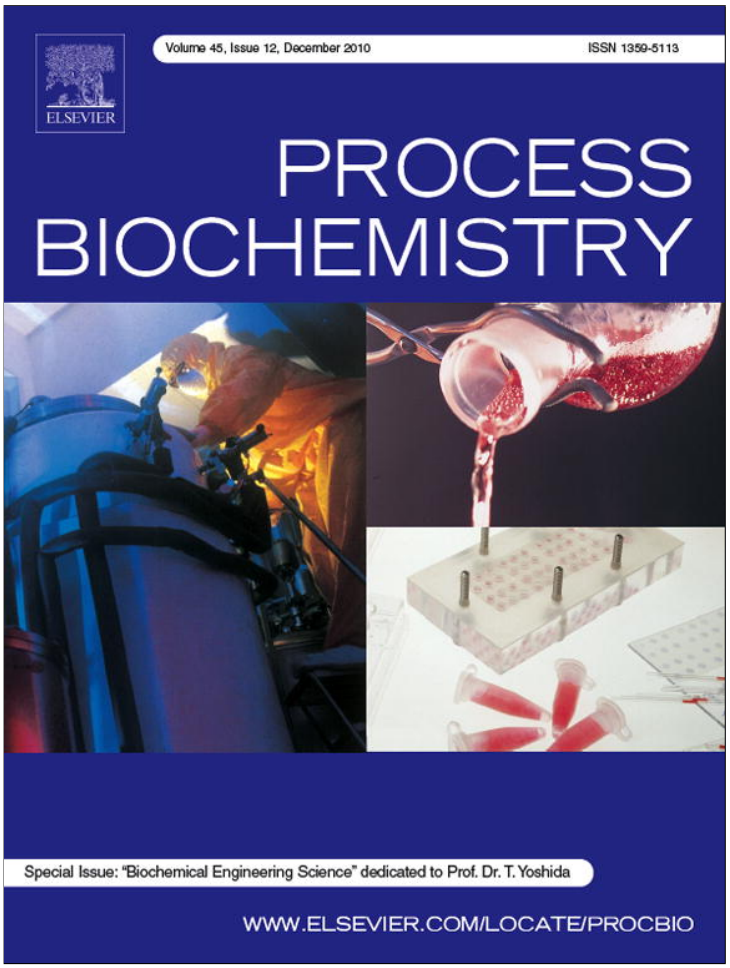

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Short communication

Photo-fermentation of *Rhodobacter sphaeroides* for hydrogen production using lignocellulose-derived organic acids

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ABSTRACT

In this study, the corn stover was pretreated, enzymatically hydrolyzed, and then fermented in the lipid production fermentation. The corn stover fermentation effluent was utilized for the photo-fermentation of a *Rhodobacter sphaeroides* ZX-5 for hydrogen production. The hydrogen production was more than twofolds greater than that in the synthetic medium under the similar organic acid concentration range. The synergism among the pure organic acids was found to facilitate cell growth and hydrogen production, although some organic acid was not utilized for hydrogen production directly. The synergism among the components in the corn stover fermentation effluent was also found. The initial pH value was found to be an important parameter for the photo-fermentation of *R. sphaeroide* ZX-5 using the corn stover fermentation effluent. The results provided a possible way to utilize lignocellulose-derived organic acids for hydrogen production, and to treat fermentation wastewater in biofuel production using lignocellulose.

1. Introduction

Lignocellulose mainly comprises cellulose, hemicellulose, and lignin. To overcome the biocalcitrance of the lignocellulose materials, the pretreatment processings such as dilute acid and steam explosion have to be applied before the enzymatic hydrolysis of lignocellulose [1,2]. During lignocellulose pretreatment processing, various organic acids are produced, such as acetic acid generated from the hydrolysis of hemicellulose, formic acid as the degradation products of pentose sugar, and levulinic acid as the degradation products of hexose sugar. When pretreated lignocellulose is used as feedstock for ethanol or lipid production, these organic acids are generally unable to be degraded and exist in the final fermentation effluent [3].

Photo-fermentation is an important method for hydrogen production by photosynthetic bacteria such as *Rhodobacter sphaeroides*, *Rhodopseudomonas*, or *Rhodospirillum* using small organic acid molecules as carbon sources [4–10]. Lignocellulose has been used as the carbon source for hydrogen production in dark fermentation [11,12], but lignocellulose-derived organic acids as the carbon source for hydrogen production via photo-fermentation have not been reported. In our previous work, a mutant photosynthetic purple non-sulfur (PNS) bacterium *R. sphaeroides* ZX-5 was developed and applied for hydrogen production using organic acids such as malic acid [13]. To widen the spectrum of cheap carbon sources for hydrogen production, lignocellulose-derived organic acid in the fermentation effluent is an important option, because the biofuel industry wastewater contains a large volume of organic acids from lignocellulose.

In this study, the individual organic acid and the mixture of the organic acids for the photo-fermentation of *R. sphaeroides* ZX-5 were tested for the potential carbon source of hydrogen production by photo-fermentation. Then the corn stover fermentation effluent containing various organic acids was applied for hydrogen production in photo-fermentation with the same strain. The results show that not only sufficient hydrogen was produced, but also the organic acids and residual sugars in the wastewater were degraded to a large extent. Under the proper initial pH value, the hydrogen accumulation using the organic acids in the corn stover fermentation effluent was up to 6–7 mL mL^{-1} of the fermentation effluent. The study provided a possible way to utilize lignocellulose-derived organic acids existing in the fermentation effluent for hydrogen production, as well as a way to treat fermentation wastewater in biofuel production using lignocellulose.

2. Materials and methods

2.1. Chemicals and enzymes

Levulinic acid was from Alfa Aesar (Ward Hill, MA, USA); DL-malic acid (SCRC, Shanghai, China); formic acid, acetic acid, ammonium chloride, and sodium glutamine were all purchased from local chemical reagent companies in Shanghai,

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China. Two industrial cellulase enzymes used were Spezyme CP from Genencor International (Rochester, NY, USA) and Novozyme 188 from Novo Industrial A/S (purchased from Sigma–Aldrich, St. Louis, MO, USA).

2.2. Strains

The photosynthetic purple non-sulfur (PNS) bacterium mutant *R. sphaeroides* ZX-5 was provided and stored by the Institute of Plant Physiology and Ecology, Shanghai Institutes of Biological Sciences, Chinese Academy of Sciences, Shanghai, China [13]. *R. sphaeroides* ZX-5 was pre-cultured in RCVB medium [14]. The RCVB medium contains a basal solution (in 1 L): 0.75 g K_2HPO_4, 0.85 g KH_2PO_4, 2.8 mg H_3BO_3, 0.75 mg $Na_2MoO_4{\cdot}2H_2O$, 0.24 mg $ZnSO_4{\cdot}7H_2O$, 2.1 mg $MnSO_4{\cdot}4H_2O$, 0.04 mg $Cu(NO_3)_2{\cdot}3H_2O$, 0.75 mg $CaCl_2{\cdot}2H_2O$, 2.0 mg EDTA, 0.2 g $MgSO_4{\cdot}7H_2O$, 11.8 mg $FeSO_4{\cdot}7H_2O$, 5 mg vitamin B1, 0.1 mg biotin, and 10 mg nicotin amide. The phosphate in the basal solution was used as buffer solution. 4 g L^{-1} of DL-malic acid and 1 g L^{-1} of ammonium chloride (for the seeds cell growth) or sodium glutamine (for the initial cell growth in photo-bioreactor) were used as the carbon source and nitrogen source, respectively [13]. The initial pH in the RCVB medium was adjusted to 7.0 using the 3 M sodium hydroxide solution. All the media were autoclaved at 115 °C for 20 min.

The oleaginous mutant strain *Trichosporon cutaneum* CX2 was isolated by our laboratory and used for lipid fermentation [15].

2.3. Enzymatic hydrolysis and lipid fermentation of corn stover

Corn stover was harvested in fall 2006 from Northeast Province Jilin, China. The corn stover was milled to pass a sieve with the pore diameter of 5 mm, and then pretreated in a steam explosion process at the condition of 200 °C, 2.0 MPa for 4 min. The pretreated corn stover was hydrolyzed using two cellulase enzymes, Spezyme CP and Novozyme 188, at the dosage of 7 FPU and 15 IU g^{-1} of the dried mass, respectively. The enzyme hydrolysis was carried out at 50 °C for 48 h. Water insoluble solid in the hydrolysate was separated by centrifugation and the liquid hydrolysate was used to culture the oleaginous yeasts. The major compositions of the corn stover hydrolysate were 60.6 g L^{-1} of glucose, 36.4 g L^{-1} of xylose, 6.6 g L^{-1} of acetic acid, and 1.5 g L^{-1} of levulinic acid. The obtained corn stover hydrolysate was autoclaved at 115 °C for 20 min and then 1.5 L hydrolysate was fed into a 3 L bioreactor (Baoxing Biotech 3BG-4, Shanghai, China). The *T. cutaneum* CX2 strain was inoculated into the hydrolysate for lipid fermentation. The lipid fermentation operation was described in our previous report [15]. Then the *T. cutaneum* cells were harvested by centrifugation and dry cell mass (DCM) was extracted for lipid oil recovery. After cells were removed, the fermentation effluent was used as medium for hydrogen production. Glucose was almost completely consumed and 4 g L^{-1} of xylose was remained in the hydrolysate. The major organic acids in the fermentation effluent were 5 g L^{-1} of acetic acid, 0.4 g L^{-1} of levulinic acid, and minor formic acid.

The lipid fermentation effluent was decoloured by 4% (w/w) activated carbon at 60 °C for 30 min and decoloured effluent was separated from the activated carbon by vacuum filtration. The supernatant was sterilized through a 0.22 μm filter and finally filled into the photo-bioreactor for hydrogen production.

2.4. Photo-bioreactor and photo-fermentation

The *R. sphaeroides* ZX-5 strain was pre-cultured in a 250 mL flask containing 100 mL RCVB media for 18 h at 30 °C, 180 rpm with orbit shaker until the OD value at 660 nm was 1.5. The hydrogen production in the RCVB medium and the corn stover fermentation effluent was carried out in the specially designed glass micro-bioreactor as described by Tao et al. [13] as shown in Fig. 1. A 40-mL glass cylinder tube bioreactor was sealed with a rubber stopper and filled with 34 mL media either the RCVB medium or the fermentation effluent, and 1 mL of the seed inoculation for photo-fermentation. The illumination was done by two 60 watts tungsten lamps at the opposite sides of the cylinder bioreactor. The light intensity was measured by an illumination photometer (ST-85, Photoelectric Instrument, Beijing, China) at the surface of the bioreactor. A 60 mL syringe with the volume reading markers was connected to the bioreactor using a stainless tube with a 0.5 mm in diameter. The hydrogen gas generated in the bioreactor was fed into the syringe spontaneously and pushed the piston of the syringe upward. In this way, the hydrogen accumulation during the photo-fermentation at the standard atmospheric pressure was measured. The temperature of the bioreactor was set to 30 °C in an incubator with the temperature control system. The light was set to the optimal point of 4500 lux for *R. sphaeroides* ZX-5 according to our previous result [13]. Samples were taken from the outlet in the rubber stopper.

2.5. Analysis

The hydrogen gas was analyzed by a gas chromatograph (GC 900C, Tianpu, shanghai, China) with a thermal conductivity detector (TCD), a 2 m (length) × 3 mm (inner diameter) stainless column packed with 5 Å molecular sieve (60–80 mesh). The temperature in oven, injector, and detector was 60, 100, and 100 °C, respectively. Electric current was 80 mA. Nitrogen was used as the carrier gas at 20 mL min^{-1}.

Glucose, xylose and organic acids including malic acid, formic acid, acetic acid, levulinic acid in the RCVB medium and the fermentation effluent were analyzed by high performance liquid chromatography (HPLC, LC-20AD, refractive index detector RID-10A, Shimadzu, Japan) with a Bio-rad Aminex HPX-87H column at the column temperature 65 °C. The mobile phase was 5 mM H_2SO_4 at the rate of 0.6 mL min^{-1}. All samples were centrifuged to remove the cell mass and other water insoluble substances, and then filtered through a 0.22-μm filter before the analysis.

The cell concentration was measured by a spectrophotometer (722N, Jingke, Shanghai, China) at 660 nm. The dry cell mass (DCM) was determined by centrifugation of 10-mL cell suspension, washing the pellet once in distilled water and drying in a vacuum oven until it reached constant weight. The calibration of DCM versus OD_{660} was determined in our previous report and approximately the DCM value at OD_{660} of 1.0 corresponds to 0.7 g L^{-1} of the dry cell mass.

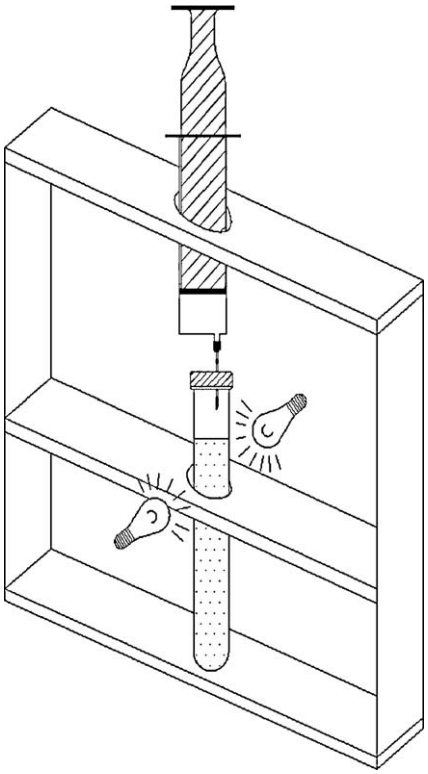

Fig. 1. Scheme of the photo-fermentation bioreactor.

3. Results and discussion

3.1. Hydrogen production from pure organic acid and their combinations

The pure organic acids, acetic acid, formic acid, and levulinic acid, were used as the carbon sources for photo-fermentation of *R. sphaeroides* ZX-5 separately, and then their mixtures were applied for photo-fermentation. The results are shown in Tables 1 and 2, respectively. The concentration of each organic acid in the photo-fermentation was given based on its concentration in the real fermentation effluent. Malic acid, the most favourable substrate for *R. sphaeroides* ZX-5, was used as the control for all the experiments [13].

Table 1 indicates that acetic acid and formic acid can be used as the carbon sources for photo-fermentation of *R. sphaeroides* ZX-5. The maximum total volumetric hydrogen accumulation in 120 h for acetic acid and formic acid was 1.36 mL H_2 mL^{-1} media and 0.67 mL H_2 mL^{-1} media, respectively. On the other hand, the best conversion efficiencies of the two acids were 30% and 36%, respectively. Levulinic acid facilitated the cell growth of *R. sphaeroides* ZX-5 (the final dry cell weight was 0.84 g L^{-1}), but no hydrogen was detected when levulinic acid was used as the sole carbon source. Table 1 also indicates that each of the three organic acids had an optimal concentration range for hydrogen production.

Table 1
Sole organic acid as carbon sources for photo-fermentation of H_2 production.

	Organic acid concentration ($g\,L^{-1}$)	Final DCM ($g\,L^{-1}$)	Final pH	Conversion efficiency (%)	Total H_2 accumulation ($mL\,mL^{-1}$)	Maximum H_2 production rate ($mL\,mL^{-1}\,h^{-1}$)
Malic acid	4.0	1.26	7.1	72	2.63 ± 0.17	0.037
Acetic acid	1.0	0.77	8.5	6	0.08 ± 0.00	0.001
	3.0	1.05	7.6	30	1.24 ± 0.05	0.016
	4.0	1.05	7.7	27	1.36 ± 0.06	0.017
	5.0	2.59	9.7	11	0.25 ± 0.00	0.003
Formic acid	1.0	0.49	9.4	0	0.00 ± 0.00	0.000
	3.0	1.12	8.8	29	0.27 ± 0.01	0.003
	5.0	1.26	8.7	36	0.67 ± 0.03	0.007
	7.0	2.17	10.0	6	0.09 ± 0.00	0.001
Levulinic acid	1.0	0.84	8.7	0	0.00 ± 0.00	0.000

34 mL medium and 1 mL inoculation seeds were filled in a 40 mL tube for photo-fermentation at 30 °C under 4500 lux. The initial pH was 7.0. Malic acid was used as the control. The conversion efficiency is the ratio of the actual moles of hydrogen produced to the theoretical amount when the sole organic acid was completely converted to hydrogen. The hydrogen accumulation is the total volume of hydrogen produced per mL of medium in 120 h of photo-fermentation for each organic acid. The maximum hydrogen productivity is the hydrogen produced per mL medium per hour.

Table 2 shows the hydrogen production performance during the photo-fermentation of *R. sphaeroides* ZX-5 using organic acid mixtures at different concentration ranges. The results show that similar to the sole organic acid substrate, each organic acid had its optimal concentration range when the concentrations of the other two organic acids were fixed. When the concentrations of formic and levulinic acid were fixed (5.0 and 1.0 $g\,L^{-1}$, respectively), both the total volumetric hydrogen accumulation and the acid conversion efficiency increased with the increase of acetic acid concentration until it reached 3.0 $g\,L^{-1}$, and then sharply decreased with further increase of acetic acid. When the concentrations of acetic acid and levulinic acid were fixed (4.0 and 1.0 $g\,L^{-1}$, respectively), the total volumetric hydrogen accumulation and the acid conversion efficiency increased with the increase of formic acid concentration until the formic acid concentration was 5.0 $g\,L^{-1}$, and then decreased with further increase of formic acid. Interestingly, when the concentration of levulinic acid in the organic acid mixture was proper (at 1.0, 4.0, and 5.0 $g\,L^{-1}$ for levulinic acid, acetic acid, and formic acid, respectively), both hydrogen accumulation and acid conversion efficiency were better than those in the organic acid mixture without levulinic acid, although no hydrogen was detected when levulinic acid was the sole carbon source for *R. sphaeroides* ZX-5 (Table 1). The result indicates that synergism among the three lignocellulose-derived organic acids enhanced hydrogen production performance in both acid conversion efficiency and hydrogen accumulation. At a suitable combination proportion of these three organic acids, both acid conversion efficiency and accumulation of hydrogen exceeded the maximum values of the sole organic acid (acetic acid, formic acid, or levulinic acid) as carbon source.

Both Tables 1 and 2 indicate that pH value increased from the original pH of 7.0 during the photo-fermentation. It was found that the improper acid concentration (low or high) was generally with an obvious increase of pH and a lower hydrogen production. This result was in agreement with other studies in photo-fermentation using pure organic acids as carbon sources [16–18]. Zhu et al. [19] assumed that the increase of pH was a result of hydrogen production suppression and pH affected hydrogen production reversely. When the acid concentration is low, the carbon source is only enough for cell growth. While the acid concentration is high and the nitrogen is limited, the organic acids are converted to PHB (poly-3-hydroxybutyric acid) which was competitively with the hydrogen formation [20,21].

3.2. Hydrogen production using lignocellulose-derived organic acids

The photo-fermentation of *R. sphaeroides* ZX-5 using the corn stover fermentation effluent was described in Section 2 and the results are shown in Figs. 2 and 3. Fig. 2 shows the time courses of the cell growth, pH change, hydrogen accumulation, as well as

Table 2
Combination of organic acids as carbon sources for photo-fermentation of H_2 production.

Organic acid concentration ($g\,L^{-1}$)			Consumption (%)			Final DCM ($g\,L^{-1}$)	Final pH	Conversion efficiency (%)	Total H_2 accumulation ($mL\,mL^{-1}$)	Maximum H_2 production rate ($mL\,mL^{-1}\,h^{-1}$)
Ace	For	Lev	Ace	For	Lev					
0.0	5.0	1.0	0.0	65.9	0.0	1.54	8.3	8	0.12 ± 0.00	0.002
1.0	5.0	1.0	99.6	60.0	12.0	1.54	8.2	45	1.34 ± 0.06	0.012
3.0	5.0	1.0	96.8	68.5	18.2	1.89	7.0	49	2.93 ± 0.01	0.026
5.0	5.0	1.0	14.0	62.4	12.0	1.96	9.6	10	0.25 ± 0.00	0.003
4.0	0.0	1.0	99.9	0.0	8.3	1.05	7.8	17	1.04 ± 0.05	0.011
4.0	3.0	1.0	100.0	37.1	12.7	1.68	7.3	23	1.49 ± 0.09	0.012
4.0	5.0	1.0	99.9	55.8	21.4	2.59	8.2	37	2.79 ± 0.11	0.020
4.0	7.0	1.0	99.0	1.3	0.0	1.68	9.3	6	0.37 ± 0.02	0.003
4.0	5.0	0.0	95.0	9.5	0.0	1.61	7.5	25	1.51 ± 0.08	0.020
4.0	5.0	1.0	99.8	59.6	18.7	2.31	7.8	37	2.75 ± 0.17	0.018
4.0	5.0	3.0	99.9	70.0	64.6	2.24	8.3	15	1.04 ± 0.01	0.008
Malic acid 4.0			99.8			1.8	7.2	75	2.98 ± 0.11	0.031

Ace: acetic acid; For: formic acid; Lev: levulinic acid.
34 mL medium and 1 mL inoculation seeds were filled in a 40 mL tube for photo-fermentation at 30 °C under 4500 lux. The initial pH was 7.0. Malic acid was used as the control test. Conversion efficiency is the ratio of the actual moles of hydrogen produced to the theoretical amount when acetic acid and formic acid were completely converted to hydrogen. The hydrogen accumulation is the total volume of hydrogen produced per mL medium in 120 h of photo-fermentation. The hydrogen productivity is the maximum hydrogen produced per mL of medium per hour.

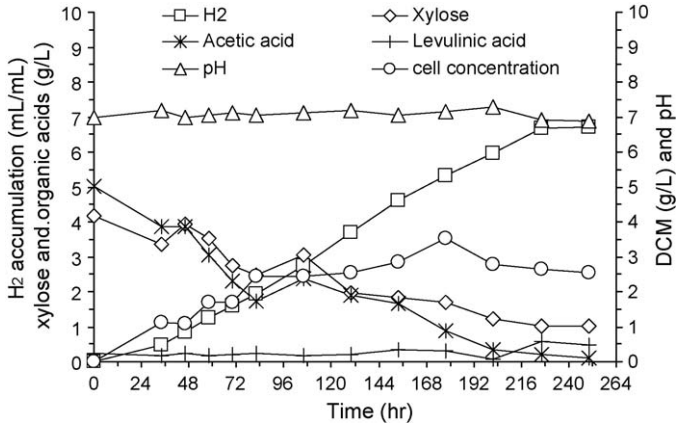

Fig. 2. Time courses of hydrogen accumulation, xylose and organic acid consumption, cell growth, and pH change in the photo-fermentation of *R. sphaeroides* ZX-5 using corn stover fermentation effluent at 30 °C under 4500 lux.

organic acids and residual xylose consumption. Only acetic acid and levulinic acid were detected in the effluent after the lipid fermentation.

Fig. 2 indicates that the *R. sphaeroides* ZX-5 cell concentration grew rapidly in the first 72 h and then kept steady around 2.2 mg L^{-1}. The pH value was kept almost constant at 7 during the 250 h fermentation. Acetic acid was utilized gradually and more than 90% of the total acetic acid was consumed. Levulinic acid maintained at a low concentration level (0.1–0.4 g L^{-1}) and did not change significantly. Xylose was the residue from the lipid fermentation and consumed steadily. The total H_2 evolved per mL effluent increased steadily with the consumption of acetic acid and xylose, and the final value was nearly 7 mL H_2 mL^{-1} of the fermentation effluent.

Proper pH is also crucial to enhance hydrogen production, due to the effects of pH on hydrogenase activity or metabolic pathways [22,23]. Fig. 3 illustrates that the initial pH affected the hydrogen production significantly during the photo-fermentation of *R. sphaeroides* ZX-5 using the corn stover fermentation effluent. In the experimental condition, the hydrogen production started at the initial pH of 6.5, and the hydrogen accumulation increased with pH value from 6.5 to 7.0, and then decreased significantly when the initial pH was 8.0. The results again indicate the importance of the initial pH and the pH value control in the photo-fermentation with *R. sphaeroides* ZX-5 using the lignocellulose-derived organic acids in the fermentation effluent.

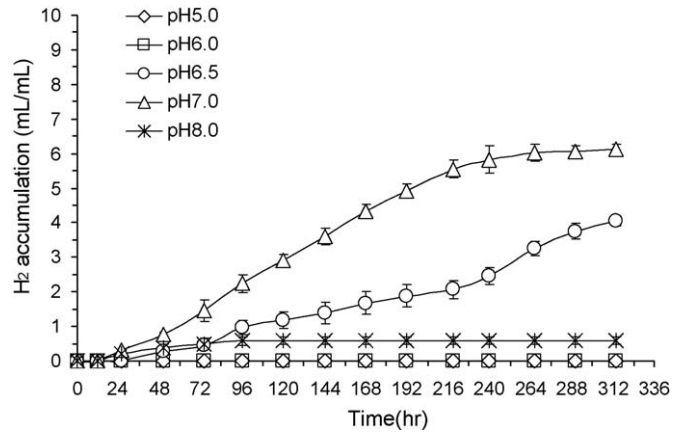

Fig. 3. Effect of initial pH on photo-fermentation of *R. sphaeroides* ZX-5 using corn stover fermentation effluent at 30 °C under 4500 lux.

The synergism among acetic acid, formic acid, and levulinic acid was observed in the pure organic acid mixture. Although levulinic acid, an important derivative from the lignocellulose degradation, could not be used as the substrate for hydrogen production, it facilitated the cell growth of *R. sphaeroides* ZX-5 and hydrogen production indirectly. However, it was found that the concentration of levulinic acid in the corn stover fermentation effluent was almost constant during the photo-fermentation with *R. sphaeroide* ZX-5. The reason could be the xylose existence in the effluent and xylose was priority to levulinic acid as carbon source for the cell growth of *R. sphaeroides* ZX-5.

Ren et al. [24] summarized the organic acid conversion in the photo-fermentation was mainly among 20–78% for photosynthetic bacteria when acetic acid was sole carbon source. In this study, the conversion of acetic acid was 30% when it was used as the sole carbon source, and the conversion of acetic and formic acid to hydrogen in the organic acid mixture of was 49%. In the corn stover fermentation effluent, the only observed organic acid consumed was acetic acid. The conversion of acetic acid to hydrogen was approximately 104% based on the hydrogen accumulation value (twofolds greater than that using pure organic acid mixture). The hydrogen accumulation using corn stover fermentation effluent under the proper initial pH value was more than doubled than the maximum hydrogen accumulation using the pure organic acid mixture under the similar organic acid concentrations (Table 2, Figs. 2 and 3). The results may indicate that substrate(s) in the corn stover fermentation effluent besides the organic acids were used for hydrogen production. Among the possible substrates, xylose in the effluent was consumed during the photo-fermentation and the consumed xylose could be utilized for hydrogen production, totally or partially. Sugars such as glucose, fructose, and sucrose have been found to be fermented by photosynthetic bacterium to hydrogen, but xylose has not been reported for hydrogen production with photosynthetic bacterium [25,26]. The further study on the xylose for hydrogen production is still under investigation. Moreover, the synergism between the organic acids and the residue sugars (xylose, arabinose, and other pentose sugars from the hemicellulose degradation), or between the organic acids and other lignocellulose degradation products such as lignin derived phenol derivatives may also contribute to the high hydrogen yield and productivity when the corn stover fermentation effluent was used as carbon source.

The present study provided a possible way to utilize lignocellulose-derived organic acids for hydrogen production, as well as a way to treat fermentation wastewater in biofuel production using lignocellulose.

Acknowledgements

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