Effect of carbon substrate on the intracellular fluxes in succinic acid producing *Escherichia coli*.

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Abstract

Metabolic engineering has become a new paradigm for the more efficient production of desired bioproducts. Metabolic engineering can be defined as directed modification of cellular metabolism and properties through the introduction, deletion, and modification of metabolic pathways by using recombinant DNA and other molecular biological tools. During the last decade, metabolic flux analysis (MFA) has become an essential tool for metabolic engineering. By MFA, the intracellular metabolic fluxes can be quantified by the measurement of extracellular metabolite concentrations in combination with the stoichiometry of intracellular reactions and mass balances.

The usefulness and functionality of MFA are demonstrated by applying to metabolic pathways in *E. coli*. First, a large-scale *in silico E. coli* model is constructed, and then the effects of carbon sources on intracellular flux distributions and succinic acid production were investigated on the basis of the uptake and secretion rates of the relevant metabolites. The results indicated that succinic acid yields increased in order of gluconate, glucose and sorbitol. Acetic acid and lactic acid were produced as major products rather than when gluconate and glucose were used carbon sources. The results indicated that among three carbon sources available, the most reduced substrate is sorbitol which yields efficient succinic acid production.

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 (Bailey, 1991; Lee and Papoutsakis, 1999; Nielsen and Villadsen, 1994). 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.

During the last decade, metabolic flux analysis (MFA) has become an essential tool for metabolic engineering. MFA technique is based on the pseudo-steady state assumption, which means no net intracellular accumulation of intermediates, considering the high turnover of intracellular metabolite pools (Nielsen and Villadsen, 1994). By MFA, the intracellular metabolic fluxes can be quantified by the measurement of extracellular metabolite concentrations in combination with the stoichiometry of intracellular reactions and mass balances (Stephanopoulos and Vallino, 1991).

Succinic acid, a member of C4-dicarboxylic acid family, has been used in many industrial applications including surfactant, ion chelator, food additive, and supplement to pharmaceuticals, antibiotics, and vitamins. Succinic acid can also be used as a precursor of several important chemicals such as 1,4-butanediol, tetrahydrofuran and other C4 chemicals (Zeikus et al., 1999). Especially, succinic acid is an intermediate of several green chemicals and materials. For example, polymerization of succinic acid and 1,4-butanediol yields the biodegradable polymer Bionelle (Showa Highpolymer Co., Tokyo, Japan). At present, succinic acid is manufactured by hydrogenation of maleic anhydride to succinic anhydride, followed by hydration to succinic acid.

Recently, much effort is being exerted for the production of succinic acid and its derivatives by microbial fermentation from renewable feedstocks (Lee et al., 1999; Zeikus, 1980). When manipulating metabolic fluxes for the production of metabolites, however, it is important to achieve the redox balance among the substrates and products (Berovic, 1999; Clark, 1989; Gill et al., 1998; Oh et al., 1998). If the redox balance is not achieved, production of desired metabolites may not be efficient.

In this study, we examined glucose, gluconate and sorbitol, which have different reducing potentials, as carbon substrates for the succinic acid production by recombinant *E. coli*. Then, the effect of reducing power on the intracellular fluxes was evaluated by MFA.

Materials and Methods

Bacterial Strain and Plasmid

E. coli strain NZN111 (F⁻ *pfl*::Cam *ldhA*::Kan) was used (Stols and Donnelly, 1997). In this strain, the anaerobic pyruvate utilization pathway is blocked because of the insertional inactivation of the *pfl* and *ldh*A genes, which encode pyruvate formate-lyase and lactate dehydrogenase, respectively. Pyruvate formate-lyase catalyzes conversion of pyruvate to acetyl-CoA and formic acid, and lactate dehydrogenase converts pyruvate to lactic acid. As a consequence,

NZN111 lost anaerobic fermentation ability. The plasmid pTrcML, which contains *trc* promoter and *E. coli sfcA* gene encoding malic enzyme, was transformed into *E. coli* NZN111 by electroporation (Hong and Lee, 2001). Malic enzyme was expressed from the *trc* promoter by inducing with isopropylthio-β-D-galactoside (IPTG).

Fermentation

Batch cultures were carried out at 37°C using a BioFlo 3000 bioreactor (5 L, New Brunswick Scientific, Edison, NJ, USA) initially containing 3 L of LB medium (tryptone 10 g/L, yeast extract 5 g/L, NaCl 5 g/L) supplemented with 20 g/L of glucose. 5 M NaOH was used to maintain the pH at 6.7. The dissolved oxygen (DO) level was maintained over 40% of air saturation during aerobic cultivation. At the OD_{600} of 5, IPTG was added to a final concentration of 0.01 mM. This allowed production of soluble malic enzyme up to 30% of total cell proteins. After IPTG induction, anaerobic conditions were achieved by lowering the agitation speed and flushing the bioreactor with oxygen-free CO_2-H_2 (molar ratio of 1:1) gas mixture (Kosock gas, Daejeon). Once anaerobic condition was achieved, there was no further gassing during fermentation.

Analysis of metabolites

Fermentation products in the medium were analyzed by high-performance liquid chromatography (Hitachi chromatography system, Tokyo, Japan) equipped with an Aminex HPX-87H column (300 mm X 7.8 mm, Bio-Rad Laboratories, Herculules, CA, USA) and a refractive index detector (L-3300, Hitachi chromatography system). The column was eluted isocratically with 0.012 N H_2SO_4.

Metabolic flux analysis

In this study, the metabolic network of *E. coli* was constructed with 127 reversible and 174 irreversible reactions and 294 metabolites, which contains all metabolic reaction pathways required for growth on glucose. MFA studies were carried out for the calculation of volumetric rates of formation of intracellular metabolites (Nielsen and Villadsen, 1994). The flux balance model was set up as follows :

$$S \cdot v = b$$

where S is the stoichiometric matrix, v is a vector for reaction rates or fluxes and b is the vector for the rates of metabolites consumption and excretion (Stephanopoulos and Vallino, 1991). Since our system is underdetermined, i.e. the number of reactions is greater than the number of metabolites or constraints, linear optimization was carried out with the following objective function :

$$\text{Maximize/minimize:} \quad Z = c_i v_i$$

where c_i are the weights and v_i are the elements of the flux vector (Stephanopoulos and Vallino, 1991). Maximum cell growth was used as an objective function. Linear optimization was carried out using MetaFluxNet (Lee et al., 2003). Experiments for various carbon sources were carried out under anaerobic condition to investigate the effect of each carbon source on the production of succinic acid. They include glucose, sorbitol and gluconate, for each of which secretion rates of metabolite products are given in Table 1.

Table 1. Constraints on the rates of metabolites uptake and excretion.

	Constraints (mM/gDCW/h)		
	Sorbitol	Glucose	Gluconate
Substrate uptake	1.78	1.21	2.63
Succinic acid excretion	2.14	0.50	0.46
Malic acid excretion	0.16	0.47	0.52
Acetic acid excretion	0.53	0.45	2.71
Lactic acid excretion	0.81	0.41	0.15

Results and Discussion

Glucose, gluconate and sorbitol were examined as carbon substrates to evaluate the beneficial effect of redox balance between the substrate and products during the production of succinic acid by recombinant *E. coli* (Fig. 1).

The time profiles of the concentrations of glucose and organic acids are presented in Fig. 1. Anaerobic condition was established after 4 h of aerobic cultivation when gluconate was examined,

and the residual gluconate concentration was 11.6 g/L at this point (Fig. 1A). Acetic acid was produced as a major product instead of succinic acid. The final concentrations of succinic, malic, acetic and lactic acids obtained were 1.1, 1.4, 5.3 and 0.3 g/L, respectively.

When glucose was examined as carbon substrate, anaerobic condition was established after 9.5 h of aerobic cultivation (Fig. 1B). At this
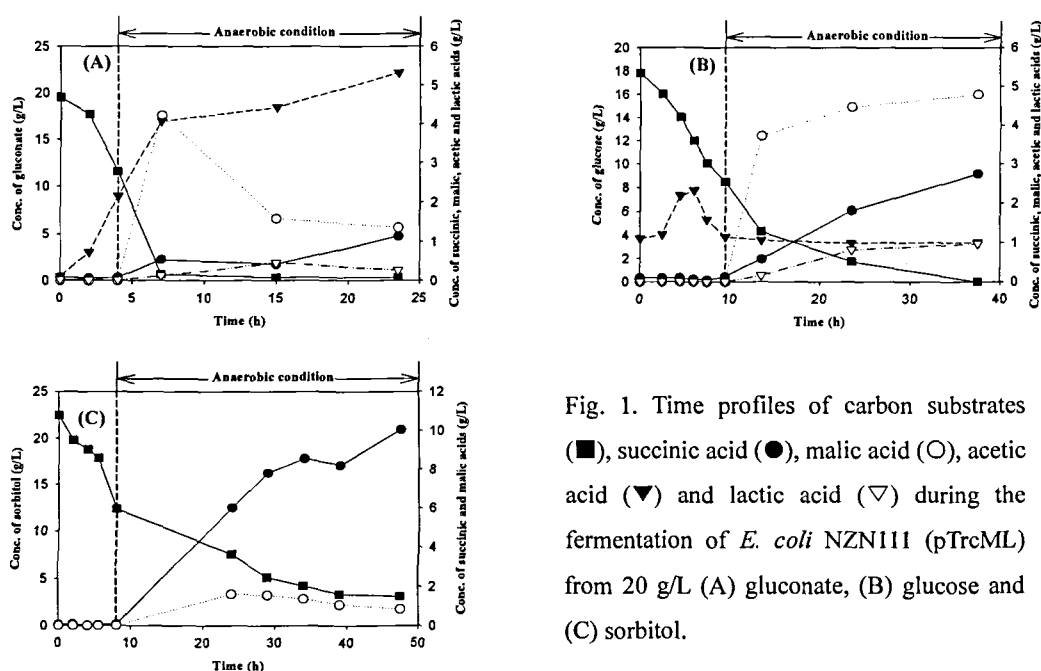

Fig. 1. Time profiles of carbon substrates (■), succinic acid (●), malic acid (○), acetic acid (▼) and lactic acid (▽) during the fermentation of *E. coli* NZN111 (pTrcML) from 20 g/L (A) gluconate, (B) glucose and (C) sorbitol.

point, the residual glucose concentration was 8.5 g/L. After 30 h of anaerobic cultivation, glucose was completely consumed and the major fermentation products remained were malic acid and succinic acid. At the end of the fermentation process, the concentrations of succinic, malic, acetic and lactic acids obtained were 2.8, 4.8, 1.0 and 1.0 g/L, respectively. Succinic acid and acetic acid were produced at the ratio of 2.8:1.

When sorbitol was used, anaerobic condition was established after 8 h of aerobic cultivation (Fig. 1C). At this point, the residual sorbitol concentration was 12.4 g/L. Concentration of

succinic acid increased steadily throughout the cultivation, and only negligible amounts of acetic and lactic acids were produced. The final concentrations of succinic and malic acid were 10.1 and 0.9 g/L, respectively.

The effect of carbon substrate on the intracellular metabolic fluxes and succinic acid production in recombinant E. coli was evaluated by MFA. Metabolic fluxes under succinic acid producing conditions were determined for three different carbon substrates such as sorbitol, glucose and gluconate (Fig. 2). One mole of sorbitol produces six moles of [H] during its
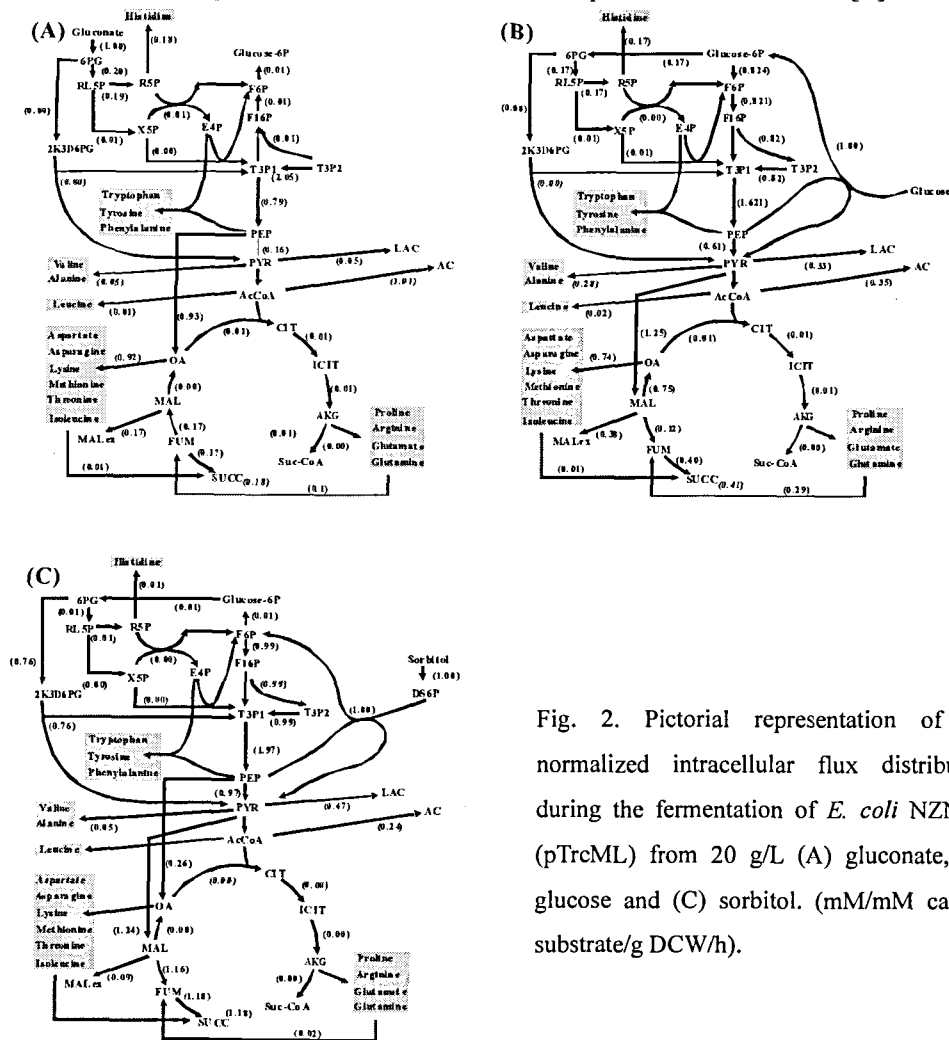

Fig. 2. Pictorial representation of the normalized intracellular flux distribution during the fermentation of E. coli NZN111 (pTrcML) from 20 g/L (A) gluconate, (B) glucose and (C) sorbitol. (mM/mM carbon substrate/g DCW/h).

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conversion to two moles of PEP while glucose and gluconate produce four and two moles of [H], respectively.

When gluconate was used as a carbon substrate, it was predicted that about 8.3% of total carbon flux was directed to succinic acid pathway, and most of it was supplied through the malic enzyme. Next, the intracellular flux distribution in recombinant *E. coli* which used glucose as a carbon source was simulated. Significant amount of carbon flux (20.5%) was directed into the succinic acid pathway. Again, it was most notable that the malic enzyme flux was highly activated under the succinic acid producing condition. These results are in good agreement with previous report suggesting pyruvate carboxylation as a optimal succinic acid production pathway (Stols and Donnelly, 1997). Finally, sorbitol was examined as a carbon substrate. Flux analysis result indicated that most of carbon flux (83.8%) was directed into succinic acid production pathway and most of other intracellular fluxes were severely reduced.

When comparing three flux analysis results, it was found that the succinic acid flux and yield were increased in the order of gluconate, glucose and sorbitol. Consumption rate of CO_2 and $FADH_2$ foramtion rate, which required for the conversion of pyruvate to oxaloacetate and for the conversion of fumarate to succinic acid, respectively, were also increased in the order of gluconate, glucose and sorbitol. These results suggesting the importance of reducing power balance.

Besides succinic acid production, production rates of other organic acids were also influenced by reducing power. When least reduced carbon substrate, gluconate, was used, acetic acid was produced as a major product. For the production of acetic acid from pyruvate, reducing power is not required (Fig. 2). When glucose was used as a carbon substrate, lactic acid, which requires NADH during conversion from pyruvate, was also produced as well as acetic acid. This result indicated that glucose could provide more reducing power. Finally, these results indicated that the most reduced carbon substrate, sorbitol, allowed efficient succinic acid production. When sorbitol was used as carbon substrate, succinic acid was produced as a major product rather than lactic and acetic acids. For the conversion of one mole of succinic acid from pyruvate, each mole of NADH and $FADH_2$ are required. Consequently, it can be mentioned that product profile can be manufactured by the control of reducing power, and succinic acid productivity could be enhanced if balance of reducing was achieved by supply of additional reducing power as predicted in previous study (Hong and Lee, 2002).

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