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Validation and Optimization of a Yeast Dynamic Flux Balance Model using a Parallel Bioreactor System

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Abstract: The availability of parallel fermentation systems comprised of miniature, independently controlled bioreactors provides new opportunities for high throughput bioprocess development. In this study, we demonstrated the use of a four bioreactor system to validate predictions from a dynamic flux balance model of *Saccharomyces cerevisiae* metabolism. First we showed that the four 250 mL bioreactors generated very reproducible aerobic batch culture data and that the parallel system results could be accurately scaled-up to a standard 1.25 L laboratory bioreactor by matching oxygen mass transfer coefficients in the different reactors. A *S. cerevisiae* dynamic flux balance model previously developed in our group was shown to produce anaerobic and aerobic batch profiles in excellent agreement with the parallel system. The validated model was used to determine the optimal aerobic-anaerobic switching time for maximal ethanol production in batch culture. An optimal switching time in agreement with parallel system experiments was obtained. We concluded that parallel fermentation is a powerful tool for batch culture optimization when used in conjunction with dynamic metabolic models.

Keywords: dynamic modeling, microbial biotechnology, biofuels, bioreactors, process optimization

1. INTRODUCTION

Dynamic flux balance analysis (DFBA) is a computational approach for analyzing and engineering cellular behavior in dynamic culture environments that predominate in batch and fed-batch biochemical reactor (Mahadevan et al., 2002; Varma et al., 1994). The basic element of DFBA is a dynamic flux balance model that combines stoichiometric mass balances on intracellular metabolites with dynamic mass balances on extracellular species through substrate uptake kinetics and the cellular growth rate. When a genome-scale reconstruction of cellular metabolism is used (Hjersted and Henson, 2009), DFBA can be expected to generate more accurate predictions than a conventional unstructured bioreactor model based on phenomenological descriptions of cellular growth and product yields. The development of customized computational tools allows DFBA to address a wide variety of problems in bioreactor analysis and design, including the dynamic simulation of batch and fed-batch cultures (Sainz et al., 2003), the dynamic optimization of fed-batch operating policies (Hjersted and Henson, 2006), the *in silico* design of metabolite overproduction mutants for batch and fed-batch bioreactors (Hjersted et al., 2007), and the simulation and optimization of microbial co-cultures (Hanly and Henson, 2010; Hanly et al., 2012). Validation of dynamic flux balance models requires the execution of batch and/or fed-batch experiments over the growth conditions of interest. Because the use of standard laboratory bioreactor systems for such experiments can be time consuming and expensive, there is considerable motivation to explore alternative cell culture technologies for dynamic metabolic model development.

Bioprocess engineering often involves the screening of many fermentation conditions to discern optimal conditions for cell growth and product formation (Kennedy and Krouse, 1999; L et al., 2009; Parekh et al., 2000). Traditionally, shake flasks and microtiter plates have been used in early stages of cell culture development and optimization. However, most of these systems lack on-line monitoring and control of crucial variables such as pH and dissolved oxygen (Archambault et al., 1996; Buchs, 2001; Szita et al., 2005). Recent advancements in bioprocess technology have focused on improving high throughput screening of fermentation operating conditions through the development of miniature, parallel bioreactors (Betts and Baganz, 2006; Weuster-Botz, 2005; Amanullah et al., 2010). Unlike shake flasks and microtiter plates that provide only surface aeration, air can be directly sparged into the culture to provide more uniform aeration and achieve more stable dissolved oxygen concentrations (Betts et al., 2006; Puskeiler et al., 2005). Parallel bioreactor systems have been used to study media formulations (Betts and Baganz, 2006), the effects of substrate perturbations (Aboka et al., 2006), the effects of aeration and agitation on oxygen limitation (Gill et al., 2008a), and the identification, characterization, and improvement of biocatalysts (Lye et al., 2003).

The objective of this study was to demonstrate the use of a parallel fermentation system for the validation and optimization of a dynamic flux balance model of *Saccharomyces cerevisiae* metabolism in batch culture. First the reproducibility of the parallel system was evaluated by operating each of the four 250 mL bioreactors at identical batch operating conditions and comparing their glucose, biomass and ethanol profiles. Then the oxygen mass transfer coefficient used in parallel fermentations was matched in a

standard 1.25 L bench-scale bioreactor to demonstrate scale-up capabilities. Batch profiles generated from the *S. cerevisiae* dynamic flux balance model were compared to those obtained from the parallel bioreactors to validate the model predictions. We have previously shown that the dynamic flux balance model predicts optimal ethanol production at a particular aerobic-anaerobic switching time (Hjersted et al., 2007). The parallel system was used to validate this computational result and to efficiently determine the sensitivity of the ethanol productivity to the switching time.

2. MATERIALS AND METHODS

2.1 Experimental Procedures

The wild-type *Saccharomyces cerevisiae* strain ATCC 32167 was used in all experiments. Cells were cultivated in a minimal medium composed of 20 g/L glucose, 1.00 g/L $MgSO_4 \cdot 7\ H_2O$, 1.10 g/L KCl, 0.15 g/L $CaCl_2 \cdot 2\ H_2O$, 1.00 g/L $(NH_4)_2HPO_4$, 8.75 g/L $(NH4)_2SO4$, 60.3 mg/L myo-inositol, 30.0 mg/L Ca-panthothenate, 6.0 mg/L thiamine-HCl (Vit. B1), 1.5 mg/L pyridoxine-HCl (Vit. B6), 0.03 mg/L biotin (Vit. H), 15.0 mg/L $FeCl_3 \cdot 6$ H2O, 10.6 mg/L $MnSO_4 \cdot H_2O$, 9.0 mg/L $ZnSO_4 \cdot 7\ H_2O$, and 2.4 $CuSO_4 \cdot 2\ H_2O$. During fermentations in which *S. cerevisiae* grew anaerobically, the medium was supplemented with 20 mg of ergosterol dissolved in 1.25 mL of a 50/50 solution of Tween 80 and ethanol for every liter of media.

Prior to bioreactor inoculation, cells from plates stored at 4°C were grown in 150 mL shake flasks containing 60 mL of minimal media and 20 g/L glucose for 18 hours at 140 RPM. The miniature stirred tank bioreactor system studied in this work was a BioXplorer four reactor system (HEL Group Ltd., Barnet, UK). The system consisted of four 250 mL glass reactors situated in one heating block that provides independent control of temperature. Each reactor also had an independent agitator. Dedicated electrochemical probes for dissolved oxygen, pH and temperature were fitted in the headplate of each reactor. Four peristaltic pumps for each reactor allowed the independent addition of acid and base for pH control, the addition of substrate for fed-batch or continuous operation, and/or the extraction of reactor medium samples. Batch operating conditions including temperature, pH and stirrer speed were set for each reactor through winISO software (HEL Group Ltd., Barnet, UK). The volumetric flow rate of air to each reactor was controlled manually by four independent rotameters. The reactors were operated at 30 °C, 5.0 pH, 500 RPM stirrer speed and 150 cm^3/min air sparging rate. Fermentations were initiated with the inoculation of 12.5 mL of shake flask culture into 200 mL of media. The shake flask media added small amounts of ethanol produced by the preculture and some unconsumed glucose to the reactor media. On average, the initial concentrations of the inoculum were 8.6 g/L glucose, 0.46 g/L biomass and 0.17 g/L ethanol. The batch time of each fermentation was determined as the time at which the glucose concentration of dropped below 0.1 g/L. The batch ethanol productivity was calculated by dividing the final ethanol concentration by the batch time.

A BioFlo 3000 (New Brunswick Scientific, Edison, NJ) with a working volume of 1.25 L was used to scale-up the fermentations performed in the parallel system. The reactor was controlled at a temperature of 30 °C, pH 5, and an aeration rate of 0.7 VVM. Oxygen transport in the two reactor systems was quantified using the static gassing out method. Dissolved oxygen was monitored as the reactor was sparged with nitrogen until anoxic and then as the reactor was sparged with air until fully saturated with oxygen. The following equation was used in the calculation of k_La:

$$\frac{dC_L}{dt} = k_L a(C^* - C_L) \quad (1)$$

where C* was the saturated oxygen concentration and C_L was the dissolved oxygen concentration. Two times *t1* and *t2* during air sparging were chosen, and a plot $\ln[(C^*-C_{Lt1})/(C^*-C_{Lt2})]$ versus time produced a line with slope equal to k_La for the given aeration and stirrer speeds. The k_La values were measured for stirrer speeds ranging from 300 to 500 RPM in intervals of 50 RPM.

A 17.5 mL sample was withdrawn from each reactor every two hours to measure the biomass, glucose and ethanol concentrations for the reproducibility study. The first 3 mL were discarded to flush the sample tubing of media that remained from the previous sample. The weight of dry biomass was measured by centrifuging 14.5 mL of the sample at 6000 rpm for 6 minutes. The resulting pellet was then resuspended in nanopure water and spun down again. The pellet was dissolved in 2 mL nanopure water, dried overnight in an oven set to 125 °C and then weighed. Prior to drying, the optical density of each sample was measured at 595 nm with a WPA UV1101 Biotech Photometer (Biochrom Ltd., Cambridge, UK). A calibration curve of OD595 to biomass produced a regressed line with correlation coefficient ($r^2 = 0.9977$) before the curve became nonlinear for OD595 greater than 0.4 (not shown). Samples with OD595 greater than 0.4 were diluted with sterile media to ensure analysis within the linear region. Once the calibration curve was established, the post-flush sample size was reduced from 14.5 to 2 mL and the biomass concentration was quantified solely by optical density.

Extracellular glucose and ethanol concentrations were measured with an YSI 2700 SELECT biochemistry analyzer (YSI Inc., Yellow Springs, Ohio) equipped with the appropriate immobilized enzyme membranes. Although not measured due to lack of the necessary membrane, glycerol was expected to be produced under the partially aerobic and anaerobic growth conditions investigated in this study. The residual 3 mL from the original 17.5 mL sample was analyzed for glucose and ethanol concentrations during the reproducibility study. Due to the time delay associated with sampling, all measurements obtained from the flush were projected back 1.5 hours as an estimate of the reactor state at the most recent sample time. This approximation provided good agreement with dynamic flux balance model predictions. Metabolite measurements for the bench-scale reactor and switching time optimization were taken from the 2 mL sample used to measure OD595. Off-gas measurements of oxygen and carbon dioxide were not available for this

study. While clearly useful for model validation, measurements of intracellular fluxes such as those obtained from C13 substrate labeling also were not available.

2.2 Dynamic Flux Balance Model

Transient predictions of the biomass, glucose and ethanol concentrations were produced from a dynamic flux balance model (Hjersted and Henson, 2009) based on the iND750 genome-scale reconstruction of *S. cerevisiae* metabolism (Duarte et al., 2004). While more recent versions of the *S. cerevisiae* metabolic reconstruction are available (Mo et al., 2009), our previous dynamic flux balance models have been based on the iND750 reconstruction and this model was deemed suitable for this study. The iND750 model consisted of 750 genes and 1149 intracellular reactions, which were divided into seven distinct intracellular compartments. Compartmentalization of the 646 unique metabolites produced a total of 1059 metabolites that were stoichiometrically balanced. The dimensions of the stoichiometric matrix were 1059 metabolites and 1264 fluxes, which included the intracellular reactions and 115 compartmental exchange fluxes.

The stoichiometric equations of iND750 were augmented by glucose and oxygen uptake kinetics as well as extracellular mass balances on biomass, glucose and ethanol as follows (Hjersted and Henson, 2009):

$$\max_{v_i} \mu = w_i^T v_i \tag{2}$$

$$Av_i = 0 \tag{3}$$

$$v_{i,min} \le v \le v_{i,max} \tag{4}$$

$$v_g = v_{gm} \frac{G}{K_g + G} \tag{5}$$

$$v_o = v_{om} \frac{O}{K_o + O} \tag{6}$$

$$\frac{dX}{dt} = \mu X \tag{7}$$

$$\frac{dG}{dt} = -v_g X \tag{8}$$

$$\frac{dE}{dt} = v_e X \tag{9}$$

Here μ is the growth rate, v_i are the intracellular fluxes, w_i are coefficients that represent the contribution of each component to biomass formation, A is a matrix of stoichiometric coefficients, $v_{i,min}$ and $v_{i,max}$ are lower and upper bounds on the fluxes, G, O and E are the extracellular concentrations of glucose, oxygen and ethanol, respectively, X is the biomass concentration, v_g and v_o are uptake rates of each substrate, v_{gm} and v_{om} are the corresponding maximum uptake rates, K_g and K_o are corresponding saturation constants, and v_e is the ethanol flux.

A fully anaerobic fermentation was performed, and the resulting biomass, glucose and ethanol profiles were used to obtain the maximum uptake and saturation constant for glucose uptake (v_{gm} = 22.4 mmol/g/h, K_g = 0.7 g/L). With the glucose uptake parameters fixed, the oxygen uptake parameters (v_{om} = 1.5 mmol/g/h, K_o = 1.0 g/L) were obtained by fitting the model to biomass, glucose and ethanol profiles obtained for a fully aerated fermentation. The dynamic flux balance model was simulated in MATLAB by solving the intracellular linear program with the MOSEK optimization code and the extracellular differential equations with the ode23 function.

3. RESULTS AND DISCUSSION

First, parallel aerobic growth experiments were performed at the same batch fermentation conditions (see Materials and Methods) to investigate reproducibility of the four bioreactors. Following inoculation at time zero, the biomass concentration, optical density, glucose concentration and ethanol concentration measured at each time point showed excellent agreement across all four reactors (Figure 1). Furthermore, similar dissolved oxygen profiles were obtained in all four reactors (not shown). Over the course of the batch fermentations, small differences in the media volume in each reactor occurred due to non-uniform addition of acid and base for pH regulation and small deviations in the volume of extracted samples. Along with inherent measurement errors, these factors can explain the small variability in biomass, glucose and ethanol concentrations between the reactors. We concluded that the parallel system was capable of precisely maintaining operating conditions in the four bioreactors and generating reliable data under well controlled fermentation conditions.

Miniature bioreactor systems are well suited for efficiently screening different cell mutants and growth conditions to determine optimal fermentation conditions. This approach requires scalability of parallel system results to larger production scale bioreactors. To examine this issue in a laboratory setting, the aerobic growth results obtained in the 0.25 L bioreactors (Figure 1) were scaled-up to a bench-scale bioreactor with 1.25 L working volume. Air was sparged into the bench-scale reactor at the same volume of gas per reactor volume per minute as used in the miniature bioreactors. Aeration was scaled-up by matching the volumetric oxygen mass transfer coefficient (k_La) in the miniature and bench-

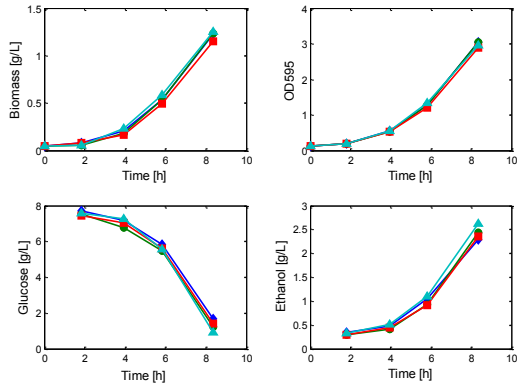

Fig. 1. Reproducibility of the parallel bioreactor system. Comparison of biomass (top left), OD595 (top right), glucose (bottom left) and ethanol (bottom right) batch profiles for each of the four parallel bioreactors under aerobic growth conditions. Each bioreactor is represented by a different symbol.

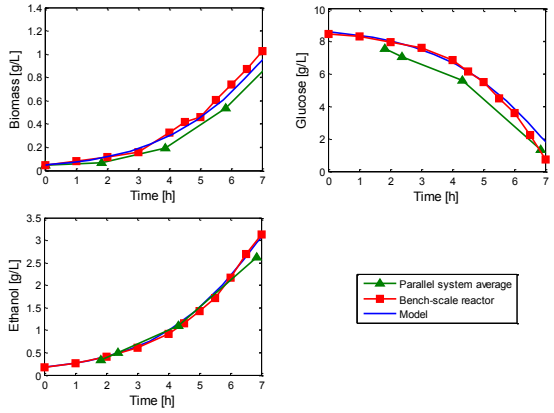

Fig. 2. Validation of the dynamic flux balance model. Comparison of biomass (top left), glucose (top right) and ethanol (bottom left) batch profiles under partially aerobic growth conditions for the bench-scale reactor, the parallel system and the dynamic flux balance model. The parallel system results were obtained by averaging individual profiles from the four miniature bioreactors.

scale bioreactors (Gill et al., 2008b; Flores et al., 1997). Following the static gassing out method (see Material and Methods), the miniature bioreactors were found to be operated with k_La = 0.0225 s^{-1}. To ensure that the scaled-up fermentation was operated under the same oxygen transport conditions, the k_La of the bench-scale bioreactor was determined at different impeller speeds using the static gassing out method. The dissolved oxygen concentration need for this calculation was very stable. The resulting graph (not shown) was used to determine that an impeller speed of 400 RPM produced the target k_La value. Because the bench-scale bioreactor has an assembly of two impellers compared to a single rotor in the miniature bioreactors, the reduced stirrer speed (400 RPM compared to 500 RPM) agreed with our expectations.

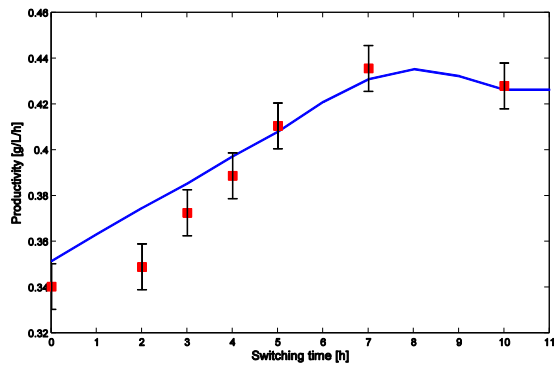

Fig. 3. Comparison of the batch ethanol productivities for different aerobic-anaerobic switching times experimentally determined using the miniature bioreactors (points) and predicted with the dynamic flux balance modeling (solid line). The error bars represent standard deviations calculated from the four independent miniature bioreactors.

Figure 2 provides a comparison of the aerobic growth profiles obtained from the bench-scale reactor, the parallel system and the dynamic flux balance model where the measured biomass concentrations were calculated from the OD595 correlation (see Materials and Methods). The parallel system results were obtained by averaging individual profiles from the four miniature bioreactors. The two experimental systems and the dynamic model produced similar biomass, glucose and ethanol concentration profiles over 7 hour batch fermentations. The procedure for matching the initial biomass concentrations in the two systems created a small disparity between the initial glucose concentrations. The shake flask used to inoculate the bench-scale reactor had a lower cell density but slightly more residual glucose than the shake flask used for the parallel system. This small discrepancy resulted in minor growth rate reduction in the parallel bioreactors as exemplified by the biomass concentration profiles. We concluded that fermentation conditions used in the parallel system could be readily scaled-up to larger bioreactors and that the metabolic model predictions were consistent with experimental results.

We have previously used the *S. cerevisiae* dynamic flux balance model to predict optimal switching times between aerobic and anaerobic growth phases to optimize ethanol production in fed-batch culture (Hjersted et al., 2007). Experimental validation of such predictions can be a time and labor intensive process when fermentations are performed in a single, laboratory scale bioreactor. We used the miniature bioreactors to validate optimal switching time predictions obtained from the dynamic flux balance model to illustrate the combined capabilities for bioprocess engineering. The model was initialized with 0.04 g/L biomass, 8.4 g/L glucose and 0.17 g/L ethanol and simulated for a range of switching times between 0 and 10 hours. The ethanol productivity for each batch simulation was calculated at the final batch time when the glucose concentration dropped below 0.1 g/L. A flat maximum productivity of 0.43 g/L/h was obtained at a switching time of approximately 8 hours (Figure 3).

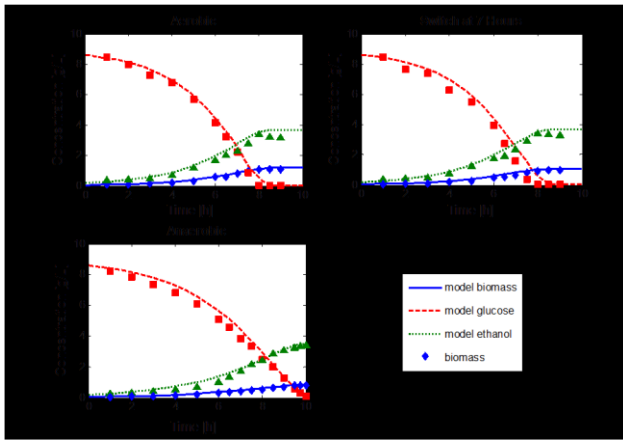

Fig. 4. Experimental (data points) and predicted (lines) glucose, ethanol and biomass concentration profiles for anaerobic growth (switching time of 0 hours), aerobic growth (switching time of 10 hours) and combined aerobic-anaerobic growth (switching time of 7 hours).

The miniature bioreactors were used to validate these *in silico* predictions by performing two sets of parallel fermentations over the same range of switching times. The seven experimentally derived productivities showed good agreement with the model predictions, with an optimal productivity of 0.43 g/L/h at a switching time of 7 hours. Relatively large deviations between experimental and predicted productivities were observed for smaller switching times and mostly anaerobic operation (Figure 3). Such deviations were not unexpected as the dynamic flux balance model does not account for known differences in glucose uptake kinetics under aerobic and anaerobic growth conditions (Akesson et al., 2004).

Figure 4 shows experimental and predicted glucose, ethanol and biomass concentration profiles for three cases: anaerobic (switching time of 0 hours), aerobic (switching time of 10 hours) and the experimentally derived optima (switching time of 7 hours). The experimental data and model predictions generally showed good agreement with the largest deviations observed for the optimal case, perhaps due to unmodeled regulatory phenomenon associated with the aerobic to anaerobic transition (Cohen et al., 2001). Both simulation and experiment demonstrated that the productivity was a weak function of the switching time if the switching time was sufficiently large (Figure 3). For example, similar productivities were obtained for aerobic growth and combined aerobic-anaerobic growth with a switching time of 7 hours (Figure 4). Due to the low maximum oxygen uptake rate (v_{om} = 1.5 mmol/g/h), fermentative pathways were still active under the partially aerobic growth conditions used and the ethanol synthesis rate was similar under aerobic and anaerobic conditions. Therefore, the advantage of anaerobic operation was partially mitigated and substantially depressed productivities were observed only for prolonged anaerobic growth (short switching times) when total biomass formation was reduced. Our previous modeling studies (Hjersted and Henson, 2006) yielded a more pronounced productivity maximum for larger maximum oxygen uptake rates (e.g. v_{om} = 8 mmol/g/h). Despite this complication, we concluded that the combined capabilities of the dynamic flux balance model and the parallel fermentation system represented a powerful tool for batch culture optimization.

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