Bioresource Technology 101 (2010) 9605–9611

Contents lists available at ScienceDirect

Bioresource Technology

journal homepage: www.elsevier.com/locate/biortech

Enhanced H_2 gas production from bagasse using *adhE* inactivated *Klebsiella oxytoca* HP1 by sequential dark-photo fermentations

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ARTICLE INFO

Article history:
Received 1 April 2010
Received in revised form 20 July 2010
Accepted 21 July 2010
Available online 29 July 2010

Keywords:
Hydrogen production
Dark-fermentation
Photo fermentation
Acetaldehyde dehydrogenase
Bagasse

ABSTRACT

Sequential dark-photo fermentations (SDPF) was used for hydrogen production from bagasse, an acetaldehyde dehydrogenase (*adhE*) gene inactivated *Klebsiella oxytoca* HP1 (Δ*adhE* HP1) mutant was used to reduce the alcohol content in dark fermentation (DF) broths and to further enhance the hydrogen yield during the photo fermentation (PF) stage. Compared with that of the wild strain, the ethanol concentration in DF broths of Δ*adhE* HP1 decreased 69.4%, which resulted in a hydrogen yield in the PF stage and the total hydrogen yield over the two steps increased by 54.7% and 23.5%, respectively. The culture conditions for hydrogen production from acid pretreated bagasse by SDPF were optimized as culture temperature 37.5 °C, initial pH 7.0, and cellulase loading 20 FPA/g in the DF stage, with initial pH 6.5, temperature 30 °C and photo intensity 5000 lux in the PF stage. Under optimum conditions, by using Δ*adhE* HP1 and wild type strain, the H_2 yields were 107.8 ± 5.3 mL H_2/g-bagasse, 96.2 ± 4.4 mL H_2/g-bagasse in DF and 54.3 ± 2.2 mL H_2/g-bagasse, 35.1 ± 2.0 mL H_2/g-bagasse in PF, respectively. The special hydrogen production rate (SHPR) were 5.51 ± 0.34 mL H_2/g-bagasse h, 4.95 ± 0.22 mL H_2/g-bagasse h in DF and 0.93 ± 0.12 mL H_2/g-bagasse h, 0.59 ± 0.07 mL H_2/g-bagasse h in PF, respectively. The total hydrogen yield from bagasse over two steps was 162.1 ± 7.5 mL H_2/g-bagasse by using Δ*adhE* HP1, which was 50.4% higher than that from dark fermentation only. These results indicate that reducing ethanol content during dark fermentation by using an *adhE* inactivated strain can significantly enhance hydrogen production from bagasse in the SDPF system. This work also proved that SDPF was an effective way to improve hydrogen production from bagasse.

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1. Introduction

Hydrogen is recognized as a promising alternative to fossil fuel because it is clean, has high energy and is renewable (Das and Verziroglu, 2001; Hallenbeck and Benemann, 2002; Chen et al., 2008; Gadhamshetty et al., 2008). Among methods for hydrogen production, biological fermentation by microorganisms to convert organic substrate into hydrogen is becoming increasingly popular (Kapdan and Kargi, 2006; Lee et al., 2007; Lata et al., 2007; Tao et al., 2008) because it is environmentally friendly and able to utilize organic waste to eliminate pollution.

Recent researches indicate that Sequential dark-photo fermentations (SDPF) can effectively enhance the biological hydrogen production yield from biomass (Chen et al., 2008; Su et al., 2009; Argun and Kargi, 2010). In SDPF systems, the organic acids such as acetate and lactic acid synthesized in dark fermentation (DF) can be further utilized to produce hydrogen via photo fermentation (PF) by photosynthetic bacteria in the presence of light (Das and Verziroglu, 2001; Oh et al., 2004; Lee et al., 2007). In contrast to organic acids, substantial ethanol in DF broth produced by fermentative anaerobic bacteria such as *Escherichia coli* and *Klebsiella oxytoca* HP1 cannot be assimilated by photosynthetic bacteria during the photo fermentation stage. Therefore, reducing the ethanol content in the dark fermentation effluent (DFE) is expected to improve hydrogen production during the photo fermentation stage in SDPF systems.

Sugarcane bagasse is a kind of waste in sugar refinery which was used as the replacer of coal in the specialized boilers. The abundant cellulose and hemicellulose in bagasse could be hydrolyzed into sugar and fermented into biofuel such as H_2 and ethanol (Singh et al., 2007). Recently, various cellulosic biomass is being used for hydrogen production by microorganisms fermentation (Lay, 2001; Fan et al., 2006a,b; Li and Chen, 2007; Zhang et al., 2007; Liu et al., 2008; Lo et al., 2008).

In this research, the hydrogen production from bagasse by SDPF was carried out using *K. oxytoca* HP1 and *Rhodopseudomonas palustris* CN as the hydrogen producer during DF and PF stage, respectively. Various factors affecting hydrogen production in SDPF system have been investigated. To enhance the hydrogen production

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doi:10.1016/j.biortech.2010.07.095

Nomenclature

Δ*adhE* HP1	acetaldehyde dehydrogenase gene (*adhE*) inactivated *Klebsiella oxytoca* HP1
DF	dark fermentation
DFE	dark fermentation effluents
DFEΔA	dark fermentation effluents of Δ*adhE* HP1
DFEK	dark fermentation effluents of *K. oxytoca* HP1
FID	flame ionization detector
FPA	filter paper activity 1 FPA cellulase can hydrolyze filtrate paper to produce 1 μmol glucose in 1 min
HPE	the hydrogen production efficiency
PF	photo fermentation
SDPF	sequential dark-photo fermentations
SHPR	special hydrogen production rate
SSF	simultaneous saccharification and fermentation (SSF)
TVS	total volatile solid

during the PF stage in a SDPF system, the acetaldehyde dehydrogenase gene (*adhE*) inactivated mutant of *K. oxytoca* HP1 (Δ*adhE* HP1) was constructed.

2. Methods

2.1. Strain and culture condition

K. oxytoca HP1 was isolated from a hot spring, as reported in our previous work (Long et al., 2005). *K. oxytoca* HP1 belongs to *Enterobacteriaceae*, its hydrogen metabolism pathway may be similar with that of *Enterobacter aerogenes*, *E. coli* and *Enterobacter cloacae*. The construction of the *adhE* inactivated *Klebsiella* HP1 mutant (Δ*adhE* HP1) was described in our previous work (Zhu et al., 2007). The growth medium and culture conditions for *K. oxytoca* HP1 and Δ*adhE* HP1 were described by Long et al. (2005).

Photosynthetic bacteria *R. palustris* CN, identified through morphometric examination and 16S rDNA sequence comparison (data not shown), was isolated from a pond sludge sample in Xiamen, Fujian, China. The growth medium of photosynthetic bacteria contained: NH_4Cl 0.53 g/L, KH_2PO_4 0.50 g/L, $Na_2HPO_4{\cdot}12H_2O$ 14.33 g/L, NaCl 2.0 g/L, $MgCl_2{\cdot}6H_2O$ 0.27 g/L, $CaCl_2$ 0.05 g/L, Na_2Ac 1.64 g/L, yeast extract 1.0 g/L, Fe-EDTA 2.0 mL and trace element solution 1.0 mL. The bacteria were grown at a light intensity 3000 lux and 30 °C.

2.2. Hydrogen production from bagasse during dark fermentation

The bagasse, derived from Zhangzhou sugar-refinery, Fujian province, China, was dried at 105 °C for 24 h and then ground to a 60 mesh powder before use. The composition of the bagasse was analyzed and shown in Table 1. The bagasse was hydrolyzed by two steps: acid-heat pretreatment and enzymatic hydrolysis. Firstly one gram of dried bagasse powder was suspended with 8 mL dilute H_2SO_4 (1% w/v) in a 140 mL serum bottle and pretreated at 121 °C for 1 h. In this process the complex crystal structure of bagasse fiber was destroyed, and about 90% hemicellulose and a small part of cellulose were degraded with a reducing sugar yield of 0.315 g/g-bagasse. Most of the cellulose in bagasse was not hydrolyzed (Table 1). To prepare the DF medium, peptone 5 g/L, $MgSO_4{\cdot}7H_2O$ 0.37 g/L, K_2SO_4 4.5 g/L, $Na_2HPO_4{\cdot}12H_2O$ 6. 6 g/L and $NaH_2PO_4{\cdot}2H_2O$ 20.5 g/L were added into the acid-heat pretreated bagasse suspension. The suspension was adjusted to pH 6.0 (unless stated otherwise) with KOH and sterilized at 121 °C for 20 min.

Then the cellulase from *Aspergillus glaucus* XC9 (Xu et al., 2006) was added into the suspension to hydrolyze the cellulose. *K. oxytoca* HP1 or Δ*adhE* HP1 cells (10^9 cells/0.5 mL) at logarithmic phase were inoculated for simultaneous saccharification and fermentation (SSF) (Li and Chen, 2007). The total volume of the suspensions was adjusted to 20 mL with sterilized water. The serum bottles were stopped with rubber stoppers and the air was removed under vacuum and replaced with argon. The cumulative hydrogen yield was monitored every 12 h from culture bottles shaken at 35 °C (unless stated otherwise) and 120 rpm in an air bath.

Table 1
Compositions of sugarcane bagasse.

Ingredients	Content (%)		
	Raw bagasse	Acid pretreated bagasse[a]	Bagasse remain after SSF[b]
Crude protein	3.8 ± 0.22	–	–
Uronic acids	3.3 ± 0.18	–	–
Cellulose	35.4 ± 2.42	45.3 ± 2.58	17.3 ± 1.21
Hemicellulose	20.6 ± 1.23	3.1 ± 0.18	3.6 ± 0.22
Starch	1.5 ± 0.08	–	–
Lignin	18.6 ± 1.02	28.1 ± 1.67	41.5 ± 1.87
Ash	8.3 ± 0.51	10.5 ± 0.52	13.2 ± 0.77
Soluble sugar	2.8 ± 0.16	–	–
Loss of dry weight	5.7 ± 0.32	–	–

[a] The dry weight of acid pretreated bagasse was 70.7% of raw bagasse.
[b] The dry weight of bagasse remain after SSF was 49.6% of raw bagasse. Each value is the mean of at least three replicates (±standard deviation).

The hydrogen production efficiency (HPE) in DF was calculated by the following formula; in this formula, all of the reducing sugars were treated as glucose.

$$\mathrm{HPE}(\%) = \frac{V_{\mathrm{Dh}}/22.4}{12M_{\mathrm{S}}/180} \times 100\%$$

HPE: the hydrogen production efficiency; V_{Dh}: the total hydrogen yield in DF (l); M_{S}: the amounts of reducing sugar comsumed in DF (g); 180: the mass of 1 M glucose (g/mol); 22.4: the gas volume (l) of 1 M hydrogen at standard condition; 12: the theoretical hydrogen yield (mol) from 1 mol glucose, refer to Eq. (a).

$$C_6H_{12}O_6 + 6H_2O = 12H_2 + 6CO_2 \quad \text{(a)}$$

2.3. Photo-hydrogen production from metabolites of dark fermentation

Photo-fermentative hydrogen production by *R palustris* CN from byproducts of the dark fermentation was carried out by the following program. First, the pH value of the broth was adjusted to 7.0 with 1 M KOH solution and then 1 mL of pre-cultured *R. palustris* CN (10^9 CUF/mL) was added. The serum bottles were stopped with rubber stoppers and the air was removed under vacuum and replaced with argon. Photo-fermentative hydrogen production was monitored every day from the culture bottles held at 30 °C under a 3000 lux light intensity measured with a photometer (TES-1332A, China).

The hydrogen production efficiency (HPE) in PF was calculated by the follow formula:

$$\mathrm{HPE}(\%) = \frac{V_{\mathrm{h}}/22.4}{6(M_{11} - M_{12}) + 4(M_{\mathrm{a1}} - M_{\mathrm{a2}})} \times 100\%$$

HPE: the hydrogen production efficiency; V_h: the total hydrogen yield produced in PF(l); M_{11}: the total lactate produced in DF(mol); M_{12}: the total remaining lactate in PFE (mol); M_{a1}: the total acetate produced in DF(mol); M_{a2}: the total remaining acetate in PFE (mol); 22.4: the gas volume (l) of 1 M hydrogen at standard condition; 6: the theory hydrogen yield (mol) from 1 mol lactate, refer to Eq. (b); 4: the theory hydrogen yield (mol) from 1 mol acetate, refer to Eq. (c).

$$C_3H_6O_3 + 3H_2O = 6H_2 + 3CO_2 \quad (b)$$

$$C_2H_4O_2 + 2H_2O = 4H_2 + 2CO_2 \quad (c)$$

2.4. Analytical methods

Bacterial cell density was estimated using a spectrophotometer at 600 nm. The components of bagasse were assayed according to the method described by Van (1998). Reducing sugars were estimated based on the method of Ghose (1987). Hydrogen accumulated in the gas phase was measured using a gas chromatograph (GC9790, Shanghai Analysis Instrument Company, China) with a thermal conductivity detector. The column (2 m × 2 mm) used for hydrogen quantification was filled with a 5 Å molecular sieve and nitrogen was used as a carrier gas. The column, injection and the detector temperatures were 100, 80 and 120 °C, respectively. Fifty microliters of the gas phase from the culture was injected directly into the gas chromatography column using a 100 μL syringe. The volume of the gas phase of the culture was measured at the same time (Long et al., 2005).

The organic acid and ethanol concentrations were also detected by GC using a flame ionization detector (FID) and a 2 m glass column packed with Unisole F-200 (30/60 mesh). The operation temperatures for the injection port, the oven and the FID were 200, 240 and 280 °C, respectively. The carrier gas was helium at a flow rate of 30 mL/min. The sample pH value was adjusted by 1 M HCl or 1 M NaOH solution to 3.0.

3. Results and discussion

3.1. Bagasse constituents

On a dry weight basis, cellulose, hemicellulose and lignin were 35.4 ± 2.42%, 20.6 ± 1.23% and 18.6 ± 1.02%, respectively, of the bagasse (Table 1). Additionally, there was 1.5 ± 0.08% starch and 2.8 ± 0.16% soluble sugars in the bagasse (Table 1). The identified constituents of bagasse were very similar to the results of previous studies (Kansoha et al., 1999; Singh et al., 2007). Cellulose and hemicellulose can be hydrolyzed to glucose and xylose under certain conditions, based on the composition of bagasse in this research (Table 1), the theoretical yield from 1 g of sugar cane bagasse being about 0.438 g glucose and 0.234 g xylose, slightly lower than 0.44 g glucose and 0.26 g xylose reported by Laser et al. (2002). These hydrolyzed sugars can further be used for alcohol or hydrogen fermentation to produce clean fuel. In addition to carbohydrate, some non-carbohydrate substances, such as 3.8 ± 0.22% crude protein, 3.3 ± 0.18% uronic acids and 8.3 ± 0.51% ash also existed in the bagasse. These non-carbohydrate ingredients can be used as nourishment for the growth of microorganisms.

In this study, the bagasse was hydrolyzed to produce reducing sugar by two steps: acid-heat pretreatment and enzymatic hydrolysis. About 90% hemicellulose, 100% starch and part of cellulose were degraded to yield reducing sugar about 0.315 g/g-bagasse, but the most of cellulose in bagasse not be hydrolyzed during acid-heat pretreatment.

Two methods were reported to enzymic hydrolysis cellulose: pre-enzymic hydrolysis and SSF (Li and Chen, 2007). In this research, the saccharification rates of cellulose in acid pretreated bagasse by pre-enzymic hydrolysis were investigated. After the addition of cellulase into acid-heat pretreated bagasse suspension to hydrolyze the cellulose, the total reducing sugar yield over two steps amount to about 0.413 g/g-bagasse (data not shown). During the two steps, about 62.5% cellulose was not hydrolyzed. The poor saccharification rate of cellulose in acid pretreated bagasse by pre-enzymic hydrolysis may caused by two facts, one is the feedback inhibition of high concentration reducing sugar to cellulase, the other is the negative effect of lignin in acid pretreated bagasse on cellulose saccharification. SSF can reduce the feedback inhibition of high concentration reducing sugar to cellulase activity, and improve the total saccharification rate of cellulose, so SSF was used in this research.

3.2. Hydrogen production from bagasse during dark fermentation

Many factors affect hydrogen production from bagasse during DF, e.g., the bagasse pretreatment method, the composition of medium, the methods of saccharification and fermentation, strain, culture conditions such as initial pH value, temperature, and enzyme loading, etc. In this research, bagasse was pretreated with dilute acid at a temperature of 121 °C and a pressure of 0.11 MPa. After the other medium content, cellulase and DF bacteria added into the acid-heat pretreated bagasse suspension, a SSF hydrogen production in DF was carried out. This research aimed to investigate the effect of different initial pH, culture temperature, enzyme loading and strain on hydrogen production by SSF during DF. Although the optimum initial pH, culture temperature for hydrolysis by cellulase from *A. glaucus* XC9 and for DF hydrogen production by *K. oxytoca* HP1 were known as pH 4.8, 50 °C (Xu et al., 2006) and pH 7.0, 35 °C (Long et al., 2005), respectively, but the optimum initial pH and culture temperature for SSF should be optimized.

To investigate the effect of initial pH on hydrogen production from bagasse by SSF in DF, acid pretreated bagasse suspension was used for biohydrogen production at different initial pH values from 5.0 to 8.0. The optimum initial pH values for hydrogen production by SSF in this research was 7.0 (Fig. 1A), as the initial pH value was low to 5.0, almost no hydrogen produced in SSF system (Fig. 1A). These results show that pH control could stimulate the microorganisms to produce hydrogen and would create a system with maximum hydrogen yield, but the hydrogen production activity would be inhibited by low or high pH values in overall hydrogen fermentation. As a large quantity of organic acid produced by *K. oxytoca* HP1 during SSF, it is important to prevent medium pH decreasing fastly by using a pH buffer during DF hydrogen production. In this study, a high concentration of Na_2HPO_4–NaH_2PO_4 (pH 7.0) buffer was used.

The effects of cellulase loading on hydrogen yield of *K. oxytoca* HP1 in SSF are presented in Fig. 1B. The cumulative hydrogen yield increased remarkably with the increase of cellulase loading, e.g., when the cellulase loading rose from 5 to 20 FPA/g, the cumulative hydrogen yield increased from 57.5 to 70.5 mL H_2/g-bagasse. Thereafter, the cumulative hydrogen yield did not increase as the cellulase loading increased, e.g., when the cellulase loading increased to 30 FPA/g, the hydrogen yield remained at 69.2 mL H_2/g-bagasse (Fig. 1B). The results show that a change in the cellulase loading obviously affected the hydrogen yield in the test, with an optimum cellulase loading for hydrogen production by SSF using *K. oxytoca* HP1 of 15 to 20 FPA/g. Excessive cellulase 277 loading did not enhance the hydrogen yield.

To investigate the effect of temperature on hydrogen production from bagasse by SSF during DF, biohydrogen productions from bagasse by SSF at different temperatures from 35 to 42.5 °C were carried out. The results (Fig. 1C) indicate that the optimum temperatures for hydrogen production from acid pretreated bagasse by

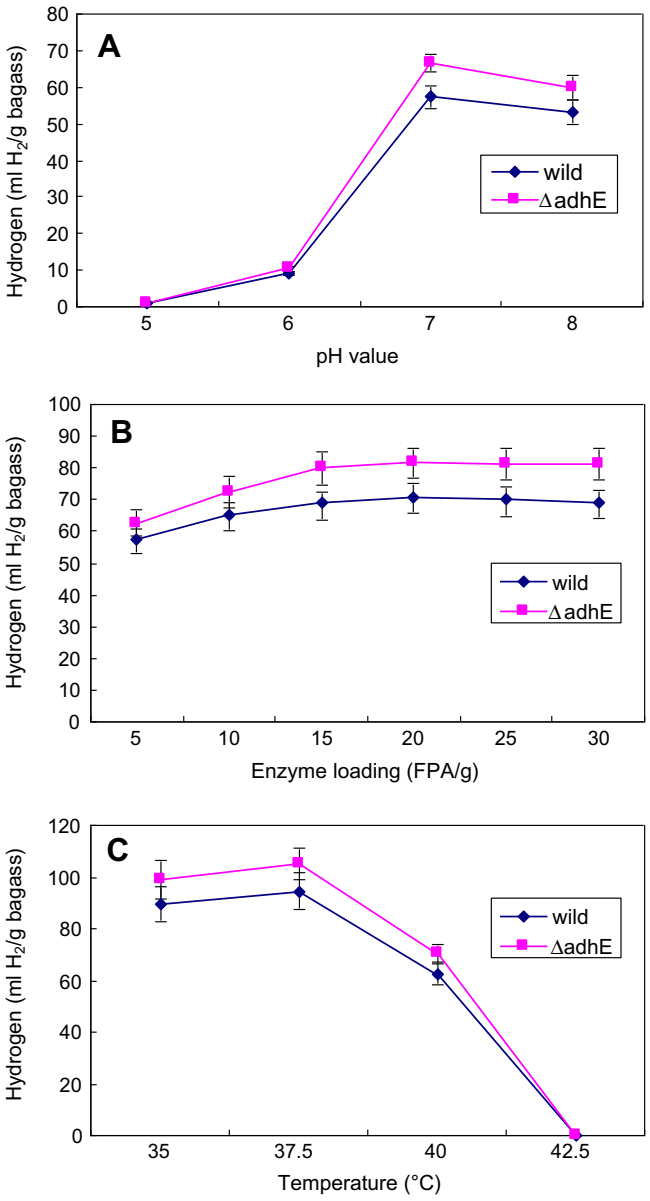

Fig. 1. Effect of pH (A), enzyme loading (B) and temperature (C) on dark fermentative hydrogen production.

SSF using both *K. oxytoca* HP1 and Δ*adhE* HP1 were 37.5 °C, when the culture temperature reached 42.5 °C, almost no hydrogen produced in SSF system. The optimum temperature for cellulose hydrolysis by cellulase from *A. glaucus* XC9 and for batch hydrogen production fermentation by *K. oxytoca* HP1 were know in our previous study as 50 (Xu et al., 2006) and 35 °C (Long et al., 2005), respectively, the results in this research show that cellulase activity was repressed in lower temperature and the hydrogen production activity of *K. oxytoca* HP1 were inhibited in higher temperature.

The optimum culture conditions for hydrogen production from acid pretreated bagasse by SSF were culture temperature 37.5 °C, initial pH 7.0 and cellulase loading 20 FPA/g. Under these optimum culture conditions, the cumulative hydrogen yield and special hydrogen production rate from acid pretreated bagasse by SSF using Δ*adhE* HP1 were 107.8 ± 5.3 mL H_2/g-bagasse and 5.51 ± 0.34 mL H_2/g-bagasse h (Table 2), respectively, which were 12.1% and 11.3% higher than that of the wild type strain, respectively. These results agree with our previous work (Zhu et al., 2007).

Hydrogen production from cellulosic biomass in DF had been reported in other literatures (Zhang et al., 2007; Lay, 2001; Fan et al., 2006a; Fan et al., 2006b; Li and Chen, 2007; Shin et al., 2007; Liu et al., 2008; Lo et al., 2008), the H_2 production performance varied from 1.09 mmol (24.4 mL) H_2/g-cellulose to 149.69 mL H_2/g-TVS were obtained (Table 3). Compared to the H_2 yields reported in literatures, a good H_2 production performance from bagasse in DF is being achieved in this work. Two improvements in this research may contribute to the good H_2 production performance. One is the high concentration of Na_2HPO_4–NaH_2PO_4 (pH 7.0) buffer used in medium, which can prevent medium pH decreasing fastly and keep a suitable pH for cellulose saccharification and hydrogen production in a long time. The other improvement was SSF be used in this research, comparison the components of bagasse remain after SSF with that of the raw bagasse, the total reducing sugar produced over the two steps of acid-heat hydrolysis and SSF can be calculated as 0.557 g/g-bagasse, which was 0.144 g/g-bagasse higher than that over the two steps of acid-heat hydrolysis and pre-enzymic hydrolysis. In fact, the H_2 yields by SSF were far higher than that by pre-enzymic hydrolysis and fermentation (data was not shown), but the results reported by Li and Chen (2007) show that the H_2 yields by SSF were not improved in comparison to that by pre-enzymic hydrolysis and fermentation. The culture conditions for SSF were optimized in this study, but not in Li and Chen's (2007) research, which may result in a different results in the two researches.

Ethanol, acetate and lactic acid were the main byproducts in DF by *K. oxytoca* HP1 (Table 2), which agree with the mixed-acid fermentation carried out by *Enterobacteriaceae* under anaerobic conditions using glycolytic carbon sources (Bagramyan et al., 2002). Under the optimum culture conditions, the concentration of ethanol in soluble metabolites of hydrogen production by Δ*adhE* HP1 was 1.18 ± 0.03 mmol/g-bagasse, 69.4% lower than that of wild type strain. The concentrations of acetate and lactic acid were 3.67 ± 0.22 mmol/g-bagasse and 1.92 ± 0.11 mmol/g-bagasse, 38.7% and 108.7% higher than that of wild type strain, respectively. The higher concentration of organic acids in Δ*adhE* HP1 soluble metabolites offered a more abundant substrate to photo hydrogen fermentation, which expected to result in a higher quantity of hydrogen produced during the photo hydrogen fermentation stage.

3.3. Hydrogen production during photo fermentation

To investigate the effect of initial pH, photo intensity and temperature on hydrogen production during PF, the pH for dark fermentation effluents (DFE) were adjusted from 5.5 to 7.5 with 1 M KOH solution, the photo intensity varied from 1000 to 9000 lux and temperatures ranging from 25 to 37.5 °C were applied.

The initial pH value of the medium affects the hydrogen production dramatically during PF (Fig. 2A). The results show that at the same pH condition, the cumulative hydrogen yields from dark fermentation effluents of *K. oxytoca* HP1 (DFEK) during PF were significantly lower than that from dark fermentation effluents of Δ*adhE* HP1 (DFEΔ*A*) (Fig. 2A). The optimum initial pH for photo-fermentative hydrogen production by *R. palustris* CN was about 6.5 (Fig. 2A), slightly different from the other *Rhodopseudomonas* which optimum pH for photo-fermentative hydrogen production reported as 7–7.5 (Chen et al., 2008; Ren et al., 2009).

The effect of photo intensity on hydrogen production during PF is shown in Fig. 2B. When the photo intensity rose from 1000 to 5000 lux, the cumulative hydrogen yields increased from 30.1 to 47.3 mL H_2/g bagasse when DFEΔ*A* were used as substrate. Thereafter, the cumulative hydrogen yields did not increase as the photo intensity increased. These results agree with that reported by Ren

Table 2
Soluble metabolite products in dark fermentation and photo fermentation.

Strain	Culture stage	Soluble metabolite roducts (mmol/g-bagasse)			[a]RS (mg/g-bagasse)	H_2 yield 0 mL/g-bagasse	[b]SHPR	[c]THY	[d]HPE (%)
		Ethanol	Acetate	Lactate					
Δ*adh*E HP1	DF	1.18 ± 0.03	3.67 ± 0.22	1.92 ± 0.11	18.3 ± 1.5	107.8 ± 5.3	5.51 ± 0.34	162.1	13.4
	PF	1.63 ± 0.04	1.26 ± 0.06	0.57 ± 0.03	4.5 ± 0.6	54.3 ± 2.2	0.93 ± 0.12		13.7
Wild	DF	3.85 ± 0.16	2.25 ± 0.14	0.92 ± 0.05	21.1 ± 1.4	96.2 ± 4.4	4.95 ± 0.22	131.3	12.0
	PF	4.18 ± 0.21	0.78 ± 0.02	0.42 ± 0.02	6.8 ± 0.5	35.1 ± 2.0	0.59 ± 0.07		17.6

[a] RS: reducing sugar.
[b] SHPR: Special hydrogen production rate (mL/g-bagasse·h).
[c] THY: Total H_2 yield in two steps (mL/g-bagasse).
[d] HPE: hydrogen production efficiency, HPE in DF was calculated based on reducing sugar consumed, in PF was calculated based on organic acid consumed. Each value is the mean of at least three replicates (±standard deviation).

Table 3
Comparison of H_2 production performance using cellulosic biomass as a substrate.

Biomass feedstock	H_2 production organism	Culture type	H_2 yield	Reference
Microcrystalline cellulose (12.5 g/l)	Anaerobic digested sludge	BDF[a]	2.18 mmol (48.8 mL) H_2/g cellulose	Lay (2001)
Wheat straw (15 g/l)	Cow dung compost	BDF	68.1 mL H_2/g TVS[b]	Fan et al. (2006a)
Beer lees (20 g/l)	Cow dung compost	BDF	68.6 mL H_2/g TVS	Fan et al. (2006b)
Corn straw (1:6)	*Clostridium butyricum* AS1.209	SSF	68 mL/g SECS[c]	Li and Chen (2007)
Cornstalk (15 g/l)	Cow dung compost	BDF	149.7 mL H_2/g TVS	Zhang et al. (2007)
Corn cob powder (0.5%)	*Clostridium thermocellum* JN4, *thermosaccharolyticum* GD17	BDF	20.4 mmol/l (91.4 mL H_2/g)	Liu et al. (2008)
Carboxymethyl cellulose (10 g/l)	*Clostridium pasteurianum*	BDF	1.09 mmol (24.4 mL) H_2/g cellulose	Lo et al. (2008)
Corn stalk powder (0.5%)	*Clostridium thermocellum* JN4, *thermosaccharolyticum* GD17	BDF	16.1 mmol/l (72.1 mL H_2/g)	Liu et al. (2008)
Bagasse (50 g/l)	Δ*adhE* HP,	BDF	107.8 mL H_2/g-bagasse	This work
Bagasse (50 g/l)	*K. oxytoca* HP1	BDF	96.2 mL H_2/g-bagasse	This work
Bagasse (50 g/l)	Δ*adhE* HP, *R. palustris* CN	SDPF	162.1 mL H_2/g-bagasse	This work
Bagasse (50 g/l)	*K. oxytoca* HP1, *R. palustris* CN	SDPF	131.3 mL H_2/g-bagasse	This work

[a] BDF: batch dark fermentation.
[b] TVS: total volatile solid, determined as (d) (Zhang et al., 2007).
[c] SECS: Steam-exploded corn straw:

$$\mathrm{TVS} = \frac{W_{\text{dry biomass}} - W_{\text{ash}}}{W_{\text{dry biomass}}}. \qquad \text{(d)}$$

et al. (2009). At the same photo intensity condition, the cumulative hydrogen yields from DFEΔ*A* (Fig. 2B). At 5000 lux photo intensity, the cumulative hydrogen yield from DFEK during PF was 31.5 mL H_2/g-bagasse, which was only 66.6% of that from DFEΔ*A* (Fig. 2B).

The culture temperature strongly affected hydrogen production during PF (Fig. 2C). The results show that under the same temperature condition, the cumulative hydrogen yields from DFEK during PF were significantly lower than that from DFEΔ*A* (Fig. 2C). The optimum temperature for photo-fermentative hydrogen production by *R. palustris* was shown as 30 °C (Table 2). At the optimum culture temperature, the cumulative hydrogen yield from DFEK during PF was 33.1 mL H_2/g-bagasse, which was only 63.4% of that from DFEΔ*A* (Fig. 2C).

The optimum culture conditions for hydrogen production from DFE during PF by *R. palustris* CN were initial pH 6.5, temperature 30 °C and photo intensity 5000 lux. Under these optimum culture conditions, the cumulative hydrogen yield and special hydrogen production rate from DFEK during PF were 35.1 ± 2.0 and 0.59 ± 0.07 mL H_2/g-bagasse·h (Table 2), respectively; The cumulative hydrogen yield from DFEΔ*A* during PF were 54.3 ± 2.2 and 0.93 ± 0.12 mL H_2/g-bagasse·h (Table 2), respectively, 54.7% and 57.6% higher than that from DFEK. These results indicated that the reducing ethanol concentration in DF effluent can effectively improve the hydrogen yield from bagasse during the PF stage.

During the PF stage, when DFEΔ*A* was used as substrate, 2.41 mmol/g-bagasse acetic acid, 1.35 mmol/g-bagasse lactic acid were consumed (Table 2), while when DFEK was used as substrate, 1.47 mmol/g-bagasse acetic acid, 0.50 mmol/g-bagasse lactic acid were consumed (Table 2). When DFEΔ*A* and DFEK used as PF substrate, the hydrogen production efficiencies (HPE) based on organic acid consumed were 13.7% and 17.6% (Table 2), respectively, which were lower performances comparison to the results that varying from 14.8% to 86.9% in PF hydrogen production reported in other literatures (Segers and Verstraete, 1983; Barbosa et al., 2001; Oh et al., 2004; Shi and Yu, 2005; Chen et al., 2006; Fang et al., 2006; Chen and Chang, 2006; Chen et al.,2008; Ren et al., 2009; Yang et al., 2010). The insufficiency of N-source in PF medium may be the major cause for poor hydrogen production efficiency in this work. In this study, no extra N-source added into the DFE, the N-source left in the DFE were used for the growth of PF bacteria and the turning-over of protein and enzyme. The C/N ratio and the N-source category were found to be important factors for photo-hydrogen production in previous reports (Eroglu et al., 1999; Oh et al., 2004), so to further improvement the PF hydrogen production from bagasse DFE, the C/N ratio and the suitable N-source category should be investigated, and the other factors (e.g. C/P ratio, concentration of substrate and the PF reactor) affecting PF hydrogen production also should be optimized.

3.4. The total hydrogen yield by sequential dark-photo fermentations

Under optimum conditions, the H_2 yields by using Δ*adhE* HP1 and wild type strain were 107.8 ± 5.3, 96.2 ± 4.4 mL H_2/g-bagasse in DF and 54.3 ± 2.2, 35.1 ± 2.0 mL H_2/g-bagasse in PF, respectively (Table 2). By using Δ*adhE* HP1 and wild type strain, the hydrogen production efficiencies were 13.4%, 12.0% (Table 2) in DF based on the total reducing sugar consumed and 13.7%, 17.6% (Table 2) in PF

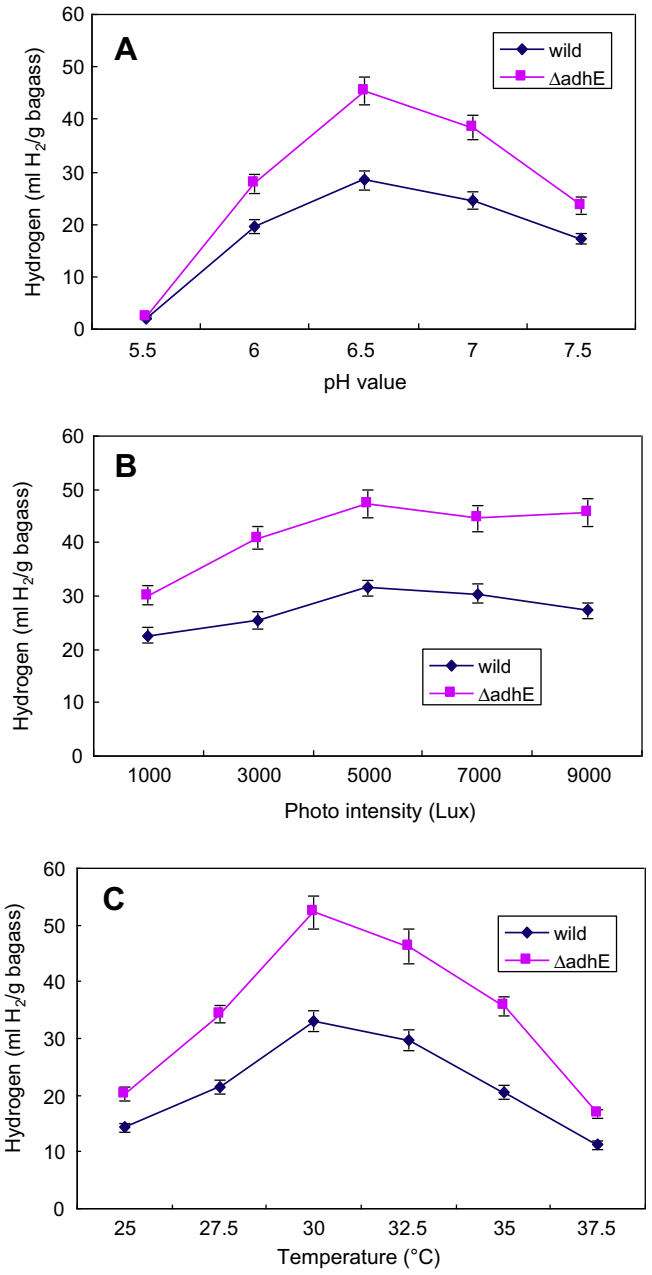

Fig. 2. Effect of pH (A), photo intensity (B) and temperature (C) on photo-fermentative hydrogen production.

based on organic acid consumed, respectively. The maximum total H_2 yield by using Δ*adhE* HP1 over two steps was 162.1 ± 7.5 mL H_2/g-bagasse (Table 2), 50.4% higher than that from DF only. While the maximum total hydrogen yield by using wild *K. oxytoca* HP1 over two steps was 131.3 ± 6.4 mL H_2/g-bagasse (Table 2), 36.5% higher than that from DF only. These results suggest that the SPDF is an effective way to enhance the hydrogen production from bagasse.

Although the hydrogen yield using the mutant strain increased significantly by 54.7% than using wild type strain in PF stage, the maximum total hydrogen yield from SPDF by using Δ*adhE* HP1 was only 23.5% higher than that by using wild strain, which can be explained because the H_2 yield in PF was the small proportion of the total H_2 yield. As the PF medium (e.g. C/N ratio, N-source category, C/P ratio and mineral elements concentration) optimized, a further improvement of the H_2 yield in PF should be achieved, which may result in a significant improvement of total H_2 yield by using the mutant strain.

4. Conclusions

This work successfully demonstrated that reducing ethanol concentration in DF effluent can effectively improve the hydrogen yield from bagasse during the PF stage in a SDPF system. Compared with that of the wild strain, the ethanol concentration in the dark fermentation broth of Δ*adhE* HP1 decreased 69.4%, which resulted in a hydrogen yield during the PF stage and the total hydrogen yield over the two steps of Δ*adhE* HP1 increased by 54.7% and 23.5%, respectively. This work also proved that SDPF was an effective way to improve hydrogen production from cellulosic biomass. Under optimum conditions, the total hydrogen yield over two steps was 162.1 ± 7.5 mL H_2/g-bagasse when Δ*adhE* HP1 was used in DF, which was 50.4% higher than that of 107.8 ± 5.3 mL H_2/g-bagasse from DF only. If wild *K. oxytoca* HP1 was used during dark fermentation, the maximum total hydrogen yield over two steps was 131.3 ± 6.4 mL H_2/g-bagasse, which was higher 36.5% than that of 96.2 ± 4.4 mL H_2/g-bagasse from DF only. The results of this work have reference value for the sequential dark-photo fermentations hydrogen production by using *Enterobacteriaceae* as DF bacteria.

Acknowledgements

This research was supported by the National Nature Science Foundation of China (30870051) and Key Project of International Collaboration of Science and Technology (2009DFA60930) and the Inventions and Innovation of Fujian, China ((2009)111).

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