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

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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, more related studies are still required. Previous work done by Suhaimi et al. [6] reported that locally isolated *E. coli* strain SS1 is able

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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%),

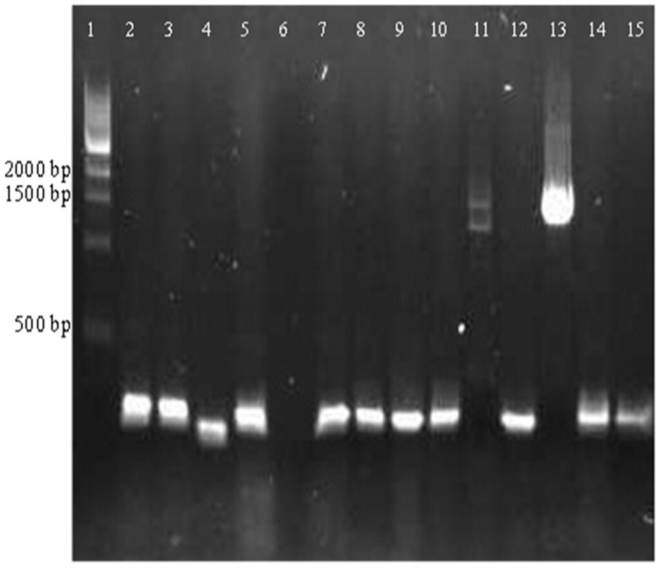

Fig. 1. Screening of the positive transformant carrying plasmid with insertion of *hybC* gene using colony PCR. Lane 1 represents 1 kb DNA ladder (New England Biolabs, USA); lanes 2–15 represent colony PCR products; lane 13 represents positive transformant.

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