HRT at the City of Davis Wastewater Treatment Plant, Davis, CA. The sludge was stored in a refrigerator (4 °C) for 24 h to allow the sludge to settle before the supernatant was decanted. The precipitate was passed through a screen with 2 mm openings to remove large particles. After processing, the sludge had a pH of 7.8 and total alkalinity of 2900 mg/L as $CaCO_3$. The characteristics of the sludge are shown in Table 1. Total carbon denoted as $C_{(Total)}$ was estimated from the total solids (TS).

2.4. Substrate

Dry whey permeate powder was obtained from Foremost Farms (Baraboom, WI). The characteristics of the powder are shown in Table 2. The feed for the bioreactors was prepared based on the COD concentration. In the continuous fermentation experiments, the following chemicals were added as supplements to enhance bacterial growth as described previously [17–19]: ammonia bicarbonate 1035 mg/L, potassium phosphate 150 mg/L, sodium chloride 1200 mg/L, sodium carbonate 1200 mg/L, magnesium sulfate 50 mg/L, zinc chloride 10 mg/L, ferrous sulfate 55 mg/L, manganese chloride 10 mg/L, and ammonium molybdate 15 mg/L. The C/N ratio was adjusted from an initial ratio of approximately 8 to a final ratio of 20. To maintain the pH in the bioreactors calcium carbonate was added as needed.

2.5. Data collection and sampling

The volume of biogas produced in each bioreactor was measured daily. The biogas produced from the batch bioreactors was analyzed for H_2, CH_4, and CO_2 concentrations at the end of the experiment and from the continuous bioreactors twice a week. Liquid samples were collected from the effluent of Systems 1 and 3 at the same time the biogas was sampled and analyzed for volatile fatty acids (VFA), alcohol, and lactate. Samples were also collected for microbial analysis from System 3 when it produced biogas with high H_2 content and from System 1 when the biogas had high and low H_2 contents.

2.6. Analytical and microbial analysis methods

The biogas composition was determined using a gas chromatograph (GC) (HP5890A, Avondale, PA) equipped with a thermal conductivity detector (TCD) and a stainless steel column (6 ft × 1/8 in × 0.085 in) packed with carbosphere at 80/100 (Alltech Associates, Inc., Deerfield, IL) using argon as the carrier gas. The Hach method (Hach Company, Loveland, CO) was used for the COD determinations. The Standard Methods for the Examination of Water and Wastewater [20] (APHA, AWWA, and WEF, 1998) were used for solids analysis. VFA and ethanol were analyzed by another GC (HP5890A) equipped with a flammable ionized detector (FID) and a 19095N-121 INNOWAX capillary column (length 15 m, ID 0.53 mm, and megabore film 1.0 μm) from J&W Scientific (Folsom, CA) using helium as the carrier gas. The lactose concentration was determined using the UV method described in the Official Method of Analysis (15th edition) of the Association of Official Analytical Chemists [21] using a DU7500 UV spectrophotometer (Beckman Coulter, Inc., Fullerton, CA) and the lactose/D-galactose kit (R-Biopharm, South Marshall, MI). Lactic acid concentrations were determined using the lactic acid kit (R-Biopharm, South Marshall, MI).

The 16S rDNA sequence analysis was performed to elucidate the predominant bacterial types in the samples. The bacteria were harvested by centrifugation at 10,000 × g for 10 min. The DNA was extracted from the resulting pellets as described previously [22]. The PCR amplification of the 16S rDNA sequences was carried out using the eubacterium-specific primers 27f (5′AGAGTTTGATCCTGGCTCAG3′) and 1392r (5′GACGGGCGGTGTGTAC3′) [23]. The PCRs were performed as recommended by Polz and Cavanaugh [24] to reduce bias in amplification. The PCR products were purified by ethanol precipitation, cloned using the Qiagen PCR cloning kit, and transformed into *E. coli* TOP10F′ cells (Invitrogen, Carlsbad, CA) by heat shock (42 °C for 30 s). Sequencing reactions were performed using the BigDye terminator v3.1 cycle sequencing kit (Applied Biosystems). Sequence electrophoresis and readouts were performed using an Applied Biosystems 3100 genetic analyzer. The predicted 16S rDNA sequences were compared to the 16S rDNA sequences in a BLASTable

database downloaded from the Ribosomal Database Project II (http://rdp.cme.msu.edu), Release 8.1. Comparisons were made using the program BLASTALL (ftp://ftp.ncbi.nih.gov) and a FASTA-formatted file containing the predicted 16S rDNA sequences. Operational taxonomic units (OTUs) were defined as clones with greater than 97% sequence identity.

2.7. Bioreactor start-up and operation

Batch bioreactors were filled with a mixture of the substrate and inocula according to selected F/M ratios. Vacuum was applied to each bioreactor, using the in-house vacuum system, to create anaerobic conditions and the bioreactors were placed in an incubator at 35 °C and continuously mixed for approximately 48 h. The total biogas produced was calculated by adding the headspace volume of each bioreactor with the volume of the biogas in the bag. Continuous bioreactors were started by filling them with a 1.5-L mixture of substrate (5.0 g COD/L) and inocula. The bioreactors were purged with Ar gas to create anaerobic conditions. Each continuous bioreactor was first operated as a batch reactor for the first 40 h and then run as a continuous stirred tank reactor (CSTR) with a HRT and SRT of 24 h. The room temperature and biogas production were recorded daily.

3. Results and discussions

3.1. Results from the batch experiments

The results of the batch experiments are shown in Table 3. More than 95% of the lactose was fermented at all of the F/M ratios. F/M ratios of 1.0 and 1.5 resulted in the greatest average H_2 yields of 8 and 10 mM/g COD fed, respectively. Over the fermentation time the pH of the fermentation solutions dropped significantly, which was likely due to the production of VFA and CO_2. At the F/M ratios of 1.0, 1.5, and 2.0 the major VFA observed were butyric and acetic acids, with butyric acid approximately two-fold greater than acetic acid. However, at the F/M ratio of 0.5, acetic acid was generated in slightly larger amounts than butyric acid. In addition, ethanol was produced in all of the bioreactors and small amounts of propionic and valeric acids were detected at the F/M ratios of 0.5 and 1.0. The pH change and biogas production during 24 h of fermentation in one of the batch experiments are shown in Fig. 2. Biogas was generated after 6.5 h of the fermentation, peaked at about 12 h, and stopped after 18 h. The pH increased slightly after biogas production ceased.

3.2. Results from the continuous hydrogen fermentation experiments

System 1 was operated with an LR of 5.0 g COD/L/d for approximately 6 weeks. During this period, biogas production varied from 0.6 to 1.8 L/d and contained a CH_4 concentration of 27–40% (v/v) and a H_2 content below 3.0% (v/v). The production of CH_4 indicated the presence of methanogens, even though the pH dropped below 5.0 which should be inhibitory to methanogenesis. It is possible that methanogens were able to persist in biofilms formed on the walls of the bioreactor or flocculent materials suspended in the bioreactor that protected them from this low pH. The performance of System 1 at an LR of 10 g COD/L/d is shown in Fig. 3. From day 17 to 27 biogas production averaged 1.8 L/d with H_2 and CH_4 contents ranging from 22% to 26% and 6% to 9%, respectively. However, the system was not stable and the biogas production fluctuated between 0.7 and 2.7 L/d over the course of the experiment with H_2 yields ranging from 0 to 2.0 mM/g COD fed with the greatest H_2 yield occurring when the pH level was between 4.0 and 5.0. The performance of System 1 at an LR of 12.0 g COD/L/d is shown in Fig. 4. During the 4 weeks of operation biogas production increased from 0.5 to 3.5 L/d and the H_2 content was approximately 30%; however, after this period the H_2 content decreased rapidly with concurrent pH and CH_4 increases. It is probable that the increase in pH promoted the growth of methanogenic archaea which consumed H_2 to produce CH_4.

The performance of System 1 at an LR of 14.0 g COD/L/d is shown in Fig. 5. Biogas was produced in the pH range between 4.0 and 5.0. When the LR increased the biogas

Table 3
Batch experiments with cheese whey wastewater as feed substrate

F/M	Test	H_2 (%) (v/v)	Yield (mM/g COD)	Acetic (mg/L)	Propionic (mg/L)	Butyric (mg/L)	Valeric (mg/L)	Ethanol (mg/L)	Lactose (mg/L)	Initial pH	Final pH
0.5	T1	39.3	6.977	1423	127	1066	101	76	188	7.29	5.92
	T2	40.9	7.688	1265	101	1047	85	105	170	7.28	5.88
	Ave.	40.1	7.333	1344	114	1057	93	91	179	7.29	5.90
1.0	T1	42.9	7.752	441	0	819	0	30	323	7.31	5.40
	T2	44.8	8.096	340	19	774	20	32	117	7.30	5.40
	Ave.	43.85	7.924	391	10	797	10	31	220	7.31	5.40
1.5	T1	50.8	9.927	370	0	786	0	39	0	7.32	4.87
	T2	48.4	10.285	423	0	839	0	30	206	7.30	5.10
	Ave.	49.6	10.106	397	0	813	0	35	103	7.31	4.99
2.0	T1	35.2	1.135	392	0	774	0	27	323	7.33	4.06
	T2	38.2	2.359	337	0	684	0	20	278	7.33	4.90
	Ave.	36.7	1.747	365	0	729	0	24	301	7.33	4.48

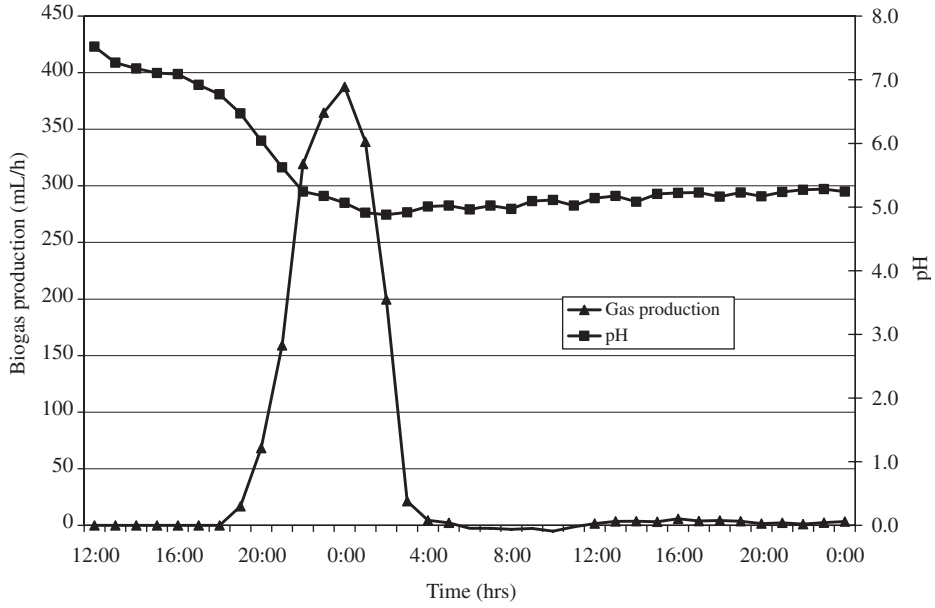

Fig. 2. pH change and biogas production during batch fermentation at the F/M ratio 1.0.

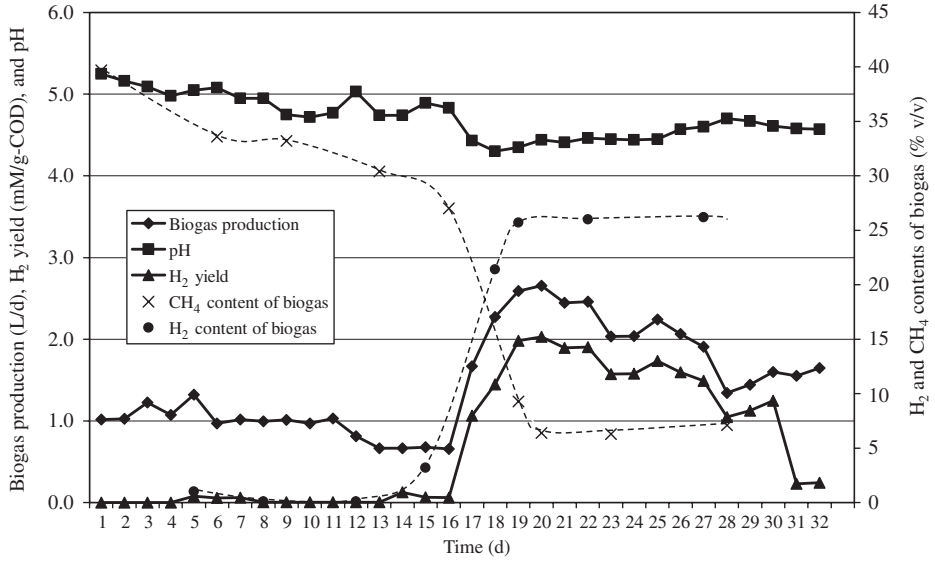

Fig. 3. Performance of System 1 at the LR of 10 g COD/L/d and HRT of 24 h.

production also increased from 3.2 to 5.1 L/d in the first week and then decreased to 1.0 L/d during the next 3 weeks. The H_2 content of the biogas increased from 6.0% on the first day to 33.2% at day 14. The CH_4 content varied from 0.9% to 16.4%. The highest H_2 yield was 2.3 mM/g COD feed. The biogas production decreased as the pH dropped below pH 4.0 but recovered when the pH increased. System 1 had the highest H_2 yield of approximately 2.0 mM/g COD feed at an LR ranging from 10.0 to 14.0 g COD/L/d.

The performance of System 2 is shown in Fig. 6. During the first 2 weeks System 2 was operated in the same way as System 1, with an LR of 5.0 g COD/L/d and an HRT of 24 h. During this period the biogas production increased and the pH stayed around 5.5, but the H_2 and CH_4 contents of the biogas were low. System 2 exhibited essentially the same behavior as System 1 in the beginning. Later, the HRT was shortened to 18 h, corresponding to an LR of 6.7 g COD/L/d. The biogas production increased, but the CH_4 content of the biogas also increased. The HRT was further shortened to 12 h, corresponding to an LR of 10 g COD/L/d that was the same as the LR for System 1 at 24 h HRT. The biogas production increased and then decreased until the LR changed. During this period, the pH decreased below 5.0, but the H_2 content in the biogas produced remained low. We concluded from these data that an HRT of less than 24 h does not favor the H_2 fermentation from cheese whey wastewater.

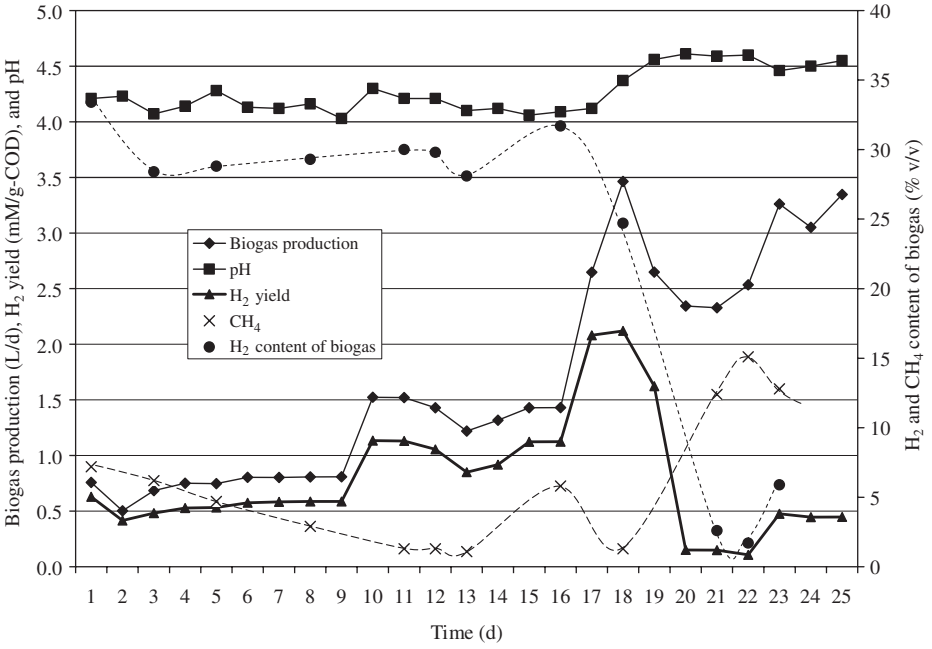

Fig. 4. Performance of System 1 at the LR of 12 g COD/L/d and HRT of 24 h.

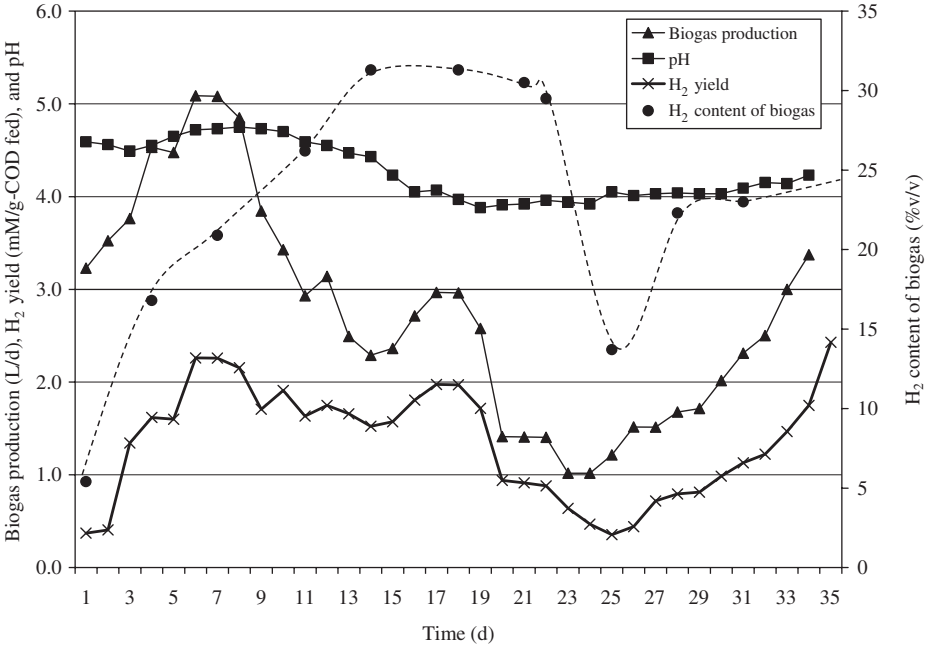

Fig. 5. Performance of System 1 at the LR of 14 g COD/L/d and HRT of 24 h.

The performance of System 3 operated at an LR of 10 or 12 g COD/L/d over 9 weeks of operation is shown in Fig. 7. One week after the bioreactor started, System 3 generated 0.96 L/d biogas on an average with 30% H_2 and less than 3.0% CH_4. The pH was in the range of 4.0–5.0. Initially, the glass bioreactor wall was clear but after 1 week it became opaque, indicating the formation of biofilms; however, no sludge build-up in the bottom of the bioreactor was noticed. We also observed oscillations in biogas production and H_2 content. The maximum H_2 yield was 2.1 mM/g COD fed at an LR of 10 and 1.8 mM/g COD fed at an LR of 12 g COD/L/d. When the pH fell below 4.0 we observed a negative impact in the reactor's performance and an increase in CH_4 content corresponding to a decrease of H_2 content similar to that observed in System 1.

3.3. Bacterial composition

DNA sequence analysis of the 16S rRNA genes isolated from System 3 revealed that the vast majority of the bacteria present were gram-type positive (88%). The 16S gene

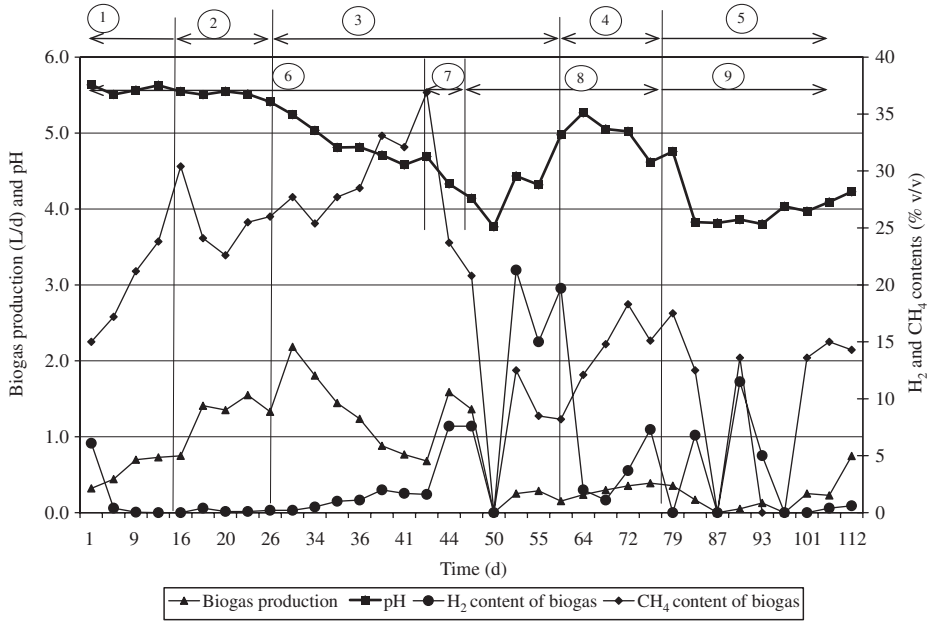

Fig. 6. Performance of System 2 at different HRTs: top line: HRT (h): (1) 24, (2) 18, (3) 12, (4) 18, (5) 24; second line: LR (g COD/L/d): (6) 5, (7) 6.7, (8) 5, (9) 10.

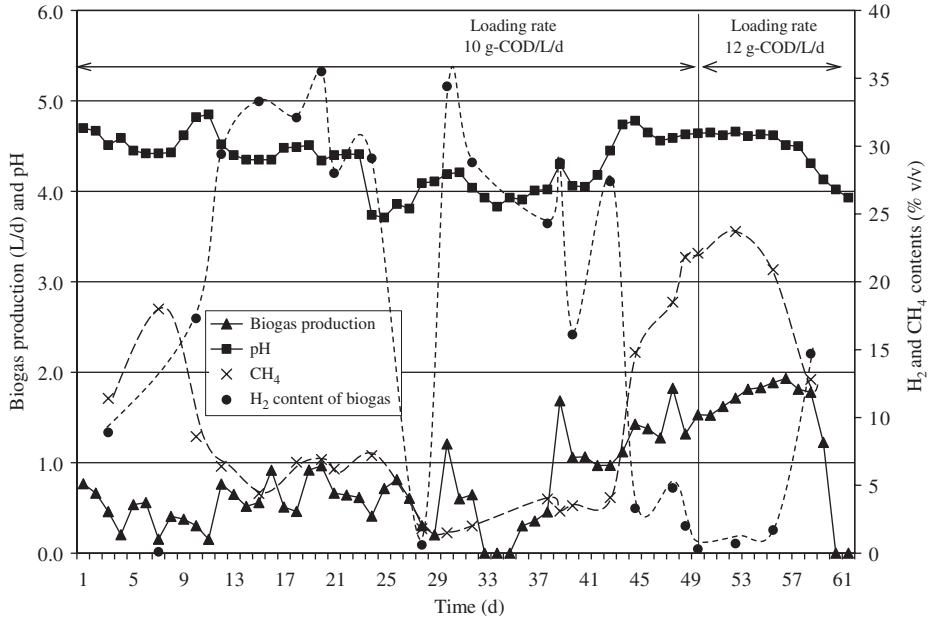

Fig. 7. Performance of System 3 at the LR of 10 and 12 g COD/L/d and HRT of 24 h.

sequences similar to those of the genus *Lactobacillus* were the most prevalent, representing approximately 50% of the total sequences analyzed, followed by those related to the genus *Olsenella* (24%), *Clostridium* (9%), and *Prevotella* (7%). The most prevalent OTU isolated represented 23% of the total sequences and was greater than 99% similar to *Lactobacillus* sp. rennanqilfy16, a highly efficient H_2-producing bacterium [25]. The second most populous OTU recovered represented 22% of the total sequences isolated and was greater than 97% similar to *Olsenella* sp. N13-17, a human oral isolate. The next two most abundant OTUs represented 10% and 7% of the total bacteria identified, respectively, and were greater than 99% similar to the *Lactobacillus coryniformis* and *Lactobacillus suebicus*. The fifth most prevalent OTU identified was greater than 99% similar to the *Clostridium tyrobutyricum*, which represented 5% of the total population identified, and has been isolated from sucrose degrading, H_2-producing anaerobic reactors [26]. These data indicated that the environmental, physical, and cultural conditions in the system were effective for the selection of the bacteria that are able to produce H_2.

Two liquid samples from System 1 were also collected on days 12 and 22 (Fig. 4), respectively. System 1 produced 1.43-L biogas with an H_2 content of 30% (v/v) and CH_4 content of 1.3% (v/v) on day 12, and 2.53-L biogas with 1.7% (v/v) H_2 and 15.1% CH_4 (v/v) on day 22. Approximately, 200 16S rDNA sequences were analyzed from each sample. The level of coverage of each sample was approximately 80%. The percentage of each phylum that was identified in each sample is shown in Table 4. These data reveal that the greatest difference in the bacterial population structure between the day 12 when there was high H_2 content and the day 22 when the H_2 content was low was within the genus *Lactobacillus*, which contains several species that have been shown to be able to produce H_2 from fermenting lactose. To confirm that the *Lactobacillus* spp. were capable of producing H_2, we isolated several of them in pure culture. When these isolates were grown on whey they produced biogas with high amounts of H_2 (data not shown).

3.4. VFA and ethanol

To identify differences in fermentation end products between samples when H_2 production was relatively high or low, VFA and alcohol contents in the liquid samples (three samples in each group) were analyzed from one of the bioreactors with an LR of 10 g COD/L/d (Table 5). Three samples (1, 2, and 3) were collected from System 1 when the bioreactor produced biogas containing high H_2 (average 26%, v/v) and low CH_4 (average 6.6%, v/v); the other samples (4, 5, and 6) were collected from the effluent of the same system when the bioreactor produced biogas containing a low H_2 (average 0.4%, v/v) and high CH_4 content (average 32.4%, v/v). The results showed several correlations between H_2 yield in the biogas and the fermentation products in the liquid. For example, we observed that the higher the H_2 yield in the biogas, the higher the ethanol, hexanoic acid, and *n*-butyric acid concentrations in the solution. It was also noticed that the higher the H_2 yield in the biogas produced, the lesser the propionic acid concentration in the solution. This pattern was also observed in the batch experiments.

Biogas production, H_2 yield, VFA, and ethanol concentrations in System 1 at different LRs are shown in Table 6. Each LR was sampled at three time points, 3–5 d apart. The results for the three samples in each group were averaged and tabulated in Table 6. The VFA and ethanol data were normalized based on the substrate LR. The normalized concentrations of ethanol, propionic, hexanoic, and heptanoic vs. H_2 yields were plotted and are shown in Fig. 8. We observed that when the H_2 yield in the biogas was high it resulted in high ethanol and hexanoic acid concentrations in the fermentation solution. However, there was an outlier for ethanol and hexanoic acid concentrations at the H_2 yield of 0.620 and 0.245 mM/g COD, respectively. We also observed that a high H_2 yield in the biogas resulted in less propionic acid in the fermentation solution. The heptanoic acid concentration in the fermentation solution appeared to be insignificant between 6 and 9 mg/L for each gram COD fed. Furthermore, the ratio of the propionate to acetate concentrations and the ratio of the propionate to *n*-butyrate concentrations were less when the bioreactor yielded higher H_2 as illustrated in Fig. 9.

The cheese whey permeate used for making feed substrate contained 83% lactose. We hypothesized that lactic acid would be generated as a fermentation end product; however, we did not

Table 4
Microbial populations in the fermentation solution and the *Lactobacillus* species in firmicutes group (% of phylum detected, refer to Fig. 5 to see the sampling days)

Phylum (%)	Sampled on day 12	Sampled on day 22
Actinobacteria	6.4	23.8
Bacteroidetes	0.6	20.7
Firmicutes	88.5	39.6
Lactobacillus	61.7	20.5
Proteobacteria	3.8	15.9
Nitrospirae	0.0	0.0
All others	0.7	0.0
Total	100	100

Table 5
Biogas production, H_2 yield, and VFA and alcohol concentrations in the fermentation solution for selected samples at the LR of 10 g COD/L/d

Sample	Biogas production (L/d)	H_2 yield (mM/g COD)	Biogas composition			pH	Alcohol and VFA						
			H_2 (%) (v/v)	CH_4 (%) (v/v)	CO_2 (%) (v/v)		Ethanol (mg/L)	Acetic (mg/L)	Propionic (mg/L)	*n*-Butyric (mg/L)	*n*-Valeric (mg/L)	Hexanoic (mg/L)	Heptanoic (mg/L)
1	2.59	1.981	25.7	6.4	67.9	4.35	157	2764	100	770	260	1062	133
2	2.46	1.904	26.0	6.3	67.7	4.46	184	2452	69	771	204	1090	95
3	1.91	1.489	26.2	7.1	66.7	4.60	157	2764	100	770	260	1062	133
Ave.	2.32	1.791	26.0	6.6	67.43	4.47	166	2660	90	770	241	1071	120
Stdev.	0.36	0.264	0.252	0.436	0.643	0.13	16	180	18	1	32	16	22
4	1.07	0.064	1.0	33.6	65.4	4.98	0	2400	215	551	262	432	45
5	1.00	0.006	0.1	33.2	66.7	4.95	0	2070	135	428	189	368	49
6	0.81	0.005	0.1	30.4	69.5	5.03	22	1610	76	461	151	618	113
Ave.	0.96	0.025	0.4	32.4	67.2	4.99	7	2027	142	480	201	473	69
Stdev.	0.13	0.034	0.5	1.7	2.095	0.04	13	397	70	64	56	130	38

Table 6
Biogas production, H_2 yield, and VFA and ethanol concentrations in the fermentation solution viewed in average from four groups of the liquid samples at each LR

Loading rate (g COD/L/d)	Sample	Biogas production (L/d)	H_2 yield (mM/g COD)	Biogas composition			pH	Alcohol and VFA						
				H_2 (%) (v/v)	CH_4 (%) (v/v)	CO_2 (%) (v/v)		Ethanol (mg/L)	Acetic (mg/L)	Propionic (mg/L)	*n*-Butyric (mg/L)	*n*-Valeric (mg/L)	Hexanoic (mg/L)	Heptanoic (mg/L)
5	Ave.	1.37	0.014	0.2	28.1	71.7	6.04	0	1527	494	253	183	123	47
	Stdev.	0.08	0.018	0.2	1.7	1.9	0.05	0	286	100	101	33	8	2
10	Ave.	1.39	0.620	20.3	12.5	67.1	4.04	312	1746	36	430	82	762	56
	Stdev.	1.42	0.372	8.0	3.1	4.9	0.34	57	114	32	302	57	576	64
12	Ave.	2.71	0.245	3.4	13.4	83.2	4.55	32	1318	157	821	315	917	85
	Stdev.	0.49	0.203	2.2	1.5	1.9	0.08	7	130	16	294	11	158	20
14	Ave.	5.65	3.210	26.7	5.7	67.6	4.79	155	2098	77	774	245	1179	83
	Stdev.	0.13	0.425	3.4	2.2	1.25	0.10	23	681	31	40	100	29	10

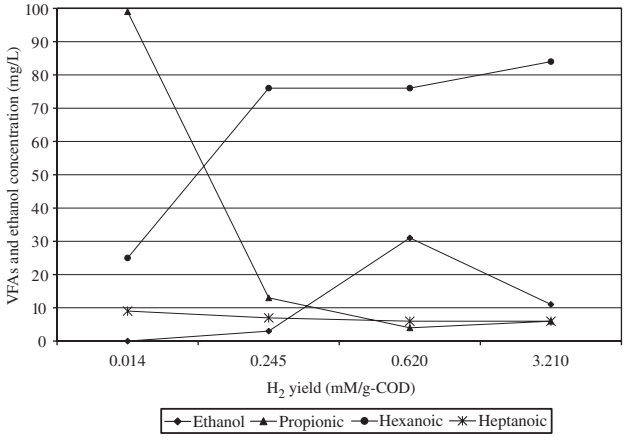

Fig. 8. Plot of H_2 yield vs. normalized VFA and ethanol concentrations in the fermentation solution.

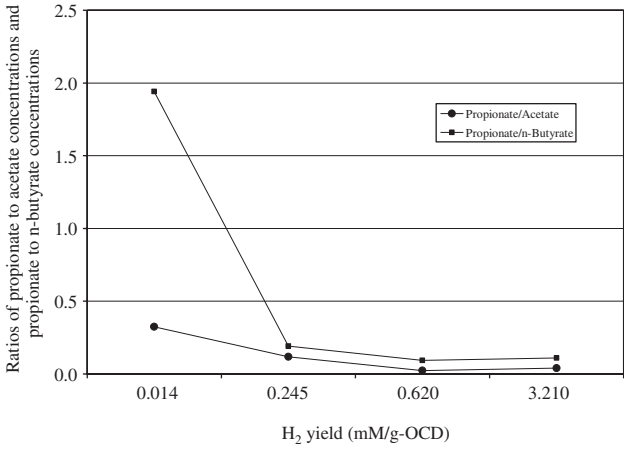

Fig. 9. Plot of H_2 yield vs. ratios of propionate to acetate and propionate to *n*-butyrate concentrations.

detect any lactate in the reactor effluents. This may be because lactate is a key intermediate of sugar fermentation [27–29] and is subsequently degraded very quickly in acidification reactors [30]. In order to determine if the cheese whey permeate (mainly lactose) was completely decomposed, two groups (three consecutive samples for each group) of liquid samples were collected when the bioreactor gave high or low H_2 in the biogas, at the LR of 12.0 g COD/L/d. The lactose determinations for the selected samples from the solutions are shown in Table 7, which indicates that the substrate was not completely utilized. In the experiments at an HRT lower than 24 we observed a greater lactose concentration in the bioreactor effluent and a greater H_2 content in the biogas. We hypothesized that this was because the 24 h HRT was not long enough for the microbial population to utilize the lactose. This phenomenon also indicated the possibility that a higher substrate concentration (or a higher LR) may be effective for a greater H_2 production (liter of H_2 per liter of reactor volume per day: L/L/d) if the bacteria are not inhibited by the substrate applied and the fermentation conditions, such as pH, are not altered. A very short HRT of 0.5–1 h has been reported as the optimum HRT in several studies [31,32], which implied that a higher H_2 production resulted from the application of a higher substrate LR. Definitely, there was almost complete decomposition of the substrate observed during the days with a higher amount of biogas produced and a higher H_2 generated.

4. Conclusions

After studying the performance of the H_2 fermentation bioreactors under different conditions, we concluded that controlling the pH in the bioreactors at a proper level is important for H_2 production. The pH of the H_2 fermentation bioreactor could be controlled at a certain level by adding alkalinity into the feed. The pH range of 4.0–5.0 was most favorable for continuous fermentation of the cheese whey wastewater. The *Lactobacillus* species were found to be the predominant microorganisms in the bioreactors when they had high H_2 yields. More than 50% of the detected bacteria were *Lactobacillus* and about 5% of the isolates were the *Clostridia* species. When the

Table 7
Lactose determination in selected samples when the H_2 bioreactor had the LR of 12 g COD/L/d

Sample	Biogas production (L/d)	pH	Biogas composition				H_2 yield (mM/g COD)	VFA, alcohol, and lactose							
			H_2 (%)	(v/v)	CH_4 (%)	(v/v) CO_2 (%) (v/v)		Ethanol (mg/L)	Acetic (mg/L)	Propionic (mg/L)	*n*-Butyric (mg/L)	*n*-Valeric (mg/L)	Hexanoic (mg/L)	Heptanoic (mg/L)	Lactose (mg/L)
1	1.520	4.21	30.0		1.3	68.7	1.131	26	523	107	222	102	173	25	2831
2	1.429	4.21	29.8		1.3	68.9	1.056	93	459	111	109	94	163	18	3378
3	1.220	4.10	28.1		1.1	70.8	0.850	33	622	137	264	125	229	23	1095
Ave.	1.390	4.17	29.3		1.2	69.5	1.012	51	535	118	198	107	188	22	2435
Stdev.	0.154	0.06	1.0		0.1	1.2	0.146	37	82	16	80	16	36	4	1192
1	2.328	4.59	2.6		12.4	85.0	0.150	33	1201	164	482	328	760	69	661
2	2.534	4.60	1.7		15.1	83.2	0.107	38	1294	169	976	306	915	79	340
3	3.261	4.46	5.9		12.8	81.3	0.477	24	1458	139	1005	312	1076	107	368
Ave.	2.708	4.55	3.4		13.4	83.2	0.245	32	1318	157	821	315	917	85	456
Stdev.	0.490	0.08	2.2		1.5	1.9	0.203	7	130	16	294	11	158	20	178

H_2 production of the bioreactor was low, the number of *Lactobacillus* was about three-fold lower, compared to the time when the H_2 production was high.

The results of batch experiments showed that H_2 yields of 8 and 10 mM/g COD fed occurred at the F/M ratios of 1.0 and 1.5, respectively. More than 95% of lactose in the cheese whey permeate was fermented under the best operating conditions. On an average, the amount of butyric acid produced in the fermentation solution was twice as much as the amount of acetic acid. Smaller amounts of propionic acid and ethanol were also produced at the lower F/M ratios and no lactic acid was detected in the batch fermentation.

In the continuous fermentation bioreactors, CH_4 was present in the biogas at various levels even when the pH was below 5. We observed that when the CH_4 content increased, the H_2 content decreased rapidly. For all the LRs tested, the fermentation was disrupted when the pH dropped below 4.0. A stable H_2 production was achieved for 2–3 weeks at a time for each LR tested, but the biogas production and H_2 content of the biogas varied over time. The maximum H_2 yield achieved was between 1.8 and 2.3 mM/g COD fed for the LR tested in System 1 and also System 3, which was operated at 24 h of HRT. This yield was lower than the yield obtained from the batch fermentation experiments. The HRT of less than 24 h was found to be non-conducive to the H_2 production. Biofilms were formed on the bioreactor walls, which together with suspended flocs likely enhanced the growth of methanogens. As methanogens can quickly convert H_2 into CH_4, the presence and growth of methanogens are not conducive for H_2 production.

It was found that in the continuous fermentation bioreactors greater H_2 yields correspond to greater ethanol and hexanoic acid yields, and low levels of propionic acid in the effluents. Hexanoic acid was not detected in the batch bioreactors and heptanoic acid concentrations were very low. It has been shown that the behaviors of anaerobic batch H_2 fermentation reactors were different from the continuous H_2 fermentation reactors based on VFA production, H_2 yield, and the substrate decomposition.

Acknowledgment

This research was conducted at the Department of Biological and Agricultural Engineering at the University of California, Davis, with the funding support from the USDA Small Business Innovation Research Program and the USDA/ARS/FCR, Albany, CA.

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