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Dynamic Flux Balance Modeling of Fed-Batch Co-Culture Fermentation of Glucose/Xylose Mixture for Improved Bioethanol

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Abstract. Lignocellulosic biomass is an attractive sustainable carbon source for fermentative production of ethanol. The lack of a microorganism which can efficiently ferment all sugars derived from lignocellulosic biomasss is the key challenge in its commercial utilization for biofuel. Many genetic and metabolic engineering of wild-type strains have resulted in specific strains that can simultaneously metabolise glucose and xylose. As an alternative strategy, it has been suggested that microbial consortia consisting of substrate-selective microbes may offer distinct advantages over single microbe strategies. We analyze the ethanol productivities from glucose/xylose mixtures in fed-batch fermentation of single microorganism that is engineered to consume both sugars and co-culture fermentation of substrate-selective microbes that selectively uptake glucose and xylose, respectively under the same operating conditions. The simulation results clearly point to the superior performance of fed-batch fermentation of microbial co-culture against fed-batch fermentation of single-organism culture.

Keywords: dynamic flux balance modeling, fed-batch, fermentation, co-culture, mixed substrate

1. Introduction

Ethanol has long been recognized as a potential alternative to petroleum-derived transportation fuels. Since the use of traditional raw materials such as sugar cane juice and corn starch for ethanol production can be uneconomical, lignocellulosic biomass such as agricultural residues and hardwood wastes has recently been identified as an attractive sustainable carbon source for fermentative production of ethanol and other value-added chemicals. Lignocellulose is essentially composed of cellulose (~45% of dry weight), hemicellulose (~30% of dry weight) and lignin (~25% of dry weight) (Wiselogel et al., 1996). While cellulose hydrolysis produces easily fermentable hexose sugars such as glucose, hemicellulose hydrolysis produces a mixture of hexose (glucose) and pentose sugars (xylose, arabinose). The key challenge for commercial utilization of lignocellulose for biofuels is the lack of microorganism that is able to ferment efficiently all sugars released by hydrolysis of lignocellulosic biomass (Zaldivar et al., 2001). The common yeast *Saccharomyces cerevisiae*, the most widely used microorganism in ethanol fermentation from glucose, is unable to ferment xylose natively. There are many microbes that are able to utilize pentose sugars, but catabolism of these sugars is suppressed by the presence of hexose, which remains a preferred substrate for many microbes. The selective and sequential uptake of mixed sugars by most microbes may effectively reduce the overall efficiency of the process. Since xylose is present in abundance in many lignocellulosic biomasses, the efficient and simultaneous conversion of glucose and xylose is essential for economic utilization of lignocellulose for production of biofuels.

Many genetic and metabolic engineering of traditional strains have focused on developing a single recombinant strain which can efficiently ferment the mixed sugars derived from lignocelluloses to produce biofuel. As a result of these attempts aimed at modifying the cellular metabolism of microbes, specific strains of *Saccharomyces cerevisisae*, *Escherichia coli*, and *Zymomonas mobilis*, etc. have been engineered for simultaneous metabolism of glucose and xylose. As an alternative strategy, it has been suggested that microbial consortia consisting of substrate-selective microbes may offer distinct advantages over single microbe strategies in simultaneous consumption of hexose and pentose sugars (Eiteman et al., 2008; Fu et al., 2009).

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The availability of genome-scale stoichiometric models for various microbes has enabled the in-silico characterization of various genetic and process engineering strategies to identify the optimal strategy in terms of increasing the yield of bioethanol. An efficient approach to analyze a genome-scale metabolic network is Flux Balance Analysis (FBA), where a linear programming problem is solved to determine the intracellular fluxes in an underdetermined stoichiometric model under the assumption that the cell distributes carbon flux to achieve maximum growth rate (Stephanopaulose et al., 1998). FBA methods assume time-invariant extracellular conditions and generate steady-state predictions consistent with continuous culture. Since large-scale production of the metabolic products is often achieved with batch and fed-batch culture, the classical FBA method has been successfully extended for such dynamic situations (Mahadevan et al., 2002). Dynamic flux balance models are obtained by combining stoichiometric equations for intracellular metabolism with dynamic mass balances on key extracellular substrates and products under the assumption of fast intracellular dynamics. The intracellular and extracellular descriptions are coupled through the cellular growth rate and substrate uptake kinetics, which can be formulated to account for known regulatory effects such as inhibition of growth by products.

The first computational study on genome-scale analysis of *S.cerevisiae* to examine various genetic engineering strategies for ethanol overproduction from glucose and glucose/xylose media in a fed-batch culture is reported by Hjersted et al. (2007). A genome-scale dynamic flux balance model is developed for a recombinant *S. cerevisiae* strain RWB 218 that is capable of consuming both glucose and xylose and the maximization of ethanol productivity in fed-batch fermentation is addressed by computing optimal aerobic-anaerobic switching times. Hanly and Henson (2010) used dynamic flux balance modeling to examine the capability of mixed culture of substrate-selective microbes (engineered *E. coli* and wild-type yeast) to improve the utilization of glucose/xylose mixtures for enhanced ethanol production in batch fermentation. The authors conclude, on the basis of batch ethanol productivities, that batch co-culture is capable of enhanced ethanol production compared to single-organism batch culture that consume both glucose and xylose. The goal of the present study is to explore dynamic flux balance modeling to investigate the efficiency of microbial co-culture in *fed-batch* fermentation of glucose/xylose mixtures for improved production of bioethanol. The fed-batch operation is attractive as the substrate levels can be varied during the operation to achieve a favorable tradeoff between cellular growth and product formation rates. To our knowledge, this study represents the first application of dynamic flux balance analysis to fed-batch fermentation of microbial co-cultures. Specifically, in this study we analyze the ethanol productivities from glucose/xylose mixtures in fed-batch fermentation of single microorganism (mono-culture) that is engineered to consume both sugars (recombinant *S. cerevisiae* RWB 218) and co-culture fermentation of substrate-selective microbes that selectively uptake glucose (wild-type and genetically modified strains of *S. cerevisiae*) and xylose (*E. coli* ZSC 113), respectively under the same operating conditions.

2. Dynamic Flux Balance Analysis

The stoichiometric models used in this study were adapted from iND750 *S. cerevesiae* and iAF1260 *E.coli* genome-scale metabolic reconstructions. The wild-type *S. cerevesiae* model iND750 is fully compartmentalized and accounts for 750 genes, 1266 fluxes and 1061 metabolites (Duarte et al., 2004). The wild-type *E.coli* model IAF1260 consists 1261 genes, 2382 fluxes and 1668 metabolites (Feist et al., 2007). The modification needed to iND750 for simulation of recombinant xylose-utilizing *S. cerevisiae* strain RWB 218 was the insertion of the reverse reaction for xylitol dehydrogenase (Hjersted et al., 2007). The modification needed for xylose-selective *E. coli* strain ZSC113 was to constrain the glucose exchange and glucose kinase fluxes to be zero. A number of genetic manipulation strategies on wild-type *S. cerevisiae* that involve gene insertions, gene overexpressions, and gene deletions have been suggested (Bro et al., 2006) for ethanol overproduction. These modifications are aimed at decreasing the glycerol yield and increasing the ethanol yield on glucose media under anaerobic conditions. We consider some of these recombinant strains as glucose-selective microbes for our fed-batch co-culture study. The details of the metabolic engineering strategies are indicated in the following section.

Under the simplifying assumption that the two microbial species are non-interacting and grow optimally under the same environmental conditions, the flux balance model for the co-culture assumes the following standard linear program (LP) form (Hanly and Henson, 2010):

$$\begin{aligned} &\min_{v_i} \mu_i = w_i^T v_i \\ &\text{subject to: } A_i v_i = 0 \\ &v_{i,min} \le v_i \le v_{i,max} \end{aligned} \qquad (1)$$

where i represents the species, A_i is the matrix of stoichiometric coefficients, v_i is the vector of intracellular reactions and exchange fluxes, $v_{i,min}$ and $v_{i,max}$ are the lower and upper flux bounds, μ_i is the specific growth rate, and w_i is the vector of weights that represent the contribution of each flux to biomass formation. The dynamic flux balance model is obtained by augmenting the stoichiometric models with the following substrate uptake kinetics for glucose, v_g (Eq. 2); xylose, v_z (Eq. 3); oxygen, v_o (Eq. 4); and the mass balance equations on extracellular species (Eq. 5-10), where G, Z, E, and O are the glucose, xylose, ethanol, and dissolved oxygen concentrations, respectively. K_g, K_z, and K_o are saturation constants, $v_{g,max}$, $v_{z,max}$, and $v_{o,max}$ are maximum uptake rates, and K_{ie} and K_{ig} are inhibition constants.

$$v_g = v_{g,max}\frac{G}{K_g+G}\frac{1}{1+\frac{E}{K_{ie}}} \quad (2)$$

$$v_z = v_{z,max}\frac{Z}{K_z+Z}\frac{1}{1+\frac{E}{K_{ie}}}\frac{1}{1+\frac{G}{K_{ig}}} \quad (3)$$

$$v_o = v_{o,max}\frac{O}{K_o+O} \quad (4)$$

$$\frac{dV}{dt} = F \quad (5)$$

$$\frac{d(VX_g)}{dt} = \mu_g VX_g \quad (6)$$

$$\frac{d(VX_z)}{dt} = \mu_z VX_z \quad (7)$$

$$\frac{d(VG)}{dt} = FG_f - v_g VX_g \quad (8)$$

$$\frac{d(VZ)}{dx} = FZ_f - v_z VX_z \quad (9)$$

$$\frac{d(VE)}{dt} = v_{e,g}VX_g + v_{e,z}VX_z \quad (10)$$

Here V is the liquid volume, F is the feed flow rate, X_g and X_z are the concentration of yeast and *E.coli*, respectively. G_f and Z_f are the glucose and xylose concentrations in the feed, respectively. The solution of the inner flux balance model provides the growth rate (μ) and the ethanol exchange flux (v_e). The oxygen balance equation is not considered as the dissolved oxygen concentration is assumed to be regulated at 0.24 mmol/L for aerobic simulation. The model parameter values used in the simulation are taken from Hanly and Henson (2010) and are listed in Table 1. Dynamic flux balance model simulations are performed in Matlab environment using ode23s to integrate the extracellular dynamic mass balance equations and the COBRA Toolbox (Becker et al., 2007) with Matlab interface to the LP code *glpk* to solve the inner linear program.

3. Results and Discussion

All the fed-batch simulations are carried out to ferment a fixed amount of total sugar using a constant feed flow rate with a fixed feed substrate concentrations and an aerobic to anaerobic switch. The total amount of inoculums is also kept the same in all simulations. The switching times are determined optimally using the single variable bounded search algorithm *fminbnd* in Matlab.

We first conduct fed-batch simulation for recombinant *S. cerevisiae* RWB 218 that is engineered for both glucose and xylose uptake with the operating conditions given in Table 1 and the results are presented in Fig. 1A. The operating conditions such as feed concentrations and fermentation time are same as used in Hjersted et al. (2007) and the simulation results agree very well with their reported results. It may be noted that glucose is consumed preferentially and xylose concentration starts decreasing sharply only when glucose is nearly exhausted. This may be due to the catabolic repression on the xylose assimilation caused by glucose. Since aerobic phase promotes cell growth and the anaerobic phase enhances the ethanol production, substantial increase in the ethanol production and a sharp decrease in biomass production follow the switching from aerobic to anaerobic regime at 16.43h. A long aerobic phase is necessary to produce enough biomass such that most of the sugars are consumed by the end of operation. This fed-batch fermentation for t_f = 20 h produces 20.24 g of ethanol with a final ethanol concentration of 16.87 g/L and predicts a productivity value (defined as ratio of total mass of ethanol produced to the duration of batch, $(VE|_{t_f})/t_f$) of 1.01 g/h.

Table 1: Substrate uptake parameters and operating conditions

Parameter	Co-culture		Mono-culture	Parameter	Co-culture	Mono-culture
	S. cerevisiae	*E. coli*	*S.cerevisiae*			
$v_{g,\max}$ (mmol/g/h)	22.4	0	7.3	F (L/h)	0.04375	0.035
K_g (g/L)	0.8	0	1.026	G_f (g/L)	50	50
$v_{o,\max}$ (mmol/g/h)	2.5	15	8	Z_f (g/L)	50	50
K_o (mmol/L)	0.003	0.024	0.003	t_f (h)	16	20
$V_{z,\max}$ (mmol/g/h)	0	12	32	G_0 (g/L)	5	5
K_z (g/L)	0	0.25	14.85	Z_0 (g/L)	5	5
$K_{i,g}$ (g/L)	0.5	0.005	0.5	V_0 (L)	0.5	0.5

$K_{i,e}$ (g/L)	10	20	10			
X_0 (g/L)	0.044	0.006	0.05			

In order to investigate the efficiency of co-culture fed-batch operation, we carry out fed-batch simulations with various strains of *S. cerevisiae* (wild-type and genetically modified) that consume only glucose and recombinant *E. coli* ZSC 113 that consumes only xylose. The operating conditions are given in Table 1. It may be noted that we keep the operating conditions such that the total amount of inoculums (0.025 g) and the total amount of sugars fed (70 g) are same in all fed-batch runs (both co-culture and mono-culture) to make a fair comparison. The ratio of inoculums concentrations and the final batch time are determined by a systematic sensitivity analysis. The fed-batch simulation for co-culture comprised of wild-type *S. cerevisiae* and recombinant *E. coli* ZSC 113 for t_f= 20 h and a switching time t_s = 7.50 h produces 23.56 g of ethanol with a final ethanol concentration of 19.64 g/L and predicts a productivity value of 1.17 g/h (data not shown). However, the simulation results reveal that the glucose gets consumed well within 15 h. Therefore, to enhance the productivity the time of operation is reduced systematically and t_f = 16 h is found to be reasonable where both the sugars are almost consumed. To keep the total amount of sugars added same, the feed rate is increased accordingly to F = 0.04375 L/h. The simulation results for co-culture study comprised of wild-type *S. cerevisiae* and recombinant *E. coli* strain ZSC 113 are shown in Fig. 1B. This fed-batch fermentation for t_f= 16 h and t_s = 8.21 h produces 22.80 g of ethanol with a final ethanol concentration of 19.0 g/L compared to 16.87 g/L of ethanol for recombinant *S. cerevisiae* RWB 218. The productivity value for the co-culture fed-batch (1.42 g/h) is much higher than that for the mono-culture fed-batch (1.01 g/h) as the co-culture produces much more ethanol in less time of opeartion. From the figure, it is clear that the consumption of glucose by yeast and xylose by *E. coli* starts simultaneously and the diauxic growth is not seen. A switch from aerobic to anaerobic phase at 8.21 h is characterized by a significant increase in the rate of production of ethanol and a small reduction in the rate of production of biomass.

Next we attempt to investigate the effect of various genetic manipulations on wild-type *S. cerevisiae* suggested by Bro et al. (2006) on the co-culture fed-batch productivity. Appropriate modification on the genome-scale reconstruction of iND750 is done to account for the genetic manipulations. Fed-batch co-culture simulations comprised of xylose consuming recombinant *E. coli* and various recombinant strains of *S. cerevisiae* that consume only glucose are then carried out and some of the results are presented in Table 2 along with the corresponding genetic manipulations. It may be noted that all the metabolic strategies improve the fed-batch ethanol productivity and the optimal aerobic-anaerobic switching time is dependent to some extent on the metabolic strategy used. All the results presented here clearly point to the superior performance of fed-batch fermentation of microbial co-culture against fed-batch fermentation of single-organism culture. We believe that further improvements can be made by carrying out simultaneous optimization of initial batch conditions, final batch time, and the substrate feed-rate policy. Our future work will address these issues.

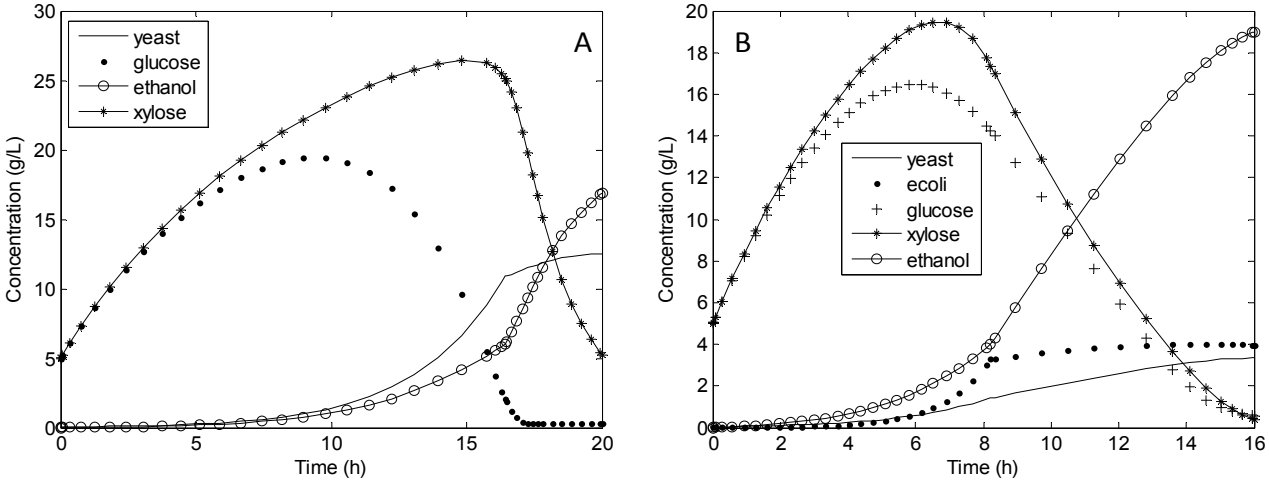

Fig. 1: (A): Fed-batch mono-culture simulation for *S. cerevisiae* strain RWB 218 on glucose/xylose media. (B): Fed-batch co-culture simulation of wild-type *S. cerevisiae* and recombinant *E. coli* strain ZSC 113 on glucose/xylose media.

Table 2: Fed-batch co-culture simulation of recombinant *S. cerevisiae* and recombinant *E. coli* strain ZSC 113 on glucose/xylose media.

No.	Metabolic strategy	Inserted/deleted/overexpressed reaction	Optimum switching time t_s, h	Mass of ethanol produced $VE\|t_f$, g	Ethanol productivity $(VE\|t_f)/t_f$, g/h
1	Deletion of *gdh1* and overexpression of *glt1* and *gln1*	2-Oxoglutarate + NADH + ATP + NH_4 → Glutamate + NAD + ADP	8.18	23.219	1.451
2	Deletion of *gdh1* and overexpression of *gdh2*	2-Oxoglutarate + NADH + NH_4 → L-Glutamate + NAD	8.31	23.262	1.453
3	Insertion of NAD dependent glycine dehydrogenase	Glyoxylate + NADH + NH_3 → Glycine + H_2O + NAD	8.31	23.294	1.455
4	Insertion of NADP dependent glycerol dehydrogenase	Glycerol + NADP → Dihydroxyacetone + NADPH	8.24	23.567	1.473
5	Insertion of NADP dependent glycerol 3-phosphate dehydrogenase	Glycerol 3-phosphate + NADP → D-Glyceraldehyde 3-phosphate + NADPH	8.31	23.294	1.455

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