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Metabolic Engineering 8 (2006) 151–159
METABOLIC ENGINEERING
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# Metabolic engineering under uncertainty. I: Framework development

Liqing Wang, Vassily Hatzimanikatis*

*Department of Chemical and Biological Engineering, Northwestern University, 2145 Sheridan Road, Room E136, Evanston, IL 60208-3120, USA*

Received 29 June 2005; received in revised form 10 November 2005; accepted 14 November 2005
Available online 18 January 2006

**Abstract**

Standard bioprocess conditions have been widely applied for the microbial conversion of raw material to essential industrial products. Successful metabolic engineering (ME) strategies require a comprehensive framework to manage the complexity embedded in cellular metabolism, to explore the impacts of bioprocess conditions on the cellular responses, and to deal with the uncertainty of the physiochemical parameters. We have recently developed a computational and statistical framework that is based on Metabolic Control Analysis and uses a Monte Carlo method to simulate the uncertainty in the values of the system parameters [Wang, L., Birol, I., Hatzimanikatis, V., 2004. Metabolic control analysis under uncertainty: framework development and case studies. Biophys. J. 87(6), 3750–3763]. In this work, we generalize this framework to incorporate the central cellular processes, such as cell growth, and different bioprocess conditions, such as different types of bioreactors. The framework provides the mathematical basis for the quantification of the interactions between intracellular metabolism and extracellular conditions, and it is readily applicable to the identification of optimal ME targets for the improvement of industrial processes [Wang, L., Hatzimanikatis, V., 2005. Metabolic engineering under uncertainty. II: analysis of yeast metabolism. Submitted].
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*Keywords:* Kinetic modeling; (Log)linear modeling; Metabolic control analysis; Bioreactors; Central carbon metabolism

## 1. Introduction

Industrial microorganisms, such as *Saccharomyces cerevisiae* and *Escherichia coli*, have been extensively used in major industrial processes including food, chemicals, pharmaceuticals and environment (Gottschalk, 1986; Walker, 1998). The microbial metabolism transforms feeding substrates into a wide variety of cellular products, many of which are essential materials for industrial and commercial purposes. Standard industrial operations have been developed to cultivate microbial cells in a controlled growth environment. Two modes of operation are currently used in industrial applications: batch and continuous (chemostat) (Bailey and Ollis, 1986; Blanch and Clark, 1996; Shuler and Kargi, 2002). In addition, significant efforts have been invested to the improvement of the performance of industrial microorganisms through Metabolic Engineering (ME) (Bailey, 1991; Cameron and Tong, 1993; Stephanopoulos et al., 1998).

Successful ME strategies rely heavily on a comprehensive understanding of the regulation underlying a metabolic network (Stephanopoulos et al., 2004). The overwhelming complexity of cellular responses largely complicates the identification of targets for predictive design and optimal ME (Kitano, 2002). As opposed to the empirical or experimental trials, a comprehensive mathematical framework that allows optimal target selection for ME appears to be rational and cost effective, and thus advantageous (Bailey, 1998; Hatzimanikatis et al., 1996; Heinrich et al., 1977). Among the developed mathematical frameworks, metabolic control analysis (MCA) quantifies the relation between genetic modifications or environmental changes and cellular process responses. MCA introduces the *control coefficients* to quantify the fractional change of cellular output, such as metabolite concentrations and metabolic fluxes, in response to fractional change of system parameters, such as enzymatic activities and growth conditions (Fell and Sauro, 1985; Hatzimanikatis and Bailey, 1996,

*Corresponding author. Fax: +1 847 491 3728.
*E-mail address:* vassily@northwestern.edu (V. Hatzimanikatis).

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doi:10.1016/j.ymben.2005.11.003

1997; Heinrich and Rapoport, 1974; Kacser and Burns, 1973; Kholodenko and Westerhoff, 1993; Reder, 1988). An immediate application of MCA on rational ME design is the ranking of potential targets based on the value of the control coefficients of the flux leading to the desired cellular product (Bowden, 1999; Cascante et al., 2002; Schuster, 1999; Westerhoff and Kell, 1996).

The precise calculation of metabolic control coefficients requires explicit knowledge of the enzyme kinetic properties. However, the deviation of the in vitro measurements from the in vivo values, the divergent experimental conditions of the various data sources, and the extensive parameter variation among individual cells introduce uncertainty into the estimation of control coefficients (Almaas et al., 2004; Alves and Savageau, 2000; Petkov and Maranas, 1997; Pritchard and Kell, 2002; Teusink et al., 2000; Thomas and Fell, 1994). In industrial bioprocess development, time and cost are two critical factors that do not allow "collection" of information about every possible enzyme and cellular process. In order to address the uncertainty in MCA and consolidate the diverse sources, we have developed a computational and statistical framework which is based on a Monte Carlo sampling method and the established (log)linear MCA formalism (Wang et al., 2004). In this work, we extended the original framework to integrate cellular biosynthesis and bioreactor conditions.

## 2. Methods

### 2.1. Generalized (log)linear MCA formalism

#### 2.1.1. Batch cultivation

For yeast cells or any other microorganism growing in a batch culture, the mass balances of intracellular metabolites can be described as

$$\frac{d\boldsymbol{x}}{dt} = \boldsymbol{N}\boldsymbol{v}(\boldsymbol{x}, \boldsymbol{p}_e, \boldsymbol{p}_s), \tag{1}$$

where $\boldsymbol{x}$ is the metabolite concentration vector, $N$ is the stoichiometric matrix, $\boldsymbol{v}$ is the metabolic flux vector, $\boldsymbol{p}_e$ is the enzyme activity parameter vector, which includes both kinetic parameters and enzyme concentrations, and $\boldsymbol{p}_s$ is the vector of other system parameters such as temperature and pH.

Due to the presence of conserved moieties in the cellular metabolism, i.e., groups of compounds such as ATP, ADP, and AMP, whose total amount is assumed to remain invariant over the characteristic response time of the metabolic network, we can divide the original set of metabolite concentrations $\boldsymbol{x}$ into two categories: an independent metabolite concentration vector, $\boldsymbol{x}_i$, and a dependent metabolite concentration vector, $\boldsymbol{x}_d$ (Reder, 1988). A third parameter set, $\boldsymbol{p}_m$, is also introduced into the system to represent the total concentration of the metabolites in each moiety group (Wang et al., 2004). The reduced form of mass balances with respect to independent metabolites can be represented as

$$\frac{d\boldsymbol{x}_i}{dt} = \boldsymbol{N}_R\boldsymbol{v}(\boldsymbol{x}_i, \boldsymbol{x}_d(\boldsymbol{x}_i, \boldsymbol{p}_m), \boldsymbol{p}_e, \boldsymbol{p}_s), \tag{2}$$

where $\boldsymbol{N}_R$ consists of the rows in the stoichiometric matrix $\boldsymbol{N}$ corresponding to the independent metabolites.

In our previous work, we have derived the following equations for the calculation of control coefficients in a intracellular metabolic system based on the (log)linear model formalism (Hatzimanikatis and Bailey, 1996, 1997; Hatzimanikatis et al., 1996; Wang et al., 2004):

$$\boldsymbol{C}_p^{x_i} = -(\boldsymbol{N}_R\boldsymbol{V}\boldsymbol{E}_i + \boldsymbol{N}_R\boldsymbol{V}\boldsymbol{E}_d\boldsymbol{Q}_i)^{-1} \times [\boldsymbol{N}_R\boldsymbol{V}\boldsymbol{\Pi}_m \vdots \boldsymbol{N}_R\boldsymbol{V}\boldsymbol{\Pi}_e \vdots \boldsymbol{N}_R\boldsymbol{V}\boldsymbol{\Pi}_s], \tag{3}$$

$$\boldsymbol{C}_p^{v} = (\boldsymbol{E}_i + \boldsymbol{E}_d\boldsymbol{Q}_i)\boldsymbol{C}_p^{x_i} + [\boldsymbol{\Pi}_m \vdots \boldsymbol{\Pi}_e \vdots \boldsymbol{\Pi}_s]. \tag{4}$$

Concentration control coefficients, $\boldsymbol{C}_p^x$, and flux control coefficients, $\boldsymbol{C}_p^v$, are defined as the fractional change of metabolite concentrations and metabolic fluxes, respectively, in response to fractional changes of system parameters. In this formalism (Eqs. (3) and (4)), $\boldsymbol{V}$ is the diagonal matrix whose elements are the steady-state fluxes; $\boldsymbol{E}_i$ and $\boldsymbol{E}_d$ are the matrices of the elasticities with respect to independent and dependent metabolites, respectively, defined as the local sensitivities of metabolic fluxes to metabolite concentrations; $\boldsymbol{\Pi}_m$, $\boldsymbol{\Pi}_e$, and $\boldsymbol{\Pi}_s$ are the matrices of the elasticities with respect to system parameters, $\boldsymbol{p}_m$, $\boldsymbol{p}_e$, and $\boldsymbol{p}_s$, respectively, defined as the local sensitivities of metabolic fluxes to the values of system parameters; and $\boldsymbol{Q}_i$ is the matrix that quantifies the relative abundance of dependent metabolites with respect to the abundance of the independent ones, and a second matrix, $\boldsymbol{Q}_m$, is also defined for the quantification of the relative abundance of depennkdzjdent metabolites with respect to the levels of their corresponding total moieties. These definitions lead to the following expression for the matrices of elasticities with respect to moiety parameters:

$$\boldsymbol{\Pi}_m = \boldsymbol{E}_d\boldsymbol{Q}_m. \tag{5}$$

#### 2.1.2. Chemostat cultivation

Cells growing in a chemostat bioreactor have a continuous supply of substrates from the feeding solution, and the reactor tank liquid is drawn off at a steady dilution rate which is equal to the rate of feeding solution feeding into the reactor (Bailey and Ollis, 1986). These steady-state conditions introduce an additional set of constraints that should be taken into account when we consider the response of cellular metabolic networks to changes in enzyme activities (Small, 1994; Snoep et al., 1994; Tan et al., 1996). Similar to Eq. (2), the mass balances of independent metabolites and biomass are described as (Fig. 1)

$$\frac{d\boldsymbol{x}_i}{dt} = \boldsymbol{N}_R\boldsymbol{v}(\boldsymbol{x}_i, \boldsymbol{x}_d(\boldsymbol{x}_i, \boldsymbol{p}_m), \boldsymbol{p}_e, \boldsymbol{p}_s, \boldsymbol{p}_b) \tag{6}$$

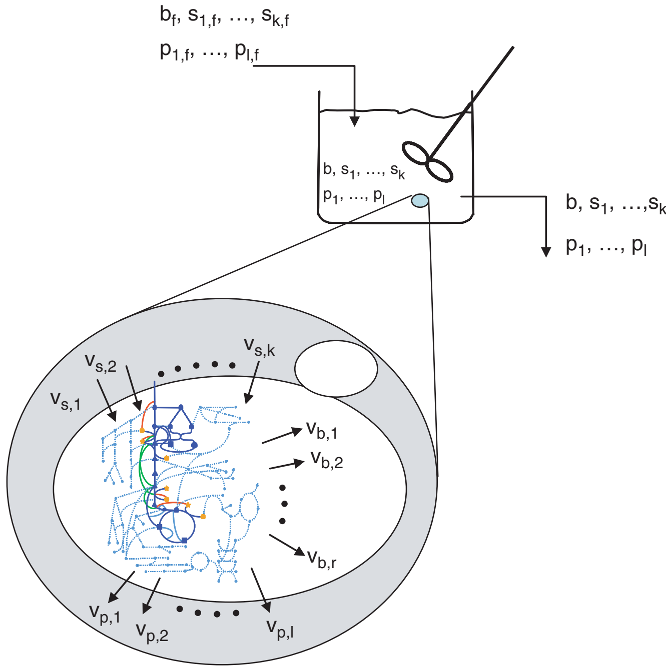


Fig. 1. A diagram of the intracellular metabolism and bioreactor processes in chemostat cultivation.

with

$$\boldsymbol{x_i} = \begin{bmatrix} \boldsymbol{x_{i,M}} \\ \boldsymbol{x_{i,B}} \end{bmatrix}, \quad \boldsymbol{x_d} = \begin{bmatrix} \boldsymbol{x_{d,M}} \\ \boldsymbol{x_{d,B}} \end{bmatrix}, \quad \boldsymbol{N_R} = \begin{bmatrix} \boldsymbol{N_{R,M}} & 0 \\ 0 & \boldsymbol{N_{R,B}} \end{bmatrix},$$

$$\boldsymbol{v} = \begin{bmatrix} \boldsymbol{v_M} \\ \boldsymbol{v_B} \end{bmatrix}, \quad \boldsymbol{p_m} = \begin{bmatrix} \boldsymbol{p_{m,M}} \\ \boldsymbol{p_{m,B}} \end{bmatrix}. \tag{7}$$

Here the subscripts "$M$" and "$B$" refer to the metabolic and bioreactor processes components, respectively, and the bioreactor parameters, $\boldsymbol{p_b}$, are also included in the equation.

Following our established (log)linear model formalism, we obtain the following expressions for the calculation of control coefficients:

$$\boldsymbol{C_p^{x_i}} = -(\boldsymbol{N_R V E_i} + \boldsymbol{N_R V E_d Q_i})^{-1} \times [\boldsymbol{N_R V \Pi_m} \vdots \boldsymbol{N_R V \Pi_e} \vdots \boldsymbol{N_R V \Pi_s} \vdots \boldsymbol{N_R V \Pi_b}], \tag{8}$$

$$\boldsymbol{C_p^{v}} = (\boldsymbol{E_i} + \boldsymbol{E_d Q_i})\boldsymbol{C_p^{x_i}} + [\boldsymbol{\Pi_m} \vdots \boldsymbol{\Pi_e} \vdots \boldsymbol{\Pi_s} \vdots \boldsymbol{\Pi_b}]. \tag{9}$$

All the elements in Eqs. (8) and (9), defined similarly to the elements in Eqs. (3) and (4), are generalized to include both the metabolic and bioreactor process components:

$$\boldsymbol{V} = \begin{bmatrix} \boldsymbol{V_M} & 0 \\ 0 & \boldsymbol{V_B} \end{bmatrix}, \quad \boldsymbol{E_i} = \begin{bmatrix} \boldsymbol{E_{x_{i,M}}^{v_M}} & \boldsymbol{E_{x_{i,B}}^{v_M}} \\ \boldsymbol{E_{x_{i,M}}^{v_B}} & \boldsymbol{E_{x_{i,B}}^{v_B}} \end{bmatrix},$$

$$\boldsymbol{E_d} = \begin{bmatrix} \boldsymbol{E_{x_{d,M}}^{v_M}} & \boldsymbol{E_{x_{d,B}}^{v_M}} \\ \boldsymbol{E_{x_{d,M}}^{v_B}} & \boldsymbol{E_{x_{d,B}}^{v_B}} \end{bmatrix}, \quad \boldsymbol{Q_i} = \begin{bmatrix} \boldsymbol{Q_{x_{i,M}}^{x_{d,M}}} & 0 \\ 0 & \boldsymbol{Q_{x_{i,B}}^{x_{d,B}}} \end{bmatrix},$$

$$\boldsymbol{Q_m} = \begin{bmatrix} \boldsymbol{Q_{p_{m,M}}^{x_{d,M}}} & 0 \\ 0 & \boldsymbol{Q_{p_{m,B}}^{x_{d,B}}} \end{bmatrix},$$

$$\boldsymbol{\Pi_m} = \begin{bmatrix} \boldsymbol{\Pi_{p_{m,M}}^{v_M}} & \boldsymbol{\Pi_{p_{m,B}}^{v_M}} \\ \boldsymbol{\Pi_{p_{m,M}}^{v_B}} & \boldsymbol{\Pi_{p_{m,B}}^{v_B}} \end{bmatrix}, \quad \boldsymbol{\Pi_e} = \begin{bmatrix} \boldsymbol{\Pi_{p_e}^{v_M}} \\ \boldsymbol{\Pi_{p_{e,M}}^{v_B}} \end{bmatrix},$$

$$\boldsymbol{\Pi_s} = \begin{bmatrix} \boldsymbol{\Pi_{p_s}^{v_M}} \\ \boldsymbol{\Pi_{p_s}^{v_B}} \end{bmatrix}, \quad \boldsymbol{\Pi_b} = \begin{bmatrix} \boldsymbol{\Pi_{p_b}^{v_M}} \\ \boldsymbol{\Pi_{p_b}^{v_B}} \end{bmatrix}, \tag{10}$$

The elements in Eqs. (8) and (9) imply that the numerical calculation of the control coefficients requires three types of information:

(1) Information about the metabolic processes:
- metabolic stoichiometry, $\boldsymbol{N_{R,M}}$;
- steady-state fluxes, $\boldsymbol{V_M}$;
- the elasticities of metabolic fluxes with respect to intracellular metabolite concentration, $\boldsymbol{E_{x_{i,M}}^{v_M}}$ and $\boldsymbol{E_{x_{d,M}}^{v_M}}$;
- the matrices for intracellular conserved moieties, $\boldsymbol{Q_{x_{i,M}}^{x_{d,M}}}$ and $\boldsymbol{Q_{p_{m,M}}^{x_{d,M}}}$;
- the elasticities of metabolic fluxes with respect to enzymatic parameters, $\boldsymbol{\Pi_{p_e}^{v_M}}$.

(2) information about the bioreactor processes:
- the stoichiometry of the bioreactor processes, $\boldsymbol{N_{R,B}}$;
- steady-state rates of the bioreactor process, $\boldsymbol{V_B}$;
- the elasticities of bioreactor fluxes with respect to extracellular metabolite concentration, $\boldsymbol{E_{x_{i,B}}^{v_B}}$ and $\boldsymbol{E_{x_{d,B}}^{v_B}}$;
- the matrices for extracellular conserved moieties, $\boldsymbol{Q_{x_{i,B}}^{x_{d,B}}}$ and $\boldsymbol{Q_{p_{m,B}}^{x_{d,B}}}$;
- the elasticities of bioreactor fluxes with respect to bioreactor parameters, $\boldsymbol{\Pi_{p_b}^{v_B}}$.

(3) Information about the metabolism–bioreactor interactions. These interations are introduced through the substrate uptake, product excretion, and cell growth:
- the elasticities of metabolic fluxes with respect to extracellular metabolite concentration, $\boldsymbol{E_{x_{i,B}}^{v_M}}$ and $\boldsymbol{E_{x_{d,B}}^{v_M}}$;
- the elasticities of bioreactor fluxes with respect to intracellular metabolite concentration, $\boldsymbol{E_{x_{i,M}}^{v_B}}$ and $\boldsymbol{E_{x_{d,M}}^{v_B}}$;
- the elasticities of metabolic fluxes with respect to bioreactor parameters, $\boldsymbol{\Pi_{p_{e,M}}^{v_B}}$;
- the elasticities of bioreactor fluxes with respect to enzymatic parameters, $\boldsymbol{\Pi_{p_b}^{v_M}}$.

### 2.2. Determination of control coefficients

In modeling cellular processes, such as glycolysis, we usually consider a subsystem composed by a *relatively* small number of physiochemical processes. However, such subsystems receive inputs *from*, and also provide inputs *to*, other cellular processes due to the explicit interactions of this subsystem with the surrounding environment. Therefore, we should take into account the effects of these interactions which are captured in the studies of subsystem responses to changes in the reactor parameters. In the following discussion, we will focus on the central carbon

pathways and the proposed method for modeling interactions between these pathways and the rest of metabolism. This method can be similarly applied in the modeling of every metabolic network.

We will first discuss about the method we have used in determining the (log)linear formalism components from the two sub-systems and their interactions.

### 2.2.1. Information about metabolic processes

*2.2.1.1. Catabolism.* Based on their distinct functions, metabolic processes in growing microbial cells are grouped into catabolism and anabolism. Enzymatic reactions involved in the catabolism are responsible for the breaking down of the substrate (e.g., glucose) to $CO_2$, a process that yields energy and biosynthetic precursors needed for cell growth and different physiological functions.

The stoichiometric matrix regarding catabolism can be constructed based on established biochemical studies and genomic information (Duarte et al., 2004; Kanehisa and Goto, 2000; Klapa et al., 2003; Lee et al., 2002; Yang et al., 2003), and the corresponding reduced stoichiometric matrix can be readily deduced through the identification of the conserved moiety groups (Schuster and Hilgetag, 1995).

The steady-state flux values for catabolic processes can be estimated from stable isotope experiments and metabolic flux analysis (dos Santos et al., 2003; Gombert et al., 2001; Klapa et al., 2003; Lee et al., 2002; Yang et al., 2003). The values of metabolic fluxes are different under various culture/bioprocess conditions, depending on the type of bioreactor, the carbon source, the growth rate, and other bioprocess parameters that impact cell physiology.

The elasticities of catabolic fluxes with respect to intracellular and extracellular metabolites depend on the local sensitivities of enzymatic reaction rates to metabolite concentrations. It has been shown that the elasticities of enzymes that follow common kinetics will be always constrained within well-defined bounds, and the specific value depends on the knowledge of the values of the metabolite concentration and kinetic parameters (Wang et al., 2004).

The elasticities of catabolic fluxes with respect to maximum enzyme activity are equal to 1 for common enzyme kinetics when the reaction rate is first-order to this parameter. However, in cases of more complex mechanism, it can be shown that these elasticities are still constrained within well-defined bounds. The elements of the conserved moiety matrix can be estimated based on knowledge about the relative concentration of the metabolites in the conserved moieties (Andersen and von Meyenburg, 1977; Ball and Atkinson, 1975; Reich et al., 1976).

*2.2.1.2. Anabolism.* Anabolism is responsible for the biosynthesis of the building blocks of cells, including protein amino acids, DNA and RNA nucleotides, lipid components, different monomers, polyamines, etc. (Gottschalk, 1986; Walker, 1998).

In this section, we will consider the central carbon pathways involved in anabolism. Anabolic pathways exhibit extraordinary complexity, but all of them originate from a limited set of precursor metabolites. Each precursor metabolite contributes a certain amount of carbon molecules into biomass and requires inputs from energy and redox cofactors (Neidhardt et al., 1990). In modeling these systems, we lump all the anabolic reactions into a set of biosynthetic fluxes in the stoichiometry, each of which originates from a corresponding precursor and integrate cofactor consumptions (details about biosynthetic cofactor precursors will be discussed in a later section).

The steady-state values for the biosynthetic fluxes can be calculated based on cell composition and yield measurement (Neidhardt et al., 1990; Sauer et al., 1996). The values of the elasticities of the biosynthetic fluxes with respect to intracellular metabolites can be calculated based on one of the following three assumptions: (1) biosynthetic fluxes are constant, i.e., do not depend on the concentration of any metabolite, (2) each biosynthetic flux depends on the concentration of its corresponding precursor only, and (3) all biosynthetic fluxes depend on every precursor and cofactor, and all the biosynthetic fluxes proceed in a coordinated manner that they share the same set of elasticities values. Although other assumptions could also be considered, we choose these three as the most basic assumptions.

### 2.2.2. Information about bioreactor processes

The extracellular environment in a chemostat bioreactor consists of the continuous flow of various substrates, products, and biomass through the feeding solution and withdrawing liquid, as well as the substrate consumption, product formation, and cell growth. As an example, the mathematical representation of the mass balances for a typical chemostat bioreactor system ($k$ substrates, $l$ products and biomass) is

$$\begin{aligned} \frac{db}{dt} &= Db_{\mathrm{f}} - Db + \mu b, \\ \frac{ds_i}{dt} &= Ds_{i,\mathrm{f}} - Ds_i - v_{s,i}b, \quad i = 1, \ldots, k, \\ \frac{dp_j}{dt} &= Dp_{j,\mathrm{f}} - Dp_j - v_{p,j}b, \quad j = 1, \ldots, l, \end{aligned} \tag{11}$$

where $b$, $s_i$, and $p_j$ denote the bioreactor concentrations of biomass, substrate $i$, and product $j$, respectively; $D$ denotes the dilution rate; the subscript $f$ denotes the concentration of the biomass, substrates, and products in the input feeding stream. $\mu$ denotes the specific growth rate, and $v_{s,i}$ and $v_{p,j}$ denote the specific consumption and production rates, respectively, of the corresponding substrates, $s_i$, and products, $p_j$. These mass balance equations of extracellular metabolites and biomass constitute the part of the bioreactor stoichiometry (Small, 1994; Snoep et al., 1994; Tan et al., 1996).

The rates of all the bioreactor processes can be readily estimated from the information of the dilution rate and the

compositions of the feeding and withdrawing solution (dos Santos et al., 2003; Gombert et al., 2001). The dilution rate and feeding solution composition are the bioreactor parameters to the system and they are subject to manipulation. The corresponding elements in the matrix of elasticities with respect to these parameters are generally equal to 1 because of the linear relationship between bioreactor processes and these parameters (Eq. (11)).

The inflow processes of metabolites and biomass are not affected by metabolite concentration in the medium, thus the values of their elasticities with respect to metabolite concentration are zero (Eq. (11)). The rates of outflow processes of the withdrawing solution are proportional to the extracellular metabolite concentration and biomass (Eq. (11)), and therefore, their concentration elasticities with respect to extracellular metabolites and biomass are equal to 1.

### *2.2.3. Information about the metabolism–bioreactor interactions*

The interactions between the metabolic subsystem and the bioreactor processes are determined by the concentration elasticities of substrate uptake, product excretion, and cell growth.

*2.2.3.1. Substrate uptake, production excretion, and metabolite transport.* Mathematically, the volumetric rates of substrate uptake and product excretion are the product of the corresponding specific transport processes with biomass concentration (Eq. (11)). Thus the concentration elasticities of the volumetric rates with respect to intracellular and extracellular metabolites are always equal to the elasticities of the corresponding specific transport fluxes. In addition, the elasticities of the volumetric uptake and production rates with respect to biomass are equal to one.

*2.2.3.2. Specific cell growth rate and biosynthetic fluxes.* - Biomass growth depends on the set of biosynthetic fluxes from the intracellular metabolism that contribute carbon compounds to biomass formation. The relation between biosynthetic fluxes and the flux representing growth can be derived as follows:

$$\sum_i n_i^{\mathrm{C}} v_{bs,i} = \mu n_{\mathrm{T}}^{\mathrm{C}}, \tag{12}$$

where $n_i^{\mathrm{C}}$ is the number of carbon moles per mole of precursor $i$; $v_{bs,i}$ is the number of moles of precursor $i$ used for biosynthesis per gram dry weight per unit time; $\mu$ is the specific growth rate; and $n_{\mathrm{T}}^{\mathrm{C}}$ is the total number of carbon moles per gram of dry weight biomass which can be calculated from the following equation:

$$n_{\mathrm{T}}^{\mathrm{C}} = \sum_i Y_{i/bs} n_i^{\mathrm{C}}, \tag{13}$$

where $Y_{i/bs}$ denotes the moles of precursor $i$ required for producing a gram of dry weight biomass (Neidhardt et al., 1990).

From Eq. (12), we can derive the following expression for the specific growth rate as a function of the precursor biosynthetic fluxes:

$$\mu = \sum_i \frac{n_i^{\mathrm{C}}}{n_{\mathrm{T}}^{\mathrm{C}}} v_{bs,i} = \sum_i \varphi_i v_{bs,i} \tag{14}$$

with $\varphi_i$ representing the fraction of carbon moles from precursor $i$ in the total carbon moles of 1 g of biomass. Note that under changing environment, the coefficient $\varphi_i$ might also change. Here we consider a reference physiological state and small perturbations around this state. In the absence of additional information, we assume that such small perturbations will not change cellular composition, and consequently $\varphi_i$, significantly.

*2.2.3.3. Elasticities of the specific growth rate.* The dependency of the specific growth rate on the precursor biosynthetic fluxes (Eq. (14)) can be described by the following vector equation:

$$\mu = \boldsymbol{\varphi}^{\boldsymbol{T}} \boldsymbol{v_{bs}}, \tag{15}$$

where $\boldsymbol{\varphi}$ is the vector of the fraction of carbon moles from all precursors in the total carbon moles of 1 g of biomass, and $\boldsymbol{v_{bs}}$ is the biosynthetic flux vector. According to this equation, the vector of the elasticities of specific growth rate with respect to metabolic intermediates, $\boldsymbol{\varepsilon^{\mu}}$, and to enzymatic parameters, $\boldsymbol{\pi^{\mu}}$, can be derived as

$$\boldsymbol{\varepsilon^{\mu}} = \boldsymbol{\varphi^T V_{bs} E^{bs}}, \tag{16}$$

$$\boldsymbol{\pi^{\mu}} = \boldsymbol{\varphi^T V_{bs} \Pi^{bs}},$$

where $\boldsymbol{V_{bs}}$ is the diagonal matrix of steady-state biosynthetic fluxes scaled by $\mu$, and $\boldsymbol{E^{bs}}$, $\boldsymbol{\Pi^{bs}}$ are the elasticity matrices of biosynthetic fluxes with respect to all metabolites and enzymatic parameters.

*2.2.3.4. Energy and redox demands/balances.* The mass balances of energy and redox carriers (e.g., ATP, NAD, and NADPH) include terms associated with the metabolite transformations, the biosynthesis requirement, and the ATP demand for maintenance (Stouthamer and Bettenhaussen, 1973; Vandijken and Scheffers, 1986). The modeling of these mass balances can be demonstrated using the ATP mass balance as an example:

$$\frac{d[\mathrm{ATP}]}{dt} = \sum_i n_i v_i - \sum_j n_j v_j - \sum_k Y_{\mathrm{ATP}/k} v_{bs,k} - v_M. \tag{17}$$

Eq. (17) involves each reaction $v_i$ in the metabolic network that produces $n_{\mathrm{i}}$ units of ATP, each reaction $v_j$ that consumes $n_j$ units of ATP, each biosynthetic flux $v_{bs,k}$ from metabolite precursor that consumes $Y_{\mathrm{ATP}/k}$ units of ATP for biosynthetic requirements, and ATP requirement for maintenance $v_M$.

The biosynthetic demand for energy and redox cofactors depends on the specific growth rate, and it can be mathematically described in two ways, depending on the available experimental information.

(a) Based on the biochemistry of biosynthesis, it is possible to assign energy requirement associated with each biosynthetic flux from precursor metabolites,

$$v_{bs,\mathrm{ATP}} = \sum_i Y_{\mathrm{ATP}/i} v_{bs,i}, \tag{18}$$

where $Y_{\mathrm{ATP}/i}$ is the moles of ATP required for transforming one mole of precursor $i$ into biomass. It can be estimated as the net moles of ATP consumed in the pathways that fix the precursors into biomass, if the detailed stoichiometry of the biosynthetic pathways is available.

(b) For most of the organisms, and in the current framework of metabolic flux analysis, the estimation of the energy requirement for biosynthesis is based on the method proposed by Stouthamer (Stouthamer and Bettenhaussen, 1973) as

$$v_{bs,\mathrm{ATP}} = Y_{\mathrm{ATP}/bs}\mu, \tag{19}$$

where $Y_{\mathrm{ATP}/bs}$ is the number of ATP moles required per gram dry weight biomass production.
Combining Eqs. (14) and (19), we obtain the following equation:

$$v_{bs,\mathrm{ATP}} = \sum_i Y_{\mathrm{ATP}/bs}\varphi_i v_{bs,i}. \tag{20}$$

Eq. (19) implies that the ATP required for each biosynthetic process is determined by the amount of biomass generated from per mole of the corresponding precursor. Therefore, the combination of Eqs. (18) and (20) yields an estimate of the amount of ATP consumption associated with each precursor used for biosynthesis:

$$Y_{\mathrm{ATP}/i} = Y_{\mathrm{ATP}/bs}\varphi_i. \tag{21}$$

Similarly, we can model the mass balances of NADH, NADPH, and other cofactors and estimate their requirement for biomass formation.

### 2.3. Extended framework of MCA under uncertainty

The quantification of the uncertainty associated with enzyme kinetic properties and metabolite concentration is critical for the calculation of control coefficients using the developed (log)linear MCA formalism (Eqs. (8) and (9)). We have established a Monte Carlo sampling methodology that allows us to access the parameter uncertainty and describe control coefficients using statistical analysis (Wang et al., 2004). The developed framework, *MCA under Uncertainty*, is extended for the application on industrial cultivations of microbial culture. The procedure is outlined below:

(1) *System setup*: In the first phase, we collect information about the system stoichiometry and the reference-state flux distribution and the bioreactor process rates, and we next separate the metabolite species into an independent-variable and a dependent-variable set.

(2) *Monte Carlo sampling and stability test*: After the initial setup, the algorithm explores the multi-dimensional space of concentration elasticities, i.e., the strength of enzymatic reactions toward reactant concentrations, and the multi-dimensional space of relative weights between independent and dependent metabolite concentrations (see (Wang et al., 2004) for details on the sampling methodology). For each generated multi-dimensional sample, the local stability of the steady state is tested.

(3) *Control coefficient calculation and statistical analysis*: For the samples that yield stable steady state to the system, the corresponding control coefficients are calculated. After a predefined number of samples are collected, various data-mining methods will be performed to study the statistical characteristics of the control coefficients.

### 2.4. Mathematical theorem for the calculation of control coefficients in bioreactors

In order to identify the origins of the differences in the calculation of the control coefficients between cells growing in batch and chemostat environment, we use control diagrams (Figs. 2A–C) which help us clarify the fundamental relationships between the parameters and variables in both batch and chemostat cultivations.

In both batch (Fig. 2A) and chemostat (Fig. 2B) conditions, the rates of metabolic fluxes are affected by enzyme activity parameters and extracellular metabolite concentrations.

$$d\ln \boldsymbol{v_i} = \boldsymbol{B}^{\boldsymbol{v_i}}_{\boldsymbol{p_e}}\, d\ln \boldsymbol{p_e} + \boldsymbol{B}^{\boldsymbol{v_i}}_{\boldsymbol{x_b}}\, d\ln \boldsymbol{x_b}, \tag{23}$$

$$d\ln \boldsymbol{v_r} = \boldsymbol{B}^{\boldsymbol{v_r}}_{\boldsymbol{p_e}}\, d\ln \boldsymbol{p_e} + \boldsymbol{B}^{\boldsymbol{v_r}}_{\boldsymbol{x_b}}\, d\ln \boldsymbol{x_b}. \tag{24}$$

Here $\boldsymbol{B}$ is used to represent the control coefficients of metabolic processes, $\boldsymbol{p_e}$ is the vector of enzyme activity parameters, $\boldsymbol{x_b}$ is the vector of extracellular metabolite concentrations, and $\boldsymbol{v_i}$ and $\boldsymbol{v_r}$ refer to intracellular and exchange fluxes (substrate uptake, product excretion, specific growth), respectively.

The difference between the control coefficients of metabolic processes within batch and chemostat reactors comes from the different conditions of extracellular metabolite concentrations, $\boldsymbol{x_b}$. In a batch culture, these concentrations are independent parameters (Fig. 2A). In a chemostat, however, extracellular metabolites are subject to constraints introduced by the mass balances of bioreactor processes (Eq. (11)). Therefore, the changes of their concentrations are determined by the exchange fluxes, $\boldsymbol{v_r}$, and bioreactor parameters, $\boldsymbol{p_b}$, including the dilution rate and feeding solution composition (Fig. 2B)

$$d\ln \boldsymbol{x_b} = \boldsymbol{G}^{\boldsymbol{x_b}}_{\boldsymbol{v_r}}\, d\ln \boldsymbol{v_r} + \boldsymbol{G}^{\boldsymbol{x_b}}_{\boldsymbol{p_b}}\, d\ln \boldsymbol{p_b}. \tag{25}$$

Here $\boldsymbol{G}$ is used to represent the control coefficients of bioreactor processes. The exchange fluxes are not only affected by the enzyme activity parameters, they are also

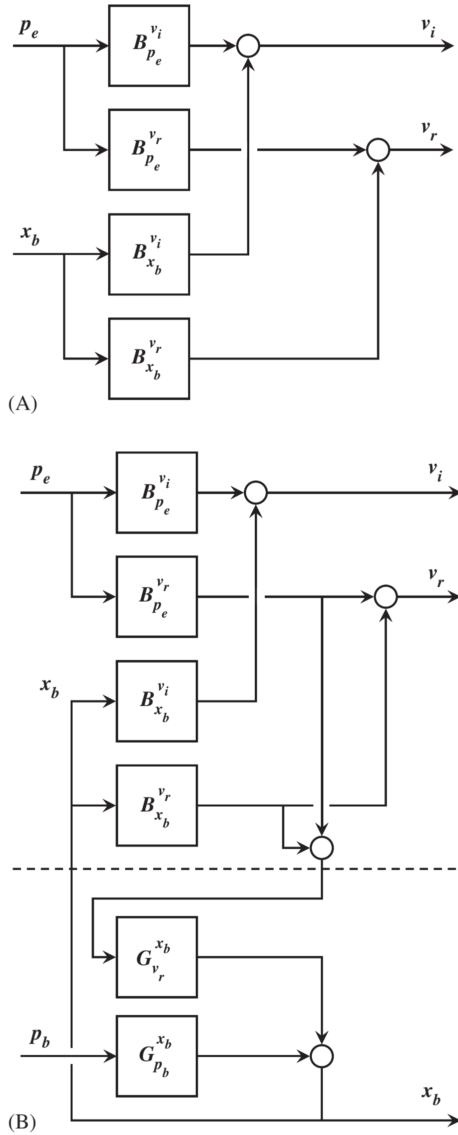


Fig. 2. A control plot of relationship among the components of microbial cells growing in a batch and chemostat reactors. (A) In a batch culture, the intracellular metabolic fluxes, $\boldsymbol{v}_i$, and exchange fluxes, $\boldsymbol{v}_r$, are affected by enzyme activity parameters, $\boldsymbol{p}_e$, and extracellular metabolite concentration, $\boldsymbol{x}_b$. $\boldsymbol{B}^{v_i}_{p_e}$ and $\boldsymbol{B}^{v_r}_{p_e}$ are the matrices of the control coefficients of intracellular and exchange fluxes, correspondingly, with respect to enzyme activity parameters. $\boldsymbol{B}^{v_i}_{x_b}$ and $\boldsymbol{B}^{v_r}_{x_b}$ are the matrices of the control coefficients of intracellular and exchange fluxes, correspondingly, with respect to extracellular metabolite concentration, which are independent parameters in the batch culture. (B) A chemostat system consists of metabolic processes (upper panel) and bioreactor processes (lower panel). In addition to the interactions illustrated in (A), the extracellular metabolite concentration, $\boldsymbol{x}_b$, is determined by bioreactor parameters, $\boldsymbol{p}_b$, and by exchange fluxes, $\boldsymbol{v}_r$, through the corresponding control coefficients, $\boldsymbol{G}^{x_b}_{p_b}$ and $\boldsymbol{G}^{x_b}_{v_r}$.

subject to feedback control from extracellular metabolite concentration (Fig. 2B)

$$d\ln \boldsymbol{v}_r = \boldsymbol{B}^{v_r}_{p_e}\, d\ln \boldsymbol{p}_e + \boldsymbol{B}^{v_r}_{x_b}\, d\ln \boldsymbol{x}_b. \tag{26}$$

Therefore, the expression of the fractional change of extracellular metabolite concentration in a chemostat (Eq. (25)) can be derived as

$$d\ln \boldsymbol{x}_b = (\boldsymbol{I} - \boldsymbol{G}^{x_b}_{v_r}\boldsymbol{B}^{v_r}_{x_b})^{-1}\boldsymbol{G}^{x_b}_{p_b}\, d\ln \boldsymbol{p}_b + (\boldsymbol{I} - \boldsymbol{G}^{x_b}_{v_r}\boldsymbol{B}^{v_r}_{x_b})^{-1}\boldsymbol{G}^{x_b}_{v_r}\boldsymbol{B}^{v_r}_{p_e} d\ \ln\ \boldsymbol{p}_e. \tag{27}$$

Eq. (27) suggests that the extracellular metabolite concentration in chemostat becomes an *output* of the chemostat as discussed above.

Introducing Eq. (27) into Eq. (23), we are able to obtain the following expression for the fractional change of metabolic fluxes in response to the fractional changes in enzyme activities and bioreactor parameters:

$$d\ln \boldsymbol{v}_i = (\boldsymbol{B}^{v_i}_{p_e} + \boldsymbol{B}^{v_i}_{x_b}(\boldsymbol{I} - \boldsymbol{G}^{x_b}_{v_r}\boldsymbol{B}^{v_r}_{x_b})^{-1}\boldsymbol{G}^{x_b}_{v_r}\boldsymbol{B}^{v_r}_{p_e})\, d\ln \boldsymbol{p}_e + \boldsymbol{B}^{v_i}_{x_b}(\boldsymbol{I} - \boldsymbol{G}^{x_b}_{v_r}\boldsymbol{B}^{v_r}_{x_b})^{-1}\boldsymbol{G}^{x_b}_{p_b}\, d\ln \boldsymbol{p}_b, \tag{28}$$

$$d\ln \boldsymbol{v}_r = (\boldsymbol{B}^{v_r}_{p_e} + \boldsymbol{B}^{v_r}_{x_b}(\boldsymbol{I} - \boldsymbol{G}^{x_b}_{v_r}\boldsymbol{B}^{v_r}_{x_b})^{-1}\boldsymbol{G}^{x_b}_{v_r}\boldsymbol{B}^{v_r}_{p_e}) d\ln \boldsymbol{p}_e + \boldsymbol{B}^{v_r}_{x_b}(\boldsymbol{I} - \boldsymbol{G}^{x_b}_{v_r}\boldsymbol{B}^{v_r}_{x_b})^{-1}\boldsymbol{G}^{x_b}_{p_b}\, d\ln \boldsymbol{p}_b. \tag{29}$$

Finally, from Eqs. (28) and (29), we were able to derive the expression for the overall control coefficients of metabolic fluxes with respect to enzyme activity parameters:

$$\boldsymbol{C}^{v_i}_{p_e} = \frac{d\ln \boldsymbol{v}_i}{d\ln \boldsymbol{p}_e} = \boldsymbol{B}^{v_i}_{p_e} + \boldsymbol{B}^{v_i}_{x_b}(\boldsymbol{I} - \boldsymbol{G}^{x_b}_{v_r}\boldsymbol{B}^{v_r}_{x_b})^{-1}\boldsymbol{G}^{x_b}_{v_r}\boldsymbol{B}^{v_r}_{p_e}, \tag{30}$$

$$\boldsymbol{C}^{v_r}_{p_e} = \frac{d\ln \boldsymbol{v}_r}{d\ln \boldsymbol{p}_e} = \boldsymbol{B}^{v_r}_{p_e} + \boldsymbol{B}^{v_r}_{x_b}(\boldsymbol{I} - \boldsymbol{G}^{x_b}_{v_r}\boldsymbol{B}^{v_r}_{x_b})^{-1}\boldsymbol{G}^{x_b}_{v_r}\boldsymbol{B}^{v_r}_{p_e}. \tag{31}$$

Mathematically, the different expressions between $\boldsymbol{B}^{v_i}_{p_e}$ and $\boldsymbol{C}^{v_i}_{p_e}$, and those between $\boldsymbol{B}^{v_r}_{p_e}$ and $\boldsymbol{C}^{v_r}_{p_e}$, explain the fact that the difference of control coefficients between batch and chemostat cultures is not simply a result of the different physiology and the different intracellular conditions (e.g., flux distribution) for microbial cells growing under different cultivations. The control coefficients of metabolic fluxes with respect to enzyme activity parameters (Eqs. (30) and (31)) suggest that the constraints of the mass balances of the extracellular metabolites, described by the matrix $\boldsymbol{G}^{x_b}_{v_r}$, are also responsible for the differences of control coefficients between batch and chemostat reactors, even if the flux distribution is the same. If matrix $\boldsymbol{G}^{x_b}_{v_r}$ is zero, which is the case in batch, the control coefficients of batch and chemostat reactors would be the same. In the companion paper (Wang and Hatzimanikatis, 2005), we build a hypothetical model where microbial cells operate in a batch culture with the same intracellular conditions as chemostat culture. The numerical results confirmed our mathematical expectation that the control coefficients are significantly different from the results from those in the cells growing in chemostat reactors. Furthermore, matrix $\boldsymbol{G}^{x_b}_{v_r}$ introduces and important coupling between the control coefficients $\boldsymbol{B}^{v_i}_{p_e}$ and $\boldsymbol{C}^{v_i}_{p_e}$, and between the control coefficients $\boldsymbol{B}^{v_r}_{p_e}$ and $\boldsymbol{C}^{v_r}_{p_e}$, as shown in Eqs. (30) and (31). The elements of matrix $\boldsymbol{G}^{x_b}_{v_r}$ are either equal to one or simple functions of metabolite concentrations (Eq. (11)). This provides a possible way to allow us estimate the

control coefficients of chemostat (or batch) based on the control coefficients of batch (or chemostat).

## 3. Conclusion

The work presented in this article extends our established computational and statistical framework—*MCA under Uncertainty*—to integrate metabolic reactions with batch and chemostat reactor conditions. Distinct from metabolic reactions, bioreactor processes are generally not catalyzed by a particular enzyme; instead, they are either physical events (e.g., dilution rate of reactor), or integration of multiple metabolic processes (e.g., cell growth). In the generalized framework, this issue has been addressed and new components in the mathematical formalism are introduced to represent the bioreactor processes.

Far from being isolated, metabolic reactions and bioreactor processes are highly interacting. These interactions between the two sub-systems are realized through the substrate uptake, product excretion, and cell growth in the bioreactor and the specific metabolite transports and biosynthetic fluxes. The modeling perspective of the interactions between metabolic reactions and bioreactor processes presented here complements previous studies (Small, 1994; Snoep et al., 1994; Tan et al., 1996).

The presented computational and statistical framework allows us to calculate the control coefficients of microbial cells growing in standard industrial bioreactors. Instead of offering exact values, the large-scale sampling procedure explores the statistical significance of metabolic control while taking into account the cellular uncertainty that is fundamental to living organisms. A specific example of the application of the generalized framework can be found in the companion article (Wang and Hatzimanikatis, 2005), in which the parameter-independent nature of our framework allows us to demonstrate that distributions of flux control coefficients are highly dependent on the growth condition under which they are calculated. Thus conclusions drawn of flux control depend on the process conditions and any genetic optimization should be carried out under conditions that closely reflect the targeted design of the final industrial process.

## Acknowledgments

The authors are grateful for the financial supported provided by the Department of Energy (DE-AC36-99GO103), the National Aeronautics and Space Administration (NAG 2-1527), and DuPont through a DuPont Young Professor Award to VH. LW received partial support by the Chinese Government through the State Excellence Scholarship program for students studying overseas.

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