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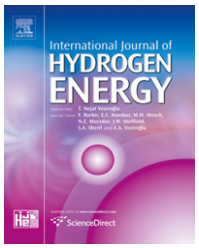

Technical Communication

Control strategies for hydrogen production through co-culture of *Ethanoligenens harbinense* B49 and immobilized *Rhodopseudomonas faecalis* RLD-53

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ABSTRACT

This study evaluated hydrogen production by co-culture of *Ethanoligenens harbinense* B49 and immobilized *Rhodopseudomonas faecalis* RLD-53 with different control strategies. To enhance cooperation of dark and photo-fermentation bacteria during hydrogen production process, the glucose concentration, phosphate buffer concentration and initial pH were controlled at 6 g/l, 50 mmol/l and 7.5, respectively. The maximum yield and rate of hydrogen production were 3.10 mol H_2/mol glucose and 17.2 mmol H_2/l/h, respectively. Ethanol from *E. harbinense* B49 in acetate medium can enhance hydrogen production by *R. faecalis* RLD-53 except the ratio of ethanol to acetate ($R_{E/A}$) among 0.8 to 1.0. Control of the proper phosphate buffer concentration (50 mmol/l) not only increased acetic acid production by *E. harbinense* B49, but also maintained stable pH of co-culture system. Therefore, the results showed that co-culture of *E. harbinense* B49 and immobilized *R. faecalis* RLD-53 was a promising way of converting glucose into hydrogen.

1. Introduction

Hydrogen has been attracting much attention as an ideal, clean and renewable energy carrier for the future. Biological processes of hydrogen production are found to be the most environmentally friendly processes of hydrogen production [1,2], and dark and photo fermentation of organic waste are considered as the most promising processes for biological hydrogen production [3]. Most dark-fermentative bacteria (*Clostridium* and *Ethanoligenens*) converted organic wastes to hydrogen at high production rate [4,5], but the hydrogen yield was inhibited by some end-products [6] such as acetic, butyric acid and ethanol etc. Photo-fermentative bacteria such as *Rhodobacter spheroides* [7] and *Rhodopseudomonas faecalis* [8], converted short chain organic acids into hydrogen with the aid of solar energy at high theoretical conversion yields, but the hydrogen production rate was limited due to low light conversion efficiency [9]. Therefore, combining different bacteria with complementary capabilities may overcome individual weaknesses and play to their strengths. At present, the combining processes included sequential two-stage and co-culture of dark and photo-fermentation.

Currently, researchers mainly focus on sequential two-stage process of dark and photo fermentation, because it can

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achieve high hydrogen yields when two-stage processes run under the respective optimal conditions [10–12]. However, the effluents from dark fermentation had to be centrifuged, adjusted to pH 7.0, diluted, and even re-flushed with argon and re-sterilized before photo-fermentation [10–12]. This greatly increased the operating costs. In addition, sequential two-stage dark and photo fermentation was uneconomical because of more reactors used and supplement space occupied [13].

In the co-culture fermentation system, organic acid produced by dark-fermentation was in-situ converted into hydrogen by photo-fermentation. This is also a way of alleviating the end-product inhibition of dark-fermentation. Therefore, higher hydrogen yields were reported by co-culture fermentation with dark and photo-fermentative bacteria compared with the dark-fermentation system alone [14–16]. However, the imbalance between the organic acid production and consumption rates, potential accumulation of organic acids and decrease of pH in the media are the major barriers for stable operation in the co-culture fermentation processes. In order to put the co-culture fermentative hydrogen production process into practice, the control strategies of co-culture fermentation should be investigated.

Ethanol-type fermentation was optimal among three fermentation types [17,18], and *Ethanoligenens harbinense* is the typical strains of H_2-ethanol coproducing fermentation [5]. Acetic acid, one of the major metabolites of ethanol-type fermentation, could be further converted into hydrogen by *R. faecalis* RLD-53 [19], which was isolated from freshwater pond sludge and had excellent ability for hydrogen production. In this work, the different control strategies of hydrogen production from glucose by co-culture of *E. harbinense* B49 and *R. faecalis* RLD-53 were investigated.

2. Material and methods

2.1. Bacteria and culture media

A fermentative hydrogen-producing bacterium, *E. harbinense* B49 [20] maintained in Research Center of Environmental Biotechnology at Harbin Institute of Technology, was used in this study. The medium used for H_2 production contained glucose as the sole carbon source. The composition of medium used for H_2 fermentation was according to Liu et al. [21]. A photosynthetic hydrogen-producing bacterium, *R. faecalis* RLD-53 [19], was isolated previously from freshwater pond sludge. The medium for photosynthetic bacterium was the same as reported earlier [19].

The medium (per liter, pH 7.0) for hydrogen production by co-culture was composed of 6 g glucose, 1.0 g sodium glutamate, 2 g yeast extract, 8.7 g K_2HPO_4, 6.8 g KH_2PO_4, 0.2 g $MgCl_2.6H_2O$, 0.1 g $CaCl_2$, 0.012 g $FeSO_4.7H_2O$, 0.1 g NaCl, 0.1 g EDTA–Na, 0.5 g L-cysteine.HCl.H_2O, 1 ml trace mineral solution, as well as 1 ml vitamin solution.

2.2. Cell immobilization of photo-fermentation bacteria

The cell immobilization was prepared as previously described [15]. The culture solution of *R. faecalis* RLD-53 was collected after its incubation by centrifugation at 5000 rpm. Agar dissolved in 20 ml of sterile deionized water at 0.4% (w/v), and was incubated at 100 °C. This solution was cooled to 45–50 °C and mixed together with 20 ml *R. faecalis* RLD-53 suspension prepared. The agar-culture solution was immediately drawn into a 50-ml syringe manually and until solidified. The cells were immobilized in agar gel, and then the agar gel containing bacterial cells was injected into the medium through a syringe. The diameter of the granule was about 3 mm and each granule contained 0.116 mg bacterial biomass on average.

2.3. Hydrogen production from glucose by co-culture

The hydrogen production experiments were conducted in triplicate with 80 ml of the medium in 100 ml serum bottles, which were sealed by rubber stopper and flushed with argon gas. The bottles and medium were sterilized at 121 °C and steam pressure of 1.05 kg/cm^2 for 15 min. *E. harbinense* B49 in the mid-exponential growth phase were inoculated into serum bottles. Subsequently, the immobilized *R. faecalis* RLD-53 were injected into the serum bottles. The bottles were shaken with 120 rpm at 35 °C in the light intensity of 4000 lx using incandescent lamps (60 W).

2.4. Analytical method

The composition in biogas was analyzed by gas chromatograph (GC) (Model SC-II, Shanghai Analysis Instrument Factory) equipped with a thermal conductivity detector and a 2-m stainless column packed with 5 Å molecular sieve. The operational temperatures at the injection port, the column oven and detector were 100, 60 and 105 °C, respectively. Argon was used as the carrier gas at a flow rate of 70 ml/min. The photosynthetic bacteria cell growth was measured in terms of OD at 660 nm by an Amersham Pharmacia Biotech ultraspec 34300 UV/Vis spectrophotometer.

Glucose concentration in the culture broth was determined by the Glucose HK kit (Sigma). Volatile fatty acids and ethanol in the supernatant of culture broth were determined by using a second GC (Model GC122, Shanghai Analysis Instrument Factory) equipped with a flame ionization detector and a 30 m × 0.25 mm × 0.25 mm fused-silica capillary column. The liquor samples were firstly centrifuged at 12,000 rpm for 5 min, and then acidified with hydrochloric acid and filtered through a 0.2-μm membrane before free acids were analyzed. The operational temperatures of the injection port, the column and the detector were 220, 190 and 220 °C, respectively. Nitrogen was used as carrier gas at flow rate of 50 ml/min. The light intensity was measured by using a digital luxmeter (TES1330A, Junkai Co.).

3. Results and discussion

3.1. Effect of ethanol concentration on photo- fermentation

The main characteristic of ethanol-type fermentation by *E. harbinense* B49 was simultaneous production of acetic acid and ethanol [22]. So, in the present studies, individual and mixed

acetate and ethanol were used as the carbon source (Table 1). Acetate was consumed completely in the all tests. This indicated the efficient utilization of acetate by *R. faecalis* RLD-53. While it appeared that acetate was readily utilized, ethanol was consumed at much slower rate, especially at the high initial concentration (40 mmol/l). There was no hydrogen detected, when ethanol was used as the sole carbon source. Comparison of the data in Table 1 suggested that the conversion rate of acetate and ethanol followed the order acetate > ethanol. The ethanol conversion varied with the ratio of ethanol to acetate ($R_{E/A}$). The maximum ethanol conversion of 34.0–34.1% was obtained at the $R_{E/A}$ of 0.8–1.0, and then it gradually decreased with increasing $R_{E/A}$. These results revealed that the change of $R_{E/A}$ may cause the change of ethanol metabolism in acetate medium by *R. faecalis* RLD-53.

The hydrogen yields and cumulative hydrogen volume were influenced by $R_{E/A}$ in the batch tests (Table 1). The hydrogen yield and cumulative hydrogen volume increased with the $R_{E/A}$ (0–0.8), and then decreased with the $R_{E/A}$ (0.8–1.0). The minimum value of 2.19 mol H_2/mol acetate was obtained at the $R_{E/A}$ 1.0. Finally, hydrogen yield and cumulative hydrogen volume increased again with the $R_{E/A}$ over the range of 1.0–1.6. These results indicated that the direct inhibition of ethanol concentration against H_2 production appear to be minimal. When the $R_{E/A}$ was 0.8–1.0, maximum ethanol conversion was 34–34.1%. However, hydrogen yield reached the minimum value (2.19 mol H_2/mol acetate). This result might suggest that while the $R_{E/A}$ in 0.8–1.0 was favorable for ethanol conversion, it was detrimental to H_2 production. Some previous studies have shown that the main effects of ethanol came from ethanol metabolism rather than ethanol per se [23], which may cause NAD/NADH imbalances, acetaldehyde accumulation, and the deactivation of replicative processes. In addition, ethanol has been shown to alter the movement of protons into and out of the plasma membrane [24], and protons transport may regulate molecular hydrogen production in the photosynthetic bacteria [25].

Products from ethanol-type fermentation have been consumed by photo fermentation. A promising way for high yield H_2 production from sole carbon source is using different control strategies to produce favorable substrates for hydrogen production by photo fermentation bacteria and maintain stable conditions in co-culture system. In this study, culture conditions should be controlled to increase production of acetate, which is suitable for hydrogen production by *R. faecalis* RLD-53. The ethanol enhanced hydrogen production by *R. faecalis* RLD-53 from acetate except that the $R_{E/A}$ was from 0.8 to 10. Therefore, in order to favor hydrogen production by strain RLD-53, the $R_{E/A}$ should be kept out of 0.8–1.0 in the co-culture system.

Table 1 – Effect of ethanol concentration on photo-H_2 production.

Initial substrate (mmol/l)		$R_{E/A}$	Substrate conversion (%)		Cumulative H_2 volume (ml/l)	H_2 yield (mol H_2/mol acetate)
EtOH	HAc		EtOH	HAc		
25	0	–	26.7	0	0	0
0	25	0	0	100	1371 ± 25	2.45
5	25	0.2	30.1	100	1443 ± 28	2.58
10	25	0.4	31.6	100	1517 ± 27	2.71
15	25	0.6	32.6	100	1593 ± 36	2.84
20	25	0.8	34.0	100	1297 ± 34	2.32
25	25	1	34.1	100	1224 ± 29	2.19
30	25	1.2	30.8	100	1505 ± 34	2.69
40	25	1.6	24.8	100	1532 ± 24	2.74

3.2. *Effect of glucose concentration on hydrogen production by co-culture*

In the mono-cultured test with B49, the hydrogen yield increased with glucose concentration from 3 to 9 g/l, and then decreased when initial glucose concentration further increased up to 15 g/l (Fig. 1a). The maximum hydrogen yield of 1.83 mol H_2/mol glucose was obtained at glucose concentration 9 g/l. Due to accumulation of acid end products, the end pH decreased with the glucose concentration from 4.83 to 3.93 (Fig. 1b). The $R_{E/A}$, on the contrary, increased with the glucose concentration. These results suggested that the percent of acetic acid in the soluble metabolites decrease with the decrease of end pH, which is conducive to ethanol fermentation [26]. However, this is harmful to hydrogen production by *R. faecalis* RLD-53 from acetic acid. Therefore, further strategies should be adopted to improve the percent of acetic acid in the soluble metabolites.

In the co-culture test of strain B49 and RLD-53, the hydrogen yield increased with increasing glucose concentration from 3 to 6 g/l, and then decreased when initial glucose concentration further increased up to 15 g/l (Fig. 1a). The hydrogen yield reached a maximum value of 2.48 mol H_2/mol glucose at 6 g/l glucose. At 6 g/l glucose, the co-culture of B49 and RLD-53 exhibited 1.5 times hydrogen yields compared with the mono-culture of B49. It has been reported that in the dark-fermentation hydrogen yield gradually decreased with increasing initial glucose concentration [27,28], and the optimum value was found to be 1.0% [28]. Initial glucose concentration of 1% was also favorable to hydrogen production by *E. harbinense* B49 [20]. However, the optimal initial glucose concentration was 0.6% (6 g/l) for co-culture process of dark and photo bacteria in this study. These results suggested that control of the appropriate glucose concentration is an effective strategy to increase hydrogen production capacity of co-culture system.

In the co-culture system, acetic acid from B49 was utilized by RLD-53, which led to the end pH was higher than that in the mono-culture of B49 (Fig. 1b). However, the end pH of 4.75 was unfavorable for photo-hydrogen production by RLD-53 at 6 g/l glucose [19]. Therefore, further strategies should be used to control the pH of co-culture system.

3.3. *Effect of phosphate buffer concentration on mono-culture of* E. harbinense *B49*

E. harbinense B49 produced acetic acid and ethanol via ethanol fermentation from glucose. As a result, the pH of the system decreased to a lower level, about 4.0–4.5. The phosphate buffer of different concentrations was used to control pH of the

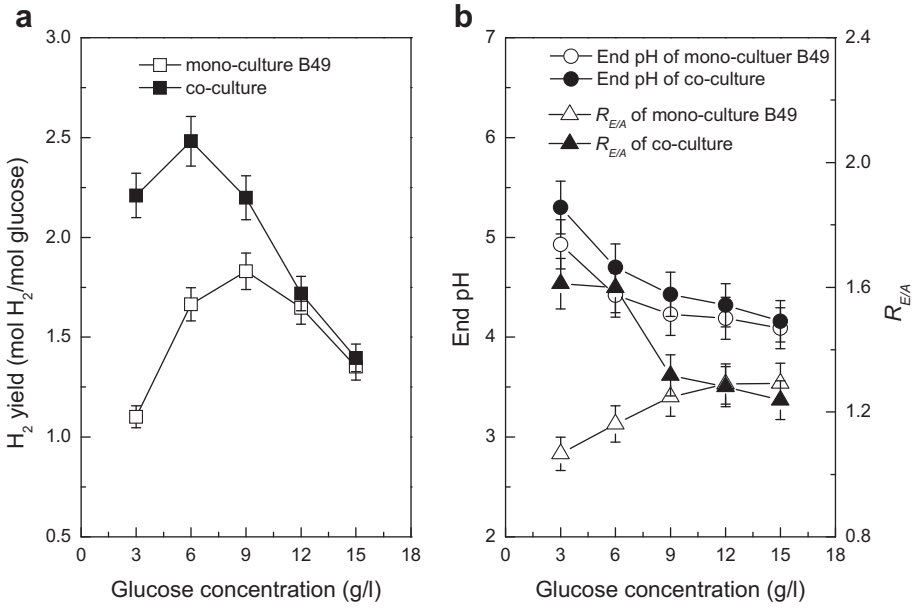

Fig. 1 – The effect of glucose concentration on co-culture hydrogen production (a.□, H_2 yield of mono-culture of B49; ■, H_2 yield of co-culture of B49 and RLD-53; b.○, End pH of mono-culture of B49; ●, End pH of co-culture of B49 and RLD-53; △, $R_{E/A}$ of mono-culture of B49; ▲, $R_{E/A}$ of co-culture of B49 and RLD-53).

fermentation system. In this study, 6 g/l glucose was used as the sole carbon source. The yield of hydrogen and composition of fatty acids produced by strain B49 were compared at different concentrations of phosphate buffer 10-50 mmol/l (Table 2).

The cumulative hydrogen volume and hydrogen yield were obviously influenced by phosphate buffer concentration in batch tests of mono-culture of *E. harbinense* B49 (Table 2). The maximum hydrogen yield of 1.95 mol H_2/mol glucose was obtained at phosphate buffer concentration of 20 mmol/l, and then it gradually decreased with the increase of phosphate concentration. A proper phosphate buffer concentration can enhance hydrogen production [29,30].

The end pH value (4.10–6.05) responsively increased with increasing concentration of phosphate buffer. The pH of system remained more stable with increasing phosphate concentration because of the buffering effect of phosphate [31]. It has been reported that rapid pH depletion resulted in a shift of metabolite production pathways [32,33]. Among these metabolites, acetate was most efficient substrate for photo-hydrogen production of *R. faecalis* RLD-53 [19]. The data in the Table 2 showed that the acetate concentration compared with ethanol concentration in soluble metabolites increased with phosphate buffer concentration over the range of 10–50 mmol/l. The $R_{E/A}$ was 0.63 at the phosphate buffer concentration of 50 mmol/l and acetate was the major metabolite under high phosphate buffer concentration. This was beneficial to hydrogen production by *R. faecalis* RLD-53. These results implied that the phosphate buffer concentration affected pH of the system, and then the pH could cause a metabolic shift of biochemical reactions of *E. harbinense* B49. The changes of metabolites by pH buffering capacity provided useful strategy for regulation co-culture system and optimization of hydrogen production.

3.4. *Effect of phosphate buffer concentration on co-culture of* E. harbinense *B49 and* R. faecalis *RLD-53*

In the co-culture test with B49 and RLD-53, hydrogen yield increased with the phosphate buffer concentration over the

Table 2 – Effect of phosphate buffer concentration on H_2 production, H_2 yield, end pH and soluble metabolites of *E. harbinense* B49.

Phosphate buffer (mmol/l)	Soluble metabolites (mmol/l)		$R_{E/A}$	Cumulative H_2 volume (ml/l)	H_2 yield (mol H_2/mol glucose)	End pH
	HAc	EtOH				
10	22.43	26.35	1.17	1287 ± 38	1.72	4.10
20	23.25	25.87	1.11	1450 ± 55	1.94	4.32
30	25.6	24.12	0.94	1338 ± 42	1.79	5.12
40	26.23	21.03	0.80	1300 ± 46	1.74	5.57
50	30.72	19.36	0.63	1125 ± 40	1.51	6.05

Table 3 – Effect of phosphate buffer concentration on H_2 production by co-culture of *E. harbinense* B49 and *R. faecalis* RLD-53.

Phosphate buffer (mmol/l)	Residual metabolites (mmol/l)		Cumulative H_2 volume (ml/l)	H_2 yield (mol H_2/mol glucose)	End pH
	HAc	EtOH			
10	2.15	25.1	1514 ± 60	2.03	4.78
20	1.36	24.86	1678 ± 56	2.25	4.82
30	2.75	22.34	1798 ± 65	2.41	5.25
40	1.64	19.52	1977 ± 80	2.65	5.63
50	2.58	17.16	2073 ± 74	2.78	6.16

range of 10–50 mmol/l (Table 3). The hydrogen yield of *E. harbinense* B49 was lower in a high phosphate buffer concentration, but B49 produced more acetate (Table 2). Acetate was subsequently converted into hydrogen by RLD-53, so hydrogen yield was higher than that obtained at low phosphate buffer concentration.

The end pH of co-culture system increased with phosphate buffer concentration. At phosphate buffer concentration of 50 mmol/l, the end pH of about 6.16 approximated to the optimum pH for photo-hydrogen production by *R. faecalis* RLD-53 [19]. Therefore, hydrogen yield of co-culture reached the maximum value of 2.78 mol H_2/mol glucose at phosphate buffer of 50 mmol/l. Compared with the mono-culture of B49, hydrogen yield of the co-culture system increased by 43.3%. For these reasons, the high phosphate buffer concentration in this study seems to favor hydrogen production by co-culture. These results indicated that control phosphate concentration at high level effectively enhanced hydrogen production performance of co-culture system.

3.5. *Effect of initial pH on hydrogen production by co-culture*

The optimum pH for photo-hydrogen production by *R. faecalis* RLD-53 was 6.5–7.5 [19]. The end pH of co-culture system 6.16 (Table 3) was unfavorable to photo-hydrogen production. In order to determine the optimum initial pH of the medium for hydrogen production by co-culture, different initial pH of 7.0, 7.5, 8.0, 8.5 and 9.0 were set at the fixed glucose of 6 g/l and phosphate buffer of 50 mmol/l.

The end pH increased with increasing initial pH (Fig. 2). The end pH was 6.52 at initial pH 7.5, which was favorable to growth and hydrogen production of *R. faecalis* RLD-53, versus 5.93 at initial pH 7.0. The low end pH of 5.93 and high initial pH 8.0–9.0 were harmful to both bacteria. The pH was found to be one of the most important parameters for hydrogen production by dark-fermentation processes [17,32]. The initial pH also had significant effects on both hydrogen yield and hydrogen production rate in the co-culture system. As shown in Fig. 3, significant increase in the cumulative hydrogen volume could be achieved using initial pH of 7.5. Further increase in initial pH to 8.0 and 9.0 resulted in a significant decrease of hydrogen production. These differences in the hydrogen yield may be due to low end pH and high initial pH value, which were unfavorable to the cooperation of the both bacteria. At the initial pH 7.5, the hydrogen yield (3.10 mol H_2/mol glucose) was higher than that at other initial pH, and hydrogen production rate reached maximum value of 17.18 ml H_2/l/h between 48 and 72 h. The results indicate that the optimal pH for co-culture hydrogen production with the phosphate-buffered medium appeared to be 7.5. This pH is higher than the reported optimal pH for hydrogen production in ethanol-type fermentation systems, where pH value of 4.0–4.5 was preferable [34]. The cooperation for hydrogen

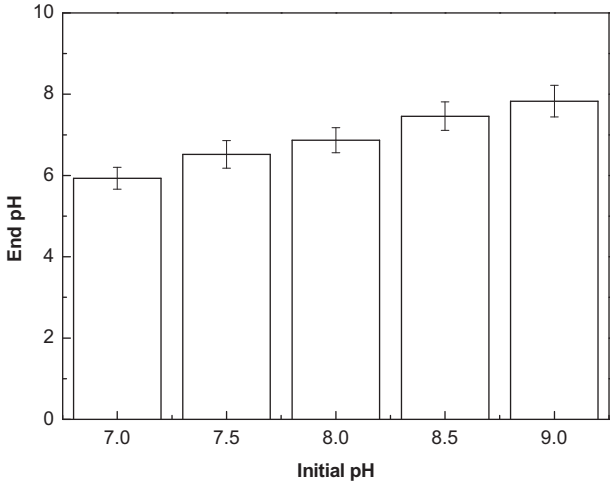

Fig. 2 – The effect of initial pH on the end pH of the co-culture system at 6 g/l glucose and 50 mmol/l phosphate buffer.

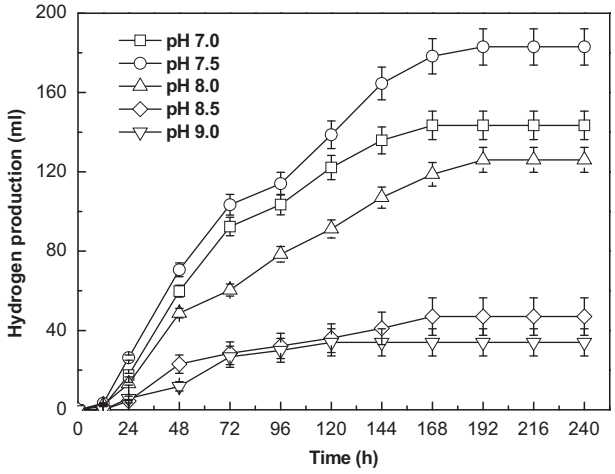

Fig. 3 – The effect of initial pH on hydrogen production of co-culture system at 6 g/l glucose and 50 mmol/l phosphate buffer (□, pH 7.0; ○, pH 7.5; △, pH 8.0; ◇, pH 8.5; ▽, pH 9.0).

production of *E. harbinense* B49 and *R. faecalis* RLD-53 was enhanced when a proper initial pH was controlled. Compared with the mono-culture of *E. harbinense* B49, the hydrogen production by co-culture is more sensitive to initial pH.

4. Conclusions

In this study, the different control strategies on hydrogen production by co-culture of *E. harbinense* B49 and immobilized RLD-53 were evaluated. Ethanol from *E. harbinense* B49 was utilized by *R. faecalis* RLD-53 for cell growth rather than hydrogen production. Ethanol can improve hydrogen production by *R. faecalis* RLD-53 from acetate except that $R_{E/A}$ was 0.8–1.0. Control of the glucose concentration at 6 g/l can effectively increase hydrogen production capacity of co-culture system. The $R_{E/A}$ (0.85–1.59) and end pH value (4.1–6.05) increased with phosphate buffer concentration over the range of 10–50 mmol/l. Control of the appropriate phosphate buffer concentration (50 mmol/l) not only increased acetic acid production by *E. harbinense* B49, but also maintained stable pH of co-culture system. Phosphate buffer concentration played an important role in the co-colure of dark and photo fermentative bacteria. The optimal pH for co-culture hydrogen production was 7.5. Proper initial pH enhanced cooperation for hydrogen production of *E. harbinense* B49 and *R. faecalis* RLD-53.

The results indicated that 3.10 mol H_2/mol glucose was produced by co-culture, when glucose of 6 g/l, phosphate buffer of 50 mmol/l and initial pH of 7.5 were controlled in the medium. The control strategies effectively enhanced cooperation of *E. harbinense* B49 and *R. faecalis* RLD-53. Co-culture of *E. harbinense* B49 and *R. faecalis* RLD-53 is a promising way of converting glucose into hydrogen and expected to close theoretical maximum value of 12 mol H_2/mol glucose.

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