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Short Communication

Co-production of Hydrogen and Ethanol of *Escherichia coli* SS1 Isolate

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ABSTRACT

Simultaneous production of hydrogen and ethanol from waste materials has potential for the development of a more cost-effective biofuels generation process. This study aimed to conduct glycerol fermentation using *Escherichia coli* SS1 to establish its hydrogen and ethanol co-production profile. Anaerobic fermentation was performed at 37°C with different concentrations of glycerol as a substrate. *E. coli* SS1 had exponential growth within 24 h (OD_{600} of 1.6), and hydrogen and ethanol were produced in abundance within 48 h of fermentation. Fermentation using 10 g/l of glycerol achieved the highest yield, 0.57 mol of hydrogen and 0.88 mol of ethanol per mol of glycerol. The highest hydrogen productivity (1.85 mmol/l/h) and ethanol productivity (3.13 mmol/l/h) were obtained at 45 g/l of glycerol. This report provides the complete data set for hydrogen and ethanol co-production yield and productivity by the wild-type *E. coli* SS1 and serves as a useful reference for other researchers working on the co-production of hydrogen and ethanol.

Keywords: hydrogen, ethanol, co-production, *Escherichia coli*, glycerol

1. INTRODUCTION

Co-production of hydrogen and ethanol is practical in one fermentation system and has been demonstrated from a variety of waste materials by the researchers [1]. Several bacteria had been reported to be able to produce both biofuels, including *Clostridium* sp. [1], *Thermoanaerobacterium* sp. [1], *Enterobacter aerogenes* [2, 3], and *E. coli* [4]. However, some of the limitations including pathogenicity and the need for strict anaerobic conditions and nutrient-rich supplementation, have restricted the development of co-production systems using these microbes. Relatively low production and yield efficiency is another potential barrier to the development of a cost-effective hydrogen and ethanol co-production system. In addition, the bioconversion efficiency in regards to substrate conversion into hydrogen and ethanol is a major concern especially in the upscaling

process, and reports on the bioconversion of biomass into biofuels by effective microbial systems are scarce. Thus, the identification and characterization of an effective single culture co-producing hydrogen and ethanol is still vastly in demand.

The biodiesel industry generates surplus glycerol. Instead of disposing of the surplus as glycerol waste, bioconversion of the glycerol into useful products such as biofuels will be beneficial from both economic and environmental perspectives. Hence, glycerol was used as the carbon source in this study. Glycerol is a better candidate for the substrate used in the co-production of hydrogen and ethanol compared to other common sugars, which may lead to the formation of by-products during fermentation [5]. Microorganisms that are capable of withstanding high glycerol concentrations and the high salinity of glycerol wastes would be beneficial in the establishment of an efficient glycerol fermentation system because they eliminate the need for dilution, which requires a high running cost. *E. coli* SS1, a potential glycerol-consuming bacterium, was found to produce hydrogen simultaneously with ethanol during fermentation using glycerol as carbon source as reported by Suhaimi et al. [6] in which the study focused on ethanol production rather than co-production of hydrogen and ethanol. The screen for and isolation of *E. coli* SS1 was conducted using a medium with glycerol added as the sole carbon source and sodium chloride added to create a high salinity condition. The medium formulation provided an environment similar to the composition of glycerol waste and suggested that utilizing the wild-type SS1 for glycerol waste degradation would be feasible. This study aimed to conduct glycerol fermentation using *E. coli* SS1 to establish its hydrogen and ethanol co-production profile. Furthermore, the effect of glycerol concentration on the hydrogen and ethanol co-production profile by *E. coli* SS1 was also examined in this paper.

2. MATERIAL AND METHODS

2.1 Microorganism and Culture Conditions

E. coli SS1 used in this study was isolated from soil [6]. SS1 was pre-cultured in LB medium consisting of 10 g/l of tryptone, 5 g/l of yeast extract, 5 g/l of NaCl, and 10 g/l of glycerol.

2.2 Batch Fermentation using Glycerol

A late log phase culture was transferred to serum bottles containing medium as described by Ito et al. [2] consisting of (per liter): 7 g of K_2HPO_4, 5.5 g of KH_2PO_4, 1.0 g of $(NH_4)_2SO_4$, 0.25 g of $MgSO_4.7H_2O$, 0.021 g of $CaCl_2.2H_2O$, 2.0 mg of nicotinic acid, 0.12 g of $Na_2MoO_4.2H_2O$, 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. Pure glycerol of different starting concentrations (10 g/l, 20 g/l, 30 g/l and 45 g/l) was used to evaluate the effect of glycerol concentration on the co-production of hydrogen and ethanol. A total volume of 75 ml of the medium was sparged with nitrogen gas for 15 min. Anaerobic fermentation was carried out at 37□ C with an agitation speed of 120 rpm. This condition had been optimized previously for ethanol production by *E. coli* SS1 isolate using Response Surface Methodology [6]. The sampling was carried out at 0, 6, 12, 24, 48, 72, 96 and 120 h during the fermentation process. The OD_{600} and pH level and the production of hydrogen and ethanol were monitored during the course of the experiments. The experiments were performed in triplicate.

2.3 Analytical Methods

The optical density was estimated by spectrophotometric analysis to measure relative biomass density indirectly. Absorbance was measured at a wavelength of 600 nm. Hydrogen gas composition and concentration were analyzed using a gas chromatograph (GC8A-Shimadzu Co., Japan) equipped with a thermal conductivity detector. Ethanol was measured using a gas chromatograph (GC17A-Shimadzu Co., Japan) equipped with a flame ionization detector and a BP21 capillary column [6]. On the other hand, glycerol concentration was measured via colorimetric detection using glycerol assay kit (Sigma-Aldrich). 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 medium per hour [3] and mol of product produced per biomass density per hour [7].

3. RESULTS AND DISCUSSION

3.1 Hydrogen and Ethanol Co-production Profile

Figure 1a shows that the exponential growth of *E. coli* SS1 ended after approximately 24 h of fermentation. Despite using different concentrations of glycerol as a substrate, the maximum OD_{600} was maintained within the range of 1.6-1.8. This indicated that the relative biomass density was not affected by glycerol concentration ranged from 10 to 45 g/l. The fact that there was no growth inhibition on *E. coli* SS1 during fermentation using 45 g/L suggested that *E. coli* SS1 could be applied in anaerobic fermentation of glycerol waste containing relatively high glycerol content. Chaudhary et al. [5] observed that the highest final dry weight was achieved by *E. coli* MG1655 at 25 g/L glycerol. Growth inhibition was observed at glycerol concentration higher than 25 g/L. This discrepancy is most likely caused by the different strains of *E. coli* used as well as the varied fermentation conditions used. The pH of the medium decreased from approximately 7.5 to between 6.4 and 6.6 during the exponential growth phase of the culture (Figure 1b), probably due to production of acidic products such as pyruvate, succinate, acetate and formate [2, 5]. After 24 hours of fermentation, pH of the medium remained relatively constant as the culture approached stationary phase. Although the difference was relatively small, the final pH of the medium decreased as glycerol concentration used increased. Figure 1c shows the cumulative hydrogen production of *E. coli* SS1. Hydrogen was produced in abundance within 48 h of fermentation regardless of the initial glycerol concentration used in this study, yielding approximately 80 to 180 ml of accumulated hydrogen. Ethanol production by *E. coli* SS1 increased proportionally with time, approaching a maximum concentration as illustrated in Figure 1d. The maximum ethanol concentration of 3.191, 6.885, 9.160, and 12.30 g/l were achieved when 10, 20, 30, 45 g/l of glycerol were supplied in the fermentation medium, respectively. Both hydrogen and ethanol production by *E. coli* SS1 started right after the onset of exponential growth. The continuous formation of products with excess glycerol (45 g/l) after 72 h indicated that glycerol concentration is affecting the yield and productivity of hydrogen and ethanol.

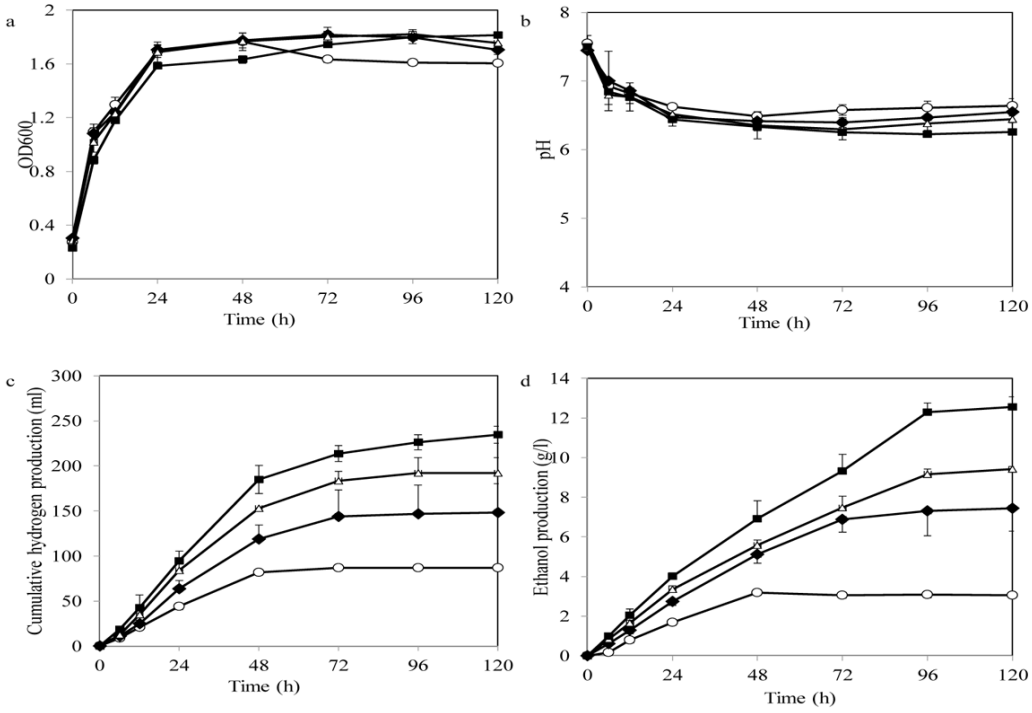

Figure 1. (a) Growth profile (OD_{600}), (b) profile of pH, (c) cumulative hydrogen production, and (d) ethanol production of *Escherichia coli* SS1 under fermentation at different concentration of pure glycerol. Open circles represent 10 g/l; closed diamonds represent 20 g/l; open triangles represent 30 g/l and closed squares represent 45 g/l.

3.2 Yield and Productivity

Table 1 shows hydrogen and ethanol yields by *E. coli* SS1 under fermentation with different concentrations of glycerol. More than 70% of the substrate was consumed in all the fermentations in the current study. As the glycerol concentration increased from 10 g/l to 45 g/l, the hydrogen yield decreased from 0.57 to 0.35 mol/mol glycerol and, the ethanol yield declined slightly from 0.88 to 0.75 mol/mol glycerol. A decrease in yield may be due to the fact that glycerol was used to produce other metabolites in addition to the hydrogen and ethanol when the glycerol concentration was high. A similar finding was reported by Ito et al. [2] in which both hydrogen and ethanol yield decreased with increasing concentrations of glycerol (1.7 g/l to 25 g/l) while the yield of lactate increased. Compared to the ethanol yield, the hydrogen yield decreased more drastically as the glycerol concentration increased. This indicated that the current fermentation conditions might not be favorable for hydrogen production. However, *E. coli* SS1 exhibited a high co-production yield at a glycerol concentration of 10 g/l achieving a hydrogen yield of 0.57 mol/mol glycerol and an ethanol yield at 0.88 mol/mol glycerol. The glycerol concentration of 10 g/l was used in most of the previous studies [2, 8, 9], and thus it is suitable for comparative studies of product yield. Moreover, Ito et al. [2] and Wu et al. [9] reported lower product yield at glycerol concentrations greater than 10 g/l. Table 2 summarizes the product yield in comparison to current findings. In comparison to *E. coli* BW25113 that obtained hydrogen yield of 0.83 mol/mol glycerol [10], the hydrogen

yield reported by *E. coli* SS1 was lower. Nevertheless, the ethanol yield of *E. coli* SS1 was the highest among the wild-type microbes reported in literature. The hydrogen yield of *E. coli* SS1 could be improved to be closer to the theoretical yield (1 mol hydrogen and 1 mol ethanol per mol glycerol) by genetic modification as shown by *E. coli* SY03 [8]. The engineered *E. coli* SY03 had inactivated fumarate reductase and phosphate acetyltransferase. Thus, the formation of the succinate and acetate by-products ceased, and carbon flux was directed towards the formation of hydrogen and ethanol. Nevertheless, the cell growth and glycerol utilization of *E. coli* SY03 were inefficient at pH 6.3. Hu et al. [4] showed that the drawback of low cell growth of *E. coli* could be overcome by adaptive evolution and chemical mutagenesis. On the other hand, wild-type strain *E. coli* SS1 has an advantage due to uninhibited growth at glycerol concentration of 45 g/l.

Table 1. Product yield and productivity of *Escherichia coli* SS1 using different glycerol concentration.

Glycerol concentration (g/l)	Hydrogen yield (mol/mol glycerol)	Ethanol yield (mol/mol glycerol)	Hydrogen productivity (mmol/l/h)	Ethanol productivity (mmol/l/h)	Hydrogen productivity (mmol/g CDW/h)	Ethanol productivity (mmol/g CDW/h)
10	0.57	0.88	0.95	1.45	1.85	2.82
20	0.45	0.85	1.38	2.31	2.68	4.50
30	0.41	0.77	1.77	2.52	3.45	4.92
45	0.35	0.75	2.14	3.13	4.52	6.61

Table 2. Product yield achieved by various microorganisms using 10 g/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. [2]
Klebsiella sp. HE1	6.0	35	0.04	0.80	Wu et al. [9]
E. coli BW25113	6.3	37	0.83	0.66	Durnin et al. [10]
E. coli MG1655	6.3	37	0.08	0.78	Chaudhary et al. [5]
E. coli SS1	7.5	37	0.57	0.88	Current study

As shown in Table 1, the hydrogen and ethanol productivity of SS1 increased with an increase in the glycerol concentration. This result indicates that the substrate concentration did not have a negative impact on the productivity of SS1 within 48 h of fermentation for glycerol under 45 g/l. The increased of hydrogen productivity of *E. coli* SS1 was most probably attributed by continuous withdrawal of gas at every sampling hour reduced the potential for adverse effects on gas production by high hydrogen partial pressure. Thus, higher amount of hydrogen could be produced at higher glycerol concentrations. On the other hand, the ethanol productivity trend in this study was in agreement to Adnan et al. [7] where their results demonstrated that ethanol productivity increased by 2.0-fold when increasing glycerol concentration was used

from 20 g/l to 45 g/l. Although glycerol fermentation using *E. coli* was widely studied, only one product, either hydrogen or ethanol was targeted. There is lack of adequate information in literature regarding productivity achieved by *E. coli* during hydrogen and ethanol co-production using glycerol, hence more relevant studies are still in demand. It is vital to bear in mind that a high glycerol concentration is the main bottleneck that needs to be overcome for hydrogen and ethanol fermentation systems using glycerol-containing wastewater from biodiesel processing plants. Using a molecular approach to construct recombinant strains that withstand high glycerol concentrations is an option for enhancing the product yield. On the other hand, the manipulation of parameters and medium composition could be considered to optimize the fermentation conditions for optimum hydrogen and ethanol co-production to enhance the yield and productivity.

4. CONCLUSION

In conclusion, hydrogen and ethanol co-production profile of *E. coli* SS1 was established. The ability of *E. coli* SS1 to consume a high amount of glycerol demonstrates the feasibility of the cell to withstand high glycerol concentration (45 g/l). *E. coli* SS1 produced the highest yield at 10 g/l of glycerol and the highest productivity at 45 g/l of glycerol, respectively. These findings would be a useful benchmark for subsequent investigations to eventually establish a very high and competitive co-production process using glycerol-containing wastewater from biodiesel industries.

5. ACKNOWLEDGEMENTS

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