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# A Review on Metabolic Pathway Analysis with Emphasis on Isotope Labeling Approach

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**Abstract** The recent progress on metabolic systems engineering was reviewed based on our recent research results in terms of (1) metabolic signal flow diagram approach, (2) metabolic flux analysis (MFA) in particular with intracellular isotopomer distribution using NMR and/or GC-MS, (3) synthesis and optimization of metabolic flux distribution (MFD), (4) modification of MFD by gene manipulation and by controlling culture environment, (5) metabolic control analysis (MCA), (6) design of metabolic regulation structure, and (7) identification of unknown pathways with isotope tracing by NMR. The main characteristics of metabolic engineering is to treat metabolism as a network or entirety instead of individual reactions. The applications were made for poly-3-hydroxybutyrate (PHB) production using *Ralstonia eutropha* and recombinant *Escherichia coli*, lactate production by recombinant *Saccharomyces cerevisiae*, pyruvate production by vitamin auxotrophic yeast *Toluropsis glabrata*, lysine production using *Corynebacterium glutamicum*, and energetic analysis of photosynthesic microorganisms such as Cyanobateria. The characteristics of each approach were reviewed with their applications. The approach based on isotope labeling experiments gives reliable and quantitative results for metabolic flux analysis. It should be recognized that the next stage should be toward the investigation of metabolic flux analysis with gene and protein expressions to uncover the metabolic regulation in relation to genetic modification and/ or the change in the culture condition.

*Keywords:* NMR, MS, isotopomer distribution, metabolic engineering, microbial cultivation, metabolic flux analysis, metabolic control analysis

## INTRODUCTION

The recent progress in biotechnology enables us to improve the quality of our lives and environment, and its research field has been expanded from basic science to engineering such as (1) bioinformatics including functional genomics and proteomics, (2) protein engineering including protein structure - function and structure – activity relationships *etc.*, (3) recombinant techniques including random mutation, DNA shuffling, and phage – display technique, (4) metabolic engineering, and (5) bioprocess engineering [1].

Metabolic engineering has been defined as "purposeful modification of intermediary metabolism using recombinant DNA techniques" [2]. Originally, the term metabolic engineering has been defined as "improvement of cellular activities by manipulation of enzymatic transport and regulatory functions of the cell with the use of recombinant DNA technology" [3]. In a broader sense, metabolic engineering can be viewed as the design of biochemical reaction networks to accomplish a certain objective. Typically, the objective is either to increase the rate of a desired product or to reduce the rate of undesired side-products [4], or to decompose the toxic or undesired substances. Of central importance to this field is the notion of cellular metabolism as a network. In other words, an enhanced perspective of metabolism and cellular function can be obtained by considering the participating reactions in their entirety, rather than on an individual basis [4]. This research field is, therefore, multidisciplinary, drawing on information and techniques from biochemistry, genetics, molecular biology, cell physiology, chemistry, chemical engineering, systems science, and computer science.

Although the term metabolic engineering has been defined as above, its definition is by no means clear-cut, and overlaps with the related terms such as physiological engineering [5], pathway engineering, in vitro evolution, direct evolution, molecular breeding, cellular engineering as listed in Cameron and Tong [2]. It should also be noted that the metabolic regulation be investigated with respect to gene and protein expressions to understand the overall picture.

The intellectual framework and the potential application of metabolic engineering have been reviewed [3,6], and yet another reviews have been made to recognize the importance of metabolic engineering [2,5,7-9]. Several books have also been published recently [10,11].

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In the present article, an overview on metabolic systems engineering approach is made for the efficient fermentation based on our recent research results with the emphasis on isotope labeling technique.

## METABOLIC SIGNAL FLOW DIAGRAM APPROACH

In principle, the metabolic networks can be expressed in terms of a directed signal flow diagram [12,13]. Therefore, the enzyme reaction of conversion of metabolite A to metabolite B can be considered as the transformation of signal A to signal B. If we consider the metabolic network of a cell as a system, there are several inputs and outputs through the boundary of the system with the environment. Then those input-output relationships may be expressed in terms of the metabolic transfer coefficients. The resulting complex signal flow diagram can be simplified through use of the equivalent transformation of the graph theory. It should be noted that the input-output relationship may be identified from the time-series data. Then we may be able to find some relationships on the metabolic transfer coefficients, and thus we may be able to estimate the activities of certain pathway networks [12,14].

In relation to this approach, an interesting network analysis method has been proposed for formulation of a metabolic model for *Escherichia coli* by several researchers [15-17]. In their works, a metabolic objective provides a physiological rationale for acetate production, which is based on mechanistic details considered as constraints on the reaction network.

In the same manner, we assumed that a microorganism has as its objective maximization of ATP production and derived the expression of the metabolic transfer coefficients [18]. Such expression enables us to understand how the important branch points are regulated based on the time-series input and output data. This method was extended for use in on-line estimation [19]. For the on-line analysis, Shimizu *et al.* [20] defined an error vector based on stoichiometric equations, and they attempted to identify the unknown metabolites based on the magnitude of such an error vector due to unbalance of material.

## METABOLIC FLUX ANALYSIS

Quantification of metabolic fluxes is an important analysis technique of metabolic engineering. A powerful technique for calculation of the fluxes through various pathways is the so-called metabolic flux analysis (MFA), where the intracellular fluxes are calculated using a stoichiometric model for all the major intracellular reactions and by applying mass balances around the intracellular metabolites. As inputs to the calculations, a set of measured fluxes, typically the specific uptake rate of substrate and the specific secretion rate of metabolites etc. are provided [21-23]. Metabolic flux distribution can then be estimated using the following stoichiometric equation:

$$Ar = q \tag{1}$$

where $A$ is an $m \times n$ matrix of stochiometric coefficients. $r$ is an $n$-dimensional flux vector, and $q$ is an $m$-dimensional vector of the specific substrate consumption rate and the specific metabolite excretion rates. The weighted least square solution to Eq. (1) is then obtained for the over-determined system as

$$r = (A^T \Phi^{-1} A)^{-1} A^T \Phi^{-1} q \tag{2}$$

provided that $A$ is of full rank, where $\Phi$ is the measurement noise variance-covariance matrix of the measurement vector $q$. Several computer programs for calculating $r$ have been developed by several researchers [24,25].

We made flux analysis based on this approach for efficient production of poly β-hydroxybutyrate (PHB) using *Ralstonia eutropha*. PHB is a homopolymer of 3-hydroxybutyrate and is most widespread and best-characterized member of poly-3-hydroxyalkanoates (PHAs). We conducted several fed-batch experiments for several substrates, and computed the flux distribution for the subdivided growth, transient, and PHB production phases.

Note that $NH_3$ concentration was relatively high during cell growth phase while it was low during the later PHB production phase. Because of this, the ammonium ion was dominantly assimilated into the cell through the reaction from α-ketoglutarate (α-KG) to glutamic acid (GLUT) during cell growth phase. It should also be noted that NADPH generated via isocitrate dehydrogenase (ICD) was mainly consumed in glutamic acid synthesis pathway during this phase. We estimated how much and where NADPH was produced and consumed in each cultivation phase based on the flux distribution obtained. The result clearly indicates that the block in the glutamic acid synthetic pathway due to low level of $NH_3$ in the later phase results in the overproduction of PHB due to increased availability of NADPH formed through ICD, since NADPH-dependent acetoacetyl-CoA reductase in the PHB producing pathway provides a sink for excess reducing equivalents. This research result demonstrates that MFA may be useful in disclosing the metabolic regulation mechanism to some extent [26]. We also made such MFA for other fermentation systems such as photosynthetic microorganisms [27,28].

Gulik and Heijinen [29] made 99-dimensional metabolic flux analysis of aerobic growth of *S. cerevisiae* on glucose/ethanol mixtures and predicted five different metabolic flux regimes upon transition from 100% glucose to 100% ethanol. Pramanik and Keasling [30] developed a stoichiometric model for *E. coli* which incorporates 153 reversible and 147 irreversible reactions and 289 metabolites from metabolic data bases for the biosynthesis of the macromolecular precursors, coenzymes, and prosthetic groups necessary for synthesis of all cel-

lular macromolecules. For such a system, the number of reactions is greater than the number of metabolites. Because multiple solutions exist, linear optimization was used to determine the fluxes. Some of the objective functions that were used included minimization or maximization of ATP usage, substrate uptake, growth rate, and product synthesis.

Takiguchi *et al.* [31] applied the metabolic pathway model to estimate the physiological state of the cells, that is, the growth and production activity, and the flux distribution of metabolites for lysine fermentation using *Corynebacterium glutamicum* from on-line measurable data.

## METABOLIC FLUX ANALYSIS WITH $^{13}C$ LABELING EXPERIMENTS

The metabolic flux analysis based on the metabolic flux distributions computed by the above method based on mass balances for the metabolites with stoichiometric eqations may be useful to find the metabolic regulation mechanism for performance improvement of fermentation etc. However, the application of the above method is limited to relatively simple case. In more complex metabolic network systems, where the system involves cyclic pathways such as TCA cycle etc. where intermediates reenter the cyclic pathway, or the large number of branching points exist or parallel reaction steps like the various anaplerotic reactions exist in the metabolic network, a detailed analysis cannot be done with the above method. Moreover, the forward and backward directions of reversible reaction steps can never be resolved with the above method. In such cases, the application of metabolite balancing either requires another sets of reactions or forced to be lumped together [21]. These problems led to the development of metabolic flux analysis based on carbon labeling experiments.

Consider a simple example as given in Fig. 1. The network consists of an input substrate $S$ with a known isotopomer distribution, intracellular intermediates $A$, $B$ and $C$, and an output metabolite $P$. $B$ is assumed to have one carbon atom, and the other metabolites are assumed to have two carbons. $V_1$ is the system input flux (and thus measurable), $V_6$ is the output flux, and the remaining fluxes $V_2$, $V_3$, $V_4$, $V_5$ are intracellular fluxes. $V_2$ and $V_3$ keep the metabolite $A$ together but with different fates of carbon atoms, while $V_4$ splits $A$ into two molecules of $B$, and $V_5$ reunites the atoms. $V_2$ is assumed to take place in both directions, and the other fluxes are unidirectional (*i.e.*, $v_1^{\sigma} = v_3^{\sigma} = v_4^{\sigma} = v_5^{\sigma} = v_6^{\sigma} = 0$), where $v_i^{\rightarrow}$ is defined as the $i$th forward flux, while $v_j^{\leftarrow}$ is the $j$th backward flux. All $v_i^{\rightarrow}$ and $v_j^{\leftarrow}$ must be nonnegative. The mass balance for each intracellular metabolite yields the following equations:

$$\begin{aligned} &\text{A:} && v_1^{\rho} + v_2^{\sigma} = v_2^{\rho} + v_3^{\rho} + v_4^{\rho} \\ &\text{B:} && v_4^{\rho} = v_5^{\rho} \\ &\text{C:} && v_2^{\rho} + v_3^{\rho} + v_5^{\rho} = v_2^{\sigma} + v_6^{\rho} \end{aligned} \tag{3}$$

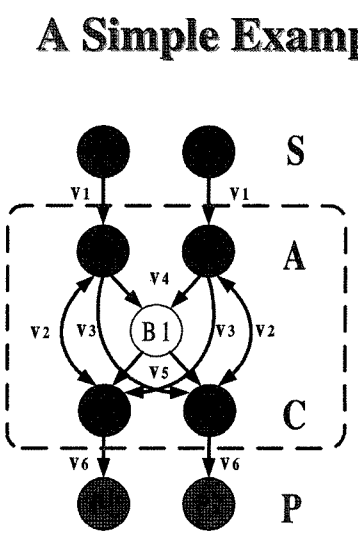


**Fig.1.** A simple example network. The isotopomer distribution of the input metabolite $S$ is known. The input flux $v_1$, the mass isotopomer distributions of metabolites $A$ ($m_0^a$, $m_1^a$, $m_2^a$) and $P$ ($m_0^p$, $m_1^p$, $m_2^p$), and the multiplet patterns of the second carbon of $A$ ($s_{C2}^a$, $d_{C2}^a$) and $P$ ($s_{C2}^p$, $d_{C2}^p$) are assumed to be measurable.

Assuming $v_1^{\rightarrow}$ to be measured, there are 3 equations for 6 variables such as $v_2^{\rightarrow}$, $v_2^{\leftarrow}$, $v_3^{\rightarrow}$, $v_4^{\rightarrow}$, $v_5^{\rightarrow}$ and $v_6^{\rightarrow}$, which indicates that the system can be determined if 3 out of 6 variables are known. For example, $v_2^{\rho}$, $v_3^{\rho}$, $v_4^{\rho}$ are known in addition to $v_1^{\rightarrow}$, then the other variables can be computed from Eq. (3) as

$$v_6^{\rho} = v_1^{\rho}$$

$$v_5^{\rho} = v_4^{\rho}$$

$$v_2^{\sigma} = v_2^{\rho} + v_3^{\rho} + v_4^{\rho} - v_1^{\rho}$$

In determining $v_2^{\rightarrow}$, $v_3^{\rightarrow}$, and $v_4^{\rightarrow}$, we need another information obtainable by carbon labeling experiment etc.

The modeling of isotope distributions may be used to evaluate intracellular fluxes in more detail and to overcome the shortcomings of the conventional MFA method. Isotopic tracer experiments are usually conducted using substrate enriched with $^{14}C$, which is radioactive, or $^{13}C$, which is not radioactive and stable and detectable by NMR and/or GC-MS. For radioactive isotopes, the specific activity is usually expressed as radioactivity per mole or per gram, while for stable isotope, the specific activity is usually expressed as the fractional enrichment of a specific atom within a molecule. The latter approach has been paid much attention during the past decade.

It should be noted that for the flux calculation, neither enzyme activities nor kinetic information on enzymes are required. Only the mass balance for the metabolites and carbon atoms are considered, while the energy balance is not considered. Several assumptions are made for flux calculation such that

1) The system of concern must be kept in a well-defined stationary physiological state during the measurement procedure, since the steady-state mass balance is applied for flux calculation. This does not, however, restrict the application to continuous culture but may be applied to batch and fed-batch culture by assuming pseudo-steady state.

2) For the metabolic pathway of interest, all relevant stoichiometric equations must be defined. Moreover, the fate of all carbon atoms must be defined. Those can be easily found in the biochemistry textbooks as far as the main metabolic pathways are considered.

3) No mass effects are assumed to present. Namely, the labeling state of a molecule does not influence the rate of its enzymatic conversion. This may be true as far as the liquid phase reactions are considered. It should, however, be careful that mass effects have been observed in certain situations for small molecules like $CO_2$ [32,33] as stated by Wiechert and Graaf [34].

It should also be noted that there is no need to preset the directionality for any of the fluxes. In general, a relatively large negative value of standard free energy may justify the assumption of irreversibility, that is, the corresponding reaction step may be considered to be unidirectional, while bi-directional reactions are considered for the other cases.

Consider the metabolite activity vector (MAV) for defining the specific activity or fractional enrichment of a metabolite, where the $i$th element of MAV contains the specific enrichment of the $i$th carbon atom in the corresponding molecule [35] such as for metabolite A

$$MAV_A = [A(1)\ A(2)]^T$$

Then Zupke and Stephanopoulos [35] introduced atom mapping matrices (AMMs) which define the transfer of atoms from reactants to products catalyzed by enzyme for each reaction. The mass balances for carbon atoms of intracellular metabolites for Fig. 1 can then be expressed using MAVs and AMMs as

$$\begin{aligned}
&\text{A: } v_1^{\rightarrow}[\text{S>A}]_{E1}S + v_2^{\leftarrow}[\text{C>A}]_{E2}C \\
&\quad = (v_2^{\rightarrow}[\text{A>C}]_{E2} + v_3^{\rightarrow}[\text{A>C}]_{E3} + v_4^{\rightarrow}[\text{A>B}]_{E4})A \\
&\text{B: } v_4^{\rightarrow}[\text{A>B}]_{E4}A = v_5^{\rightarrow}[\text{B>C}]_{E5}B \qquad (5) \\
&\text{C: } v_2^{\rightarrow}[\text{A>C}]_{E2}A = v_3^{\rightarrow}[\text{A>C}]_{E3}A + v_5^{\rightarrow}[\text{B>C}]_{E5}B \\
&\quad = v_6^{\rightarrow}[\text{C>P}]_{E6}C
\end{aligned}$$

Where $[S>A]_{E1}$ is the AMM from S to A catalyzed by E1 and so forth.

The MAVs contain the information of fractional enrichments at individual carbon atom positions. The above approach is based on the so-called positional representation. From the practical application point of view using NMR and/or GC-MS, another representation may be convenient.

As an alternative to MAVs, another vector was introduced by Schmidt *et al.* [36] paying attention to mole fractions of metabolite molecules that are labeled in a specific pattern. This vector is called as the isotopomer distribution vector (IDV), and it contains mole fractions of the individual isotopomers. Since a carbon atom is either labeled or nonlabeled, the labeling patterns of a metabolite can be coded with the sequence of ones and zeros. Therefore, the IDV of a molecule having $n$ carbon atoms has $2^n$ elements. Let A have 2 carbon atoms. Then IDV for A is expressed as

$$\begin{aligned}
I_A &= [I_A(0)\ I_A(1)\ I_A(2)\ I_A(3)]^T \\
&= [I_A(00_{bin})\ I_A(01_{bin})\ I_A(10_{bin})\ I_A(11_{bin})]^T
\end{aligned}$$

with $\sum_{i=0}^{3} I_A(i) = 1$

where the subscript "bin" implies the binary representation. Note that the first element of IDV corresponds to unlabeled molecule, while the last element corresponds to uniformly labeled molecule.

Consider the reaction system as mentioned above in Eq. (5). The isotopomer distribution of the product molecules is determined by the isotope distribution of the reactants and the reaction mechanism, which is specified by the AMMs of the molecular conversion. Schmidt *et al.* [36] introduced isotopomer mapping matrices (IMMs) which can be constructed to sum up all pairs of reactant isotopomers that produce the respective product isotopomer in all positions of the product IDV. There is one IMM defined for each pair of reactant and product molecules in a biochemical carbon exchange reaction. The number of rows in IMMs equals the number of vector elements of the reactant IDV.

In IMMs, columns are associated with the individual labeling patterns of the respective substrate molecule, and the individual rows are associated with the product isotopomer labeling patterns. The $i$th column corresponds to the $i$th substrate IDV element. Hence, the first column of $IMM_{A>B}$ marks those product isotopomers that can evolve from unlabeled A molecules ($00_{bin}$). The second IMM column marks those that can evolve from substrate molecules labeled A in the second position ($01_{bin}$), and so forth.

Consider the following example: A + B → C

Where

$$AMM_{A\rangle C} = \begin{bmatrix} 01 \\ 10 \end{bmatrix}, \qquad AMM_{B\rangle C} = \begin{bmatrix} 0 \\ 0 \\ 1 \end{bmatrix}$$

Then

$$IDV_A = \begin{bmatrix} 00 \\ 01 \\ 10 \\ 11 \end{bmatrix}, \qquad IDV_B = \begin{bmatrix} 0 \\ 1 \end{bmatrix}$$

$$IDV_C = \begin{bmatrix} 000 \\ 001 \\ 010 \\ 011 \\ 100 \\ 101 \\ 110 \\ 111 \end{bmatrix}, \quad IMM_{A\rangle C} = \begin{bmatrix} 1000 \\ 0010 \\ 0010 \\ 0100 \\ 0100 \\ 0001 \\ 0001 \end{bmatrix}, \quad IMM_{B\rangle C} = \begin{bmatrix} 01 \\ 10 \\ 01 \\ 10 \\ 01 \\ 10 \\ 01 \end{bmatrix}$$

Using IMMs, one can calculate the isotopomer distribution of the product molecule C as

$$IDV_C = (IMM_{A>C} \cdot IDV_A) \odot (IMM_{B>C} \cdot IDV_B) \quad (6)$$

where operator $\odot$: $R^8 \times R^8 \rightarrow R^8$ is defined as the elementwise multiplication of two equally long column vectors.

From the practical application point of view using the measured data obtained from MS, it may be better to convert the IDV to mass distribution vector (MDV)[37]. Let $MDV_A$ be the MDV of A, and let $x_A(m)$, $x_A(m+1)$, $x_A(m+2)$, ... be the elements of $MDV_A$ and molecular fraction of a group of isotopomers with same mass. Then IDV can be converted to MDV by the following conversion matrix:

$$\begin{bmatrix} A_{m+1} \\ A_{m+2} \end{bmatrix} = \begin{bmatrix} 110 \\ 001 \end{bmatrix} \begin{bmatrix} A_{01} \\ A_{10} \\ A_{11} \end{bmatrix} \quad (7)$$

where $A_{ij}$ is the concentration of the ij isotopomer with $ij \in \{0,1\}$, and $A_{m+i}$ is the mass spectrometry signal produced by the A molecule of molecular weight m+i, $i=\{1,2\}$, where m is the molecular weight of the nonlabeled molecule. In the above equation, $A_{00}$ and $A_m$ are not shown, since those are obtained using the condition that the sum of all the sotopomers are unity, and therefore, $A_{00}$ and $A_m$ are the compliments to 1's of the sum of the labeled isotopomers.

Mass spectrometry measurements lead to complex mass spectra, which are caused by partial fragmentation of the analyzed compound and isotope distribution. The isotopomer analysis using GC-MS spectra obtained by some fragmentation was proposed by Christensen and Nielsen [38].

Consider next the case of using NMR data. Let A be constituted of 3 carbon atoms such as alanin as shown in Fig. 2. Then matrices that map the isotopomer distribution on the relative contribution of singlet, doublet, doublet of doublet, and triplet signals on the expected multiplet pattern can be devised from the covalent structure of the respective molecule. Then matrices that map the isotopomer distribution on the relative contribution of singlet, doublet, doublet of doublet, and triplet signals on the expected multiplet pattern can be devised from the covalent structure of the respective molecule (see Fig. 2).

$$\begin{bmatrix} 00001100 \\ 00100000 \\ 01000100 \end{bmatrix} \quad I_A = \begin{bmatrix} S'_{C1} \\ S'_{C2} \\ S'_{C3} \end{bmatrix} \quad (8) \qquad I_A = \begin{bmatrix} 000 \\ 001 \\ 010 \\ 011 \\ 100 \\ 101 \\ 110 \\ 111 \end{bmatrix}$$

where the 5th and 6th IDV elements of A contribute to a

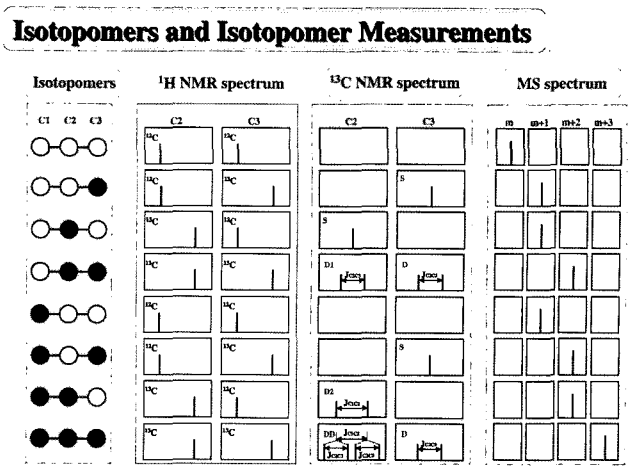


**Fig. 2.** The isotopomers of a $C_3$ molecule and the corresponding $^{13}C$ NMR and MS spectra. The first carbon is not considered for NMR because its $^{13}C$ peak is generally very weak. In the $^{13}C$ NMR spectrum as observed along $\bullet_1$ in 2D $^1H$-$^{13}C$ NMR spectroscopy, the centrally located carbon atom give rise to multiplet signals in form of singlet (S), doublets (D1, D2), and doublet of doublets (DD), while the peripheral carbon atom give only singlet (S) and doublet (D) signals. In the case of equal coupling constants D1 and D2 cannot be distinguished and doublet of doublets become triplets. The MS spectrum as analyzed by GC-MS separate isotopomers with different molecular mass.

singlet signal in C1, and so on. Relative contributions of doublet (D1 and D2) signals and doublet of doublets DD (including triplets) can be calculated in a similar manner as

$$\begin{bmatrix} 00000000 \\ 00010000 \\ 00010001 \end{bmatrix} \quad I_A = \begin{bmatrix} D1'_{C1} \\ D1'_{C2} \\ D1'_{C3} \end{bmatrix} \quad (9)$$

$$\begin{bmatrix} 00000011 \\ 00000010 \\ 00000000 \end{bmatrix} \quad I_A = \begin{bmatrix} D2'_{C1} \\ D2'_{C2} \\ D2'_{C3} \end{bmatrix} \quad (10)$$

$$\begin{bmatrix} 00000000 \\ 00000010 \\ 00000000 \end{bmatrix} \quad I_A = \begin{bmatrix} DD'_{Ci} \\ DD'_{C2} \\ DD'_{C3} \end{bmatrix} \quad (11)$$

The relative contributions to the overall carbon signal must be scaled to the sum of the individual multiplet signals. The relative contribution of the singlet signal to the overall A C2 signal is therefore expressed as

$$S_{C2} = \frac{S'_{C2}}{S'_{C2} + D1'_{C2} + D2'_{C2} + DD'_{C2}} \quad (12)$$

Consider the example in Fig. 1 again to understand the analysis based on isotopomer representation. The isotopomer fractions of a metabolite may be denoted using

an index notation corresponding to the isotopomer name. For example, the isotopomer fractions of $A$ are written as $A_{00}$, $A_{01}$, $A_{10}$, $A_{11}$, where a **1** indicates that the corresponding carbon atom position is labeled and a **0** indicates that it is not labeled. Then the balance equations for all the intracellular isotopomers are given as:

$$\begin{aligned}
A_{01}: &\quad (\overset{\rho}{\nu_2}+\overset{\rho}{\nu_3}+\overset{\rho}{\nu_4})a_{01} = \overset{\sigma}{\nu_2}\, c_{01} + \overset{\rho}{\nu_1}\, s_{01}\\
A_{10}: &\quad (\overset{\rho}{\nu_2}+\overset{\rho}{\nu_3}+\overset{\rho}{\nu_4})a_{10} = \overset{\sigma}{\nu_2}\, c_{10} + \overset{\rho}{\nu_1}\, s_{10}\\
A_{11}: &\quad (\overset{\rho}{\nu_2}+\overset{\rho}{\nu_3}+\overset{\rho}{\nu_4})a_{11} = \overset{\sigma}{\nu_2}\, c_{11} + \overset{\rho}{\nu_1}\, s_{11}\\
B_1: &\quad 2\overset{\rho}{\nu_5}\, b_1 = \overset{\rho}{\nu_4}\, a_{01} + \overset{\rho}{\nu_4}\, a_{10} + 2\overset{\rho}{\nu_4}\, a_{11}\\
C_{01}: &\quad (\overset{\sigma}{\nu_2}+\overset{\rho}{\nu_6})c_{01} = \overset{\rho}{\nu_2}\, a_{01} + \overset{\rho}{\nu_3}\, a_{10} + \overset{\rho}{\nu_5}(1-b_1)b_1\\
C_{10}: &\quad (\overset{\sigma}{\nu_2}+\overset{\rho}{\nu_6})c_{10} = \overset{\rho}{\nu_2}\, a_{10} + \overset{\rho}{\nu_3}\, a_{01} + \overset{\rho}{\nu_5}(1-b_1)b_1\\
C_{11}: &\quad (\overset{\sigma}{\nu_2}+\overset{\rho}{\nu_6})c_{11} = \overset{\rho}{\nu_2}\, a_{11} + \overset{\rho}{\nu_3}\, a_{11} + \overset{\rho}{\nu_5}\, b_1^2
\end{aligned} \tag{13}$$

where $a_{ij}$, $b_i$, $c_{ij}$, $s_{ij}$ $(i, j = 0, 1)$ are the isotopomer fractions of metabolites $A$, $B$, $C$ and $S$, respectively. The isotopomer distribution of $C$ is the same as that of output metabolite $P$. The balances for the pools $A_{00}$, $B_0$, $C_{00}$ are not included in Eq. (13), since the sum of all isotopomer fractions corresponding to one metabolite is **1**.

The balance equations can now be used to represent all isotopomer distributions in terms of fluxes. From Eq. (13), we obtain

$$a_{01} + a_{10} + 2a_{11} = 2b_1 = c_{01} + c_{10} + 2c_{11} = s_{01} + s_{10} + 2s_{11} \tag{14}$$

The first and third terms of Eq. (14) represent the sum of the positional labeling data (*i.e.* $^1$H-NMR measurements) for the two carbons of metabolites $A$ and $C$, respectively. Thus it means that the $^{13}$C enrichment of the first carbon atom is redundant with that of the second carbon atom. Consequently, there is no chance to determine the three free fluxes from positional enrichment data of metabolites $A$ and $C$. However, the isotopomer measurements contain additional information on the fluxes so that all free fluxes can be determined. From Eq. (13), it can be obtained that

$$\begin{aligned}
a_{01} - a_{10} &= (\overset{\rho}{\nu_1}\overset{\rho}{\nu_2} + \overset{\rho}{\nu_1}\overset{\rho}{\nu_3} + \overset{\rho}{\nu_1}\overset{\rho}{\nu_4}) \cdot (s_{01}-s_{10})/\delta\\
c_{01} - c_{10} &= (\overset{\rho}{\nu_1}\overset{\rho}{\nu_2} - \overset{\rho}{\nu_1}\overset{\rho}{\nu_3}) \cdot (s_{01}-s_{10})/\delta\\
a_{11} &= \left[\begin{matrix} 4(\overset{\rho}{\nu_1}\overset{\rho}{\nu_2} + \overset{\rho}{\nu_1}\overset{\rho}{\nu_3} + \overset{\rho}{\nu_1}\overset{\rho}{\nu_4})s_{11} + (\overset{\rho}{\nu_2}\overset{\rho}{\nu_4} + \overset{\rho}{\nu_3}\overset{\rho}{\nu_4} + \overset{\rho^2}{\nu_4} - \overset{\rho}{\nu_1}\overset{\rho}{\nu_4}) \\ (s_{01} + s_{10} + 2s_{11})^2 \end{matrix}\right]/\zeta\\
c_{11} &= \left[4(\overset{\rho}{\nu_1}\overset{\rho}{\nu_2} + \overset{\rho}{\nu_1}\overset{\rho}{\nu_3})s_{11} + (\overset{\rho}{\nu_2}\overset{\rho}{\nu_4} + \overset{\rho}{\nu_3}\overset{\rho}{\nu_4} + \overset{\rho^2}{\nu_4})\,(s_{01} + s_{10} + 2s_{11})^2\right]/\zeta
\end{aligned} \tag{15}$$

where $\delta = 2\overset{\rho^2}{\nu_3} + \overset{\rho^2}{\nu_4} + 2\overset{\rho}{\nu_2}\overset{\rho}{\nu_3} + \overset{\rho}{\nu_2}\overset{\rho}{\nu_4} + 3\overset{\rho}{\nu_3}\overset{\rho}{\nu_4} + \overset{\rho}{\nu_1}\overset{\rho}{\nu_2} - \overset{\rho}{\nu_1}\overset{\rho}{\nu_3}$

and $\zeta = 4(\overset{\rho^2}{\nu_4} + \overset{\rho}{\nu_2}\overset{\rho}{\nu_4} + \overset{\rho}{\nu_3}\overset{\rho}{\nu_4} + \overset{\rho}{\nu_1}\overset{\rho}{\nu_2} + \overset{\rho}{\nu_1}\overset{\rho}{\nu_3})$

Combining Eqs. (14) and (15), all the isotopomer fractions of $A$ and $C$ can be represented by the free fluxes. However, as shown in Fig. 2, the isotopomer measurement techniques do not enable isotopomer fractions to be measured directly. Instead, they yield linear combinations of such fractions. In this example, the mass isotopomer distributions of metabolites $A$ ($m_0^a$, $m_1^a$, $m_2^a$) and $P$ ($m_0^p$, $m_1^p$, $m_2^p$) are measured by using GC-MS, and the multiplet patterns of the second carbon of $A$ ($s_{C2}^a$, $d_{C2}^a$) and $P$ ($s_{C2}^p$, $d_{C2}^p$) are determined by NMR. The mass isotopomer distribution ($m_0^c$, $m_1^c$, $m_2^c$) and multiplet pattern ($s_{C2}^c$, $d_{C2}^c$) of metabolite $C$ are the same as those of $P$, since the isotopomer distributions of both metabolites are identical. Based on the relationship as shown in Fig. 2, we can express the different measurement data by isotopomer fractions.

$$\begin{aligned}
&a_{01} + a_{10} = m_1^a\\
&a_{11} = m_2^a\\
&c_{01} + c_{10} = m_1^c\\
&c_{11} = m_2^c\\
&\frac{a_{01}}{a_{01} + a_{11}} = s_{C2}^a\\
&\frac{c_{01}}{c_{01} + c_{11}} = s_{C2}^c
\end{aligned} \tag{16}$$

The equations for the measurements $m_0^a$, $m_0^c$, $m_{C2}^a$, $d_{C2}^c$ are not included in Eq. (16). This is because the measurement data are the relative abundance of different mass fractions or multiplet signals, and $n$ measurements give only $n$-1 constraints on the isotopomer distribution. From Eqs. (14)-(16), the functional relationship:

$$\gamma: (\overset{\rho}{\nu_2}, \overset{\rho}{\nu_3}, \overset{\rho}{\nu_4}) \rightarrow (m_1^a, m_2^a, m_1^c, m_2^c, s_{C2}^a, s_{C2}^c) \tag{17}$$

can be constructed. For example, $m_1^a$ can be expressed as

$$\begin{aligned}
m_1^a = {} & s_{01} + s_{10} + 2s_{11} - \\
& [8(\overset{\rho}{\nu_1}\overset{\rho}{\nu_2} + \overset{\rho}{\nu_1}\overset{\rho}{\nu_3} + \overset{\rho}{\nu_1}\overset{\rho}{\nu_4})s_{11} + 2(\overset{\rho}{\nu_2}\overset{\rho}{\nu_4} + \overset{\rho}{\nu_3}\overset{\rho}{\nu_4} + \overset{\rho^2}{\nu_4} - \overset{\rho}{\nu_1}\overset{\rho}{\nu_4}) \\
& (s_{01} + s_{10} + 2s_{11})^2]/\zeta
\end{aligned} \tag{18}$$

Thus, for the flux estimation, a set of fluxes has to be found that reproduces all the measured fluxes and isotopomer labeling data. This flux determination problem cannot always be solved explicitly because of the nonlinear nature of the system. In this situation a nonlinear least squares fitting approach may be considered suitable for flux estimation. That is, the intracellular fluxes are determined such that the deviation between the measured data and the calculated values is minimized.

It should be noted that both the forward and backward fluxes of bidirectional reactions can be computed from isotopomer measurements as implied in the above example. This is one of the advantages over the conventional metabolic flux analysis based on metabolite balancing, which can estimate only the net fluxes. Moreover, the isotopomer measurements are superior as compared with positional enrichment measurements,

since the former contain more information and pose more constraints on intracellular fluxes.

The above simple example demonstrated that the isotopomer measurements enabled the fluxes of both directions to be quantitated. For better description of the bidirectional reactions, they may be expressed in terms of their net flux $v^{net}$ and their exchange flux $v^{exch}$ that quantitates the amount of flux common to forward and backward fluxes (see Eq. (19) below). Nonlinear mapping of the exchange fluxes $v^{exch}$ to exchange coefficients $v^{exch[0,1]}$ [34,39] as given below in Eq. (20) may be introduced to overcome the numerical problems arising from very large parameter values. Here, $\beta$ is a constant on the order of magnitude of the net fluxes.

$$v^{\text{exch}} = min(\overset{\rho}{v}, \overset{\sigma}{v}) \tag{19}$$

$$v^{\text{exch}[0,1]} = \frac{v^{\text{exch}}}{\beta + v^{\text{exch}}} \tag{20}$$

The strategy of flux determination in a complex metabolic network is now explained by generalizing the concepts presented for the simple example. Similar to the conventional metabolite balancing, balances are taken around all isotopomers of a particular metabolite. Then the mathematical framework relating intracellular fluxes and isotopomer measurements is developed. The intracellular fluxes can thus be estimated by a nonlinear least squares fitting procedure. One of the procedures to solve this problem may be is as follows:

1) The vector of free fluxes ($v_{\text{free}}^{\text{net}}$, $v^{exch[0,1]}$) is given with an arbitrary value. The linear constraints on the net fluxes provided by the stoichiometry of metabolic networks are not usually sufficient for a complete determination of all net fluxes. Therefore, in order to fix the remaining degrees of freedom, some net fluxes must be identified that enable the metabolite balance equations to be solved when values are supplied for them. These fluxes, together with all the exchange coefficients that can not be resolved by metabolite balancing, must be taken as the free fluxes to be optimized.

2) All the net fluxes are determined by using the stoichiometric equations for key intracellular metabolites and the measured extracellular flux data.

3) The vector ($v^{net}$, $v^{exch[0,1]}$) is transformed into the vector $(\overset{\varpi}{v}, \overset{\varpi}{v})$ by using Eqs. (19) and (20).

4) The set of isotopomer balance equations is solved iteratively via computer to obtain the isotopomer distribution of each metabolite. In the isotopomer balance equations, the sum of incoming isotopomer fluxes is set equal to the sum of isotopomer fluxes out of the pool. The flux of isotopomer into a metabolite is the sum of the substrate isotopomers that are required to produce the individual product isotopomers in biochemical reactions, weighted by the corresponding reaction rate $(\overset{\varpi}{v}, \overset{\varpi}{v})$ [35,36]. The isotopomer fractions of substrates should be initialized. Then each of the isotopomer balance equations is solved sequentially for the isotopomer distribution vector of products, and the above procedure is repeated until convergence is achieved.

5) The calculated isotopomer distributions are transformed into multiplet intensities and mass isotopomer distributions based on the relationship as shown in Fig. 2.

6) Steps 1-5 are incorporated into an optimization program to find the optimal free fluxes that generate the estimated isotopomer measurement data and extracellular fluxes fitted best to the experimental results. The optimal estimation for free net fluxes and exchange coefficients is obtained by minimizing the sum of squares of the deviations between estimated values and measured data. The objective function to be minimized may be defined as

$$F(v) = \sum_{i=1}^{M} \left(\frac{W_i - E_i(v)}{\delta_i}\right)^2 + \sum_{j=1}^{N} \left(\frac{Y_j - v_k}{\delta_j}\right)^2 \tag{21}$$

where $v$ is the vector of free fluxes to be optimized in the program, and $W_i$ are the $M$ individual isotopomer measurement data and $E_i$ their corresponding estimated values computed by the assumed values of $v$. $Y$ is a vector containing the measured data of $N$ extracellular fluxes. $v_k$ is the element of $v$, which corresponds to the extracellular flux measurement $Y_j$. $\delta_i$ is the absolute measurement error[40].

It should be noted that the labeling patterns of the intracellular metabolites are difficult to measure due to the small pool sizes of these metabolites. However, since the amino acids reflect the labeling patterns of a number of important central metabolites through their precursors from the central metabolism, and relatively abundant, the labeling patterns of amino acids have been used for elucidation of labeling patterns in the central metabolism [41]. Thus $^{13}$C-NMR and GC-MS have been employed to identify indirectly the labeling patterns of the intracellular metabolites. In the isotope labeling experiments, the mixture of 90% of unlabeled or naturally labeled carbon source such as glucose and 10% of uniformly labeled carbon source [U-$^{13}$C] and/or the first carbon labeled carbon source [1-$^{13}$C] is often employed, but it should be noted that there are many other possible combinations, and this will affect the relaibility of the computed fluxes from the statistical point of view. In the analysis of carbon labeling patterns of amino acids, it is also necessary to make a correction for the contribution of labeling arising naturally labeled species of $^{15}$N, $^{17}$O, $^{18}$O, $^{13}$C, $^{2}$H etc. This correction may be carried out using matrix-based methods as given by Lee *et al.* [42,43] and Wittman and Heinzle [37].

Marx *et al.* [23] combined the information of $^{1}$H-detected $^{13}$C NMR spectroscopy to follow individual carbons with carbon balances for cultivation of lysine-producing strain of *C. glutamicum*. The result shows that the flux through pentose phosphate pathway is 66.4% (relative to the glucose input flux of 1.49 mmol/g dry weight h), that the entry into TCA cycle 62.2%, and the contribution of the succinylase pathway of lysine synthesis 13.7%.

For the systems having cyclic pathways, Klapa *et al.* [44] presented a mathematical model to analyze iso-

topomer distributions of TCA cycle intermediates following the administration of $^{13}C$ (or $^{14}C$) labled substances. Such theory provides the basis to analyze $^{13}C$ NMR spectra and molecular weight distributions of metabolites. This method was applied to the analysis of several cases of biological significance [45].

Wiechert and his co-workers develop a model for positional carbon labeling systems, which was further extended to general isotopomer labeling systems with statistical treatment [34,46,47]. Mollney *et al.* [48] compared the different measurement techniques such as $^{1}H$ NMR, $^{13}C$ NMR and mass spectrometry (MS) as well as two-dimensional $^{1}H$-$^{13}C$ NMR techniques to characterize in more detail with respect to the formulation of measurement equations. Based on these measurement equations, a statistically optimal flux estimator was established. Having implemented these tools, different kinds of labeling experiments were compared using statistical quality measures.

Although many applications have been reported for the flux analysis using NMR, GC-MS is also quite useful and has several advantages over NMR such that GC-MS require relatively small amount of samples as compared with NMR, and GC-MS gives rapid analysis etc. In GC-MS, the compounds are separated by the gas chromatography, and the mass spectrometry step analyzes the labeling patterns of the compounds as they elute. The mass spectrum of a compound usually contains ions that are produced by fragmentation of the molecular ion (*i.e.*, the ionized intact molecule). These fragments contain different subsets of the original carbon skelton, and the mass isotopomer distributions of these fragments contain information that can, in addition to the information from the molecular ion, be used for analyzing the labeling pattern of the metabolites [38]. Christiensen and Nielsen [49] quantified the intracellular fluxes of Penicillium chrisogenum using GC-MS. They found that glycine was synthesized not only by serine hydroxymethyltransferase, but also by threonine aldorase. The formation of cytosolic acetyl-CoA was also found to be synthesized both via the citrate lyase-catalysed reaction and by degradation of the penicillin side-chain precursor, phenoxyacetic acid.

We recently used both $^{1}H$-$^{13}C$ 2D NMR and GC-MS to quantify the flux distributions of cyanobacteria at different culture conditions such as autotrophic, mixotrophic, and heterotrophic conditions, and obtained some insight into the metabolic regulation with respect to culture environment [50]. The fractionally $^{13}C$-labeled *Synechocystis* sp. PCC6803 was harvested from the culture, subjected to hydrolysis, and the labeling patterns of the amino acids in the hydrolysate were analyzed using two-dimensional NMR spectroscopy and GC-MS. For GC-MS, the mass spectrum of a compound can contain ions that are produced by fragmentation of the molecular ion (*i.e.*, the ionized intact molecule). The fragment ions contain different subsets of the original carbon skeleton, thus the mass isotopomer distributions of these fragments can provide additional information to analyze the labeling pattern of the metabolite. Since the substrate uptake and the biomass effluxes were directly measured, the metabolite balances leave two degrees of freedom for net fluxes. As the free net fluxes, we have chosen the flux of $CO_2$ fixation in Calvin cycle ($rbc^{net}$) and the gluconeogenic flux of malic enzyme ($me^{net}$). The set of free fluxes is completed by all exchange coefficients of reaction steps that are assumed to be bidirectional based on thermodynamic considerations. The estimation of free net fluxes and exchange coefficients was then performed with the least squares fitting approach described previously.

The result shows that the flux of $CO_2$ reduction through the Calvin cycle was 211.4% of the glucose input flux. The reaction mediated by the fructose-1,6-bisphosphatase was found to be present in *Synechocystis* grown mixotrophically, demonstrating the presence of an ATP-dissipating futile cycle via ATP-consuming phosphofructokinase and fructose-1,6-bisphosphatase. The $CO_2$ fixation through the phosphoenolpyruvate carboxylase was 73.4%, which represented about 25% of the assimilated $CO_2$. The reaction catalyzed by the malic enzyme was identified by the labeling experiments, and the backward flux from the TCA cycle to glycolysis was found to be 84.6%. This explains the significant increase of C2-C3 carbon bond cleavage in pyruvate when compared to the conservation of C2-C3 connectivity in PEP. High exchange rates in the glucose-6-phosphate isomerase, ribose-5-phosphate isomerase, glyceraldehyde-3-phosphate to phosphoenolpyruvate conversions were found, and the phosphoenolpyruvate synthase were identified to be inactive during growth mixotrophically.

The statistical analysis of the estimated intracellular flux distributions was performed to check the reliability of the flux estimates and investigate the sensitivity of the estimated parameter values with respect to the measurement inaccuracies. A total of 500 data sets were generated by addition of normally distributed measurement noise to the estimated isotopomer labeling. The standard deviations in the measurement data were computed from multiple measurements, from redundant labeling measurements, and from the signal-to-noise ratio. From these 500 data sets, the same parameter estimation procedure as was used for the estimation of the best fit flux distribution was applied to estimate 500 flux distributions. From the probability distribution of the 500 flux distributions, confidence regions for the individual flux estimates can then be computed.

All free net fluxes were well determined from the measured data with small confidence regions. On the other hand, relatively large confidence intervals were found for the exchange coefficients. This indicates that the data do not supply the sufficient information for the quantification of these parameters, since the exchange coefficients of these reactions have no or only an insignificant influence on the value of the objective function in the error minimization procedure.

In conclusion, it was shown that the combination of the information available from such isotopomer measurements as GC-MS and two dimensional NMR spec-

troscopy with the information obtained from metabolite balancing enabled a refined analysis of the metabolic fluxes in a detailed metabolic network.

## SYNTHESIS OF METABOLIC PATHWAYS AND OPTIMIZATION OF METABOLIC FLUX DISTRIBUTION

The synthesis of metabolic pathways involves the construction of pathways, *i.e.*, sets of enzyme-catalyzed bioreactions whose stoichiometry is given, to meet certain specifications. Systematic synthesis of pathways that satisfy a set of specifications is relevant in the early steps of the conception and design of a bioprocess, where a pathway must be chosen for the production of the desired product. The first effort for systematic synthesis of metabolic pathways was made by Seressiotis and Bailey [51]. They presented an approach for synthesizing pathways that start from a given substrate and produce a target product with calculation of yield etc.

Mavrovouniotis *et al.* [52] developed a computer-aided synthesis method of metabolic pathways, where the algorithm satisfies each stoichiometric constraint by recursively transforming a base-set of pathways. They applied the algorithm to the problem of lysine synthesis from glucose and ammonia and discovered that the yield of lysine over glucose cannot exceed 67% in the absence of enzymatic recovery of carbon dioxide.

Another approach is to find the optimal flux distribution based on known metabolic network structure and the objective function such as yield etc. We considered this problem for PHB production using recombinant *E. coli*. The predicted flux distributions obtained by the application of linear programming indicate that in order to achieve the maximum PHB yield, about half of the carbon flow should be metabolized via the pentose-phosphate (PP) pathway and the flux to TCA cycle should be shut down. This is easy to understand since there are two pathways significantly affect the availability of two substrates for PHB synthesis, NADPH and acetyl-CoA. The predicted flux from glucose 6-phosphate to the PP pathway was, however, 46% which is much higher than the normal value [53]. Based on our analysis, the metabolic pathway that metabolizes glucose through part of the PP pathway and the Entner-Doudoroff (ED) pathway may be the best pathway for a metabolic variant of *E. coli* to overproduce PHB [54]. This kind of analysis gives the upper bound for the yield etc. and may give the motivation on whether further effort should be made or not.

## MODIFICATION OF METABOLIC PATHWAYS

### Gene-engineering Technique

Consider lactic acid fermentation as an example of modifying metabolic pathways by gene-engineering technique. In lactic acid fermentation, lactic acid production increasingly inhibits cellular metabolic activities. Several processes have, therefore, been developed to remove the lactic acid *in situ*. Among them, extractive fermentation has recently been paid most attention. The problem with this process, however, is that only undissociated lactate is extracted. Since in lactic acid fermentation, the culture pH is generally maintained at around 6-7, the direct application of extractive fermentation yields very low productivity due to large fraction of dissociated lactate at such pH values.

We, therefore, considered to modify the metabolism of *Saccharomyces cerevisiae* by expressing the lactate dehydrogenase (LDH) gene to produce a relevant amount of lactic acid at low pH. Using this recombinant *S. cerevisiae* strain, several fermentations were conducted at several pH values (4.5-3.5). Since the recombinant *S. cerevisiae* produced a considerable amount of ethanol as well as lactate (about 10 g/L), we then disrupted several pyruvate decarboxylase (PDC) genes to suppress the ethanol formation. Among the *PDC* genes, *PDC1*, *PDC5*, and *PDC6*, *PDC1* showed the greatest effect on the cell growth and ethanol production. The plasmid which contains the *LDH-A* structure gene was then transformed into the mutant strain lacking the *PDC1* gene. Cultivation of this strain improved the lactate yield from glucose while suppressing ethanol formation to some extent [55].

Those results indicate that the metabolic pathway modification can be made by gene-engineering technique locally, but it is not an easy task to modify metabolic flux distribution to the desired one.

### Control of Culture Environment

Another means of modifying the metabolic flux distribution is to manipulate the culture environment. We considered this problem for pyruvate fermentation. There is an increasing demand for pyruvic acid since it is an important raw material for the production of many amino acids such as tryptophan and tyrosine, and for the synthesis of many drugs and agrochemicals. Only a small amount of pyruvate is, however, secreted into the culture broth from wild-type microorganisms. Some auxotrophic strains, such as thiamine auxotrophs or lipoic acid auxotrophs have been utilized for pyruvate overproduction.

We investigated the metabolism and fermentation characteristics of *Torulopsis glabrata* IFO 0005, a vitamin-auxotrophic pyruvate-producing yeast screened by Yonehara and Miyata [56]. The strain used is auxotrophic for four vitamins such as thiamine hydrochloride, nicotinic acid (NA), biotin and pyridoxine hydrochloride. Since dissolved oxygen (DO) is an important environmental factor which affects the tricarboxylic acid (TCA) cycle activity, and thiamin hydrochrolide plays a key role in the strain [57], attention was focused on analyzing the effects of these culture conditions on the cellular metabolism of this strain. Several batch experi-

ments were conducted at different DO concentrations and metabolic flux distributions were computed for each experiment. The point in this research is to control the specific pathway pinpoint by manipulating culture environment and by addition of vitamins [58-60].

## METABOLIC CONTROL ANALYSIS

The concept of metabolic flux analysis is useful for quantification of flux distribution, but it does not allow for evaluation of how the fluxes are controlled. One of the most important aspects of metabolic engineering may be control of flux. The main objective is to find rate-limiting step and bottleneck enzyme. Delgado and Liao [61] proposed an idea of inverse flux analysis which allows the prediction of the flux distribution when some of the manipulable fluxes were perturbed. The application of this method to *E. coli* suggested that the increase in the flux of the anaplerotic pathways, indicating the reactions catalyzed by phosphoenol pyruvate carboxylase and the glyoxylate bypass will decrease acetate production while increasing the growth yield.

The concept of metabolic control analysis (MCA) was developed by Kacser and Burns [62] and Heinrich and Rapoport [63]. Several reviews have been made with some extensions [7,64,65]. In MCA, several kinds of coefficients play important roles. Elasticity coefficient (EC) is defined as

$$\varepsilon_k^i = \frac{dv_i}{dx_k}\frac{x_k}{v_i} = \frac{d \ln v_i}{d \ln x_k} \tag{22}$$

where $v_i$ is the i-th reaction rate and $x_k$ a k-th variable that modifies the rate. This coefficient is modulated by metabolites, which is of practical concern in enzyme regulation. If parameter $p_k$ is used instead of $x_k$, it may be called as π-elasticity [66] instead of ε-elasticity.

Flux control coefficient (FCC) is defined [62] as

$$C_i^{Jk} = \frac{dJ_k}{de_i}\frac{e_i}{J_k} = \frac{d \ln J_k}{d \ln e_i} \tag{23}$$

where FCCs express the fractional change in the steady state flux through the pathway ($J_k$) that results from an infinitesimal change in the activity of enzymes (or reaction rates). It should be noted that whereas the elasticity coefficients are properties of the individual enzymes, the FCCs are properties of the system. The FCCs are, therefore, not fixed but change with the environmental conditions [5].

Concentration control coefficient (CCC) is defined as

$$C_i^k = \frac{dx_k}{de_i}\frac{e_i}{x_k} = \frac{d \ln x_k}{d \ln e_i} \tag{24}$$

We applied MCA for lysine fermentation. We studied how FCC changed as fermentation proceeds. It was found that the bottleneck enzyme changes: aspartokinase during lysine formation phase while permease at the late stage fermentation. We could increase the lysine production rate by increasing those enzyme activities by constructing gene-engineered *C. glutamucum* [67,68]. Nielsen and Jorgensen [69] have made the similar analysis for penicillin biosynthesis using *Penicillium chrisogenum*.

Although MCA approach has been enthusiastically employed by many researchers [70], the application to practical experimental systems is quite limited. Liao and Delgado [7] pinpointed the gaps still remaining between mathematical treatment and experimental implementations.

Stephanopoulos and Simpson [71] proposed a means for calculating group control coefficients as measures of the control exercised by groups of reactions on the overall network fluxes and intracellular metabolites pools. The concepts of this method were illustrated through the simulation of a case study involving aromatic amino-acid biosynthetic pathway. It was further demonstrated that the optimal strategy for the effective increase of network fluxes was through the coordinated amplification of a small number of steps in order to maintain maximum throughput while ensuring an uninterrupted supply of intermediate metabolites. Simpson *et al.* [72] also proposed a method of determining group flux control coefficients based on many experimental data without using mathematical models.

Dynamic extension of metabolic control analysis has been made with dynamic model for the metabolic change on the order of minutes [73-75].

MCA is not a modeling framework, but it is a set of postulates that allows the systematic computation of network sensitivities to single perturbations in the environmental or network parameters. Thus its predictive capability is limited by the quality of the model employed. Since traditional kinetic models lack the description of the regulatory component, the sensitivity coefficients for MCA is suspect. The cybernetic framework developed by Ramkrishna and his co-workers may offer some advantage over conventional methodologies [76,77]. The framework hypothesizes that metabolic systems have evolved optimal goal oriented strategies as a result of evolutionary processes. The inclusion of a goal-oriented regulatory strategy gives the cybernetic description of a metabolic network, the key feature of regulatory responsiveness, an element that is missing from many other contemporary metabolic network analysis and modeling frameworks.

As stated above, MCA is based on information of the kinetics of individual reactions. The lack of *in vivo* kinetic information on the individual pathway reactions are limiting a widespread use of this concept. To overcome such a problem, there are yet alternative approaches based on thermodynamic considerations. The relevant thermodynamic variable for a thermodynamic feasibility analysis is the Gibb's free energy of a reaction (ΔG). A strict requirement for flow of carbon through cellular pathways is that ΔG is negative for all individual reactions. Mavrovouniotis [78,79] used this concept in order to develop a procedure that seeks the range of metabolic concentrations where all the reactions are feasible ($\Delta G < 0$). This procedure is based only on the

knowledge of the value of $\Delta G^{0'}$, the standard Gibbs free energy and the concentration of cofactors. Pissara and Nielson [80] applied the thermodynamic feasibility algorithm of Mavrovouniotis [78,79] to the α-aminoadipic acid pathway in *P. chrysogenum*. Thereafter they considered the penicillin biosynthetic pathway, for which they calculated the standard Gibbs free energies using the concept of group contributions [78], and they used measurements of the pathway metabolite concentrations to calculate the ΔG values for the reactions during a fed-batch cultivation.

## DESIGN OF METABOLIC REGULATORY STRUCTURES

It has recently been shown that genetic modification of metabolic control systems can significantly enhance the process performance. However, past modifications of enzyme properties in metabolic system were usually based on trial and error methods, which becomes ineffective when the system becomes large. Since the recent genetic engineering techniques allow modifications of both gene-level (expression of genes) and protein-level (activity of enzymes) regulations, it is desired to develop a useful method for finding the optimal metabolic structure. However, the optimization techniques for metabolic system design are not well established now. One metabolic optimization method is to maximize the objective metabolic flux based on the stoichiometric information of a metabolic system. This approach may provide the optimal flux distributions for individual pathways of a metabolic network. However, it does not suggest the effects of the modification of metabolic regulatory structure, since no kinetics are considered in this method. Another conventionally used optimization method is the utilization of the kinetic model deried by the S-system formalism [81,82]. This approach does not also address the effect of the change in regulatory structure, although the optimum manipulation of external inputs can be provided [83-85]. The only way to find the optimal regulatory structures for a metabolic network may be to use the kinetic model obtained from enzymatic reaction mechanism. Since the mechanism-based kinetic expressions are usually nonlinear, and many data are required for the optimization of nonlinear system, a (log)linearized model formulation has been developed by Hatzimanikatis *et al.* [86]. This type of (log)linearized model has been verified to approximate the original nonlinear model much better than the general linearized model [87]. By constructing a set of regulatory structures in which every metabolite is considered to be capable of regulating any enzyme in the system, and by considering every possible combination, the optimization can be carried out effectively for the nonlinear systems, and the optimal regulatory structures obtained can give the direction on how genetic engineering will be applied.

We attempted to find the optimal metabolic regulation structure by applying the mixed integer linear programming method to the log (linearized) model for the lysine synthetic pathway. The results indicate that more than 20% increase of internal lysine flux can be obtained when only the inhibitory regulation was allowed, and eight optimal structures with one regulatory loop were adopted. When regulation of enzyme activation was allowed, internal lysine flux can be increased by more than 70%. Changes of participating precursor and cofactor concentrations may not improve lysine flux significantly in this system [88].

## IDENTIFICATION OF UNKNOWN PATHWAYS

Historically, the isotopomer has been used to find the unknown pathways. However, the method itself is not systematic. It is expected that the metabolic synthesis methodology be extended for identifying unknown pathways.

When propionibacteria are grown under anaerobic condition, propionate, acetate, and $CO_2$ are produced through the randomizing pathway. Moreover, vitamin $B_{12}$ is accumulated intracellularly under anaerobic condition. The accumulation of propionate causes strong inhibition on cell growth as well as vitamin $B_{12}$ synthesis. In contrast, if cultivation was switched from anaerobic to aerobic, the propionate and vitamin $B_{12}$ ceased to be produced while acetate continued to be produced. Noting the experimental fact that the propionate accumulated during anaerobic cultivation was decomposed when the cultivation was shifted to an aerobic condition, we considered to change the DO concentration periodically between 0 ppm and 1 ppm to keep the propionate concentration at low level and thus to show the improvement of vitamin $B_{12}$ productivity [89].

Although the metabolic pathway of propionibacterium grown under anaerobic condition has been well investigated, its pathway under aerobic condition is not fully investigated yet. The randomizing pathway may function in a reversed direction in the presence of oxygen, through which the propionate is oxidized. We analyzed this based on $^{13}C$ experiments [90].

## FUTURE PERSPECTIVES

Recent rapid progress in molecular biology unveiled the intricacies and mechanistic details of genetic information transfer and determined the structure of DNA and the nature of the gene code, establishing DNA as the source of heredity containing the blueprints form which organisms are built. These scientific revolutionary endeavors have rapidly spawned the development of new technologies and emerging fields of research based around genome sequencing efforts (*i.e.*, functional genomics, structural genomics, proteomics and bioinfomatics) [91].

Once presented with the sequence of a genome, the

first step is to identify the location and size of genes and their open reading frames (ORF's). DNA sequence data then need to be translated into functional information, both in terms of the biochemical function of individual genes, as well as their systematic role in the operation of multigenic functions.

Genomic technologies can be broadly divided into two groups, namely those that alter gene structure and deduce information from altered gene function and those that observe the behavior of intact genes [92]. The former methods involve the random or systematic alteration of genes across an organism's genome to obtain functional information. Current genome-altering technologies are distinguished by their strategies for mutagenizing and analyzing cell populations in a genome-wide manner.

For the latter, several methods have been developed to efficiently monitor the behavior of thousands of intact genes. DNA chips are tools that fractionate a heterogeneous DNA mixture into unknown components, while a complementary method, called SAGE (Serial Analysis of Gene Expression), has been important for identifying transcripts not predicted by sequence information alone [93]. The two commonly available DNA chips are oligonucleatide chips and DNA microarrays. A primary technical difference between oligonucleotide chips and DNA microarrays is the size of their DNA targets. DeRishi *et al.* [94] showed how the metabolic and genetic control of gene expression could be studied on a genome scale using DNA microarray technology. The temporal changes in genetic expression profiles that occur during the diauxic shift in *S. cerevisiae* were observed for every known expressed sequence tag in this genome.

One of the key issues in functional genomics is to relate linear sequence information to nonlinear cellular dynamics. Toward this end, significant scientific effort and resources are directed at mRNA expression monitoring methods and analysis, and at the same time the field of proteomics (the simultaneous analysis of total gene expression at the protein level) represents one of the premiere strategies for understanding the relationship between various expressed genes and gene products [95].

From annotated genomes, we can directly construct the stoichiometric matrix for the entire metabolic network of an organism, which allows us to determine metabolic pathways. The genome-specific stoichimetric matrix can be obtained for any recently sequenced and annotated genome, and it may be used to synthesize *in silico* organisms. With this thinking, Palsson and his coworkers [91,96] constructed an *in silico* strain of *E. coli* K-12 from annotated sequence data and from biochemical information. Using this *in silico* microorganism, one can study the relation between *E. coli* metabolic genotype and phenotype in the *in silico* knockout study [96]. At the heart of this persective is the study of the system as a whole rather than the detailed study of individual components and their direct interactions.

It should be noted that it is important to understand the regulation mechanism of gene and protein expressions as well as metabolic regulation. Recently, we investigated how culture environments affect those regulations for *Synechocystis* using RT-PCR and 2-dimensional electrophoresis as well as NMR and GC-MS [97]. We found that many genes are differentially regulated according to different mechanism, and the regulation of metabolic fluxes may be exerted at the transcriptional, post-transcriptional, translational, post-translational, and metabolic levels. Although at present, the transcriptomics, proteomics, and metabolic flux analysis allow high-throughput analysis of gene expression profiles, each of these techniques has its own advantages and limitation,, and only their integration may provide us with a detailed gene expression phenotype at each level and allow us to tackle the great complexity underlying biological processes.

Finally, it should be stated that a fusion of concepts from biological and nonbiological disciplines, including mathematics, computer science, physics, chemistry and engineering is required to address the theoretical and experimental challenges facing the field of genomics, and together promise great breakthroughs in our understanding and engineering cellular systems [92].

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