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Improvement of hydrogen yield of ethanol-producing *Escherichia coli* recombinants in acidic conditions

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ABSTRACT

Background: An effective single culture with high glycerol consumption and hydrogen and ethanol coproduction yield is still in demand. A locally isolated glycerol-consuming *Escherichia coli* SS1 was found to produce lower hydrogen levels under optimized ethanol production conditions. Molecular approach was proposed to improve the hydrogen yield of *E. coli* SS1 while maintaining the ethanol yield, particularly in acidic conditions. Therefore, the effect of an additional copy of the native hydrogenase gene *hycE* and recombinant clostridial hydrogenase gene *hydA* on hydrogen production by *E. coli* SS1 at low pH was investigated.
Results: Recombinant *E. coli* with an additional copy of *hycE* or clostridial *hydA* was used for fermentation using 10 g/L (108.7 mmol/L) of glycerol with an initial pH of 5.8. The recombinant *E. coli* with *hycE* and recombinant *E. coli* with *hydA* showed 41% and 20% higher hydrogen yield than wild-type SS1 (0.46 ± 0.01 mol/mol glycerol), respectively. The ethanol yield of recombinant *E. coli* with *hycE* (0.50 ± 0.02 mol/mol glycerol) was approximately 30% lower than that of wild-type SS1, whereas the ethanol yield of recombinant *E. coli* with *hydA* (0.68 ± 0.09 mol/mol glycerol) was comparable to that of wild-type SS1.
Conclusions: Insertion of either *hycE* or *hydA* can improve the hydrogen yield with an initial pH of 5.8. The recombinant *E. coli* with *hydA* could retain ethanol yield despite high hydrogen production, suggesting that clostridial *hydA* has an advantage over the *hycE* gene in hydrogen and ethanol coproduction under acidic conditions. This study could serve as a useful guidance for the future development of an effective strain coproducing hydrogen and ethanol.

1. Introduction

Utilization of glycerol waste to produce hydrogen and ethanol by microbial fermentation has been extensively studied in recent years [1,2,3]. Coproduction of hydrogen and ethanol is considered a beneficial strategy from both economic and environmental perspectives compared to fermentation focusing on either hydrogen or ethanol production alone. Glycerol serves as a promising carbon source to coproduce hydrogen and ethanol because it produces less byproducts than other common sugars [4]. From the equation $C_3H_8O_3 \rightarrow C_2H_5OH + H_2 + CO_2$, 1 mol of hydrogen and 1 mol of ethanol could be yielded from 1 mol of glycerol.

Microorganisms play a key role in the fermentation system. *Clostridium* sp. are well-known as hydrogen producers and are primarily used in research regarding hydrogen fermentation because of their high productivity [5]. However, the presence of oxygen could limit hydrogen production by these strict anaerobes. Hydrogen production is possible using facultative anaerobes such as *Escherichia coli*. Moreover, *E. coli* had been reported to produce hydrogen simultaneously with ethanol [6]. Shams Yazdani and Gonzalez [6] is possibly the first to demonstrate the feasibility of hydrogen and ethanol coproduction by *E. coli*. In their study, engineered *E. coli* SY03 was constructed by inactivating fumarate reductase and phosphate acetyltransferase and achieved a product yield value approaching the theoretical yield value during glycerol fermentation. However, a limitation was that cell growth and glycerol utilization of *E. coli* SY03 were inefficient. *E. coli* SY03 took 120 h to consume approximately 8 g/L (86.96 mmol/L) of glycerol, and fermentation rate was low. The use of microaerobic conditions, adaptive evolution, and chemical mutagenesis can increase the cell growth of *E. coli* [6,7]. However, an effective single culture with high glycerol consumption and product yield is in demand. In our

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previous study, a locally isolated glycerol-consuming *E. coli* SS1 was reported to produce ethanol at the theoretical yield value under optimized ethanol fermentation conditions [8]. Coincidentally, *E. coli* SS1 produced a lower amount of hydrogen simultaneously during the fermentation. According to the preliminary study, the wild-type strain *E. coli* SS1 could consume approximately 80% of the glycerol within 48 h when 10 g/L (108.7 mmol/L) of glycerol was used as the substrate. Moreover, *E. coli* SS1 has an advantage due to uninhibited growth at glycerol concentration of 45 g/L (489.1 mmol/L).

Using a molecular approach to construct ethanol-producing recombinant *E. coli* SS1 with improved hydrogen yield could be promising to develop an effective strain that coproduces hydrogen and ethanol. Hydrogenases are generally present in microorganisms that catalyze the reversible redox reactions of hydrogen [9]. Genetic modification of the hydrogenase gene was hypothesized to improve hydrogen production by *E. coli* SS1. *E. coli* possess multiple hydrogenases. These hydrogenases belong to [NiFe]-hydrogenases that consist of at least two distinct subunits [10]: one large subunit of the core enzyme containing a heterobimetallic active site and additional subunits. Several *E. coli* hydrogenase gene-knockout mutants have been constructed for hydrogenase characterization [11,12,13]. There is lack of information regarding recombinant *E. coli* strains with additional copies of native hydrogenase genes. According to Maeda et al. [14] and Sanchez-Torres et al. [11], *E. coli* hydrogenase 3 is associated with formate dehydrogenase (FDH-H) to form formate hydrogen lyase (FHL) system that is responsible for hydrogen synthesis. The FHL system is activated at low pH.Oxygen-sensitive [FeFe]-hydrogenases, which are present in *Clostridium* sp., exhibit 10 times more active hydrogen-producing activity than [NiFe]-hydrogenases [9]. Subudhi and Lal [15] showed that recombinant *E. coli* BL-21 harboring the hydrogenase gene *hydA* isolated from *Clostridium butyricum* produced a hydrogen yield of 3.2 mol H_2/mol glucose, whereas the host strain did not produce any hydrogen. To date, there was no research report regarding the effect of recombinant *hydA* gene on glycerol fermentation using a host strain harboring *hydA*.

The performance of the recombinant strain highly depends on the applied fermentation conditions [16]. For instance, pH could influence the cell enzyme activity and metabolism, thus affecting the composition of fermentation end products. In a previous study, the optimized ethanol production by *E. coli* SS1 was achieved at pH 7.61, a slightly alkaline condition [17]. According to Murarka et al. [18], the optimum pH for the conversion of glycerol into hydrogen and ethanol was 6.3. According to some studies, hydrogen production by *E. coli* could be improved under acidic condition [16,19]. Chong et al. [5] and Masset et al. [20] stated that optimal hydrogen production by *Clostridium* sp. was approximately at pH 5.5. In this study, the effect of an additional copy of *hycE* (which encodes the large subunit of hydrogenase 3) on hydrogen production by ethanol-producing *E. coli* SS1 under acidic condition was investigated. Moreover, recombinant *E. coli* SS1 containing the *hydA* gene from *Clostridium acetobutylicum* ATCC 824 was also constructed in this study to examine the effect of the *hydA* gene on hydrogen and ethanol coproduction by *E. coli* SS1 under acidic conditions.

2. Materials and methods

2.1. Microorganisms and growth conditions

E. coli SS1 was isolated from soil [8]. The recombinant strains with additional copy of *hycE* and clostridial *hydA* were constructed in this study. The strains were precultured in LB medium (10 g/L (kg/m^3) tryptone, 5 g/L (kg/m^3) yeast extract, and 5 g/L (kg/m^3) NaCl). *C. acetobutylicum* ATCC 824 was grown on reinforced clostridial medium broth under strict anaerobic condition [21].

2.2. Construction of recombinant strains

Genomic DNA of *E. coli* SS1 and *C. acetobutylicum* ATCC 824 were extracted using DNeasy Blood and Tissue Kit (QIAGEN). For the PCR amplification of the *hycE* gene from the genomic DNA of *E. coli* SS1, the oligonucleotide primers were designed according to the nucleotide sequences of *hycE* available in the NCBI database (GenBank accession number: **AAC75763.1**). Each PCR reaction mixture had a total volume of 25 μL containing 1× PCR buffer (10 mM Tris/HCl, 50 mM KCl, 1.5 mM $MgCl_2$), 200 μM of dNTP mix, 0.2 μM each of forward and reverse primers, 1 unit of *Taq* polymerase, and 1.0 μL of the DNA template. The following PCR conditions were used for the amplification: initial denaturation at 95°C (368 K) for 2 min, followed by 30 cycles of denaturation at 95°C (368 K) for 1 min, annealing temperature at 50°C (323 K) for 1 min, elongation at 72°C (345 K) for 1 min, and a final elongation step at 72°C (345 K) for 5 min. The nucleotide sequence analysis for the amplification of full fragments of *hycE* resulted in 1710 bases, and the sequence was found to have about 99% similarity with the sequences of *hycE* in the NCBI database.

After the confirmation of the *hycE* sequence, recombinant plasmids containing the *hycE* and *hydA* genes were constructed using pETDuet (Novagen) vector. The *hycE* and *hydA* genes were PCR amplified using primers listed in Table 1. A *BamH*I restriction site was added to the forward primer, and a *Not*I restriction site was added to the reverse primer. The *hydA* gene with a length of 1749 bases (GenBank accession number: **AAB03723.1**) was PCR amplified from the genomic DNA of *C. acetobutylicum* ATCC 824 [22]. Referencias [23] y [24] citadas en Table 2

The PCR product was then digested with the restriction enzymes *BamH*I and *Not*I. The plasmids were obtained by ligating the resultant digests of the *BamH*I and *Not*I sites into the pETDuet. The plasmids were then transformed by heatshock into the host strain SS1 and then grown on agar plate containing 50 μg/mL (0.05 kg/m^3) ampicillin. The selection for the presence of plasmids was performed by colony PCR. Positive transformants carrying plasmid with *hycE* and *hydA* produced a single band of approximately 2 kb as shown in Fig. 1. The plasmid was extracted using QIAprep Spin Miniprep Kit, and the presence of an insert was confirmed by nucleotide sequencing. Vector pETDuet-1 was driven by the T7-*lac* promoter; *lac* expression systems are typically induced using Isopropyl β-D-1-thiogalactopyranoside (IPTG). In this study, expression of recombinant protein using IPTG was not demonstrated because the lactose present in the tryptone that was used in the preparation of the medium could induce the expression systems.

2.3. Batch fermentation using glycerol

A late log-phase culture (approximately 12 h) was transferred to serum bottles containing medium as described by Ito et al. [1], consisting of (per liter) 0.1 M of potassium phosphate buffers, 1.0 g of $(NH_4)_2SO_4$, 0.25 g of $MgSO_4 \cdot 7H_2O$, 0.021 g of $CaCl_2 \cdot 2H_2O$, 2.0 mg of nicotinic acid, 0.12 g of $Na_2MoO_4 \cdot 2H_2O$, 0.172 mg of Na_2SeO_3, 0.02 mg of $NiCl_2$, 6.8 g of yeast extract, 6.8 g of tryptone, and 10 mL of trace element solution. The trace element solution contained (per liter) 0.5 g of $MnCl_2 \cdot 4H_2O$, 0.1 g of H_3BO_4, 0.01 g of $AlK(SO_4)_2 \cdot H_2O$, 1.0 mg of $CuCl_2 \cdot 2H_2O$, and 0.5 g of Na_2EDTA. Pure glycerol (10 g/L; 108.7 mmol/L) was used as substrate. Then, 75 mL of the medium was

Table 1
Primers used in this study.

Primers	Sequence
hycE-Fw	GCGGATCCATGTCTGAAGAAAAATTAGGTC
hycE-Rv	GATATGCGGCCGCTTATTTCAGCGGCGAG
hydA-Fw	GCGGATCCATGAAAACAATAATCTTAAATGGCAAT
hydA-Rv	GATATGCGGCCGCTTATTCATGTTTTGAAACATT

[a]Fw, forward; Rv, reverse; underlined sequence in primers indicate introduced restriction enzyme sites (*BamH*I and *Not*I).