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Metabolic Engineering for Systematic Organization and Analysis of Complex Metabolic Networks

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Abstract: Metabolic pathway analysis is increasingly promising for assessing inherent network properties in biochemical reaction networks. Metabolic pathways are series of chemical reactions occurring within a cell. Pathways are the reaction sets linked by having the product of one reaction be the reactant of the next reaction in the chain. Enzymes catalyze these reactions, and often require dietary minerals, vitamins, and other cofactors in order to function properly. The collection of pathways is called metabolic network. A challenging task for the future is the calculation and study of the complete set of pathways at a genomic scale, and its combination with cellular regulation to obtain the whole picture. Metabolic engineering strives to use this knowledge to manipulate metabolic reaction networks in order to achieve some objectives of complex biochemical reaction networks. The ultimate goal of metabolic engineering is to be able to produce valuable substances on reaction networks in a cost effective manner. Various metabolic engineering strategies have been widely applied for the more efficient production of desired metabolites and biomolecules. In this paper, we demonstrate some methodologies have been developed to describe for systematic organization and to analyze the metabolic behavior (networks) of an organism or a living cell depending on the goals of the metabolic pathway analysis to understanding the complex metabolic network.

Key words: Metabolic Engineering, Metabolic Flux Analysis, Flux Balance Analysis, ^{13}C-Metabolic Flux Analysis, Elementary Mode Analysis, Extreme Pathway Analysis.

1. Introduction

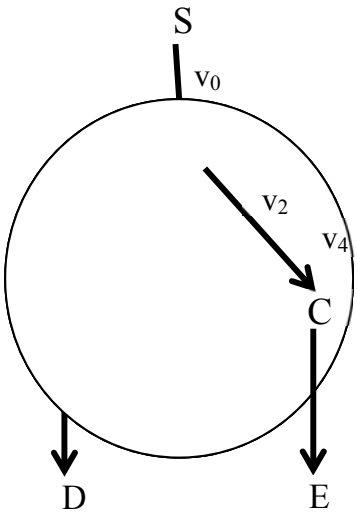
A cell is a complex system composed of a large number of molecular components, including nucleic acids, proteins, and metabolites (Caspi et al., 2010). Metabolic pathway analysis is the discovery and analysis of meaningful routes in metabolic networks. Metabolic pathway is a series of chemical reactions occurring within a cell, catalyzed by enzymes, and resulting in either the formation of a metabolic product to be used or stored by the cell or the initiation of another metabolic pathway. Intuitively, a pathway should be a set of connected reactions; it is rather more complicated to give an exact definition of 'meaningful', which should cover physiological as well as biotechnological aspects (Klamt and Stelling 2003). Pathway analysis undoubtedly has great potential for biotechnology and metabolic engineering. It helps us gain a better understanding of the cellular metabolism and to find possible targets for manipulation in complex metabolic networks. First attempts to analyze the pathway structure in complex networks, partially by subdividing them, reveal interesting results (e.g. on pathway redundancy) that are useful for comparing different networks. Cellular metabolism is most often described and interpreted in terms of the biochemical reactions that make up the metabolic network. The underlying pathway structure that is determined from the set of extreme pathways now provides us with the ability to analyze, interpret, and perhaps predict metabolic function from a pathway-based perspective in addition to the traditional reaction based perspective. Metabolism is broadly defined as the complex of physical and chemical processes involved in the maintenance of life. It is comprised of a vast repertoire of enzymatic reactions and transport processes used to convert thousands of organic compounds into the various molecules necessary to support cellular life. Metabolic engineering specifically seeks to mathematically model these networks, calculate a yield of useful products, and pin point parts of the network that constrain the production of these products(Yang et al., 1998).

2. Metabolic Engineering for Complex Metabolic Networks

In the past, to increase the productivity of a desired metabolite, a microorganism was genetically modified by chemically induced mutation, and the mutant strain that overexpressed the desired metabolite was then chosen. However, one of the main problem with this technique was that the metabolic pathway affecting the metabolite production was not analyzed, and as a result, constraints to production and relevant pathway enzymes to be modified were unknown (Voit and Torres 2002). In 1990s, a new technique called metabolic engineering emerged. Metabolic engineering is the directed improvement of product formation or cellular properties through the modification of specific biochemical reactions or the introduction of new ones with the use of recombinant DNA technology (Stephanopoulos et al., 1998). Some examples of successful metabolic engineering are the following: (i) Identification of constraints to lysine production in corynebacterium*glutamicum*and insertion of new genes to relieve these constraints to improve production (Stephanopoulos et al., 1998). (ii) Engineering of a new fatty acid biosynthesis pathway, called reversed beta oxidation pathway which can potentially be catalytically converted to chemicals and fuels (Dellomonaco 2011) (iii) Improved production of DAHP (3-deoxy-D-arabino-heptulosonate 7-phosphate), an aromatic metabolite produced by *E.coli*that is an intermediate in the production of aromatic amino acids (Patnaik and Liao1994). In general, metabolic pathway analysis identifies the topology of cellular metabolism based on only the stoichiometric structure and thermodynamic constraints of reactions where kinetic parameters are not explicitly revealed and/or required for the calculations. A variety of biological data from all levels of metabolism, from genome to metabolome, are combined in order to view the studied organism as a whole rather than investigating the single components of the system (Kesson et al., 2004). A metabolic flux vector, also known as metabolic flux distribution, defines cellular phenotypes under a given growth condition. Several mathematical modeling frameworks have been developed to describe and to analyze the metabolic behavior of an organism or a living cell depending on the goals of the analysis, these tools can be grouped into three categories including (i) Metabolic Flux Analysis (Stephanopoulos et al. 1998)(ii) Flux Balance Analysis (Price et al. 2004) and (iii) Metabolic Pathway Analysis (Klamt and Stelling 2003).

3. Metabolic Flux Analysis

Metabolic flux analysis (MFA) is a powerful and essential tool for the determination of metabolic pathway fluxes. In this approach, the intracellular fluxes are calculated by using a stoichiometric model for the major intracellular reactions and applying mass balances around intracellular metabolites. Consider a simple example reaction network occurring in a constant volume cell shown in the following diagram:



To our current example, suppose the reaction network follows the following reactions

Reaction 0 : $S \xrightarrow{v_0} A$

Reaction 1 : $A \xrightarrow{v_1} B$

Reaction 2 : $A \xrightarrow{v_2} C$

Reaction 3 : $B \xrightarrow{v_3} D$

Reaction 4 : $C \xrightarrow{v_4} E$

Fig.1: Example pathways of stoichiometric modeling. The circle represents the system boundary. Where A, B and C are the intracellular metabolites, and S, D and E are the extracellular metabolites.

In the above reaction network, S is changed into A (this may consider as the intake of substrate from outside of the cell), A is converted to B and C through reaction v_1 and v_2, B is converted to D by reaction v_3, and C is converted to E through reaction v_4. Therefore, the mas balance equation for intracellular metabolites A, B and C can be written as a matrix form as follows:

$$\frac{d}{dt}\begin{bmatrix} A \\ B \\ C \end{bmatrix} = \begin{bmatrix} 1 & -1 & -1 & 0 & 0 \\ 0 & 1 & 0 & -1 & 0 \\ 0 & 0 & 1 & 0 & -1 \end{bmatrix} \begin{bmatrix} v_O \\ v_1 \\ v_2 \\ v_3 \\ v_4 \end{bmatrix} = 0 \Rightarrow \mathbf{SV} = 0 \qquad (1)$$

Where **S** is the stoichiometric matrix and **V** is the reaction rector or flux vector. We illustrate the procedures used for the calculation of MFA using the simple example pathway shown in **Fig.1**. In the case where v_0 and v_3 are measurable (known) in the intracellular metabolites A, B and C, the stoichiometric matrix can be separated into known and unknown parts:

$$\begin{bmatrix} 1 & 0 \\ 0 & -1 \\ 0 & 0 \end{bmatrix} \begin{bmatrix} v_O \\ v_3 \end{bmatrix} + \begin{bmatrix} -1 & -1 & 0 \\ 1 & 0 & 0 \\ 0 & 1 & 0 \end{bmatrix} \begin{bmatrix} v_1 \\ v_2 \\ v_4 \end{bmatrix} = 0 \qquad (2)$$

By moving the known part to the right side of the equation and multiplying by the inverse of the unknown part of the stoichiometric matrix on both sides of the equation, the unknown fluxes (v_1, v_2, and v_4) can be expressed as a function of the measurable (known) fluxes (v_0 and v_3):

$$\begin{bmatrix} v_1 \\ v_2 \\ v_4 \end{bmatrix} = -\begin{bmatrix} -1 & -1 & 0 \\ 1 & 0 & 0 \\ 0 & 1 & 0 \end{bmatrix}^{-1} \begin{bmatrix} 1 & 0 \\ 0 & -1 \\ 0 & 0 \end{bmatrix} \begin{bmatrix} v_O \\ v_3 \end{bmatrix} \qquad (3)$$

At this stage, the unknown flux vector can, in principle, be solved using the stoichiometry and the known measurable fluxes. However, before actually solving for the fluxes, we must consider the degrees of freedom of the network (Stephanopoulos et al., 1998).The number of degrees of freedom is the number of independent fluxes and is calculated as follows:

$$d=n-k-m \qquad (4)$$

where d is the degrees of freedom, n is the number of fluxes, k is the number of constraints, and m is the number of measurable fluxes. If the number of degrees of freedom is 0 (a "determined system"), the fluxes are determined as a unique solution; that is, the solution is the intersection of the lines which represent constraints. Furthermore, if the number of degrees of freedom is less than 0 (an "overdetermined system"), then the minimum norm and least-squares solution can be calculated using the Moore-Penrose pseudo inverse method (Penrose, 1955 and Stephanopoulos et al., 1998). However, if the number of degrees of freedom is greater than 0 (an "underdetermined system"), then immense solutions exist because of the lack of constraints. Therefore, we can determine the unknown fluxes using MFA quite easily when the system is either determined or overdetermined. Underdetermined systems require more constraints to reach a particular confined solution.

The primary challenge in the use of MFA is that many biological networks are underdetermined systems. To overcome this problem we can apply Flux Balance Analysis (FBA) and ^{13}C-Metabolic Flux Analysis (^{13}C-MFA), both of which are frequently used to solve such underdetermined systems. There are two major approaches to applying MFA to such underdetermined systems: (1) prediction of flux distribution based on an objective function by flux balance analysis (FBA) and (2) the monitoring of flux distribution by MFA employing a ^{13}C-labeling technique, alternatively known as ^{13}C-metabolic flux analysis.

4. Flux Balance Analysis

Flux Balance Analysis (FBA) is a constraint-based approach used to predict the (quasi-) steady state fluxes by applying mass balance constraints and objective functions (Varma and Palsson 1994; Feist and Palsson, 2010). To predict fluxes in the exponential growth phase, maximization of the biomass yield orAdenosine-5'-triphosphate (ATP) yield is frequently used as the objective function (Van Gulik and Heijnen, 1995). We can evaluate the maximum yield of the specific compound using maximization of the target production rate as the objective function. Because FBA can be performed from the network information alone and without the enzyme kinetics, many FBA studies use genome-scale metabolic pathways rather than a small pathway alone, such as the central carbon metabolism pathway. To predict metabolic fluxes using FBA, a stoichiometric model is first constructed, as in general MFA. The solution space is then limited by the addition of constraints, such as the upper or lower bounds of each flux, and a unique flux distribution is then predicted by applying an objective function. Because a linear objective function is generally used, the flux distribution that maximizes or minimizes the objective value can be solved by linear programming.

$$\text{maximize} Z = \sum_{k=1}^{n} c_k^T \cdot v_k$$

$$s.t \qquad \sum_{k=1}^{n} S_{jk} \cdot v_k = 0 \quad ; \quad v_k^{lb} < v_k < v_k^{ub} \tag{5}$$

Where c_k is a weight coefficient for flux v_k, and the superscripts *lb* and *ub* represent lower and upper boundaries, respectively. Although FBA is a powerful method of solving underdetermined systems, the choice of an appropriate objective function can be subjective and requires careful consideration. While the maximization of biomass yield is frequently used as the objective function in many studies, it is not certain whether a single objective function can be universally applicable, especially for gene knockout mutants (Toya et al., 2010). To solve this problem, advanced FBA have been proposed. Minimization of metabolic adjustment (MOMA)(Segre et al., 2002) and regulatory on/off minimization (ROOM)(Shlomi et al., 2005) are such methods that can be used for gene knockout mutants, building from a wild type solution obtained previously by FBA. However, it should be noted that these methods are based on some assumptions. How, then, can more reliable fluxes be obtained? In this context, we introduce another method that employs a ^{13}C-labeling technique to measure the metabolic fluxes indirectly known as ^{13}C-Metabolic Flux Analysis (^{13}C-MFA).

5. ^{13}C-Metabolic Flux Analysis

^{13}C-Metabolic Flux Analysis (^{13}C-MFA) is frequently used when the systems of stoichiometric modeling are underdetermined (lack of constraints). Carbons atoms are naturally found in 3 forms; ^{12}C with 6 protons and 6 neutrons, ^{13}C with 7 neutrons and ^{14}C with 8 neutrons exist in trace amounts. ^{13}C is useful in MFA, because it can be distinguished from ^{12}C through techniques such as nuclear magnetic resonance (NMR) spectroscopy and mass spectrometry (MS) (Shimizu, 2004). A typical ^{13}C-MFA procedure consists of the following steps: (1) determine the analytical pathways and the substrate-labeling pattern from the prospective metabolism (2) conduct a ^{13}C-labeling experiment under the steady state condition, and measure the labeling patterns of proteinogenic amino acids using gas chromatography-mass spectrometry (GC-MS) and/or nuclear magnetic resonance (NMR) spectroscopy (Shimizu, 2004); (3) construct a stoichiometric model and the isotopomer balance equations for intracellular metabolites based on the network determined in step 1 and (4) optimize the flux distribution by determining the labeling patterns of proteinogenic amino acids computed from the assumed fluxes in step 3 as the best fit to the experimental data obtained in step 2 (Zhao and Shimizu 2003). Several methods have been proposed to express the isotope labeling pattern of compounds, including the isotopomer method (Schmidt et al., 1997) and the elementary metabolite units method (Antoniewicz et al., 2007). In this section, we describe the isotopomer method using a simple example (**Fig.2**) pathway (Toya, et al., 2011). The pathway consists of two reactions (v_1 and v_2), that metabolizes two substrates (S_1 and S_2) to the same product (P) via the same intermediate (X). In this model, the ^{13}C-labeling pattern of each compound is expressed as an isotopomer distribution vector (IDV). Each IDV contains the molar fractions of the 2^nisotopomers and the sum of each vector is 1.

We show the IDVs of S_1, S_2, and X, and P (the subscripts 0 and 1 represent ^{12}C and ^{13}C atoms, respectively). Then, the transition of isotopomers by reaction is expressed as an isotopomer mapping matrix (IMM). The IMMs from S_1 to X and from S_2 to X are shown here, where $IMM_{A\rightarrow B}$ represents the IMM from metabolite A to B. The column and row in an IMM correspond to the vector elements in the substrate IDV and product IDV, respectively. Each element in the IMM expresses the possibility of isotopomer transition by the reaction as 0 (not possible) or 1 (possible). For example, the element of $IMM_{S1\rightarrow X}$ in the third row and second column indicates the transition of the first labeled S_1 to the first labeled X. Because this transition is possible, the element is valued at 1. Then, the time-derivative of each intermediate IDV (isotopomer balance equation) is expressed using the fluxes, the IDVs, and the IMMs.

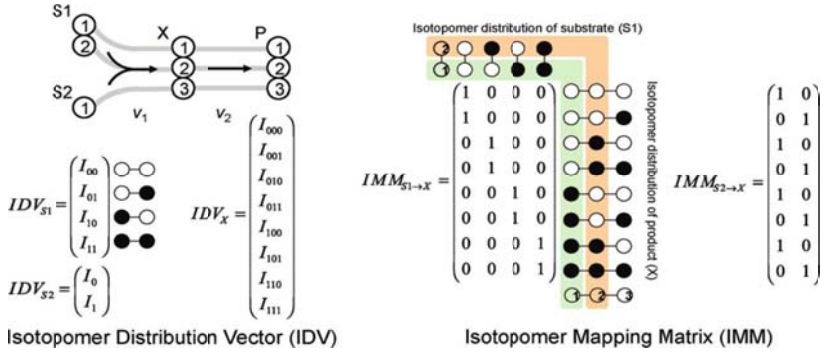


Fig.2: Isotopomer modeling of the metabolic network for ^{13}C-MFA.Blank circles represent ^{12} C atoms and dark circles represent ^{13}C atoms.

6. Metabolic Pathway Analysis

Metabolic pathway analysis is important for assessing network properties in biochemical reaction networks. It has two promisingways: elementary modes and extreme pathways.Elementary mode (EM) analysis (Schuster and Hilgetag 1994) is one of the most popular and essential techniques in metabolic pathway analysis of metabolic networks. EM analysis is potentially effective in integrating transcriptome or proteome data into metabolic network analyses and in exploring the mechanism of how phenotypic or metabolic flux distribution is changed with respect to environmental and genetic perturbations (Zhao and kurata 2010). A quantitative measure of fluxes carried by individual elementary modes is of great help to identify dominant metabolic processes, and to understand how these processes are redistributed in biological cells in response to changes in environmental conditions, enzyme kinetics, or chemical concentrations. Biological networks can be represented by a stoichiometric matrix (**S**). The rows of **S** correspond to metabolites in a reaction network. The columns of **S** correspond to the reactions in a network, with elements corresponding to stoichiometric coefficients of the associated reactions (Kurata et al., 2007). At steady-state, mass balance provides the flux-balance equations defined as before in equation (1) rewritten as:

$$\mathbf{SV}=0,$$

where$\mathbf{V} = (v_1, v_2, ..., v_n)^t$is the vector whose elements correspond to fluxes through the associated reactions in **S**. The set of all possible solutions can be described by a set of basis vectors. The elementary mode (EM) is the minimal set of enzymes that can operate at steady-state with all the irreversible reactions operating properly. The elementary mode matrix (**P**) is uniquely determined from the stoichiometric matrix and the flux vector is provided by:

$$\mathbf{V} = \mathbf{P}\cdot\boldsymbol{\lambda}, \quad (6)$$

where$\boldsymbol{\lambda} = (\lambda_1, \lambda_2, ..., \lambda_m)^t$is the elementary mode coefficient (EMC) vector, *n* is the number of reactions, and *m* is the number of EMs. The ingredients of these vectors and matrix are displayed as:

$$\begin{bmatrix} v_1 \\ v_2 \\ . \\ . \\ . \\ v_n \end{bmatrix} = \begin{bmatrix} e_{11} & e_{12} & \dots & e_{1m} \\ e_{21} & e_{22} & \dots & e_{2m} \\ . & . & \dots & . \\ . & . & \dots & . \\ . & . & \dots & . \\ e_{n1} & e_{n2} & \dots & e_{nm} \end{bmatrix} \begin{bmatrix} \lambda_1 \\ \lambda_2 \\ . \\ . \\ . \\ \lambda_m \end{bmatrix} \quad (7)$$

The *i*-th column for the **P** matrix is the *i*-th EM vector: $\mathbf{e}_i = (e_{1i}, e_{2i}, \ldots, e_{ni})^t$. The flux distribution can be also represented as a superposition of the EM vectors with non-negative

EMCs as follows:

$$\mathbf{V} = \sum_{i=1}^{m} \boldsymbol{\lambda}_i \mathbf{e}_i \qquad (8)$$

EM analysis is a useful metabolic pathway analysis tool to identify the structure of a metabolic network. The EM analysis can decompose the intricate metabolic network comprised of highly interconnected reactions into uniquely organized pathways. These pathways, consisting of a minimal set of enzymes that can support steady state operation of cellular metabolism, represent independent cellular physiological states (Trinh et al., 2009). However, EM analysis does not decompose the reversible reactions into two irreversible reactions in calculating EMs and introduces a systematic way of extracting biologically meaningful pathways from an intricate metabolic network. In this context, an alternative approach is Extreme pathway analysis (Schilling et al., 2000).

Extreme pathway analysis concept is closely related to EM analysis because extreme pathways are a subset of elementary modes. The extreme pathway analysis can be considered as a hybrid between stoichiometric network analysis and EM analysis. In calculating extreme pathways (ExPas), the analysis splits only the internal reversible reaction into two irreversible reactions while it does not decompose reversible exchange reaction (Trinh et al., 2009). Different from EM analysis, extreme pathway analysis contains one additional constraint to make all extreme pathways systematically independent (Schilling et al., 2000). The metabolic flux vector can be expressed as a nonnegative linear combination of extreme pathways or elementary modes in metabolic reaction networks. The extreme pathways are the systematically independent subset of elementary modes; that is, no extreme pathways can be represented as a nonnegative linear combination of any other extreme pathways. The two sets extreme pathways and elementary modes are identical when all reactions including both internal and exchange reactions are irreversible in metabolic networks. Therefore, the identification of extreme pathways depends on the reconfiguration of the metabolic network analyzed, while the identification of elementary modes does not. For instance, extreme pathways identified in a metabolic network whose reversible exchange reactions split into two irreversible reactions may not be extreme pathways to any further extent in the original metabolic networks whose reversible exchange reactionsdo not split (Klamt and Stelling 2003). EM analysis used in order to understand regulation of a human red blood cell metabolism using singular value decomposition (price et al., 2003).

7. Concluding Remarks

Metabolic engineering provide clear and insightful information regarding the activity of metabolic complex reaction networks from an individual reaction based perspective. The metabolic pathway is a collection of step by step modification. The initial substance used as substrate by the first enzyme is transformed into a product. This product will then be the substrate for the next reaction. MFA is a promising method for quantitative estimation of the flux distribution of the enzymatic pathway network and provides important clues for the understanding of metabolism. MFA is performed using the stoichiometric model of the pathway network, which is constructed from pathway databases and does not require a priori knowledge of detailed enzyme kinetics. Therefore, we can determine the unknown fluxes using MFA quite easily when the system is either determined or overdetermined. In biological pathways, the majority are underdetermined systems. In this context, there are two approaches; FBA and ^{13}C-MFA are commonly applied to such underdetermined systems. FBA is a convenient means for the prediction of the flux distribution from network information alone, but it is based on an empirically selected objective function. Although ^{13}C-MFA requires expensive labeling experiments and complex calculations using an isotopomer model, it provides much more reliable results, revealing more realistic intracellular fluxes of enzymatic reactions. Metabolic pathway analysis can identify all metabolic flux vectors that exist in a metabolic network without requiring knowledge of any fixed flux or imposing any objective function to cellular metabolism. Two most promising concepts for pathway analysis, one relies on elementary mode analysis and other on extreme pathway analysis. These concepts are closely related but not identical although extreme pathways are a subset of elementary modes. Two are identical when all reactions including both internal and exchange reactions are irreversible in a metabolic networks.

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