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Prediction of Maximum Yields of Metabolites and Optimal Pathways for Their Production by Metabolic Flux Analysis

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Abstract The intracellular metabolic fluxes can be calculated by metabolic flux analysis, which uses a stoichiometric model for the intracellular reactions along with mass balances around the intracellular metabolites. In this study, metabolic flux analyses were carried out to estimate flux distributions for the maximum *in silico* yields of various metabolites in *Escherichia coli*. The maximum *in silico* yields of acetic acid and lactic acid were identical to their theoretical yields. On the other hand, the *in silico* yields of succinic acid and ethanol were only 83% and 6.5% of their theoretical yields, respectively. The lower *in silico* yield of succinic acid was found to be due to the insufficient reducing power, but this lower yield could be increased to its theoretical yield by supplying more reducing power. The maximum theoretical yield of ethanol could be achieved, when a reaction catalyzed by pyruvate decarboxylase was added in the metabolic network. Furthermore, optimal metabolic pathways for the production of various metabolites could be proposed, based on the results of metabolic flux analyses. In the case of succinic acid production, it was found that the pyruvate carboxylation pathway should be used for its optimal production in *E. coli* rather than the phosphoenolpyruvate carboxylation pathway.

Key words: Metabolic engineering, metabolic flux analysis, *in silico* yield, optimal pathway

Metabolic engineering can be defined as the 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 [1, 13]. Various metabolic engineering strategies have been applied for the more efficient production of desired metabolites and biomolecules [13]. Even though enhanced production of some biomolecules has been successful, many other attempts have failed due to the lack of rational strategies based on a predictable technique. Therefore, metabolic flux analysis, which allows the calculation of intracellular metabolic fluxes based on the stoichiometry of intracellular reactions and mass balances around the intracellular metabolites, has become an essential tool for metabolic engineering [4, 16].

Metabolic flux analysis is based on the pseudo-steady state assumption, which means no net intracellular accumulation of intermediates, considering the high turnover of intracellular metabolite pools [19, 21]. Metabolic flux analysis has been applied to calculate the maximum theoretical yield of a desired metabolite to be produced, and to identify the rigidity of branch points in the metabolic pathways [11, 20]. Another possible application is the identification of alternative metabolic pathways that lead to the desired product [14, 22]. Detailed theories and applications of the flux analysis can be found in recent reviews [4, 19].

Determination of the achievable maximum yield and optimal metabolic pathways is essential for the engineering of the metabolic pathways and for the redirection of metabolic fluxes towards the desired bioproducts. In this study, metabolic flux analyses were carried out for the estimation of the maximum *in silico* yields of four metabolites in *Escherichia coli* and for the determination of the optimal flux distribution.

Theoretical Backgrounds

Construction of *In Silico* Metabolic Network. From the results of *E. coli* genome sequencing project, about 600 to 700 metabolic reactions were identified [2]. Most of the reactions such as amino acid transport and utilization are, however, not essential for cell growth, and consequently, 224 metabolic reactions can support growth of *E. coli* on glucose [3, 17]. Therefore, in this study, a metabolic network was constructed consisting of 127 reversible and

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174 irreversible reactions and 294 metabolites, containing all metabolic pathways required for the growth of *E. coli* on glucose. The list of reactions and metabolites can be found from our website (http://mbel.kaist.ac.kr).

Metabolic Flux Analysis. Metabolic flux analyses were carried out for the calculation of the volumetric rates of formation of intracellular metabolites [13, 16]. For the calculation of intracellular metabolic fluxes, the composition of *E. coli* biomass was assumed to be 55% protein, 20.5% RNA, 3.1% DNA, 9.1% lipids, 3.4% lipopolysaccharides, 2.5% peptidoglycan, 2.5% glycogen, 0.4% polyamines, and 3.5% other metabolites, cofactors, and ions [15]. The types and amounts of precursors required to synthesize these macromolecules were determined from the average composition of each of the macromolecules in *E. coli*: Protein: Ala 9.6%, Arg 5.53%, Asn 4.51%, Asp 4.51%, Cys 1.71%, Glu 4.92%, Gly 4.92%, His 1.77%, Ile 5.43%, Leu 8.42%, Lys 6.42%, Met 2.87%, Phe 3.46%, Pro 4.13%, Ser 4.03%, Thr 4.74%, Trp, 1.06%, Tyr 2.58%, Val 7.91%; RNA: ATP 26.2%, GTP 32.2%, CTP 20.0%, UTP 21.6%; DNA: dATP 24.7%, dGTP 25.4%, dCTP 25.4%, TTP 24.7%; phospholipids: phosphatidylethanolamine 72.8%, phosphatidylglycerol 19.8%, cardiolipin 3.4% [15].

The flux balance model was set up as follows:

$$\mathbf{S \cdot v = b} \tag{1}$$

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 [4]. Since this study's 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:

$$\text{Maximize: } Z = \sum_i c_i v_i \tag{2}$$

where c_i are the weights and v_i are the elements of the flux vector [21]. Linear optimization was carried out using the program LP_solve (State University of New York, NY, U.S.A.). To obtain feasible solutions, constraints on the rates of metabolites uptake and excretion were applied (Table 1). As seen in Table 1, cells are allowed to operate both aerobic and anaerobic pathways with no limit of nutrient.

Table 1. Constraints on the specific rates of metabolite uptake and excretion.

	Specific rate (mM/g DCW/h)
Glucose uptake	<1
Oxygen uptake	>0
NH_3 uptake	>0
Phosphate uptake	>0
H_2SO_4 uptake	>0
CO_2 uptake	>0
CO_2 excretion	>0

Definition of *In Silico* Yield and Theoretical Yield. The *in silico* yield is defined as the yield calculated by metabolic flux analysis, which represents the actual amount of metabolite that can be formed through the metabolic reactions in operation from one mole of substrate. The theoretical yield is defined as the yield which can be achieved by the complete conversion of substrate to metabolite.

RESULTS

Maximum Metabolite Yields

The maximum capacities of metabolites production were estimated by metabolic flux analysis. The maximum theoretical and *in silico* yields of acetic acid, lactic acid, succinic acid, and ethanol on glucose are shown in Table 2. As expected, the maximum *in silico* yields of acetic acid and lactic acid were 2 mole/mole glucose, which are identical to their theoretical yields.

However, the maximum *in silico* yield of succinic acid and ethanol were only 1.65 mole/mole glucose (83% of the maximum theoretical yield) and 0.13 mole/mole glucose (6.5% of the maximum theoretical yield), respectively. It was found that the CO_2 availability for maximum production of succinic acid was 0.82 mole/mole glucose/h even though the CO_2 consumption rate was not restricted (Table 1). CO_2 is required for the carboxylation of C_3-compounds

Table 2. The maximum *in silico* yields of various metabolites.

	Metabolites			
	Acetic acid	Lactic acid	Succinic acid	Ethanol
Maximum theoretical yield	0.67* (2**)	1 (2)	1.31 (2)	0.51 (2)
Maximum *in silico* yield	0.67 (2)	1 (2)	1.08 (1.647)	0.03 (0.13) 0.51*** (2****)

*Mass yield (g metabolites/g glucose).
**Molar yield (mole metabolites/mole glucose).
***Mass yield when an additional reaction catalyzed by pyruvate decarboxylase was introduced.
****Molar yield when an additional reaction catalyzed by pyruvate decarboxylase was introduced.

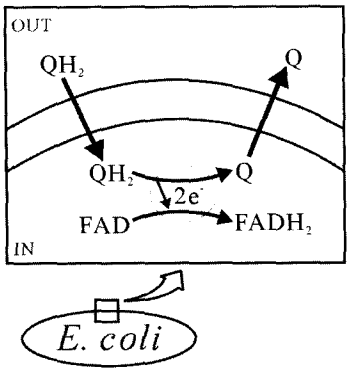

Fig. 1. External supply of reducing power.
Ubiquinol or menaquinol (QH_2) is oxidized to ubiquinone or menaquinone (Q), during which the electron acceptor (shown here is FAD) is reduced.

(phosphoenolpyruvate and pyruvate) to C_4-compounds (oxaloacetate and malic acid), which are further converted to succinic acid [10]. Therefore, 2 moles of CO_2 are theoretically required for each mole of glucose to achieve the maximum yield of succinic acid. These results indicate that there exists a limiting factor. As stated earlier, however, cells are operating metabolic pathways without any nutrient limitation. Examination of the metabolic reaction network that was constructed suggested that there was no reaction which incorporated external reducing power. It was therefore reasoned that the limiting factor for succinic acid production might be reducing power. To examine this hypothesis, the following reaction was added to the metabolic reaction network:

$$QH_2 \rightarrow Q + 2H^+ + 2e^- \quad (3)$$

where QH_2 is ubiquinol or menaquinol and Q is ubiquinone or menaquinone. The electrons generated can be transferred to several different acceptors including FAD (Fig. 1) and fumaric acid. After incorporating this reaction, intracellular flux distribution was estimated again for all four cases. The maximum *in silico* yield of acetic acid, lactic acid, and ethanol were the same as before. However, the maximum *in silico* yield of 2 moles of succinic acid/mole glucose was achieved, instead of 1.65 mole/mole glucose, by supplying more reducing power. This result is consistent with our previous report showing that succinic acid flux was controlled by reducing power in *E. coli*, and that the succinic acid production could be enhanced by using a more reduced carbon substrate such as sorbitol [7].

Ethanol production is rather inefficient in *E. coli*. Good ethanol producers such as yeast and *Zymomonas* possess pyruvate decarboxylase for the conversion of pyruvate to acetaldehyde, which in turn is converted to ethanol. Interestingly, when this pyruvate decarboxylase reaction was added to the metabolic network of *E. coli*, the maximum ethanol yield of 2 moles/mole glucose could be achieved. It has already been reported that the introduction of the *Zymomonas mobilis* pyruvate decarboxylase gene in *E. coli* allowed the efficient production of ethanol [9].

Optimal Flux Distributions for Maximum Metabolite Production

The optimal metabolic flux distributions for the production of acetic acid, lactic acid, succinic acid, and ethanol are shown in Fig. 2. The optimal flux distribution for the production of lactic acid is quite simple. All glucose is converted to pyruvate through glycolysis and further converted

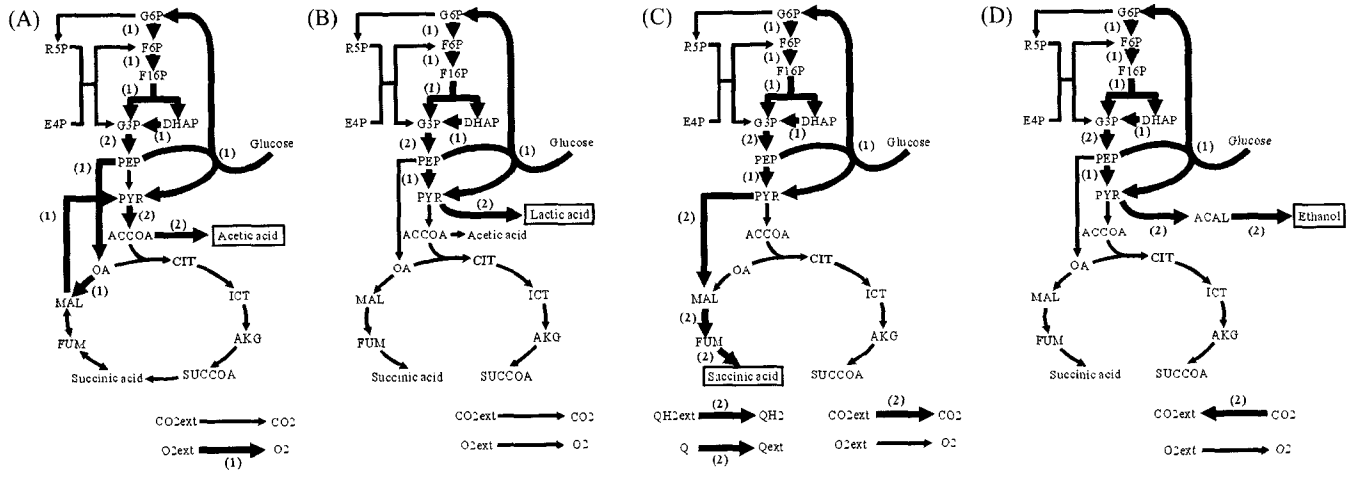

Fig. 2. Optimized metabolic flux distributions during the production of (A) acetic acid, (B) lactic acid, (C) succinic acid, and (D) ethanol.
Abbreviations: ACCOA, acetyl-CoA; AKG, α-ketoglutarate; CIT, citrate; CO2, carbon dioxide; DHAP, dihydroxyacetone phosphate; E4P, erythrose-4-phosphate; F16P, fructose-1,6-bisphosphate; F6P, fructose-6-phosphate; FUM, fumaric acid; G3P, glyceraldehyde-3-phosphate; G6P, glucose-6-phosphate; ICT, isocitrate; MAL, malic acid; O2, oxygen; OA, oxaloacetate; PEP, phosphoenolpyruvate; PYR, pyruvate; R5P, ribose-5-phosphate; SUCCOA, succinyl-CoA; Q, ubiquinone or menaquinone; QH2, ubiquinol or menaquinol; ext, external.

to lactic acid. No fluxes to the pentose phosphate pathway nor to the macromolecule biosynthesis, which included amino acids and lipid, were detected from flux analysis.

For acetic acid production, all glucose is converted to phosphoenolpyruvate (PEP) in the same manner as in lactic acid production. However, half of PEP is carboxylated to oxaloacetate by PEP carboxylase and further converted to pyruvate by malate dehydrogenase and malic enzyme:

$$\text{Phophoenolpyruvate}+CO_2 \rightarrow \text{Oxaloacetate}+\text{Phosphate} \quad (4)$$

$$\text{Oxaloacetate}+\text{NADH} \leftrightarrow \text{Malic acid}+\text{NAD} \quad (5)$$

$$\text{Malic acid}+\text{NAD} \rightarrow CO_2+\text{NADH}+\text{Pyruvate} \quad (6)$$

Through this pathway, free energy of PEP is wasted as inorganic phosphate while ATP can be generated by pyruvate kinase.

$$\text{PEP}+\text{ADP} \rightarrow \text{Pyruvate}+\text{ATP} \quad (7)$$

Even though half of the PEP's free energy was wasted, the ATP produced through acetic acid production was three times higher than that obtained through other metabolite production pathways (Table 3):

$$\text{Pyruvate}+\text{CoA} \rightarrow \text{Acetyl-CoA}+\text{Formate} \quad (8)$$

$$\text{Acetyl-CoA}+\text{Phosphate} \rightarrow \text{Acetylphosphate}+\text{CoA} \quad (9)$$

$$\text{Acetylphosphate}+\text{ADP} \rightarrow \text{Acetic acid}+\text{ATP} \quad (10)$$

The optimal metabolic pathway for succinic acid production is shown in Fig. 2C. Glucose is converted to pyruvate and finally to succinic acid by sequencial reactions of malic enzyme, fumarase, and fumarate reductase. The proposed optimal *in silico* pathway is not consistent with the traditionally known succinic acid production pathway, which consists of PEP carboxylase, malate dehydrogenase, fumarase, and fumarate reductase [8, 12]. Moreover, the optimal pathway indicates that the direction of the malic enzyme reaction should be opposite to the normal direction, since the malic enzyme nomally catalyzes the decarboxylation of malic acid to pyruvate due to the kinetic characteristics (K_m=0.4 mM for malic acid, 16 mM for pyruvate) [18].

Optimal flux distribution for the production of ethanol was quite simple, when a reaction catalyzed by pyruvate decarboxylase was added to the metabolic network (Fig. 2D). As in lactic acid production, all glucose was converted to pyruvate through glycolysis and further converted to ethanol.

Table 3. Formation rates of various metabolites and cofactors.

	Metabolites		Cofactors		
	Acetyl-CoA	Pyruvate	ATP	NADH	$FADH_2$
Acetic acid	2*	2	6.3	3	0
Lactic acid	0	2	3	2	0
Succinic acid	0	2	3	2	2
Ethanol	2	2	3	2	0

*Formation rates: sum of metabolites producing fluxes (mM/g DCW/h).

DISCUSSION

Through metabolic flux analysis, the maximum *in silico* yields and the optimal metabolic pathways for the production of four metabolites could be determined. We used well-known single primary metabolites as model systems to demonstrate the usefulness of this analysis. Based on the yields and optimal pathways predicted *in silico*, rational metabolic engineering strategies can be established. For example, resting cell could be applied for the efficient production of lactic acid, succinic acid, and ethanol, since any macromolecule biosynthesis fluxes are not detected from the optimal pathways. However, growing cell might be more suitable for acetic acid production, since large amount of ATP which may exceed the capacity of the *E. coli* metabolic network is produced. It can be utilized through macromolecule biosynthesis, and thus ATP balance could be achieved in the growing cell. Furthermore, metabolic flux analysis can also suggest suitable culture conditions for metabolites production. As shown in Fig. 2, aerobic culture condition is suitable for acetic acid and ethanol production, while anaerobic culture condition is more appropriate for the production of lactic acid and succinic acid.

As mentioned above, the proposed optimal pathway for succinic acid production is different from the typical succinic acid production pathway. Usually, succinic acid is produced from PEP by PEP carboxylation. However, considering the low energy efficiency of the PEP carboxylation pathway (loss of high energy phosphate bond), pyruvate is a more appropriate substrate for succinic acid production. These results suggest that the metabolism of *E. coli* has not been optimized for succinic acid production. This was supported by previous reports that efficient production of succinic acid could be achieved by the amplification of the malic enzyme in the *ldhA pfl* double-mutant *E. coli* strain NZN111 [6, 7]. Moreover, it was found that succinic acid productivity could be enhanced if redox potential was balanced by the supply of additional reducing power [7].

The maximum theoretical yield can be defined as the yield which can be achieved by complete conversion of substrate to metabolite. However, the maximum theoretical yield is different from the real maximum yield achievable under physiological conditions. To estimate a reasonable *in silico* maximum yield, ATP requirements for maintenance can be incorporated. Maintenance energy can be classified into growth associated and non-growth associated fractions. For the maximum metabolites production, the growth

Table 4. The maximum *in silico* yield of various metabolites when maintenance energy was considered.

	Metabolites			
	Acetic acid	Lactic acid	Succinic acid	Ethanol
Maximum *in silico* yield	2*	2	1.647 (2**)	0.073 (1.16***)

*Molar yield (mole metabolites/mole glucose).
**Molar yield with sufficient supply of external reducing power.
***Molar yield when an additional reaction catalyzed by pyruvate decarboxylase was introduced.

associated fraction can be ignored. It was reported that the non-growth associated maintenance requirement is 7.6 mmol of ATP/g DCW/h in *E. coli* under normal growth condition [21]. Under anaerobic condition, however, the non-growth associated ATP requirement is only 0.69 mmol of ATP/g DCW/h [6]. As mentioned above, optimal culture conditions for metabolites production can be formulated based on flux analysis. Using these proposed culture conditions, metabolic flux analyses were carried out again to predict the optimal metabolic pathway and the maximum

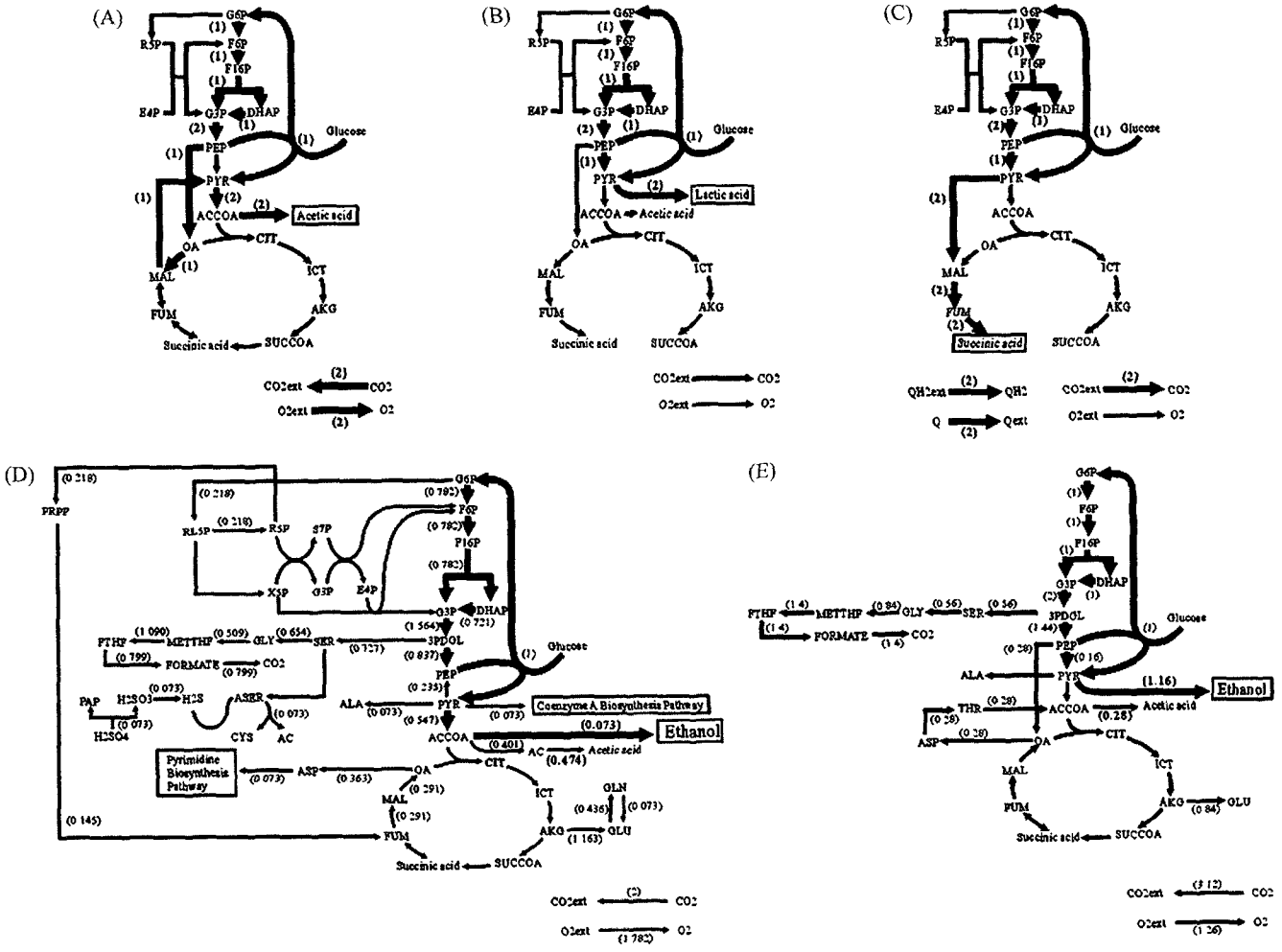

Fig. 3. Optimized metabolic flux distributions re-estimated by considering ATP requirement during the production of (A) acetic acid, (B) lactic acid, (C) succinic acid, and ethanol (D) without and (E) with additional reaction catalyzed by pyruvate decarboxylase.
Abbreviations: 3PDGL, 3-phosphoglycerate; AC, acetate; ACCOA, acetyl-CoA; AKG, α-ketoglutarate; ALA, alanine; ASER, O-acetylserine; ASP, aspartate; CIT, citrate; CO2, carbon dioxide; CYS, cysteine; DHAP, dihydroxyacetone phosphate; E4P, erythrose-4-phosphate; F16P, fructose-1,6-bisphosphate; F6P, fructose-6-phosphate; FORMATE, formate; FTHF, 10-formyl-tetrahydrofolate; FUM, fumaric acid; G3P, glyceraldehyde-3-phosphate; G6P, glucose-6-phosphate; GLN, glutamine; GLU, glutamate; GLY, glycine; H2S, hydrogen sulfide; H2SO3, hydrogen sulfite; H2SO4, hydrogen sulfate; ICT, isocitrate; MAL, malic acid; METTHF, 5,10-methylene tetrahydrofolate; O2, oxygen; OA, oxaloacetate; PAP, adenosine-3,5-diphosphate; PEP, phosphoenolpyruvate; PRPP, phosphoribosyl pyrophosphate; PYR, pyruvate; Q, ubiquinone or menaquinone; QH2, ubiquinol or menaquinol; R5P, ribose-5-phosphate; RL5P, ribulose 5-phosphate; S7P, sedo-heptulose; SER, serine; SUCCOA, succinyl-CoA; THR, threonine; X5P, xylulose-5-phosphate; ext, external

in silico yield with consideration of maintenance requirements. The results of metabolic flux analyses are presented in Table 4 and Fig. 3. The optimal metabolic pathway for acetic acid production was the same as that obtained when not considering the ATP requirement. However, the oxygen uptake rate increased 2-fold. The CO_2 excretion rate was 2 mole/g DCW/h when considering ATP requirement, while there was no excretion of CO_2 when the maintenance energy was not considered (Fig. 3A). These results indicate that respiration plays an important role in ATP generation. The optimal metabolic pathway for lactic acid production was also the same as that obtained when not considering the maintenance energy. Similarly, metabolic flux distribution for succinic acid production under sufficient supply of external reducing power was equal to the flux distribution without considering the maintenance energy. The optimal metabolic pathway for ethanol production was somewhat different when the ATP requirement was considered. A major difference found was the production of acetic acid. With consideration of ATP requirement, 0.47 mole of acetic acid/mole glucose was produced while only 0.073 mole of ethanol/mole glucose was produced. The molar ratio of acetic acid to ethanol is 6.5 mole acetic acid/mole ethanol. This result again indicates that wild-type *E. coli* is not suitable for the production of ethanol. The effect of introducing pyruvate decarboxylase into *E. coli* was estimated by metabolic flux analysis with maintenance requirement (Fig. 3E). When the reaction catalyzed by pyruvate decarboxylase was added to the metabolic reaction network, the maximum yield increased to 1.16 mole ethanol/mole glucose, which is 16 times higher than that obtained without introducing this reaction. Furthermore, acetic acid production decreased to 0.28 mole ethanol/mole glucose from 0.47 mole ethanol/mole glucose by introducing this reaction into the metabolic network. From these results, it can be concluded that pyruvate decarboxylase is an attractive enzyme as it provides two beneficial effects: enhanced metabolite (ethanol) production and decreased byproduct (acetic acid) formation.

In this study, we applied metabolic flux analysis technique to estimate the maximum metabolic capacities of the metabolic network of *E. coli* and to evaluate the achievable *in silico* maximum theoretical yields. In some cases, the maximum theoretical yield cannot be achieved even under ideal conditions due to the lack of metabolic limitation inherent in the metabolic network. It was found that this limiting factor could be identified by flux analysis, and thus, the optimal metabolite production fluxes could be proposed. Additionally, the effects of introducing new reaction pathways into metabolites production can be predicted by metabolic flux analysis. Based on the optimal metabolic pathways predicted *in silico*, new efficient metabolite production systems can be suggested. Therefore, this type of *in silico* metabolic flux analysis will reduce the time, money, and effort required to develop metabolically engineered strains for the enhanced production of various bioproducts.

Acknowledgments

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References

1. Bailey, J. E. 1991. Towards a science of metabolic engineering. *Science* **252:** 1668-1674.
2. Blattner, F. R., G. Plunkett, C. A. Bloch, N. T. Perna, V. Burland, M. Riley, J. Collado-Vides, J. D. Glasner, K. Rode, G. F. Mayhew, J. Gregor, N. W. Davis, H. A. Kirkpatrick, M. A. Goeden, D. J. Rose, B. Mau, and Y. Shao. 1997. The complete genome sequence of *Escherichia coli* K-12. *Science* **277:** 1453-1462.
3. Burgard, A. P., S. Vaidyaraman, and C. D. Maranas. 2001. Minimal reaction sets for *Escherichia coli* metabolism under different growth requirements and uptake environments. *Biotechnol. Prog.* **17:** 791-797.
4. Edwards, J. S., R. Ramakrishna, C. H. Schilling, and B. O. Palsson. 1999. Metabolic flux balance analysis, pp. 13-57 *In* S. Y. Lee and E. T. Papoutsakis (eds.), *Metabolic Engineering*. Marcel Dekker, New York, U.S.A
5. Hong, S. H. and S. Y. Lee. 2000. Metabolic flux distribution in a metabolically engineered *Escherichia coli* strain producing succinic acid. *J. Microbiol. Biotechnol.* **10:** 496-501.
6. Hong, S. H. and S. Y. Lee. 2001. Metabolic flux analysis for succinic acid production by recombinant *Escherichia coli* with amplified malic enzyme activity. *Biotechnol. Bioeng.* **74:** 89-95.
7. Hong, S. H. and S. Y. Lee. 2002. Importance of redox balance on the production of succinic acid by metabolically engineered *Escherichia coli*. *Appl. Microbiol. Biotechnol.* **58:** 286-290.
8. Hong, Y. K., W. H. Hong, and Y. K. Chang. 2001. Effect of pH on the extraction characteristics of succinic and formic acids with tri-n-octylamine dissolved in 1-octanol *Biotechnol. Bioprocess Eng.* **6:** 347-351.
9. Ingram L. O., P. F. Gomez, X. Lai, M. Moniruzzaman, B. E. Wood, L. P. Yomano, and S. W. York. 1998. Metabolic engineering of bacteria for ethanol production. *Biotechnol. Bioeng.* **58(2-3):** 204-214.
10. Kang, K. H. and H. W. Ryu. 1999. Enhancement of succinate production by organic solvents, detergents, and vegetable oils. *J. Microbiol. Biotechnol.* **9:** 191-195.
11. Lee, J. W., A. Goel, M. M. Ataai, and M. M. Domach. 2002. Flux regulation patterns and energy audit of *E. coli* B/r and K-12. *J. Microbiol. Biotechnol.* **12:** 248-258.

12. Lee, P. C., W. G. Lee, S. Y. Lee, Y. K. Chang, and H. N. Chang. 2000. Fermentative production of succinic acid from glucose and corn steep liquor by *Anaerobiospirillum succiniciproducens*. *Biotechnol. Bioprocess Eng.* **5:** 379-381.
13. Lee, S. Y. and E. T. Papoutsakis. 1999. *Metabolic Engineering*. Marcel Dekker, New York, U.S.A.
14. Lee, T. H., M. Y. Kim, Y. W. Ryu, and J. H. Seo. 2001. Estimation of theoretical yield for ethanol production from D-xylose by recombinant saccharomyces cerevisiae using metabolic pathway synthesis algorithm. *J. Microbiol. Biotechnol.* **11:** 384-388.
15. Neidhardt, F. C., R. Curtiss, J. L. Ingraham, E. C. C. Lin, K. B. Low, B. Magasanik, W. S. Reznikoff, M. Riley, M. Schaechter, and H. E. Umbarger. 1996. *Escherichia coli and Salmonella*. ASM Press, Washington D.C., U.S.A.
16. Nielsen, J. and J. Villadsen 1994. *Bioreaction Engineering Principles*. Plenum Press, New York, U.S.A.
17. Pramanik, J. and J. D. Keasling. 1997. Stoichiometric model of *Escherichia coli* metabolism: Incorporation of growth-rate dependent biomass composition and mechanistic energy requirements. *Biotechnol. Bioeng.* **56:** 398-421.
18. Samuelov, N. S., R. Lamed, S. Lowe, and J. G. Zeikus. 1991. Influence of CO_2-HCO_3-level and pH on growth, succinate production, and enzyme activities of *Anaerobiospirillum succiniciproducens*. *Appl. Environ. Microbiol.* **57:** 3013-3019.
19. Stephanopoulos, G. 1999. Metabolic fluxes and metabolic engineering. *Metab. Eng.* **1:** 1-11.
20. Stephanopoulos, G. and J. J. Vallino. 1991. Network rigidity and metabolic engineering in metabolite overproduction. *Science* **252:** 1675-1681.
21. Varma, A. and B. O. Palsson. 1994. Metabolic flux balancing: Basic concepts, scientific and practical use. *Nat. Biotech.* **12:** 994-998.
22. Wong, H. H., R. J. van Wegen, J. Choi, S. Y. Lee, and A. P. J. Middelberg. 1999. Metabolic analysis of poly(3-hydroxybutyrate) production by recombinant *Escherichia coli*. *J. Microbiol. Biotechnol.* **9:** 593-603.