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Enhancing hydrogen production of *Enterobacter aerogenes* by heterologous expression of hydrogenase genes originated from *Synechocystis* sp.

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HIGHLIGHTS

- The *hoxEFUYH* genes were heterologously expressed in *Enterobacter aerogenes*.
- SDS-PAGE and western blot analysis confirm the successful expression of *hoxEFUYH*.
- Hydrogenase activity of the recombinant is 152% higher than the wild strain.
- H_2 yield of the recombinant is 298.3 ml/g-glucose, 88% higher than the wild strain.
- The recombinant produces more acetate and butyrate as by-product, but less ethanol.

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ABSTRACT

The hydrogenase genes (*hoxEFUYH*) of *Synechocystis* sp. PCC 6803 were cloned and heterologously expressed in *Enterobacter aerogenes* ATCC13408 for the first time in this study, and the hydrogen yield was significantly enhanced using the recombinant strain. A recombinant plasmid containing the gene in-frame with Glutathione-S-Transferase (GST) gene was transformed into *E. aerogenes* ATCC13408 to produce a GST-fusion protein. SDS-PAGE and western blot analysis confirm the successful expression of the *hox* genes. The hydrogenase activity of the recombinant strain is 237.6 ± 9.3 ml/(g-DW·h), which is 152% higher than the wild strain. The hydrogen yield of the recombinant strain is 298.3 ml/g-glucose, which is 88% higher than the wild strain. During hydrogen fermentation, the recombinant strain produces more acetate and butyrate, but less ethanol. This is corresponding to the NADH metabolism in the cell due to the higher hydrogenase activity with the heterologous expression of *hox* genes.

1. Introduction

Hydrogen is the ideal secondary energy with high energy density and higher heating value per unit than hydrocarbons and alcohol compounds, and water as its combustion product without any pollution. Recently, hydrogen fermentation by microorganism draws more and more attention, for its low cost, rapid bacteria growth, high hydrogen producing capacity and abundant feedstocks (Xia et al., 2015; Yokoi et al., 1995; Yu et al., 2002). *Bacterium* and *Clostridium* are efficient producers of hydrogen through fermentation among a large number of microorganisms (Chu et al., 2009; Jo et al., 2008; Nandi and Sengupta, 1998). *Enterobacter* strains are considered to be more suitable for industrial scale hydrogen production because they grow faster, can use a wide range of substrate, and have higher tolerance to dissolved oxygen, hydrogen partial pressure and pH (Kumar and Das, 2000; Ren et al., 2009). *Enterobacter aerogenes* is a kind of natural hydrogen producing bacteria with high hydrogen producing efficiency, but the hydrogen yields of the wild strains are only about 1 mol/mol-glucose (Rachman et al., 1997; Tanisho et al., 1987; Yokoi et al., 1995). Based on the theoretical yield of hydrogen which is 4 mol/mol-glucose by dark fermentation, it is necessary to increase the hydrogen producing ability of *E. aerogenes*. It is feasible and effective to enhance the hydrogen production of *E. aerogenes* via gene engineering (Goyal et al., 2012; Hallenbeck and Ghosh,

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2012; Lu et al., 2010; Zhang et al., 2011). However, many wild strains of *E. aerogenes* have some specific antibiotic resistance, and it is more difficult to do genetic modification with *E. aerogenes* compared with *Escherichia coli* (Song et al., 2011; Zhao et al., 2010).

The hydrogenase genes (*hoxEFUYH*) of *Cyanobacteria Synechocystis* sp. PCC 6803 which have 6493 bp is consisted of five open reading frames. They were cloned and heterologously expressed in *E. coli* TG1 for the first time by Maeda et al., and the hydrogen yield of the recombinant strain was 41-folder higher than the wild strain, because the heterologous expression of *hoxEFUYH* suppressed the activity of uptake hydrogenase in the recombinant strain without affecting the growth of the cell (Maeda et al., 2007). They were heterologously expressed in *E. coli* DH5α by Zheng et al. and the hydrogen yield of the recombinant strain was 1.89 mol/mol-glucose while the yield of the wild strain was 1.32 mol/mol-glucose. The expression of *hoxEFUYH* not only suppressed the activity of uptake hydrogenase, but also produced hydrogen through NADH pathway. Furthermore, formic acid assimilation was accelerated (Zheng et al., 2012). In order to increase the hydrogen yield of *E. aerogenes* which is more suitable for practical hydrogen production, it should be effective to heterologously express *hoxEFUYH* in *E. aerogenes*.

In this article, the hydrogenase genes (*hoxEFUYH*) of *Cyanobacteria Synechocystis* sp. PCC 6803 were amplified and heterologously expressed in *E. aerogenes* ATCC13408 for the first time to improve hydrogen production. Hydrogenase activities of the wild and recombinant strains were tested. Hydrogen production of the two strains from glucose as substrate was carried out to study the hydrogen yields and redistribution of fermentative fluxes.

2. Materials and methods

2.1. Bacterial strains and plasmids

E. aerogenes ATCC13408 was purchased from China General Microbiological Culture Collection Center. The coding region of the *hoxEFUYH* genes were amplified from the genome of *Synechocystis* sp. PCC 6803 with forward (*hoxEFUYH*-F 5′-CCCGGG ATGACCGTTGCCACCGAT-3′) and reverse (*hoxEFUYH*-R 5′-CTCGAG CCATTGACATTGAGTTCTCC-3′) primers, which had a *Sma* I site and an *Xho* I site, respectively. The plasmid of pGEX-4T-2-Cat was reconstructed by Zhao et al. (2010) in the former study. Then the *hoxEFUYH* genes were subcloned into the multiple cloning sites digested with *Sma* I and *Xho* I, and positive clones were selected in the presence of ampicillin and chloramphenicol. The recombinant plasmid pGEX-4T-2-Cat/*hoxEFUYH* was confirmed by sequencing and then transformed into the competent cells of *E. aerogenes* ATCC13408 via electroporation. The wild strain, the recombinant strain, and *E. coli* DH5α (the host for genetic manipulation) were grown aerobically at 37 °C in Luria Bertani (LB) medium in an incubator shaker at 220 rpm until the cultures reached mid-log phase. 1 mM Isopropyl β-D-1-thiogalactopyranoside (IPTG) was used as an inducer in hydrogen producing medium.

2.2. SDS-PAGE and Western blot analysis

The recombinant strain containing the *hoxEFUYH* genes (pGEX-4T-2-Cat/*hoxEFUYH*) was grown aerobically overnight with 20 μg/ml chloramphenicol. 100 μl of this seed culture was inoculated into 5 ml LB medium and cultured for 2 h. The cells were induced with 1 mM IPTG and further grown for 4 h hermetically. The cells were then centrifuged and lysed by sonication. The lysates were separated by 12% SDS–PAGE. Heterologous expression of *hoxEFUYH* was confirmed by western blot analysis with anti-GST polyclonal antibody.

2.3. Hydrogenase assay

The hydrogenase activities of the wild strain and the recombinant strain were quantified by the amount of H_2 evolved from Na-dithionite reduced methyl viologen (MV). The method was the same as that in the former article (Song et al., 2011). The hydrogenase released from the lysed cells was used to produce hydrogen from the reduced MV. Hydrogen gas was detected on a GC (Agilent 7820A, USA). The triplicate experiments for all the conditions were performed to give the average data.

2.4. Analysis of bio-hydrogen

The recombinant strain was cultured for more than ten generations before it was used for the fermentative experiment. 100 ml of the cells which reached mid-log phase were added into 200 ml of fermentative medium composed of 3 g of glucose (carbon source), 2 g of malt extract, 0.8 g of yeast extract, 0.03 g of $FeCl_2$, 1 ml of vitamin liquid, and 1 ml of microelement liquid (Chittibabu et al., 2006; Dutta et al., 2009; Xie et al., 2008). A 300-ml reactor for fermentation was purged with N_2 gas for 5 min to create an anaerobic environment. The pH value was adjusted to 6 ± 0.2 by adding 6 M NaOH into the reactor during hydrogen fermentation at 37 °C. The concentrations of H_2 and CO_2 were analyzed on a gas chromatography (GC, Agilent 7820A, USA) equipped with a thermal conductivity detector and a 5A column (Φ 3 mm × 3 m, Agilent, USA). The temperatures of injection port and thermal conductivity detector were 200 °C and 300 °C, respectively. The initial column temperature was set at 65 °C for 1 min, then increased to 145 °C at a heating rate of 25 °C/min, and then held at 145 °C for 3 min. Argon gas was used as the carrier gas at a flow rate of 27 ml/min.

2.5. Analysis of liquid metabolic byproducts

The liquid metabolites were analyzed on another GC (Agilent 7820A, USA) equipped with a flame ionization detector and a DB-FFAP column (Φ 0.32 mm × 50 m, Agilent, USA). The temperatures of injection port and flame ionization detector were both 250 °C. The initial column temperature was set at 100 °C for 1 min, then increased to 200 °C at a heating rate of 10 °C /min, and then held at 200 °C for 2.5 min.

3. Results and discussion

3.1. Heterologous expression of hoxEFUYH in E. aerogenes ATCC13408

The *hoxEFUYH* genes were amplified and inserted into a reconstructed vector pGEX-4T-2-Cat to produce GST-tagged recombinant HoxEFUYH protein. The PCR product of *hoxEFUYH* ORF with the specific primers was shown in Fig. 1A. With the strong promoter provided by the vector pGEX-4T-2-Cat, the *hoxEFUYH* genes are successfully expressed in *E. aerogenes* ATCC13408. The GST-tag is 26 kDa, and the HoxE is 18 kDa, so the fusion protein should be 44 kDa. A specific protein of about 40 kDa was observed when 1 mM IPTG was added into the recombinant strain. To confirm this result, the whole cell lysate was exposed to Western blot analysis by using an anti-GST polyclonal antibody. The result demonstrated that a strong reactive band was detected in the recombinant strain (Fig. 1B), suggesting that GST-HoxE was successfully expressed in *E. aerogenes* ATCC13408 strain.

3.2. Hydrogenase activity

After 36 h of H_2 fermentation, liquid samples of the wild strain and the recombinant strain were removed into 13 ml sealed vials

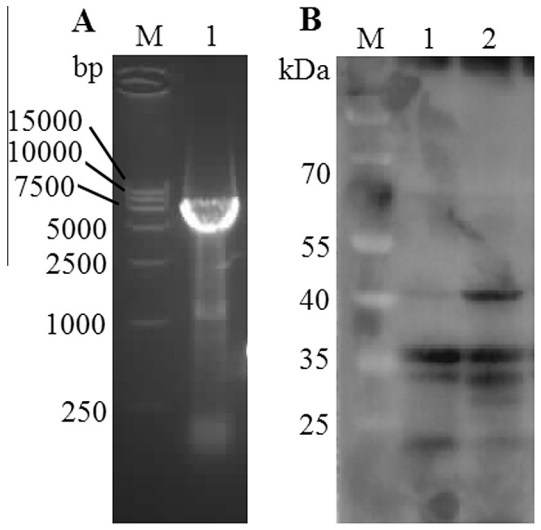

Fig. 1. PCR product and western blot analysis. A: the PCR product of *hoxEFUYH* ORF with the specific primers hoxEFUYH-F and hoxEFUYH-R. Lane-M: mol. wt. marker, 15,000 bp DNA ladder; Lane-1: PCR product of 6493 bp. B: immunoblot analysis with anti-GST polyclonal antibody. Lane-M: protein marker; Lane-1: the wild strain *E. aerogenes* ATCC13408; Lane-2: the recombinant strain *E. aerogenes*/HoxEFUYH.

after the pH of the solutions were adjusted to 6, respectively. After being purged with N_2 for 2 min, the samples were cultured anaerobically for another 2 h, and then used for hydrogenase assays. Hydrogenase activity is defined as the hydrogen yield from one gram of dry cells per hour. As shown in Fig. 2, the slopes of the fitting curves represent the hydrogenase activities. Each data point is a single measurement from one of three triplicate vials. Hydrogenase activity of the recombinant strain with the heterologously expressed HoxEFUYH is 237.6 ± 9.3 ml-H_2/(g-DW·h), which is 152% higher than the wild strain of 94.2 ± 3.7 ml-H_2/(g-DW·h). It demonstrates that the expression of *hoxEFUYH* is efficient for increasing active hydrogenase in the recombinant strain. The protein of interest is effective in promoting hydrogen production, not only by acting as a hydrogenase itself, but also by suppressing the activity of uptake hydrogenase.

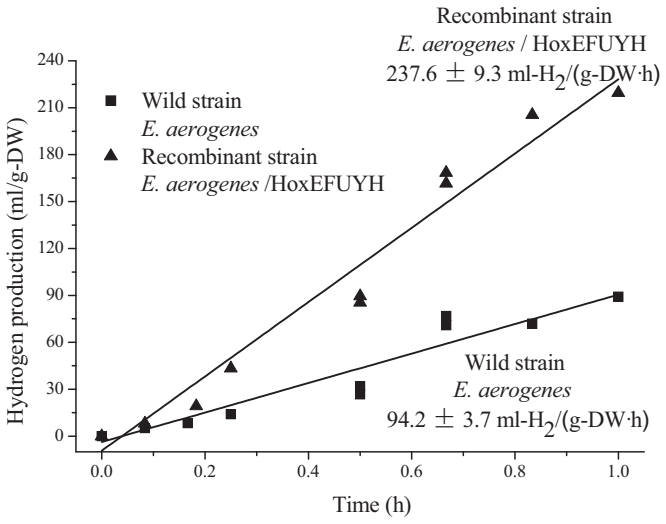

Fig. 2. Hydrogenase activity of the wild strain (*E. aerogenes*) and the recombinant strain (*E. aerogenes*/HoxEFUYH).

3.3. Hydrogen production from glucose

Hydrogen fermentation from glucose using the wild strain and the recombinant strain was performed respectively with the results shown in Figs. 3 and 4. The volume of H_2 produced is calculated from volume of biogas multiplied by composition ratio of the H_2 fraction. It is found by experiments that the peak hydrogen concentration in the biogas is 56–68% (v/v). Fig. 3 shows the hydrogen producing rates of the wild strain and the recombinant strain. The wild strain gives the peak rate of 63.6 ml/(L·h) at around 48 h. It takes more time for the recombinant strain to give a peak hydrogen rate of 124 ml/(L·h), which is obviously higher than that of the wild strain. It may be attributed to a higher demand in energy for synthesis of HoxEFUYH for the recombinant strain, which leads to a lower reproduction speed of the cells and the later peak rate. After more HoxEFUYH expressed, the recombinant strain gives a much higher peak rate, with a higher hydrogenase activity. Besides, the recombinant strain with HoxEFUYH could produce hydrogen through NADH pathway, which leads to a lower NADH/NAD^+ ratio and the improvement of glucose uptake. The pathways

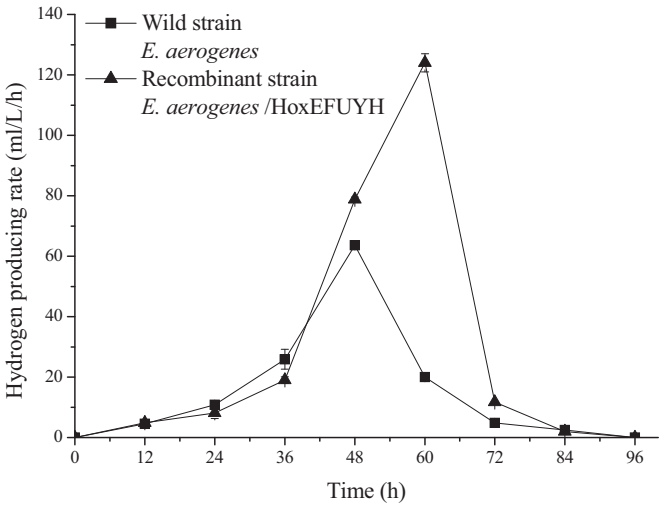

Fig. 3. Hydrogen producing rate of the wild strain (*E. aerogenes*) and the recombinant strain (*E. aerogenes*/HoxEFUYH).

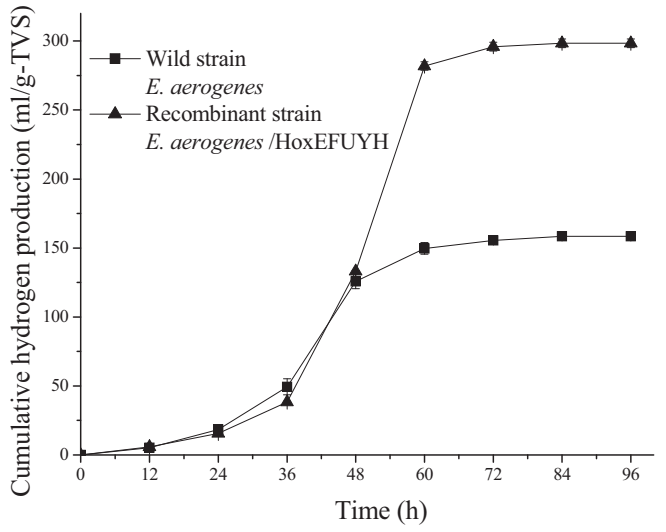

Fig. 4. Cumulative hydrogen production of the wild strain (*E. aerogenes*) and the recombinant strain (*E. aerogenes*/HoxEFUYH).

Table 1
Soluble metabolites of the wild strain (*E. aerogenes*) and the recombinant strain (*E. aerogenes*/HoxEFUYH) after hydrogen fermentation (φ%).

	Ethanol	Acetic acid	Propionic acid	Butyric acid	Valeric acid
Wild strain *E. aerogenes*	0.183 ± 0.014	0.126 ± 0.022	0.002 ± 0.0003	0.208 ± 0.016	0.011 ± 0.005
Recombinant strain *E. aerogenes*/HoxEFUYH	0.102 ± 0.013	0.172 ± 0.018	0.002 ± 0.0006	0.428 ± 0.029	0.005 ± 0.001

that need more NADH also accelerate the hydrogen production (Wang et al., 2013).

Fig. 4 shows that the cumulative H_2 yield of the recombinant strain with the heterologously expressed HoxEFUYH is promoted from 158.5 ml/g-TVS of the wild strain to 298.3 ml/g-TVS, increased by 88%. This result could be ascribed to the following reasons. (1) Hydrogenase activity for hydrogen evolution is increased. HoxEFUYH is effective in promoting hydrogen production by acting as a hydrogenase itself. The recombinant strain produces more hydrogen with a higher hydrogenase activity because of the expressed HoxEFUYH (Maeda et al., 2007). (2) The expression of hoxEFUYH suppresses the transcription of native hydrogenase which is responsible for hydrogen uptake activity (Zheng et al., 2012), and more hydrogen will be released. (3) The hydrogen production in *Enterobacter* species is contributed by two routes (Zhang et al., 2009): from formate and excess intracellular NADH. With the expression of HoxEFUYH, formic acid assimilation is accelerated, resulting in the enhancement of hydrogen production (Zheng et al., 2012). (4) The expression of HoxEFUYH altered the metabolic fluxes, hydrogen production via both the formate pathway and the NADH pathway is enhanced (Wells et al., 2011; Zhao et al., 2015). Besides, a control condition in which only void GST was expressed was carried out. The fermentative hydrogen producing rate and the cumulative hydrogen yield in the control condition were almost the same as the results using the wild strain.

3.4. Redistribution of soluble metabolites due to HoxEFUYH

The soluble liquid by-products after hydrogen fermentation using the two strains were determined. Aqueous solutions containing ethanol, acetic acid, propionic acid, butyric acid, and valeric acid at known concentrations were prepared and measured by GC, and standard calibration curves were generated according to the results. The concentrations of metabolites during H_2 fermentation were calculated based on the standard calibration curves. As shown in Table 1, final metabolites mainly include acetic acid, butyric acid, and ethanol. It can be seen that the final concentration of ethanol is less: from 0.183% of the wild strain to 0.102% of the recombinant strain, while that of acetate and butyrate is larger: from 0.126% and 0.208% to 0.172% and 0.428%, respectively. This transformation of metabolism corresponds to the improvement of hydrogen yield with the recombinant strain. According to the metabolism pathways of glucose as substrate, pyruvate is produced from glucose and subsequently converted into acetyl coenzyme A, which is the precursor of ethanol, acetate, and butyrate. During this degradation process, NADH is released as reducing equivalents, which need to be re-oxidation by producing reduced products such as hydrogen, ethanol, and organic acids. For generation of hydrogen, ethanol, and butyrate, the consumption of NADH is 1 mol/mol, 2 mol/mol, and 2 mol/mol, respectively, while generation of acetate needs no NADH. But butyrate consumes less NADH per carbon because butyrate is a 4-carbon-product, while ethanol is only 2. In another word, 2 ethanol or 1 butyrate can be produced from per glucose consumed, and 2 ethanol consume 4 NADH while 1 butyrate only consumes 2 NADH. With more hydrogen produced, the recombinant strain produces more acetate and butyrate, which consumes no or less NADH, to maintain the balance of reducing equivalents inside the cell. In this way, the heterologous expression of HoxEFUYH modifies the metabolic pathways. In the wild strain *E. aerogenes*, less hydrogen is produced and the metabolism favors ethanol production to maintain the balance of reducing equivalents inside the cell (Zhang et al., 2009). Correspondingly, less acetate or butyrate is produced.

4. Conclusion

Heterologous expression of hydrogenase genes (*hoxEFUYH*) originated from *Synechocystis* sp. PCC 6803 could effectively improve the hydrogen producing ability of *Enterobacter aerogenes*. The hydrogenase activity of the recombinant strain is 237.6 ± 9.3 ml/(g-DW·h), 152% higher than the wild strain. The hydrogen yield of the recombinant strain is 298.3 ml/g-glucose, 88% higher than the wild strain. During hydrogen fermentation, the recombinant strain produces more acetate and butyrate, but less ethanol, which is corresponding to the NADH metabolism. It is necessary to further enhance the hydrogen yield of *E. aerogenes* through metabolic engineering for large-scale hydrogen production.

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