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Strain screening and optimization of biohydrogen production by *Enterobacter aerogenes* EB-06 from glycerol fermentation

Yifeng Li, Yongqiu Qiu, Xu Zhang*, Minglong Zhu and Wensong Tan

Abstract

Biohydrogen technology has drawn much attention due to its many advantages. However, it is still necessary to screen much more strains with stronger hydrogen-producing capacity for future commercialization processes. In this paper, a biohydrogen-producing strain *Enterobacter aerogenes* EB-06 was isolated, identified, and named. It could convert glycerol to biohydrogen by microorganism fermentation. The effects of oxygen content, initial pH, initial glycerol concentration, and initial nitrogen source content on biohydrogen production process were investigated. The results have shown that biohydrogen generation was more favorable under anaerobic conditions. The optimum specific biohydrogen production rate (Q_{H2}) was obtained as 41.47 mmol H_2/g DCW h at 40 g/L initial glycerol concentration. The optimum volume H_2 yield (C_{H2}) was 83.76 mmol H_2/L at initial pH 7.0. It was found that nitrogen source content (0–4 g/L) could promote biohydrogen production and cell growth. The biohydrogen production of *Enterobacter aerogenes* EB-06 from glycerol was optimized by the orthogonal experimental design. The optimal yield coefficient of biohydrogen from glycerol fermentation ($Y_{H2/glycerol}$) of EB-06 was obtained as 1.07 mmol H_2/ mol glycerol at 10 g/L initial glycerol concentration, initial pH 5.0, and initial C/N ratio 5/3.

Keywords: Biohydrogen production, Condition optimization, Strain screening, *Enterobacter aerogenes*, Glycerol

Introduction

Hydrogen gas is a multipurpose energy source that can change the usage of hydrocarbon-based fossil fuels since the higher energy yield (122 kJ/g per unit mass), which is 2.75-fold greater than that of fossil fuels. Only water is generated as a major by-product (Asadi and Zilouei 2017; Azman et al. 2016) after hydrogen combustion. Currently, molecular hydrogen has been mainly generated from fossil fuel-based resources. Hydrogen gas production through biological pathways from biomass is one of the rising technologies due to its eco-friendly and sustainable nature (Niu et al. 2011; Sørensen 2011; Trchounian and Trchounian 2015). Dark fermentation of biohydrogen production is a promising method because of high hydrogen-producing rate, simple fermentation equipment, and bioconversion feasibility from the recyclable resources (Dessì et al. 2018). It is essential to choose inexpensive substrates, such as glucose, xylose, glycerol, and cellulose, for the development of biohydrogen production technology (Asadi and Zilouei 2017; Pachapur et al. 2015). Recently, aviation diesel has been partly replaced by biodiesel due to the energy conservation and environmental protection (Faber and Ferreira-Leitão 2016). However, a large amount of glycerol can be produced as by-product during biodiesel production process (Sivaramakrishnan and Incharoensakdi 2018). How to use these glycerol has become an urgent problem to be solved (Jitrwung and Yargeau 2011; Pott et al. 2014). The comprehensive utilization of these glycerols has been studied extensively. A wide diversity of microorganisms from environment including *archaea*, *cyanobacteria*, and *bacteria* (facultative aerobic and anaerobic) have been studied. Besides, lower *eukaryotes* are also reported as hydrogen producers, such as *Thermotoga*, *Rhodopseudomonas palustris*, *Escherichia*, *Enterobacter aerogenes* ATCC35029, *Klebsiella* sp. TR17

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Clostridium pasteurianum, and *Chlamydomonas,* which could also produce hydrogen through glycerol fermentation (Chookaew et al. 2014; Jitrwung and Yargeau 2011; Liu et al. 2015; Maru et al. 2012; Sarma et al. 2016; Zhang et al. 2015). The advantages of biohydrogen production through glycerol by microorganisms are low energy consumption, environment-friendly and high production efficiency. Ito et al. (2005) studied H_2 and ethanol production through glycerol from biodiesel wastes by *Enterobacter aerogenes* HU-101 fermentation. It has revealed that the optimum $Y_{H2/glycerol}$ of pure glycerol and raw glycerol are 1.05 mmol H_2/ mol pure glycerol and 1.12 mmol H_2/mol raw glycerol, respectively. Murarka et al. (2008) investigated the mechanism of biohydrogen production process by *Escherichia coli* via nuclear magnetic resonance analysis of cultures. Either 50% U-C-13-labeled or 100% unlabeled glycerol has been used. The $Y_{H2/glycerol}$ of 0.94 mmol H_2/mol glycerol was obtained. Selembo et al. (2010) obtained the $Y_{H2/glycerol}$ of 0.28 mol H_2/mol glycerol from glycerol metabolization by heat-treated mixed bacteria. (Liu and Fang 2010) has found that the $Y_{H2/glycerol}$ of 0.53 mmol H_2/mol glycerol was obtained by *Klebsiella* from treated biodiesel waste.

It has certain advantages for *Enterobacter aerogenes* in biohydrogen production by glycerol fermentation compared with the other bacteria. For example, a large proportion of the energy in glycerol can be converted to hydrogen energy due to a relatively high $Y_{H2/glycerol}$ obtained by *Enterobacter aerogenes*. Theoretically, $Y_{H2/glycerol}$ can reach 1.00 mol H_2/mol glycerol. Genetic modification (Ito et al. 2005) and bacterial isolation (Mangayil et al. 2015, Poleto et al. 2016) were investigated to improve the biohydrogen production capacity. Therefore, bacteria with high biohydrogen production capacity still should be continuously screened and the key parameters of biohydrogen production through glycerol metabolism should also be optimized.

In this paper, several biohydrogen-producing strains could metabolize glycerol to produce hydrogen at high production efficiency were isolated by Hungate anaerobic rolling tube technology and identified by 16S rDNA gene sequence. The effects of environmental factors, including oxygen, initial glycerol concentration, initial pH, and initial nitrogen source concentration, on the biohydrogen production process from glycerol fermentation were investigated. And finally, the optimization of cultural condition was studied using orthogonal experiment. The results could provide technical support for the development of industrialization process of biohydrogen production.

Materials and methods

Strain and culture

Strain isolation and identification

The bacteria were isolated from river sludge of Shanghai Botanical Garden, Shanghai, China. Sludge samples were collected in the sterile test tubes and stored at 4 °C. The anaerobic bacteria were enriched twice in a modified Hungate technique in combination with the sera bottle technique under anaerobic conditions at 37 °C, which could inhibit the growth of aerobic bacteria. Bacterial morphology, physiology and biochemistry, molecular biology, and other characteristics were also investigated.

Preparation of culture medium

The batch culture medium (pH 6.0–6.2) was composed of glycerol 10 g/L, peptone 4 g/L, yeast extract 2 g/L, NaCl 4 g/L, beef extract 4 g/L, K_2HPO_4 1.5 g/L, $MgCl_2$ 0.1 g/L, $FeSO_4{\cdot}7H_2O$ 0.1 g/L, and trace elements 10 mL. The medium was sterilized at 121 °C for 20 min. The 50% (w/v) glycerol was sterilized at 115 °C for 30 min. The composition of trace elements included $MnSO_4{\cdot}7H_2O$ 0.01 g/L, $ZnSO_4{\cdot}7H_2O$ 0.05 g/L, H_3BO_3 0.01 g/L, $N(C_{H2}COOH)_3$ 4.5 g/L, $CaCl_2{\cdot}2H_2O$ 0.01 g/L, Na_2MnO_4 0.01 g/L, $CoCl_2{\cdot}6H_2O$ 0.2 g/L, and $KAl(SO_4)_2$ 0.01 g/L.

Biohydrogen production experiments

The isolated strain was inoculated in the sterile medium and cultured in a 250 mL sealed flask with working volume of 100 mL. Batch fermentations were carried out in one 500 mL anaerobic sera bottle with 50 mL of working volume by shaking at 180 rpm. The sera bottle was sealed by a rubber stopper after inoculation and flushed with N_2 for 30 min. The biogas was collected by a syringe and measured by gas chromatography immediately. The controlled pH was measured by a pH meter (FE20, Mettler-Toledo, Shanghai, PR China) at every sampling interval. All experiments were carried out in triplicate.

Analytical methods

Cell concentration

The bacteria concentration was evaluated by the turbidimetric method. The optical density (OD) of cell growth was measured by a spectrophotometer (752 N, Shanghai Precision & Scientific Instrument Co. Ltd., Shanghai, PR China) at 660 nm. The DCW was calculated through the correlation between OD_{600} and DCW. One OD_{660} unit was estimated as 0.41 g/L dry cell weight, as shown in the following equation:

$$1\ OD_{660} = 0.41\ g\ DCW/L. \quad (1)$$

Gas composition

Gas-phase products (mainly H_2 and CO_2) were analyzed by gas chromatography (GC, 900C, Shanghai Sky Spectrum Analysis Instrument Co., Ltd., Shanghai, China). High purity nitrogen (99.999%) was used as carrier gas. The column model was TDX-01 and the detector was TCD thermal conductivity detector. The temperatures of the chromatographic column, sampler, and detector were 80 °C, 100 °C, and 100 °C, respectively. During the test, the headspace gas of the culture bottle was sampled to analyze the composition and content. The amount of hydrogen accumulated was calculated by the percentage content of hydrogen and volume of headspace gas.

Liquid-phase composition

High-performance liquid chromatography (SHIMADZU 10A, Shimadzu International Treading Co. Ltd., Japan) was used for the analysis of composition of biohydrogen fermentation broth. The model of liquid chromatographic column was Aminex HPX-87 h. The temperature of chromatographic column was 65 °C. The flow phase was 5 M dilute sulfuric acid with the flow rate of 0.6 mol/min. The detector model was RID-10A, 8.6 Pa. The amount of sampling was 20 μL. The UV spectrophotometric was used to determine the glycerol concentration.

Physiological and biochemical characteristics of isolates

The bacterial physiological and biochemical characteristics were analyzed to identify the isolated strain. The extraction of bacterial genomic DNA, electrophoretic detection, PCR amplification, and the analysis of 16S rDNA gene sequence were performed by Shanghai Major biological Medicine Technology Co. Ltd. The sequence was compared with the NCBI database. Sequence alignment was performed with phylogenetic analysis which was accomplished using the MEGA 6.0 software (Zhang and Sun 2008).

Effect of environmental factors on biohydrogen production

The effects of the key environmental factors, including oxygen, initial glycerol concentration, initial pH, and initial nitrogen source content, on the biohydrogen production process through glycerol fermentation were investigated.

Orthogonal experimental design was used to examine the interaction among factors. The orthogonal experiments of three factors and three levels were conducted to investigate the effects of the interaction of factors on the bacterial growth and the biohydrogen production under the anaerobic condition. The experimental error of all data in this paper was no more than 5%.

Results and discussion

Results of strains screening

For the primary isolation, 108 strains were isolated and numbered through initial enrichment, anaerobic plate culture, and anaerobic fermentation of glucose (to expand the substrate utilization range of hydrogen-producing microorganisms). It was observed that 98 strains could produce gas through the anaerobic fermentation of glucose.

For the secondary isolation, 16 strains with high biohydrogen production capacity were screened through the results of glucose fermentation from the 98 strains. The amounts of biohydrogen production of No. 6, No. 63, and No. 104 were higher than those of others. Therefore, they were selected for further investigation and named as EB-06, EB-63, and EB-104. The results of biohydrogen production of them are shown in Table 1.

It is demonstrated that the volume of biohydrogen production per milliliter of fermentation broth (C_{H2}) from EB-06 was the largest in the three experimental groups. Therefore, EB-06 strain would be studied in detail as the model strain for biohydrogen production through glycerol fermentation in this paper.

Identification of strain

The bacterial morphological characteristics, physiology and biochemistry, were analyzed in this paper. It was observed that EB-06 was a facultative anaerobe, Gram-negative, rod-shaped, moving, no spore, single or paired, as shown in Fig. 1. According to the results of Petri dish experiment, it was shown that the colony was milky white, neat, round, smooth, and opaque, as shown in Fig. 1a. It was pink or mauve colony on the EMB identification medium, as shown in Fig. 1b.

The results of the physiological and biochemical characteristics of EB-06, EB-104, and EB-63 are shown in Table 2. It was demonstrated that the physiological and biochemical characteristics of EB-06 were consistent with that of *Enterobacter aerogenes*.

The results of sequence of 16S rDNA of EB-06 indicated that there were 99% homology similarity between the 16S rDNA sequences of *Enterobacter aerogenes* LN623608,1. The phylogenetic tree of EB-06 based on 16S rDNA sequences was shown in Fig. 2. According to the physiological and biochemical characteristics, and the phylogenetic

Table 1 Partial results of the secondary screening

Strains	EB-06	EB-104	EB-63
C_{H2} (mL H_2/mL)	1.00 ± 0.001	0.75 ± 0.003	0.63 ± 0.01

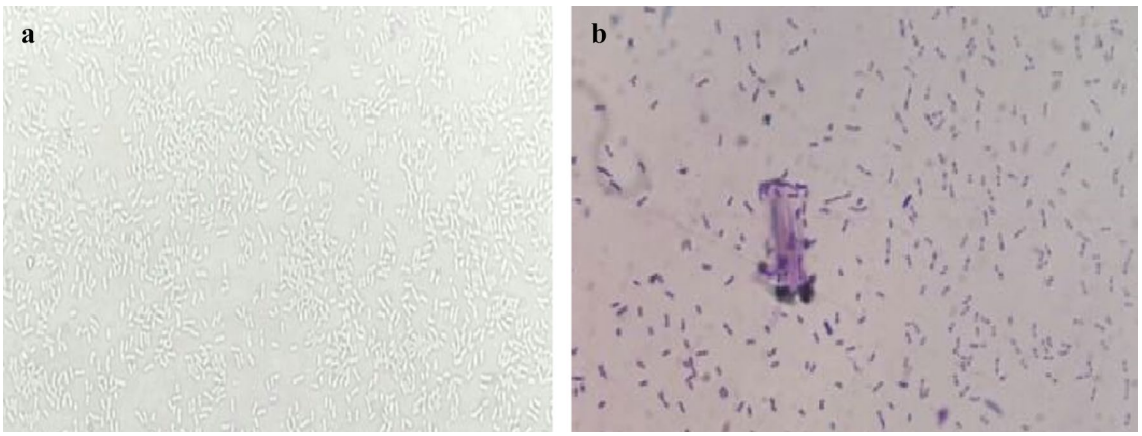

Fig. 1 Microscope picture of the EB-06 strain: **a** unstained bacterial morphology (10×1000); **b** stained bacterial morphology (10×1000)

Table 2 Results of the physiological and biochemical experiment of the strains

Test name/strains	EB-06, *α*	EB-104, *β*	EB-63, *γ*	*Enterobacter aerogenes*
Catalase test	+	+	+	+
Methyl red test	–	–	+	–
Voges-Proskauer test	+	+	+	+
Indole test	–	–	–	–
Citrate utilization test	+	+	+	+
Reduction of nitrate test	+	+	+	+
Production of H_2S test	–	–	–	–
Urease test	–	+	–	–
Gelatin liquefaction test	+	+	+	+
Glucose U	+	+	+	+
Glycerol U	+	–	+	+

tree of EB-06, the target strain EB-06 was identified as *Enterobacter aerogenes* and named as *Enterobacter aerogenes* EB-06.

Characteristics of biohydrogen production by *Enterobacter aerogenes* EB-06 through glycerol fermentation

The *Enterobacter aerogenes* EB-06 strain could produce biohydrogen through glycerol fermentation. The experiment was conducted in a 500 mL sera bottle under anaerobic conditions at 37 °C, 180 rpm. The initial glycerol concentration was 10 g/L. The initial pH was 6.0.

The change of amount of biohydrogen per liter of broth (C_{H2}), pH, amount of biomass per liter of broth (C_x), and the residual glycerol concentration ($C_{glycerol}$) during the biohydrogen production process by EB-06 through glycerol fermentation were shown in Fig. 3. It was shown that C_{H2} and C_x in the process increased with time, while $C_{glycerol}$ decreased and pH maintained constantly, which means that the EB-06 strain could utilize glycerol under anaerobic conditions, accompanied by the generation of biohydrogen and acidic metabolites in the fermentation broth. In addition, the properties of biohydrogen production through glucose anaerobic fermentation by EB-06 in batch culture were also shown in Fig. 4.

It was demonstrated that EB-06 was able to utilize glycerol to produce more hydrogen and biomass than glucose from Figs. 3 and 4. Therefore, the characteristics of biohydrogen production of EB-06 by glycerol fermentation were worthy of further study. As shown in Fig. 3, it was demonstrated that C_{H2}, pH, C_x, and $C_{glycerol}$ changed apparently from the 4th to the 8th cultural hour. After the 8th hour, the C_x, pH, and $C_{glycerol}$ tended to be invariant. However, C_{H2} continued to increase, which means that biohydrogen production was lagging behind the growth of EB-06 and glycerol consumption.

Figure 5 shows the trend of μ (the specific growth rate, h^{-1}), $Q_{glycerol}$ (the specific rate of glycerol consumption, g/g DCW h), and Q_{H2} (the specific rate of hydrogen production, mmol/g DCW h) with cultural time. It was observed that the trend of $Q_{glycerol}$, μ, and Q_{H2} was similar during whole biohydrogen production process. All of them increased first and then decreased. The corresponding time of the maximum value of them was different.

The corresponding time of the maximum of Q_{H2} significantly lagged behind those of the maximum of $Q_{glycerol}$ and μ. It was indicated that the biohydrogen production was lagging behind the bacteria growth process. The corresponding time of the maximum $Q_{glycerol}$ appeared earlier than those of growth and biohydrogen generation. It was indicated that a large amount of glycerol was consumed in advance for the preparation

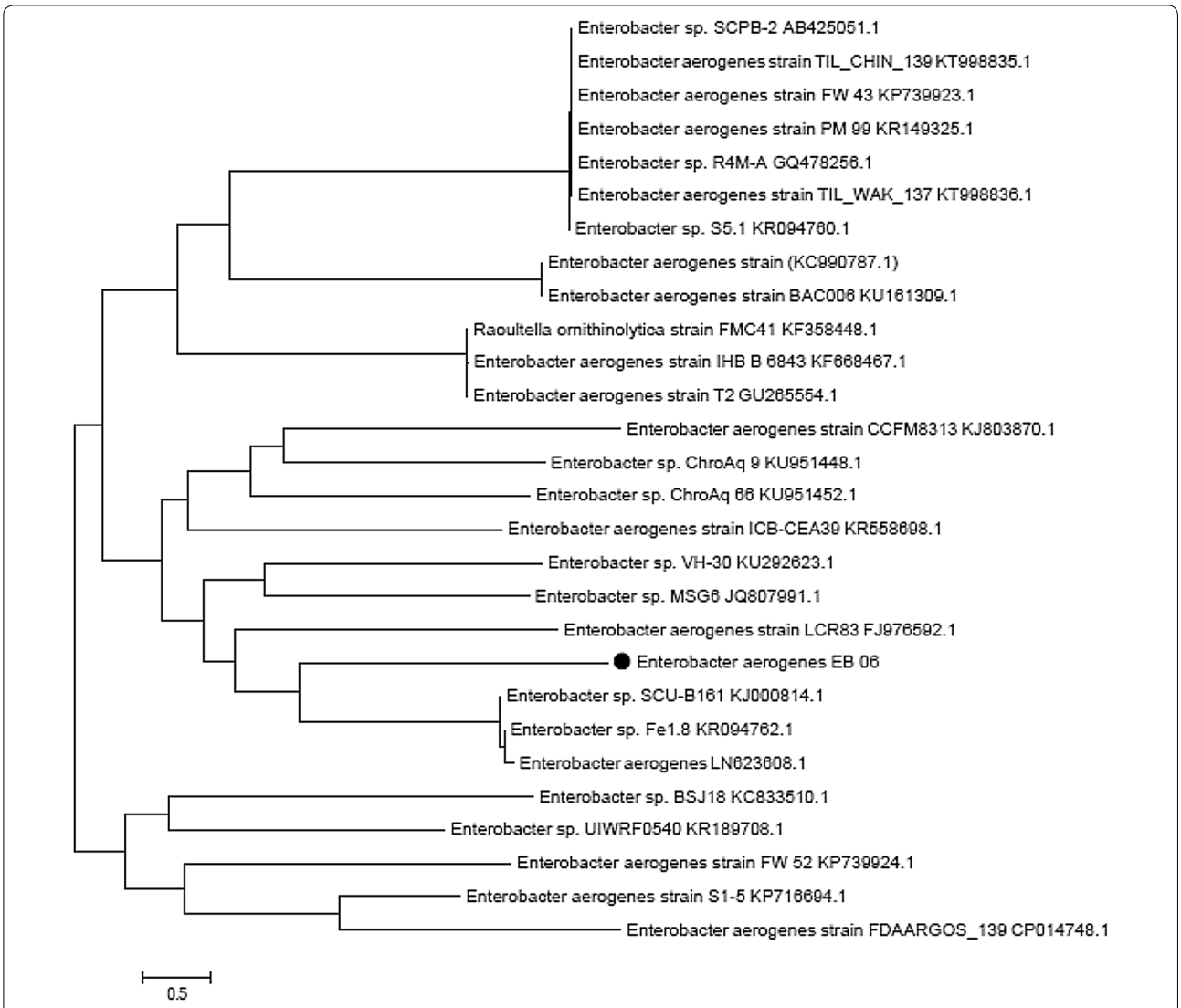

Fig. 2 The phylogenetic tree of EB-06 based on 16S rDNA sequences

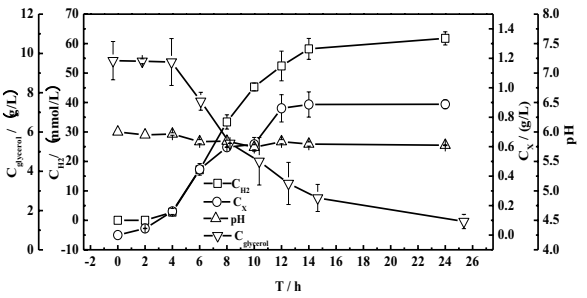

Fig. 3 The properties of biohydrogen production through glycerol anaerobic fermentation by EB-06 in batch culture

of bacterial growth and biohydrogen production during the 0–2nd hours of cultural time.

Effect of oxygen on biohydrogen production by EB-06 through glycerol fermentation

To study biohydrogen production characteristics of *Enterobacter aerogenes* under anaerobic and aerobic conditions, the effect of oxygen on the biohydrogen production by EB-06 through glycerol fermentation was explored. Figure 6 shows the change of C_x and C_{H2} with time by EB-06 through glycerol fermentation under anaerobic and aerobic conditions, respectively. It was observed that C_x and C_{H2} increased with time under both the anaerobic and aerobic conditions. The $Y_{H2/glycerol}$ under anaerobic conditions was calculated as 0.81 mol H_2/mol glycerol, which was 4.5 times higher than that under aerobic conditions.

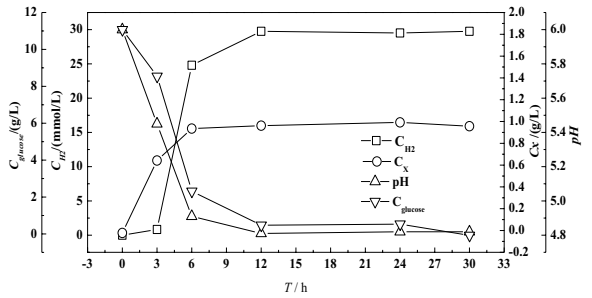

Fig. 4 The properties of biohydrogen production through glucose anaerobic fermentation by EB-06 in batch culture

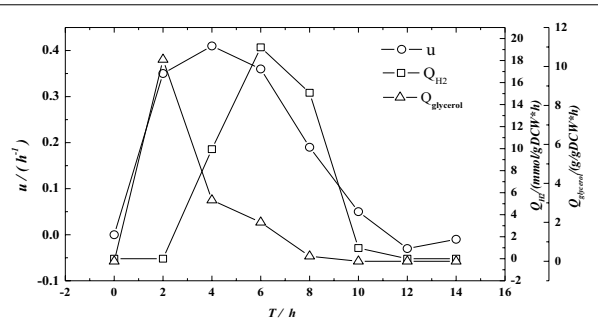

Fig. 5 The changing trend of μ, $Q_{glycerol}$, and Q_{H2} during EB-06 biohydrogen production process

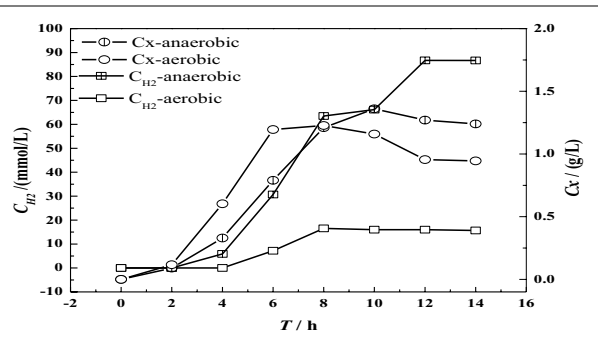

Fig. 6 Cell growth (C_x) and hydrogen production (C_{H2}) of EB-06 in anaerobic and aerobic fermentation

It seems that C_{H2} under anaerobic conditions was much smaller than that under aerobic conditions shown in Fig. 6. It is well known that there were two biohydrogen-producing pathways in the genus of *Enterobacter*, i.e., formic acid hydrogen production pathway and NADH hydrogen production pathway (Kurokawa and Tanisho 2005; Leonhartsberger et al. 2002). For the former, the activity of the formate dehydrogenase could be inhibited by oxygen (Vidal-Limón et al. 2017). For the latter, NADH was converted to hydrogen through hydrogenase. And the oxygen was used as the electron acceptor, which could efficiently provide more NADH reduction capacity and further produce more biohydrogen. Thus, the presence of oxygen will have the opposite effect on these two biohydrogen production pathways (Liu and Fang 2010). Moreover, it could also be deduced that the amount of biohydrogen produced by the formic acid pathway was different from NADH pathway. The formic acid biohydrogen production pathways might play a major role under anaerobic conditions.

On the other hand, the formate dehydrogenase activity was inhibited by O_2, so that the hydrogen-producing pathway of formic acid was heavily blocked under aerobic condition (Selembo et al. 2010). Similarly, the hydrogenase could also be inhibited partly by O_2. Meanwhile, NADH hydrogen-producing pathway was also affected. The electrons from NADH pathway could not flow to hydrogen production pathway, but to biomass (Chu et al. 2013). However, a small amount of biohydrogen was still produced under aerobic conditions, as shown in Fig. 6. This phenomenon might due to the fact that the activities of the above two enzymes were severely inhibited in the presence of oxygen rather than being completely inactivated.

According to the results shown in Fig. 6, C_x was 0.33 g/L under anaerobic conditions, while it was 0.60 g/L under aerobic conditions. It means that EB-06 could grow better under aerobic condition than that of anaerobic condition just because more NADH and ATP might be obtained for the biosynthetic pathway of EB-06 when O_2 was the electron acceptor.

The trends of μ and Q_{H2} during the biohydrogen production by EB-06 glycerol fermentation under aerobic and anaerobic conditions were shown in Fig. 7. It was observed that the trends of them with time were similar, whether it is under the condition of aerobic or anaerobic. All of them increased first and then decreased. It was also shown that the presence of oxygen was beneficial to the growth of EB-06, while was harmful to the biohydrogen production capacity of EB-06.

The μ and $Q_{glycerol}$ were shown in Fig. 8 during the biohydrogen production by EB-06 glycerol fermentation under aerobic and anaerobic conditions. The presence of oxygen was harmful to the consumption of glycerol by EB-06 due to the Pasteur effect.

Effects of initial pH on biohydrogen production by EB-06 through glycerol fermentation

The fermentative biohydrogen production could be effected by different initial pH (Nikhil et al. 2014). To investigate the effects of pH on biohydrogen production process by EB-06 through glycerol fermentation, C_{H2}, C_x,

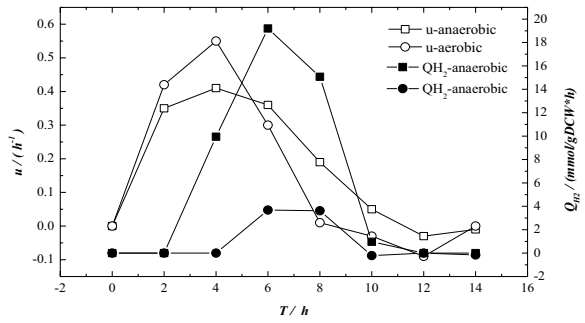

Fig. 7 The relationship with μ and Q_{H2} during EB-06 biohydrogen production process under aerobic and anaerobic conditions

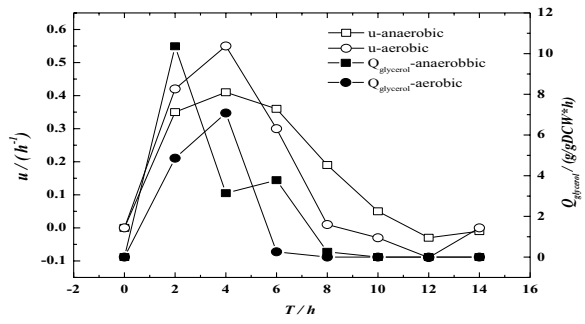

Fig. 8 The relationship with μ and $Q_{glycerol}$ observed in EB-06 biohydrogen production under aerobic and anaerobic conditions

pH, and $C_{glycerol}$ under different initial pH were studied with results shown in Fig. 9.

The C_{H2} under different initial pH are shown in Fig. 9a. It was found that C_{H2} increased with time under different pH. The C_{H2} increased first and then decreased with pH. They were significantly higher at pH 6 and 7 than that of others. It is indicated that amount of biohydrogen generated by EB-06 would be heavily inhibited at too lower or too higher pH. The optimum C_{H2} was 83.76 mmol H_2/L, and the $Y_{H2/glycerol}$ was 0.78 mol H_2/mol glycerol under initial pH 7.0.

The effects of initial pH values on biomass of EB-06 were shown in Fig. 9b. It was found that C_x in all the experimental groups increased with time, and reached to a stable stage after the 10th cultural hour. However, C_x were first increased and then decreased with the increasing of initial pH values. The optimum C_x was obtained as 1.34 g/L at pH 6.0.

The influence of initial pH on the pH of the fermentation broth could be seen in Fig. 9c. It was shown that the pH of broth had gradually decreased over time. In addition, the pH in the broth increased gradually with the increase of the initial pH. It was indicated that acidic metabolites gradually produced with the glycerol/glucose fermentation progresses.

The major liquid metabolites and concentrations in biohydrogen production process from EB-06 fermentation of glucose and glycerol are shown in Table 3. It could be seen that the major liquid metabolites were lactic acid and acetic acid for glucose fermentation, while ethanol for glycerol fermentation. Therefore, the pH of the fermentation broth during the biohydrogen production through glycerol fermentation was more stable than those of glucose fermentation.

It was found that C_x increased without any biohydrogen produced from 0 to 2nd cultural hour at pH 6.0 and 7.0 from Fig. 9. The $C_{glycerol}$ was decreased and approached zero from 8th to 12th cultural hour. However, C_{H2} continued to increase even at the 12th cultural hour. It was reported that formic acid might be an important intermediate metabolite (Ghimire et al. 2015). Thus, to a certain extent, the amount of intracellular formic acid was likely to accumulate before hydrogen could be produced.

Another possible explanation was that there existed biohydrogen-producing step and the biohydrogen-releasing step for biohydrogen production process. The hydrogen would be released to outside of the cell when the intracellular hydrogen accumulated to a certain level (Ghimire et al. 2015).

The curves of Q_{H2} with time under different initial pH are shown in Fig. 10. All of these Q_{H2} increased first and then decreased with time. However, the maximum Q_{H2} and the corresponding time under different initial pH were different. The optimum of Q_{H2} was obtained as 22.11 mmol H_2/g DCW h at initial pH 6.

In regards to the biohydrogen production of EB-06 through glucose fermentation. The optimum C_x, C_{H2} and $Y_{H2/glycerol}$ was obtained as 1.42 g/L, 46.13 mmol H_2/L and 0.71 mmol/mol glycerol at initial pH 8.0, respectively.

It could be found that the effects of the optimum initial pH on biohydrogen production process were different when glycerol or glucose was used as fermentation substrate. Glucose fermentation tends to occur under alkaline conditions, while glycerol fermentation tends to occur under acidic or neutral conditions.

Effect of initial glycerol concentration on biohydrogen production by EB-06 through glycerol fermentation

The effects of different initial glycerol concentrations on biohydrogen production by EB-06 were studied by batch culture at initial pH 6.0. The effects of different initial glycerol concentration on C_{H2}, C_x, pH, and $C_{glycerol}$ are shown in Fig. 11.

As shown in Fig. 11a, C_{H2} increased gradually and eventually became invariant with time during the whole

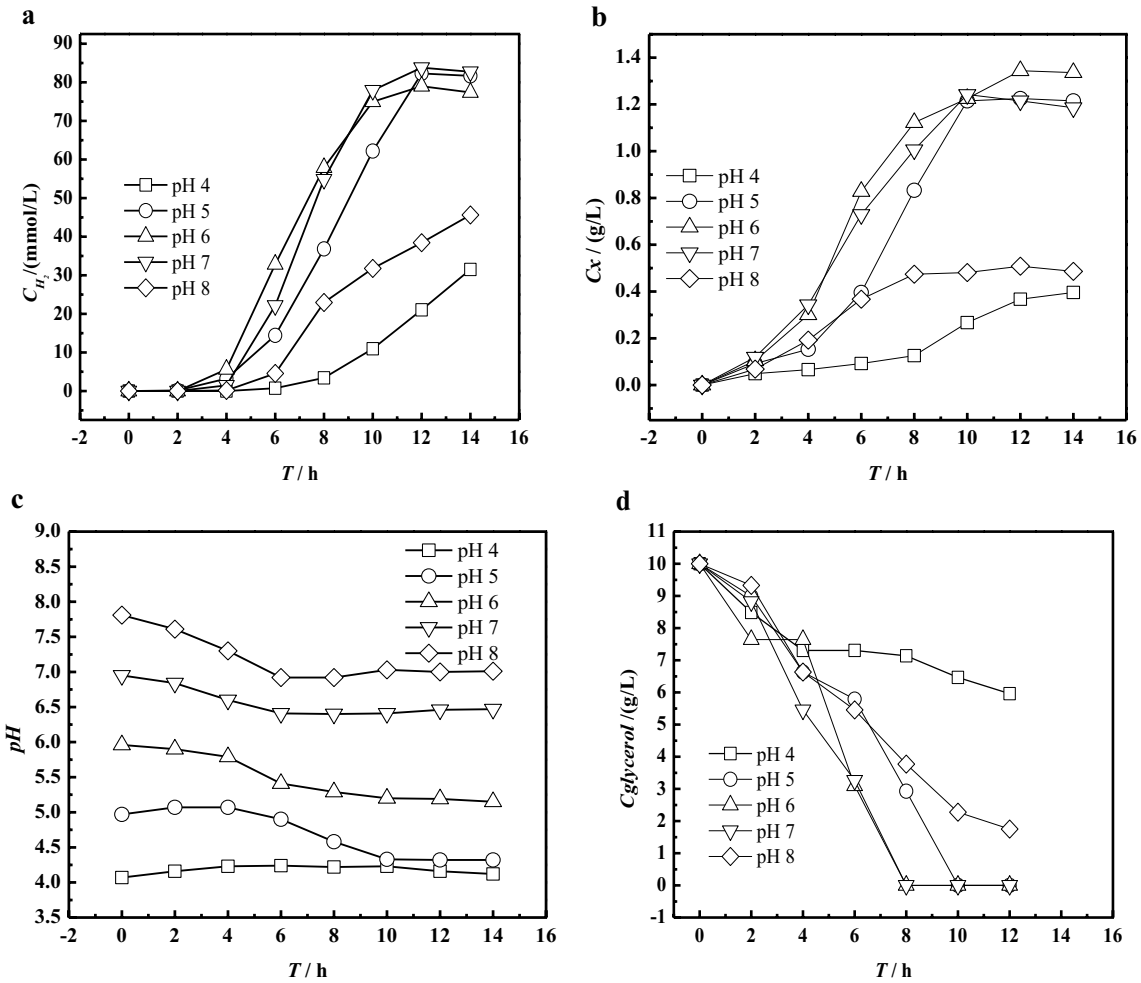

Fig. 9 The effect of different initial pH on C_{H2}\$C_x$\pH\$C_{glycerol}$ by EB-06 glycerol fermentation. **a** C_{H2}, **b** C_x, **c** pH, and **d** $C_{glycerol}$

Table 3 The major liquid products and their concentrations of biohydrogen production from EB-06 fermentation of glucose and glycerol

Substrate/product	Formic acid (g/L)	Acetic acid (g/L)	ethanol (g/L)	2,3-butanediol (g/L)	lactate (g/L)
Glucose	0.000	0.612	1.624	A little	0.083
Glycerol	0.000	0.005	3.873	A little	0.006

Culture conditions: initial pH 6.0, glycerol or glucose (1%), 37 °C, 180 rpm

fermentation process. However, C_{H2} increased first and then decreased with initial glycerol concentration. The optimum C_{H2} was obtained as 87.24 mmol H_2/L at the initial glycerol concentration of 10 g/L. It was also reported that C_{H2} would be increased with substrate concentration at the low concentration range of substrate. However, it was inhibited at high concentrations of substrate.

The cell growth curve is also shown in Fig. 11b. It was shown that the C_x was gradually increased during whole fermentation process. However, the C_x increased first and then decreased with the initial glycerol concentration. The growth of EB-06 was inhibited by the high initial glycerol concentrations. The optimum biomass concentration was about 1.10 g/L at the initial glycerol concentration of 10 g/L. It was found that the Han–Levenspiel model could well describe the effect of substrate concentration on biohydrogen production.

The pH in the fermentation broth decreased with the fermentation time, as shown in Fig. 11c. Among them, the pH in the fermentation broth was decreased when the initial glycerol concentration was 10 g/L, which indicated that the amount of acidic metabolites produced were relatively large.

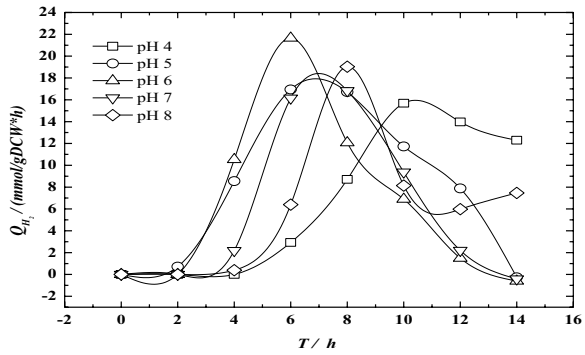

Fig. 10 The effect of different initial pH values on Q_{H2} by EB-06 glycerol fermentation

The $C_{glycerol}$ would gradually decrease due to substrate consumption, as shown in Fig. 11d. The $C_{glycerol}$ increased with initial glycerol. Glycerol was completely consumed at the 8th cultural hour when the initial glycerol concentration was 5 g/L and 10 g/L. However, glycerol had not been completely consumed at the initial glycerol concentration from 20 to 40 g/L. $Q_{glycerol}$ increased with the initial glycerol concentration. The $Q_{glycerol}$ at the initial glycerol concentration of 40 g/L was obtained as 7.73 g/g DCW h, as shown in Table 4. However, the total amount of glycerol consumption is less due to the lower C_x at the higher initial concentrations of glycerol.

It was shown that the initial glycerol concentration for the optimum C_{H2} was different from that of the optimum Q_{H2} shown in Fig. 11a and Table 4. It was revealed that EB-06 biohydrogen production metabolism and glycerol metabolism pathway were affected by the initial glycerol concentration in Table 4. $Y_{x/glycerol}$ (yield coefficient of

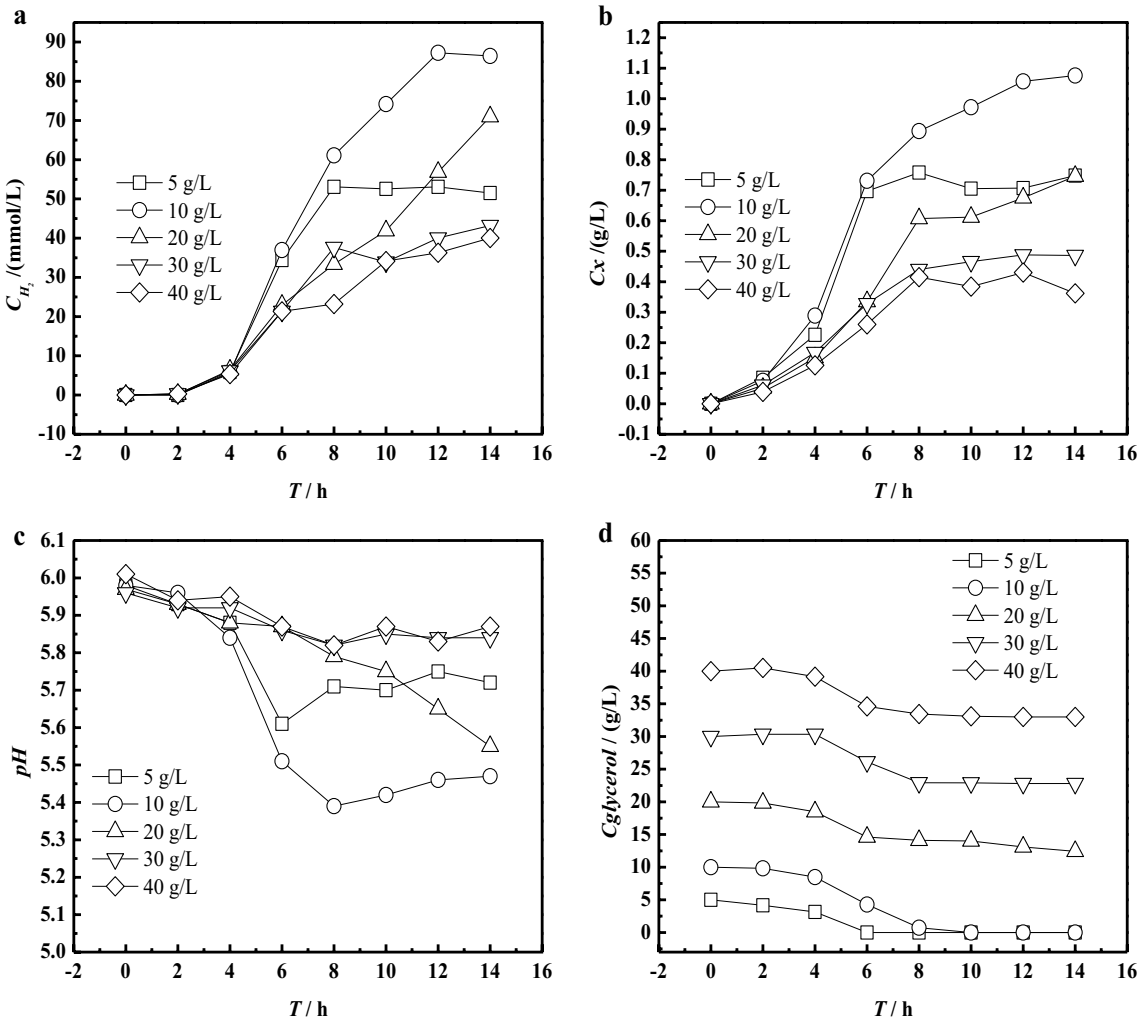

Fig. 11 The effect of different initial glycerol concentration on H_2\$C_x$\pH\$C_{glycerol}$ during EB-06 glycerol fermentation: **a** C_{H2}, **b** C_x, **c** pH, and **d** $C_{glycerol}$

Table 4 Parameters of biohydrogen production under different initial glycerol concentration

Glycerol concentration (g/L)	5	10	20	30	40
$Y_{x/glycerol}$ (g/mol)	8.40	6.60	6.00	5.56	5.26
$Y_{H2/glycerol}$ (mol H_2/mol)	0.99	0.81	0.77	0.63	0.56
Q_{H2} (mmol H_2/g DCW h)	30.93	30.76	32.04	30.66	41.47
$Q_{glycerol}$ (g/g DCW h)	2.95	3.44	5.76	6.05	7.73

biomass from glycerol, g/mol) and $Y_{H2/glycerol}$ (yield coefficient of biohydrogen from glycerol consumed, mol H_2/mol) would decrease with the increasing of initial glycerol concentration, while Q_{H2} and $Q_{glycerol}$ would increase with the initial glycerol concentration. The C_{H2} is the integral value of the product of Q_{H2} and C_x to the fermentation time. Therefore, the optimum C_{H2} was obtained when the initial glycerin concentration was 10 g/L, as shown in 11A.

The Q_{H2} was related with the bacteria species and substrate type. A large number of hydrogen-producing bacteria have been isolated and screened to obtain efficient hydrogen-producing strains. (Kumar and Das 2000) isolated *Enterobacter clocae* IIT-BT08 from the tree leaf extract. It was found that the maximum Q_{H2} was 29.63 mmol H_2/g DCW h at pH 6.0 and 37 °C; the Q_{H2} was from 25.0 to 28.0 mmol H_2/g DCW h at pH 6.0 and 36 °C. (Xing et al. 2006) screened two ethanol-type hydrogen-producing bacteria *Ethanoligenens harbinense* sp. R3 and *Ethanoligenens harbinense* sp.Y3 from CSTR reactor. The maximum Q_{H2} was 35.74 mmol H_2/g DCW h when glucose was the substrate. It could be seen that the optimum specific hydrogen production rate would reach to 41.47 mmol H_2/g DCW h when the initial glycerol concentration was 40 g/L seen from Table 4. It was indicated that EB-06 had a potential in biohydrogen production.

Effect of initial nitrogen source on hydrogen production by EB-06 through glycerol fermentation

It was well known that the growth and metabolism of microorganisms would be affected by the initial nitrogen source content of the medium. In this experiment, the peptone as nitrogen source contained in medium was set as 4.0 g/L, 2.0 g/L, and 0.0 g/L, respectively.

Figure 12 shows that the final C_{H2}, C_x, and Q_{H2} of the EB-06 biohydrogen production process under the different initial nitrogen source concentration. Moreover, both the final C_{H2} and C_x were gradually decreased with the decrease of initial nitrogen source concentration, as shown in Fig. 12a, b. The Q_{H2} of EB-06 also decreased with the decrease of initial nitrogen source concentration, as shown in Fig. 12c. The results shown that initial nitrogen source played an important role in cell growth and hydrogen generation during biohydrogen production through glycerol fermentation.

Optimization of biohydrogen production by EB-06 of glycerol fermentation through orthogonal design

The growth and the biohydrogen production by EB-06 through glycerol fermentation could be affected by the medium composition and environmental conditions. Orthogonal experiments can be used to optimize the interaction of multiple factors. The initial glycerol concentration, initial pH, and initial C/N ratio were selected as three key factors to investigate their effects on EB-06 growth and biohydrogen production according to the literature (Poleto et al. 2016). Three-factor

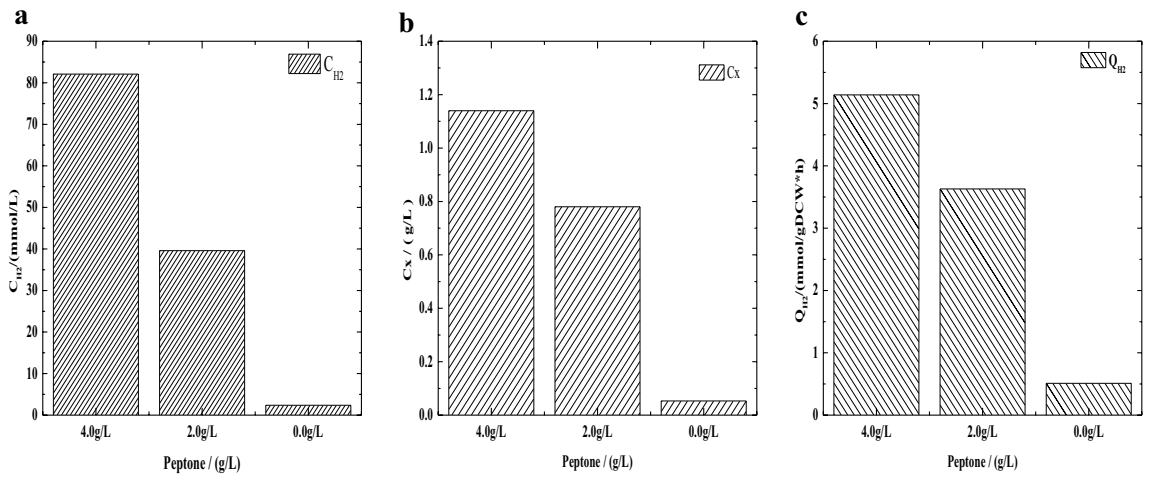

Fig. 12 The effect of nitrogen source on C_{H2}\$C_x$\$Q_{H2}$ by EB-06 glycerol fermentation

Table 5 Levels of selected factors for orthogonal experimental design

Level	Factors		
	$C_{glycerol}$ (g/L) [A]	pH [B]	C/N ratio [C]
1	5	5	5/2
2	10	6	5/3
3	15	7	5/4

Table 6 The results of orthogonal test for glycerol concentration, pH, and C/N ratio

Factors/levels	Glycerol concentration (g/L) [A]	pH [B]	C/N ratio (g/g) [C]	Y_{H2} (mol H_2/mol glycerol)
1	5	5	5/2	0.85
2	5	6	5/3	0.76
3	5	7	5/4	0.93
4	10	5	5/3	1.07
5	10	6	5/4	0.70
6	10	7	5/2	0.84
7	15	5	5/4	1.05
8	15	6	5/2	0.95
9	15	7	5/3	1.06

and three-level orthogonal experiments were adopted to study the optimal medium composition and optimal culture conditions. All the orthogonal experimental design data are shown in Table 5.

The results of the orthogonal experiment are shown in Table 6. The comprehensive score was characterized by Y_{H2} (mol/mol). It shown that the optimum Y_{H2} was obtained as 1.07 mol H_2/mol glycerol at 10 g/L initial glycerol, initial pH 5.0, and initial C/N ratio 5/3.

The range analysis method was used to analyze the orthogonal experimental results of biohydrogen production through EB-06 fermented glycerol with the results shown in Table 7. The range R values of the three factors are 0.19 (B), 0.17 (A), and 0.08 (C), respectively. Therefore, it could be concluded that the main factor was initial pH, followed by the initial glycerol concentration and the initial C/N ratio.

It was shown that the optimum Y_{H2} of 1.07 mol H_2/mol glycerol was obtained by EB-06 in this paper. The results were close to 1.12 mol H_2/mol glycerol reported by (Ito et al. 2005), which was the maximum value of Y_{H2} reported so far. And the yield of biohydrogen to glycerol obtained by this paper was higher than that of others shown in Table 8.

Table 7 The results of analysis of orthogonal experimental design

Calculation	Glycerol concentration (g/L) [A]	pH [B]	C/N ratio (g/g) [C]
K1	2.54	2.97	2.64
K2	2.61	2.41	2.89
K3	3.06	2.83	2.68
k 1	0.85	0.99	0.88
k 2	0.87	0.80	0.96
k 3	1.02	0.94	0.89
R	0.17	0.19	0.08
Primary and secondary order	B > A > C		
Optimal levels	A_2	B_1	C_2
Optimal combination	10 g/L, pH5.0, C/N 5/3		

Table 8 Biohydrogen characteristics of some reported microorganisms from glycerol fermentation

Substrate type	Microbial source	Reactor type	T/ (°C)	pH	Max Y_{H2}	References
Biodiesel	*Enterobacter aerogenes* HU-101	Batch	37 °C	–	1.12 mol H_2/mol glycerol	Ito et al. (2005)
Glycerol	*Enterobacter aerogenes* EB-06	Batch	37 °C	6.0	1.07 mol H_2/mol glycerol	This study
Waste glycerol	*Enterobacter. aerogenes* ATCC 35029	Batch	37 °C	–	0.85 mol H_2/mol glycerol	Jitrwung et al. (2013)
Biodiesel-based glycerol	*Enterobacter aerogenes*	Batch	37 °C	6.8	0.84 mol/mol of glycerol	Fan et al. (2010)
Crude glycerol	*Clostridium pasteurianum*	Batch	36 °C	6.7	0.63 mol H_2/mol glycerol	Sarma et al. (2016)

Conclusion

1. A highly efficient biohydrogen-producing strain was isolated, identified, and named as *Enterobacter aerogenes* EB-06.
2. The characteristics of the biohydrogen production of *Enterobacter aerogenes* EB-06 through glycerol fermentation were analyzed in this paper. The paper also studied effects of the key environmental factors, such as oxygen, initial pH, initial glycerol concentration, and nitrogen source on the biohydrogen production process.
3. Based on the analysis of orthogonal experiment, it was found that the main factor affecting biohydrogen production was initial pH, followed by the initial glycerol concentration, and, finally, the initial C/N ratio. Simultaneously, the optimum Y_{H2} of 1.07 mol H_2/mol glycerol was obtained at the initial glycerol concentration 10.00 g/L, the initial pH value 5, and the C/N ratio 5/3.

Abbreviations

C_{H2}: amount of hydrogen production per volume of fermentation broth (mmol /L); C_x: cell concentration per volume of fermentation broth (g/L); OD_{660}: the optical density of bacteria at 660 nm; DCW: dry cell weight; $C_{glucose}$: residual glucosel concentration per volume of fermentation broth (g/L); $C_{glycerol}$: residual glycerol concentration per volume of fermentation broth (g/L); $Q_{glycerol}$: specific glycerol consumption rate [(g glycerol consumed) (g dry cell formed)$^{-1}$ h^{-1}]; Q_{H2}: specific H_2 formation rate [(mmol H_2 formed) (g dry cell formed)$^{-1}$ h^{-1}]; μ: specific growth rate of biomass (h^{-1}); $Y_{H2/glycerol}$: yield coefficient of H_2 from glycerol [(mmol H_2 formed) (g glycerol consumed)$^{-1}$]; $Y_{x/glycerol}$: yield coefficient of biomass from glycerol [(g dry cell formed) (g glycerol consumed)$^{-1}$]; ATCC: American Type Culture Collection; PR: People's Republic of China; TCD: thermal conductivity detector; RID: refractive index detector; MEGA: molecular evolutionary genetic analysis; UV: ultraviolet; PCR: Polymerase Chain Reaction; NCBI: National Center for Biotechnology Information; NADH: nicotinamide adenine dinucleotide; ATP: adenosine triphosphate.

Authors' contributions

WT and XZ conceived and designed the experiments. YL performed the experiments and analyzed the data. YL wrote the paper. YQ helped in manuscript writing. XZ and MZ revised the manuscript. All authors read and approved the final manuscript.

Acknowledgements

The authors are grateful for the financial support of this research by the Open Project Funding of the State Key Laboratory of Bioreactor Engineering of China.

Competing interests

The authors declare that they have no competing interests.

Availability of data and materials

All data generated or analyzed during this study are included in the main manuscript file.

Consent for publication

The authors approved the consent for publishing the manuscript.

Ethics approval and consent to participate

Not applicable.

Funding

Not applicable.

Publisher's Note

Received: 13 December 2018 Accepted: 19 April 2019
Published online: 02 May 2019

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