OPTIMAL SELECTION OF ENZYME LEVELS USING LARGE-SCALE KINETIC MODELS

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Abstract: A hybrid optimization framework is introduced to identify enzyme sets and levels to meet overproduction requirements using kinetic models of metabolism. A customized Simulated Annealing Algorithm is employed to navigate through the discrete space of enzyme sets while a Sequential Quadratic Programming method is utilized to identify optimal enzyme levels. The framework is demonstrated on a model of *E.coli* central metabolism for serine biosynthesis. Computational results show that by identifying relatively small optimal enzyme sets, a substantial increase in serine production can be achieved. The proposed approach thus provides a versatile tool for the elucidation of controlling enzymes with implications in biotechnology and medicine.

Keywords: biotechnology, global optimization, and mathematical models.

1. INTRODUCTION

The systematic development of optimal microbial strains in biotechnology and efficient therapeutic interventions in medicine is a fundamental challenge for metabolic engineering in the post-genomic era (Kholodenko and Westerhoff 2004). Mathematical modeling is an indispensable tool in this endeavor by providing a *systematic* quantitative description of how changes in the system's properties (i.e., metabolic fluxes, concentrations, or cell growth) respond to changes in the system's components and environment (i.e., gene knockouts, enzyme activities, or gene expression). A variety of modeling frameworks to study metabolism are available. Stoichiometric models have been extensively used in biotechnology (Stephanopoulos, *et al.*, 1998; Palsson 2004) due to their relative simplicity to devise strategies of genetic modifications for targeted overproductions (Burgard, *et al.*, 2003; Pharkya, *et al.*, 2003; Pharkya, *et al.*, 2004). While successful in many instances (Ibarra, *et al.*, 2002; Burgard, *et al.*, 2004), stoichiometric models cannot capture regulatory effects mediated by metabolite concentrations and modulated enzyme levels. Metabolic Control Analysis (MCA) is an alternative modeling approach that employs a local log-linear approximation around the original steady-state (Kacser and Burns 1973; Heinrich and Rapoport 1974). MCA has been extensively used to provide deep insights as to how metabolism responds to small changes in metabolite concentrations or enzyme levels (Cornish-Bowden and Cardenas 1990; Heinrich and Schuster 1996; Fell 1997). Furthermore, MCA allows for the quantitative characterization of controlling enzymes and their activities, a crucial knowledge in biomedical applications (Cornish-Bowden and Cardenas 2000; Comin-Anduix, *et al.*, 2001). Because MCA is based on a log-linear approximation of inherently nonlinear kinetic models, MCA-based predictions tend to be valid only locally. However, typically genetic manipulations cause metabolic networks to allocate flux distributions that depart significantly from the original steady states. In response to these limitations, a number of research groups are exploring Michaelis-Menten type kinetic models (Rizzi, *et al.*, 1997; Chassagnole, *et al.*, 2002). Prominent examples are models developed at the ECell International Project (Tomita 2001), the minimal cell model (Castellanos, *et al.*, 2004), and virtual cell models (Slepchenko, *et al.*, 2003). The

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key challenges in these developments are the unraveling of regulatory structures and identification of kinetic constants needed for the model parameterization.

Given these challenges, various approaches and techniques have been developed to study kinetic models of moderate size and complexity. Specifically, evolutionary strategies have proved to be effective for fitting model parameters (Moles, *et al.*, 2003). Recently, genetic algorithms have been employed to understand the evolution of oscillatory reactions using a small kinetic model of glycolysis (Tsuchiya and Ross 2003). Minimization of internal concentrations of metabolites in biochemical systems has also been deployed to identify distinct time hierarchies in the corresponding kinetic models (Heinrich and Schuster 1996). To reduce the complexity of highly nonlinear kinetic models, an S-system approximation was developed (Savageau 1969) and then successfully used for the optimization of the integrated cellular performance (Voit 1992) and the optimal redesign of metabolic regulatory architectures (Hatzimanikatis, *et al.*, 1996). Recently, large-scale kinetic models have been used to assess capabilities of microbial strains for the overproduction of certain biochemicals (Mauch, *et al.*, 2001).

In this paper, a hybrid optimization framework is introduced to identify minimal enzyme sets to meet overproduction requirements in the context of large-scale kinetic models of metabolism. Since in most cases it is impractical to modulate levels of *all* enzymes in a pathway, the identification of relatively small enzyme sets (e.g., one, two, or three), whose manipulation leads to significant improvements in the production of useful biochemicals, is an important biotechnological target. Alternatively, this framework can also be used in biomedical studies to identify enzymes that control undesired large metabolite concentrations and fluxes. Such enzymes can then be ranked as candidates for potential biomarkers of the underlying diseases or drug targets. Because metabolism plays an important role in cellular systems by supplying them with energy and biosynthetic precursors, the large-scale kinetic model of the primary metabolism of *Escherichia coli* (Chassagnole, *et al.*, 2002) is chosen as the basis for benchmarking and presenting the developed optimization framework.

2. MATHEMATICAL DESCRIPTION OF KINETIC MODEL

A kinetic model of metabolic processes is usually postulated as a set of species mass balances and additional homeostasis constraints (Reich and Selkov 1981; Heinrich and Schuster 1996; Stephanopoulos, *et al.*, 1998). The kinetic dynamics is described by equation (1), where C_i is the concentration of species i, $i \in$ N, S_{ij} is the stoichiometric coefficient of species i in reaction j, $j \in$ M, and $r_j(r_j^{\max}, \mathbf{C}, \mathbf{K})$ is the rate of reaction j. Here $r_j^{\max}$ is the maximal reaction rate, **C** is the vector of metabolite concentrations, and **K** is the vector of kinetic parameters. N = {1,…,*N*} and M = {1,…,*M*} are sets of metabolites and reactions, respectively.

$$\frac{dC_i}{dt} = \sum_{j=1}^{M} S_{ij} \cdot r_j(r_j^{\max}, \mathbf{C}, \mathbf{K}), \ \forall i \in \mathrm{N}, \tag{1}$$

$$\frac{1}{M}\sum_{j=1}^{M}\frac{r_j^{\max}}{r_j^{\max,0}} = 1, \tag{2}$$

$$\frac{1}{N}\sum_{i=1}^{N}\frac{|C_i - C_i^0|}{C_i^0} \le d \ . \tag{3}$$

Since $r_j^{\max}$ represent the maximal specific enzyme activities (Stephanopoulos, *et al.*, 1998), ratios $r_j^{\max} / r_j^{\max,0}$ can be interpreted as the ratios of enzyme levels e_j and e_j^0 (i.e., $r_j^{\max} / r_j^{\max,0} = e_j / e_j^0$) for engineered and reference organisms, respectively. Constraint (2) thus assures that an increase in the levels of some enzymes will be compensated by the decrease in the rest enzymes' levels. This allows for the maintenance of physiologically meaningful protein levels excluding protein crowding and stresses due to the increased amino acids production and a limited amount of mRNA available (Mauch, *et al.*, 2001).

Kinetic models are valid only for the concentration ranges used in the estimation of model parameters. Specifically, constraint (3) enforces this restriction (Mauch, *et al.*, 2001) with d describing allowable concentration changes relative to the reference steady state concentrations $\mathbf{C_0}$ at the original enzyme levels $\mathbf{e_0}$.

3. SOLUTION METHOD

3.1 Nonlinear programming formulation

Optimal enzyme sets and levels can be identified by solving the following nonlinear formulation

$$\left(\begin{array}{ll} \textbf{maximize} & r_{j_0}(r_{j_0}^{\max}, \mathbf{C}) \\ \textbf{subject to} & \sum_{j=1}^{M} S_{ij} \cdot r_j(r_j^{\max}, \mathbf{C}) = 0 \\ & \frac{1}{M}\sum_{j=1}^{M}\frac{r_j^{\max}}{r_j^{\max,0}} = 1 \\ & \frac{1}{N}\sum_{i=1}^{N}\frac{|C_i - C_i^0|}{C_i^0} \le d \end{array}\right) ? \tag{5}$$

Here reaction rate $r_{j_0}(r_{j_0}^{\max}, \mathbf{C})$ is optimized to increase the production of a biochemical of interest (i.e., a product of reaction j_0). Each reaction rate $r_j(r_j^{\max}, \mathbf{C})$ explicitly depends on its own enzyme level $r_j^{\max}$ and steady state concentrations **C**. For simplicity, the dependence of $r_j(r_j^{\max}, \mathbf{C})$ on the other fixed kinetic parameters **K** is omitted. Note that

both steady state metabolite concentrations and reaction rates are implicitly coupled with *all* enzyme levels $r_j^{\max}$. In formulation (5), the indices of modulated enzymes (i.e., $E = \{j_1,..., j_K\}$) are integer variables and the enzyme levels (i.e., $r_{j_1}^{\max}, \ldots, r_{j_K}^{\max}$) are continuous variables. While the levels of modulated enzymes can be arbitrarily varied, the levels of non-modulated enzymes can either be kept constant or adjusted to account for limited amounts of mRNA available for transcription (see Sect. 4).

3.2 Search for Optimal Enzyme Sets

In the following pseudo-code, the simulated annealing algorithm (SA) is implemented to navigate through the discrete space of enzyme sets E until the optimal enzyme set is found

1. Generate an initial E_t
2. Set $E_b = E_c = E_t$
3. $r_b = r_c = r_t$ = Optimize(E_t)
4. `for` i = 1:*MaxIter*
5. E_t = Select(E_c)
6. r_t = Optimize(E_t)
7. `if` $r_t > r_b$
8. $r_b = r_c = r_t$
9. `else`
10. *anneal* = $e^{(r_t - r_c)/T}$
11. Generate a random $d \in (0,1)$
12. `if` $d <$ *anneal*
13. $E_c = E_t$
14. `end if`
15. `end if`
16. `if` [i/L] = 0
17. $T = \mathrm{a} \cdot T$
18. `end if`
19. Check_Stop_Condition
20. `end loop`

Here, E_b, is the "best" set found thus far with the best reaction rate r_b, E_c, is the current set with rate r_c, and E_t is the trial set with reaction rate r_t. T is the annealing temperature, reduced by factor a after L random moves, and *MaxIter* is the maximal number of allowable iterations. The heuristics utilized in the move class Select relies on a random swap between two enzymes, one chosen from the trial set (i.e., E_t) and another one chosen from the rest of all non-modulated enzymes (i.e., M\ E_t).

The SA algorithm belongs to a general class of direct search algorithms and is based on the analogy between the annealing of solids and solving combinatorial problems (Kirkpatrick, *et al.*, 1983). It guarantees the asymptotic convergence of trials E_t toward the optimal set E_b. Indeed, the move class Select is based on a uniform (i.e., *unbiased*) random search, followed by the Boltzmann-Metropolis acceptance criterion (see step 17) that adds *bias* toward a stationary Boltzmann distribution of enzyme sets accumulated around the optimal set E_b (Kirkpatrick, *et al.*, 1983; Salamon, *et al.*, 2002).

3.3 Elucidation of Optimal Enzyme Levels

The optimal enzyme levels for every given set E_t (see steps 3 and 6 in Sect. 3.2.), which maximize the given production rate $r_{j_0}(r_{j_0}^{\max}, \mathbf{C})$ (see (5)), can be computed standard nonlinear programming methods. The evaluation of $r_{j_0}(r_{j_0}^{\max}, \mathbf{C})$ relies on the calculation of stable steady state concentrations **C** using equation (1). Specifically, **C** is calculated in a two-step procedure, the first step of which is the direct integration of (1) over the time interval [0, t_{end}] until either condition $d\mathbf{C}_i(t)/dt \le e$ or $k \cdot t_{\mathrm{end}} = T_{\mathrm{stop}}$ is satisfied. Subsequently, the end condition of the integration step is used as an initial guess for Newton-based solvers to compute the solution, **C**, of nonlinear equations $\sum S_{ij} \cdot r_j(r_j^{\max}, \mathbf{C}) = 0$.

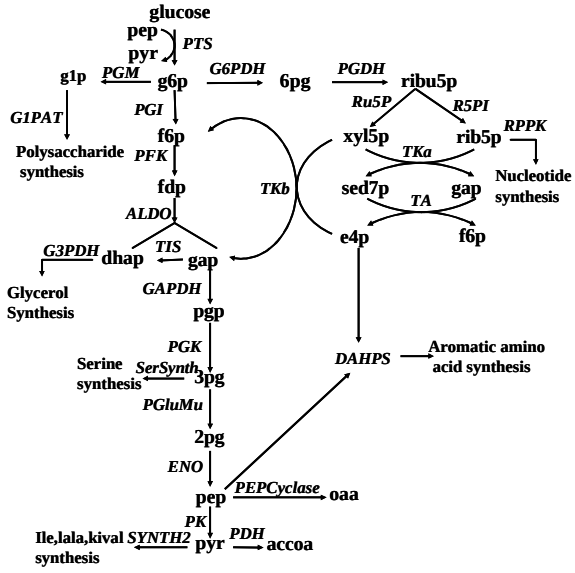

Fig. 1. Central metabolism of *Escherichia coli*.

3.3 Computational implementation

The kinetic model (Chassagnole, *et al.*, 2002) includes 30 enzymes and 17 metabolites (see Fig. 1). In all studies, only 10% variation in concentrations is allowed (i.e., d = 0.1). After some experimentation, the following parameters of the numeric procedures are set: *MaxIter* = 200, T = 0.5, L = 20, a = 0.9, e = 10^{-2}, $t_{\mathrm{end}} = 10^2$, and $T_{\mathrm{stop}} = 10^5$. Because of the very large variations in the maximal reaction rates $r_j^{\max,0}$, ranging from 10^{-4} to $5.1 \cdot 10^5$, the normalized rates $\tilde{r}_j^{\max}$ (i.e., $\tilde{r}_j^{\max} = r_j^{\max}/r_j^{\max,0}$) are used in all numeric procedures. Random multistarts have also been performed to check the robustness of the local search. The entire framework is implemented in Matlab©.

4. RESULTS AND DISCUSSION

Maximal serine overproduction using the *E.coli* model (Chassagnole, *et al.*, 2002) are examined for

the following two cases: 1) all enzyme levels are modulated, and 2) only glycolytic enzyme levels are modulated while the other levels are kept constant. Fig. 2*b* shows that in case 1, a 20-fold increase in serine production can be achieved, while in case 2 only a 7-fold increase is possible.

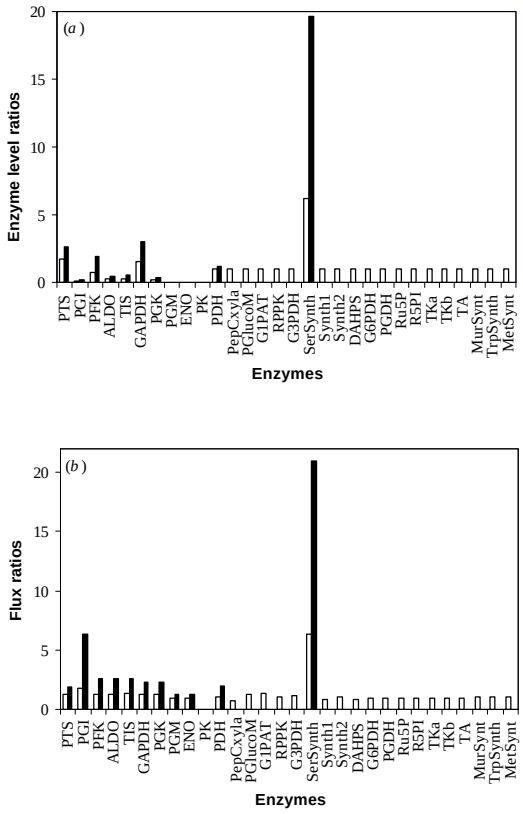

Fig. 2. (*a*) Enzyme level ratios and (*b*) reaction rate ratios. The solid and white bars correspond to the two cases, where all or only glycolytic enzyme levels are modulated, respectively.

The analysis of the optimal enzyme levels (see Fig.2*a*) shows that in case 1, the activities of all enzymes that do not contribute toward serine biosynthesis are shut down. For example, the activity the glycolytic pathway below the branching point at 3-phosphoglycerate (i.e., 3PG in Fig. 1) is blocked. Additionally, the pentose-phosphate pathway, the biosynthesis of polysaccharides, and aromatic amino acids are suppressed.

To investigate the predictive strength of Metabolic Control Analysis (MCA), the flux control coefficients for serine biosynthesis are computed and the FCC-based predictions are then compared with the optimization results, where only two enzyme levels are modulated. Specifically, for every pair of modulated enzymes i and i', the maximal serine production flux versus the sum $|FCC_i| + |FCC_{i'}|$ (see Fig. 3) is presented in Fig. 3. Calculation of FCCs leads to the following values: $FCC = 0.949$ for serine synthesis, $FCC = 0.192$ for phopshotransferase transport system, and the other FCCs have absolute values around 0.1 or much less. All enzyme sets form two clusters (see Fig. 3). The first cluster corresponds to the sets which do not include serine synthesis, while the second cluster corresponds to the sets where serine synthesis is present. Based on these observations, only a minor correlation between FCCs and serine production is established. The modulation of the enzyme levels with small FCCs leads to a small increase in serine production, while the modulation of the enzyme levels where serine biosynthesis is present leads to a considerably increase in serine production. This means that the FCC values alone do not necessarily pinpoint optimal enzyme manipulations, which becomes even more pronounced when more than two enzymes are modified simultaneously.

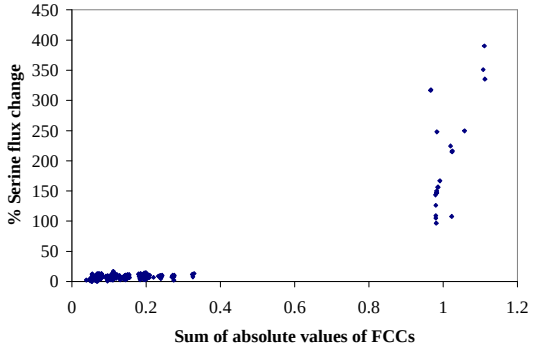

Fig. 3. Relative changes in serine production versus the sums of FCC absolute values.

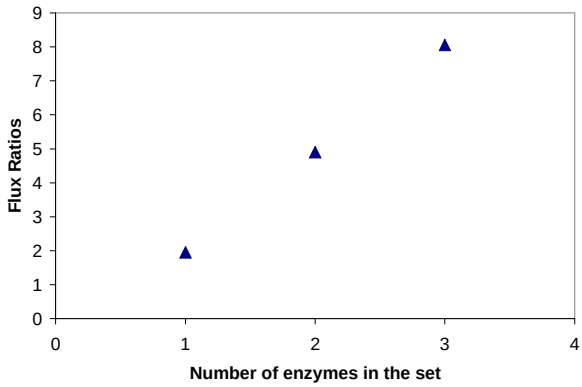

Fig. 4. The maximal ratios $r_{\mathrm{SerSynth}} / r^0_{\mathrm{SerSynth}}$ are plotted versus the number of modulated enzymes.

Subsequently, the best enzyme sets of one, two, and three enzymes are investigated (see Fig. 4). In this optimization study, the fixed ratios for non-modulated enzyme levels are used to accounts for homeostasis in transcriptional rates. Note that the optimal selection of three enzymes leads to the 8-fold increase in the serine production rate (i.e., 40% of the maximal theoretic prediction). This case corresponds to the 4-fold increase in the level of PFK (FCC=0.14), 10-fold increase in the level of serine biosynthesis (FCC=0.949), and complete elimination of PEPCxlylase (FCC=-0.126). The elimination of PEPCxlylase can be favorable because this enzyme is located below the branching point leading to the serine biosynthesis and hence its elimination can contributes toward the release of the additional

amount of mRNA utilized in the transcription of the increased enzyme levels.

In Table 1, alternative enzyme sets leading to a substantial increase in serine production are presented. Clearly, substantial improvements in serine production are feasible by manipulating only a small set of enzymes.

Table 1. Alternative best enzyme sets leading to increased serine production

Size of the Set	Enzyme Sets	Flux Ratio
1	SerSynth	1.95
	PGM	1.09
	ENO	1.08
	DAHPS	1.08
	Synth1	1.07
2	SerSynth, PTS	4.9
	SerSynth, PK	4.51
	SerSynth, PepCyclase	4.35
	SerSynth, PDH	4.18
	SerSynth2, Synth2	4.17
3	SerSynth, PFK, PepCyclase	8.06
	SerSynth, Synth2, Ru5P	7.49
	SerSynth, G1PAT, PFK	7.45
	SerSynth, Pglucom, PFK	7.29
	SerSynth, PK, PepCyclase	6.03

5. CONCLUSIONS

This paper has presented a hybrid optimization framework for optimal selection of enzyme levels and sets to enhance or suppress capabilities of cellular systems using large-scale kinetic models of metabolism. The simulating annealing algorithm (Kirkpatrick, *et al.*, 1983; Salamon, *et al.*, 2002) is employed to navigate through the discrete space of enzyme sets, while general nonlinear programming methods are used to pinpoint the optimal enzyme levels for the selected sets.

The proposed framework has been demonstrated on a large-scale kinetic model of central metabolism in *Escherichia coli* (Chassagnole, *et al.*, 2002) with the objective of serine overproduction. Computational results show that by systematically pinpointing relatively small enzyme sets significant many-fold improvements in serine production can be achieved. It is important to note that serine is not only an important target in biotechnology but also can be related to certain human diseases. Specifically, serine can act as a toxicant resulting in serious kidney damage (Bandara, *et al.*, 2003). Therefore in certain situations, it can be important to pinpoint and then diminish the activities of enzymes controlling serine biosynthesis. Thus, the framework and modeling studies described can serve as a basis for the further development of systematic approaches and frameworks to predict and control global effects of metabolic activities that can potentially contribute toward biosynthesis of important biochemicals.

5. NOMENCLATURE

Enzymes: aldolase (ALDO), DAHP synthases (DAHPS), enolase (ENO), glucose-1-phosphate adenyltransferase (G1PAT), glycerol-3-phosphate dehydrogenase (G3PDH), glucose-6-phosphate dehydrogenase (G6PDH), glyceraldehyde-3-phospahte dehydrogenase (GAPDH), isloleucine synthesis (IleSynth), methionine synthesis (MetSynth), mureine synthesis (MurSynth), phosphofructokinase (PFK), 6-phosphogluconate dehydrogenase (PGDH), glucose-6-phosphate isomerase (PGI), phosphoglycerate kinase (PGK), pyruvate dehydrogenase (PDH), PEP carboxylase (PEPCyclase), phosphoglucomutase (PGM), pyruvate kinase (PK), phopshotransferase system (PTS), ribose-phosphate isomerase (R5PI), ribose-phosphate pyrophosphokinase (RPPK), ribulose-phosphate epimerase (Ru5P), synthesis1 (Synth1), synthesis2 (Synth2), transaldolase (TA), triosephosphate isomerase (TPI), transketolase A (TKa), transketolase B (TKb), tryptophan synthesis (TrpSynth). *Metabolites*: 1,3-diphosphoglycerate (pgp), 2-phosphoglycerate (2PG), 3-phosphoglycerate (3PG), 6-phosphogluconate (6PG), acetyl-coenzyme A (accoA), dihydroxyacetonephophate (dhap), erythrose-4-phosphate (e4p), fructose-6-phosphate (f6p), fructose-1,6-bisphosphate (fdp), glucose-1-phosphate (g1p), glucose-6-phosphate (g6p), glyceraldehyde-3-phosphate (gap), glucose(glc), oxaloacetate (oaa), phosphoenolpyruvate (pep), pyruvate (pyr), ribose-5-phosphate (rib5P), ribulose-5-phospahte (ribu5p), sedoheptulose-7-phosphate (sed7p), xylulose-5-phosphate (xyl5p).

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