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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).

Table 2
Product yield achieved by various microorganisms using 10 g/L (108.7 mmol/L) pure glycerol.

Culture	pH	(°C)	Hydrogen yield (mol/mol glycerol)	Ethanol yield (mol/mol glycerol)	Source
E. aerogenes HU-101	6.8	37	0.71	0.67	Ito et al. [1]
Klebsiella sp. HE1	6.0	35	0.04	0.80	Wu et al. [28]
E. coli BW25113	6.3	37	0.83	0.66	Durnin et al. [29]
E. coli MG1655	6.3	37	0.08	0.78	Chaudhary et al. [4]
E. coli SS1	5.8	37	0.46	0.70	Current study

sparged with nitrogen gas for 15 min. Anaerobic fermentation was carried out at 37°C (310 K) with an agitation speed of 120 rpm for 72 h. The experiments were performed in triplicate.

2.4. Analytical method

Evolved gas was collected in Hungate tubes using the water displacement method. Hydrogen gas concentration was analyzed using a gas chromatograph (GC8A-Shimadzu Co., Japan) equipped with a thermal conductivity detector. The amount of hydrogen produced was calculated from the headspace measurement of gas composition and total biogas composition at each sampling interval under standard temperature and pressure conditions (298 K, 101.3 kPa) [5]. Fermentation broths were collected and centrifuged at 10,000 × g for 10 min. The diluted supernatant was filtered through 0.2-μm membranes. The filtered supernatant was then used to measure ethanol and glycerol contents. Ethanol was measured using a gas chromatograph (GC7890A-Agilent) equipped with a flame ionization detector and a DB-WAX column. Helium was used as a carrier gas. Glycerol concentration was measured by colorimetric detection using a glycerol assay kit (Sigma-Aldrich). The hydrogen produced was expressed in terms of yield and productivity. Product yield was calculated by dividing the amount of product (mol) by the amount of glycerol consumed (mol). Productivity was expressed as mol of product produced per liter of medium per hour, calculated by the maximum of product yield (mol/L) divided by time in hours [2]. The data were analyzed by one-way analysis of variance to obtain the mean value, standard deviation, and the p-value of the tested sample data. Differences of $P < 0.05$ were considered significant.

3. Results and discussion

3.1. Hydrogen production by E. coli SS1 and the recombinant with additional copy of the hycE gene

E. coli SS1 consumed glycerol and produced hydrogen and ethanol simultaneously within 48 h of fermentation as shown in Fig. 2. *E. coli* SS1 showed a hydrogen yield of 0.46 ± 0.01 mol/mol glycerol and ethanol yield of 0.70 ± 0.02 mol/mol glycerol when the initial pH was 5.8. This result was comparable to those of the hydrogen and ethanol coproducing wild-type strains reported in the literature (Table 2).

Recombinant *E. coli* with an additional copy of the *hycE* gene, which encoded the large subunit of hydrogenase 3, showed a hydrogen yield of 0.65 ± 0.04 mol/mol glycerol with an initial pH of 5.8 (Table 3). This was approximately 41% higher than wild-type SS1. This indicated that an additional copy of *hycE* could improve the hydrogen yield of SS1 in acidic conditions. This result is in agreement with those in the literature where it was stated that hydrogenase 3 is preferable to produce hydrogen at acidic pH but catalyzes the oxidation of hydrogen during glycerol fermentation at an initial pH of 7.5 [12,13,14]. According to Sanchez-Torres [11], *hycE* knockout mutant had reduced hydrogen production (approximately 10 μmol/mg protein) after 48 h at pH 6.5 compared to wild-type *E. coli* BW25113 (approximately 100 μmol/mg protein). There are no studies regarding *E. coli* strain with an additional copy of native hydrogenase gene; the present study is believed to be the first to reveal that an additional copy of the *hycE* gene could increase the hydrogen yield of *E. coli* at low pH. *E. coli* SS1 with an additional copy of the *hybC* gene, which encoded the large subunit of hydrogenase 2, was also used for fermentation using 10 g/L (108.7 mmol/L) of glycerol as carbon source with an initial pH of 5.8 (data not shown). The recombinant *hybC* was

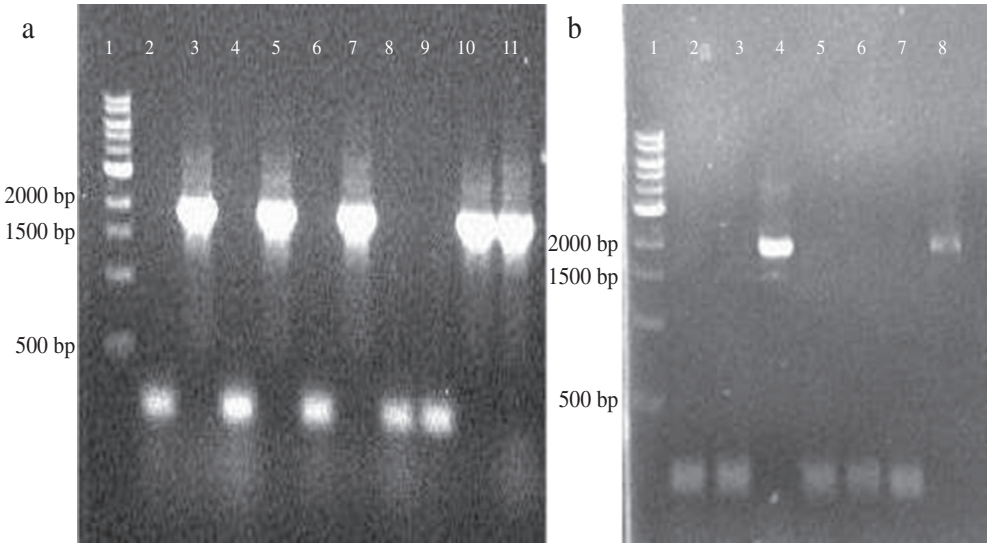

Fig. 1. Screening of the positive transformants using colony PCR. (a) Screening of the positive transformants carrying plasmid with the *hycE* gene (lanes 2 to 11: colony PCR products; lanes 3, 5, 7, 10 and 11: positive transformants carrying plasmid with the *hycE* gene; lanes 2, 4, 6, 8, and 9: negative transformants carrying plasmid without the insert DNA), and (b) *hydA* gene (lanes 2 to 8: colony PCR products; lanes 4 and 8: positive transformants carrying plasmid with the *hydA* gene; lanes 2, 3, 5, 6, and 7: negative transformants carrying plasmid without the insert DNA). Lane 1:1 kb DNA ladder (New England Biolabs, USA).

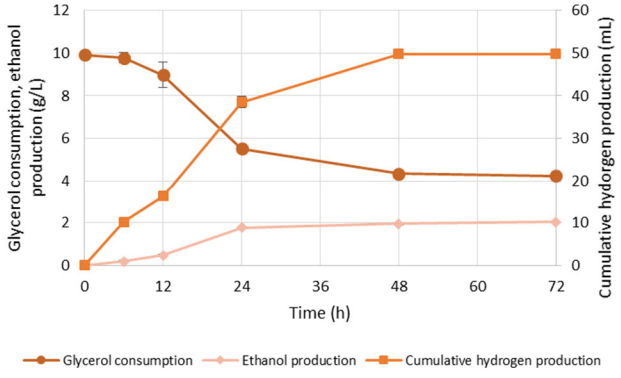

Fig. 2. Fermentation profile of *E. coli* SS1 during glycerol fermentation with an initial pH of 5.8.

found to produce a hydrogen yield of 0.04 ± 0.03 mol/mol glycerol, which was approximately 90% lower than that of wild-type *E. coli* SS1 and recombinant *E. coli* with *hycE*. The impaired hydrogen production by recombinant *E. coli* with *hybC* was most likely due to the hydrogen uptake characteristic of hydrogenase 2 [14].

Although recombinant *E. coli* with *hycE* resulted in higher hydrogen yield when the initial pH was 5.8 than wild-type SS1, the hydrogen productivity of recombinant *E. coli* with *hycE* was 0.49 ± 0.02 mmol/L/h, which was lower than that of wild-type SS1 (0.58 ± 0.01 mmol/L/h) (Table 3). This observation could be because of the lower glycerol consumption rate. Wild-type SS1 consumed 5.59 ± 0.05 g/L (60.76 ± 0.54 mmol/L) of glycerol when the initial pH was 5.8, whereas total glycerol consumed by recombinant *E. coli* with *hycE* was barely 3.30 ± 0.08 g/L (35.87 ± 0.87 mmol/L) (Table 3). It was suggested that glycerol consumption was correlated with hydrogen production. Under anaerobic conditions, glycerol is metabolized into hydrogen and ethanol through several pathways (Fig. 3). Glycerol is first broken down to dihydroxyacetone phosphate followed by glyceraldehyde-3-phosphate and phosphoenolpyruvate. The phosphoenolpyruvate is then broken down to pyruvate, which is the central intermediate to yield acetyl-CoA and formate subsequently. Acetyl-CoA is be converted into either acetate or ethanol, whereas formate is converted into hydrogen and carbon dioxide [19]. It was suggested that an additional copy of the *hycE* gene could regulate glycerol metabolism and thus decrease glycerol consumption by *E. coli* SS1. However, more studies are required to identify the true role of the *hycE* gene in glycerol metabolism.

Moreover, the ethanol yield of recombinant *E. coli* with *hycE* during fermentation when the initial pH was 5.8 was 0.50 ± 0.02 mol/mol glycerol, which was approximately 30% lower than that of wild-type SS1. This indicated that an additional copy of *hycE* significantly affected the ethanol yield of SS1 in acidic conditions. This may be due to higher hydrogen production, which inhibits the production of ethanol. Hydrogen production by hydrogenase 3 is associated with FHL complex where FDH-H first decomposes formate to H^+, 2 e^-, and CO_2. The electrons derived from formate oxidation are then used for the reduction of protons to hydrogen gas, which is catalyzed by

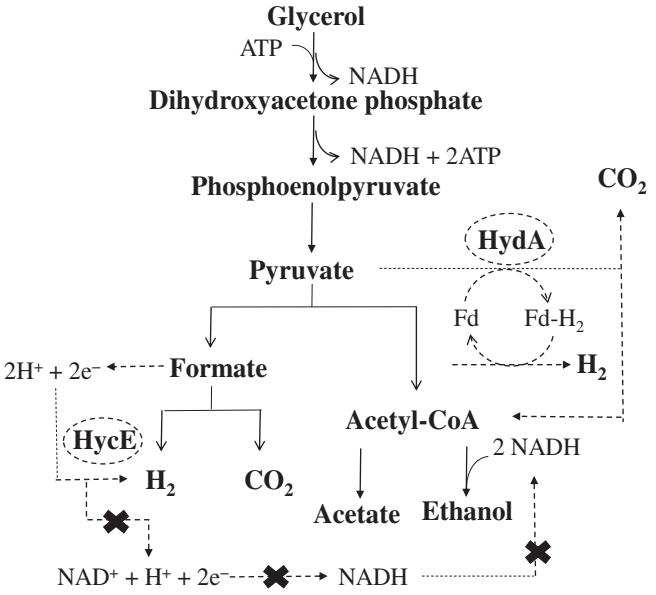

Fig. 3. Glycerol metabolic pathways in recombinant *E. coli* with an additional copy of *hycE* gene or the clostridial *hydA* gene. Additional copy of the *hycE* gene in SS1 decreased H^+ and electrons at higher levels during the conversion of formate into hydrogen; thus, there are limited H^+ and electrons for the formation of NADH, which is required in the reactions catalyzed by acetaldehyde dehydrogenase and ethanol dehydrogenase to produce ethanol. Recombinant clostridial *hydA* gene in SS1 could generate hydrogen by pathways facilitated by pyruvate:ferredoxin oxidoreductase, where pyruvate is broken down into acetyl-CoA and carbon dioxide accompanied by the reduction of oxidized ferredoxin (Fd). Solid line (—) represents hydrogen and ethanol synthesis pathways in wild-type SS1; dashed line (···) represents the effect of recombinant on the metabolic pathways.

hydrogenase 3 [23,24]. The additional copy of *hycE* could have decreased H^+ and electrons at higher level (Fig. 3). Consequently, there are limited H^+ and electrons for the formation of NADH that is required in the reactions catalyzed by acetaldehyde dehydrogenase and ethanol dehydrogenase to produce ethanol. Therefore, higher hydrogen yield of recombinant *E. coli* with *hycE* under acidic conditions may limit ethanol synthesis, leading to lower ethanol yield. It was noted that the inhibition of NADH formation could reduce the activity of both glycerol dehydrogenase and glyceraldehyde-3-phosphate dehydrogenase; thus, glycerol consumption of recombinant *E. coli* with *hycE* was also affected as discussed earlier.

3.2. Hydrogen production by the recombinant with clostridial hydA gene

With an initial pH of 5.8, recombinant *E. coli* with clostridial *hydA* gene showed a hydrogen yield of 0.58 ± 0.01 mol/mol glycerol, which was approximately 20% higher than that of wild-type SS1 (Table 3). The reason for the increase in the hydrogen yield of recombinant *E. coli* with *hydA* was possibly the activation of the clostridial hydrogenase gene for hydrogen production under acidic conditions [5,20]. There

Table 3
Hydrogen yield, hydrogen productivity, ethanol yield, ethanol productivity, and glycerol consumption achieved by *E. coli* SS1 and recombinant *E. coli* with *hycE* or h*ydA* when the initial pH was 5.8.

Strain	Hydrogen yield (mol/mol glycerol)	Hydrogen productivity (mmol/L/h)	Ethanol yield (mol/mol glycerol)	Ethanol productivity (mmol/L/h)	Glycerol consumed (g/L)
E. coli SS1	0.46 ± 0.01^{c}	0.58 ± 0.01^{a}	0.70 ± 0.02^{a}	0.85 ± 0.03^{a}	5.59 ± 0.05^{a} (60.76 ± 0.54 mmol/L)
Recombinant with *hycE*	0.65 ± 0.04^{a}	0.49 ± 0.02^{b}	0.50 ± 0.02^{b}	0.63 ± 0.01^{c}	3.30 ± 0.08^{b} (35.87 ± 0.87 mmol/L)
Recombinant with *hydA*	0.58 ± 0.01^{b}	0.55 ± 0.04^{a,b}	0.68 ± 0.09^{a}	0.73 ± 0.05^{b}	4.86 ± 0.06^{a} (52.83 ± 0.65 mmol/L)

^{abc} Different superscript small letters within the same column indicate significant differences ($P < 0.05$) between different strains at the same initial pH.

were previous studies that reported on the overexpression of clostridial hydrogenase in native host that resulted in enhanced hydrogen production. Morimoto et al. [17] had constructed a recombinant *Clostridium paraputrificum* overexpressing the *hydA* gene that achieved 1.7-fold increase in hydrogen yield from N-acetylglucosamine. The recombinant overexpressing *hydA* showed abolished lactate production and increased acetate production. Moreover, Jo et al. [25] demonstrated the homologous overexpression of the *hydA* gene in *Clostridium tyrobutyricum* JM1. In comparison to the wild-type, the hydrogen yield of the recombinant had increased by 1.5-fold during fermentation using 15 g/L (83.08 mmol/L) of glucose. The lactate production was reduced and butyrate production was increased in the recombinant, indicating that the overexpression of *hydA* manipulated the metabolic pathway of *C. tyrobutyricum*. In recent years, *E. coli* harboring the *hydA* gene has been constructed for gene expression study purpose; however, there is lack of information regarding recombinant *hydA* gene in *E. coli* for increased hydrogen production by the host strain [15]. The improved hydrogen yield of recombinant *E. coli* with *hydA* during glycerol fermentation at low pH was first demonstrated in this study. This could provide a better understanding of the effect of clostridial *hydA* on hydrogen production from glycerol.

Recombinant *E. coli* with *hydA* gave an ethanol yield of 0.68 ± 0.09 mol/mol glycerol, which was comparable to that of wild-type SS1 when the initial pH was 5.8. It was suggested that the recombinant clostridial *hydA* gene can retain the ethanol production in acidic conditions despite higher hydrogen production. [FeFe]-hydrogenase is a ferredoxin-dependent enzyme. Ferredoxin is reduced during the oxidative decarboxylation of pyruvate by a pyruvate ferredoxin oxidoreductase. The reduced ferredoxin serves as the physiological electron donor of [FeFe]-hydrogenase [26]. The recombinant clostridial *hydA* gene may provide an alternate pathway that utilizes reduced ferredoxin as an electron donor instead of producing hydrogen through the FHL complex (Fig. 3). In this way, hydrogen could be produced without affecting the H^+ and electron levels. Thus, the formation of NADH and the reactions of acetaldehyde dehydrogenase and ethanol dehydrogenase might be not influenced. Therefore, ethanol yield of recombinant *E. coli* with *hydA* was not constricted by high hydrogen yield under acidic conditions. This suggests that recombinant *E. coli* SS1 with clostridial hydrogenase could be a better option for hydrogen and ethanol coproduction under acidic conditions.

According to Suhaimi et al. [8], ethanol production by *E. coli* SS1 was optimum at slightly alkaline conditions. Thus, glycerol fermentation using recombinant *E. coli* with *hydA* was performed with an initial pH of 7.5 to examine its product yields as compared to that of wild-type SS1 under alkaline condition. The hydrogen yield of recombinant *E. coli* with *hydA* was only half of that achieved by wild-type SS1 when the initial pH was 7.5, and the ethanol yield of wild-type SS1 was higher than that of recombinant *E. coli* with *hydA* (Table 4). The pH of crude glycerol generated from biodiesel industry is in a broad range of 2 to 11 depending on the plant production process [27]. Although wide-type SS1 gave higher product yields under alkaline conditions, recombinant *E. coli* with *hydA* would be more preferable for biofuel production using crude glycerol at low pH. It was proposed that using a mixed culture of wild-type SS1 and recombinant *hydA* in a fermentation system might eliminate the effect of initial pH on hydrogen and ethanol coproduction compared to fermentation using a single batch culture. The result obtained by mixed culture is shown in Table 4 and was fairly the same when the initial pH was 5.8 or 7.5. The recombinant *E. coli* with *hydA* might rectify the low hydrogen yield of wild-type SS1 at lower pH, whereas wild-type SS1 might rectify the low hydrogen yield of recombinant *hydA* at higher pH. This suggested that mixed culture could retain relatively similar hydrogen and ethanol yields regardless of initial pH. Eventually, this can be possibly applied in fermentation system that utilize glycerol waste with a wide range of pH.

4. Conclusion

Additional copy of native hydrogenase gene *hycE* and recombinant clostridial *hydA* could improve the hydrogen yield of *E. coli* SS1 during glycerol fermentation when the initial pH is 5.8. Nevertheless, clostridial *hydA* could retain ethanol yield, suggesting that recombinant *E. coli* with *hydA* is a better option for hydrogen and ethanol coproduction by *E. coli* SS1 under acidic conditions. Improvement in the development of a culture system for efficient hydrogen and ethanol coproduction is still in the infancy stage. Substantial research is required for the future development of coproduction of both biofuels using glycerol-containing wastewater from biodiesel industries.

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Conflict of interest

The authors declare that they have no conflict of interest.

Ethical approval

This article does not contain any studies with human participants or animals performed by any of the authors.

Informed consent

This article does not involve any informed consent.

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Table 4
Hydrogen and ethanol yields and productivities achieved by mixed culture of *E. coli* SS1 and recombinant *E. coli* with *hydA* when the initial pH is 5.8 and 7.5.

Culture		*E. coli* SS1	Recombinant *hydA*	Mixed culture
Initial pH 5.8	Hydrogen yield (mol/mol glycerol)	0.46 ± 0.01[a]	0.58 ± 0.01[a]	0.57 ± 0.09[a]
	Hydrogen productivity (mmol/L/h)	0.58 ± 0.01[b]	0.55 ± 0.04[b]	0.70 ± 0.14[a]
	Ethanol yield (mol/mol glycerol)	0.70 ± 0.02[a]	0.68 ± 0.09[a]	0.79 ± 0.06[a]
	Ethanol productivity (mmol/L/h)	0.85 ± 0.03[a,b]	0.73 ± 0.05[b]	0.98 ± 0.12[a]
Initial pH 7.5	Hydrogen yield (mol/mol glycerol)	0.57 ± 0.02[a]	0.28 ± 0.06[c]	0.49 ± 0.01[b]
	Hydrogen productivity (mmol/L/h)	0.95 ± 0.04[a]	0.42 ± 0.07[c]	0.82 ± 0.02[b]
	Ethanol yield (mol/mol glycerol)	0.88 ± 0.04[a]	0.76 ± 0.05[a]	0.80 ± 0.18[a]
	Ethanol productivity (mmol/L/h)	1.45 ± 0.04[a]	1.01 ± 0.04[a]	1.34 ± 0.32[a]

[abc] Different superscript small letters within the same row indicate significant differences ($P < 0.05$) between different strains at the same initial pH.

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