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Dynamic metabolic flux analysis (DMFA): A framework for determining fluxes at metabolic non-steady state

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ABSTRACT

Metabolic flux analysis (MFA) is a key tool for measuring *in vivo* metabolic fluxes in systems at metabolic steady state. Here, we present a new method for dynamic metabolic flux analysis (DMFA) of systems that are not at metabolic steady state. The advantages of our DMFA method are: (1) time-series of metabolite concentration data can be applied directly for estimating dynamic fluxes, making data smoothing and estimation of average extracellular rates unnecessary; (2) flux estimation is achieved without integration of ODEs, or iterations; (3) characteristic metabolic phases in the fermentation data are identified automatically by the algorithm, rather than selected manually/arbitrarily. We demonstrate the application of the new DMFA framework in three example systems. First, we evaluated the performance of DMFA in a simple three-reaction model in terms of accuracy, precision and flux observability. Next, we analyzed a commercial glucose-limited fed-batch process for 1,3-propanediol production. The DMFA method accurately captured the dynamic behavior of the fed-batch fermentation and identified characteristic metabolic phases. Lastly, we demonstrate that DMFA can be used without any assumed metabolic network model for data reconciliation and detection of gross measurement errors using carbon and electron balances as constraints.

1. Introduction

Metabolic flux analysis (MFA) has emerged as a tool of great significance in metabolic engineering, biotechnology and systems biology. Determining *in vivo* metabolic fluxes provides useful information about the cellular metabolic phenotype: it offers insight into the regulation of product biosynthesis pathways (Antoniewicz et al., 2007c; Moxley et al., 2009; Reed et al., 2010), allows identification of genetic targets for metabolic engineering (Park et al., 2007), and can be used for metabolic network validation (Crown et al., 2011). MFA has become a standard tool for measuring steady state metabolic fluxes in many academic labs. However, MFA is still not commonly used in the industry. One reason is that MFA relies on the assumption that the biological system is at metabolic steady state, specifically that intracellular metabolic fluxes are constant in time. This assumption is approximated during early exponential growth in batch cultures and in steady state continuous cultures. However, industrial bioprocesses are predominantly fed-batch fermentations and these cultures are inherently dynamic in nature, as the cells continually adapt to a changing environment characterized by decreasing nutrient levels, increasing cell density and accumulation of products and by-products (Ahn and Antoniewicz, 2011; Hjersted et al., 2007; Meadows et al., 2010). In these systems, classical MFA techniques cannot be directly applied to determine fluxes.

In recent years, initial attempts have been undertaken to address this problem through the development of dynamic metabolic flux analysis (DMFA) techniques (Lequeux et al., 2010; Llaneras and Pico, 2007; Niklas et al., 2011). The goal of DMFA is to determine changes in fluxes during a culture from analysis of time-series of extracellular measurements. Current DMFA methods assume that flux transients are relatively slow, on the order of hours, compared to the time required to reach pseudo steady state for intracellular metabolites, which is typically on the order of seconds to minutes. With this assumption, determining flux transients from time-series of extracellular measurements involves the following three steps: (i) divide the experiment into discrete time intervals; (ii) calculate average external rates for each time interval by taking derivatives of external concentration measurements; and (iii) calculate fluxes for each time interval using classical MFA (Niklas et al., 2011). The results of these steady state models, evaluated at different time points, are then combined to obtain a time profile of flux transients. While these methods provide useful information, they have a number of drawbacks and limitations. First, current methods do not allow the complete time-series of data to be analyzed as a whole, but rather as unconnected sub-problems. Thus, important

Abbreviations: DMFA, dynamic metabolic flux analysis; MFA, metabolic flux analysis; PDO, 1,3-propanediol; SSR, variance-weighted sum of squared residuals

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dynamic information is ignored. Furthermore, there are missed opportunities for data reconciliation, detection of gross measurement errors, and enforcement of additional constraints, such as an overall carbon and electron balance for the culture. Finally, substantial errors are introduced in the analysis when concentration measurements are transformed into average external rates. Taking derivatives of concentration measurements results in loss of information, e.g. two or more concentration measurements provide one average flux, and more significantly, measurement errors are amplified. It has been suggested that data smoothing techniques can reduce the latter problem (Lequeux et al., 2010; Niklas et al., 2011); however, this comes at a cost, namely further loss of information and the potential to introduce biases.

Here, we present a new mathematical approach for DMFA that directly fits the complete time-series of concentration measurements all at once to determine metabolic transients for the entire culture. Our method does not depend on manually selecting time intervals to describe flux transients. Instead, rigorous statistical criteria are used to automatically detect the level of dynamic information present in the data. Our DMFA algorithm returns the simplest flux solution that yields a statistically acceptable fit of the data. This is a new and unique feature of our approach. Finally, our method is generic, and thus can be applied to any biological system, e.g. microbial, mammalian, plant, etc. In this contribution, we demonstrate the application of DMFA in three example systems. First, we demonstrate the basic concepts of DMFA using a simple three reaction model. Next, we investigate a realistic model of *Escherichia coli* metabolism using data from a fed-batch fermentation taken from literature where 1,3-propanediol was produced from glucose (Antoniewicz et al., 2007c). Lastly, we evaluate the application of DMFA without an assumed metabolic network model for detecting gross measurement errors in fermentation data and for data reconciliation using only carbon and electron balances as constraints.

2. Methods

2.1. Metabolic flux analysis at metabolic steady state

The key to quantifying intracellular metabolic fluxes is to analyze the biological system as an integrated biochemical network, rather than a set of individual reactions. The classical MFA method relies on balancing fluxes around metabolites within an assumed network stoichiometry. At metabolic steady state, intracellular fluxes (v) are constrained by the stoichiometry matrix (S):

$$S \cdot v = 0 \tag{1}$$

To determine intracellular fluxes from measured external rates (r_m), the following least-squares problem is solved:

$$\min SSR = \sum (r_i - r_{i,m})/{\sigma_i}^2 \tag{2}$$

$$\text{s.t.} \quad r_i = R_i \cdot v$$
$$0 = S \cdot v$$

The MFA objective is to find a set of intracellular fluxes that minimizes the variance-weighted sum of squared residuals (SSR) between the measured and predicted external rates. Here, the measurement matrix R contains unity values for fluxes corresponding to external rates, and σ denotes standard deviations of external rate measurements. Because the model equations are linear with respect to the metabolic fluxes, a simple solution to the MFA problem can be derived, shown here in matrix form (Appendix A)

$$v = K \cdot (K^T \cdot R^T \cdot W \cdot R \cdot K)^{-1} \cdot K^T \cdot R^T \cdot W \cdot r_m \tag{3}$$

With

$$K = null(S) \text{ and } v = K \cdot u \tag{4}$$

here W is a weighting matrix with the inverses of measurement variances ($1/\sigma_i^2$) on the diagonal, K is the kernel (i.e. null space) of the stoichiometry matrix, and u is the vector of the so-called free fluxes (Antoniewicz et al., 2006).

2.2. Equations for describing dynamics of concentrations and fluxes at metabolic non-steady state

The goal of this work is to extend MFA to metabolic non-steady state cases, that is, to allow the estimation of fluxes in experiments where metabolite pools and fluxes changed with time. Our method is based on non-steady state mass balances for metabolite pools (i.e. a more general form of Eq. (1)):

$$dc/dt = S \cdot v(t) = S \cdot K \cdot u(t) \quad \text{with} \quad K = null(S_{bal}) \tag{5}$$

We classify metabolites in the model as either being balanced or non-balanced. Typically, we will assume that intracellular metabolites are balanced and external metabolites are non-balanced. To model flux dynamics in cultures we need an expression for describing flux changes as a function of time. The simplest assumption is that fluxes change linearly between two time points t_0 and t_1:

$$v(t) = K \cdot u_0 \cdot \left(1 - \frac{t - t_0}{t_1 - t_0}\right) + K \cdot u_1 \cdot \left(\frac{t - t_0}{t_1 - t_0}\right) \tag{6}$$

By combining Eqs. (5) and (6) we can rewrite the mass balances explicitly as a function of free fluxes u_0 and u_1, time points t_0 and t_1, and time t

$$dc/dt = S \cdot K \cdot u_0 \cdot \left(1 - \frac{t - t_0}{t_1 - t_0}\right) + S \cdot K \cdot u_1 \cdot \left(\frac{t - t_0}{t_1 - t_0}\right) \tag{7}$$

Explicit integration of Eq. (7) yields the following expression for metabolite pool sizes as a function of time:

$$c(t) = c_0 + S \cdot K \cdot u_0 \cdot \left(t - t_0 - \frac{1}{2}\frac{(t - t_0)^2}{t_1 - t_0}\right) + S \cdot K \cdot u_1 \cdot \left(\frac{1}{2}\frac{(t - t_0)^2}{t_1 - t_0}\right) \tag{8}$$

Eq. (8) is the exact algebraic solution to the original ODE problem stated in Eq. (5). A useful characteristic of Eq. (8) is that it is linear with respect to model parameters that will be estimated, namely u_0, u_1, and c_0. We have further generalized Eq. (8) by allowing the multiple so-called DMFA time points to be defined for an experiment (t_1, t_2, …, t_n), and derived expressions that describe the dynamics of fluxes and metabolite pools as a function of time, shown here in matrix form

$$v(t) = K \cdot U \cdot \kappa(t, t_i) \tag{9}$$

$$c(t) = c_0 + S \cdot K \cdot U \cdot \gamma(t, t_i) \tag{10}$$

Here, c_0 is a vector of initial pool sizes, U is a matrix that contains column vectors of free fluxes at the DMFA time points, $U = [u_1, u_2, \ldots, u_n]$, and the matrices γ and κ contain all of the time-dependent terms. These matrices are calculated using the expressions given in Table 1. Eqs. (9) and (10) form the basis for our DMFA method that is described next.

2.3. Metabolic flux analysis at metabolic non-steady state

In order to describe flux transients mathematically, the time domain of a culture is divided into multiple smaller time intervals where fluxes can be assumed to change at a constant rate between the various DMFA time points. For illustration purposes, the time domain in Fig. 1 was divided into three intervals where the flux changed linearly between the initial time point (t_1), two inflection time points (t_2 and t_3), and the end time point (t_4). Two important things to note are that we do not require the inflection