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ORIGINAL PAPER

Effects of temperature and initial pH on biohydrogen production from food-processing wastewater using anaerobic mixed cultures

Yen-Hui Lin · Mu-Ling Juan · Hsin-Jung Hsien

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Abstract This study attempted to determine the optimal temperature and initial cultivation pH by conducting a series of batch tests in stirred-tank bioreactor using fructose-producing wastewater as an organic substrate. The bioreactor temperature was controlled at 35–55°C with an initial pH of 4–8. Hydrogen production efficiency was assessed using specific hydrogen production potential (SHPP) and the maximum specific hydrogen production rate ($SHPR_m$). Experimental results indicated that temperature and initial pH markedly affected SHPP and $SHPR_m$, volatile fatty acids distribution as well as the ratio of butyrate/acetate (BHu/HAc). Two-fold higher SHPP and $SHPR_m$ were obtained at thermophilic condition (55°C) than those at mesophilic condition (35°C). The optimal initial pH was 6 for hydrogen production with peak values of SHPP of 166.8 ml-H_2/g-COD and $SHPR_m$ of 26.7 ml-H_2/g-VSS-h for fructose-processing wastewater. Molasses-processing wastewater had a higher SHPP (187.0 ml-H_2/g-COD) and $SHPR_m$ (42.7 ml-H_2/gVSS-h) than fructose-processing wastewater at pH 6. The DGGE profiles indicated that molasses-processing wastewater is a better substrate than fructose-processing wastewater for growth of hydrogen-producing bacteria due to the high staining intensity of bands.

Keywords Temperature · Initial pH · Biohydrogen · Food-processing wastewater · Butyrate/acetate ratio

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Introduction

Alternative uses of wastes are encouraged to reduce environmental pollution. Previously, organic wastes were discharged directly into the environment (Van Ginkel et al. 2005). Approximately 600 food-processing companies in Taiwan produce 43 million liters of wastewater annually. This food wastewater can be characterized as nontoxic because it contains few hazardous compounds, has high biological oxygen demand (BOD) compared with many other industrial wastewaters, and most organic compounds consists of simple and complex sugars (Speece 1996). Due to increased enforcement of discharge regulations, many food processing companies are taking steps to reduce, recycle or treat their wastewater prior to discharge. One alternative use for food-processing wastewater is to use it as raw material for fermentative processes (Damasceno et al. 2003).

Attention is focusing on hydrogen production from microbial conversion of industrial wastes and residues, not only because hydrogen is a clean energy but also because it can be a process for wastes reduction and reclamation (Eroğlu et al. 2004; Han and Shin 2004). The possibility of using various industrial and agricultural wastes as carbon sources for hydrogen production by microorganisms had been explored in different studies. These waste-based hydrogen production processes mainly include milk industry wastewater (Monteoliva-Sanches et al. 1996), lactic acid fermentation plant wastewater (Sasikala et al. 1991), distillery wastewater (Sasikala et al. 1992) and sewage sludge (Sunita and Mitra 1993) as well as municipal solid waste (Fascetti et al. 1998).

Useful applications of fructose and molasses wastewaters are currently being investigated. Fructose, a simple reducing sugar, is found in honey, fruit and plant parts. Fructose is formed when sucrose is hydrolyzed by the enzyme invertase. High-fructose syrup has taken the place of sucrose, and is widely used in the production of many products such as frozen foods, dairy products, and canned foods. Fructose wastewater has a chemical oxygen demand (COD) of 3000–6000 mg/l (Chao 2004). Molasses, a thick syrup, is by-product of processing sugarcane or sugar beets into sugar and often used as raw material by alcohol distilleries. Untreated molasses wastewater from alcoholic fermentation has a high organic content with COD in the range of 50–100 g/l (Jiménez et al. 2004). Therefore, fructose and molasses wastewaters as organic substrates have a great potential for hydrogen production. Fructose and molasses wastewater from food processing, which contain high concentrations of carbohydrate-rich materials, were examined in batch tests to determine their hydrogen production potential in this study.

During the fermentation process, hydrogen is produced during the exponential growth phase of hydrogen-producing bacteria (Khanal et al. 2004). When the population reaches the stationary growth phase, the reactions shift from a hydrogen/acid production phase to a solvent production phase. This shift occurs when the pH drops to 4.5 or below. Other researchers found that the shift occurred at pH levels above 5.7 due to enzyme synthesis or enzyme activation, which is necessary for solvent production (Dabrock et al. 1992). Results so far indicated that the control of pH is crucial to the hydrogen production, due to the effects of pH on the hydrogenase activity and/or on the metabolism pathways (Fang and Liu 2002). Thus, it is important to control pH at an optimal range to maintain hydrogen production. On the other hand, if the pH is not maintained in the desired range, it could inhibit hydrogen production or cause a microbial population shift resulting in cessation of hydrogen production (Khanal et al. 2004). To what extent pH affects hydrogen production for sugar-based substrates has been widely studied (Fang and Liu 2002; Lin et al. 2006). However, the optimum pH to maintain hydrogen production for fructose and molasses wastewater used as organic substrates has not been reported. In practice, both butyrate and acetate are formed during fermentation process and the ratio may vary with pH within thermodynamically determined limit (Thauer et al. 1977). The changes in butyrate/acetate ratio implied a metabolic alteration due to environmental changes such as pH, partial pressure of hydrogen and the accumulation of intermediate products in the fermentation process (Van Andel et al. 1985). Thus, the ratio of butyrate to acetate was used to indicate the progress of hydrogen production in this study.

Based on these backgrounds, the objectives of this study were intended to (1) investigate the effects of different temperatures on biohydrogen production with the initial cultivation pH range of 4–8 using fructose as an organic substrate; (2) examine the effects of temperature and initial pH on specific hydrogen production potential (SHPP) and maximum specific hydrogen production rate ($SHPR_m$); (3) observe the effects of variation of the butyrate/acetate (HBu/HAc) on hydrogen production at different initial pHs; and (4) observe and identify the diversity of microbial communities from hydrogen fermentation.

Materials and methods

Feedstocks

The fructose and molasses wastewaters were obtained from the Fonen Sugar Refinery and Taiwan Sugar, respectively. Starch and sugarcane are the raw materials for fructose and molasses production at these two factories, respectively. Fructose wastewater obtained from an equalization basin after being subjected to an aerobic biological treatment process.

Molasses wastewater was obtained from a mixing basin followed by an anaerobic–aerobic treatment process. Feedstocks were collected from the same locations at two different times during the day to obtain a range of substrates concentration from the effluents of wastewater treatment facility. In the fructose-processing or sugar-beet-processing units, water is used in preparing fructose product or molasses by-product at several plants locations. Water was collected at these units and then discharged into a central line connected to a wastewater treatment plant. Fructose and molasses effluents obtained from equalization basin and mixing basin, respectively, during one of two normal production shifts, as well as during a late-night shift primarily focused on cleaning operations. Thus, these feedstocks represent a monthly-averaged wastewater samples. All feedstocks were collected in duplicate, sent by overnight courier to the laboratory, and then were stored at −4°C prior to use. The precipitates of feedstocks were filtered through cheese-cloth (Yetis et al. 2000). Samples were analyzed within 2 days for their general characteristics. Analysis of the volatile fatty acids (VFAs) was performed to verify that samples had not undergone substantial anaerobic degradation prior to their use in batch tests. Table 1 lists the characteristics of fructose and molasses wastewaters.

Seed inoculums

The seed sludge, used to determine the optimal temperature and initial cultivation pH, was obtained from anaerobic sludge digester at the fructose-processing wastewater treatment plant. After collection, the anaerobic sludge was filtered through a No. 8 sieve (diameter 2.35 mm) to eliminate large particulates materials (Lin et al. 2008). Before being seeded into the bioreactors, the screened sludge was heat-treated at 100°C for 2 h to inhibit methane-producing bacteria activity and selected as heat-resistant, hydrogen-producing, and spore-forming bacteria (Lin et al. 2008).

Table 1 Characteristics of fructose and molasses wastewaters

Parameter	Fructose wastewater	Molasses wastewater
COD_t (mg/l)	4110 ± 381	6525 ± 263
COD_s (mg/l)	3790 ± 325	5050 ± 214
SS (mg/l)	1015 ± 276	1060 ± 236
Total carbohydrate (mg/l)	3017 ± 196	3256 ± 225
VSS (mg/l)	248 ± 18	610 ± 38
TKN as N (mg/l)	145 ± 28	327 ± 23

Experimental procedures

The BHP test employed in this study was modified from the biochemical methane potential (BMP) test developed by Owen et al. (1979). The batch experiments were conducted in stirred-tank bioreactor with a working volume of 5 l seeded with 600 ml heat-pretreated sludge and fed with 2500 ml substrate solution and 1900 ml nutrient solution. The tested temperatures were 35–55°C at an interval of 10°C and the tested pH values were 4–8 in an interval of 1 unit. The reactor was operated with a rotating shaft with a rotation speed of 90 rpm to enhance the contact between substrate and the nutrient solution. In each batch bioreactor, following nutrient stock solution (per liter) were added: 1 g resazurin; 26.7 g $(NH_4)_2HPO_4$; 16.7 g $CaCl_2{\cdot}2H_2O$; 26.6 g NH_4Cl; 120 g $MgCl_2{\cdot}6H_2O$; 86.7 g KCl; 1.33 g $MnCl_2{\cdot}4H_2O$; 2 g $CoCl_2{\cdot}6H_2O$; 0.38 g H_3BO_3; 0.18 g $CuCl_2{\cdot}2H_2O$; 0.17 g $Na_2MoO_4{\cdot}2H_2O$; 0.14 g $ZnCl_2$; 370 g $FeCl_2{\cdot}4H_2O$; 500 g $C_2H_3NaO_2S$; 0.002 g Biotin; 0.002 g folic acid; 0.01 g pyridoxine hydrochloride; 0.005 g riboflavin; 0.005 g thiamin; 0.005 g pantothenic acid; 0.005 g nicotinic acid. After adjusting the pH to the desired initial levels by adding a few drops of concentrated HCl or NaOH, the wastewater was then transferred to batch reactor for fermentative hydrogen production. The batch reactor was gassed with nitrogen gas to remove dissolved oxygen to retain an anaerobic environment.

Analytical methods

The biogas was measured using gas flow meter, and biogas volume was calibrated to a temperature of 25°C and pressure of 760 mmHg. Hydrogen in biogas was analyzed using a Hewlett-Packard model 5890 gas chromatograph (Hewlett-Packard Development Co., Palo Alto, CA, USA) equipped with a thermal conductivity detector (TCD) and a 2 m × 1/8″ stainless column-a Molesieve 5A (60/80 mesh). Nitrogen,

the carrier gas, had a flow rate of 23 ml/min. The operational temperature of the injection port, oven and detector was 110, 60 and 150°C, respectively. The volatile fatty acids, including acetate, propionate and butyrate, were analyzed using an Agilent model 6850 gas chromatograph equipped with an FID detector. The column was a $6' \times 8'$ stainless column-10%SP-1200/1% H_3PO_4 (80/100 mesh). The operational temperature of the injection port, oven and detector were maintained at 140, 100 and 140°C, respectively. Helium, the carrier gas, had a flow rate of 40 ml/min. Chemical oxygen demand (COD), suspended solids (SS) and volatile suspended solids (VSS), pH, ORP, TKN and alkalinity were measured according to Standard Methods (APHA 2005). The total sugar was analyzed using phenol–sulfuric acid method (Jungermann et al. 1973; Li et al. 2007).

Adequate amounts of sludge samples were collected, frozen immediately and stored at −20°C. Prior to DNA extraction, waste sludge samples were thawed, dispersed, and washed twice in phosphate buffer saline. Total genomic DNA was obtained and purified using the Blood and Tissue Genomic DNA extraction Miniprep System (Viogene, Taiwan). The DNA samples were stored at −20°C prior to PCR reaction. Two sets of primers for 16s rDNA gene amplification, 341f-gc with 534r and M13f with M13r, were used for monitoring bacterial community swift and bacterial identification (Girbal et al. 1995; Zigova and Sturdik 2000). Before cloning PCR-amplified extracted rDNA fragments, sequence variations were assessed by DGGE. The DGGE profile of PCR-amplified DNA was obtained via the method developed by Muyzer et al. (1993) using a DCodeTM Universal Mutation Detection System (Bio-Rad). The 6% (w/v) acrylamide solution was utilized to cast a gel with denaturant gradients of 35–65%. Electrophoresis was conducted in a 1× TAE buffer solution at 180 V and 60°C for 5 h. The gels were stained for 10 min with ethidium bromide and visualized using UV radiation. The number of operational taxonomic units in each sample was defined as the number of DGGE bands. Cloning of DNA fragments was performed using a TA Cloning Kit (Yeastern Biotech Corporation, Taiwan). In total, 60 colonies were selected for inoculation on a Luria–Bertani agar plate containing 50 mg/l ampicillin. Colony PCR of the primer set M13r/M13f was used to amplify the inserted rDNA fragments carried on the plasmid. The DGGE profiles of all clones, amplified using the primer set of 341f-gc and 534r, were compared. Clones containing correct DNA insertion were identified and sequenced by direct amplification of the inserted DNA fragments with a vector-specific primer set (M13r and M13f). Analysis of DNA sequences and homology searches were done with standard DNA sequencing programs and the BLAST server of the National Center for Biotechnology Information (NCBI) using the BLAST algorithm (Lee 1997).

Experimental data analysis

Hydrogen gas production was calculated from headspace measurements of the gas composition and total volume of biogas produced at each time interval using the following mass balance equation:

$$V_{H,i} = V_{H,i-1} + C_{H,i}\left(V_{G,i} - V_{G,i-1}\right) + V_H\left(C_{H,i} - C_{H,i-1}\right) \quad (1)$$

where $V_{H,i}$ and $V_{H,i-1}$ are cumulative hydrogen gas volumes at the current (i) and previous (i − 1) time intervals, respectively; $V_{G,i}$ and $V_{G,i-1}$ are the total biogas volumes at the current and previous time intervals, respectively. $C_{H,i}$ and $C_{H,i-1}$ are the fractions of hydrogen gas in the current and previous intervals, respectively; and V_H is the total headspace volume in the reactor. The hydrogen gas composition between sampling times was calculated assuming a linear change in a concentration over the sampling interval (Oh and Logan 2005).

Cumulative biogas production curves were obtained over the course of the batch experiment and analyzed using the modified Gompertz model (Mu et al. 2007) to acquire the hydrogen production potential (P), the hydrogen production rate (R_m) and lag phase (λ)

$$H(t) = P\exp\left\{-\exp\left[\frac{R_m * e}{H_{\max}}(\lambda - t) + 1\right]\right\} \quad (2)$$

where H(t) is the cumulative hydrogen production (ml); P is the hydrogen production potential (ml); R_m is the maximum hydrogen production rate; λ is the lag-phase time (h); t is time (h) and e is exp(1) = 2.71828. Parameters (P, R_m and λ) were

determined by fitting the cumulative hydrogen production curves by minimizing the ratio of the sum of square error (SSE) to the correlation coefficient (r^2) using the "Solver" function in Microsoft Excel version 5.0 (Microsoft, Inc., USA). The specific hydrogen production potential (SHPP) was obtained by dividing P by the substrate COD applied (Khanal et al. 2004). The maximum specific hydrogen production rate ($SHPR_m$) was determined by dividing R_m by volatile suspended solids (VSS) added. Hydrogen conversion efficiencies for fructose and molasses wastewaters were compared using SHPP and $SHPR_m$ values.

Results and discussion

Effects of temperature

A series of batch tests were conducted using fructose wastewater as an organic substrate to evaluate hydrogen production with different temperatures. Figure 1 presents the time course of hydrogen production during 120 h fermentation for fructose wastewater at temperatures of 35–55°C and initial pH range of 4–8. After a lag period of 5–15 h, hydrogen production proceeded as an exponential phase during the transient period and then reached the stationary phase. As shown in Fig. 1, hydrogen production increased as temperature increased, except when initial pH was 8. Hydrogen production was low at mesophilic condition (35°C) but was rather efficient and high at thermophilic condition (55°C). This experimental finding is consistent with the experimental results obtained by Lin et al. (2008), who examined hydrogen production from xylose by mixed anaerobic cultures at temperatures of 30–55°C. They found that thermophilic fermentation at 50–55°C has higher hydrogen yield and hydrogen production rate values than those of mesophilic fermentation at 30–40°C. Figure 2 shows the variation in hydrogen production at different temperatures and initial pH values. Hydrogen production was maximal at 55°C and pH 6 for fructose wastewater as an organic substrate for the growth of hydrogen-producing bacteria.

Figure 3 depicts the hydrogen production for experimental data and modified Gompertz model simulation. The correlation coefficient (r^2) range was

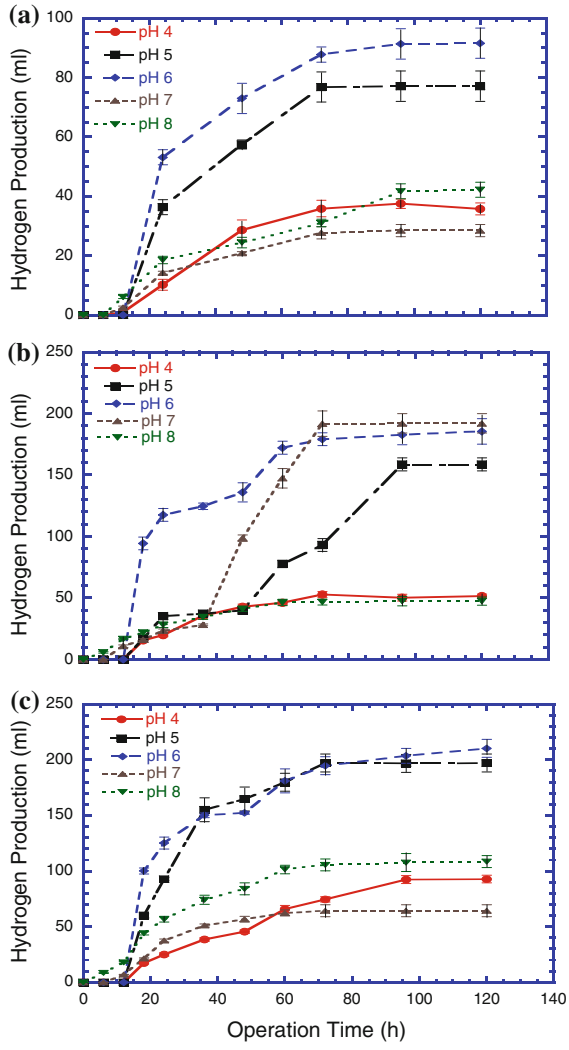

Fig. 1 Hydrogen production at different temperatures: **a** 35°C **b** 45°C **c** 55°C

0.948–0.975 for fructose wastewater. Comparing the experimental data with model simulation, the parameters of hydrogen production potential (P), the maximum hydrogen production rate (R_m) and lag-phase time (λ) was determined (Table 2). The P and R_m increased as temperature increased from 35 to 55°C for fructose wastewater. However, it was found that temperature did not have an effect on lag phase for hydrogen production. Figure 4 depicts the variation in SHPP and $SHPR_m$ along with operating temperatures. The trend of hydrogen production parameters observed when operated under different temperatures were similar. In general, thermophilic fermentation had higher hydrogen yield (HY),

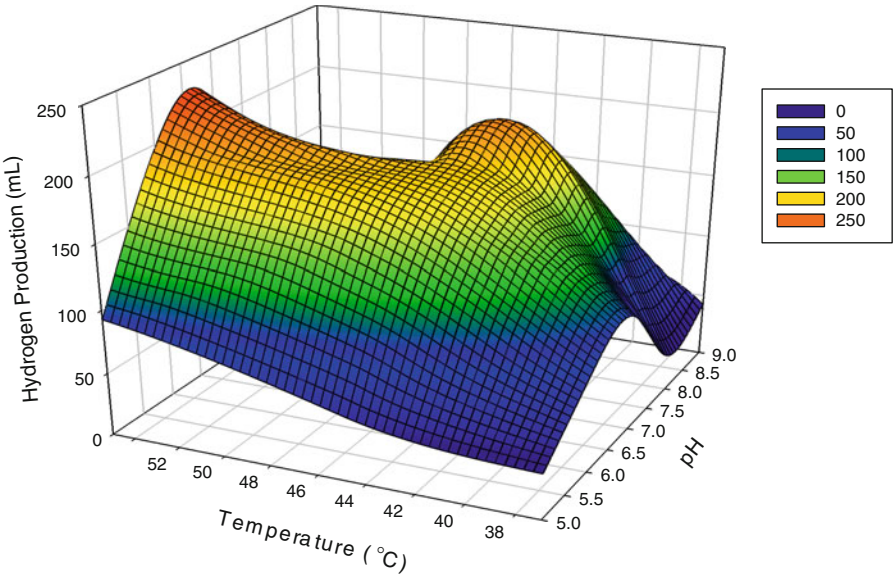

Fig. 2 Variation of hydrogen production with different temperatures and initial pHs

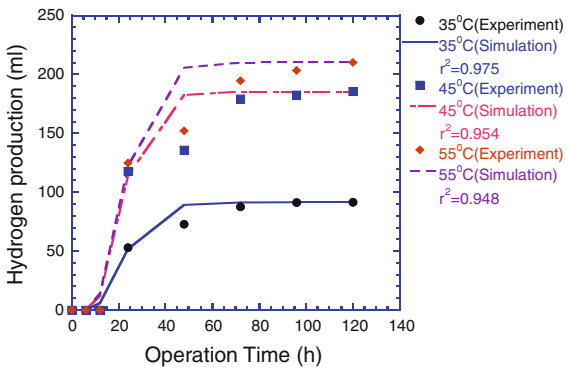

Fig. 3 Cumulative hydrogen production fitted by modified Gompertz equation at different temperatures

Table 2 Modified Gompertz model parameters values for fructose wastewater at different temperatures

Temperature (°C)	P (ml)	R_m (ml h^{-1})	λ (h)	r^2
35	91.6	4.4	12	0.975
45	185.5	9.8	12	0.954
55	210.2	10.4	12	0.948

hydrogen production rate (HPR) and specific hydrogen production rate (SHPR) values than those of mesophilic fermentation by 200% (Lin et al. 2008). Similar low hydrogen production was experienced at 35°C in this study with 78.3 ml-H_2/g-COD and 15.9 ml-H_2/g-VSS-h for SHPP and $SHPR_m$, respectively. The highest values of SHPP and $SHPR_m$, 188.8 ml-H_2/g-COD and 32.4 ml-H_2/g-VSS-h, respectively, were obtained when operated at 55°C. The experimental results indicated that a two-fold higher SHPP and $SHPR_m$ was obtained at thermophilic condition (55°C) than that at mesophilic condition (35°C).

Effects of initial pH

In the previous study by Van Ginkel et al. (2001), it was concluded that initial pH did not have as profound effect on hydrogen production rate as it has on specific hydrogen production potential. To confirm their experimental results, a series of batch tests were conducted using two different organic substrates–fructose and molasses wastewaters with different initial pHs. Table 3 summarizes the experimental results obtained for fructose wastewater at various initial cultivation pH values during 120 h of hydrogen fermentation. The experimental results showed that the oxidation–reduction potential (ORP) values ranged from −195 to −418 mv and initial alkalinity values were in the range of 468–6852 mg/l (as $CaCO_3$), which favored fermentative hydrogen production for fructose wastewater (Hawkes et al. 2002; Lin and Lay 2005; Lin et al. 2006). Therefore,

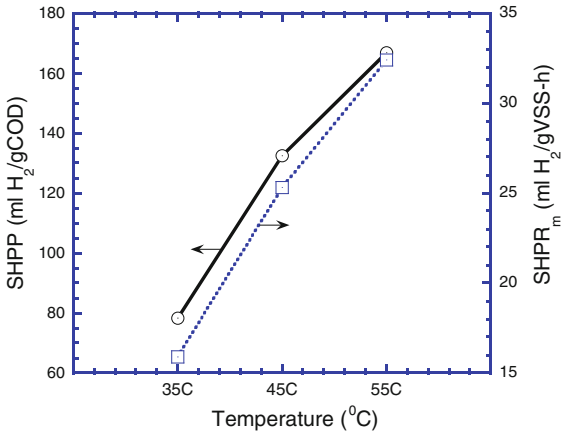

Fig. 4 Specific hydrogen production potential (SHPP) and maximum specific hydrogen production rate ($SHPR_m$) at different temperatures for fructose wastewater

the initial cultivation pH is the only environmental factor to affect hydrogen production for batch tests at a constant temperature of 55°C. A pH value of 6 was optimal for a mixed culture to produce the highest amount of hydrogen (210 ml) with the highest hydrogen content in the biogas (42%). Table 4 shows hydrogen production from molasses wastewater at various initial pH values during hydrogen fermentation for 120 h. The amount of hydrogen produced at the end of fermentation was 129–236 ml and was dependent on the initial cultivation pH. The variation in hydrogen production and hydrogen content with the initial pH from molasses wastewater was the same as that from fructose wastewater. At an initial pH of 6, the highest amount of hydrogen produced was 236 ml with the highest hydrogen content (52%) in the biogas.

Figure 5a and b shows comparisons of hydrogen production for experimental data and modified Gompertz model simulation from fructose and molasses wastewaters. The correlation coefficient (r^2) range was 0.937–0.995 for fructose wastewater and 0.792–0.992 for molasses wastewater at the same pH range of 4–8. The hydrogen production curves using fructose wastewater as an organic substrate has a better model fit than molasses wastewater. The modified Gompertz model parameters (P, R_m and λ) were initially pH-dependent for fructose and molasses wastewaters (Table 5). The P and R_m values increased as pH increased from 4 to 6 for both fructose and molasses wastewater. The P and R_m values peaked at pH 6 for fructose wastewater but P and R_m peaked at 6 and 7 for molasses wastewater, respectively. The optimal pH for hydrogen production was found to be in the range of 6 (Table 5). This is in close agreement with the earlier work (Lin et al.

Table 3 Hydrogen production from fructose wastewater at various initial cultivation pH values during 120 h fermentation at 55°C

pH		ORP (−mV)	VSS added (g)	Alkalinity (mg/l as $CaCO_3$)		Biogas (ml)	H_2 (ml)	H_2 (%)
Initial	Final			Initial	Final			
4	3.8 ± 0.1	195 ± 14	0.32	468 ± 42	258 ± 12	304	93	31
5	4.5 ± 0.1	214 ± 6	0.46	683 ± 48	327 ± 28	532	197	37
6	4.8 ± 0.1	308 ± 25	0.39	2360 ± 32	625 ± 56	488	210	43
7	5.5 ± 0.1	332 ± 18	0.22	5875 ± 157	1892 ± 43	183	64	35
8	7.2 ± 0.1	418 ± 32	0.46	6852 ± 136	4328 ± 121	327	108	33

Table 4 Hydrogen production from molasses wastewater at various initial cultivation pH values during 120 h fermentation at 55°C

pH		ORP (−mV)	VSS added (g)	Alkalinity (mg/l as $CaCO_3$)		Biogas (ml)	H_2 (ml)	H_2 (%)
Initial	Final			Initial	Final			
4	3.5 ± 0.1	245 ± 8	0.24	328 ± 32	168 ± 9	607	170	28
5	4.2 ± 0.1	255 ± 11	0.13	567 ± 56	365 ± 15	449	202	45
6	4.3 ± 0.1	408 ± 16	0.11	3874 ± 117	1738 ± 56	454	236	52
7	6.5 ± 0.1	432 ± 24	0.15	4582 ± 73	2248 ± 66	383	161	42
8	7.4 ± 0.1	318 ± 28	0.26	6742 ± 85	3572 ± 68	358	129	36

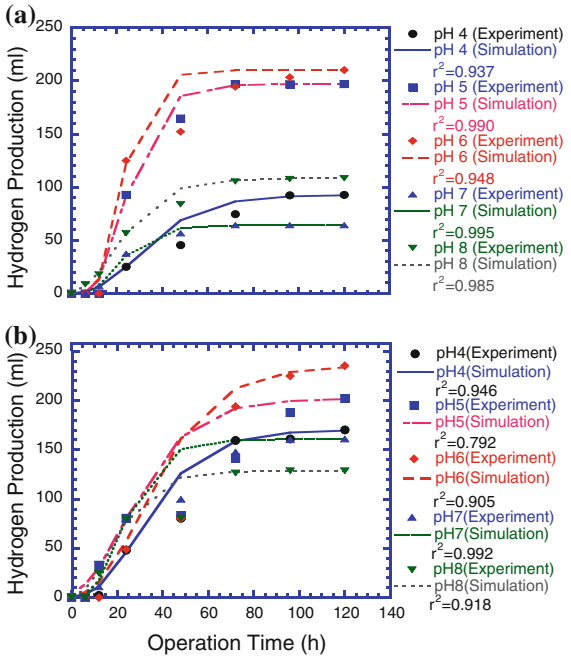

Fig. 5 Cumulative hydrogen production fitted by modified Gompertz equation at different initial pH levels: **a** Fructose wastewater. **b** Molasses wastewater

2006) where pH of 6.5 was deemed the optimum initial pH for hydrogen production As to a lag time (λ), both fructose and molasses wastewaters had the same lag time, except when initial pH was 5. The stirred-tank bioreactor with an initial pH of 4–6 had the longest lag time of 12 h for fructose and molasses wastewaters. For fructose wastewater, the lag phase decreased when initial pH decreased from 6 to 8. Maximum hydrogen production potential occurred at lag time of 12 h.

Figure 6 plots the variation in SHPP with initial cultivation pH. The graph shows that SHPP is affected by initial pH. The batch tests of fructose

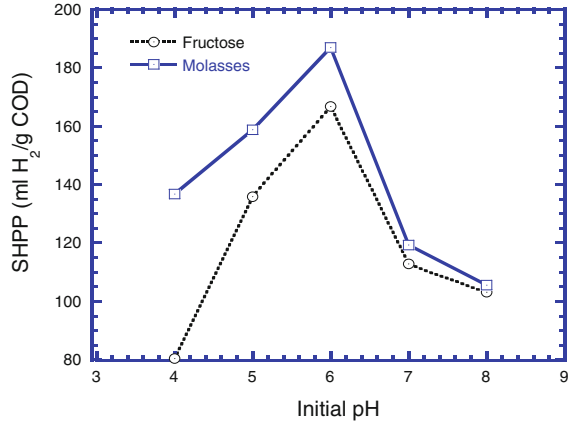

Fig. 6 Specific hydrogen production potential (SHPP) at different initial pH levels for food-processing wastewater

and molasses wastewaters had similar trends for SHPP. The SHPP increased and then declined when initial pH was 4–6 and 6–8, respectively. The SHPP peaked at 166.8 ml-H_2/g-COD for fructose and at 187.0 ml-H_2/g-COD for molasses at an initial pH of 6. Experimental observations indicated that SHPP for molasses wastewater was higher than that for fructose wastewater at different initial pH of 4–8. Figure 7 shows the variation in $SHPR_m$ with initial cultivation pH. Experimental results demonstrate that $SHPR_m$ occurred at an initial pH of 6 for both fructose and molasses wastewaters. The peak value of $SHPR_m$ was 26.7 ml-H_2/g-VSS-h and 42.7 ml-H_2/g-VSS-h for fructose and molasses wastewaters, respectively.

Intermediate products and their effects

Hydrogen production is usually accompanied by production of volatile fatty acids (VFAs) and solvents. Production of these intermediates reflects

Table 5 Modified Gompertz model parameters values for fructose and molasses wastewater at various initial cultivation pH values at 55°C

pH	P (ml)		R_m (ml h^{-1})		λ (h)		r^2	
	Fructose	Molasses	Fructose	Molasses	Fructose	Molasses	Fructose	Molasses
4	92.5	170.2	2.1	3.8	12	12	0.937	0.946
5	197.0	202.2	7.7	4.5	12	6	0.990	0.792
6	210.2	235.5	10.4	4.7	12	12	0.948	0.905
7	64.3	161.0	2.6	5.8	10	10	0.995	0.992
8	108.4	128.8	3.2	4.4	6	6	0.985	0.918

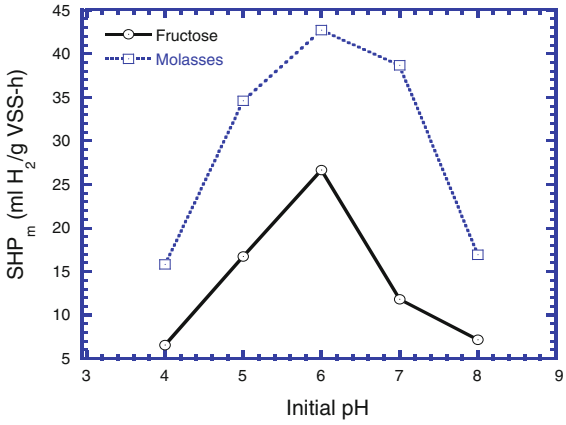

Fig. 7 Maximum specific hydrogen production rate ($SHPR_m$) at different initial pH levels for food-processing wastewater

changes in the metabolic pathway of microorganisms involved in hydrogen production. Such changes can improve the understanding of favorable conditions for hydrogen production. Thus, during the course of hydrogen production, liquid samples were collected and analyzed for volatile fatty acids (VFAs) in batch tests.

The major VFAs in the fermentation process were acetate, propionate and butyrate. Figure 8a and b present hydrogen production, hydrogen content, pH and VFAs production profiles for fructose and molasses wastewaters, respectively. These experimental results were obtained with a stirred-tank bioreactor with an initial pH of 6.0. A gradual production of acids depleted buffering capacity, resulting in a concomitant decline in pH to about 5.0 for fructose wastewater and 5.2 for molasses wastewater before hydrogen production began. This was followed by a rapid production of hydrogen with an increase in VFAs production until hydrogen production peaked at 210 ml for fructose wastewater and 236 ml for molasses wastewater when the stationary phase was achieved. At the stationary phase, a lower final pH was observed for molasses wastewater than that for fructose wastewater. It reflects that more carbohydrate in molasses wastewater was used by hydrogen producing bacteria than that in fructose wastewater. This finding was in close agreement with the experimental results obtained by Chen et al. (2006). The hydrogen content in biogas reached a maximum value of 43% for fructose wastewater and 52% for molasses wastewater as maximum hydrogen production peaked. In the exponential phase, the butyrate concentration increased dramatically after 50 h and then butyrate reached the highest concentration in the stationary phase. The lowest propionate concentrations for fructose and molasses wastewaters were observed at an initial pH of 6. In the stationary phase, the productions of acetate and butyrate decreased slightly but an increase in propionate production was noticed. Nevertheless, acetate and butyrate levels were independent of pH in the range studied. This observation was in close agreement with the results obtained by Van Andel et al. (1985) based on a study using pure culture of *Clostridum butyricum* for hydrogen production.

The ratio of the butyrate concentration to the acetate concentration can vary with microbial growth during fermentation process. Figure 9a and b show the ratio of butyrate (HBu) to acetate (HAc) and cumulative hydrogen production varied with initial pH. As to fructose wastewater, the HBu/HAc ratio during hydrogen production had a similar pattern for all batch tests—the maximum HBu/HAc ratio occurred during the exponential phase, followed by the stationary phase of hydrogen production at which the ratio of HBu/HAc declined except for an initial pH of 8. However, for molasses wastewater, the maximum ratio was observed during the exponential growth phase and it showed a declining trend at stationary phase, which only occurred at an initial pH of 6. The maximum HBu/HAc ratio for fructose wastewater increased when initial pH was 4–6, and then decreased when initial pH was 6–8. For molasses wastewater, the maximum HBu/HAc ratio increased when initial pH was 4–5 then declined when initial pH was 5–8. The HBu/HAc ratio was in the range of 1.4–3.1 for fructose wastewater and 1.4–2.3 for molasses wastewater. Lin et al. (2006) found the ratio of HBu/HAc ranging 2.6–4.0 elevated the efficient hydrogen production. The HBu/HAc ratio value obtained in their study was higher than that in this study since they used a pure substrate (e.g. xylose) for the growth of *Clostridium* sp. The changes in HBu/HAc ratio indicated a metabolic alteration due to environmental factors changes such as pH, hydrogen content in the biogas and the accumulation of intermediate products in the batch tests.

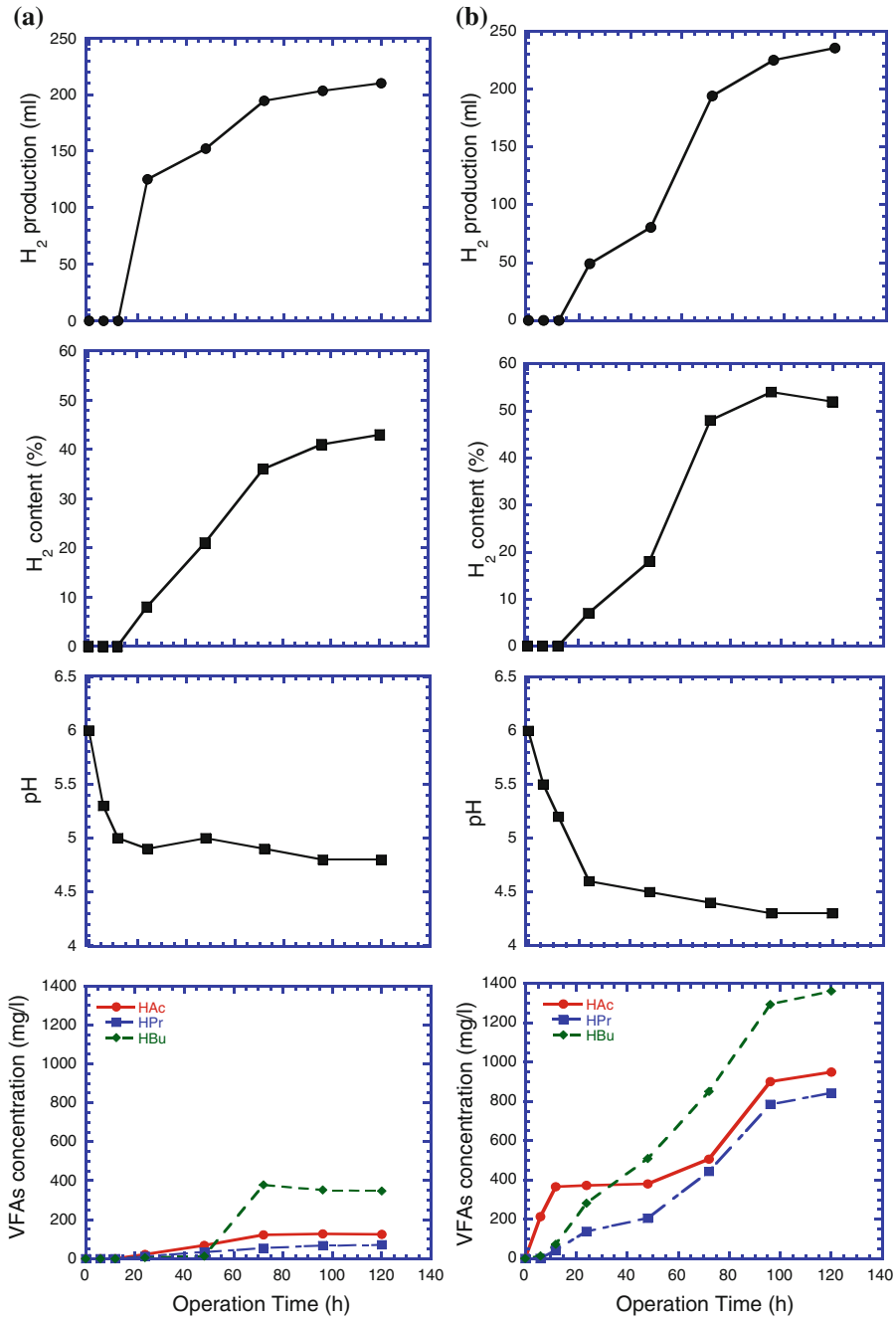

Fig. 8 Hydrogen production, hydrogen content, pH and volatile fatty acids profiles at a pH of 6 and 55°C: **a** Fructose wastewater. **b** Molasses wastewater

Microbial community

The compositions of the microbial community in hydrogen-producing mixed culture from fructose and molasses wastewaters were analyzed using PCR-DGGE method. Figure 10 shows the DGGE profiles of the 16S rDNA gene fragment amplified from the mixed culture of hydrogen-producing bacteria. Each band on the DGGE profile corresponds to a gene fragment of unique 16S rDNA sequences, and, thus, represents a specific species in the microbial community. The staining intensity of a band represents the relative abundance of that microbial species (Zhang and Fang 2000). The DGGE profiles clearly

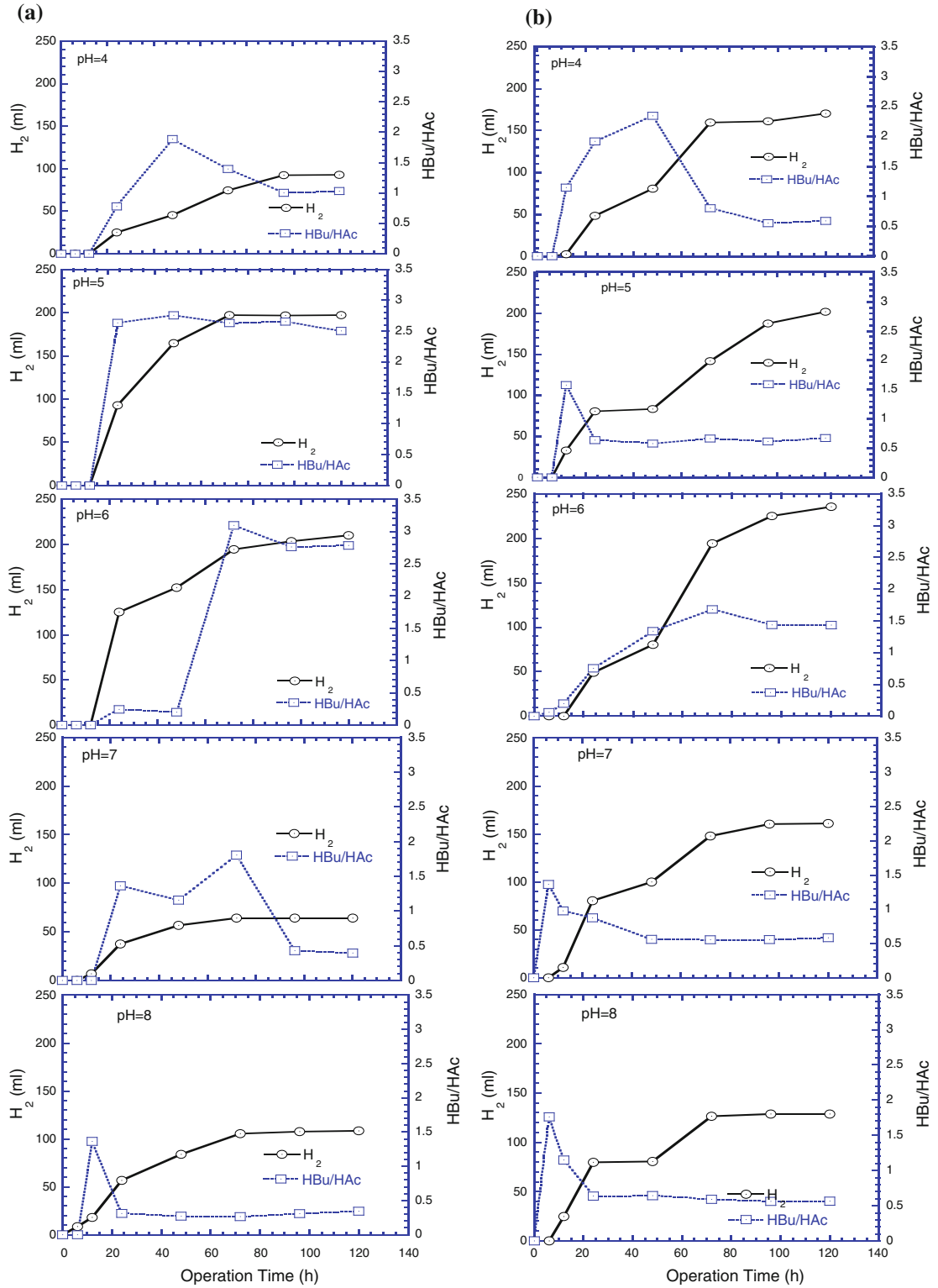

Fig. 9 The profile of HBu/HAc ratio and cumulative hydrogen production at different initial pH levels at 55°C: **a** Fructose wastewater. **b** Molasses wastewater

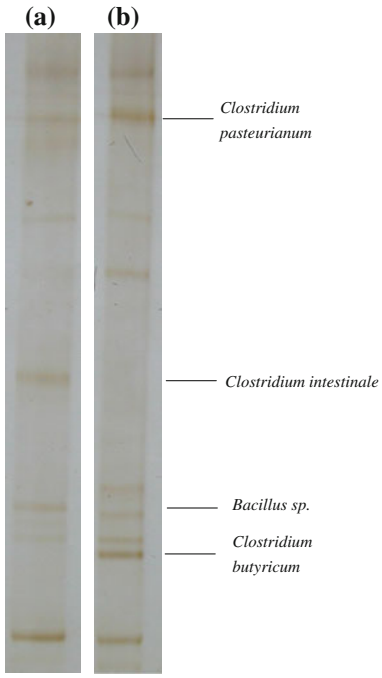

Fig. 10 DGGE profiles of 16S rDNA obtained from food processing wastewater after 120 h fermentation at initial pH 6: lane **a**, fructose; lane **b**, molasses

show that the microbial community changed for different food-processing wastewater. Molasses wastewater as a substrate has a higher staining intensity of the bands than fructose wastewater. Based on the gene sequencing results, it was concluded that *Clostridium* species was the dominant species of hydrogen-producing bacteria in the bioreactor. For fructose wastewater, it was observed that *Clostridium intestinale* and *Bacillus* sp. dominated the sludge samples collected from fructose wastewater with a pH of 6. Two distinct bands were identified as *Clostridium butyricum* and *Clostridium pasteurianum* are dominant species from molasses wastewater. The *Clostridium* sp. for hydrogen production found in this study was in close agreement with the earlier works (Lin et al. 2008) where *Clostridium pasteurianum* and *Clostridium intestinale* became dominant species in their study. The differences in distribution of specific species for fructose and molasses wastewaters might relate to the ratio of butyrate to acetate concentration (HBu/HAc) that may vary with microbial growth conditions during fermentation process and has been used to indicate the progress of hydrogen production (Lin et al. 2006).

Conclusions

The pH and intermediate products especially volatile fatty acids drive the hydrogenase reaction during hydrogen fermentation. Besides, pH control is also important to suppress hydrogen consumers and to obtain an enriched culture of hydrogen producing clostridia. A series of batch tests were conducted to examine the effects of temperature and initial pH on hydrogen production using fructose and molasses wastewater as organic substrates. This work demonstrates that both fructose and molasses-processing wastewaters are effective organic substrates for producing biohydrogen. The initial cultivation pH markedly affects SHPP, $SHPR_m$, liquid fermentation products concentrations and the HBu/HAc ratio. The maximum SHPP was 166.8 ml-H_2/g-COD and 187.0 ml-H_2/g-COD at an initial pH of 6 for fructose and molasses-processing wastewaters, respectively. The $SHPR_m$ was 26.7 ml-H_2/g-VSS-h and 42.7 ml-H_2/g-VSS-h for fructose and molasses-processing wastewaters, respectively, at an initial pH of 6. The stationary phase of hydrogen production for all batch tests occurred after the HBu/HAc ratio decreased. The DGGE profiles indicate that molasses-processing wastewater as a substrate has a higher staining intensity band than fructose-processing wastewater. *Clostridium* sp. became dominant species for both fructose and molasses wastewaters in this study. This experimental result suggests that molasses-processing wastewater as a substrate produces more hydrogen than fructose-processing wastewater. The findings of this study can be applied when designing high-rate hydrogen-production bioreactors using food-processing wastewater as organic substrates.

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