Parametric Sensitivity of Stoichiometric Flux Balance Models Applied to Wild-Type *Escherichia coli* Metabolism

Amit Varma and Bernhard O. Palsson*
Department of Chemical Engineering, University of Michigan, Ann Arbor, Michigan 48109-2136

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Stoichiometrically based flux balance models provide a method to quantify the metabolic pathway fluxes within a living cell. Predictions of flux balance models are expected to have applications in pathway engineering as well as in bioprocess design and control. These models utilize optimality principles applied to metabolic pathway stoichiometry along with the metabolic requirements for growth to determine the flux distribution in a metabolic network. A flux balance model has been developed for *Escherichia coli* W3110 using five experimentally determined strain-specific parameters. In this report, we determine the sensitivity of the predictions of the flux balance model to these five strain-specific parameters. Model predictions are shown to be sensitive to the two parameters describing metabolic capacity, while they are relatively insensitive to the three parameters that describe the metabolic requirements for growth. Thus, when stoichiometrically based models are formulated for additional strains one needs to measure the metabolic capacity (maximum rates of nutrient and oxygen utilization) accurately. Determination of metabolic capacity from batch experiments is relatively easy to perform. On the other hand, the harder to determine maintenance parameters need not be as accurately determined.
Key words: *E. coli* • linear optimization • metabolic fluxes • stoichiometry • sensitivity analysis

INTRODUCTION

Quantitative descriptions of metabolic fluxes inside living cells have long been sought to understand metabolic physiology as well as to engineer metabolic flux distributions. Many attempts to systematically model metabolic dynamics have been carried out and several reviews are available on this topic.[5,6,12,14] Much effort has also been devoted toward the development of a theoretical framework for the analysis of metabolic regulation, mostly through the use of logarithmic sensitivity coefficients[7,9,15] and overviews are available.[3,17] However, these developments have been limited by the lack of kinetic and regulatory information on the function of all enzymes in a particular cell.

Recently, a new approach for describing metabolic fluxes has been formulated that relies on the stoichiometry of metabolic networks.[4,10,16,18,25] This approach requires easily obtainable metabolic pathway stoichiometry along with the metabolic requirements for growth without requiring enzyme kinetic information. Flux balance models have been found to provide valuable information about metabolic pathway utilization. A flux balance model has been formulated for the industrially and scientifically important bacterium *Escherichia coli* that includes both the catabolic[21,22] as well as the biosynthetic network of reactions.[19] The flux balance model has been used to successfully explain metabolic physiology under different environmental conditions.[20] Predictions of the flux balance model are expected to be useful for bioprocess design and control in industry.[19,23]

The flux balance model has been specified for a wild-type *E. coli* W3110 strain using five experimentally determined strain-specific parameters.[24] The model has been shown to provide accurate predictions for growth, and uptake and secretion of metabolites under several environmental conditions.[24] These predictions naturally lead to questions about their sensitivity to model parameters and the robustness of flux balance models. In this report we have examined the parametric sensitivity of the flux balance model to the five experimentally determined strain-specific parameters. Model predictions, using different parameter values, are used to simulate aerobic chemostat, batch, and fed-batch experimental data. These predictions are compared with experimental data reproduced from other sources.[24]

MATERIALS AND METHODS

Culture

An *E. coli* K-12 strain W3110 (ATCC # 27325) was used for all the experimental results reproduced from elsewhere.[24] The strain has been described as a nearly wild-type strain and is able to grow on glucose mineral medium. The defined glucose limited culture conditions are described elsewhere.[24]

Flux Balance Model

A metabolic steady state is assumed, in which metabolic fluxes leading to the formation and degradation of a metab-

* To whom all correspondence should be addressed.

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olite must balance, leading to the flux balance equation[4,16,21]:

$$\mathbf{S} \cdot \mathbf{v} = \mathbf{b} \tag{1}$$

where **S** is a matrix comprising the stoichiometry of the catabolic reactions, **v** is a vector of metabolic fluxes, and **b** is a vector containing the net metabolite uptake by the cell. Eq. (1) is typically underdetermined, because the number of fluxes (or metabolic pathways) normally exceeds the number of metabolites. Thus, a plurality of solutions exists and a particular solution may be found using linear optimization by stating an objective and seeking its maximal value within the stoichiometrically defined domain. In other words, specifying an objective, such as to optimize the growth rate, would determine the best metabolic pathway utilization that would fulfill the stated objective.

Objective

A maximal growth objective has been used in the present study,[21,22] i.e., Eqs. (2) and (3).

$$\text{Minimize } Z = -V_{\text{gro}} \tag{2}$$

$$\sum_{\text{all } M} d_M \cdot M \xrightarrow{V_{\text{gro}}} \text{biomass} \tag{3}$$

where d_M are the requirements in millimoles per gram biomass of the M biosynthetic precursors and cofactors for biomass production. These metabolic requirements for growth are based on biomass composition.[8,22] As noted later we have also incorporated a scaling factor to allow for strain-specific variations in biomass and maintenance. V_{gro} is the growth flux (g biomass produced), which with the basis of 1 g DW-h reduces to the growth rate (g biomass produced/g DW-h). Z is the objective.

To fulfill the objective of optimizing the growth rate we do not constrain the model's ability to secrete byproducts such as acetate, ethanol, formate, lactate, and succinate. Thus, the amount of byproduct secreted is determined by the objective of optimizing the growth rate. We then compare model predictions of growth rates and byproducts to experimental data.

Predictive Algorithm

For the prediction of the unsteady state buildup of metabolic byproducts or the consumption of substrates we have divided the experimental time into increments of Δt. For the first time step the initial concentration values are specified. Starting with the first time step the flux balance model is used to predict concentrations for the next step using the following algorithm:

1. Substrate concentration is given by the substrate concentration predicted from the previous step plus any additional substrate provided in a fed-batch mode.

$$S_c = S_{co} + \frac{\text{supply} \cdot \Delta t}{\text{volume}} \quad (\text{mmol/L}) \tag{4}$$

2. The substrate concentration is appropriately scaled to define the substrate available *per* unit biomass *per* unit time.

$$\text{Substrate available} = \frac{S_c}{X \cdot \Delta t} \quad (\text{mmol/g DW-h}) \tag{5}$$

3. The flux balance model is used to evaluate the net substrate uptake (S_u) (may be less than the substrate available), the growth rate (μ), and potential byproduct secretion.
4. Concentrations for the next time step are calculated from the standard differential equations using the above-determined growth rate (μ), and substrate uptake rate (S_u):

$$\frac{dX}{dt} = \mu X \Rightarrow X = X_o \cdot e^{\mu \Delta t} \tag{6}$$

$$\frac{dS_c}{dt} = -S_u \cdot X \Rightarrow S_c = S_{co} + \frac{S_u}{\mu} + X_o\,[1 - e^{\mu \Delta t}] \tag{7}$$

In the above algorithm we denote both glucose and byproducts as substrates that can be used by cells. Thus, as a result of implementing the above algorithm we predict the concentration time profiles of cells, glucose, and byproducts.

RESULTS AND DISCUSSION

The flux balance model formulated for glucose metabolism and growth of the *E. coli* W3110 strain incorporates five experimentally determined parameters in addition to the metabolic network stoichiometry. The first two parameters relate to the enzymatic capacity of the cell to utilize the carbon substrate (glucose) as well as oxygen. Enzymatic capacity limits for the utilization of glucose as well as oxygen are incorporated into the flux balance model to define a finite upper limit of metabolism *per* unit biomass *per* unit time.

Two parameters for maintenance and one for biomass scaling are incorporated into the model to account for those activities not included in the metabolic demands for growth. Maintenance is incorporated as a non-growth-associated as well as a growth-associated requirement of energy by the bacterium.[20] Biomass requirements for growth have been estimated from published composition analyses.[8] To apply the flux balance model for our bacterial strain we have found it necessary to apply a biomass scaling parameter that scales the metabolic requirements for growth which may be strain and environment sensitive.

We now consider the sensitivity of the flux balance model to these five parameters that were experimentally determined for the *E. coli* W3110 strain.[24] Significant ±20% variations in the parameters were used to determine

the effect of the parameters on predictive capability of the flux balance model. Experimental data[24] is also plotted to provide a reference to the sensitivity analysis.

Enzymatic Capacity for Oxygen Utilization

The enzymatic capacity for oxygen utilization was determined from batch experiments with oxygen and glucose present in excess in accordance with literature.[1] For the strain *E. coli* W3110 the value was determined to be 15 mmol O_2/g DW h.[24]

Aerobic batch and fed-batch cultures were simulated using 20% variations in the oxygen utilization capacity. Plots for computed cell density, glucose, and acetate concentration varying with culture time are shown in Figure 1. The

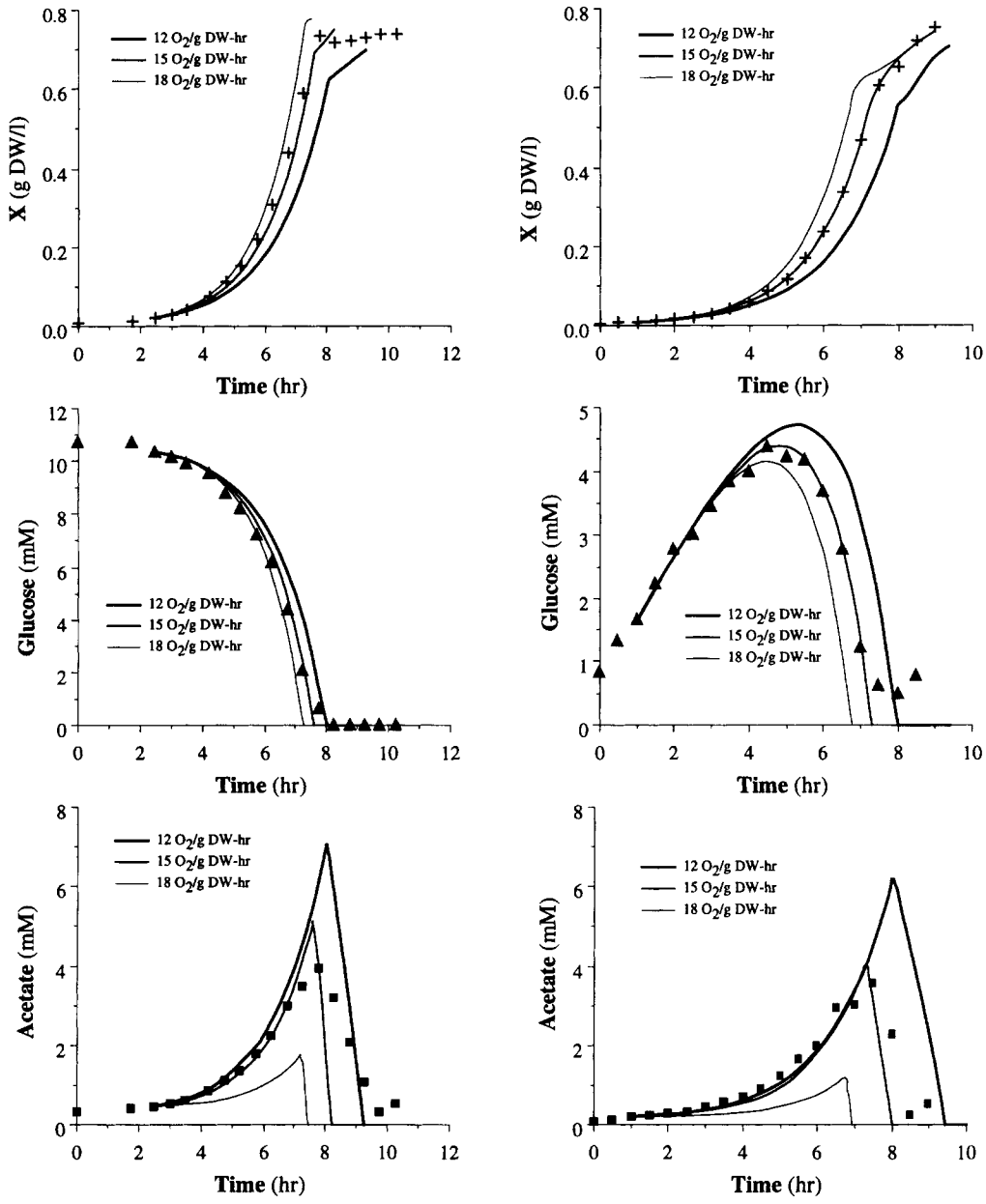

Figure 1. Sensitivity of the flux balance model to the enzymatic capacity for oxygen utilization. Simulated predictions for ±20% variations in oxygen utilization capacity are shown as continuous lines against the plotted experimental data. The left panels represent a batch experiment, while the right panels represent a fed-batch experiment with a glucose feed rate of 0.2 g glucose/L h. Experimental data is from ref. 24.

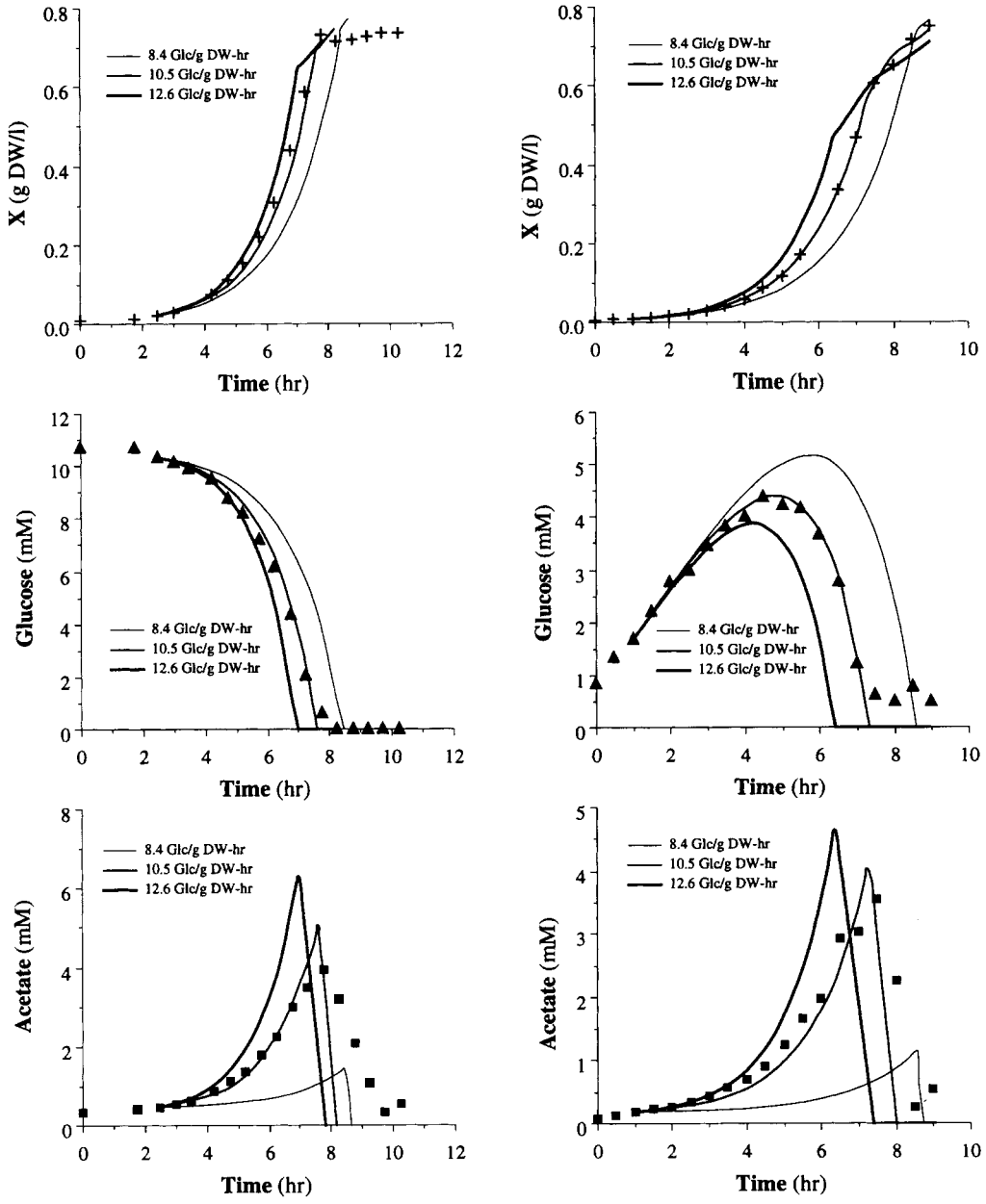

Figure 2. Sensitivity of the flux balance model to the enzymatic capacity for glucose utilization. Simulated predictions for ±20% variations in glucose utilization capacity are shown as continuous lines against the plotted experimental data. The left panels represent a batch experiment, while the right panels represent a fed-batch experiment with a glucose feed rate of 0.2 g glucose/L h. Experimental data is from ref. 24.

results show that the maximum growth rate changes significantly with the maximum oxygen utilization capacity. The final biomass concentration (or net yield) was observed to be more sensitive to an underestimation in the oxygen utilization capacity for the fed-batch simulation.

Glucose concentration depends on the ability of the flux balance model to utilize glucose which is limited by the glucose utilization capacity times the cell density. Thus, the glucose concentration profiles shown in Figure 1 directly reflect the differences in the cell density profile. The acetate accumulation in culture medium shows the greatest sensitivity to the oxygen utilization capacity. The flux balance

model predicts that acetate secretion occurs primarily due to surplus redox generation.[20] Thus, the oxygen utilization capacity has a direct and pronounced effect on the acetate secretion rate.

Enzymatic Capacity for Glucose Utilization

For aerobically growing cells, the maximum glucose utilization rate was determined from batch experiments in which glucose was present in excess. *E. coli* W3110 was observed to have a maximum glucose uptake rate of 10.5 mmol glucose/g DW-h.[24]

Aerobic batch and fed-batch cultures were simulated for 20% variations in the glucose utilization capacity. Plots for cell density, glucose, and acetate concentration are shown in Figure 2. An increase in the glucose utilization capacity is directly observed as a faster glucose consumption. Growth rate is also accelerated with a higher glucose utilization capacity.

As with the oxygen utilization capacity, the acetate concentration profiles were found to show the greatest sensitivity to the maximum glucose utilization rate, (Fig. 2). Because the availability of the terminal oxidant, oxygen, is enzymatically limited, the surplus glucose is degraded to acetate to produce metabolic energy. Thus, increased glucose uptake is directly evidenced by an increased acetate secretion.

Maintenance and Biomass Scaling

Maintenance is generally described as those activities required to maintain the viable state of the cell regardless of the growth conditions. Non-growth-associated maintenance can be reduced to an energy equivalent that is used for activities such as gradient maintenance, protein turnover, and so forth.

Non-growth-associated maintenance was determined from a plot of the chemostat data shown in Figure 3a. Glucose uptake and acetate secretion data were acquired in a chemostat that was not limited for minerals. The y-intercept of glucose uptake extrapolated to a zero dilution or growth rate was used to determine the glucose substrate required for nongrowth-associated maintenance. The glucose requirement was then converted into its energy equivalent of 7.6 mmol ATP/g DW h as the non-growth-associated maintenance energy.

As evidenced by the plots for 20% variations in the non-growth-associated maintenance (Fig. 3a), predictions of the flux balance model are not very sensitive to this parameter. We have also determined the effect of variations in non-growth-associated maintenance on batch and fed-batch predictions (Fig. 4). Once again, we note that the model predictions are not very sensitive to the non-growth-associated maintenance.

Biomass scaling is a parameter used to account for any differences in the metabolic requirements for growth for the specific strain and conditions used in our experiments. Growth has been defined in the flux balance model[22] as a requirement of metabolites based on the biomass composition for *E. coli* published in the literature.[8] Because biomass composition may vary with the specific strain as well as the growth environment, we used a multiplicative scaling factor to define the metabolic requirements for the conditions in this study. Because the flux balance model is not very sensitive to any particular metabolite[22] the biomass scaling

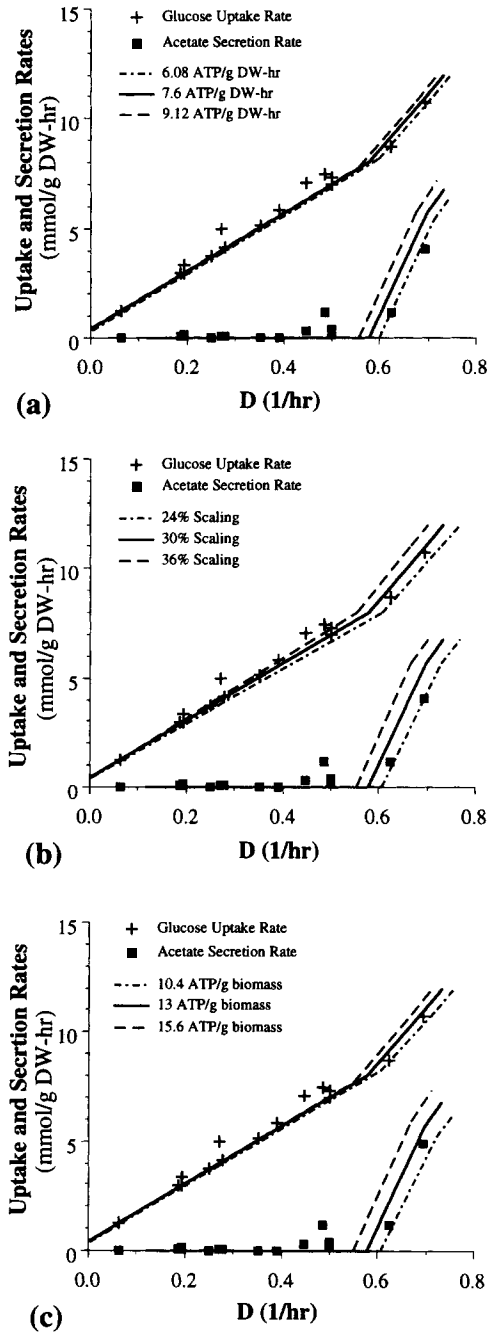

Figure 3. Glucose uptake and acetate secretion as a function of dilution rate in a chemostat. Flux balance model predictions are plotted for ±20% variations in the model parameters: (a) non-growth-associated maintenance; (b) biomass scaling; and (c) growth-associated maintenance. Experimental data is from ref. 24.

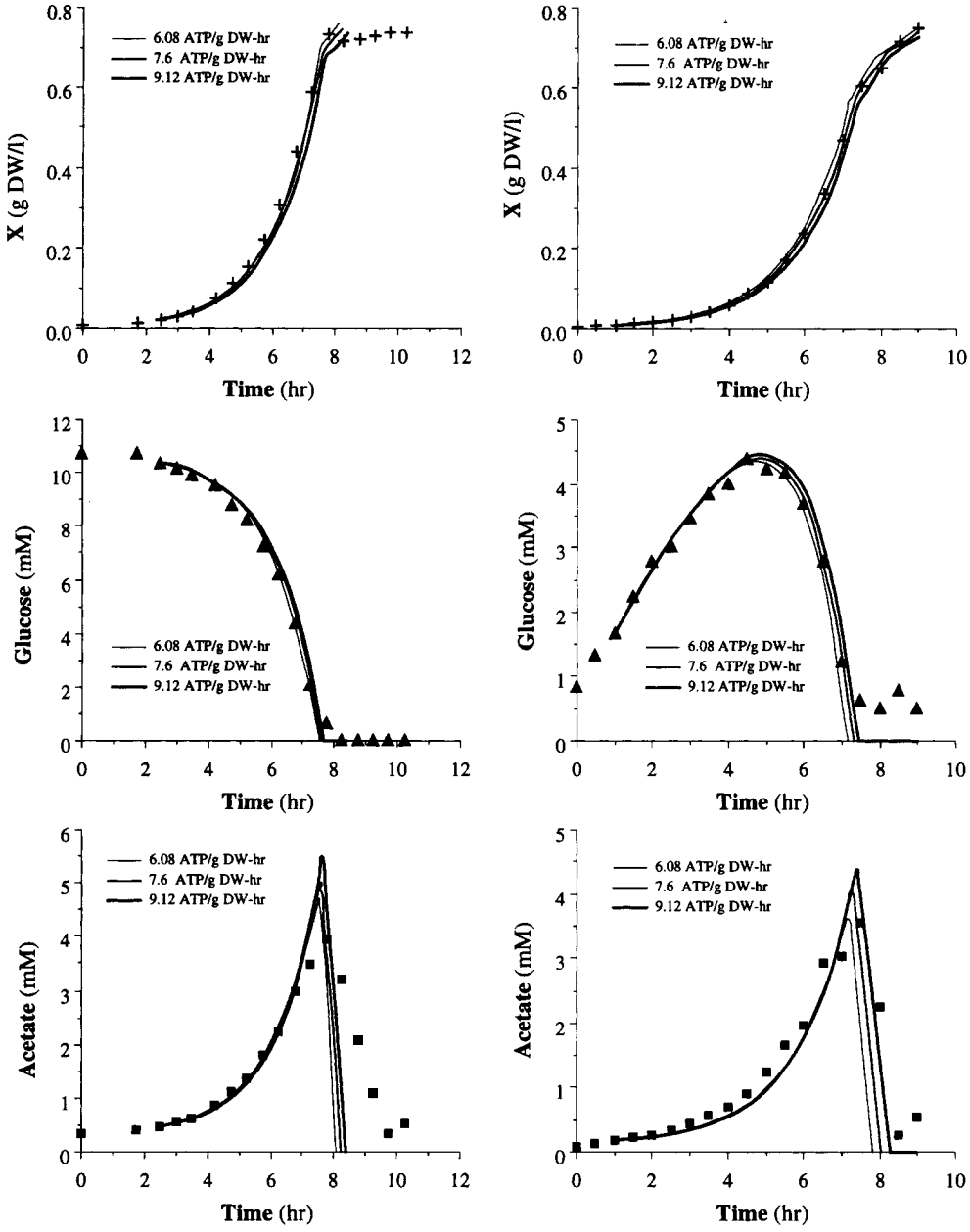

Figure 4. Sensitivity of the flux balance model to the non-growth-associated maintenance requirements. Simulated predictions for ±20% variations in non-growth-associated maintenance are shown as continuous lines against the plotted experimental data. The left panels represent a batch experiment while the right panels represent a fed-batch experiment with a glucose feed rate of 0.2 g glucose/L h. Experimental data is from ref. 24.

parameter is expected to provide an adequate representation of the metabolic requirements for growth.

The biomass scaling parameter was determined from chemostat experiments, Figure 3b. Biomass scaling has a direct impact on the biomass yield because it determines the amount of metabolites needed to produce biomass. Thus, the parameter was estimated from the slope (which reflects the biomass yield) of the glucose uptake line in Figure 3b. Biomass scaling for the *E. coli* W3110 strain was estimated as 30% greater metabolites required for growth than the nominal value from the literature.[8,22]

Sensitivity of the flux balance model predictions toward biomass scaling was determined for chemostat operation as shown in Figure 3b. Moderate to small differences are

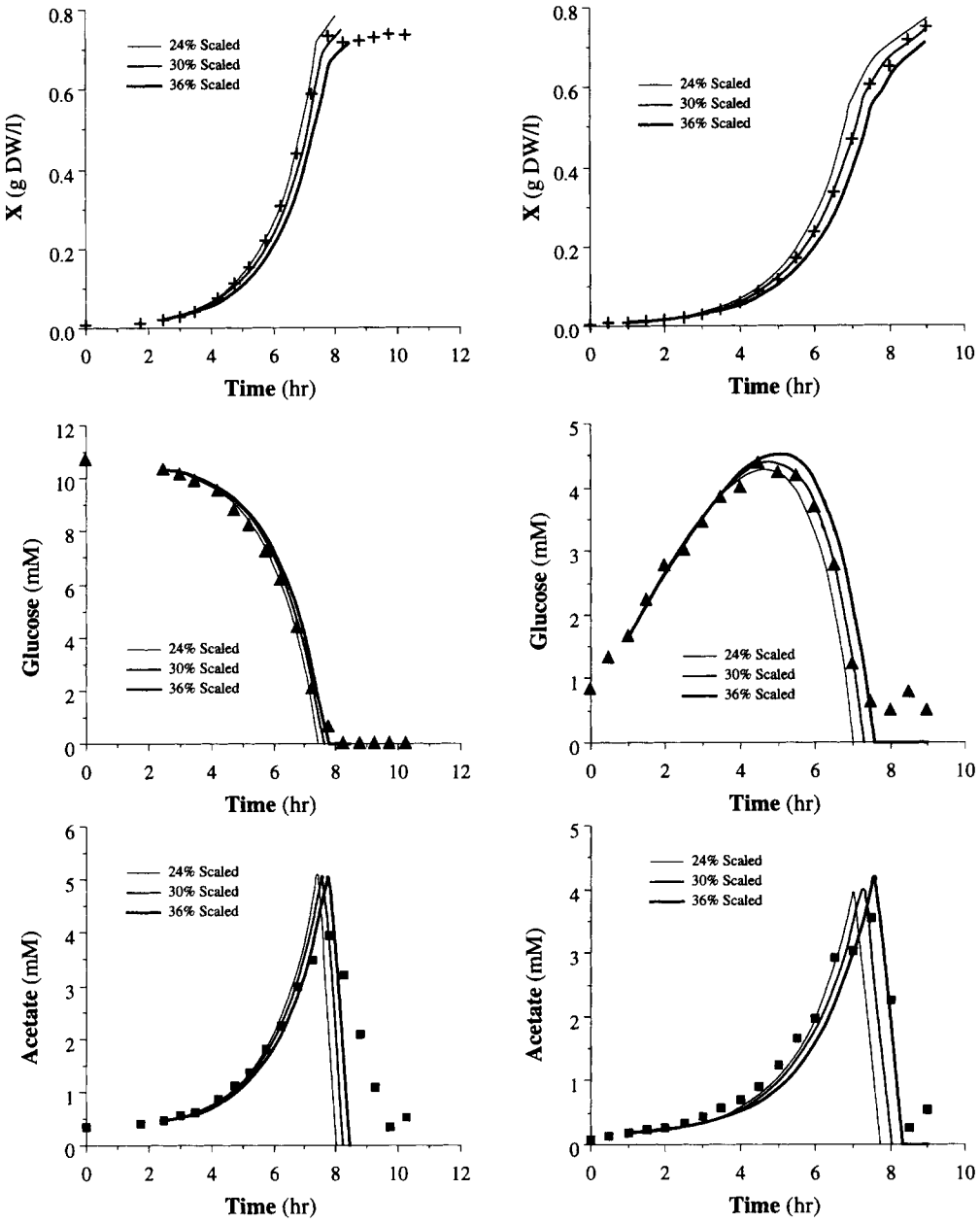

Figure 5. Sensitivity of the flux balance model to the biomass scaling. Simulated predictions for ±20% variations in biomass scaling are shown as continuous lines against the plotted experimental data. The left panels represent a batch experiment, while the right panels represent a fed-batch experiment with a glucose feed rate of 0.2 g glucose/L h. Experimental data is from ref. 24.

found in the model predictions for 20% differences in biomass scaling. Simulations for batch and fed-batch experiments were also carried out and are shown in Figure 5. Once again, whereas biomass scaling was found to affect the biomass yield, the model predictions were quite similar.

In addition to non-growth-associated maintenance activities there are some maintenance functions of a living cell that increase with the growth rate. Thus, a growth-associated maintenance energy term has been defined[20] that accounts for such activities. The growth-associated maintenance parameter is determined from chemostat data as shown in Figure 3c. The figure also shows a plot of model predictions for 20% variations in the parameter. Growth-associated maintenance was determined from the critical

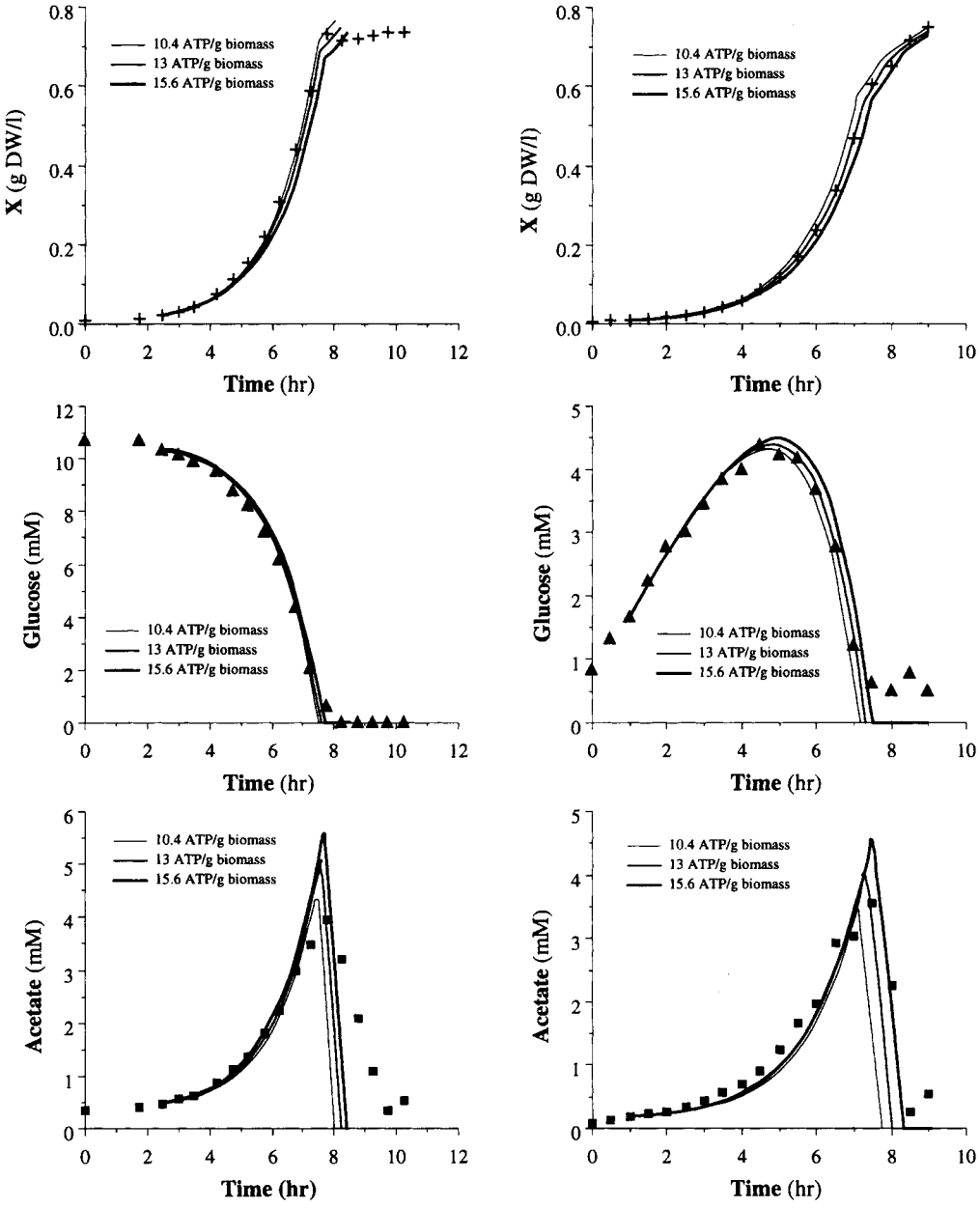

Figure 6. Sensitivity of the flux balance model to the growth-associated maintenance requirements. Simulated predictions for ±20% variations in growth-associated maintenance are shown as continuous lines against the plotted experimental data. The left panels represent a batch experiment, while the right panels represent a fed-batch experiment with a glucose feed rate of 0.2 g glucose/L h. Experimental data is from ref. 24.

growth rate at which acetate is secreted or the point at which the glucose uptake line changes slope. This critical growth rate is also the point at which the oxygen utilization capacity is reached. For the bacterial strain and conditions used here growth-associated maintenance was estimated at 13 mmol ATP/g DW h.

From the simulations of chemostat experiments, shown in Figure 3c, we observed that the flux balance model was not sensitive to the growth-associated maintenance. Batch and fed-batch cultures were also simulated for ±20% variations in the growth associated maintenance and are shown in Figure 6. Similar to the chemostat results the flux balance model predictions were not found to be sensitive to the growth-associated maintenance parameter.

In summary, the flux balance model was not found to be very sensitive to ±20% variations in the maintenance and biomass scaling parameters. Although the determination of these parameters is open to some subjective interpretation, due to slight scatter in the chemostat data, it may be re-emphasized that small differences in the parameters do not affect the flux balance model predictions.

Another noteworthy observation from the chemostat data in Figure 3 relates to the acetate secretion rate. The acetate secretion rate may be mathematically described as[2]:

$$\nu = \begin{cases} 0 & \text{if } \mu < \mu_c \\ \nu_i(\mu - \mu_c) & \text{if } \mu > \mu_c \end{cases} \quad (8)$$

where ν is the byproduct formation rate in units of millimoles acetate/g DW-h. It was observed that whereas the maintenance and biomass scaling parameters affect μ_c, the critical growth rate at which acetate secretion occurs, the parameters have no effect on ν_i the slope of the acetate secretion line. The slope of the acetate secretion line depends only on the condition of optimality of growth assumed in the flux balance model. The slope can be larger or smaller if suboptimal acetate secretion occurs at any growth rate. Thus, a fit of the acetate secretion predictions to the chemostat data provides a test for the validity of the flux balance model formulated for a particular strain.

Sensitivity to the P/O Ratio

Little ambiguity remains about the stoichiometry of the metabolic pathways used to formulate the flux balance model.[21] The electron transfer system and the concomitant chemiosmotic generation of high energy phosphate bonds is one area in which alternate stoichiometries have been proposed. For the well-studied *E. coli* cell we have used the stoichiometry of P/O = 1.3 to formulate the flux balance model which is supported by some experimental literature; e.g., see refs. 11, 13, and 21. Because there exists some debate on this issue we have investigated the effect of a different P/O stoichiometric ratio on the flux balance model for *E. coli*.

Simulations for chemostat growth were carried out using P/O ratios of 1.3 and 2.0. The results are shown in Figure 7. A small difference is observed in the slope of the glucose uptake rate, which indicates a small increase in the yield due to the additional metabolic energy generated at the higher P/O ratio of 2. However, a more dramatic result was observed for acetate secretion where the critical growth rate for the onset of acetate secretion was significantly higher. Indeed, the critical growth rate is much higher than the maximum growth rate of the bacterium on glucose and thus, the flux balance model does not predict acetate secretion to occur for *E. coli* using the P/O ratio of 2.

The above deductions are reinforced by simulations for batch and fed-batch experiments shown in Figure 8. At the higher P/O ratio of 2, a higher growth rate is observed along with a faster rate of glucose consumption. The resulting

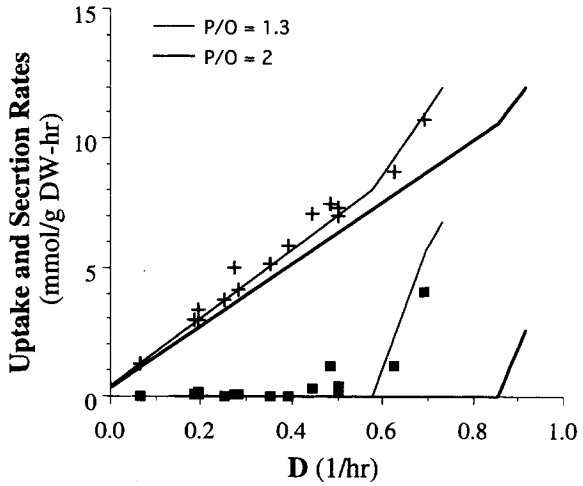

Figure 7. Effect of the P/O ratio on the predictions of the flux balance model for chemostat growth. Model predictions for P/O ratios of 1.3 and 2.0 are plotted against experimental data. Experimental data is from ref. 24. + represents glucose uptake rate, ■ represents acetate secretion rate.

biomass yield was slightly higher for the P/O ratio of 2. Acetate was again not found to be secreted at the higher P/O ratio. Thus, the flux balance model permits a discrimination between alternate P/O stoichiometries.

It is interesting to note that, whereas the flux balance model was not very sensitive to the maintenance energy, it was very sensitive to the P/O ratio, although both quantities primarily affect the ATP energy pool. The situation may be explained by pointing out that the maintenance energy forms only a small fraction of the cells total energy needs, which is approximately 70 mmol ATP/g DW. Thus, the model is not very sensitive to the maintenance requirements. On the other hand, most of the metabolic energy is produced by the overall process of aerobic respiration. Therefore, it may be expected that the P/O ratio would have a significant impact on the flux balance model.

CONCLUSION

In this report we have examined the sensitivity of the flux balance model formulated for *E. coli* W3110. The model was found to be very sensitive to the parameters describing maximum enzymatic utilization capacity for oxygen and glucose. These parameters are readily determined for any strain and processing condition of interest. On the other hand, the model was found to be relatively insensitive to the parameters describing the metabolic requirements for growth, namely non-growth-associated maintenance, growth-associated maintenance, and biomass scaling. The stoichiometry of the respiratory chain, the P/O ratio, was determined to influence predictions of growth and metabolic flux distribution significantly. The experimentally determined value of 1.3 reported in literature was found to result in accurate predictions of experimental data.

Given the robustness of the predictions obtained from the

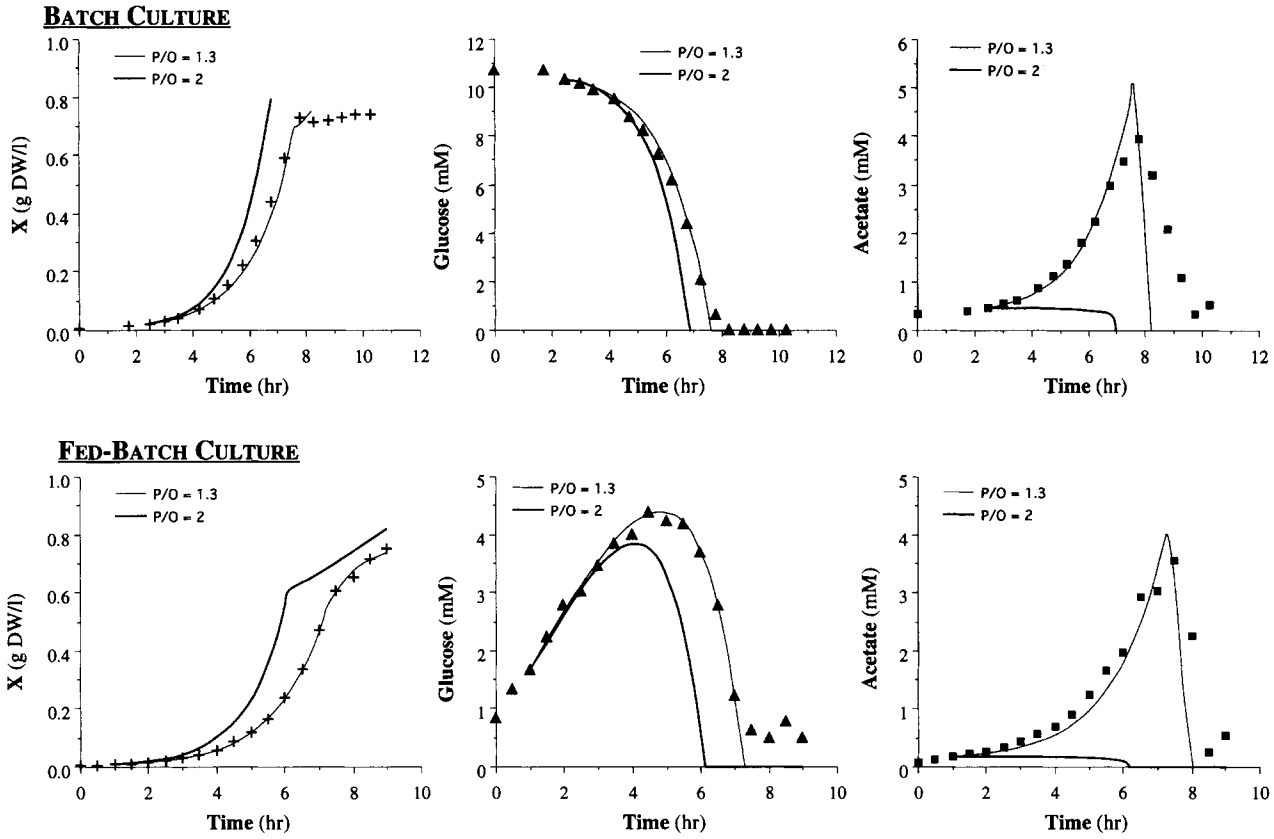

Figure 8. Sensitivity of the flux balance model to the P/O ratio. Simulated predictions for P/O ratios of 1.3 and 2.0 are shown as continuous lines against the plotted experimental data. The upper panels represent a batch experiment, and the lower panels represent a fed-batch experiment with a glucose feed rate of 0.2 g glucose/L h. Experimental data is from ref. 24.

flux balance based models, the relative ease with which the key strain-specific parameters are obtained, and the straightforwardness of the model formulation, it is likely that stoichiometric models will find wide use in strain and bioprocess development.

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