

Enhanced Production of Succinic Acid by Metabolically Engineered *Escherichia coli* with Amplified Activities of Malic Enzyme and Fumarase

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Abstract A *pfl ldhA* double mutant *Escherichia coli* strain NZN111 was used to produce succinic acid by overexpressing the *E. coli* malic enzyme gene (*sfcA*). This strain, however, produced a large amount of malic acid as well as succinic acid. After the analyses of the metabolic pathways, the *fumB* gene encoding the anaerobic fumarase of *E. coli* was co-amplified to solve the problem of malic acid accumulation. A plasmid, pTrcMLFu, was constructed, which contains an artificial operon (*sfcA-fumB*) under the control of the inducible *trc* promoter. From the batch culture of recombinant *E. coli*/NZN111 harboring pTrcMLFu, 7 g/L of succinic acid was produced from 20 g/L of glucose, with no accumulation of malic acid. From the metabolic flux analysis the strain was found under reducing power limiting conditions by severe reorientation of metabolic fluxes.

Keywords: succinic acid, malic enzyme, fumarase, metabolic engineering

INTRODUCTION

Succinic acid is a member of the C₄-dicarboxylic acid family, and has a wide range of applications in the fields of agriculture, medicine, polymer synthesis and chemistry. It is produced by many kinds of bacteria under anaerobic conditions [1,2]. *Anaerobiospirillum succiniciproducens*, *Bacteroides fragilis*, *Mannheimia succiniciproducens* and *Wolinella succinogenes* are known as good succinic acid producers. *Escherichia coli* also produce a small amount of succinic acid as an anaerobic end product [3-7].

Recently, metabolic engineering strategies [8-10] have been employed to create recombinant *E. coli*, which has enhanced succinic acid production ability due to the re-orientation of the metabolic fluxes in *E. coli* (Fig. 1). In one example, the *E. coli* phosphoenolpyruvate (PEP) carboxylase gene (*ppc*) was overexpressed for the enhanced production of succinic acid [11]. In another study, the fumarate reductase (FrdABCD) gene was overexpressed in *E. coli* for the conversion of fumaric acid to succinic acid [12,13]. In a more recent example, the *E. coli* malic enzyme gene was overexpressed in the *ldhA* and *pfl* double mutant strain of *E. coli* (NZN111) for the conversion of pyruvate to succinic acid *via* malic acid [14,15]. Normal *E. coli* strains produce malic acid from PEP in two

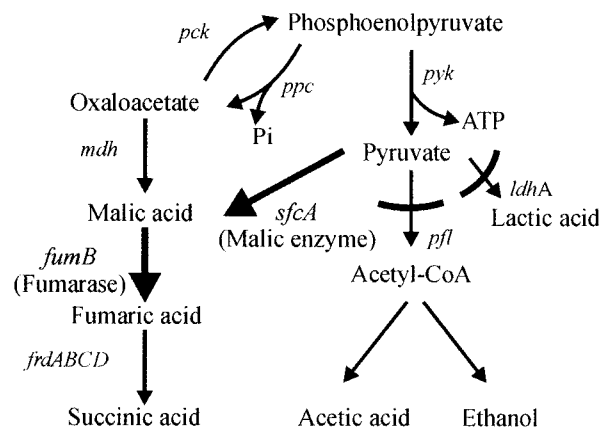


Fig. 1. Anaerobic metabolic pathways in *E. coli* NZN111. The formation and consumption of reducing power, [H], are shown for three carbon substrates. Enzyme coded by the genes shown are: *frdABCD*, fumarate reductase; *fumB*, fumarase B; *ldhA*, lactate dehydrogenase; *mdh*, malate dehydrogenase; *pck*, PEP carboxylase; *pfl*, pyruvate formate-lyase; *ppc*, PEP carboxylase; *pyk*, pyruvate kinase and *sfcA*, malic enzyme. Black bar represents knock-out of the corresponding gene.

step reactions catalyzed by PEP carboxylase and malate dehydrogenase (Mdh), but the free energy of the PEP is wasted through this pathway. Therefore, the NZN111 strain was designed to produce malic acid from pyruvate, while conserving the free energy of the PEP [15]. When

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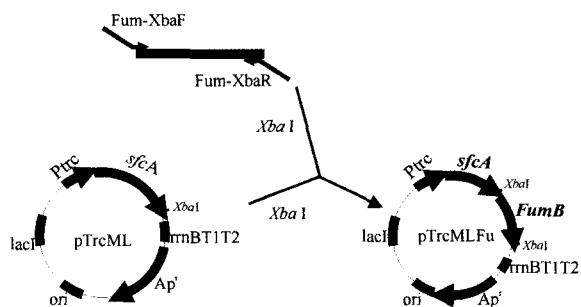


Fig. 2. Construction of pTrcMLFu. The PCR amplified fumarase gene (*fumB*) was cloned downstream of the malic enzyme gene (*sfcA*), forming an operon under the control of the *trc* promoter.

the *E. coli* malic enzyme gene was overexpressed in this strain, 9.4 g/L succinic acid could be produced from 31 g/L glucose during 120 h of anaerobic cultivation [14]. However, a significant amount of malic acid (8.4 g/L), the precursor of succinic acid, was also produced. Therefore, it was assumed that fumarase, which converts malic acid to fumaric acid, might become a new bottleneck in this engineered pathway. In this study, the *E. coli* fumarase gene was overexpressed to solve the problem of malic acid accumulation, and subsequently improve the succinic acid production. A metabolic flux analysis was also performed to examine the effect of fumarase overexpression on the overall metabolic network.

MATERIALS AND METHODS

Bacterial Strain and Plasmid

E. coli NZN111 (F' *pfl*::Cam *ldhA*::Km) was used as a host strain, which lost its lactate dehydrogenase and pyruvate-formate lyase activities because of the insertional inactivation of the *ldhA* and *pfl* genes. As a result, the NZN111 strain can not metabolize pyruvate under anaerobic conditions.

The construction of the plasmids used in this study is shown in Fig. 2. The *fumB* gene, which encodes the anaerobic fumarase of *E. coli*, was cloned from XL1-Blue (*supE44 hsdR17 recA1 endA1 gyrA96 thi relA1 lac F' (proAB lacI^q lacZ Δ M15 Tn10(tet^r))*) (Stratagene Cloning Systems, La Jolla, CA, USA) using PCR. The PCR primers were designed based on the full genomic sequence of *E. coli* [16]. The primers, FumB-*Xba*F (5'-T GCTCTAGACGCCATTTTCGAATAACAAATAC-3') and FumB-*Xba*R (5'-TGCTCTAGATTATTACTTAGTGCAGT TCGCGC-3'), were designed to contain *Xba*I sites (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, containing the *fumB* gene and its ribosomal binding site, was ligated into pTrcML [14] using *Xba*I restriction enzyme sites to construct pTrcMLFu (Fig. 2). In this plasmid, genes are

transcribed in the order of *sfcA* and *fumB* on their induction with isopropylthio-β-D-galactoside (IPTG). The DNA sequence of the *fumB* gene was confirmed by sequencing with the automatic DNA sequencer (ABI Prism model 377, Perkin Elmer Co., IL, USA). The direction of the *fumB* gene was confirmed by *Pst*I digestion, since the *Pst*I enzyme site is located 666 bp downstream of the start codon of the *fumB* gene. The *E. coli* strain NZN111 was transformed with pTrcMLFu by electroporation.

Fermentation

Fermentation was carried out at 30°C in a 5-L bioreactor (BioFlo 3000, New Brunswick Scientific, Edison, NJ, USA) containing 3 L of LB medium supplemented with 20 g/L glucose. The pH was controlled at 6.7 with 5 M NaOH. The dissolved oxygen (DO) level was maintained at over 40% during the aerobic cultivation. When the culture OD₆₀₀ reached 5, IPTG was added to a final concentration of 0.01 mM. After induction, anaerobic conditions were achieved by lowering the agitation speed and flushing the bioreactor with oxygen-free CO₂-H₂ (molar ratio of 1:1) gas mixture (Kosock gas, Daejeon).

Analytical Procedure

The cell growth was monitored by measuring the absorbance at 600 nm (OD₆₀₀). Fermentation products were analyzed by high-performance liquid chromatography (Hitachi chromatography system, Tokyo, Japan), equipped with an Aminex HPX-87H column (300 mm × 7.8 mm, Bio-Rad Laboratories, Hercules, CA, USA) and a refractive index detector (L-3300, Hitachi). The column was eluted isocratically with 5 mM H₂SO₄.

Metabolic Flux Analysis

Metabolic flux analysis was carried out for the calculation of the volumetric rates of formation of intracellular metabolites [10,17,18]. The metabolic network was constructed with 154 reversible and 156 irreversible reactions and 295 metabolites, similar to that reported by Pramanik and Keasling (1997) [19]. The maximum growth rate was used as an objective function to analyze this under-determined system. The MetaFluxNet program package was used for the calculations [20].

RESULTS AND DISCUSSION

Solving the Problem of Malic Acid Accumulation

In our previous study, a new flux analysis strategy was proposed for the prediction of intracellular metabolite concentrations [14]. It was found that malic acid was excreted to the medium and pyruvate intracellularly accumulated during the fermentation. The measured intracellular pyruvate concentration increased to levels as high as 5.6 mM, which was in accordance with the result of the metabolic flux analysis, which predicted intracellular

Table 1. Results of metabolic flux analysis

	Metabolites formation rates (mM/gDCW/h)				Ratio of production rates	
	Glucose	Succinic acid	Malic acid	Reducing power	Malic acid /glucose	Reducing power /malic acid
Period 1	0.747	0.395	0.874	2.65	1.17	3.03
Period 2	0.580	0.182	0.331	1.56	0.57	4.71

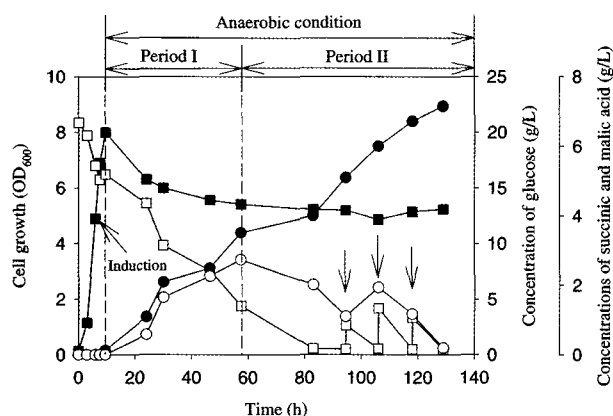


Fig. 3. Time profiles of the culture at OD_{600} (■) and concentrations of glucose (g/L, □), succinic acid (g/L, ●) and malic acid (g/L, ○) during fermentation of the NZN111 harboring pTrcMLFu with 20 g/L of glucose. The head-space gas was replaced with a CO_2 - H_2 gas mixture under anaerobic condition. Arrows indicate the time points of IPTG induction and glucose addition.

pyruvate accumulation. However, fumaric acid, which is converted from malic acid by fumarase, was not excreted to the medium and/or intracellularly accumulated. From these results, it could be concluded that fumarase might have become a new bottleneck to the production of succinic acid. To solve this problem, the overexpression of the fumarase gene was investigated. The pTrcMLFu plasmid was constructed, as described in Materials and Methods.

Fermentation of the recombinant *E. coli* NZN111 harboring pTrcMLFu was carried out. The time profiles of the cell density and concentrations of glucose, succinic acid and malic acid are presented in Fig. 3. After 9.5 h of aerobic cultivation, the culture OD_{600} reached 8. At this point, anaerobic conditions were achieved by flushing the fermentor with an oxygen-free CO_2 - H_2 gas mixture. The residual glucose concentration was 16 g/L. After the glucose had been completely consumed, glucose was supplemented occasionally (arrows in Fig. 3) to supply a carbon source. At the end of the fermentation, the concentrations of succinic acid and malic acid were 7 and 0 g/L, respectively. The concentration of malic acid increased during the early period of anaerobic cultivation, but after 57.5 h it started to decrease and finally became zero. As it proceeded, the equilibrium of the fumarase reaction severely shifted to malic acid (K_m value of 1.2

and 0.3 mM for malic acid and fumaric acid, respectively), which seems to be the reason for the transient accumulation of malic acid during the early period of cultivation as well as upon glucose feeding.

Therefore, the problem of malic acid accumulation and excretion could be solved by the overexpression of the anaerobic fumarase gene, which allowed the conversion of the accumulated malic acid to fumaric acid due to the kinetically shifting the flux. However, the apparent yield of succinic acid during anaerobic cultivation was only 0.3 g succinic acid/g glucose, which was rather low than the maximum theoretical yield (1.3 g succinic acid/g glucose).

Identification of Further Limiting Factor by Metabolic Flux Analysis

To gain an insight into the intracellular metabolism and identify the factor causing the reduced succinic acid yield, metabolic flux analysis was carried out (Table 1). Considering the time profile for the malic acid concentration, the fermentation phase was divided into two periods. In Period I ($9.5 < h < 57.5$), the malic acid accumulating period, only 33% of the carbon flux was directed to the PP pathway and used for the biosynthesis of building blocks, such as amino acids, membrane and cofactors. Most of the residual carbon substrate (58.5% of total carbon flux) was directed toward the succinic acid pathway, but only 45% of the malic acid was converted to succinic acid. In Period II ($57.5 < h < 129$), the glucose consumption and succinic acid formation rates both decreased. Most of the carbon substrate (81.2% of total carbon flux) was used for the biosynthesis of building blocks through the PP pathway, and 28.5% of the carbon substrate was converted to malic acid. Especially, the ratio of the reducing power, [H], the ration of the formation (summation of NADH, NADPH, and $FADH_2$ formation rates) to the malic acid formation rates increased, indicating that malic acid could be more efficiently converted to succinic acid by increasing the reducing power supply.

These results suggested that the carbon flux was severely shifted toward the succinic acid pathway by the *pfl* *ldhA* double mutation and the overexpression of the malic enzyme. However, this reoriented carbon flux exceeded the reducing power supply capacity of the recombinant strain during Period I. Therefore, excess malic acid was excreted into the culture medium in a similar manner to that of acetic acid during the cultivation in excess glucose. This was supported by the finding that all the $FADH_2$,

which is required for the conversion of fumaric acid to succinic acid, was used for the production of succinic acid, resulting in reducing power limiting conditions. This insufficient reducing power seems to be due to the fact that eight moles of [H] are required to convert pyruvate to succinic acid, while only four moles of [H] were produced through glycolysis [3]. Even though an oxygen-free CO₂-H₂ (molar ratio of 1:1) gas mixture was flushed to provide extra reducing power, and to achieve a balance of the redox potential, it was not able to provide enough reducing power to the fermentor scale culture.

In conclusion, it was found that severe modification of metabolic flux can cause side effects, such as accumulation of precursors, *e.g.* malic acid. This problem could be solved by debottlenecking the new rate controlling enzyme, fumarase. The metabolic flux analysis suggested that the reducing power is a limiting factor in the production of succinic acid by this metabolically engineered *E. coli*. This was recently shown to be true by examining several carbon sources with different redox states: more reduced carbon sources allowed the production of greater amounts of succinic acid [21].

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