

# *In Silico* Metabolic Pathway Analysis and Design: Succinic Acid Production by Metabolically Engineered *Escherichia coli* as an Example

Sang Yup Lee

leesy@mail.kaist.ac.kr

Soon Ho Hong

totenkof@mail.kaist.ac.kr

Soo Yun Moon

moonsy@mail.kaist.ac.kr

Metabolic and Biomolecular Engineering National Research Laboratory, Department of Chemical & Biomolecular Engineering and BioProcess Engineering Research Center, Bioinformatics Research Center, Center for Ultramicrochemical Process Systems, Korea Advanced Institute of Science and Technology 373-1 Guseong-dong, Yuseong-gu, Daejeon 305-701, Republic of Korea

## 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, we have constructed *in silico* metabolic pathway network of *Escherichia coli* consisting of 301 reactions and 294 metabolites. Metabolic flux analyses were carried out to estimate flux distributions to achieve the maximum *in silico* yield of succinic acid in *E. coli*. The maximum *in silico* yield of succinic acid was only 83% of its theoretical yield. The lower *in silico* yield of succinic acid was found to be due to the insufficient reducing power, which could be increased to its theoretical yield by supplying more reducing power. Furthermore, the optimal metabolic pathways for the production of succinic acid could be proposed based on the results of metabolic flux analyses. In the case of succinic acid production, it was found that pyruvate carboxylation pathway should be used rather than phosphoenolpyruvate carboxylation pathway for its optimal production in *E. coli*. Then, the *in silico* optimal succinic acid pathway was compared with conventional succinic acid pathway through minimum set of *wet* experiments. The results of *wet* experiments indicate that the pathway predicted by *in silico* analysis is more efficient than conventional pathway.

**Keywords:** metabolic flux analysis, *in silico* simulation, metabolic engineering, succinic acid

## 1 Introduction

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 [1, 6]. Various metabolic engineering strategies have been widely applied for the more efficient production of desired metabolites and biomolecules [6, 14]. Even though enhanced production of some biomolecules has been successful, many other attempts have failed due to the lack of rational strategies based on predictable technique. Therefore, metabolic flux analysis, which allows calculation of the 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, 10].

Metabolic flux analysis 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 [13, 16]. 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. Another possible application is the identification of alternative metabolic pathways leading to a desired product. The detailed theories and applications of the flux analysis can be found in the recent reviews [4, 13].

Succinic acid is a member of the C<sub>4</sub>-dicarboxylic acid family and can be used as a precursor of numerous products including pharmaceuticals, fine chemicals and biodegradable polymers. Succinic acid has been produced by chemical processes. Recently, much effort is being exerted for the production of succinic acid by microbial fermentation using renewable feedstocks, because of pollution problems associated with chemical processes [5]. The best known succinic acid producing bacterium is *Anaerobiospirillum succiniciproducens*, which can produce a mixture of succinic acid and acetic acid at a molar ratio of 2:1 from glucose [5, 12]. *Escherichia coli* produces several metabolic products by fermentation: acetic acid, ethanol, formic acid, lactic acid, and also a small amount of succinic acid. The ratio of these fermentation products varies depending on the culture condition employed.

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, we report construction of *in silico* metabolic network of *E. coli*, and as an example, its use in the estimation of the maximum *in silico* yield of succinic acid and the determination of optimal flux distribution.

## 2 Methods

### Theoretical Backgrounds

#### Construction of *in silico* metabolic network

From the result of genome sequence project, it is known that *E. coli* possesses 600 to 700 hundred metabolic reactions [2]. Most of reactions such as amino acids transport and utilization are, however, not essential for cell growth, and it was reported that 224 metabolic reactions can support growth on glucose [3, 11]. Our metabolic network was constructed with 127 reversible and 174 irreversible reactions and 294 metabolites, which contains all metabolic reaction pathways required for growth on glucose (Table 1).

#### Metabolic flux analysis

Metabolic flux analyses were carried out for the calculation of volumetric rates of formation of intracellular metabolites [6, 10]. 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 [9]. The types and amounts of precursors required to synthesize these macromolecules were determined from the average composition of each 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%); phospholipid (72.8% phosphatidylethanolamine, 19.8% phosphatidylglycerol, 3.4% cardiolipin) [9].

The flux balance model was set up as follows:

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

where  $\mathbf{S}$  is the stoichiometric matrix,  $\mathbf{v}$  is a vector for reaction rates or fluxes and  $\mathbf{b}$  is the vector for the rates of metabolites consumption and excretion [4]. 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:

$$\text{Maximize : } Z = c_i v_i$$

where  $c_i$  are the weights and  $v_i$  are the elements of the flux vector [16]. In this study, maximum metabolite production were used as objective functions. Linear optimization was carried out using the program LP\_solve (State university of New York, NY, USA). To obtain feasible solutions, constraints on the rates of metabolites uptake and excretion were applied (Table 2). As can be seen from Table 2, cells are allowed to operate both aerobic and anaerobic pathways with no limiting nutrient.

Table 1. Reactions and metabolites involved in the *in silico* metabolic network of *E. coli*

Reaction #	Gene	Enzyme	R eaction	Reaction #	Gene	Enzyme	R eaction
<b>Glycolysis</b>				<b>Biosynthesis of aspartate</b>			
1	ptsI, ptsH	Phosphotransferase system	GL C + PE P => PYR + G6P	56	aspC	Aspartate transaminase	OA + GL U <=> AS P + AKG
2	pgi	Phosphoglucose isomerase	G6P <=> F6P	<b>Biosynthesis of asparagines</b>			
3	pfk	Phosphofructokinase	F6P + AT P <=> AD P + F16P	57	asnB	Glutamine-dependent asparagine synthetase	AS P + AT P + GL N => GLU + ASN + AMP + PPI
4	fbp	Fructose 1,6-bisphosphatase	F16P + PI => F6P	58	asnA	Ammonia-dependent asparagine synthetase	AS P + AT P + NH3 => ASN + AMP + PPI
5	fba	Fructose 1,6-bisphosphatase	F16P <=> T3P1 + T3P2	<b>Biosynthesis of glutamate</b>			
6	tpi	Triosephosphate isomerase	T3P1 <=> T3P2	59	gdhA	Glutamate dehydrogenase	AKG + NH3 + NADPH => GLU + NADP
7	gap	Glyceraldehyde-3-phosphate dehydrogenase	T3P1 + PI + NAD <=> NADH + 13P2DG	60	glnA	Glutamine synthetase	GL U + NH3 + AT P => GLN + AD P + PI
8	pgk	Phosphoglycerate kinase	13P2DG + ADP <=> AT P + 3PDGL	61	gltBD	Glutamate synthase	AKG + GLN + NADPH => NADP + 2 GLU
9	gpm	Phosphoglycerate mutase	3PDGL <=> 2PDGL	<b>Biosynthesis of alanine</b>			
10	eno	Enolase	2PDGL <=> PEP	62		Glutamic-pyruvic transaminase	PYR + GL U <=> AKG + AL A
11	pyk	Pyruvate kinase	PEP + ADP => AT P + PYR	<b>Biosynthesis of arginine, putrescine, and spermidine</b>			
12	pck	PEP carboxykinase	OA + AT P <=> PEP + CO2 + ADP	63	argA	N-Acetylglutamate synthase	GL U + ACCO A => COA + NAGLU
13	ppc	PEP carboxylase	PEP + CO2 => OA + PI	64	argB	N-Acetylglutamate kinase	NAGLU + AT P => AD P + NAGLUYP
14	lpdA	Pyruvate dehydrogenase	PYR + CO A + NAD => NADH + CO2 + ACCO A	65	argC	N-Acetylglutamate phosphate reductase	NAGLUYP + NADPH <=> NADP + PI + NAGLUSAL
15	pps	PEP synthase	PYR + AT P => PEP + AMP + PI	66	argD	Acetylornithine aminotransferase	NAGLUSA L + GLU <=> AKG + NAARON
<b>Pentose phosphate shunt</b>				67	argE	N-Acetylornithinase	NAARON => AC + ORN
16	zwf	Glucose-6-phosphate dehydrogenase	G6P + NADP <=> D6PGL + NA DPH	68	carAB	Carbamoyl phosphatesynthetase	GL N + 2 AT P + CO2 => GLU + CAP + 2 ADP + PI
17	pgi	6-Phosphogluconolactonase	D6PGL => D6PGC	69	argF1	Ornithine carbamoyl transferase	ORN + CA P <=> CI TR + PI
18	gnd	6-Phosphogluconate dehydrogenase	D6PGC + NADP <=> NADPH + CO2 + RL 5P	70	argG	Argininosuccinatesynthase	CI TR + AS P + AT P <=> AMP + PPI + ARGSUCC
19	rpi	Ribose-5-phosphate isomerase	RL 5P <=> R5P	71	argH	Argininosuccinase	ARGSUCC <=> FUM + ARG
20	rpe	Ribose-5-phosphate epimerase	RL 5P <=> X5P	72	speC	Ornithine decarboxylase	ORN => PTRSC + CO2
21	tktA	Transketolase 1	R5P + X5P <=> T3P1 + S7P	73	speE	Spermidinesynthase	PTRSC + DSAM => SPRMD + 5MT A
22	tal	Transaldolase	T3P1 + S7 P <=> E4P + F6P	74	speD	Adenosylmethionine decarboxylase	SAM <=> DSAM + CO2
23	tktB	Transketolase 2	X5P + E4P <=> F6P + T3P1	75		Unknown pathway	5MTA => ADN + MET
24	edd	6-Phosphogluconate dehydrase	D6PGC => 2K3D6PG	76	speA	Agmatine decarboxylase	ARG => CO2 + AGM
25	eda	2-Keto-3-deoxy-6-phosphogluconate aldolase	2K3D6PG => T3P1 + PY R	77	speB	Agmatine ureohydrolase	AGM => URE A + PTRSC
<b>Glycogen metabolism</b>				<b>Biosynthesis of proline</b>			
26	pgm	Phosphoglucomutase	G6P <=> G1P	78	proB	Glutamyl kinase	GLU + AT P => ADP + GLU P
27	glgA	Glycogen synthase	G1P + AT P => ADP + PPI + GLYCOGE N	79	proA	Glutamate-5-semialdehyde dehydrogenase	GL UP + NADPH <=> NADP + PI + GLUGS AL
28	glgP	Glycogen phosphorylase	GLYCOGEN + PI => G1P	80	proC	Pyroline-5-carboxylate reductase	GLUGS AL + NADPH <=> PR O + NADP
29	ldh	Dissimilation of pyruvate	PYR + NADH <=> NAD + LAC	81		Ornithine oxoacid transaminase	ORG + AKG <=> GLU + GLUGSAL
30	adh	Lactate dehydrogenase	ACAL + NADH <=> ET HANO L + NAD	<b>Biosynthesis of branched-chain amino acids</b>			
31	adh	Alcohol dehydrogenase	ACCO A + NADH <=> NAD + ACAL	82	leuA	Isopropylmalate synthase	ACCO A + OI VA L => COA + CB HCAP
32	pfk	Acetaldehyde dehydrogenase	PYR + COA => ACCO A + FORMA TE	83	leuCD	Isopropylmalate isomerase	CBHCAP <=> IPPMAL
33	pta	Pyruvate formate lyase	ACCO A + PI <=> ACTP + COA	84	leuB	3-Isopropylmalate dehydrogenase	IPPMAL + NAD => NADH + OI CA P + CO2
34	ackA	Phosphotransacetylase	ACTP + ADP <=> AT P + AC	85	ilvE	Transaminase C	OI CA P + GL U => AKG + LE U
35	fhl	Acetate kinase	FORMA TE => CO2	86	ilvB	Acetylhydroxy acid synthase	2 PYR => CO2 + AC LA C
<b>TCA cycle and glyoxylate bypass</b>				87	ilvC	Acetylhydroxy acid isomeroreductase	AC LA C + NADPH => NADP + DH VA L
36	gltA	Citrate synthase	ACCO A + OA <=> COA + CIT	88	ilvD	Dihydroxy acid dehydratase	DH VA L => OI VA L
37	can	Aconitase	CIT <=> ICIT	89	ilvE	Transaminase C	OI VA L + GLU <=> AKG + VA L
38	idh	Isocitrate dehydrogenase	ICIT + NADP <=> CO2 + NADPH + AKG	90	ilvA	Threonine deaminase	THR => NH3 + OBUT
39	sucAB	2-Ketoglutarate dehydrogenase	AKG + NAD + CO A <=> CO2 + NADH + SUCCO A	91	ilvB	Acetylhydroxy acid synthase	OBUT + PYR => ABUT + CO2
40	sucCD	Succinate thiokinase	SUCCO A + GDP + PI <=> GTP + CO A + SUCC	92	ilvC	Acetylhydroxy acid isomeroreductase	ABUT + NADP => DHM VA
41	sdhABCD	Succinate dehydrogenase	SUCC + F AD => FA DH2 + FUM	93	ilvD	Dihydroxy acid dehydratase	DHM VA => OM VA L
42	frdABCD	Fumarate reductase	FUM + F ADH2 => SUCC + F AD	94	ilvE	Transaminase B	OM VA L + GL U <=> AKG + ILE
43	fumAB	Fumarase	FUM <=> MA L	95		Amino acid oxidase	O2 + ILE => OMV AL + NH3
44	mdh	Malate dehydrogenase	MA L + NAD <=> NADH + OA	<b>Biosynthesis of aromatic amino acids</b>			
45	mez	Malic enzyme	MA L + NADP => CO2 + NADPH + PYR	96	aroFGH	3-Deoxy-D-arabinoheptosonate-7-phosphatase	E4P + PEP => PI + 3DDAH7P
46	mez	Malic enzyme	MA L + NAD <=> CO2 + NADH + PYR	97	aroB	3-Dehydroquinate synthase	3DDAH7P => DQT + PI
47	aceA	Isocitrate lyase	ICIT => GL X + SUCC	98	aroD	3-Dehydroquinate dehydratase	DQT <=> DHSK
48	aceB	Malate synthase	ACCO A + GL X => COA + MAL	99	aroE	Shikimate dehydrogenase	DHSK + NADPH <=> SM E + NADP
<b>Respiration</b>				100	aroKL	Shikimate kinase	SM E + AT P => ADP + SMESP
49	ndh	NADH dehydrogenase II	NADH + Q => NAD + QH2	101	aroA	5-Enolpyruvoylshikimate-3-phosphatesynthase	SMESP + PEP <=> 3PSME + PI
50	ndh	NADH dehydrogenase I	NADH + Q => NAD + QH2 + 4 HEXT	102	aroC	Chorismatesynthase	3PSME => PI + CHOR
51	fdnGHI	Formate dehydrogenase	FORMA TE + Q => 2 HE XT + QH2 + CO2	103	pheA	Chorismate mutase	CHOR => PHEN
52	cyoABC D	Cytochrome oxidase bo3	QH2 + 1/2 O2 => Q + 4 HEXT	104	pheA	Prephenatedehydratase	PHEN => CO2 + PHP YR
53	cydABC D	Cytochrome oxidase bd	QH2 + 1/2 O2 => Q + 2 HEXT	105	tyrB	Phenylalanine aminotransferase	PHPYR + GL U => AKG + PHE
54	sdhABCD	Succinate dehydrogenase complex	FADH2 + Q <=> FAD + QH2	106	tyrA	Prephenatedehydrogenase	PHEN + NADP => HPHPYR + CO2 + NADPH
<b>AT P synthesis</b>				107	tyrB	Tyrosine aminotransferase	HPHPYR + GL U <=> AKG + TY R
55	unc	F0F1-ATPase	AT P <=> ADP + PI + 3 HEXT				

(Table 1. Continued)

Reaction #	Gene	Enzyme	R eaction
108	trpDE	Anthranylatesynthase	CHOR + GLN => GLU + PYR + AN
109	trpD	Anthranylate phosphoribosyl transferase	AN + PRPP => PPI + NPRAN
110	trpC	Phosphoribosyl anthranilate isomerase	NPRAN => CP AD5P
111	trpC	Indoleglycerol phosphate synthetase	CPAD5 P => CO2 + IG P
112	trpAB	Tryptophansynthetase	IG P + SER => T3P1 + TRP
<b>Biosynthesis of histidinesynthase</b>			
113	prs	Phosphoribosyl pyrophosphatesynthetase	R5P + AT P <=> PRPP + AMP
114	hisG	Phosphoribosyl pyrophosphate	PRPP + AT P => PPI + PRBA TP
115	hisI	PR-A TP pyrophosphodrolase	PRBA TP => PPI + PRBAMP
116	hisI	PR-AMP cyclohydrolase	PRBAMP => PRFP
117	hisA	5-ProFAR isomerase	PRF P => PRLP
118	hisFH	Imidazoleglycerol phosphatesynthase	PRLP + GL N => GLU + AI CAR + DI MGP
119	hisB	IG P dehydratase	DI MGP => IM ACP
120	hisC	L-Histidinol phosphate aminotransferase	IM ACP + GLU => AKG + HI SOLP
121	hisB	Hol-P-phosphatase	HI SOLP => PI + HI SOL
122	hisD	Hol dehydrogenase	HI SOL + 2 NAD => 2 NADH + HIS
<b>Biosynthesis of serine, glycine, and 1-carbon units</b>			
123	serA	3-Phosphoglyceratedehydrogenase	3PDGL + NAD => NADH + PHP
124	serC	Phosphoserine transaminase	PHP + GLU => AKG + 3P5ER
125	serB	Phosphoserine phosphatase	3P5ER => PI + SER
126	glyA	Serine hydroxymethyltransferase	GLY + METTHF <=> THF + SER
127	gev/HTP	Glycine cleavage system	GLY + THF + NAD => METTHF + NADH + CO2 + NH3
128	thd	Threonine dehydrogenase	THR + NAD <=> AABK + NADH
129	kbl	Amino-b-ketobuoyrase	AABK + COA <=> GLY + ACCCOA
130		Formate dehydrogenase	FORMA TE + NAD => NADH + CO2
131		Formate THF li gase	THF + FORMA TE + AT P => AD P + PI + FTHF
132		Formyl THF defo rmylase	FTHF => FORMA TE + THF
133		Dihydrofolate reductase	DHF + NADPH <=> NADP + THF
<b>Biosynthesis of cysteine</b>			
134	cysDN	AT P sulfhydrolase	H2SO4 + AT P => PPI + APS
135	cysC	AT S kinase	APS + AT P => ADP + PAPS
136	cysH	PAPS sulfotransferase	PAPS + NADPH => NADP + H2SO3 + PAP
137	cysGU	NAHPH-sulfite redu ctase	H2SO3 + 3 NADPH <=> 3 NADP + H2S
138	cysE	Serine transaceylase	SER + ACCCOA <=> COA + ASER
139	cysKM	O-Acetylserine (thiol)-lyase	ASER + H2S => AC + CYS
140		Sulfotransferase	PAP + H2SO3 <=> PAPS
141		Adenylyl sulfate kinase	PAPS + ADP => AT P + APS
<b>Biosynthesis of threonine andysine</b>			
142	thrA	Aspartate kinase	ASP + AT P <=> AD P + BASP
143	asd	Aspartate semialdehyde dehydrogenase	BASP + NADPH <=> NADP + PI + ASPSA
144	thrA	Homoserine dehydrogenase	ASPSA + NADPH <=> NADP + HSER
145	thrB	Homoserine kinase	HSER + AT P => AD P + PHSER
146	thrC	Threonine synthase	PHSER => PI + THR
147	dapA	Dihydrodipicolinatesynthase	ASPSA + PYR => D23P IC
148	dapB	Dihydrodipicolinate reductase	D23PIC + NADPH => NADP + PIP26DX
149	dapD	Tetrahydrodipicolinate succinylase	PIP26DX + SUCCOA => COA + NS2A6O
150	dapC	Succinyl diaminopimelateaminotransferase	NS2A6O + GLU <=> AKG + NS26DP
151	dapE	Succinyl diaminopimelate desuccinylase	NS26DP => SUCC + D26P IM
152	dapF	Diaminopimelate epimerase	D26PIM <=> MDAP
153	lysA	Diaminopimelate decarboxylase	MDAP => CO2 + LY S
<b>Biosynthesis of methionine</b>			
154	metA	Homoserine transsuccinylase	HSER + SUCCOA => COA + OS LHSER
155	metB	Cystathioninesynthase	OS LH SER + CYS => SU CC + HCYS + PYR + NH3
156	metC	Cystathionase	HCYS + ADN <=> SAH
157	metEH	Methioninesynthase	HCYS + MTHF => ME T + THF
158	metK	Methionyl adenylyl transferase	MET + AT P => PPI + PI + SAM
<b>Biosynthesis of purine nucleotides</b>			
159	purF	Glutamine PRPP amidotransferase	PRPP + GL N => PPI + GLU + PRAM
160	purD	GAR synthetase	PRAM + AT P + GLY <=> AD P + PI + GAR
161	purNT	GAR transformylase	GAR + FTHF => THF + FGAR
162	purL	FGAM synthetase	FGAR + AT P + GLN => GLU + ADP + PI + FGAM

Reaction #	Gene	Enzyme	R eaction
163	purM	AI R synthetase	FGAM + AT P => AD P + PI + AI R
164	purK	RCA IM synthetase	AI R + CO2 <=> RCAIM
165	purE	PRSCAI M synthetase	RCAIM + AT P + AS P <=> AD P + PI + PRSCAI M
166	purB	Adenylosuccinatelyase	PRSCAI M <=> FUM + AI CAR
167	purH	AI CAR transformylase	AI CAR + FTHF <=> THF + PRFICA
168	purH	IM P cyclohydrolase	PRFI CA <=> IM P
169	purA	Adenylosuccinatesynthetase	IM P + GTP + ASP => GDP + PI + ASUC
170	purB	Adenylosuccinatelyase	ASUC <=> FUM + AM P
171		AMP phosphatase	AMP => PI + ADN
172	adk	Adenylate kinase	AT P + ADN => ADP + AMP
173	adk	Adenylate kinase	AT P + AMP => 2 ADP
174	guaB	IM P dehydrogenase	IM P + NAD => NADH + XMP
175	guaA	GMP synthetase	XMP + AT P + GL N => GLU + AMP + PPI + GMP
176	gmK	GMP kinase	GMP + AT P <=> GDP + ADP
177	gmK	GDP kinase	GDP + AT P <=> GTP + ADP
178	deoD	Ribonucleotide reductase (ADP)	ADP + NADPH => DADP + NADP
179	deoD	Ribonucleotide reductase (GDP)	GDP + NADPH => DGDP + NADP
180	deoD	Ribonucleotide reductase (ATP)	AT P + NADPH => NADP + DATP
181	deoD	Ribonucleotide reductase (GTP)	GTP + NADPH => NADP + DGTP
182	ndk	dADP kinase	DADP + AT P <=> DATP + ADP
183	ndk	dGDP kinase	DGDP + AT P <=> DGTP + ADP
184	ndk	dAMP kinase	DAMP + AT P <=> ADP + DADP
185	ndk	dGMP kinase	DGMP + AT P <=> DGDP + ADP
<b>Biosynthesis of pyrimidines</b>			
186	pyrBI	Aspartate carbamoyl transferase	CAP + ASP => CAASP + PI
187	pyrC	Dihydroorotase	CAAS P <=> DOROA
188	pyrD	Dihydroorotate dehydrogenase	DOROA + NAD <=> NADH + OROA
189	pyrE	Orotate phosphoribosyl transferase	OROA + PRPP <=> PPI + OMP
190	pyrF	OMP decarboxylase	OMP => CO2 + UMP
191	pyrH	UMP kinase	UMP + AT P <=> UDP + ADP
192	ndk	UDP kinase	UDP + AT P <=> UTP + ADP
193	pyrG	CTP synthetase	UTP + GLN + AT P => GLU + CTP + ADP + PI
194	ndk	CMP kinase	CMP + AT P <=> CDP + ADP
195	ndk	CDP kinase	CDP + AT P <=> CTP + ADP
196	dd	Deoxycytidilate deaminase	DCMP => NH3 + DUMP
197	nrdB	Ribonucleotide reductase (CDP)	CDP + NADPH => DCDP + NADP
198	nrdB	Ribonucleotide reductase (UDP)	UDP + NADPH => DUDP + NADP
199	nrdB	Ribonucleotide reductase (CTP)	CT P + NADPH => DCTP + NADP
200	nrdB	Ribonucleotide reductase (UTP)	UTP + NADPH => NADP + DUTP
201	ndk	dCMP kinase	DCMP + AT P <=> ADP + DC DP
202	ndk	dCDP kinase	DCDP + AT P <=> DCTP + ADP
203	ndk	dUDP kinase	DUDP + AT P <=> DUTP + ADP
204	dut	dUTP pyrophosphatase	DUTP => PPI + DUMP
205	ndk	dUMP kinase	DUMP + AT P <=> ADP + DUDP
206	thyA	Thymidilate synthetase	DUMP + METTHF => DHF + TMP
207	tmk	TMP kinase	TMP + AT P <=> ADP + TDP
208	ndk	TDP kinase	TDP + AT P <=> AD P + TTP
<b>Biosynthesis of THF</b>			
209	metF	Methylene THF redu ctase	METTHF + NADH => NAD + MTHF
210	foiD	Methylene THF dehydrogenase	METTHF + NADP <=> METHF + NADPH
211	foiD	Methenyl tetrahydrofolate cyclehydrolase	METHF <=> FTHF
<b>Biosynthesis of membrane lipids</b>			
212	acc	Acetyl-CoA carboxylase	ACCOA + AT P + CO2 <=> MALCOA + ADP + PI
213	mta	Malonyl-CoA:ACP transacylase	MA LC OA + ACP <=> MA LA CP + COA
214	kasI	b-Ketoacyl-ACP synthase I	MA LA CP => ACACP + CO2
215	ata	Acetyl-CoA:ACP transacylase	ACACP + COA <=> ACCCOA + ACP
216		b-Ketoacyl-ACP synthase I (C14:0)	ACACP + 6 MA LA CP + 12 NADP H => C 14:0AC P + 6 CO2 + 12 NADP + 6 ACP
217	fab	b-Ketoacyl-ACP synthase I (C14:1)	ACACP + 6 MA LA CP + 11 NADP H => C14:1AC P + 6 CO2 + 11 NADP + 6 ACP
218	fab	b-Ketoacyl-ACP synthase I (C16:0)	ACACP + 7 MA LA CP + 14 NADP H => C16:0AC P + 7 CO2 + 14 NADP + 7 ACP
219	fab	b-Ketoacyl-ACP synthase I (C16:1)	ACACP + 7 MA LA CP + 13 NADP H => C16:1AC P + 7 CO2 + 13 NADP + 7 ACP

(Table 1. Continued)

Reaction#	Gene	Enzyme	R eaction
220	fab	b-Ketoacyl-ACP synthase I (C18:1)	ACACP + 8 MA LA CP + 15 NADPH H => C18:1AC P + 8 CO2 + 15 NADP + 8 ACP
221	gpsA	Glycerol-3-phosphate dehydrogenase	NADH + T3P2 <=> GL3P + NAD
222	pls	1-Acyl-G3P acyltransferase	GL 3P + 0.03 C14:0ACP + 0.086 C14:1ACP + 0.607 C16:0ACP + 0.12 C16:1AC P + 0.85 C18:1ACP => PA + 1.694 ACP
223	cdsA	CDP-Diacylglycerol synthetase	PA + CTP <=> CDP DG + PP I
224	pssA	Phosphatidylserine synthase	CDPDG + SER <=> CMP + PS
225	psd	PS decarboxylase	PS => PE + CO2
226	pgsA	Phosphatidylglycerol phosphatase	CDPDG + GL 3P <=> CMP + PGP
227	pgpA	Phosphatidylglycerol phosphate phosphatase	PGP => PI + PG
228	cls	Cardiolipin synthase	PG + CDPDG <=> CL + CMP
<b>Biosynthesis of isoprenoids</b>			
229		Aldose reductase	GL + NADP <=> NADPH + GL AL
230		Glyceraldehyde kinase	GL AL + AT P => AD P + T3P1
231		Hydroxymethyl-glutaryl-CoA synthase	3 ACCO A => 2 CO A + HMGCOA
232		3-Methyl-glutaconyl-CoA hydratase	HMGCOA <=> TMGCOA
233		IPP synthase	HMGCOA + 2 NADPH + 3 AT P => CO A + 2 NADP + 3 AD P + PI + CO2 + IPPP
234		GGP P synthase	4 IPPP => 3 PPI + GGPP
235		Methylcrotonyl-CoA carboxylase	MCCOA + AT P + CO2 <=> ADP + TMGCOA + PI
236		Acyl-CoA dehydrogenase	IS OVCO A + Q <=> QH2 + MCCOA
237		2-Keto-isocaproate decarboxylase	OICAP + COA + NADP => NADPH + CO2 + IS OVCOA
<b>Biosynthesis of quinone</b>			
238	ubiC	Chorismate pyruvate-lyase	CHOR => 4HBZ + PYR
239	ubiADX	Hydroxybenzoate octapentenyltransferase	4HBZ + GGPP => 2PPP + CO2 + PP I
240	ubiB	2O6H synthetase	2PPP + Q2 => 2O6H
241	ubiEFGH	QH2 synthetase	2O6H + 2 O2 + 3 SAM => 3 SAH + QH2
<b>Biosynthesis of riboflavin</b>			
242	ribA	GTP cyclohydrolase	GTP => D6RP5P + CO2 + PP I
243	ribD	Pyrimidine deaminase	D6RP5P => A6RP5 P + NH3
244	ribD	Pyrimidine reductase	A6RP5P + NADPH => A6RP5 P2 + NADP
245		Phosphatase	A6RP5P2 => A6RP + PI
246	ribB	3,4-Dihydroxy-2-butanone-4-phosphatase	A6RP => DB4P + FORMA TE
247	ribE	6,7-Dimethyl-8-ribityllumazine synthase	DB4P + A6RP => D8R L + PI
248	ribC	Riboflavin synthase	D8R L => RI BOFL AV IN + A6RP
249	ribF	Riboflavin kinase	RI BOFL AV IN + AT P => FMN + ADP
250	ribF	FAD synthetase	FMN + AT P => FAD + PP I
<b>Biosynthesis of folate</b>			
251	folE	GTP cyclohydrolase	GTP => FORMA TE + AHTD
252		H2Neopterin triphosphat pyrophosphatase	AHTD => 3 PI + DHP
253		H2Neopterin aldolase	DHP => AHMMP + GL AL
254	folK	6-Hydroxymethyl H2pterin pyrophosphokinase	AHMMP + AT P => AM P + AHM D
255	folP	H2pteroatesynthase	AN + AHMMD => PP I + DHD
256	folA	Dihydrofolate reductase	DHD + AT P + GL U => AD P + PI + DHF
<b>Biosynthesis of coenzyme A</b>			
257	panBCDE	CoA Synthase	OIVL + METHFH + NADPH + AL A + CTP + 4 AT P + CY S => THF + NADP + AMP + 2 PP I + 2 ADP + CO2 + COA
258	acpS	ACP Synthase	COA => 35ADP + ACP
259		3,5-ADP phosphatase	35ADP => AMP + PI
<b>Biosynthesis of NAD</b>			
260	nadAB	Quinolatesynthase	ASP + FAD + T3P2 => FA DH2 + PI + QNL
261	nadC	Quinolate phosphoribosyl transferase	QNL + PRPP => PPI + NICNT + CO2
262	nadD	NAMN adenylil transferase	NICNT + AT P => PPI + DANAD
263	nadE	Deamido-NAD ammonia ligase	DANAD + AT P + NH3 => AMP + PPI + NAD
264		NAD kinase	NAD + AT P => NADP + ADP
265		NADP phosphatase	NADP => NAD + PI
<b>Biosynthesis of porphyrins and heme</b>			
266	gltX, hemA	GS A synthetase	GL U + AT P + NADPH => GSA + AMP + PPI + NADP
267	hemL	GSA aminotransferase	GSA => AL AV
268	hemBCD	Porphyrinogensynthetase	AL AV => PORPH + NH3

<b>Biosynthesis of porphyrins and heme</b>			
266	gltX, hemA	GS A synthetase	GL U + AT P + NADPH => GSA + AMP + PPI + NADP
267	hemL	GSA aminotransferase	GSA => AL AV
268	hemBCD	Porphyrinogensynthetase	AL AV => PORPH + NH3
271	glmU	Acetyl glucosamine mutase	AGA6P <=> AGA1P
272		UDP N-acetylglucosamine pyrophosphorylase	AGA1P + UTP <=> PPI + UDPGA
273		UDP N-acetylglucosamine 4-epimerase	UDPGA <=> UDPG LN
274	kdsA	N-Ac ylglucosamine-6-P2-epimerase	UDPGA + PEP <=> PI + UDPGC
275		UDP-N-acetylmuramatedehydrogenase	UDPGC + NADH => UDPGA M + NAD
276	kdsB	CMP-2-keto-3-deoxyoctanoatesynthase	R5P + PEP + CTP => 2 PI + PPI + CMPKDO
277		Isomerase+mutase+pyrophosphorylase+ epimerase	S7P + AT P <=> ADPHEP + PPI
278		Ethanolamine phosphotransferase	PE + CMP <=> CDPETN + DGR
279		Phosphatidate phosphatase	PA <=> PI + DGR
280		Lyposaccharidesynthetase	UDPG LN + 5 C14:0ACP + 2 AT P + 3 CMPKDO + CDPETN + 3 ADPHEP + 2 UDPG + PE + 2 UDPGA L => 2 AC + UM P + 6 UDP + 5 AD P + 3 CM P + DGR + LP S
<b>UDP glucose synthase</b>			
281		UDP glucose synthase	G1P + UTP => PPI + UDPG
282		UDP galactosesynthase	G1P + UTP <=> PPI + UDPGAL
283		Murein synthetase	UDPGA + UDPAM + 2 AL A + D26PIM + GLU + 5 AT P => PEPTI DO + 5 ADP + 5 PI + 2 UDP
<b>Polyphosphate an pyrophosphate metabolism</b>			
284	ppa	Pyrophosphatase	PPI => 2 PI
285	ppk	Polyphosphate kinase	1000 AT P <=> 1000AD P + POLY P
286	ppx	Polyphosphatase	POLY P => 1000 PI
<b>Glycerol metabolism</b>			
287		Glycerol kinase	GL + AT P <=> GL3P + ADP
288		Glycerol-3-phosphate dehydrogenase	GL 3P + FAD => T3P2 + FADH2
<b>Transport reactions</b>			
289		Ammonia transport	NH3ext + Hext <=> NH3
290		Sulfate transport	H2SO4ext <=> H2SO4
291	pit	Phosphate transport	PIext + Hext <=> PI
292		Acetate transport	ACext + Hext <=> AC
293		Lactate transport	LACext + Hext <=> LAC
294		Formate transport	FORMA T ext + Hext <=> FORMA TE
295		Ethanol transport	ETHANOLE xt <=> ETHANOL
296		Succinate transport	SUCCext + Hext <=> SUCC
297		Malate transport	MALe xt <=> MAL
298		D-Glyceraldehyde transport	GL AL ext + Hext <=> GL AL
299		Glucose transport	GL Cext <=> GL C
300		Carbon dioxide transport	CO2ext <=> CO2
301		Oxygen transport	O2ext <=> O2

Table 2: Constraints on the rates of metabolites uptake and excretion.

	Constraints (mM/gDCW/h)
<b>Glucose uptake</b>	< 1
Oxygen uptake	> 0
NH <sub>3</sub> uptake	> 0
Phosphate uptake	> 0
H <sub>2</sub> SO <sub>4</sub> uptake	> 0
CO <sub>2</sub> uptake	> 0
CO <sub>2</sub> excretion	> 0

### Bacterial strain and culture condition

*E. coli* strain NZN111 (F- *pft::Cm ldhA::Kn*) and WGS-3 (W3110 *ldhA::Cm pta::Kn adhE::Tc*) were used. The mutant strain NZN111 lost the activities of pyruvate formate-lyase and lactate dehydrogenase, and WGS-3 strain lost the activities of lactate dehydrogenase, phosphotransacetylase and alcohol dehydrogenase [15]. Therefore, both strain lost anaerobic pyruvate utilization metabolism.

The *sfcA* gene encoding malic enzyme was ligated into pTrc99A using *NcoI* and *XbaI* restriction enzyme sites to construct pTrcML. The plasmid pTrcML, in which the *sfcA* gene is under the control of *trc* promoter, was transformed into NZN111 and applied to examine *in silico* succinic acid pathway [8]. The the *Mannheimia succiniciproducens* MBEL55E *pckA* gene encoding phosphoenolpyruvate (PEP) carboxykinase was ligated into p10499A using *NcoI* and *XbaI* restriction enzyme sites to construct p104ManPck. The plasmid p104ManPck, in which the *pckA* gene is under the control of *gntT104* promoter, was transformed into WGS-3 and applied to examine conventional succinic acid pathway (Figure 1).

Fermentation studies were carried out in a 5 L fermentor (BioFlo 3000, New Brunswick Scientific, Edison, NJ) containing 3 L of LB medium at 37°C. The medium pH was controlled at 6.7 by the addition of 5 M NaOH. The DO level was maintained over 40% of oxygen saturation during aerobic cultivation. When the OD<sub>600</sub> reached 5, isopropylthio-b-D-galactoside (IPTG) was added to a final concentration of 0.1 mM. After induction, three different oxygen-free gases (Kosock gas, Taejon, Korea) were flushed to the fermentor until anaerobic condition was achieved. Once anaerobic condition was achieved, there was no further gassing during fermentation.

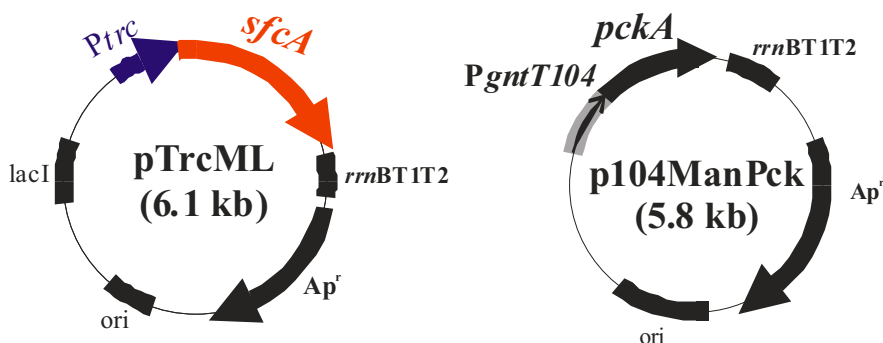


Figure 1: Construction of pTrcML and p104ManPck.

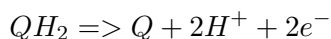
### Analytical procedure

Cell growth was monitored by measuring the absorption at 600 nm (OD<sub>600</sub>). 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, Hercules, CA) and a refractive index detector (L-3300, Hitachi chromatography system). The column was eluted isocratically with 5 mM H<sub>2</sub>SO<sub>4</sub>.

### 3 Results

#### Maximum yields

The maximum capacity for succinic acid production was estimated by metabolic flux analysis. The maximum *in silico* yield of succinic acid is listed in Table 3. The maximum theoretical yield was also calculated for comparison. The maximum *in silico* yield of succinic acid was only 1.65 mole/mole glucose (83% of the maximum theoretical yield). It was found that the CO<sub>2</sub> consumption rate was 0.82 mole/mole glucose/h even though the CO<sub>2</sub> consumption rate was not restricted (Table 1). CO<sub>2</sub> is required for the carboxylation of C3-compounds (phosphoenolpyruvate and pyruvate) to C4-compounds (oxaloacetate and malic acid), which are further converted to succinic acid. Therefore, 2 moles of CO<sub>2</sub> 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 metabolic reaction network we constructed suggested that there was no reaction for incorporating 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.



where QH<sub>2</sub> is ubiquinol or menaquinol and Q is ubiquinone or menaquinone.

Table 3: The maximum *in silico* yield of succinic acid.

Succinic acid	
Maximum theoretical yield	1.31 g succinic acid/g glucose (2 M succinic acid/M glucose)
Maximum <i>in silico</i> yield	1.08 g succinic acid/g glucose (1.647 M succinic acid/M glucose)

The electrons generated can be transferred to several different acceptors including FAD and fumaric acid. After incorporating this reaction, intracellular flux distribution was re-estimated. The maximum *in silico* yield of 2 mole succinic acid/mole glucose was achieved. This result is consistent with our previous report showing that succinic acid flux was controlled by reducing power in *E. coli* and succinic acid production could be enhanced by using more reduced carbon substrate such as sorbitol [7].

#### Optimal flux distributions

The optimum metabolic pathway for succinic acid production predicted *in silico* is shown in Figure 2. Glucose is converted to pyruvate and finally to succinic acid by sequential reactions of malic enzyme, fumarase and fumarate reductase. However, the proposed optimal pathway is different from the conventional succinic acid production pathway, which consists of PEP carboxylase, malate dehydrogenase, fumarase and fumarate reductase.

Through this pathway, free energy of PEP is wasted as inorganic phosphate while ATP can be generated by pyruvate kinase. Moreover the optimal pathway indicates that direction of malic enzyme reaction should be opposite of normal direction since usually malic enzyme catalyze decarboxylation of malic acid to pyruvate because of kinetic characteristics (K<sub>m</sub> = 0.4 mM for malic acid, 16 mM for pyruvate) [12].

#### Succinic acid production

To examine the *in silico* optimum succinic acid production pathway, the minimum set of *wet* experiments were carried out. First, conventional succinic acid pathway was tested by the overexpression of the *pckA* gene of *M. succinicroducens* MBEL55E in mutant *E. coli* strain WGS-3 (Table 4).

After 9 h of aerobic cultivation, the culture OD<sub>600</sub> reached 6 and anaerobic condition was established. At this point, the residual glucose concentration was 4.7 g/L. After 18 h of anaerobic cultivation, glucose was completely consumed. Concentrations of succinic acid and acetic acid increased steadily throughout the cultivation. The final concentrations of succinic acid and acetic acid were 3.4 and 0.9 g/L, respectively. Glycerol was also tested as a carbon substrate since it is non-PTS

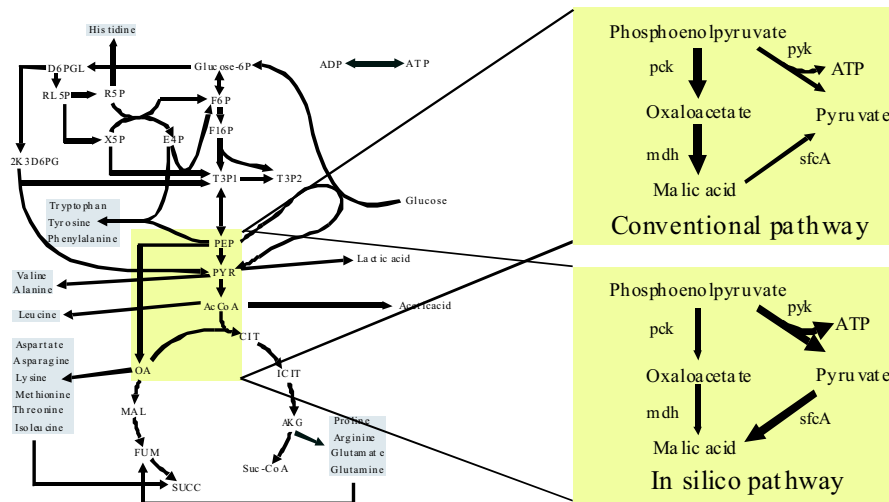


Figure 2: Conventional and *in silico* metabolic pathway for the production of succinic acid.

carbon substrate and it can more efficiently provide PEP which is very important for succinic acid production. When glycerol was supplied instead of glucose, anaerobic condition was established in 7 h cultivation. At this time, the culture  $OD_{600}$  was 5.2 and the residual glycerol concentration was 15.4 g/L. After 19 h of anaerobic cultivation, glycerol concentration decreased slightly and most of glycerol was not utilized. Concentrations of succinic acid and acetic acid increased steadily throughout the cultivation. The final concentrations of succinic acid and acetic acid were 1.1 and 0.5 g/L, respectively.

Second, the *in silico* succinic acid pathway was tested by the overexpression of the *sfcA* gene of *E. coli* in mutant *E. coli* strain NZN111 (Table 4). After 9 h of aerobic cultivation, the culture  $OD_{600}$  reached 9 and the residual sorbitol concentration was 12.4 g/L. At this point, anaerobic condition was achieved by replacing the head-space gas with  $CO_2$  gas. The final concentration of succinic acid at the end of fermentation was 10.1 g/L.

In Table 4, the results of *wet* experiments are summarized. The highest succinic acid yield (1.3 g succinic acid/g glycerol) was obtained through conventional pathway with glycerol, but succinic acid concentration and succinic acid productivity were low. The highest succinic acid concentration and productivity were obtained through the *in silico* pathway with sorbitol, and succinic acid yield (1.1 g succinic acid/g sorbitol) was as high as that obtained with glycerol. Therefore, it can be concluded that more efficient succinic acid production can be achieved through the pathway predicted by *in silico* analysis.

The effect of reducing power on the production of succinic acid was simulated since the balance of redox potential is important for succinic acid production (see above). To evaluate the effect of reducing power on succinic acid production, sorbitol, glucose and gluconate were examined as carbon substrates. It was found that the succinic acid concentration, yield and productivity were increased in the order of gluconate, glucose and sorbitol, which supports the results of *in silico* simulation suggesting the importance of reducing power balance.

## 4 Discussion

In this study, linear optimization strategy was applied to carry out *in silico* simulation. Considering the large number of degree of freedom and lack of experimental data, the calculated *in silico* metabolic flux distributions may not represent actual intracellular flux values. However, this approach is very useful to understand the overall metabolic flux distribution under various conditions. For example, the maximum *in silico* yields and the optimal metabolic pathways for succinic acid could be proposed, and the importance of reducing power on the succinic acid was predicted. Based on the yields and pathways, rational metabolic engineering strategies as well as efficient fermentation strategies could be established. Consequently, the approaches taken in this study should be useful in analyzing the

Table 4: Effects of substrates and head-space gas on the production of succinic acid.

		Consumed substrates (g/L)	Succinic acid concentration (g/L)	Succinic acid yield (g succinic acid /g carbon source)	Succinic acid productivity (g/L/h)
Conventional pathway	Glucose	4.7	3.4	0.72	0.18
	Glycerol	0.84	1.1	1.3	0.06
<i>In silico</i> pathway	Sorbitol	20	10.1	1.1	0.25
	Glucose	20	3.4	0.3	0.11
	Gluconate	20	1.2	0.1	0.06

metabolic pathways under particular circumstances, and consequently, designing metabolic engineering strategies for the improved production of various metabolites of biotechnological interest.

As mentioned above, the proposed optimal pathway for succinic acid production is different from normal succinic acid production pathway. Usually succinic acid is produced from PEP by PEP carboxylation. However, considering the low energy efficiency of PEP carboxylation pathway (see above), pyruvate is more appropriate substrate for succinic acid production. It can be supported by previous report that efficient production of succinic acid could be achieved by amplification of malic enzyme in *ldhA pft* double mutant strain NZN111 [8]. When glucose was used as substrate, however, the proposed optimal pathway did not provided enhanced succinic acid productivity compare with normal pathway because of imbalance of reducing power. Then, succinic acid productivity could be enhanced if balance of reducing was achieved by supply of additional reducing power [7].

The maximum theoretical yield can be defined as the yield which can be ideally achieved. However, the maximum theoretical yield cannot be achieved even under ideal condition due to the lack of metabolic capacity of metabolic network. In this study, we applied metabolic flux analysis technique to estimate the maximum metabolic capacity of metabolic network of *E. coli* and to evaluate the achievable *in silico* maximum yield. Additionally, it was found that the limiting factor could be identified by flux analysis, and optimal metabolite production fluxes could be proposed. Based on these optimized metabolic pathways, new efficient metabolite production systems can be suggested, and enhanced production of metabolites might be achieved. In conclusion, as demonstrated in this paper, the *in silico* metabolic flux analysis will reduce time, money and effort required to develop metabolically engineered strains for the enhanced production of various bioproducts.

## Acknowledgments

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Appendix : Optimum metabolic flux distribution for succinic acid production.

