Electronic Journal of Biotechnology 30 (2017) 64–70

Contents lists available at ScienceDirect

Electronic Journal of Biotechnology

Research article

Co-production of hydrogen and ethanol by *Escherichia coli* SS1 and its recombinant

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ARTICLE INFO

Article history:
Received 30 May 2017
Accepted 5 September 2017
Available online 20 September 2017

Keywords:
Alkaline
Fermentation
Glucose
Glycerol
hybC
Hydrogenase
Industrial application
Recombinant

ABSTRACT

Background: The development of a potential single culture that can co-produce hydrogen and ethanol is beneficial for industrial application. Strain improvement via molecular approach was proposed on hydrogen and ethanol co-producing bacterium, *Escherichia coli* SS1. Thus, the effect of additional copy of native hydrogenase gene *hybC* on hydrogen and ethanol co-production by *E. coli* SS1 was investigated.
Results: Both *E. coli* SS1 and the recombinant *hybC* were subjected to fermentation using 10 g/L of glycerol at initial pH 7.5. Recombinant *hybC* had about 2-fold higher cell growth, 5.2-fold higher glycerol consumption rate and 3-fold higher ethanol productivity in comparison to wild-type SS1. Nevertheless, wild-type SS1 reported hydrogen yield of 0.57 mol/mol glycerol and ethanol yield of 0.88 mol/mol glycerol, which were 4- and 1.4-fold higher in comparison to recombinant *hybC*. Glucose fermentation was also conducted for comparison study. The performance of wild-type SS1 and recombinant *hybC* showed relatively similar results during glucose fermentation. Additional copy of *hybC* gene could manipulate the glycerol metabolic pathway of *E. coli* SS1 under slightly alkaline condition.
Conclusions: HybC could improve glycerol consumption rate and ethanol productivity of *E. coli* despite lower hydrogen and ethanol yields. Higher glycerol consumption rate of recombinant *hybC* could be an advantage for bioconversion of glycerol into biofuels. This study could serve as a useful guidance for dissecting the role of hydrogenase in glycerol metabolism and future development of effective strain for biofuels production.

1. Introduction

Microbial fermentation using low cost sustainable waste as substrates for renewable biofuels production has been extensively studied due to its contribution for environmental advantages and commercial benefits. Biodiesel production generates abundant waste glycerol, which serves as one of the popular carbon sources used in microbial fermentation. Microorganisms are able to degrade glycerol into metabolite products such as 1,3-propanediol, ethanol, acetic acids, lactic acids, succinic acids, hydrogen and carbon dioxide under fermentation conditions [1]. Among these fermentation products, hydrogen and ethanol have enormous value and great potential as alternative fuels for future. Hydrogen is well-known as an efficient energy that can be used for many applications including alternates for fossil fuels, electricity and thermal energy generation. On the other hand, ethanol is widely used as major substitute to gasoline as alternative fuel. Therefore, simultaneous production of both hydrogen and ethanol using waste glycerol has received increasing attention in biofuel industry.

Microorganisms play a key role in fermentation system to yield desired products. *Escherichia coli* was identified as one of the microorganisms that able to ferment glycerol into hydrogen and ethanol [2]. *E. coli*, which belongs to facultative anaerobes that are tolerant to oxygen, has an advantageous over strict anaerobes such as *Clostridium* sp. Besides that, its well-studied characterization and ease of molecular engineering compared to other species such as *Klebsiella* and *Enterobacter* further elucidate the reason for developing researches in simultaneous production of hydrogen and ethanol using *E. coli* [3]. Theoretically, 1 mol of glycerol could produce 1 mol of hydrogen and 1 mol of ethanol, respectively [4]. Yazdani and Gonzalez [5] performed genetic modification on *E. coli* to co-produce hydrogen and ethanol approaching theoretical yield during glycerol fermentation. Nevertheless, in their study, the engineered *E. coli* SY03 was inefficient in cell growth and glycerol utilization. Fermentation using *E. coli* to co-produce hydrogen and ethanol is still at its infancy stage to accomplish the feasibility in industrial applications. Thus,

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Peer review under responsibility of Pontificia Universidad Católica de Valparaíso.

https://doi.org/10.1016/j.ejbt.2017.09.002

more related studies are still required. Previous work done by Suhaimi et al. [6] reported that locally isolated *E. coli* strain SS1 is able to consume glycerol at high concentration to produce ethanol at theoretical yield under optimized fermentation condition. Based on the preliminary study, *E. coli* SS1 has an advantage due to uninhibited growth at glycerol concentration of 45 g/L. However, concurrent hydrogen production was rather low.

Hydrogenase is the enzyme identified to catalyze the reversible redox reactions of hydrogen. According to previous study [7], recombinant *E. coli* SS1 with an additional copy of *hycE* gene which encoded large subunit of Hydrogenase 3 showed 1.4-fold higher hydrogen yield at initial pH 5.8, while the wild-type SS1 exhibited 1.4-fold higher ethanol yield than recombinant *hycE*. Hydrogenases 2 was claimed to play a role for increased hydrogen production by *E. coli* at slightly alkaline condition under glycerol fermentation [8]. Hydrogenase 2 of *E. coli* is transcribed from the *hyb* operon which composed of eight genes (*hybOABCDEFG*) and *hybC* encodes the large subunit [9]. Trchounian and Trchounian [8] reported that *E. coli hybC* knockout mutant had diminished hydrogen production rate about 100% compared to wild-type. According to Maeda et al. [10], the role of Hydrogenase 2 is responsible for the hydrogen uptake activity in *E. coli* during glucose fermentation. The role of this hydrogenase in hydrogen metabolism is still ambiguous. Hence, further study regarding Hydrogenase 2 is vital to develop a superior hydrogen producing recombinant strain. To date, there was no research report regarding *E. coli* recombinant strain with additional copy of *hybC* gene. In the present work, the effect of *hybC* gene on hydrogen and ethanol co-production by *E. coli* strain SS1 under glycerol fermentation was investigated. Glucose fermentation was also demonstrated for comparison study.

2. Materials and methods

2.1. Culture conditions

The *E. coli* SS1 used in this study was isolated from soil [6]. The recombinant strain with additional copy of *hybC* was constructed in this study. The strains were pre-cultured in LB medium consisting of 10 g/L of tryptone, 5 g/L of yeast extract, and 5 g/L of NaCl.

2.2. Construction of recombinant strains

Expression vector pETDuet-1 (Novagen) was used for cloning and sequencing of *hybC* gene in *E. coli* strain SS1. The *hybC* gene used was isolated from genomic DNA of *E. coli* strain SS1 and was PCR amplified using forward primer designed with the addition of *BamH*I restriction site 5′-GCGGATCCATGAGCCAGAGAATTACTATTGATC-3′ and reverse primer designed with the addition of *Not*I restriction site 5′-GATATGCGGCCGCTTACAGAACCTTCACTGAAACCA-3′ (restriction sites are underlined). The oligonucleotide primers were designed according to the nucleotide sequences of *hybC* available in NCBI database (GenBank accession number: **AAA21591.1**). 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 $MgCl_2$), 0.2 mM of dNTP mix, 0.2 μM of each of forward and reverse primers, 0.04 U/μL of *Taq* polymerase, and approximately 200 ng of the DNA template. The following PCR conditions were employed for the amplification; initial denaturation at 95°C for 2 min, followed by 30 cycles of denaturation at 95°C for 1 min, annealing temperature at 50°C for 1 min, elongation at 72°C for 1 min and a final elongation step at 72°C for 5 min. The nucleotide sequence analysis for the amplification of full fragments of *hybC* gene resulted in 1704 bases, which was found to be 100% similarity with the sequence of *hybC* that is available in the NCBI database.

The plasmid was obtained by digesting the PCR product with restriction enzyme *BamH*I and *Not*I, and then ligating with the resulting digest within the *BamH*I and *Not*I sites of pETDuet-1. The plasmid was then transformed via heatshock into host strain SS1. Selection for the presence of plasmids was carried out in the presence of 50 μg/ml ampicillin. Colonies grown on the agar plate in the presence of ampicillin were selected randomly for colony PCR to determine the presence of insert DNA in plasmid. Plasmid extraction was performed using QIAprep Spin Miniprep Kit. The positive transformants carrying plasmid with insertion of *hybC* produced a single band with approximately 2 kb as shown in Fig. 1. Upon nucleotide sequencing of plasmids, the DNA inserts were confirmed as *hybC*. Vector pETDuet-1 is driven by the T7-*lac* promoter, *lac* expression systems are typically induced using IPTG. In this study, expression of recombinant *hybC* protein using IPTG was not demonstrated due to the lactose which present in the tryptone that was used in the preparation of medium could induce the expression systems.

2.3. Batch fermentation

The late log phase culture (approximately 12 h) was transferred to serum bottles containing medium consisted of (per liter): 0.1 M potassium phosphate buffer (pH 7.5), 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 [11]. 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. According to previous study [12], *E. coli* SS1 showed the highest hydrogen and ethanol co-production yield at glycerol concentration of 10 g/L. Thus, pure glycerol of 10 g/L was used as substrate in this study. The medium with a total volume of 75 mL was sparged with nitrogen gas for 15 min. The anaerobic fermentation was carried out at temperature of 37°C with an agitation speed of 120 rpm. The sampling was done for fermentation time at (h): 0, 6, 12, 24, 48, and 72. The OD_{600}, pH level and gas production were monitored during the course of experiments. The experiments were performed in triplicate. Anaerobic fermentation was repeated using glucose as substrate to compare the glycerol fermentation and glucose fermentation by wild-type *E. coli* SS1 and the recombinant *hybC*. Glucose was sterilized separately from medium by using membrane filtration through 0.2 μm membranes. The medium was prepared by substituting glycerol to glucose of 10 g/L. Noted that 1 mol of glycerol and glucose carry same percentage of carbon atoms (40%),

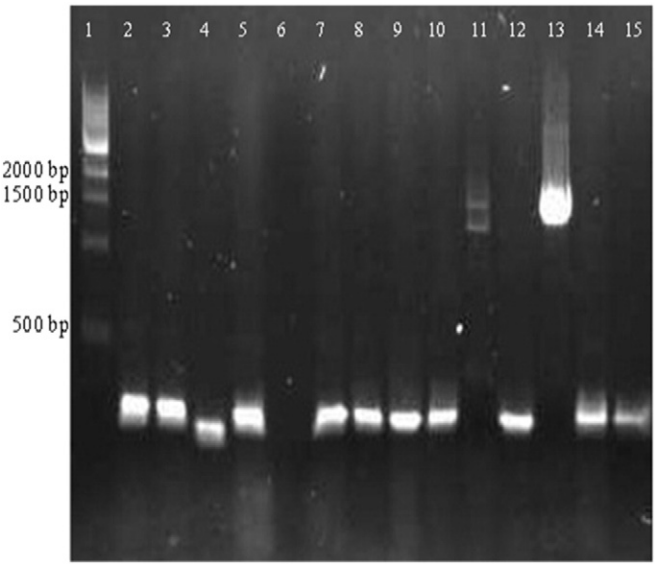

Fig. 1. Screening of the positive transformant carrying plasmid with insertion of *hybC* gene using colony PCR. Lane 1 represents 1 kb DNA ladder (New England Biolabs, USA); lanes 2–15 represent colony PCR products; lane 13 represents positive transformant.

concentration of the glucose used was similar as glycerol to ensure the amount of carbon source supplied in both fermentation was same (0.11 mol).

2.4. Analytical methods

The optical density was estimated by spectrophotometric analysis to measure relative cell mass indirectly. Absorbance was measured at a wavelength of 600 nm. Evolved gas was collected in Hungate tubes using water displacement method. Hydrogen gas composition and concentration were analyzed using gas chromatography (GC7890A-Agilent) equipped with a thermal conductivity detector and a CP-carboPLOT P7 column. Fermentation broth was collected and centrifuged at 10,000 rpm for 10 min. The supernatant was used for both ethanol and glycerol analysis. The diluted supernatant was filtered through 0.2 μm membranes. Ethanol was measured by gas chromatography (GC7890A-Agilent) equipped with a flame ionization detector and a DB-WAX column. Helium was used as a carrier gas. Glycerol concentration was measured using glycerol assay kit (Sigma-Aldrich). Glucose concentration was monitored by performing glucose assay analysis (Biovision).The hydrogen and ethanol produced were expressed in terms of yield and productivity. Product yield was calculated by dividing the amount of product (mol) by the amount of substrate 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 hour [13].

3. Results and discussion

3.1. Cell growth of E. coli SS1 and its recombinant during fermentation

The *E. coli* wild-type SS1 and recombinant strain with additional copy of *hybC* were subjected to glycerol and glucose fermentation at initial pH 7.5, respectively to study their hydrogen and ethanol co-production profile. Wild-type SS1 and recombinant *hybC* had exponential growth at first 12 h during glycerol fermentation as shown in Fig. 2a. The recombinant *hybC* reached maximum OD_{600} as high as 3.04 which was approximately 2-fold higher than wild-type SS1 (OD_{600} = 1.70). During glucose fermentation, both of wild-type SS1 and recombinant *hybC* had exponential growth at first 6 h approaching OD_{600} approximately 3.30 and reached stationary phase, respectively. The recombinant *hybC* had approximately 2-fold higher cell growth than wild-type SS1 during glycerol fermentation probably due to *hybC* gene plays a role for anaerobic cell growth using glycerol as carbon source under slightly alkaline condition. The observation was in agreement to the previous study of Sanchez-Torres et al. [14] suggested that Hydrogenase 2 is required to have optimum growth using glycerol as sole carbon source. The authors reported that *hybC* knockout mutant had 2-fold lower cell growth compared to wild-type after 120 h of glycerol fermentation at initial pH 7.5. Nevertheless, wild-type SS1 and recombinant *hybC* showed fairly the same cell growth indicating *hybC* gene did not play a role in anaerobic cell growth during glucose fermentation at initial pH 7.5. The cell growth of wild-type SS1 during glucose fermentation had doubled in comparison to glycerol fermentation. This observation was in agreement with Chaudhary et al. [15] who reported that the cell growth rate of *E. coli* K12 in glucose fermentation was higher than that of glycerol fermentation.

3.2. Glycerol consumption of E. coli SS1 and its recombinant during fermentation

Substrate consumption was monitored during the course of experiment. At initial pH 7.5, wild-type SS1 consumed glycerol proportionally with time for a duration of 72 h, while glycerol consumption of recombinant *hybC* occurred predominantly within the

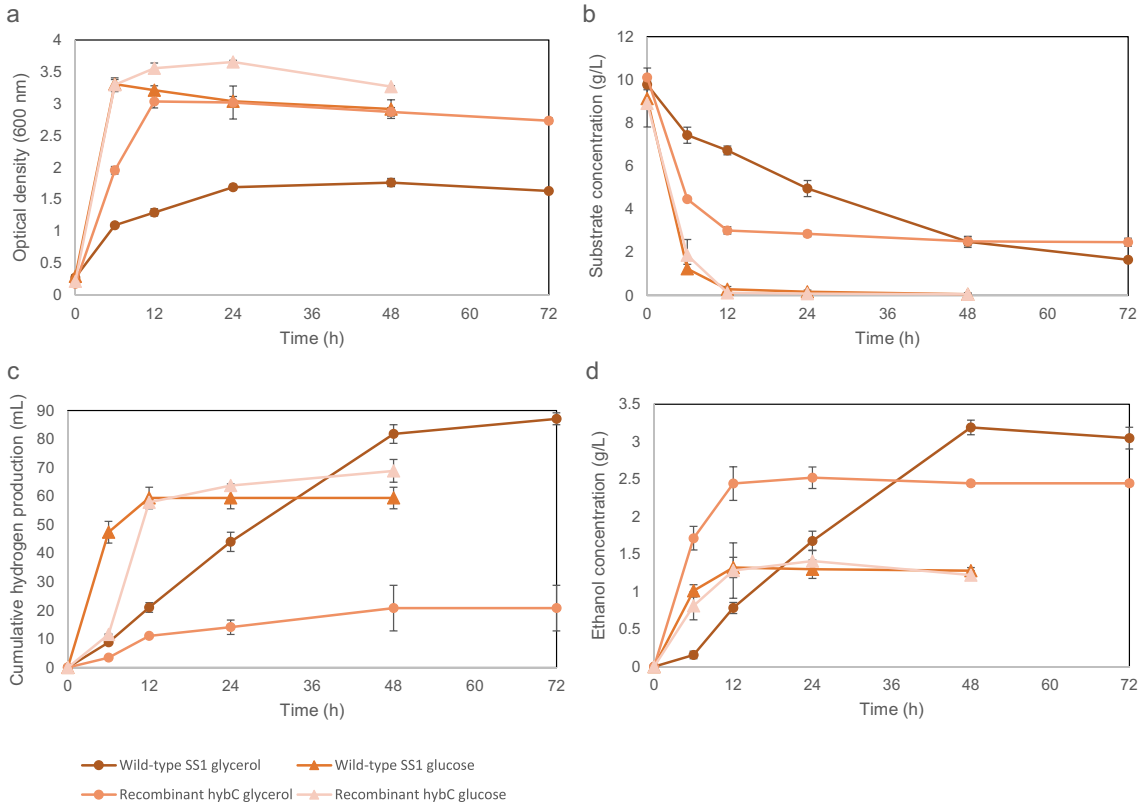

Fig. 2. Growth profile (OD_{600}) (a), substrate consumption (b), cumulative hydrogen production (c), and ethanol production (d) of wild-type SS1 and recombinant *hybC* during fermentation at initial pH 7.5.

first 12 h as shown in Fig. 2b. Total glycerol consumed by both strains was approximately in the range of 7.30 to 7.60 g/L after 48 h. Approximately 8.8 g/L of glucose was consumed rapidly by both wild-type SS1 and recombinant *hybC* within 12 h of fermentation at initial pH 7.5, respectively. As for glycerol fermentation, the recombinant *hybC* (0.591 g/L/h) had about 5.2-fold higher glycerol consumption rate than wild-type SS1 (0.113 g/L/h) at initial pH 7.5. Thus, it was proposed that additional copy of *hybC* could accelerate glycerol metabolism (further discussion in Section 3.5) at slightly alkaline condition. However, both strains had about the same glucose consumption rate suggesting that an additional copy of *hybC* did not play a role in glucose metabolism at initial pH 7.5.

3.3. Hydrogen production of E. coli SS1 and its recombinant during fermentation

Fig. 2c illustrates the cumulative hydrogen production of wild-type SS1 and recombinant *hybC* during glycerol and glucose fermentation at initial pH 7.5. Wild-type SS1 produced gas gradually accumulating 80 mL of hydrogen within 48 h of fermentation when glycerol was used. On the other hand, the recombinant *hybC* produced 11 mL of accumulated hydrogen within 12 h of fermentation, and gas was scarcely produced after 12 h. This was probably due to glycerol consumption that occurred predominantly within the first 12 h. As for glucose fermentation, the cumulative hydrogen production had an exponential phase within 12 h for both wild-type SS1 and recombinant *hybC*, accumulating 59 and 55 mL of hydrogen, respectively. The hydrogen yield and productivity of wild-type SS1 and recombinant *hybC* are presented in Table 1. The recombinant *hybC* had reduced hydrogen yield by 4-fold compared to wild-type SS1 during glycerol fermentation at initial pH 7.5, probably due to the fact that the additional copy of *hybC* had negatively affected the hydrogen production by SS1 when glycerol was used as substrate. Trchounian and Trchounian [8] revealed that *hybC* knockout mutant had lower hydrogen production rate at pH 7.5 in comparison to wild-type, and thus hypothesized that Hydrogenase 2 was responsible for hydrogen production during glycerol fermentation under alkaline condition. However, it was later opposed by Sanchez-Torres et al. [14] whom proposed that the diminished glycerol utilization capability was the primary factor of low hydrogen production by *hybC* mutant. Moreover, variation of this study and the findings by Trchounian and Trchounian [8] might be due to differences in medium composition and the characteristic of microorganism used. At initial pH 5.8, the recombinant *hybC* was found to produce hydrogen yield of 0.04 ± 0.03 mol/mol glycerol which was about 11-fold lower than wild-type SS1. In contrary, recombinant *hycE* in the previous study [7] showed improved hydrogen yield (0.65 mol/mol glycerol). This indicated that additional copy of *hybC* gene could reduce hydrogen yield regardless of pH, probably due to the mechanism involved which will be discussed in Section 3.5.

For glucose fermentation in the present study, the recombinant *hybC* showed comparable hydrogen yield and productivity with wild-type SS1. It was suggested that recombinant *hybC* gene does not play a major role in glucose fermentation at initial pH 7.5. According to Fan et al. [16], *hybC* knockout mutant had comparable hydrogen production rate and 1.3-fold higher hydrogen yield (0.70 mol/mol glucose) compared to wild-type *E. coli* W3110 (0.54 mol/mol glucose) at pH 7.6. On the other hand, Trchounian and Trchounian [8] reported that *hybC* knockout mutant had about 10% increased hydrogen production rate during glucose fermentation at pH 7.5 in comparison to wild-type. The outcome of these studies indicated hydrogen uptake characteristic of Hydrogenase 2 at slightly alkaline condition when glucose was used as substrate, which reflects the conflict with the observation of this study. Yet, the increment in hydrogen yield and productivity of mutants as reported by these studies (10–30%) were considered minor.

In comparison to glycerol fermentation, hydrogen yield in glucose fermentation was higher. As for reason, 1 mol of glucose could generate 2 mol of hydrogen theoretically, which is higher than the theoretical yield when glycerol is used as carbon source. The higher hydrogen productivity in glucose fermentation in both wild-type SS1 and recombinant *hybC* was probably attributed by higher cell growth. This was in agreement with Trchounian and Trchounian [8] that demonstrated hydrogen production by *E. coli* BW25113 using glycerol was 2.5-fold lower than that using glucose.

3.4. Ethanol production of E. coli SS1 and its recombinant during fermentation

As shown in Fig. 2d, ethanol was produced proportionally by wild-type SS1 approaching a maximum concentration of 3.20 g/L after 48 h during glycerol fermentation at initial pH 7.5. The recombinant *hybC* produced ethanol achieving approximately 2.44 g/L within 12 h of fermentation. As for glucose fermentation at initial pH 7.5, the ethanol production of both wild-type SS1 and recombinant *hybC* occurred exponentially within 12 h yielding 1.30 g/L and plateaued after 12 h. The ethanol yield and productivity of wild-type SS1 and recombinant *hybC* at initial pH 7.5 are presented in Table 1. The ethanol yield of recombinant *hybC* obtained in glycerol fermentation was lower than the wild-type SS1, whereas no prominent change of ethanol yield was observed between both strains during glucose fermentation. This indicated that the bioconversion of glycerol into ethanol was affected by the additional copy of *hybC* gene. The ethanol yield of recombinant *hybC* was found to decrease further under acidic condition and achieved approximately 0.31 mol/mol glycerol at initial pH 5.8 (data not shown). The lower ethanol yield (0.50 mol/mol glycerol) was also observed in the recombinant *hycE* at initial pH 5.8 [7]. Albeit lower ethanol was yielded by recombinant *hybC* during glycerol fermentation, the ethanol productivity was 3-fold higher in comparison to wild-type SS1 at initial pH 7.5, probably due to higher consumption rate of glycerol and higher cell growth. It was noted that the ethanol yield of wild-type SS1 obtained in glucose fermentation was lower than glycerol fermentation. In theory, both glycerol and glucose could yield similar molar of ethanol which is one mole of ethanol per mole substrate. However, wild-type SS1 tends to produce higher ethanol yield from glycerol rather than glucose. Yet, the ethanol productivity of glucose fermentation was higher than glycerol probably due to higher cell growth.

Table 1
Hydrogen and ethanol yields and productivities achieved by wild-type SS1 and recombinant *hybC*.

Strain	Hydrogen yield (mol/mol substrate)		Hydrogen productivity (mmol/L/h)		Ethanol yield (mol/mol substrate)		Ethanol productivity (mmol/L/h)	
	Gly	Glu	Gly	Glu	Gly	Glu	Gly	Glu
Wild-type SS1	0.57 ± 0.02[a]	0.67 ± 0.06[a]	0.95 ± 0.04[a]	2.75 ± 0.17[a]	0.88 ± 0.04[a]	0.59 ± 0.06[a]	1.45 ± 0.04[b]	2.40 ± 0.25[a]
Recombinant *hybC*	0.14 ± 0.06[b]	0.67 ± 0.07[a]	0.51 ± 0.01[b]	2.68 ± 0.02[a]	0.64 ± 0.02[b]	0.58 ± 0.20[a]	4.42 ± 0.41[a]	2.32 ± 0.67[a]

Different superscript small letters within the same column indicate significant differences ($P < 0.05$) between different strains at initial pH 7.5.
* Gly, glycerol; Glu, Glucose.
** Values represent means ± standard deviation.

The outcome of this study elucidated the remarkable effect of *hybC* in glycerol consumption at initial pH 7.5. For comparison, Yazdani and Gonzalez [5] constructed a recombinant strain by simultaneously overexpressing *gldA* and *dhaKLM* genes in combination with deletion of *frdA* and *pta* genes. The hydrogen and ethanol yield of the engineered strain approached theoretical yield. However, the fermentation rate was low and the engineered strain took 120 h to consume about 8 g/L (86.96 mmol/L) of glycerol. Thapa et al. [17] reported that ethanol production of the recombinant (3.01 g/L) overexpressed with alcohol dehydrogenase and pyruvate fumarate-lyase increased by 2-fold over wild-type (1.45 g/L). Tran et al. [18] had successfully created the engineered *E. coli* (0.67 mol/mol glycerol) that was able to produce 4.5-fold higher hydrogen yield than parent strain (0.15 mol/mol glycerol) after 24 h of fermentation and achieve the theoretical yield after 48 h. The engineered strain was constructed by multiple knockout of fumarate reductase, lactate dehydrogenase, formate dehydrogenase, phosphoenolpyruvate carboxylase, nitrate reductase, methylglyoxal synthase, and a regulator of the transcriptional regulator *FhlA*. In comparison to the recombinant strains in literature, recombinant *hybC* exhibited comparable performance in terms of glycerol consumption rate and ethanol productivity.

3.5. Metabolism involved in hydrogen and ethanol co-production using glycerol and glucose

It was observed that cell growth, glycerol consumption rate, hydrogen and ethanol productivity of wild-type SS1 during glucose fermentation was higher in comparison to glycerol. These observations could be explained by the difference in glucose metabolic pathway of *E. coli* from that of glycerol [4]. Glucose is converted into fructose-1,6-bisphosphate followed by glyceraldehyde-3-phosphate. The glyceraldehyde-3-phosphate is then converted into 2 mol of phosphoenolpyruvate, yielding 2 mol of NADH. A mole of pyruvate is produced from each mole of phosphoenolpyruvate. In contrary to glycerol metabolism that produces 1 mol of pyruvate, there are 2 mol of pyruvate yielded from glucose. However, the NADH yielded is similar in both glucose and glycerol metabolism. For glucose metabolism, the available NADH is insufficient for conversion of 2 mol of pyruvate into 2 mol of ethanol. Instead of producing 2 mol of ethanol, one of the pyruvate is converted into acetate in which no NADH required during the process. Concurrently, 2 mol of hydrogen are generated from 2 mol of pyruvate. Hence, 1 mol of glucose could yield 1 mol of ethanol, 1 mol of acetate, and 2 mol of hydrogen theoretically. This explained the higher hydrogen yield of wild-type SS1 when glucose was used as substrate in comparison to glycerol fermentation. Production of high levels of acetate might clarify the drop of medium pH in greater extent to pH under 6.0 for glucose fermentation by wild-type SS1 (not shown). According to Trchounian and Trchounian [8], medium pH decreased scarcely from 7.50 to 7.25 for glycerol fermentation, whereas pH decreased from 7.50 to 6.78 during glucose fermentation due to higher levels of organic acids formation. The conversion of glucose to hydrogen and ethanol in addition to acetate production produce 3 mol of net ATP. In contrast to glycerol metabolism that produce only 1 mol of net ATP, glucose fermentation yields higher ATP leading to higher cell density. Subsequently, the higher cell density leads to higher substrate consumption rate as well as hydrogen and ethanol productivity of wild-type SS1 during glucose fermentation.

The recombinant with additional copy of *hybC* which encoded large subunit of Hydrogenase 2 had impaired hydrogen and ethanol yield during glycerol fermentation at initial pH 7.5. There was noticeable drop of medium pH at the early stage of fermentation using recombinant *hybC*. The additional copy of *hybC* gene seems leading to high accumulation of acidic products after 12 h of fermentation that inhibited cell growth and eventually affected the glycerol consumption as well as hydrogen and ethanol production as shown in Fig. 3. Hydrogenase 2 is a membrane-bound hydrogenase that involved in periplasmic hydrogen uptake by catalyzing the oxidation of H_2 to H^+ and have an optimal gene expression at slightly alkaline condition [19]. It was believed that additional copy of *hybC* gene had converted the H_2 into H^+ at higher rate during fermentation at initial pH 7.5. This statement explains the drastic decrease of medium pH during fermentation using recombinant *hybC*. The increased acidity of medium had eventually disrupted enzyme activity as well as cell growth of recombinant *hybC*. As a result, glycerol consumption was halted and hydrogen as well as ethanol yield were impaired. Another potential reason is that additional copy of *hybC* resulted in lower pH in the cytoplasm. The increase of acidity in the cell could lead to activation of formate transporter to reduce formate concentration [20]. Thus, formation of hydrogen from formate via formate hydrogen lyase was inhibited, and subsequently *hybC* activity, glycerol consumption as well as ethanol production were repressed.

Nevertheless, the recombinant *hybC* exhibited remarkably cell growth, glycerol consumption rate and ethanol productivity in comparison to wild-type SS1 at initial pH 7.5. This suggested that additional copy of *hybC* had altered the glycerol metabolic pathway of SS1 at slightly alkaline condition. Glycerol dehydrogenase and glyceraldehyde-3-phosphate dehydrogenase are enzymes catalyzing the conversion of glycerol to dihydroxyacetone phosphate and glyceraldehyde-3-phosphate to phosphoenolpyruvate, respectively. Both of the enzymes involve in the glycerol metabolism by reducing NAD^+ to NADH. The additional copy of *hybC* gene had apparently triggered high oxidation levels of H_2 to H^+ in the periplasm and thus generated excess electrons that could be used to reduce NAD^+ to NADH on the cytoplasmic (Fig. 4). Subsequently, both the glycerol dehydrogenase and glyceraldehyde-3-phosphate dehydrogenase were activated, thus increasing the rate of glycerol consumption by the recombinant *hybC*. This might explain the reason in the previous study done by Sanchez-Torres et al. [14] that claimed Hydrogenase 2 might be important for glycerol metabolism at slightly alkaline conditions. Sanchez-Torres et al. [14] reported that *hybC* mutants exhibited impaired glycerol consumption at pH 7.5 accompanied with lower gene expression of *gldA* and *dhaK*. *GldA* and *dhaK* are genes encoded glycerol dehydrogenase and dihydroxyacetone kinase, respectively. Both genes have optimum activity at slightly alkaline condition, as well as *hybC* gene. Apparently, *hybC* gene had contributed in glycerol metabolism pathway by regulating related genes with hydrogen redox reactions. The conversion of glycerol to pyruvate by *E. coli* would synthesize one mole of ATP to provide energy by substrate-level phosphorylation. The acceleration of glycerol dissimilation to pyruvate may allow higher ATP production, thus recombinant *hybC* achieved higher cell density in comparison to wild-type SS1. The higher rate of glycerol degradation by recombinant *hybC* had apparently accelerated fermentation process, subsequently led to higher ethanol productivity to recycle high levels of NADH to NAD^+. As mentioned earlier, glycerol metabolism in *E. coli* generates hydrogen and ethanol simultaneously. It was expected that hydrogen productivity would increase proportionally with ethanol productivity in recombinant *hybC*. However, hydrogen accumulated by recombinant *hybC* was diminished attributed by the characteristic of hydrogen uptake of Hydrogenase 2.

As for comparison study, the cell growth, glucose consumption, hydrogen and ethanol yield as well as productivity were fairly the same for both wild-type SS1 and recombinant at initial pH 7.5 when glucose was used as substrate. This finding demonstrated that glucose fermentation using *E. coli* SS1 was not affected by the additional copy of *hybC* gene under this condition. In comparison to glycerol metabolism, the glucose metabolism in *E. coli* involves more enzymatic reactions including glucokinase, phosphoglucose isomerase, and phosphofruckinase. The additional copy of *hybC* might increase the level of H^+ as well as NADH synthesis, hence stimulating

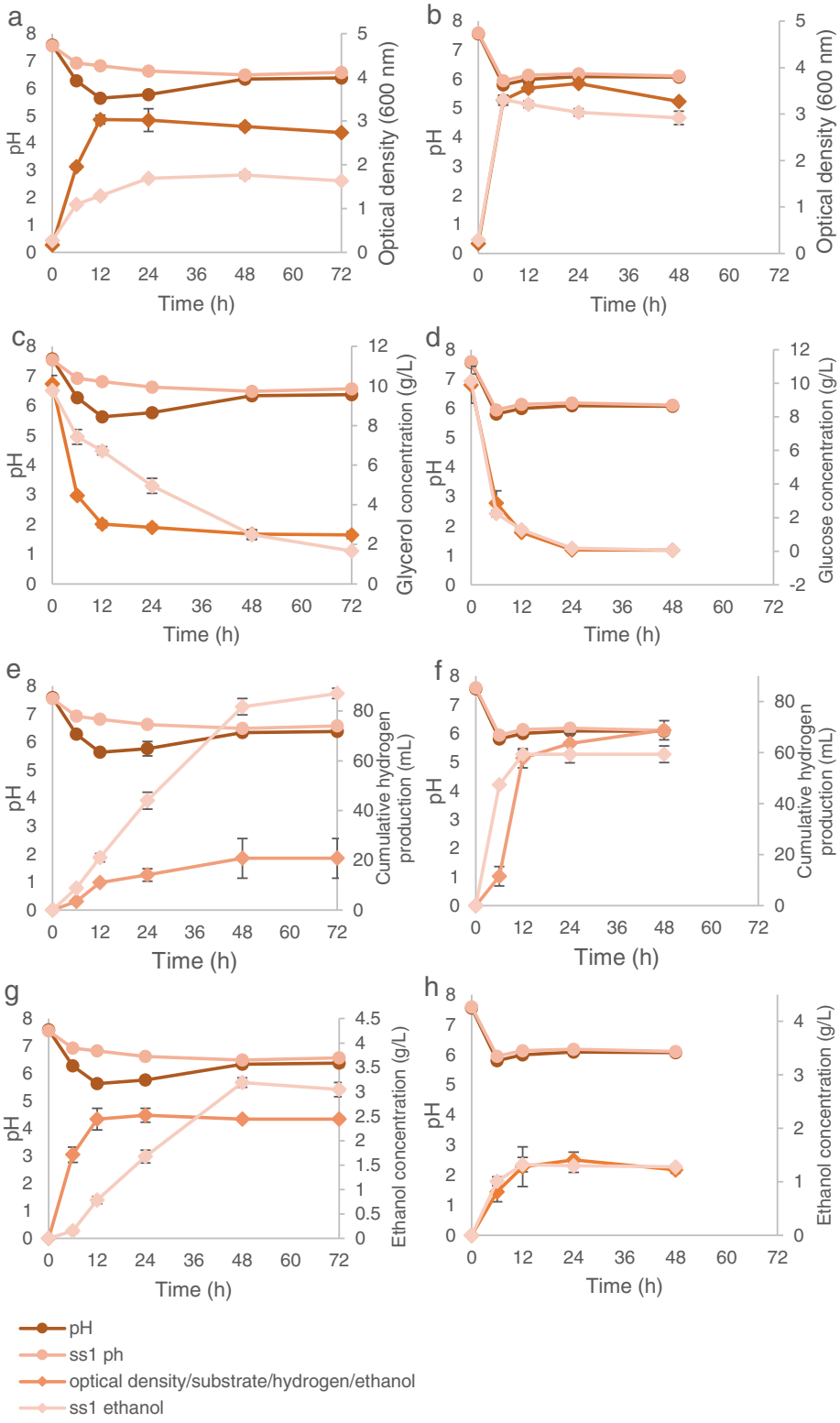

Fig. 3. pH profile and growth profile (OD_{600}) during glycerol fermentation (a) and glucose fermentation (b), pH profile and substrate consumption during glycerol fermentation (c) and glucose fermentation (d), pH profile and cumulative hydrogen production during glycerol fermentation (e) and glucose fermentation (f), and pH profile and ethanol production during glycerol fermentation (g) and glucose fermentation (h) at initial pH 7.5.

conversion of glyceraldehyde-3-phosphate into phosphoenolpyruvate. Nevertheless, the former pathways that convert glucose into glyceraldehyde-3-phosphate were apparently not being stimulated. As a result, the rate of glucose consumption was not as fast as in glycerol.

4. Conclusion

Additional copy of native hydrogenase gene *hybC* could improve substrate consumption rate and ethanol productivity by *E. coli* SS1

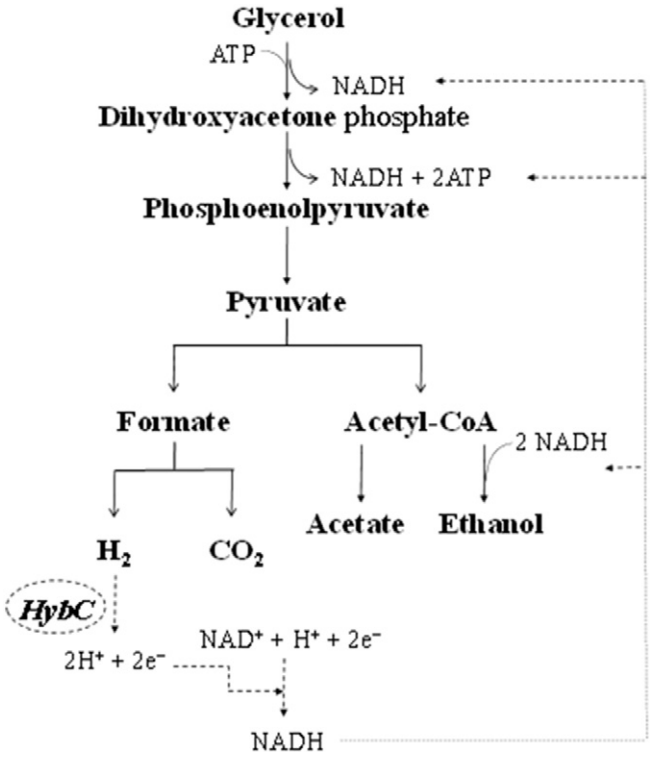

Fig. 4. Glycerol metabolic pathways in the recombinant with additional copy of *hybC* gene. Additional copy of *hybC* gene in SS1 could trigger high levels of the oxidation of H_2 to H^+ and generate excess electrons that could activate the reduction of NAD^+ to NADH and subsequently the pathways catalyzed by glycerol dehydrogenase, glyceraldehyde-3-phosphate dehydrogenase, acetaldehyde dehydrogenase and ethanol dehydrogenase. Solid line (—) represents hydrogen and ethanol synthesis pathways in wild-type SS1; dashed line (---) represents the effect of additional copy of *hybC* gene on the metabolic pathways.

despite lower product yields under glycerol fermentation at initial pH 7.5. Hydrogen and ethanol co-production of *E. coli* SS1 and recombinant *hybC* showed about the same performance in terms of product yields, cell growth and substrate consumption when glucose was used as substrate. This suggested *hybC* gene could play a significant role in glycerol consumption and it could be worth to investigate ethanol production by recombinant *hybC*. The acidic products which caused the drastic drops of medium pH during fermentation could be eliminated through genetic modification. Further study on strain improvement would be a potential strategy for future development of biofuel production using glycerol-containing wastewater from biodiesel industries.

Ethical approval

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

Conflict of interest

The authors declare that they have no conflict of interest.

Informed consent

This article does not involve any informed consent.

Financial support

The project was funded by Fundamental Research Grant Scheme (FRGS/1/2012/SG06/UCSI/02/1).

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