Metabolic Engineering 15 (2013) 25–33

Contents lists available at SciVerse ScienceDirect

Metabolic Engineering

journal homepage: www.elsevier.com/locate/ymben

Dynamic modeling of aerobic growth of *Shewanella oneidensis*. Predicting triauxic growth, flux distributions, and energy requirement for growth

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ARTICLE INFO

Article history:
Received 30 March 2012
Received in revised form
23 June 2012
Accepted 7 August 2012
Available online 26 September 2012

Keywords:
Shewanella oneidensis
Lumped hybrid cybernetic model (L-HCM)
Dynamic metabolic model
ATP requirement for growth
^{13}C-MFA

ABSTRACT

A model-based analysis is conducted to investigate metabolism of *Shewanella oneidensis* MR-1 strain in aerobic batch culture, which exhibits an intriguing growth pattern by sequentially consuming substrate (i.e., lactate) and by-products (i.e., pyruvate and acetate). A general protocol is presented for developing a detailed network-based dynamic model for *S. oneidensis* based on the Lumped Hybrid Cybernetic Model (L-HCM) framework. The L-HCM, although developed from only limited data, is shown to accurately reproduce exacting dynamic metabolic shifts, and provide reasonable estimates of energy requirement for growth. Flux distributions in *S. oneidensis* predicted by the L-HCM compare very favorably with ^{13}C-metabolic flux analysis results reported in the literature. Predictive accuracy is enhanced by incorporating measurements of only a few intracellular fluxes, in addition to extracellular metabolites. The L-HCM developed here for *S. oneidensis* is consequently a promising tool for the analysis of intracellular flux distribution and metabolic engineering.

1. Introduction

Shewanella oneidensis MR-1 is a facultative anaerobe that can be found in aquatic and sediment ecosystems. This strain preferentially grows on three-carbon sources (such as lactate) instead of six-carbon sugars. There has been particular attention to *S. oneidensis* because of its ability to respire with solid electron acceptors including Mn and Fe oxides. This feature makes *S. oneidensis* MR-1 an attractive tool for bioremediation, microbial fuel cell applications and biofuel production (Fredrickson et al., 2008; Hau and Gralnick, 2007).

The biotechnological potential of *S. oneidensis* MR-1 can greatly benefit from metabolic modeling and simulations which not only promote fundamental understanding, but also point to new rational strategies for performance improvements (Bailey, 1998). To do so, the model must provide reliable descriptions of key system properties. Two major components of metabolism are the network of metabolic pathways and their dynamic regulation. No models of *S. oneidensis* that incorporate both of the foregoing components have been reported.

Modeling studies of *S. oneidensis* have been conducted on metal reduction kinetics but these lack a connection to internal metabolism (Bonneville et al., 2006; Lall and Mitchell, 2007; Viamajala et al., 2003). Tang et al. (2007) developed a kinetic model for aerobic growth of *S. oneidensis* on lactate in batch culture. This lumped model neglected the internal structure of the metabolic network but focused on simulations of external metabolites, and described chromate reduction simply with link to biomass concentration only. Pinchuk et al. (2010) has conducted constraint-based analysis of *S. oneidensis* growth on numerous carbon sources using a genome-scale network model and discussed various metabolic traits under steady state conditions.

Our interest here is to develop a detailed network-based *dynamic* model. In this regard, the Lumped Hybrid Cybernetic Model (L-HCM) developed by Song and Ramkrishna (2010, 2011) provides an appropriate framework. L-HCMs have the following attributes: (i) they use cybernetic laws to account for dynamic cellular regulation (Kompala et al., 1986; Young and Ramkrishna, 2007), (ii) fairly large-scale networks can be handled using metabolic pathway analysis, and (iii) model identification can be made from limited data as the number of adjustable parameters is minimal due to the concept of pathway lumping. In short, L-HCM is a dynamic modeling framework which has a balanced consideration of both structural and functional components of metabolic systems (i.e., metabolic network and dynamic regulation) but avoids over-parameterization. In their recent papers, Song and Ramkrishna have demonstrated the L-HCM's capability of predicting complex dynamic response of *Escherichia coli* (as well as yeast) not only to environmental changes (Song and Ramkrishna, 2010; 2011), but also to genetic perturbations (Song and Ramkrishna, 2012). We use L-HCM to investigate *S. oneidensis* MR-1

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http://dx.doi.org/10.1016/j.ymben.2012.08.004

Nomenclature

Abbreviations

EM	elementary mode
FBA	flux balance analysis
GAR	growth rate-dependent ATP requirement
HCM	hybrid cybernetic model
L-EM	lumped elementary mode
L-HCM	lumped hybrid cybernetic model

Symbols

a	coefficient of yield term appearing in the structural return-on-investment (ROI)
b	coefficient of flux term appearing in the structural ROI
c	concentration of biomass (g/L)
e	enzyme level
K	Michaelis–Menten constant (mM)
k	uptake rate constant (mmol/gDW/h)
k_La	volumetric gas transfer coefficient (1/h)
$L(J_A)$	the set of indices of ATP-producing EMs of the Jth family
$L(J_B)$	the set of indices of biomass-producing EMs of the Jth family
n_{eta}	parameter controlling the sensitivity of L-EM to structural ROI
n_F	number of EM families
n_r	number of reactions
n_x	number of extracellular metabolites
p	return-on-investment
r	individual flux
$\mathbf{r}$	vector of individual fluxes
r_F	flux through EM family
$\mathbf{r_F}$	vector of fluxes through EM families
r_M	flux through individual EM
$\mathbf{S_x}$	stoichiometric coefficient matrix for extracellular metabolites
t	time (h)
u	cybernetic variable controlling synthesis of enzyme
v	cybernetic variable controlling activity of enzyme
x	concentration of extracellular metabolites (mM)
x_O^*	dissolved oxygen solubility limit (mM)
Y_A	ATP yield
Y_B	biomass yield
$\mathbf{z_F}$	L-EM
$\mathbf{z_A}$	ATP-producing L-EM
$\mathbf{z_B}$	biomass-producing L-EM
$\mathbf{Z_F}$	L-EM matrix

Greek letters

α	constitutive synthesis rate of enzyme (1/h)
β	degradation rate of enzyme (1/h)
ε	correction term to structural ROI
η	efficiency of EMs
μ	specific growth rate (1/h)
ρ	correlation coefficient

Subscripts

A	ATP or acetate
B	biomass
E	enzyme
F	family of EMs
j	index for individual EMs
J	index for EM families
L	lactate
M	individual EM
O	oxygen
P	pyruvate

Superscripts

kin	kinetic
max	maximum
rel	relative
st	stoichometric

aerobic growth by respiration of lactate. A complex (triauxic) growth pattern of *S. oneidensis* in batch culture, presumably driven by intricate regulatory circuits, poses a serious challenge to modeling.

In this article, we first developed a general protocol for constructing an L-HCM from batch data exhibiting sequential consumption of different alternative carbon sources. Second, we demonstrated the power of L-HCM in predicting intracellular flux distribution and their dynamic shift with time. Model performance is evaluated by comparing with constraint-based approaches, as well as with experimental data of our own and those available in the literature. Third, we discuss model's ability to estimate the energy requirement for cellular growth as a critical input parameter. Through this work, it is shown that modeling cellular regulation with cybernetic control laws provides an efficient route to describing the complex dynamic behavior of *S. oneidensis* particularly when considering large-scale metabolic networks.

2. Modeling framework

L-HCM is a dynamic metabolic modeling framework built on concepts including the cybernetic control laws, metabolic pathway analysis and pathway lumping. The mathematical essence is provided in the Appendix A, and the concepts are presented here. For the full description of L-HCM, the original papers of Song and Ramkrishna (2010, 2011) should be referred to.

The cellular network of biochemical reactions is orchestrated by a sophisticated regulatory machinery through controls upon both enzyme expression and activity. Instead of considering the mechanistic details of complex cellular regulation (e.g., as in Hardiman et al., 2010), the cybernetic modeling approach provides a dynamic description of regulation based on a view that cellular reactions are optimally driven towards maximizing a metabolic objective (Ramkrishna and Song, 2012). It is assumed here that the metabolic objective is the maximization of the carbon uptake flux into the cell. This hypothesis is translated into that the whole metabolism of microorganisms can undergo a drastic change whenever the total or individual uptake fluxes are significantly affected by any genetic modifications (Young et al., 2012) or environmental perturbations (Kim et al., 2012). Based on this perspective, a rational description of dynamic regulation is given in the form of the cybernetic control laws, which replace mechanistic details of regulation. While earlier formulations have focused on predicting various growth patterns on mixed substrates with a lumped description of networks (e.g.,

Kompala et al., 1986; Ramakrishna et al., 1996), current developments of cybernetic models based on quasi steady state approximation are able to systematically handle expanded networks.

Under quasi steady state approximation for intracellular metabolites, metabolic networks can be decomposed into elementary modes (EMs). In a simple sense, EMs can be viewed as subnetworks or pathways composed of a minimal set of reactions operating in steady state (Schuster et al., 1999; Schuster and Hilgetag, 1994). Any physiologically feasible flux distribution in steady state can be represented by nonnegative combinations of EMs. The cybernetic modeling framework incorporates EMs based on the following perspectives: (i) EMs are metabolic options for converting inputs (i.e., substrates) into outputs (i.e., products) and (ii) fluxes through EMs are optimally regulated towards maximizing a metabolic objective. These lead to the development of Hybrid Cybernetic Model (HCM) (Kim et al., 2008; Song et al., 2009). However, as larger metabolic networks are simulated, the number of EMs increases exponentially and this could lead to over-parameterization. To avoid this in HCM, EMs are reduced to a smaller subset through Metabolic Yield Analysis as developed by Song and Ramkrishna (2009). Thus, a systematic reduction of EMs to their minimal set is an essential part of a standard formulation of HCM.

The basic structure of HCM is inherited by L-HCM. The latter, however, resolves the over-parameterization issue by a different approach, EM lumping. In L-HCMs, EMs are lumped by taking a weighted average of EMs in a family characterized by some common feature, e.g., EMs consuming the same kind of substrate(s). The resulting lumped pathway is termed as Lumped EM (L-EM). L-HCM accounts for dynamic regulation over L-EMs, instead of individual EMs. Consequently, L-HCM features a small number of parameters which can be identified from limited experimental data (Song and Ramkrishna, 2010). In subsequent analysis, it becomes possible to identify significant EMs from the lumped entities by tuning lumping parameters through comparison with experimental data (Song and Ramkrishna, 2011).

3. Experiments

Shewanella oneidensis MR-1 was routinely maintained in aerobic tryptic soy broth at 30 °C. Controlled batches were conducted in modified M1 medium (Pinchuk et al., 2011) supplemented with 90 mM lactate and 1.5 mM Na_2SO_4. Batch experiments were performed in 6-liter Bioflo 3000 reactors (New Brunswick Scientific, Edison, NJ) at 3 l working volume. Agitation and gas flow rate were maintained at 450 rpm and 4 l/min, respectively. Dissolved O_2 tension was kept at 50% of air saturation by automatically controlling concentration of N_2, air, and O_2 in sparging gas. Other conditions were maintained as described elsewhere (Pinchuk et al., 2011). Samples (from 20 to 50 ml) were withdrawn periodically for measurements of optical density at 600 nm, dry weight biomass, and organic acids concentrations as described previously (Pinchuk et al., 2010).

4. Results and discussion

4.1. Model formulation

Using the framework described in the Appendix A, we developed an L-HCM for aerobic batch growth of *S. oneidensis* on lactate. L-HCM formulation generally goes through the following stages (Fig. 1): (i) reconstruction of a metabolic network, (ii) network decomposition into EMs, (iii) EM classification into different families, followed by EM lumping in each family, (iv) set-up of dynamic balance equations for metabolites of interest within the cybernetic modeling framework, and (v) parameter identification and model validation.

Metabolic network for the central metabolism of *S. oneidensis* was based upon the *E.* coli network reconstructed by Stelling et al. (2002). Biomass synthesis equation for *S. oneidensis* was formulated from Pinchuk et al. (2010). Similarly to *E. coli*, *S. oneidensis* MR-1 has gene sequences for the enzymes of the central carbon metabolism including Entner–Doudoroff pathway, pentose phosphate pathway, pyruvate dehydrogenase complex, tricarboxylic acid cycle, glyoxylate bypass, and anaplerotic reactions among others (Serres and Riley, 2006). Further, both *S. oneidensis* and *E. coli* have in common complete reaction routes for the synthesis of amino acids, nucleotides and cofactors. The use of carbon and energy sources in *S. oneidensis* is, however, more restricted than in *E. coli*. That is, experimental observation shows that *S. oneidensis* MR-1 does not grow on glucose. Rather, *S. oneidensis* prefers three-carbon sources such as lactate and pyruvate as well as two-carbon substrate such as acetate (Serres and Riley, 2006). The resulting metabolic network contained 119 reactions, and 113 metabolites (16 extracellular and 97 intracellular) (Tables S1 and S2). The network is decomposed using METATOOL v5.1 (von Kamp and Schuster, 2006), resulting in 112,545 EMs.

A simplified network is provided in Fig. 2. While included in Table S1 providing the full list of reactions, production of formate and ethanol is not displayed here as they are inactive in *S. oneidensis*

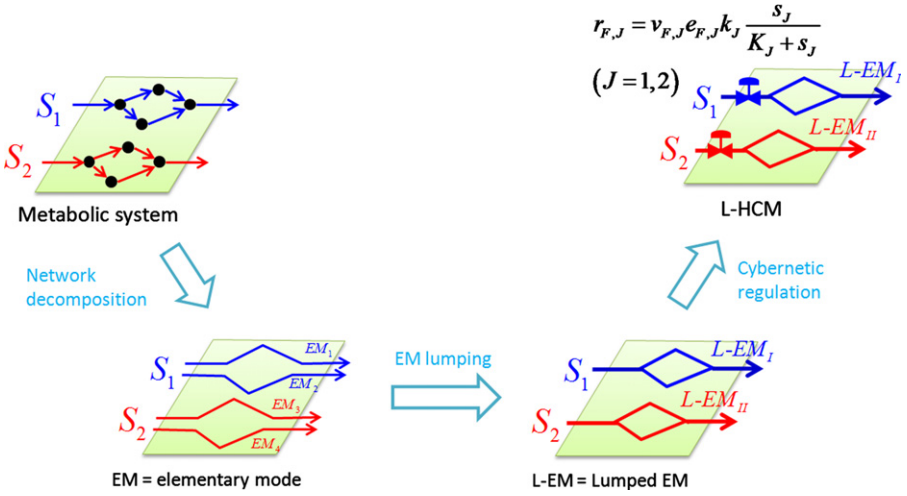

Fig. 1. Schematic illustrating the procedures of L-HCM construction.

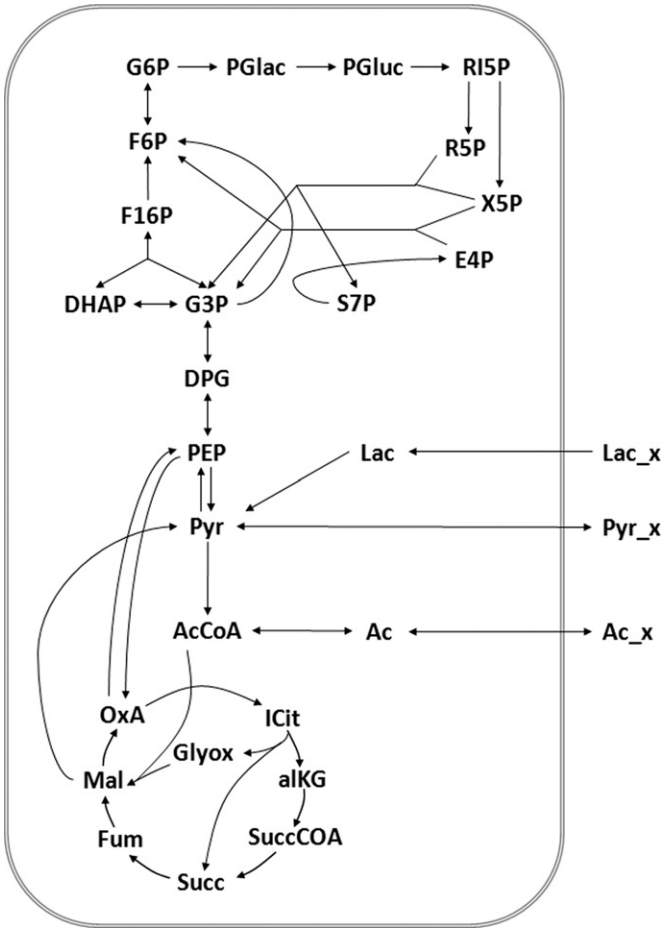

Fig. 2. Simplified metabolic network for the central metabolism of *S. oneidensis* (see Table S2 for full names of metabolites).

under aerobic conditions (Pinchuk et al., 2010; Sawers and Watson, 1998). Suppression of these reactions is automatically reflected in the model through the tuning of lumping parameters. That is, fluxes through EMs producing formate or ethanol are set to be negligibly low as a consequence of fitting to experimental data on pyruvate and acetate.

During the initial period of aerobic batch culture, *S. oneidensis* grew on lactate, producing pyruvate and acetate as by-products. In the next phase where the preferred substrate (i.e., lactate) is depleted, pyruvate was utilized as an alternative carbon source for further growth with acetate produced. When pyruvate is depleted, acetate was finally consumed. Based on these experimental observations, EMs are classified into three different families (Table 1): aerobic growth on (i) lactate, (ii) pyruvate, and (iii) acetate. In L-HCM, this sequential growth of *S. oneidensis* is described as the outcome of optimal modulation of three lumped pathways (identified in Table 1) towards maximizing the carbon uptake flux into the cell.

Model equations along with parameter values are presented in Table 2. Most of the parameters are fixed to their standard values typically used in cybernetic modeling without seeking their optimal values. Our justifications for this are as follows. (i) The parameters associated with enzyme synthesis equations (such as $k_{E,J}$, $\alpha_{F,J}$, and $\beta_{F,J}$) and Michaelis–Menten constants do not affect model predictions of batch growth in a certain range (Baltes et al., 1994; Song and Ramkrishna, 2011). (ii) O_2 solubility limit and volumetric mass transfer coefficient for O_2 are parameters which should be determined from thermodynamic and operating conditions. (iii) Yield coefficients (denoted by $Y_{X,J}$ where the subscript X can be any metabolites) are given from EM lumping. Thus, we have only three parameters to optimize from data fitting, i.e., maximum uptake rate constants, $k_J^{\max}$ $(J=I,II,III)$.

It is important to note that the number of parameters to be optimized in L-HCM is significantly small. An attempt to simulate triauxic growth on multiple carbon sources including external substrates and metabolic products solely using ad hoc inhibition kinetics would unavoidably result in a large number of parameters. Not surprisingly, the black-box model by Tang et al. (2007) optimized 8 parameters (of 18 in total) to describe the simpler case involving only lactate and acetate. Consequently, extensive experimental data were needed to determine optimal values of parameters. A further increase in parameters would have been warranted had production and consumption of pyruvate also been included in their model.

Table 1
EM classification according to their main substrates: lacate (family I), pyruvate (family II), and acetate (family III). Y denotes stoichiometric coefficients of metabolites.

EM family	Main substrate	Secondary substrate	No. of EMs	L-EM
I[a]	Lactate (L)	Oxygen (O)	35,647	$L+Y_{O,I}O\rightarrow Y_{B,I}B+Y_{P,I}P+Y_{A,I}A+\cdots$
II	Pyruvate (P)		24,526	$P+Y_{O,II}O\rightarrow Y_{B,II}B+Y_{A,II}A+\cdots$
III	Acetate (A)		4312	$A+Y_{O,III}O\rightarrow Y_{B,III}B+\cdots$

[a] In sorting out Family I, EMs consuming D-lactate are neglected assuming that all lactates are of the L-form.

Table 2
Collection of model equations and parameters.

Dynamic balances for extracellular metabolites, biomass and enzymes[a]

Extracellular metabolites:

$$\frac{dx_L}{dt}=-\nu_{F,I}e_{F,I}^{rel}r_{F,I}^{kin}c \quad \text{(Lactate)}$$

$$\frac{dx_P}{dt}=(Y_{P,I}\nu_{F,I}e_{F,I}^{rel}r_{F,I}^{kin}-\nu_{F,II}e_{F,II}^{rel}r_{F,II}^{kin})c \quad \text{(Pyruvate)}$$

$$\frac{dx_A}{dt}=\left(\sum_{J=I}^{II}Y_{A,J}\nu_{F,J}e_{F,J}^{rel}r_{F,J}^{kin}-\nu_{F,III}e_{F,III}^{rel}r_{F,III}^{kin}\right)c \quad \text{(Acetate)}$$

$$\frac{dx_O}{dt}=-\left(\sum_{J=I}^{III}Y_{O,J}\nu_{F,J}e_{F,J}^{rel}r_{F,J}^{kin}\right)c+k_La(x_O^*-x_O) \quad \text{(Oxygen)}$$

Biomass:

$$\frac{dc}{dt}=\mu c \quad \left(\text{where } \mu=\sum_{J=I}^{III}Y_{B,J}\nu_{F,J}e_{F,J}^{rel}r_{F,J}^{kin}\right)$$

Enzymes:

$$\frac{de_{F,J}}{dt}=\alpha_{F,J}+u_{F,J}r_{FE,J}^{kin}-(\beta_{F,J}+\mu)e_{F,J} \quad (J=I,II,III)$$

Kinetics

$$r_{F,J}^{kin}=k_J^{\max}[r_{F,J}^{kin}]_c;\quad r_{FE,J}^{kin}=k_{E,J}[r_{F,J}^{kin}]_c \quad (J=I,II,III)$$

where

$$[r_{F,J}^{kin}]_c=\begin{cases}\frac{x_Lx_O}{(K_L+x_L)(K_O+x_O)} & (J=I)\\ \frac{x_Px_O}{(K_P+x_P)(K_O+x_O)} & (J=II)\\ \frac{x_Ax_O}{(K_A+x_A)(K_O+x_O)} & (J=III)\end{cases}$$

Parameter values fixed a priori

$$K_L=K_P=K_A=0.02\ (\text{mM}),\quad K_O=0.006\ (\text{mM}),$$
$$k_{E,J}=1(J=I,II,III),\quad \alpha_{F,J}=0.01\ (J=I,II,III),\quad \beta_{F,J}=0.05\ (J=I,II,III),$$
$$k_La=40\ (1/\text{h}),\quad x_O^*=0.238\ (\text{mM})$$

[a] The meaning of symbols used in the equations is defined in Appendix A and nomenclature section.

4.2. Guidelines for data-incorporated EM lumping

The performance of L-HCM depends on the accuracy with which L-EMs can represent actual flux distributions in a cell. An L-EM is acquired by taking a weighted average of individual EMs

in each family; fluxes through EMs are used as weighting factors. We assume that EM fluxes are proportional to their stoichiometric efficiency, as determined by the yield of biomass or ATP produced. This initial estimate is corrected by tuning the weighting factors through the incorporation of experimental measurements into EM lumping (see the Appendix A for details). While both extracellular and intracellular metabolite data can theoretically be used, here we considered only dynamic measurements on extracellular metabolites.

When lumping parameters are tuned, the apparent number of parameters for optimization increases. However, no additional data are required for the identification of lumping parameters, other than batch culture data which are used for determining kinetic parameters, i.e., $k_J^{\max}$ ($J=I,II,III$). It is precisely this feature that gives L-HCM its control on parameterization without compromise of its predictive capabilities.

In this system of sequential growth on multiple alternative carbon sources, a strategy was needed for EM lumping due to difficulty in extracting product yields from batch growth data. Thus, we provide basic guidelines for EM lumping as follows:

- First, dynamic batch data are fitted using *empirical* algebraic equations. Fitted equations instead of raw data are used to extract experimental information. Empirical equations are useful in smoothing out raw data which may suffer from significant fluctuations. For this purpose, we use logistic equations suggested from Goudar et al., (2005).
- Growth of *S. oneidensis* in batch culture was separated into three phases: lactate, pyruvate; and acetate. Yields of products and biomass in each regime were determined using logistic equations fitted in the previous step. These must be viewed as *rough* initial estimates due to uncertainties in distinguishing different phases.

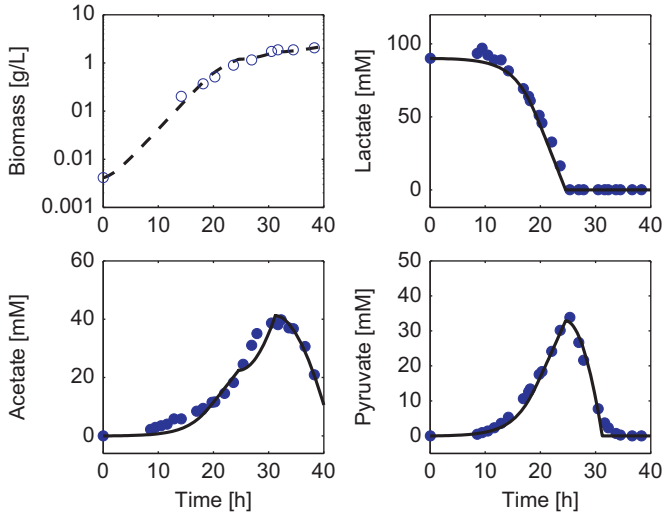

Fig. 3. Aerobic batch data of *S. oneidensis* MR-1 along with model fit (solid lines) and prediction (dashed lines).

- More accurate values of product yields are sought from dynamic curve fitting *using a lumped cybernetic model* similar to the one used by Jones and Kompala (1999) which can be readily constructed at a low cost (see Supp Text A).
- Finally, the resulting products yields are used for tuning the lumping parameters.

Detailed procedures of yield extraction for parameter tuning are provided in Supp Text A by employing batch data collected under the conditions described in Experiments section. The same method has been used to develop models for other similar experimental systems (Tang et al., 2009). In all cases, the effect of data incorporation into EM lumping is shown to be significant.

4.3. Basic analysis of aerobic batch growth

Experimental data of *S. oneidensis* growth on lactate are presented in Fig. 3 along with model simulations. Following the modeling protocol established in the previous section, we extract yield data from dynamic trajectories of extracellular metabolite concentrations, tune lumping parameters, and finally, determine optimal values of three maximum uptake rate constants. Among four measured biochemical species, only lactate, pyruvate and acetate data are used for parameter optimization while biomass data is compared with prediction for model validation. With adjustment of only three parameters (i.e., maximal uptake rate constants), L-HCM provides excellent fit and prediction as measured by the coefficient of determination or R^2(see Table 3). The growth rate-dependent ATP requirement (GAR) is input to L-HCM (Appendix A) and was set based upon Pinchuk et al. (2010). Implication of GAR is discussed in detail in a subsequent section.

4.4. Simulation of growth under salt-stress conditions

L-HCM was also used to model *S. oneidensis* growth under salt-stress using experimental data provided from Tang et al. (2009). First, for comparison, we modeled normal growth of *S. oneidensis* using the same source of data following the procedures shown in Supp Text A. Then, the response to salt-stress was modeled by modifying kinetic equations to include the effect of sodium chloride in the culture medium. An underlying assumption for this is that addition of sodium chloride to the medium results in reduction in growth and production rates, with no prominent effect on the product yields. The dynamic response of *S. oneidensis* to salt-stress is simply simulated by refitting only three maximum uptake rate constants, i.e., $k_J^{\max}$ ($J=I,II,III$).

Simulation results are consistent with experimental data as shown in Fig. 4 and Table 3. As the salt-stress data are limited to the first phase of growth on lactate, the uptake rate constants for pyruvate and acetate (i.e., $k_{II}^{\max}$ and $k_{III}^{\max}$) may not be accurate.

Unlike the previous case shown in Fig. 3 where biomass data is predicted (as denoted by dashed line) with a specific value of GAR given from the literature suggestion, the GAR value in Fig. 4 was adjusted such that the model can generate an experiment-consistent biomass curve by fitting other data. While not providing

Table 3
Kinetic parameters optimized through data fitting and goodness of fit.

Fig.	$k_J^{\max}$			Coefficient of determination (R^2)			
	$J=I$	II	III	Lactate	Pyruvate	Acetate	Biomass
3	22.1 ± 0.35	8.19 ± 0.58	4.39 ± 0.35	0.995	0.976	0.964	0.978
4 (normal)	16.0 ± 0.14	11.5 ± 1.79	9.38 ± 1.34	0.950	0.991	0.954	0.966
4 (salt-stress)	10.0 ± 11.8	$0.094 \pm 7.9e3$	$0.099 \pm 5.1e3$	0.909	0.988	0.803	0.749

a prediction of biomass (as denoted by solid line) in a true sense, this scenario offers a way to estimate a GAR value.

4.5. Prediction of intracellular flux distribution

The L-HCM, although identified from limited analyses of extracellular metabolites, is able to predict intracellular flux distribution. Fig. 5 compares flux values from L-HCM and ^{13}C-Metabolic Flux Analysis (^{13}C-MFA) (Tang et al., 2009) for the growth under normal and salt-stress conditions. Model predictions of flux distribution are made during the balanced growth phase, i.e., at 15 h for the normal growth, and at 20 h for the salt-stress data. The correlations between L-HCM prediction and ^{13}C-MFA results (denoted by ρ) are 0.951 (for normal condition) and 0.952 (for salt-stress condition). No appreciable change in flux distribution took place under normal and salt-stress conditions, not only in L-HCM prediction (Fig. S3(a)), but also in ^{13}C-MFA (Fig. S3(b)). This provides additional support for model prediction.

Fig. 6 compares predictions by L-HCM and flux balance analysis (FBA) (Orth et al., 2010) for flux distribution under normal growth condition. The FBA estimation was made such that the biomass yield is maximized under the following constraints: experimentally measured uptake fluxes of lactate, pyruvate, and acetate (Tang et al., 2009), and ATP requirement for growth (i.e., GAR value). Under these typical constraints, L-HCM shows a higher correlation with ^{13}C-MFA fluxes than FBA, while the latter shows progressive improvement as an increasing number of constraints are considered (Fig. S4). Details on model estimation of flux distribution are provided in Supp Text B.

The most striking difference between L-HCM and FBA is the ability of the former to predict dynamic shifts in flux distribution with time. Video S1 illustrates how L-HCM predicts dynamic changes in flux distribution as *S. oneidensis* goes through the three different growth phases.

Supplementary material related to this article can be found online at http://dx.doi.org/10.1016/j.ymben.2012.08.004.

L-HCM prediction can be further improved by tuning weights in L-EMs with a few intracellular measurements. Fig. 7 shows the improvement in correlation between measurement and prediction by implicating "outliers" in the tuning process.

4.6. Implication of GAR and its estimation

Finally, we discuss how GAR value affects L-HCM prediction and to what extent it could be estimated using available data. As presented in the Appendix A, GAR is an essential input to computation of L-EMs. GAR value of *S. oneidensis* was previously estimated using constraint-based analysis by accounting for the energy expenditure on unknown maintenance processes that may include protein and mRNA turnover or repair, proton leakage, and maintenance of membrane integrity (Pinchuk et al., 2010). In our work, GAR accounts for ATP requirements for polymerization reactions

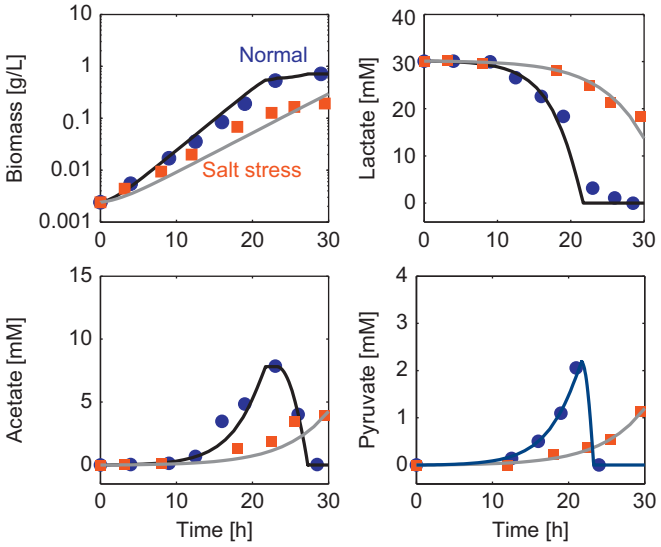

Fig. 4. Aerobic batch data of *S. oneidensis* MR-1 under normal (circle) and salt-stress conditions (square) and L-HCM simulation curves. Data from Tang et al. (2009).

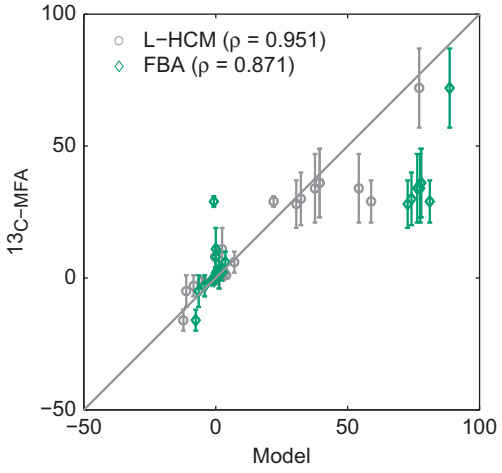

Fig. 6. Prediction of flux distribution by L-HCM (circle) and FBA (diamond). Data from Tang et al. (2009).

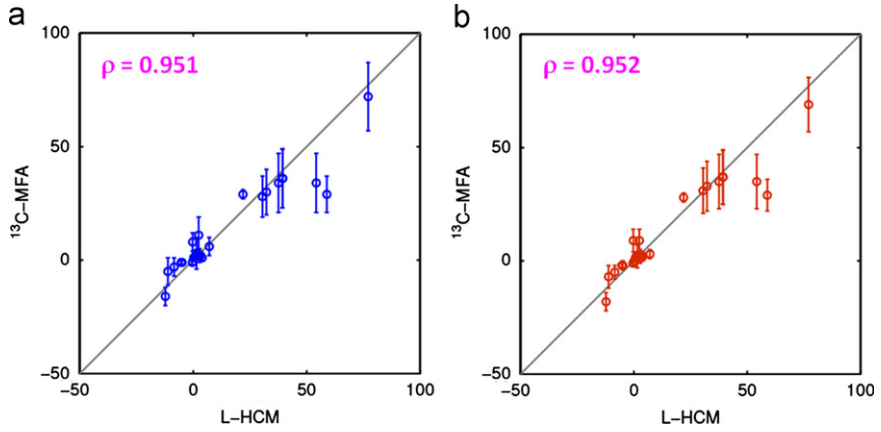

Fig. 5. Flux distribution predicted by L-HCM: (a) normal growth condition, (b) salt-stress condition. Data from Tang et al. (2009).

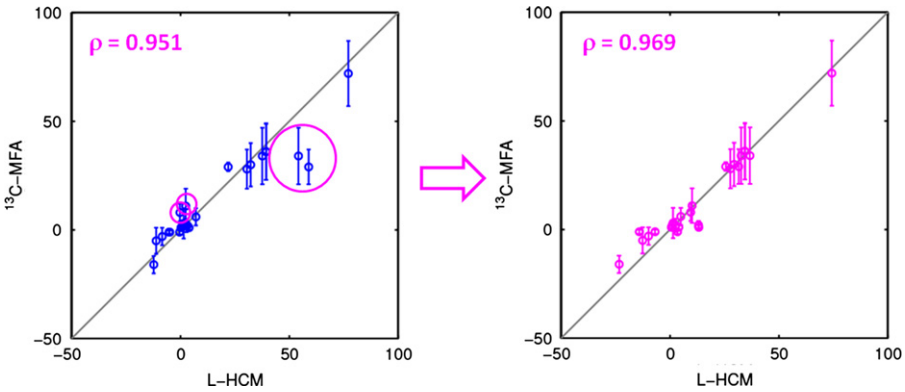

Fig. 7. Improvement of L-HCM prediction by incorporating four outliers into EM lumping. Data from Tang et al. (2009).

(GAR_p) and (growth-associated) maintenance (GAR_m). The former denotes the amount of ATP needed for the synthesis of macromolecular biomass components from carbon sources while the latter corresponds to GAR described by Pinchuk et al. (2010). Thus, here the total GAR is the sum of GAR_p and GAR_m.

At first Pinchuk et al. (2010) estimated GAR_m to be 220 [mmol ATP/gDW], which is significantly higher in comparison to that of *E. coli* and other bacteria. Subsequently, gene knock-out experiments and comparative analysis of different *Shewanella* species led to the conclusion that three most energetically efficient enzymes for H^+ pumping (i.e., Nuo, Cco and Cox) were inactive under tested experimental conditions, and a revised estimate of 81 for GAR_m was calculated. As GAR_p is calculated to be 36 from the network, the GAR_m values of 220 and 81 are converted to the total GAR values of 256 and 117, respectively. With this correction, the latter value of energy requirement for growth in *S. oneidensis* is now in the range of GAR for *E. coli* which has been reported as 97 (Hempfling and Mainzer, 1975; Stephanopoulos et al., 1998) and 118 (Shuler and Kargi, 2002).

Interestingly, L-HCM prediction of biomass is highly accurate with the corrected value of GAR (i.e., 117), while poor with the initial estimation (i.e., 256). In other words, reliable prediction by L-HCM requires a physiologically meaningful value of GAR in the current setting. This also suggests that, should information of ATP requirement for growth be unavailable, GAR can be estimated using L-HCM by *fitting* biomass data.

Two sets of normal growth data shown in Figs. 3 and 4 differ in their metabolic features. Biomass yields (at the end of batches), and values of pyruvate and acetate (at the peaks) normalized with respect to initial lactate concentration are 0.023, 0.37 and 0.46 in Fig. 3, while 0.024, 0.073 and 0.26 in Fig. 4. That is, pyruvate production is significantly suppressed when initial concentration of lactate is relatively low as in Fig. 4. Due to this qualitative dissimilarity, data in Fig. 4 are fitted with completely different values of parameters and GAR is estimated at 165 (for normal growth), which is higher than the one used in Fig. 3 (i.e., 117). The same value of GAR was used for simulating salt-stress condition in Fig. 4 for which data are limited to growth on lactate only. Estimation of more reasonable values of GAR requires collection of complete set of data including gaseous components such as CO_2 which is, however, missing in cases considered in the present work.

5. Conclusion

L-HCM used to investigate the growth dynamics of *S. oneidensis* on lactate in an aerobic batch culture is able to predict experimental intracellular fluxomic data as well as their dynamic redistribution. While prediction of significant dynamic change in flux distribution has not been fully validated due to the lack of data, obviously, it is beyond the scope of constraint-based approaches. Systematic procedures are established in this paper for modeling of metabolic systems exhibiting sequential growth on different carbon compounds. This work also provides the basis for modeling *S. oneidensis* under more complex environments subject to electron acceptor and/or carbon limitations.

Acknowledgments

This research was supported by the Genomic Science Program (GSP), Office of Biological and Environmental Research (OBER), U.S. Department of Energy (DOE), and is a contribution of the Pacific Northwest National Laboratory (PNNL) Foundation and Biofuels Scientific Focus Areas. Partial support was also provided by the Center for Science of Information (CSoI), an NSF Science and Technology Center, under Grant agreement CCF-0939370.

Appendix:A. The L-HCM framework

Mathematical aspects of the L-HCM framework are provided here. Material balances for extracellular metabolites are given as follows:

$$\frac{1}{c}\frac{d\mathbf{x}}{dt} = \mathbf{S_x r} \tag{A1}$$

where t is time, $\mathbf{x}$ is the vector of n_x concentrations of extracellular components including biomass (c), $\mathbf{S_x}$ is the $(n_x \times n_r)$ stoichiometric matrix, and $\mathbf{r}$ is the vector of n_r fluxes. In L-HCM, $\mathbf{r}$ is represented as nonnegative combinations of L-EMs, i.e.,

$$\mathbf{r} = \mathbf{Z_F r_F} \tag{A2}$$

where the vector of weights ($\mathbf{r_F}$) is composed of n_F fluxes through L-EMs and $\mathbf{Z_F}$ is $(n_r \times n_F)$ L-EM matrix. A methodology for formulating $\mathbf{Z_F}$ is provided in a subsequent section. Substitution of (A2) into Eq. (A1) leads to

$$\frac{1}{c}\frac{d\mathbf{x}}{dt} = \mathbf{S_x Z_F r_F} \tag{A3}$$

Normalization of each column of $\mathbf{S_x Z_F}$ (with respect to the uptake flux of the reference substrate which is always nonzero) defines $\mathbf{r_F}$ as the "uptake" fluxes through L-EMs.

Description of regulation using cybernetic control laws

The L-HCM takes an L-EM as a functional unit for describing dynamic cellular regulation. In its view, uptake fluxes through L-EMs are optimally regulated by adjusting enzyme level and their

activity such that a metabolic objective (i.e., the total carbon flux into the cell) is maximized. Thus, uptake flux through the Jth L-EM is modeled as follows:

$$r_{F,J} = v_{F,J} e^{rel}_{F,J} r^{kin}_{F,J} \tag{A4}$$

where $e^{rel}_{F,J}$ and $v_{F,J}$ denote the relative enzyme level and cybernetic variable (controlling enzyme activity), respectively, and $r^{kin}_{F,J}$ is the unregulated flux term described only by kinetics. If we take a simple Michaelis–Menten (M–M) form,

$$r^{kin}_{F,J} = k^{\max}_J \frac{s_J}{K_J + s_J} \tag{A5}$$

where s_J is the concentration of the Jth substrate, and $k^{\max}_J$and K_J are the maximum uptake rate constant and M–M constant, respectively, for the Jth family of EMs.

Relative enzyme level in Eq. (A4) is defined as

$$e^{rel}_{F,J} = \frac{e_{F,J}}{e^{\max}_{F,J}} \tag{A6}$$

where $e_{F,J}$ is the level of enzyme catalyzing EM throughput flux and $e^{\max}_{F,J}$ is the maximal value of $e_{F,J}$ at steady state. Enzyme level ($e_{F,J}$) is determined by solving the following dynamic balance equation, i.e.,

$$\frac{de_{F,J}}{dt} = \alpha_{F,J} + u_{F,J} r^{kin}_{FE,J} - (\beta_{F,J} + \mu) e_{F,J} \tag{A7}$$

The four terms of the right-hand side in the above denote constitutive and inducible synthesis rates, degradation rate, and dilution rate by growth, respectively, $u_{F,J}$ is the cybernetic variable regulating the induction of enzyme synthesis, and $r^{kin}_{FE,J}$ is the kinetic part of inducible enzyme synthesis rate. From Eq. (A7), the expression for $e^{\max}_{F,J}$ is given as follows:

$$e^{\max}_{F,J} = \frac{\alpha_{F,J} + k_{E,J}}{(\beta_{F,J} + Y_{F,J} r^{kin,\max}_{F,J})} \tag{A8}$$

Optimal control of enzyme level and their activity towards maximizing the total carbon uptake flux is implemented by the cybernetic variables $u_{F,J}$ and $v_{F,J}$ in Eqs. (A4) and (A7) as given in the following, i.e.,

$$u_{F,J} = \frac{p_J}{\sum_K p_K} \quad \text{and} \quad v_{F,J} = \frac{p_J}{\max_K (p_K)} \tag{A9}$$

where p_J denotes the return-on-investment (ROI) such as the carbon uptake rate through the Jth family. Optimality properties of Eq. (A9) have been demonstrated by Young and Ramkrishna (2007).

EM lumping

The essence of L-HCM lies in getting L-EMs, i.e., $\mathbf{Z_F}$ in Eqs. (A2) and (A3). It stars from EM classification into different families according to their commonalities (such as substrate(s) shared among EMs). In each family, EMs are dividing into biomass-producing group (including both biomass and ATP-producing modes), and ATP-only-producing group (referred to as B- and A-groups, hereafter). EMs in each group are lumped by taking a weighted average as follows:

$$\mathbf{z}_{\mathbf{B},J} \text{ (or } \mathbf{z}_{\mathbf{A},J}) \equiv \frac{\sum_{j \in L(J_B) \text{ (or } L(J_A))} \mathbf{z}_j r_{M,j}}{\sum_{j \in L(J_B) \text{ (or } L(J_A))} r_{M,j}} \tag{A10}$$

where $\mathbf{z}_j$ denote the jth EM, $r_{M,j}$ is the uptake flux through $\mathbf{z}_j$, and $L(J_B)$ and $L(J_A)$ are the set of indices of EMs belonging to the B- and A-groups, respectively, of the Jth family. Subsequently, L-EM for each family is obtained by combining $\mathbf{z}_{\mathbf{B},J}$ and $\mathbf{z}_{\mathbf{A},J}$ defined in Eq. (A10), i.e.,

$$\mathbf{z}_{\mathbf{F},J} = w_J \mathbf{z}_{\mathbf{B},J} + (1 - w_J)\, \mathbf{z}_{\mathbf{A},J}, \quad 0 \le w_J \le 1 \tag{A11}$$

where the parameter w_J is determined such that energy requirement for growth (i.e., GAR) is satisfied.

Note that L-HCM selects dominant modes according to their actual rates instead of yields. Thus, all EMs with appreciable fluxes contribute to formulating L-EMs. This is a striking contrast with flux balance analysis which takes only a single optimal pathway (among many alternatives) with the highest yield of biomass. We discuss an idea of identifying EM fluxes in the following section.

Estimation of individual EM fluxes

As regulation of individual EM fluxes within each family is synchronized through the dynamic regulation of L-EM, the flux through the jth EM (i.e., $r_{M,j}$) is formulated as follows:

$$r_{M,j} = v_{F,J} e^{rel}_{F,J} r^{st}_{M,j} \tag{A12}$$

where the first two variables on the right-hand side in Eq. (A12) account for the dynamic modulation among EM families. The static element of $r_{M,j}$ (i.e., $r^{st}_{M,j}$) is given in a typical cybernetic modeling form as follows:

$$r^{st}_{M,j} = v_{M,j} e^{rel}_{M,j} r^{kin}_{M,j} \tag{A13}$$

where $e^{rel}_{M,j}$, $v_{M,j}$, and $r^{kin}_{M,j}$ denote relative enzyme level, cybernetic variable (controlling enzyme activity), and unregulated flux term described by kinetics, respectively, for the jth EM. Under the conditions specified in Song and Ramkrishna (2010), relative enzyme level $e^{rel}_{M,j}$ of Eq. (A13) can be approximated by the cybernetic variable $u_{M,j}$, i.e.,

$$r^{st}_{M,j} \approx v_{M,j} u_{M,j} r^{kin}_{M,j} \tag{A14}$$

In L-HCM, we assume that $r^{kin}_{M,j}$ in Eq. (A14) is assumed to be proportional to the *efficiency* of the jth mode (which is denoted by η_j) in B- and A-groups, i.e.,

$$r^{kin}_{M,j} = k^{\max}_{jJ} \eta_j \frac{s_J}{K_J + s_J}, \quad k^{\max}_{jJ} = \begin{cases} k^{\max}_{B,J} & (\text{for } B\text{-group}) \\ k^{\max}_{A,J} & (\text{for } A\text{-group}) \end{cases} \tag{A15}$$

Note that the functional form of $r^{kin}_{M,j}$ above is related to $r^{kin}_{M,J}$ given in Eq. (A5).

Cybernetic variables $u_{M,j}$ and $v_{M,j}$ in Eqs. (A13) and (A14) are obtained from the cybernetic control laws in B- and A-groups, separately, without having to identify the parameters $k^{\max}_{B,J}$ and $k^{\max}_{A,J}$, i.e.,

$$u_{M,j} = \frac{\eta_j}{\sum_k \eta_k}, \quad v_{M,j} = \frac{\eta_j}{\max_k \eta_k}, \quad k \in \begin{cases} L(J_B) & (\text{for } B\text{-group}) \\ L(J_A) & (\text{for } A\text{-group}) \end{cases} \tag{A16}$$

Substitution of Eq. (A12) together with Eqs. (A14), (A15) and (A16) into Eq. (A10) leads to

$$\mathbf{z}_{\mathbf{B},J} \text{ (or } \mathbf{z}_{\mathbf{A},J}) = \frac{\sum_{j \in L(J_B) \text{ (or } L(J_A))} \mathbf{z}_j \eta_j^3}{\sum_{j \in L(J_B) \text{ (or } L(J_A))} \eta_j^3} \tag{A17}$$

As a basic hypothesis, the parameter η_j is defined as being proportional to yields of biomass (for B-group) or ATP (for A-group) of individual EMs. As this very initial hypothesis may be invalid in general, we introduce a correction term to η_j, i.e.,

$$\eta_j = \begin{cases} (Y_{B,j} + \varepsilon_j)^{n_{eta}}, & j \in L(J_B) \\ (Y_{A,j} + \varepsilon_j)^{n_{eta}}, & j \in L(J_A) \end{cases} \tag{A18}$$

where $Y_{B,j}$ and $Y_{A,j}$ denote the yields of biomass and ATP of the jth mode, respectively, ε_j is the correction to η_j, and n_{eta} is a tuning

parameter. Through ε_j, we are able to adjust η_j so that the resulting L-EM provides suitable estimations matching with measured yields and fluxes. The correction term ε_j is formulated as follows:

$$\varepsilon_j = \sum_i a_i Y_{i,j} + \sum_k b_k z_{k,j} \quad \text{(A19)}$$

where $Y_{i,j}$ and $z_{k,j}$ are the yield of the ith metabolite and the kth flux, respectively, of the jth EM. Constant coefficients a_is and b_ks are optimally determined from the comparison with experimental yield and flux data of the WT strain, in the absence of which they are set to zero as a default strategy (i.e., no modification to η_j).

Appendix B. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.ymben.2012.08.004.

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