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Metabolic Flux Distribution in a Metabolically Engineered *Escherichia coli* Strain Producing Succinic Acid

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Abstract *Escherichia coli* NZN111, which is known as a *pfl ldhA* double mutant strain, was metabolically engineered to produce succinic acid by overexpressing malic enzyme into the *E. coli* controlled by a *trc* promoter. Fermentation studies were carried out in a LB medium by first growing cells aerobically to an OD_{600} of 5. At this point, 0.01 mM IPTG was added to induce the overexpression of malic enzyme and the agitation speed was gradually lowered. When the culture OD_{600} reached 11, a complete anaerobic condition was achieved by flushing with a CO_2-H_2 gas mixture. When NZN111(pTrcML) was cultured at 37°C, the final succinic acid concentration of 2.8 g/l could be obtained after 30 h of anaerobic cultivation. The fermentation results were analyzed by the calculation of metabolic fluxes. Metabolic flux analysis showed that about 85% of phosphoenolpyruvate (PEP) was converted to pyruvate, and further converted to malic acid by malic enzyme.

Key words: Succinic acid, malic enzyme, metabolic engineering, metabolic flux analysis, *Escherichia coli*

Metabolic engineering, which is the directed alteration of an organism's metabolism through genetic manipulation, often involves the amplification or introduction of naturally occurring metabolic pathways [1, 7]. In the past, the trial-and-error approach was taken for metabolic engineering of cells since only little information on the metabolic status of cells was available. In recent years, however, the metabolic flux analysis has been placed at the core of metabolic engineering. By metabolic flux analysis, the metabolic status of the strain can be evaluated, and numerous reaction sequences from substrates to products can also be monitored. Moreover, metabolic flux analysis is useful for calculating the maximum theoretical yield and for the characterization of specific metabolic pathways [12].

Succinic acid is an intermediate of cellular metabolism belonging to the C_4-dicarboxylic acid family. Succinic acid and its derivatives are widely used as common chemicals to synthesize polymers, along with some applications in foods, pharmaceuticals, and cosmetics. Succinic acid is currently manufactured by the hydrogenation reaction of maleic anhydride to succinic anhydride, followed by hydration to succinic acid. Production of succinic acid through fermentation represents an alternative synthetic route *via* the utilization of renewable feedstocks [6, 11, 16, 17].

Metabolic engineering strategies have been employed to create a recombinant *E. coli* strain for enhancing succinic acid production from glucose. For example, phosphoenolpyruvate carboxylase (PEP carboxylase), which converts PEP to oxaloacetate, was overexpressed for the production of succinic acid [8]. In addition to glucose, fumaric acid has been used as a substrate for the synthesis of succinic acid by recombinant *E. coli* strains with amplified fumarate reductase [5, 14]. In *E. coli*, however, a PEP carboxylase channels PEP towards succinic acid and dissipates the energy of PEP through hydrolysis to inorganic phosphate [4, 9]. The free energy of PEP is conserved in the major routes of PEP metabolism in *E. coli*, *via* conversion to pyruvate by pyruvate kinase (PyK) or by the phosphotransferase system (PTS). A possible alternative route was recently proposed; conversion of pyruvate to dicarboxylic acid by the reductive carboxylation of pyruvate to malic acid catalyzed by malic enzyme [13]. Here, we evaluate the possibility of constructing an unnatural metabolic pathway to dicarboxylic acids through expression of the *E. coli* NAD^+-dependent malic enzyme, in a host which is deficient in its normal fermentation metabolism of pyruvate. The metabolic flux analysis was also carried out to thoroughly examine the extent of involvement of various pathways in the overall metabolic pathways.

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MATERIALS AND METHODS

Bacterial Strain and Plasmid

E. coli strain NZN111 (F *pfl*::Cam *ldhA*::Kan) was used. In this strain, the anaerobic pyruvate utilization pathway is blocked due to the insertional inactivation of the *pfl* and *ldh*A genes. As a result, NZN111 lost its ability to ferment anaerobically [3]. *E. coli* NZN111 was transformed with pTrcML by electroporation. The pTrcML contains the *trc* promoter and the *sfcA* gene which encodes the anaerobic NAD^+ specific malic enzyme 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, U.S.A.) [2].

Fermentation

Fed-batch culture was carried out at 37°C using the BioFlo 3000 bioreactor (5 l, New Brunswick Scientific, Edison, U.S.A.) 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. NaOH (5 M) was used to maintain the pH level at 6.7. Antibiotics were added: 100 μg/ml ampicillin, 30 μg/ml kanamycin, and 30 μg/ml chloramphenicol. 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. After IPTG induction, the anaerobic condition was achieved by lowering the agitation speed and flushing the bioreactor with an oxygen-free CO_2-H_2 (molar ratio of 1:1) gas mixture (Kosock gas, Taejon, Korea). A completely anaerobic condition was achieved when the culture reached to OD_{600} of 11.

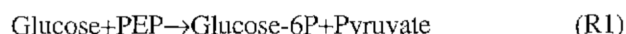
Product Analysis

Fermentation products were analyzed by HPLC (Hitachi chromatography system, Tokyo, Japan) equipped with an Aminex HPX-87H column (300 mm×7.8 mm, Bio-Rad Laboratories, Hercules, U.S.A.) and a refractive index detector (L-3300, Hitachi chromatography system). The column was eluted isocratically with 5 mM H_2SO_4.

MODELLING AND THEORETICAL ASPECTS

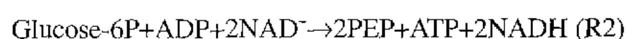
Construction of the Flux Network

Glucose import. *E. coli* can import glucose by using the phosphotransferase system (PTS) and by glucokinase. In this study, it was assumed that *E. coli* takes in glucose only by the PTS. PEP is converted to pyruvate as soon as glucose is taken up by the PTS.



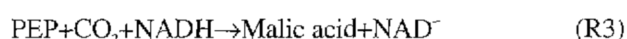
Glucose+PEP→Glucose-6P+Pyruvate (R1)

Glycolysis. Reactions from glucose-6P to PEP in the glycolysis pathway are carried out by a one-step reaction.



$Glucose\text{-}6P+ADP+2NAD^+ \rightarrow 2PEP+ATP+2NADH$ (R2)

PEP catabolism. PEP can be converted to pyruvate not only by the PTS but also by PyK. Two moles of PEP are produced by glycolysis from one mole of glucose. One mole of PEP is used to import one mole of glucose by the PTS, while the residual PEP can be converted to pyruvate or oxaloacetate. PEP can be converted to oxaloacetate and further converted to malic acid by PEP carboxylase and malate dehydrogenase (MDH). These two reactions are lumped into the reaction R3. NADH and CO_2 are required for the conversion of PEP to malic acid, and ATP is not produced.



$PEP+CO_2+NADH \rightarrow Malic\ acid+NAD^+$ (R3)

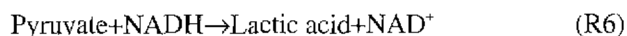
PEP is converted to pyruvate by Pyk with concomitant production of equimolar ATP.

PEP+ADP→Pyruvate+ATP (R4)

Pyruvate pool. In this system, accumulation of pyruvate is necessary to produce succinic acid and, for this reason, the size of the pyruvate pool was not constant. The concept of intracellular pyruvate pool was introduced to analyze this system with the satisfaction of a pseudo-steady state assumption.

Pyruvate→Pyruvate pool (R5)

Pyruvate catabolism. Pyruvate can be catabolized to several different metabolites such as acetic acid, lactic acid, and succinic acid. Formation of lactic acid has an important role for NAD^+ regeneration under anaerobic condition. This reaction is catalyzed by lactate dehydrogenase (LDH), the expression of which is enhanced by low pH and pyruvate accumulation.



$Pyruvate+NADH \rightarrow Lactic\ acid+NAD^+$ (R6)

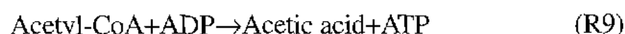
Under anaerobic condition, PEP is converted to malic acid by PEP carboxylase and Mdh. Then, malic acid is used as a substrate for malic enzyme to produce pyruvate. However, in the current host strain we are employing, pyruvate can be converted to malic acid because the metabolic pathway is reoriented by the *pfl ldhA* double mutation. Consequently, malic enzyme converts pyruvate to malic acid as follows.

$Pyruvate+CO_2+NADH \rightarrow Malic\ acid+NAD^+$ (R7)

E. coli has two enzymes to convert pyruvate to acetyl-CoA. Under aerobic condition, pyruvate is decarboxylated to acetyl-CoA by pyruvate dehydrogenase (PDH) and equi-molar NADH is generated. Under anaerobic condition, pyruvate is converted to acetyl-CoA by pyruvate formate-lyase (PFL), with a concomitant formation of formic acid.

Pyruvate→Formic acid+Acetyl-CoA (R8)

Acetyl-CoA catabolism. Acetyl-CoA is converted to acetic acid in two enzymatic reactions catalyzed by phosphotransacetylase (PTA) and acetate-kinase (ACK).



Acetyl-CoA+ADP→Acetic acid+ATP (R9)

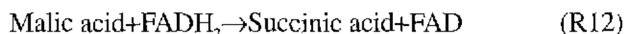
Under anaerobic condition, acetyl-CoA can also be converted to ethanol by alcohol dehydrogenase (ADH) with concomitant regeneration of $2NAD^+$.

Acetyl-CoA+2NADH→Ethanol+$2NAD^+$ (R10)

Malic acid excretion. Because a considerable amount of malic acid was excreted to the medium (see below), this excretion pathway is included in the metabolic network. Excreted malic acid is designated as extracellular malic acid (ext-Mal).

Malic acid→ext-Mal (R11)

Succinic acid formation. Malic acid is converted to succinic acid by fumarase (FumB) and FrdABCD enzymes.



Malic acid+$FADH_2$→Succinic acid+FAD (R12)

Maintenance energy and other ATP usage are simply shown by the equation of R13. The amount of ATP consumed at 37°C was determined by the black box method [10] using the data collected from other fermentation studies.

ATP→ADP (R13)

To simplify, NADH is used to represent a general electron carrier in the proposed metabolic pathways. Anabolic reactions are not considered since no cell growth was observed under this anaerobic condition.

Metabolic Flux Analysis

Here, we used metabolic flux analysis for the calculation of volumetric rates of formation for intracellular metabolites [7, 10, 15]. This metabolic network consisted of twelve reactions along with seven metabolic intermediates. Five reaction rates (glucose, succinic acid, ext-Mal, acetic acid, and lactic acid) and maintenance energy were measured, although only four reaction rates were needed to calculate volumetric rates of intracellular metabolites (glucose, succinic acid, ext-Mal, and acetic acid). The redundant information on the lactic acid formation rate was used to check the validity of pseudo-steady state assumption by comparing the calculated lactic acid formation rate with the measured lactic acid formation rate.

The vector $\mathbf{q}$ was defined as the net volumetric rates of formation for substrates, metabolic products, and biomass component. $\mathbf{T}$ is the total stoichiometric matrix for all the reactants and products of the reactions. Relating to the equation below,

$$\mathbf{q}=\mathbf{T}^{T}\mathbf{r}x \tag{F1}$$

$\mathbf{r}$ represents the unknown vector for forward reaction rates and x is for biomass concentration (mM). The total volumetric rate vector $\mathbf{q}$ can be divided into 3 parts:

$$\mathbf{q}=\begin{pmatrix}\mathbf{q}_m\\ \mathbf{q}_c\\ \mathbf{q}_x\end{pmatrix}=\begin{pmatrix}\mathbf{q}_m\\ \mathbf{q}_c\\ \mathbf{0}\end{pmatrix}=\begin{pmatrix} q_{glu}\\ q_{suc}\\ q_{ext-Mal}\\ q_{ace}\\ \underline{q_{lac}}\\ q_{for}\\ q_{etha}\\ \underline{q_{pyr-pool}}\\ q_{glu-6P}\\ q_{PEP}\\ q_{pyr}\\ q_{ace-CoA}\\ q_{malic}\\ q_{ATP}\\ q_{NADH}\end{pmatrix} \tag{F2}$$

where $\mathbf{q}_m$ is the vector of measured reaction rates (glucose, succinic acid, ext-Mal, and acetic acid) and $\mathbf{q}_c$ is of calculated reaction rates (lactic acid, formic acid, and ethanol). The vector $\mathbf{q}_x$ is a zero vector because it is assumed that the metabolic intermediates are in pseudo-steady states (glucose-6P, PEP, pyruvate, acetyl-CoA, malic acid, ATP, and NADH).

Similar to the arrangement of $\mathbf{q}$ in equation (F2), the system of linear algebraic equation in equation (F1) can be written as follows.

$$\begin{pmatrix}\mathbf{q}_m\\ \mathbf{q}_c\\ \mathbf{0}\end{pmatrix}=\begin{pmatrix} q_{glu}\\ q_{suc}\\ q_{ext-Mal}\\ q_{ace}\\ \underline{q_{lac}}\\ q_{for}\\ q_{etha}\\ \underline{q_{pyr-pool}}\\ q_{glu-6P}\\ q_{PEP}\\ q_{pyr}\\ q_{ace-CoA}\\ q_{malic}\\ q_{ATP}\\ q_{NADH}\end{pmatrix}=\left(\begin{array}{ccccc|ccccccc}
-1 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0\\
0 & 1 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0\\
0 & 0 & 1 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0\\
0 & 0 & 0 & 1 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0\\
0 & 0 & 0 & 0 & 1 & 0 & 0 & 0 & 0 & 0 & 0 & 0\\
\hline
0 & 0 & 0 & 0 & 0 & 1 & 0 & 0 & 0 & 0 & 0 & 0\\
0 & 0 & 0 & 0 & 0 & 0 & 1 & 0 & 0 & 0 & 0 & 0\\
0 & 0 & 0 & 0 & 0 & 0 & 0 & 1 & 0 & 0 & 0 & 0\\
\hline
1 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & -1 & 0 & 0 & 0\\
-1 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 2 & -1 & -1 & 0\\
1 & 0 & 0 & 0 & -1 & -1 & 0 & -1 & 0 & 1 & 0 & -1\\
0 & 0 & 0 & -1 & 0 & 1 & -1 & 0 & 0 & 0 & 0 & 0\\
0 & -1 & -1 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 1 & 1\\
0 & 0 & 0 & 1 & 0 & 0 & 0 & 0 & 1 & 1 & 0 & 0\\
0 & -1 & 0 & 0 & -1 & 0 & 2 & 0 & 2 & 0 & -1 & -1
\end{array}\right)$$

$$\begin{pmatrix} r_1\\ r_{12}\\ r_{11}\\ r_9\\ \underline{r_6}\\ r_8\\ r_{10}\\ r_5\\ r_2\\ r_4\\ r_3\\ r_7\end{pmatrix}=\begin{pmatrix}\mathbf{T}_1 & \mathbf{T}_2\\ \mathbf{T}_3 & \mathbf{T}_4\\ \mathbf{T}_5 & \mathbf{T}_6\end{pmatrix}\begin{pmatrix}\mathbf{r}_m x\\ \mathbf{r}_c x\end{pmatrix} \tag{F3}$$

Whereas the partitioning of $\mathbf{q}$ into measured rates $\mathbf{q}_m$, calculated rates $\mathbf{q}_c$, and zero net-rate elements has a clear meaning, the corresponding partitioning of $\mathbf{r}$ into $\mathbf{r}_m$ and $\mathbf{r}_c$ does not imply that the part of $\mathbf{r}$ is known. All elements of $\mathbf{r}$ as well as the vector $\mathbf{q}_c$ can be calculated as shown below.

$$\mathbf{r}_c x = -\mathbf{T}_6^{-1}\mathbf{T}_5\mathbf{T}_7^{-1}\mathbf{q}_m \quad \text{(F4)}$$

where

$$\mathbf{T}_7 = \mathbf{T}_1 - \mathbf{T}_2\mathbf{T}_6^{-1}\mathbf{T}_5 \quad \text{(F5)}$$

Obviously, the inverse of the matrices $\mathbf{T}_6$ and $\mathbf{T}_7$ should exist for the above calculations.

RESULTS AND DISCUSSION

Fermentation of NZN111(pTrcML) was carried out at 37°C. The culture was induced with 0.01 mM IPTG at the OD_{600} of 5. The induction profiles of cell density and concentrations of glucose and organic acids are presented in Figs. 1A and 1B. After 10 h of aerobic cultivation, the OD_{600} reached to 11 and an anaerobic condition was achieved. At this point, the residual glucose concentration was 9 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 acid, lactic acid, and acetic acid were 2.8, 0.98, and 1.0 g/l, respectively. Succinic acid and acetic acid were produced at the ratio of 2.8:1, which is higher than that typically obtained of the best succinic acid producers, *A. succiniciproducens* (2:1). While 2.1 g/l of malic acid was produced in 1 h, only 0.8 g/l of malic acid was converted to succinic acid in that same period. This caused accumulation and excretion of malic acid. In fact, this unbalanced situation might be due to the overexpression of malic enzyme and metabolic flux reorientation.

Based on the time profiles of metabolite concentrations, the anaerobic cultivation phase can be divided into two periods. Period I (10<t<13 h) is the early stage (adaptation stage) of the anaerobic cultivation and, during this period, the culture OD_{600} decreased rapidly. The rate of malic acid formation was high, and succinic acid and lactic acid started to accumulate during this period. In Period II (13<t<40 h), the glucose consumption rate and malic acid formation rate decreased. During this period, the concentration of succinic acid and malic acid increased steadily. Acetic acid concentration was rather constant, while lactic acid first increased slowly to reach a constant value.

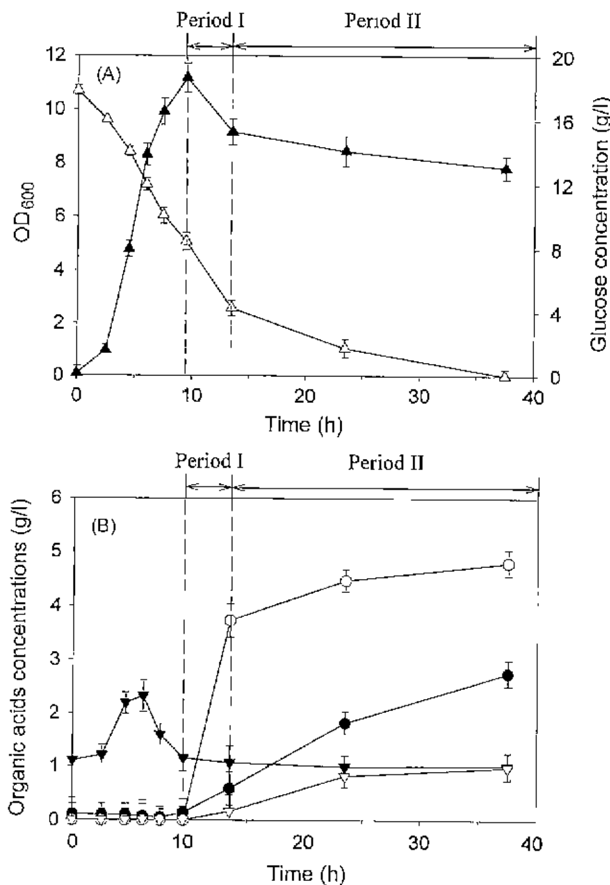


Fig. 1. Time profiles of (A) the culture OD_{600} (▲) and glucose concentration (g/l, △), and (B) concentrations of succinic acid (g/l, ●), malic acid (g/l, ○), acetic acid (g/l, ▼), and lactic acid (g/l, ▽).

Metabolic Flux Analysis

The result of fermentation was analyzed and shown in Fig. 2. Figure 2A represents the average intracellular metabolic flux distribution during the entire anaerobic cultivation process, while Figs. 2B and 2C represent those of Period I and II, respectively. When the metabolic flux analysis was carried out during the entire anaerobic cultivation period, it was observed that 85% of PEP was converted to pyruvate with concomitant production of ATP. The flux of reaction R9 has a minus sign, since a small amount of acetic acid was produced and the acetic acid produced during aerobic period was consumed.

In Period I, 73% of PEP was converted to malic acid. Malic acid produced in a large amount followed three alternative destinations: excretion to the medium, conversion to pyruvate, or conversion to succinic acid. During Period I, most of the malic acid produced was excreted to the medium, while only a small amount converted to succinic acid. In Period II, the glucose consumption rate decreased significantly, and so did almost all of the intracellular metabolic fluxes. In contrast to Period I, nearly all PEP was converted to pyruvate and little pyruvate (13%) was accumulated in the pyruvate pool (63% in Period I). These results suggest that pyruvate was not accumulated to a high enough concentration to change the direction of reaction R7 in Period I. Pyruvate concentration increased to the level which allowed the reversal of the R7 reaction during Period II. More than 70% of malic acid was converted to succinic acid. Since 30% of malic acid was still excreted (wasted in the view of succinic acid production), the rate of malic acid formation should be reduced to enhance the succinic acid production.

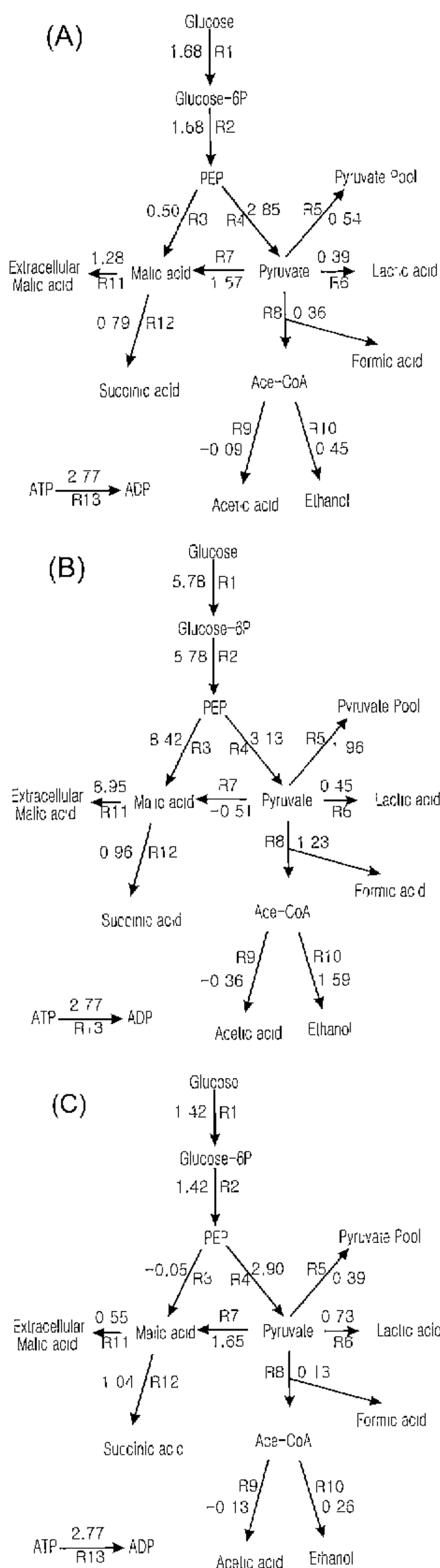


Fig. 2. Pictorial representation of intracellular flux (mM/h) distribution with reaction numbers (R1-13) (A) during the entire anaerobic cultivation, (B) during Period I, and (C) during Period II.

Several approaches were possible to achieve an enhanced succinic acid production: (1) reduction of malic enzyme expression, (2) overexpression of fumarase, whereby malic acid can be converted to fumaric acid, and (3) use of other substrates which have more reducing power than glucose. All these possibilities are currently under investigation.

NOMENCLATURES

$\mathbf{q}$	the net volumetric rates of formation for substrates, metabolic products
$\mathbf{q}_m$	the vector of measured reaction rates
$\mathbf{q}_c$	the vector of calculated reaction rates
$\mathbf{q}_x$	the vector of net formation rates of metabolic intermediates
$\mathbf{T}$	the total stoichiometric matrix for all the reactants and products of reactions
x	biomass concentration (mM)
$\mathbf{r}$	the unknown vector of net forward reaction rates
$\mathbf{r}_m$	the unknown vector of measured forward reaction rates
$\mathbf{r}_c$	the unknown vector of calculated forward reaction rates

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