Effect of introduction of fumarase on the production of succinic acid

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Abstract

The *fumB* gene encoding anaerobic fumarase of *Escherichia coli* XL1-Blue was introduced to solve the malic acid accumulation problem. When NZN111 harboring pTrcMLFu was cultured, 7 g/L of succinic acid was produced and malic acid was not accumulated.

Introduction

Many organic acids such as lactic acid, acetic acid, succinic acid etc. were produced through fermentation in *Escherichia coli*.[2), 5)] 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.[3), 4)]

Until now, most efficient succinic acid producing recombinant *E. coli* is NZN111. This strain lost fermentation ability by insertional inactivation of *ldhA* and *pfl* gene. NZN111 was designed to produce malic acid from pyruvate for conservation of free energy of PEP. The malic enzyme was overexpressed in recombinant *E. coli* NZN111 which lost fermentation ability by insertional inactivation of *ldhA* and *pfl* gene, and head-space gas was replaced with oxygen-free CO_2-H_2 (molar ratio of 1:1) gas mixture. The strain produced successfully produced significant amount of succinic acid with little byproduct. When fermentation study, however, was carried out, significant amount of malic acid the precursor of succinic acid was produced. In this study, the fumarase which convert malic acid to fumaric acid was overexpressed to solve malic acid accumulation problem.

Materials and Methods

E. coli strain NZN111 (F⁻ *pfl*::Cam *ldhA*::Kan) was used as a host. The strain lost activities of lactate dehydrogenase and pyruvate-formate lyase because of insertional inactivation of *ldhA* and *pfl* genes. As a result, NZN111 could not metabolite pyruvate under anaerobic condition.

The *fumB* gene which encode anaerobic fumarase of *E. coli* XL1-Blue (*supE44 hsdR17 recA1 endA1 gyrA96 thi relA1 lac* F'(*proAB lacI*^{q} *lacZ* Δ *M15* Tn*10*(*tet*^{r}))) (Stratagene Cloning Systems, La Jolla, CA, USA) was cloned by PCR. Two sets of PCR primers were designed base on the full genomic sequence of *E. coli*.[1)] A set of primers, FumB-*Xba*F (5`-

TGCTCTAGACGCCATTTTCGAATAACAAATAC-3`) and FumB-*Xba*R (5`-TGCTCTAGATTATTACTTAGTGCAGTTCGCGC-3`), contain *Xba*I restriction enzyme site (underlined). PCR was performed using a PCR Thermal Cycler MP TP3000 (Takara Shuzo Co., Shiga, Japan) and High Fidelity PCR System (Boeringer Mannheim, Mannheim, Germany). The PCR product contains *fumB* and ribosomal binding site of fumarase, was ligated into pTrcML using *Xba*I restriction enzyme sites to construct pTrcMLFu. DNA sequence of the *fumB* gene was confirmed by sequencing with the automatic DNA sequencer (ABI Prism model 377, Perkin Elmer Co., IL). The direction of *fumB* gene was conformed by *Pst*I restriction enzyme digestion, since *Pst*I enzyme site is located in 666 bp downstream of start codon of *fumB* gene. The other set of primer, FumB-*Nco*F (5`-CATGCCATGGCAAACAAACCCTTTATCTACCA-3`) and FumB-*Nco*R (5`-AATTCCATGGTCTGTTTCCTCCAGGCGCTGGGCCGAA-3`), contain *Nco*I restriction enzyme site (underlined). The PCR product contains *fumB* gene and ribosomal binding site of pTrc99a plasmid (underlined), was ligated to pTrcML using *Nco*I restriction enzyme site to construct pTrcFuML. *E. coli* strain NZN111 was transformed with pTrcMLFu and pTrcFuML by electroporation.

NZN11 harboring pTrcFuML and pTrcMLFu were used as host strains, Fermentation studies were carried out at 30°C using BioFlo 3000 bioreactor (5 L, New Brunswick Scientific, Edison, NJ) containing 3 L of LB medium. Glucose and sorbitol were supplemented as carbon sources, and 5 M NaOH was used to maintain medium pH at 6.7. Dissolved oxygen (DO) level was maintained over 40% of oxygen saturation during aerobic cultivation. When OD_{600} was reached to 5, isopropylthio-β-D-galactoside (IPTG) was added to a final concentration of 0.01 mM. After induction, oxygen-free CO_2-H_2 (molar ratio of 1:1) gas mixture (Kosock gas, Taejon, Korea) was flushed to achieve anaerobic condition when glucose was used as a carbon source.

Fermentation products were analyzed by high-performance liquid chromatography (Hitachi chromatography system, Tokyo, Japan) equipped with Aminex HPX-87H column (300 mm X 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

E. coli NZN111 harboring pTrcFuML was cultivated. The time profiles of cell density and the concentrations of glucose, succinic acid, and malic acid were shown in Figure 1.After 10 h of aerobic concentration, the culture OD_{600} was reached to 6.5 and anaerobic condition was achieved. At this point residual glucose concentration was 15 g/L. After 120 h of anaerobic cultivation, glucose was completely consumed. The concentration of succinic acid and malic acid were increased steadily during anaerobic cultivation. The final concentrations of succinic acid and malic acid were 8.7 and 6.5 g/L, respectively. Glucose was more slowly consumed, while the time profiles and final concentrations of organic acids were similar with fermentation of NZN111 harboring pTrcML. It was supposed that the activity of malic enzyme was not

sufficient since *sfcA* gene follows *fumB* gene.

E. coli NZN111 harboring pTrcMLFu was cultivated to certify above supposion. The time profiles of cell density and concentrations of glucose, succinic acid, and malic acid were presented in Figure 2. After 10 h of aerobic cultivation, culture OD_{600} was reached to 8. At this point anaerobic condition was achieved by flushing of mixed gas and residual glucose concentration was 16 g/L. After glucose was completely consumed, glucose was supplemented occasionally (arrows in Figure 2) to supply the carbon source. At the end of fermentation, the concentrations of succinic acid and malic acid were 7 and 0 g/L, respectively. The concentration of malic acid increased during early period of anaerobic cultivation. Then, after 50 h of anaerobic cultivation, it started to decrease to zero. Besides, glucose was completely consumed within 70 h of anaerobic cultivation and glucose consumption rate was higher than that of NZN111 harboring pTrcFuML. With these results, it was concluded that the activity of malic enzyme was sufficient to metabolite pyruvate to malic acid and fumarase had activity to metabolite malic acid to fumarate. Therefore, accumulation and excretion problem of malic acid is resolved by use of pTrcMLFu.

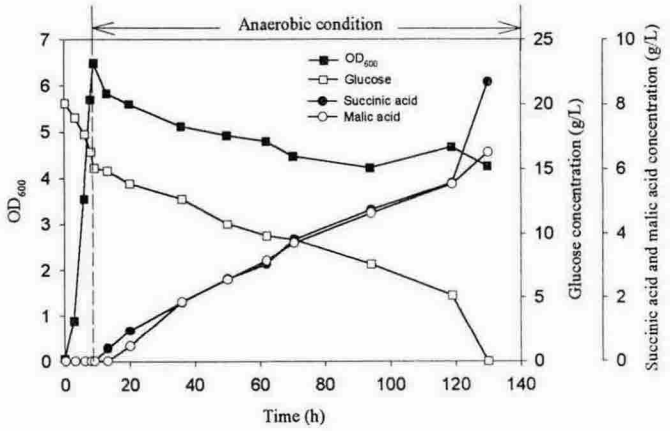

Figure 1

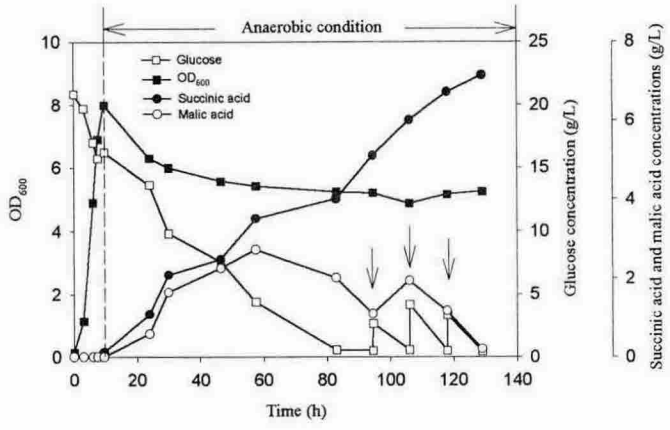

Figure 2

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