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Dynamic Flux Balance Analysis of a Genetic Engineered Cyanobacterium for Ethanol Production. Parameter Estimation

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Chapter 1 In this work, we present a parameter estimation problem for Dynamic Flux Balance Analysis to study the production of ethanol by a mutant strain of the cyanobacterium *Synechocystis* sp. PCC 6803, as well as experimental data. This modified strain harbors the genes *pdc* and *adhB* from *Zymomonas mobilis*. The model includes two major components: (1) a dynamic model with mass balances for biomass, ethanol, nitrate, phosphate, internal nitrogen and phosphorus, and (2) a steady state genome-scale metabolic Linear Problem (LP) model. The biomass equation includes limiting functions for temperature and kinetics of growth inhibition by ethanol toxicity. Limitation of light by biomass accumulation is also taken into account. We formulate the dynamic parameter estimation problem with a weighted least-squares objective function, subject to dynamic mass balance equations at the bioreactor level and the intracellular LP model. The problem is solved in GAMS through a simultaneous optimization approach. The data sets for parameter estimation were obtained in experiments performed over 73 hours in batch liquid cultures. Numerical results provide useful insights on the ethanol production by the genetically modified strain within the context of genomic-scale cyanobacterial metabolism.

1. Introduction

Cyanobacteria or blue-green algae constitute an ancient and diverse group (150 genera with about 2000 species) of autotrophic prokaryotes that played a crucial role in the atmosphere change from oxidative to reductive during the Precambrian period (Komarek, 2003). These microorganisms have developed adaptive mechanisms that allowed them to survive within a wide range of habitats, including freshwater, marine and terrestrial environments. They have a wide variety of morphologies, metabolism and cell structures and are very important in the nitrogen cycle in marine environments and in the dynamics of nitrogen and CO_2 in the biosphere (Zehr *et al*., 2001). Cyanobacteria have been studied for a long time, they are considered as models of biological processes such as oxygenic photosynthesis which is the same process performed by higher plants. In the last decades they have attracted attention due to their potential to obtain commercially interesting products, such as biofuels and pharmaceuticals. Advances in metabolic engineering and synthetic biology based on gene sequence, biochemical and physiological data in public databases together with the constant improvement of mathematical tools can help speed the development of desirable phenotypes for production of several interesting bio-products (Picataggio, 2009).
In this work we formulate a parameter estimation problem for Dynamic Flux Balance Analysis approach to study the production of ethanol by a mutant strain of the cyanobacterium *Synechocystis* sp. PCC 6803. We formulate a dynamic parameter estimation problem with a weighted least-squares objective function subject to dynamic mass balance equations at the bioreactor level and an intracellular LP model. The problem is solved in GAMS through a simultaneous dynamic optimization approach.

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2. System

Chapter 2 The system under study in this paper is the photosynthetic ethanol production by a mutant strain of *Synechocystis* sp. PCC 6803 obtained by Vidal Vidal (2009) in batch liquid culture. This modified strain harbors the genes *pdc* and *adhB* encoding for the enzyme pyruvate descarboxylase (EC 1.2.4.1), which catalyzes the non-oxidative decarboxylation of pyruvate to acetaldehyde and CO_2 and the alcohol deshydrogenase II (EC 1.1.1.1), which participates in the reduction of acetaldehyde to ethanol. Both heterologous genes from *Zymomonas mobilis* are cloned under the control of the promoter of the endogenous gene *petE* in the *Synechocystis* mutant.

1. Methods

1.1 Dynamic Flux Balance Analysis

Flux Balance Analysis (FBA) is a genome-scale constraint- based modelling approach for metabolic networks (Höffner *et al*. 2012) where the constraints are the steady state mass balances corresponding to metabolic fluxes (reactions) around each node (metabolite). The steady state assumption is justified by high reaction rates within the microorganism. In this way, the mass balances are described by a set of lineal equations,

$$Av = 0 \tag{1}$$

where A is the *m* (metabolites) x *n* reactions) stoichiometric matrix and v are the vector of flux (metabolic reactions of the network). To solve the intracellular fluxes and the uptake and secretion rates of the cell, a linear programming problem (LP) is formulated where the objective function is the maximization of the growth rate (Varma and Palsson, 1994). Solving the LP problem we obtain the metabolic flux distribution of the cell (Paulo *et al*., 2011). In order to describe the dynamics of the substrates, products and biomass concentrations and metabolic fluxes inside the cell we have extended FBA to a Dynamic Flux Balance Analysis approach, which allows modeling the interaction between the cellular metabolism and the environment (Höffner et al., 2012). Therefore, an LP problem is embedded within a dynamic model that takes into account mass balances for main substrates, products and biomass at the bioreactor level, allowing the inclusion of kinetic expression.

3.1.1 Dynamic mass balances

We formulate mass balances for external metabolites and biomass to model the environmental changes during *Synechocystis* growth by substrate consumption and biomass and product accumulation. We also include mass balances for internal phosphorus and nitrogen to model the storage of the main nutrients. The resulting Differential Algebraic Equation (DAE) problem is as follows:

$$\frac{dX}{dt} = \mu X - k_d X \tag{2}$$

$$\frac{dPO_4}{dt} = -v_{PO_4} AX \tag{3}$$

$$\frac{dNO_3}{dt} = -Y_{xn} \mu X \tag{4}$$

$$\frac{dE}{dt} = v_E AX \tag{5}$$

$$\frac{dPI}{dt} = UP_{max}\left(\frac{PO_4}{PO_4 + K_P}\right)\left(\frac{P_{max} - PI}{P_{max} - P_{min}}\right) - \mu PI \tag{6}$$

$$\frac{dNI}{dt} = UN_{max}\left(\frac{NO_3}{NO_3 + K_N}\right)\left(\frac{N_{max} - NI}{N_{max} - N_{min}}\right) - \mu NI \tag{7}$$

$$\mu = v^{*}_{growth} f(T) f(E) \tag{8}$$

$$f(E) = \frac{1}{1 + \frac{E}{KI}} \tag{9}$$

$$f(T) = \frac{T}{T_{opt}} exp\left(1 - \frac{T}{T_{opt}}\right) \tag{10}$$

Where, X, PO_4, NO_3, E, PI and NI represent biomass, phosphate, ethanol, nitrate, internal phosphate and internal nitrate, respectively. The net growth rate (μ) is calculated affecting the maximum growth rate (v^{*}_{growth}) by limiting functions for ethanol concentration and temperature. Table 1 shows description and values of the model parameters.

3.1.2 LP problem

The internal metabolism is represented by the following optimization problem:

$$\max_{v,b} \; v_{growth} \tag{11}$$

s.t.

$$Av = 0 \tag{12}$$

$$v^{L} \le v \le v^{U}, \forall \; v \ne v_{APF}, v_{PO_4} \tag{13}$$

The intracellular and extracellular models are linked by growth rate, absorbed photon flux (v_{APF}) and phosphorus uptake rate (v_{PO_4}). The model includes a limiting function for the light uptake that takes into account the decrease of light availability by biomass accumulation in the reactor. The phosphate uptake incorporates a kinetic expression depending on both external and internal phosphorus concentration. Bounds over these fluxes represent additional constraints for the inner problem.

$$v_{APF} \le v^{*}_{APF} f(I) \tag{14}$$

$$v_{PO_4} \le v^{*}_{PO_4} f(N) \tag{15}$$

$$f(N) = \frac{PI - P_{min}}{P_{max} - P_{min}} \tag{16}$$

$$f(I) = \frac{I_o}{I_{opt}} exp\left(1 - \frac{I_o}{I_{opt}}\right) \tag{17}$$

$$I_o = \frac{1 - exp(K_{ext} pX)}{(K_{ext} pX)} \tag{18}$$

1.2 Experimental data

Synechocystis sp. PCC 6803 was cultivated in BG-11 medium at 30 °C under continuous light (100 μE m^{-2} s^{-1}) and air bubbling enriched with 1% CO_2, which is considered as a CO_2 rich medium. Liquid batch cultures were performed by duplicate for wild type and ethanol mutant strains for 73 h from the beginning of the exponential growth phase of growth. Biomass was estimated by OD_{730}, Chlorophyll a concentration and total organic carbon. Nitrate and phosphate in the medium were measured by spectrophotometric methods and ethanol by an enzymatic method described by Kaplan and Ciotti (1957).

1.3 Parameter estimation problem

Chapter 3 The parameter estimation problem for *Synechocystis* sp. PCC 6803 has the following general formulation:

$$min\,\varphi(p) = \sum_{i=1}^{N_p} \left(x(t_l) - \overline{x_l}\right)^T W \left(x(t_l) - \overline{x_l}\right) \tag{19}$$

Chapter 4 Subject to DAE system Eqs. (2)-(10), which represent extracellular environment and the LP Eqs. (11)-(18) representing *Synechocystis* sp. PCC 6803 metabolism.

$$p^L \leq p \leq p^U \tag{20}$$

$$X_{(0)} = 0.078 g/l, E_{(0)} = 0.046 mM, PO_{4(0)} = 0.056 mM, NO_{3(0)} = 6.868 mM \tag{21}$$

Chapter 5 Where $\overline{x}_l$ are N_D sets of experimental measurements obtained at time t_l, and $x(t_l)^T = \left[z(t_l)^T\right]$ are the corresponding calculated differential variables for measured components. *W* is the weighting matrix for the least squares function (Eq. (19)). In this typical parameter estimation problem were defined data sets in each period, the state variables remains continuous over all periods, and parameters (*p*) are the same in all periods (Bard, 1974). p^L and p^U are the lower and upper bounds over the estimated parameter while $X_{(0)}$, $E_{(0)}$, $PO_{4(0)}$ and $NO_{3(0)}$ stand for initial conditions for the biomass, ethanol, phosphate and nitrate concentrations respectively.

Chapter 6 In order to embed the outer optimization problem and the inner one, it is well known that the Karush-Kuhn-Tucker (KKT) optimality conditions are necessary, to represent the intracellular model in this work, and transform the original bilevel problem into a single level one. The dynamic parameter estimation problem corresponding to component mass balance equations and path constraints is transformed into a NLP through orthogonal collocation (Biegler *et al*., 2006) the problem and is solved in (General Algebraic Modelling System, Brooke *et al*., 2011) with the CONOPT solver.

1. Results and Discussion

We present the numerical results from solving the parameter estimation problem with a dynamic simultaneous approach for a batch ethanol production system for *Synechocystis* sp. PCC 6803 mutant. The batch time was 73 h. The time horizon was discretized with 73 finite elements and 2 collocation points, resulting a NLP of 88,337 equations and 58,063 variables. Table 1 shows the optimal values for the estimated parameters. Figures 1-4 show state variables profiles as compared to experimental data. There is good agreement between experimental data for *Synechocystis* biomass, nitrate and ethanol concentrations and predicted values. During the initial phase, the model accurately reproduces the behaviour of the phosphate concentration. However, from after 30 hours, the model predictions do not exactly agree with experimental data from phosphate dynamics. Pitt *et al*., (2010) and Burut-Archanai *et al*., (2011) reported that *Synechocystis* sp. PCC 6803 has two phosphate transport systems, Pst1 and Pst2. Pst1 is a low-affinity and high-velocity transporter, which predominates in high phosphate concentrations, while Pst2 is characterized by a high-affinity and low-velocity. In this study, we take into account only one phosphate uptake kinetic for high concentrations. Both kinetic will be included in future work through complementarity constraints (Raghunathan *et al*., 2006).

Table 1: Parameters values used in model simulation and estimated parameter values.

Symbol	*Description*	*Value*	*Units*	
kd	*[mortality rate]*	*0.033*	*[1/h]*	*Estimated value*
Kext	*[light extintion coefficient]*	*16.748*	*[m^2/g]*	*Estimated value*
Ki	*[constant inhibition constant]*	*310.135*	*[mM]*	*Estimated value*
Ynx	*[nitrogen biomass yield]*	*1.228*	*[mM/g]*	*Estimated value*
UPmax	*[maximum phosphate uptake]*	*0.244*	*[mM/h]*	*Fixed*
UNmax	*[maximum nitrogen uptake]*	*0.184*	*[mM/h]*	*Fixed*
KP	*[half saturation constant for phosphate uptake]*	*4.44*	*[mM]*	*Fixed*
KN	*[half saturation constant for nitrogen uptake]*	*2.82*	*[mM]*	*Fixed*
$v^0_{PO_4}$	*[maximum phosphate velocity rate]*	*0.184*	*[1/h]*	*Fixed*
v^0_{PAR}	*[maximum light velocity rate]*	*150*	*[1/h]*	*Fixed*

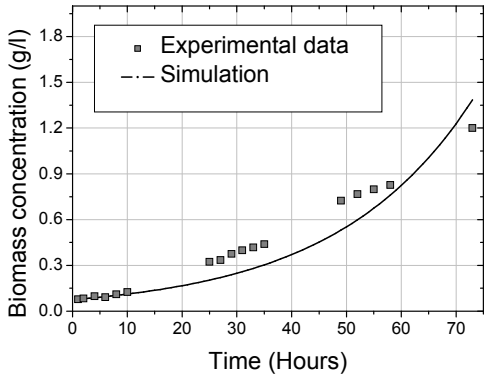

Figure 1. Biomass concentration for measured and predicted profiles.

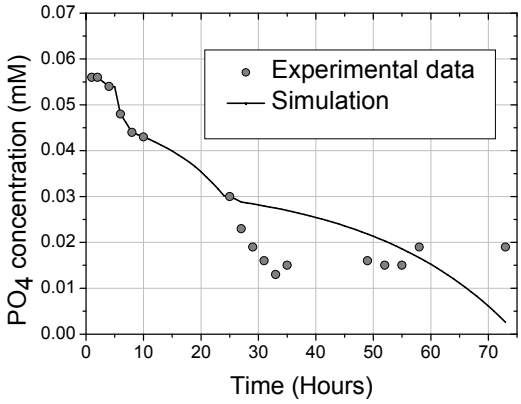

Figure 2. Phosphate concentration for measured and predicted profiles.

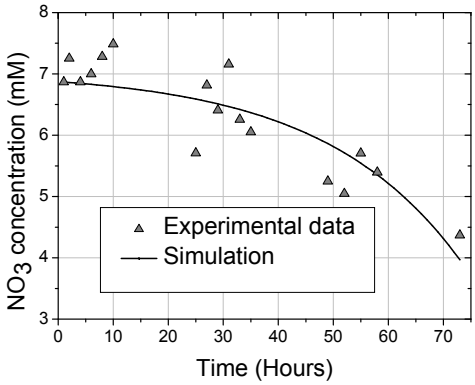

Figure 3. Nitrate concentration for measured and predicted profiles.

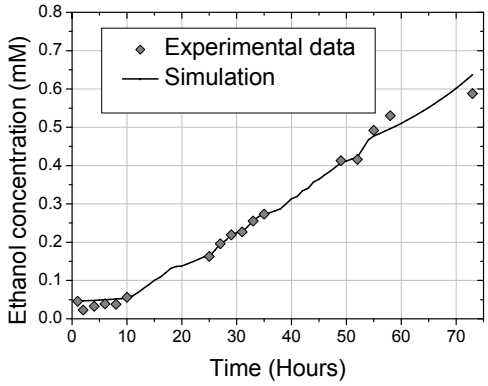

Figure 4. Ethanol production for measured and predicted profiles.

2. Conclusions

In this work, we have coupled in a simultaneous framework the interaction between environment and intracellular metabolism of an ethanol producer mutant of *Synechocystis* sp. PCC 6803. The parameter estimation problem has been formulated within a simultaneous dynamic optimization framework within GAMS 23.0, with CONOPT shows good agreement with experimental data.

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