FBA-BASED PREDICTION OF BIOMASS AND ETHANOL CONCENTRATION TIME PROFILES IN *Saccharomyces cerevisiae* FED-BATH CULTURES

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Abstract: A Flux Balance Analysis (FBA)-based dynamical model is proposed for predicting biomass and ethanol concentration time profiles in *Saccharomyces cerevisiae* fed-batch cultures, with glucose and ammonium uptake rates as inputs. It is based on a metabolic network compiling the necessary internal fluxes for reproducing respiratory as well as respiro-fermentative metabolisms. While the objective cost function classically accounts for biomass growth maximization, additional linear constraints are used to reproduce overflow metabolism phenomena. New conditional equality constraints are introduced in the FBA linear programs to account for variable biomass composition (based on protein mass fraction identification with or without ammonium feeding). The proposed FBA-based model contains only 7 parameters which are estimated with the experimental data. A two-step procedure for separately solving the FBA linear programs and the macroscopic mass balance ODEs significantly lowers the computational load for parameter identification. Direct and leave-one-out cross-validation results are provided.

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**Keywords**: *Saccharomyces cerevisiae*, dynamical model, Flux Balance Analysis, underdetermined systems, overflow metabolism, variable biomass composition.

1. INTRODUCTION

Cell cultures represent currently one of the most important and promising sources for biopharmaceuticals and agri-food industries. These processes can be simulated with dynamical models which reproduce the concentration time profiles of the main species: substrates, products and biomass (Richelle, Fickers and Bogaerts, 2014). These biotechnological processes can considerably benefit from modeling, since it allows their monitoring, optimization and control. Bioprocess models are often unstructured, i.e. they capture the metabolism macroscopically without considering intracellular metabolism. These models require the determination of a macroscopic reaction scheme and the structural and parametric estimation of the reaction rates. The knowledge of metabolic networks allows describing details of the intracellular metabolism. However, in many cases, determining the intracellular fluxes corresponds to an underdetermined problem in the sense that the number of unknown fluxes is higher than the number of available equations (e.g., mass balances of internal metabolites, measured external fluxes) (Richelle, Mhallem Gziri and Bogaerts, 2016). To try overcoming this underdeterminacy issue, Flux Balance Analysis (FBA) predicts the distribution of metabolic fluxes using linear programming to maximize an objective cost function based on the assumption of optimal overall behavior such as biomass maximization (Simeonidis et al., 2010). FBA can be inserted in dynamical models aiming at predicting extracellular species concentration time profiles (Bogaerts, Mhallem Gziri and Richelle, 2017). Plaza and Bogaerts (2018) proposed a metabolic network-based predictor of biomass growth and ethanol production rates in *S. cerevisiae* fed-batch cultures using FBA (aiming at maximizing biomass growth), time profiles of the input fluxes (glucose and ammonium) and additional constraints for explaining glucose overflow metabolism. However, this predictor does not allow the prediction of concentration time profiles and does not consider changes in biomass composition according to the culture medium feeding. The feasibility of the proposed biological network and inequality constraints accounting for overflow metabolism has been shown in Plaza and Bogaerts (2018) without parameter identification (parameter values were only based on trial-and-errors and/or literature) and only for predicting output (biomass and ethanol) fluxes.

The goal of this study is to propose a macroscopic FBA-based model for predicting biomass and ethanol concentration time profiles based on the estimation of glucose and ammonium uptake rates. Besides the prediction of concentration time profiles instead of only specific production rates, the main new contributions with respect to Plaza and Bogaerts (2018) concern: (i) a modified metabolic network that allows reproducing biomass growth when ethanol is consumed through respiratory metabolism, (ii) new conditional equality constraints in the FBA linear programs to account for variable biomass composition (based on protein mass fraction identification with or without ammonium feeding), and (iii) an efficient parameter estimation of a low complexity model (involving a two-step procedure for separately solving the FBA linear programs and the macroscopic mass balance ODEs) and its direct and leave-one-out cross validation.

The text is organized as follows: Section 2 presents the case study on *Saccharomyces cerevisiae* fed-batch cultures. Section 3 proposes the metabolic network under consideration, the stoichiometric model and the methodology to calculate the external uptake fluxes (glucose and ammonium). Section 4 describes the FBA-based model for predicting concentration...
time profiles of biomass and ethanol, the parameter identification procedure and the model validation. Finally, section 5 presents final conclusions and perspectives.

2. CASE STUDY: *Saccharomyces cerevisiae* FED-BATCH CULTURES

This case study comes from (Richelle, Fickers and Bogaerts, 2014), where baker’s yeast fed-batch cultures were performed for building a macroscopic dynamical model without using metabolic networks. The glucose concentration in the feeding ($G_{feed}$) was 300 g/L and the concentration of ammonium sulfate ($N_{feed}$) varied between the different experiments: 0 g/L (Exp 1), 33 g/L (Exp 2), 16.5 g/L (Exp 3) and 33 g/L during the first 15 h and 0 g/L afterwards (Exp 4). The volumetric feeding rate was set to mimic the different industrial baker’s yeast production conditions: a first phase with glucose feeding where respiratory metabolism predominates; a second phase with a high glucose feeding where fermentation with ethanol production predominates, and finally, a third phase with the consumption of the ethanol produced. More experimental details can be found in (Richelle, Fickers and Bogaerts, 2014).

3. METABOLIC NETWORK AND STOICHIOMETRIC MODEL

The *S. cerevisiae* metabolic network used in this work (Fig. 1) is a representation of the main central carbon metabolism, tricarboxylic acid cycle, pentose phosphate pathways, fermentative metabolism with ethanol production under aerobic conditions (Nissen et al., 1997; Sainz et al., 2003; Varela, Pizarro and Agosin, 2004), central nitrogen metabolism, aminoacids metabolism and precursors for biomass production (proteins, lipids, nucleic acids and carbohydrates) (Quiros et al., 2013), gluconeogenesis and glyoxylate cycle (Vanrolleghem et al., 1996; Gonzalez et al., 2003) and transport reactions (external transport to the cell and cytosomal/mitochondrial transport). Modifications with respect to the metabolic network proposed in Plaza and Bogaerts (2018) have been introduced so that, in case of ethanol consumption, biomass production takes place by the activation of the glyoxylate cycle and gluconeogenesis (inverse operation of the glycolytic pathway), providing the different precursor metabolites for biomass growth.

The synthesis of biomass is calculated using the following growth reaction (see $\gamma_{B}$ in Fig. 1):

$$a \, \text{Prot} + b \, \text{Lip} + \gamma \, \text{NA} + \delta \, \text{Carb} + \epsilon \, \text{ATP} \rightarrow 1 \, \text{g Biomass}$$

(1)

where Prot, Lip, NA and Carb stand for the basic precursors proteins, lipids, nucleic acids and carbohydrates respectively. The coefficients $a$, $b$, $\gamma$ and $\delta$ are the necessary mass fractions of these precursors (g precursor/g biomass) needed to produce 1 g of biomass and $\epsilon$ the growth energy requirement expressed as g ATP/g biomass.

*S. cerevisiae*’s network of Fig. 1 is represented by a stoichiometric matrix $N_{S} \in \mathbb{R}^{56 \times 85}$ with 59 rows corresponding to the balanced internal metabolites and 85 columns to the metabolic fluxes.

The mass balance of the internal metabolites included in the metabolic network is:

$$C_{int \, met} = N \cdot v (t) - \mu \cdot C_{int \, met} (t)$$

(2)

where, $\mu$ is the specific growth rate, $v \in \mathbb{R}^{85}$ the vector of metabolic fluxes, $C_{int \, met} \in \mathbb{R}^{59}$ the vector of internal metabolite concentrations and $\mu \cdot C_{int \, met}$ the dilution term accounting for biomass growth. Assuming that the intracellular metabolites are at pseudo-steady state and the...
dilution term $\mu \cdot C_{int, met}$ may be neglected in comparison with the reaction term $N \cdot v(t)$, the mass balances reduce to:

$$N \cdot v(t) = 0$$

This system of equations is underdetermined with 85 (fluxes) - 59 (equations) = 26 degrees of freedom.

The links between $S.\ cer evisiae$’s network and the external concentration profiles are given by the transport reactions of the inputs (consumption of glucose and ammonium) and the outputs (production of ethanol and biomass). The input fluxes $v_G$ and $v_N$ are estimated based on smoothing splines of the substrate concentrations $G(t)$ and $N(t)$ and the mass balances:

$$\dot{G}(t) = -v_G X(t) + D(t) (G_{feed} - G(t))$$

$$\dot{N}(t) = -v_N X(t) + D(t) (N_{feed} - N(t))$$

where $D$ is the dilution rate, $X$, $G$ and $N$ the biomass, glucose and ammonium concentrations, $G_{feed}$ and $N_{feed}$ the glucose and ammonium feeding concentrations. Smoothing splines of $G(t)$ and $N(t)$ are computed with the function spaps in MATLAB.

The concentration measurements are depicted in Fig. 2 with their smoothing splines. The time derivatives $\dot{G}(t)$ and $\dot{N}(t)$ are computed with the analytical expression of the corresponding smoothing splines (function fnder in MATLAB). These estimated input fluxes (represented in Fig. 3) are lumped in the vector $v_{in} = [v_G \ v_N]^T$ which is linked to the metabolic network as follows (see Fig. 1):

$$v_{in}(t) = [v_G \ v_N]^T = [v_{49} \ v_{49}]^T = N_{in} \ v(t)$$

with $N_{in} \in \mathbb{R}^{2 \times 85}$.

The output fluxes correspond to biomass and ethanol production and are linked to the metabolic network as follows (see Fig. 1):

$$v_{out}(t) = [v_X \ v_E]^T = [v_{79} \ v_{69}]^T = N_{out} \ v(t)$$

with $N_{out} \in \mathbb{R}^{2 \times 85}$.

Fig. 2. Measured concentrations of glucose and ammonium and their corresponding smoothing splines (in blue): a. Exp 1, b. Exp 2, c. Exp 3 and d. Exp 4.

Section 4 will explain how to compute the predicted time profiles of $v_{out}(t)$ (and, in turn, the corresponding concentration profiles $X(t)$ and $E(t)$) based on the metabolic network proposed in Fig. 1 and the estimated inputs $v_{in}(t)$.

4. FBA-BASED MODEL FOR PREDICTING OUTPUT PROFILE CONCENTRATIONS

Plaza and Bogaerts (2018) used FBA for predicting biomass growth and ethanol production fluxes ($v_{out}$) based on the estimation of the uptake fluxes ($v_{in}$) and inequality constraints explaining glucose overflow metabolism in $S.\ cer evisiae$. These constraints are linked to the two main (respiratory and respiro-fermentative) operating regimes. When glucose uptake rate $v_G$ is smaller than the maximum respiratory capacity of glucose $v_{G,SAT}$, respiration of glucose (Fig. 4a) and/or ethanol (Fig. 4c) takes place in the respiratory regime. An upper bound ($v_{eth}$) has to be set regarding the ethanol consumption:

$$-v_{eth} < v_{69} < 0$$

When glucose uptake rate $v_G$ is greater than the maximum respiratory capacity of glucose $v_{G,SAT}$ (Fig. 4b), the excess of glucose is going through the fermentative pathway. In this latter, the transformation of acetaldehyde ($v_{10}$) is split into ethanol ($v_{11} + v_{12}$) and acetate ($v_{13}$) and the produced acetate does not exceed the ethanol produced (Franzén, 2003; Quirós et al., 2013; Plaza and Bogaerts, 2018):

$$v_{13} < (v_{11} + v_{12})$$

The constraints (8) and (9) will be grouped in the conditional inequality constraints (12) used in FBA below.

Fig. 3. External input (glucose, ammonium) fluxes based on smoothing splines and mass balances: a. Exp 1, b. Exp 2, c. Exp 3, and d. Exp 4.
Florianópolis - SC, Brazil, April 23-26, 2019
2019 IFAC DYCOPS

the inputs (consumption of glucose and ammonium feeding concentrations). Smoothing splines of the links between the glucose and ammonium concentrations, and their corresponding smoothing splines (in blue): a. Exp 1, (see Fig. 1): v

v

\text{with} \quad N \quad \text{and} \quad \mu \quad \text{involving ethanol and fluxes} \ j \in \{11,12,69\} \quad \text{involving amino acid metabolisms, (iii) an additional inequality constraint guarantees that the main use of glucose is through glycolysis (v}_2 \) \text{compared to carbohydrate formation (v}_7 \) \text{and the pentose phosphate pathway (v}_k \) \text{Franzén, 2003; Gonzalez et al., 2003; Quirós et al., 2013). Finally, (iv) \alpha_1 \text{and} \alpha_2 \text{are mass fractions for proteins (see (1)) in, respectively, absence and presence of ammonium concentration in the feeding rate. Note that the other parameters corresponding to biomass composition (\beta \text{and} \gamma) \text{were assumed constant. Different experiments were carried out changing the four precursors of biomass compositions with the presence or absence of ammonium in the feeding rate, and the results showed minimal changes in nucleic acids (\gamma) and lipids (\beta) compositions under the different conditions (results not shown). Given the strong underdeterminacy of system (3) highlighted in Section 3, the use of the objective cost function (10) and the constraints \{(11),(12),(13)\} in FBA does not lead to a unique flux distribution. Hence, lower and upper bounds of the ethanol specific production rate (v}_E \text{MIN}(t_k) \text{and} v}_E \text{MAX}(t_k) \text{are computed by solving, still at each time instant} t_k, \text{2 additional LPs:} v}_E \text{MIN}(t_k) = \text{Min,Max} v}_E \text{still under the constraints \{(11),(12),(13)\} augmented with the additional equality constraint:} v}_E opt(t_k) \text{has been determined thanks to the first LP \{(10),(11),(12),(13)\}.}

4.1 First step: FBA accounting for overflow metabolism and for variable biomass composition

Based on the estimation of the input fluxes v_in(t) = [v_G v_N] (6) computed in Section 3 (Fig. 3), we propose to first determine the output fluxes v_out(t) = [v_E v_L] (7) through FBA. At each time instant t_k for which v_in(t_k) has been estimated, a first linear program (LP) is solved (linprog in MATLAB):

\begin{equation}
\text{v}_G opt(t_k) = \max_v v_79 \tag{10}
\end{equation}

under the constraints,

\begin{align}
Nv = 0; & \quad N_0 v \leq (1 + \epsilon_v) v_in(t_k); \tag{11} \\
N_1 v & \geq (1 - \epsilon_v) v_in(t_k); \\
v_{16} + v_{76} < v_2; \\
v_j \geq 0;
\end{align}

coupled with the conditional inequality constraints accounting for overflow metabolism

\begin{align}
\text{if} \quad v_G > v_Gsat & \quad v_{13} < (v_{11} + v_{12}) \tag{12} \\
\text{else} \quad v_G \leq v_Gsat & \quad -v_{eth} < v_{69} < 0
\end{align}

and with the following conditional equality constraints accounting for variable biomass composition:

\begin{align}
\text{if} \quad N_0 = 0 & \quad \alpha = \alpha_1 \tag{13} \\
\text{else} & \quad \alpha = \alpha_2
\end{align}

\delta = 1 - (\alpha + \beta + \gamma)

where (i) \epsilon_v is the smallest variation coefficient (1% in this work) which allows feasible solutions to the LP \{(10),(11),(12),(13)\} at each time instant t_k, (ii) \nu_j \geq 0 are the positivity inequality constraints (j being the indices of fluxes which are assumed to be positive, i.e. j \in \{1,\ldots,85\}

\begin{align}
\nu_G & \leq \nu_G SAT \tag{14} \\
\nu_G & > \nu_G SAT \tag{15}
\end{align}

c. Ethanol and nearly zero glucose are produced. d. Respiration: glucose is fully oxidized. b. Respiro-fermentation: overflow metabolism with glucose excess and ethanol production.

4.2 Second step: FBA-based dynamical model

In this study, FBA is used for predicting the concentration time profiles of biomass \(X(t)\) and ethanol \(E(t)\) from the external inputs fluxes \(v_{in}(t)\). To this end, the mass balance equations of biomass and ethanol are introduced and involve the production rates \(v_{out} = [v_E v_L] \) (16) predicted by FBA in the above subsection 4.1. The macroscopic FBA-based model is given by the following set of ODE’s:

\begin{align}
\dot{X}(t) = F(t) & \quad \tag{16} \\
\dot{E}(t) = v_{E opt}(t) X(t) - D(t) X(t) & \quad \tag{17} \\
\dot{E}_{min}(t) = v_{E \text{MIN}}(t) X(t) - D(t) E_{min}(t) & \quad \tag{18} \\
\dot{E}_{max}(t) = v_{E \text{MAX}}(t) X(t) - D(t) E_{max}(t) & \quad \tag{19}
\end{align}

where, at each time instant \(t\), \(V\) is the volume; \(X, E_{max}\) and \(E_{min}\) are the concentrations of biomass and ethanol; \(D=F/V\) is the dilution rate; \(F\) is the volumetric feed flow rate; \(v_{E \text{opt}}\) is the optimal (maximum) growth rate and \(v_{E \text{MIN,MAX}}\) are the minimum and maximum values of \(v_E\).

Note that, at each time instant \(t\) considered by the ODE solver (ode15s in MATLAB) used for integrating \{(16),(17),(18),(19)\}, the fluxes \(v_{E \text{opt}}(t)\) and \(v_{E \text{MIN,MAX}}(t)\) are known from the LPs solved in step 1 of sub-section 4.1 and from linear interpolation between the results obtained at the closest time instants \(t_{k-1}\) and \(t_k\) for which these fluxes have been determined and such that \(t_{k-1} \leq t \leq t_k\). This two-step procedure allows separately solving the FBA LPs and the macroscopic mass balance ODEs, which lowers the
computational load in comparison with FBA LPs nested in the ODE solver.

4.3 Parameter identification and model validation

The Nelder-Mead simplex algorithm (function fminsearch in MATLAB) is used to minimize a least-squares criterion (sum of squared differences between model predictions and experimental measurements):

\[
J(\theta) = \sum_{i=1}^{n_{exp}} \sum_{j=1}^{N_j} (Y_{ij}(\theta) - Y_{m,ij})^2 (W_{ij}^{-1}) (Y_{ij}(\theta) - Y_{m,ij})
\] (20)

where \( \theta \) is the vector of parameters to be identified \( \theta^T = [\alpha_1 \alpha_2 \beta \gamma \varepsilon \nu_{gSat} \nu_{eth}] \), \( Y_{ij}(\theta) = [X_{ij} E_{ij}] \) is the vector of simulated variables calculated from mass balance ODEs \{16), (17), (18), (19)\} at the \( i \)th time instant of the \( j \)th experiment, \( Y_{m,ij} = [X_{m,ij} E_{m,ij}] \) is the vector corresponding to the measurements of biomass and ethanol concentrations and \( W_{ij} \) is a weighting matrix defined as:

\[
W_{ij} = \text{diag}(\sigma^2(X_{m,ij}), \sigma^2(E_{m,ij}))
\] (21)

where \( \sigma^2 \) are the variances of the corresponding measurement errors. 100 uniformly distributed pseudo-random values over a given range (Table 1) were used as multistart strategy for the initialization of the algorithm to circumvent local minima and convergence problems. The identified parameter values based on all the experiments are presented in Table 1. Note that minimizing (20) is a bi-level optimization problem: for each parameter set \( \theta \) to be assessed by (20) at a given iteration of the Nelder-Mead simplex algorithm, the LPs described in sub-section 4.1 have first to be solved before the ODE solver integrates system \{16),17),(18),19\}. The low computational load of the two-step procedure highlighted at the end of sub-section 4.2 plays here a key role.

Table 1. Initialization and identified values of the 7 parameters

| Parameter | Initialization values | Identified Values |
|-----------|-----------------------|------------------|
| \( \alpha_1 \) | 0.20 - 0.30 | 0.2404 |
| \( \alpha_2 \) | 0.40 - 0.60 | 0.4887 |
| \( \beta \) | 0.05 - 0.15 | 0.1536 |
| \( \gamma \) | 0.05 - 0.15 | 0.1265 |
| \( \varepsilon \) | 0.05 - 0.15 | 0.1096 |
| \( \nu_{gSat} \) | 0.001 - 0.01 | 0.0075 |
| \( \nu_{eth} \) | 0.001 - 0.01 | 0.0042 |

To evaluate the goodness of fit of measured values versus the simulated ones, the correlation coefficient \( R^2 \) is computed for each simulated variable in direct and cross validation of the model:

\[
R^2 = 1 - \frac{\sum_{i=1}^{N_{exp}} \sum_{j=1}^{N_j} (Y_{ij}(\theta) - Y_{m,ij})^2 (Y_{ij}(\theta) - Y_{m,ij})}{\sum_{i=1}^{N_{exp}} \sum_{j=1}^{N_j} (Y_{ij}(\theta) - Y_{m,ij})^2 (Y_{ij}(\theta) - Y_{m,ij})}
\] (22)

where \( \theta, Y_{ij}(\theta), Y_{m,ij} \) are defined as above and \( Y_{m,ij} \) is the mean of the corresponding measurements.

The direct validation and leave-one-out cross validation are presented in Table 2. The biomass and the associated maximum and minimum ethanol concentration time profiles are shown in Fig 5. The proposed model gives a good prediction since the regression coefficients are greater than 0.92 (for biomass concentration) and 0.83 (for ethanol concentration) in all sets of experiments. This suggests that the simulated values are in good accordance with the experimental results. In addition, the results of the parameter estimation obtained with the different sets of experiments are quite similar, showing a same order of magnitude for the SSE and similar values for the regression coefficient values computed on all the experiments. The set of parameters for Exp 2-3-4 has the highest value for SSE and the lowest values for the correlation coefficients. This can be explained as Exp. 1 contains essential information regarding nitrogen limitation conditions.

5. CONCLUSION AND PERSPECTIVES

The macroscopic FBA-based model proposed in this contribution for predicting biomass and ethanol concentration time profiles in fed-batch cultures of \( S. \) cerevisiae exhibits key features:

- a metabolic network representing both respiratory and respiro-fermentative operating regimes;
- conditional inequality constraints in FBA accounting for overflow metabolism;
- conditional equality constraints in FBA accounting for variable biomass composition (as a function of the presence or absence of ammonium concentration in the feeding rate);
- a two-step procedure for separately solving FBA LPs and macroscopic mass balance ODEs;
• a final low structural complexity of 7 parameters: 5 parameters concerning biomass composition and 2 parameters for overflow metabolism.

In future works, the glucose and ammonium specific uptake rates could be replaced with appropriate kinetic structures, such as those described in Richelle and Bogaerts (2015), in the FBA-based model. This would result in a genuine simulation model capable to reproduce the dynamics of the main species (glucose, ammonium, ethanol and biomass) concentrations based only on the initial concentrations and the feeding rate.

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Fig. 5. Direct validation results with the 4 experiments: in the top, biomass concentration time profiles and, in the bottom, maximum (blue) and minimum (red) ethanol concentration time profiles. Comparison of 95 % confidence intervals of the measurements (red bars) with the estimation of the concentration time profiles obtained with the FBA-based model.