Effect of redox potential on the production of succinic acid by metabolically engineered *Escherichia coli*

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Abstract

Recombinant *Escherichia coli* NZN111 (F- *Δpfl*::*Cam ldhA*::*Kan*) harboring pTrcML, a plasmid containing the *E. coli* malic enzyme gene, produced considerable amount of malic acid along with the desired product succinic acid. This seemed to be due to the unmatched redox states between glucose and succinic acid. Therefore, a more reduced carbon substrate sorbitol was examined for the possibility of matching the potential during succinic acid production. When NZN111 (pTrcML) was cultured in LB medium containing 20 g/L sorbitol under CO_2 atmosphere, 10 g/L of succinic acid was produced. The apparent yield of succinic acid was 1.1 g succinic acid per g sorbitol, which is 85% of the maximum theoretical yield.

Introduction

Metabolic engineering can be defined as the directed modification of cellular metabolic properties through the modification of specific metabolic pathways or introduction of new ones by using recombinant DNA technology.[6] Metabolic engineering can be applied to improve the productivity and yield of native products, and to produce new products that are not produced in the natural hosts. Recently, much effort is being exerted for the fermentative production of chemicals from renewable resources. When manipulating metabolic fluxes for the production of metabolites, it is important to achieve the redox balance among the substrates and products.

Many organic acids such as lactic acid, acetic acid, succinic acid etc. were produced through fermentation in *Escherichia coli*. The succinic acid is a member of C_4-dicarboxylic acid family. Since succinic acid can be used as a precursor of numerous chemicals such as pharmaceuticals and biodegradable polymers, it has wide application on agriculture, medicine, and polymer synthesis. It is currently produced by chemical processes. Recently, much researches are being carried out for the fermentative production of succinic acid from renewable feedstock using anaerobic and facultative bacteria.[4), 5)]

Recently, recombinant *E. coli* NZN111 overexpressing the *E. coli* malic enzyme was developed for the production of succinic acid. The head-space gas was replaced with oxygen-free CO_2-H_2 (molar ratio of 1:1) gas mixture. H_2 was included to achieve redox balance since the redox potential can not be regulated by controlling the DO level under anaerobic condition. CO_2 was

included because it is essential for the conversion of pyruvate (C_3) to malic acid (C_4). The recombinant strain successfully produced 9.4 g/L succinic acid from 31 g/L glucose after 120 h of anaerobic cultivation at 30°C. However, a considerable amount of malic acid (8.4 g/L), the precursor of succinic acid, was also produced. It was thought to be due to the insufficient reducing power of glucose since four moles of H^+ produced through glycolysis are completely consumed during conversion of pyruvate to malic acid.[2), 3)] It was postulated that the use of more reduced carbon source might solve this problem. In this study, sorbitol was examined as a carbon substrate to achieve the redox balance.

Materials and Methods

E. coli strain NZN111 (F^- *pfl*::Cam *ldhA*::Kan) harboring pTrcML, which contains the *E. coli* *sfcA* gene encoding malic enzyme[1)] under the control of *trc* promoter, was used as a host strain. Fermentation studies were carried out in a 5 L fermentor (BioFlo 3000, New Brunswick Scientific, Edison, NJ) containing 3 L of LB medium at 37°C. The medium pH was controlled at 6.7 by the addition of 5 M NaOH. The DO level was maintained over 40% of oxygen saturation during aerobic cultivation. When the OD_{600} reached 5, isopropylthio-β-D-galactoside (IPTG) was added to a final concentration of 0.1 mM. After induction, three different oxygen-free gases were flushed to the fermentor until anaerobic condition was achieved. Fermentation products were analyzed by high-performance liquid chromatography (Hitachi chromatography system, Tokyo, Japan) equipped with Aminex HPX-87H column (300 mm × 7.8 mm, Bio-Rad Laboratories, Herculules, CA) and a refractive index detector (L-3300, Hitachi chromatography system). The column was eluted isocratically with 5 mM H_2SO_4.

Results and Discussion

When the head-space gas was replaced with oxygen-free CO_2-H_2 (molar ratio of 1:1) gas mixture (Kosock gas, Taejon, Korea), the recombinant *E. coli* NZN111 (pTrcML) produced 2.75 g/L succinic acid and 4.8 g/L malic acid from 20 g/L glucose. The apparent yield of succinic acid during anaerobic cultivation was only 0.23 g of succinic acid per g of glucose, which is considerably lower than the maximum theoretical yield (1.3 g of succinic acid per g of glucose). It seemed to be due to the unmatched redox states between glucose and succinic acid. Eight moles of H^+ are needed to metabolize pyruvate to succinic acid while only four moles of H^+ are produced through glycolysis when glucose is used. Even though the head-space was filled with CO_2-H_2 gas mixture, it was not able to provide enough reducing power to fermentor scale culture. Therefore, another strategy was needed to achieve redox balance for the enhanced production of succinic acid.

To achieve redox balance and subsequently to enhance succinic acid production, sorbitol was examined as a carbon substrate. One mole of sorbitol produces six moles of H^+ during its conversion to PEP. The recombinant *E. coli* NZN111 (pTrcML) was cultured in LB medium

supplemented with 20 g/L sorbitol (Fig. 1). After 9 h of aerobic cultivation, anaerobic condition was achieved by replacing the head-space gas with oxygen-free CO_2-H_2 gas mixture. At this point, the culture OD_{600} was 9 and the residual sorbitol concentration was 16 g/L. After 40 h of anaerobic cultivation, sorbitol was completely consumed. The concentration of succinic acid increased gradually and reached 8.4 g/L. Malic acid was accumulated during the early stage of anaerobic cultivation, but decreased to zero during the later phase. By using sorbitol as a carbon substrate and by providing CO_2-H_2 gas environment, succinic acid production could be improved.

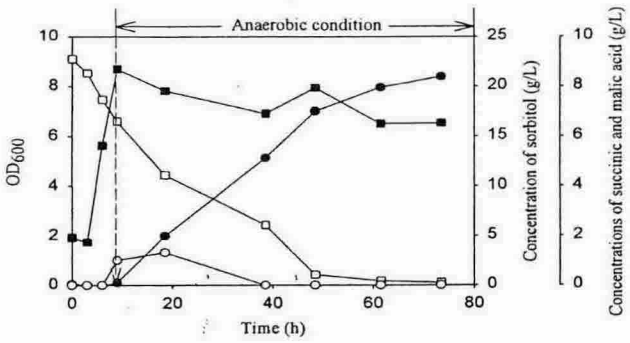

Figure 1

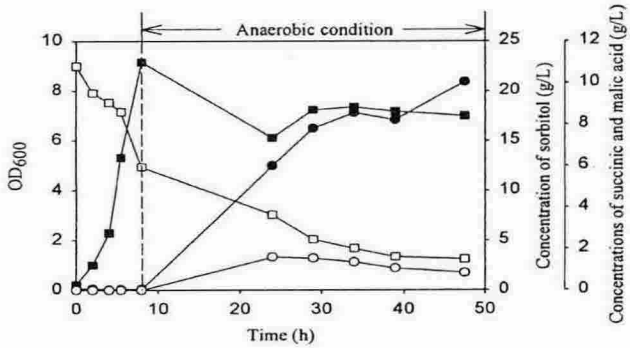

Figure 2

To evaluate the effect of head-space gas on the achievement of redox balance, CO_2-H_2 (molar ratio of 1:1) gas mixture was replaced with N_2 or CO_2 gas. When the head-space gas was replaced with N_2 gas, sorbitol was not consumed and little amount of succinic acid was produced under anaerobic condition while the recombinant strain grew well under aerobic condition. This seemed to be due to the deficiency of CO_2, since one mole of CO_2 is needed to convert one mole of pyruvate to one mole of malic acid. Therefore, the head-space gas was replaced with CO_2 gas. Cells were cultured in LB medium supplemented with 20 g/L sorbitol (Fig. 2). After 9 h of aerobic cultivation, the culture OD_{600} reached 9 and the residual sorbitol concentration was 12.4 g/L. At this point, anaerobic condition was achieved by replacing the head-space gas with CO_2 gas. The final concentration of succinic acid at the end of fermentation was 10.1 g/L. The apparent yield of succinic acid during anaerobic cultivation was 1.1 g of succinic acid per g of sorbitol. On a molar basis, this corresponds to the apparent yield of 1.7 mol of succinic acid per mole of sorbitol, which is 85% of theoretical maximum yield. This is even better than that achieved under CO_2-H_2 head-space gas.

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