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A simple structured model for biomass and extracellular enzyme production with recombinant *Saccharomyces cerevisiae* YPB-G

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A simple structured model is proposed for simulating batch cultivation data on growth, substrate utilization, and heterologous enzyme production of recombinant *Saccharomyces cerevisiae* YPB-G. The enzyme is a fusion protein displaying α-amylase and glucoamylase activities. Cell growth is modulated mainly by intracellular substrate and ethanol concentrations. Intracellular substrate concentration is evaluated by means of the extracellular substrate and biomass concentrations. Extracellular α-amylase and glucoamylase activities are taken to depend on biomass concentration. The nine parameters of the proposed model are determined using nonlinear estimation techniques, and the model is validated against experiments not used in parameter determination. The model developed simulates glucose consumption, cell mass, α-amylase and glucoamylase production in a batch system. Simulation and experimental results are found to be in good agreement.
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Keywords: recombinant yeast; structured modeling; α-amylase; glucoamylase; batch cultivation

Introduction

The yeast *Saccharomyces cerevisiae* has proved an excellent model microorganism for the production of both homologous and heterologous proteins and has a number of advantages over procaryotes [13]. Progress in biotechnology has created a need to quantify metabolic processes of native and recombinant microorganisms including yeast so that they can be most thoroughly and efficiently exploited [15]. Models describing the behavior of recombinant microorganisms are required for the organization of experimental and literature information, the design of experiments, and for the control and optimization of recombinant processes [7]. One approach used in model development involves designing independent experiments specifically for the purpose of parameter determination. The model includes sufficient structure so that it adequately reflects the kinetics of biochemical regulatory mechanisms. This type of approach tends to balance model complexity with practicality.

The simplest representation of microbial kinetics is the *unstructured model*, which describes biomass growth, substrate consumption and extracellular metabolic product formation. The unstructured model includes the most fundamental microbial processes: the rate of cell mass production is proportional to biomass concentration there is a saturation limit for growth rate on each substrate, the cells need substrate and may synthesize products even when they do not grow. This kind of model does not recognize any internal structure of the cell, nor diversity between cell forms, which may be an important feature of certain cell cultures. These models are quite satisfactory in many situations, e.g., when balanced growth is prevalent or in the control and optimization of cultivation processes with minimum mathematical complexity.

When the cell composition and/or the morphology of the cell culture are important and strongly time-dependent variables, the solution is to use *structured models* that include key aspects of microbial physiology and structure for the mathematical description of the metabolism of microorganisms [10,11]. Some authors emphasize the use of simple structured models against highly structured models [14]. A complete metabolic description of the microbial kinetics may not be required, and a simple description of transport, diffusion and excretion phenomena, together with some intracellular concentrations, may be sufficient to describe correctly the dynamic behavior of the process [10].

The recombinant *S. cerevisiae* YPB-G strain constructed by de Moraes [8], de Moraes *et al* [9] and secreting a bifunctional fusion protein that contains both the *Bacillus subtilis* α-amylase and the *Aspergillus awamori* glucoamylase activities was used by our research group in a series of cultivation experiments with glucose or starch as the carbon source. Ethanol production and growth characteristics of this strain under various conditions have been reported elsewhere [5]. In the present work, biomass and extracellular enzyme (α-amylase and glucoamylase) productions by recombinant *S. cerevisiae* YPB-G from glucose are modeled through a simple structured model in order to simulate the dynamic behavior of the process. Cell growth is modulated by the intracellular substrate and ethanol concentrations. Intracellular substrate concentration is evaluated by means of the measured extracellular substrate and biomass concentrations. Extracellular α-amylase and glucoamylase activities are taken to depend on the biomass concentration. The results indicate that the model proposed is adequate for describing cell growth and enzyme production. This study constitutes a first step that provides the basis for constructing structured models for starch fermentation by recombinant yeast cells.

Materials and methods

Microorganism

A *S. cerevisiae* strain, YPB-G, which expresses the *B. subtilis* α-amylase and *A. awamori* glucoamylase (BsAAase/GAase) as a

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fusion protein was kindly provided by Dr Stephen G Oliver (UMIST, UK).

Media and cultivation

The medium employed was a complex medium (YPG) containing 1% yeast extract, 2% peptone, 0.002% histidine, and 0.5–5% glucose (all w/v). The preculture was grown in YNB-G that contained 0.3% YNB (yeast nitrogen base) without amino acids, 0.002% histidine, 0.5% $(NH_4)_2SO_4$, and 2% glucose (all w/v). The first set of experiments were conducted in shake flasks located in an orbital shaker at 180 rpm and 30°C. pH was allowed to follow its natural course. The second set of experiments was performed in a New Brunswick Bioflo III bioreactor (New Brunswick, England) operated at 30°C with an agitation speed of 400 rpm. The pH of the medium was kept constant at 5.6 with either 12.5% (v/v) NH_4OH or 3% (w/v) succinic acid. The culture volume in these experiments was 1 L and the inoculum size was 10% (v/v). To eliminate problems with structural instability of the plasmid constructs, a selection procedure was applied for the preparation of the preculture and is described elsewhere [5] for both sets of experiments.

Analyses

The growth of yeast cells was followed by measuring the optical densities at 600 nm using a calibration chart to correlate dry weights to optical densities.

Glucose concentrations in the culture supernatants were determined by the enzymatic oxidation of NADH to NAD^+ at 340 nm, using a Boehringer-Mannheim D-glucose test kit (Boehringer-Mannheim, Germany). The ethanol concentration was determined both by a Boehringer-Mannheim ethanol kit and by gas chromatography using a Porapak Q column. Protein concentrations were determined using a protein assay kit (BioRad, USA) based on the method of Bradford [6].

Extracellular activities of α-amylase and glucoamylase in samples taken at different phases of growth were assayed as described by de Moraes *et al* [9]. One unit of α-amylase activity was defined as the quantity of enzyme required to hydrolyze 0.1 mg starch in 10 min at 40°C when 2 mg starch was present at the start of the reaction. One unit of glucoamylase was defined as the amount of enzyme required to release 1 μmol glucose per minute from starch.

Experimental observations

In the first set of experiments, several shake-flask cultivations were carried out in duplicate to study the effect of initial glucose concentration on α-amylase and glucoamylase secretion as well as on ethanol production; the results are summarized in Table 1. A typical set of experimental data on the time course of biomass and glucose concentrations, α-amylase and glucoamylase activities are presented in Figure 1, for the cases of 5, 10 and 25 g l^{-1} initial glucose concentration. Observation of cell growth revealed that the experimental maximum specific growth rate, μ_{max}, increases with increasing initial glucose concentration. The overall biomass/substrate yield observed was constant at a value close to 0.1 biomass per glucose (g g^{-1}). The S-shaped behavior of biomass formation and glucose consumption was observed in all experiments. No ethanol has been detected when starting with 5 g l^{-1} of initial glucose concentration. Ethanol reached detectable levels in the medium toward the stationary phase of the batch growth when starting with 10 and 25 g l^{-1} of initial glucose. Higher ethanol production was associated with high levels of initial glucose concentrations as a general tendency. The glucoamylase activities showed a sharp increase at the mid-exponential growth phase while α-amylase activities stayed relatively constant until the stationary phase and then increased rapidly. The insufficient level of glucose towards the end of the batch cultivation may contribute to the triggering of enzyme production by inducing the PGK promoter in the plasmid.

Table 1 Summary of experimental results

Initial glucose concentration (±1%) (g l^{-1})	μ_{max} (±1%) (h^{-1})	Final ethanol concentration (±1%) (g l^{-1})
5*	0.258	nd
10*	0.265	3
25*	0.268	5
50**	0.250	25

nd: not detected.
*Shake flask experiments.
**Bioreactor experiments.

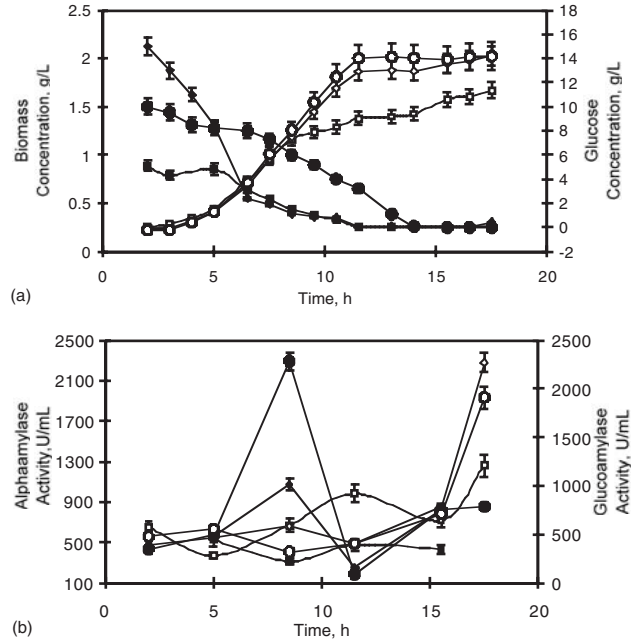


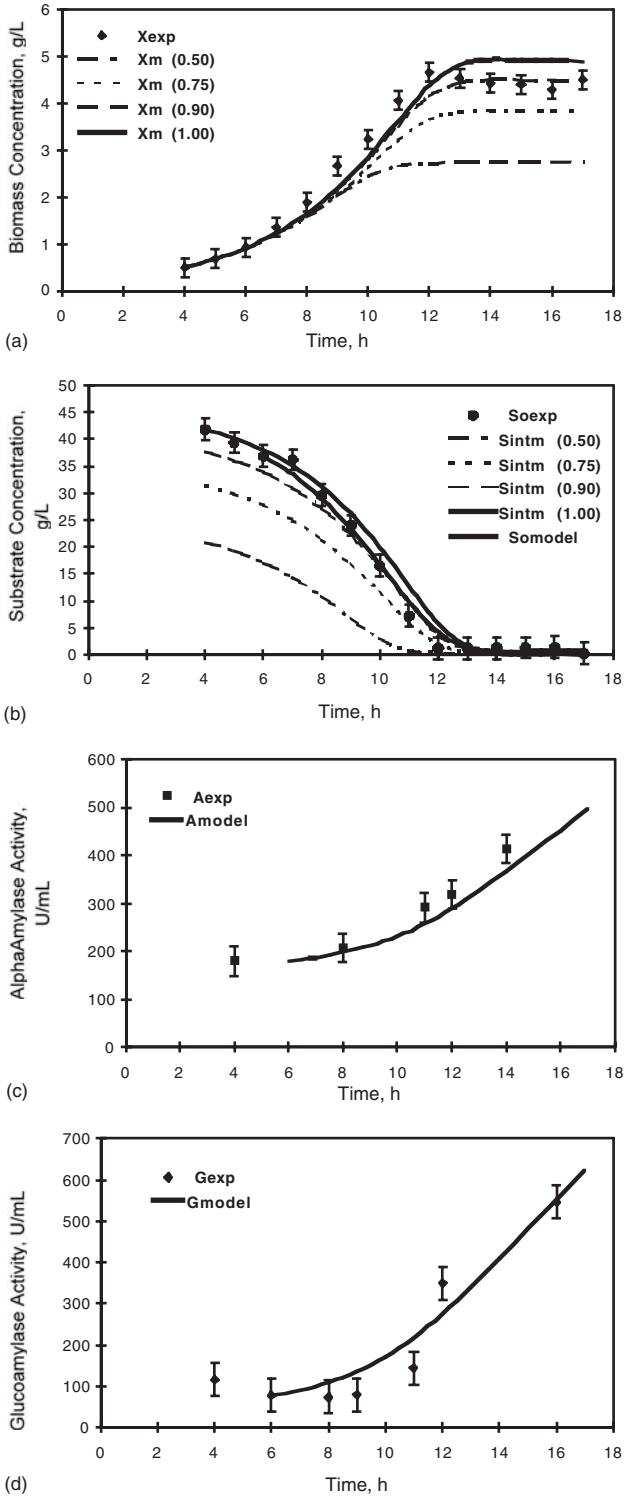
Figure 1 Time Course of (a) biomass formation (□), (○), (◇) and substrate utilization (■), (●), (◆), (b) α-amylase (□), (○), (◇) and glucoamylase activities (■), (●), (◆), for YPB-G grown on YPG containing 5, 10 and 25 g l^{-1} initial glucose in shake flasks, respectively. Duplicated experimental data is averaged.

In the second set of experiments, YPB-G cells were grown in a New Brunswick Bioflo III bioreactor operating in the batch mode, and increasing glucose concentrations were tested. Experimental data on the time course of biomass and glucose concentrations, α-amylase and glucoamylase activities are presented in Figure 2, for the case of 50 g l^{-1} initial glucose concentration. Maximum specific growth rate, enzyme activities, final biomass and ethanol concentrations are reported in Table 1.

Reaction scheme

In the simple structured model developed, the initial substrate decomposes into an internal substrate and an external substrate. The total concentration of the substrate is considered equal to the summation of the extracellular substrate, intracellular substrate and

the consumed substrate. Extracellular substrate enters the cell through the cell membrane via active transport and is used for biomass formation and ethanol formation. Biomass formation is associated with the production of α-amylase and glucoamylase, which are secreted into the medium through the cell membrane at the same rate as they are produced. A plausible representation of the reaction scheme is presented in Figure 3. The dynamic model consists of a set of five differential equations describing the growth of the microorganism, transport of the substrate into the cell, consumption of the internal substrate, excretion of α-amylase and glucoamylase activities. One hundred percent plasmid stability is assumed throughout the entire growth phase, since this was confirmed by experiments [2–4].



Cell growth: Cellular growth is assumed to be modulated by the intracellular concentration of substrate (S) and is described by Monod's equation [1].

$$\mu = \frac{\mu_{max} S}{K_s + S} \quad (h^{-1}) \tag{1}$$

where $\mu_{max} = \mu_{max}'(1 - KP)$.

$$\frac{dx}{dt} = \mu x \quad (\text{g biomass } l^{-1}\ h^{-1}) \tag{2}$$

Glucose is transported into the cell by active transport with saturation and is consumed there [10]. Active transport, i.e., transport of nutrient through the cell membrane against its chemical gradient, is a process that requires metabolic energy [12]. KP term in maximum specific growth rate expression is used to include the toxic effect of ethanol at high concentrations and $\mu_{max}'KP$ can be treated as a death rate.

Substrate utilization: The total substrate (S_T) added to the medium in the bioreactor is composed of the extracellular substrate (S_o), the intracellular substrate ($S = xS_i$) and the substrate consumed during cultivation ($xY_{x/s}$). See the nomenclature for the dimensional consideration of the terms defined in the intermediate steps.

$$S_T = S_o + xS_i + xY_{x/s} \tag{3}$$

Assuming that the biomass yield on substrate ($Y_{x/s}$) and the total substrate (S_T) charged to the bioreactor are constant, the rate of total substrate consumption can be written as;

$$\frac{dS_T}{dt} = \frac{dS_o}{dt} + (S_i + Y_{x/s})\frac{dx}{dt} + x\frac{dS_i}{dt} = 0 \tag{4}$$

Rearranging Eq. (4) gives;

$$\frac{dS_i}{dt} = -\frac{1}{x}\frac{dx}{dt}(S_i + Y_{x/s}) - \frac{1}{x}\frac{dS_o}{dt} \tag{5}$$

Figure 2 Time course of (a) biomass formation, (b) substrate utilization, (c) α-amylase and (d) glucoamylase activities for YPB-G grown on YPG containing 50 g l^{-1} glucose in a New Brunswick bioreactor. Duplicated experimental data are averaged. Model parameters are optimized from batch cultivation experiments starting with an initial glucose concentration of 50 g l^{-1} at a constant pH of 5.6, 400 rpm and 30°C and the optimized parameter set is used to simulate data obtained from different experimental conditions (shake flask, natural course of pH, 30°C, 180 rpm; see Figure 1). Xm (0.5), Sintm (0.5), Xm (0.75), Sintm (0.75), Xm (0.90), Sintm (0.90), Xm (1), Sintm (1) represent the model simulations for biomass and intracellular glucose concentrations for the ratio of intracellular to extracellular substrate concentration of 0.5, 0.75, 0.90 and 1, respectively. Xexp, Soexp are the experimental data for biomass and extracellular glucose concentrations, respectively. Somodel, Amodel and Gmodel are the model simulations of extracellular glucose concentration, extracellular α-amylase and glucoamylase activities, respectively.

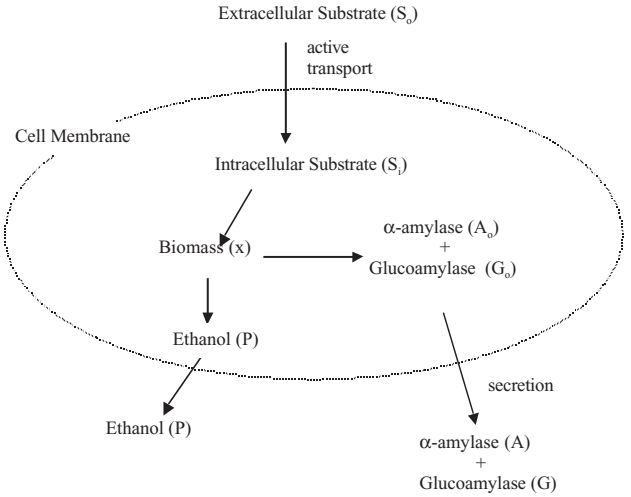


Figure 3 Representation of the reaction scheme.

Here, $(1/x)(dx/dt)=\mu$ and

$$\frac{dS_i}{dt} = -\frac{1}{x}\frac{dS_o}{dt} - Y_{x/s}\mu - \mu S_i$$

$$(\text{g intracellular substrate g biomass}^{-1}\ \text{h}^{-1}) \qquad (6)$$

The extracellular substrate utilization rate is of the Monod type:

$$\frac{dS_o}{dt} = -\frac{k_{so}S_o}{K_{so}+S_o}x$$

$$(\text{g extracellular substrate l}^{-1}\ \text{h}^{-1}) \qquad (7)$$

Extracellular enzyme production: Both α-amylase and glucoamylase are produced and secreted through a bifunctional fusion protein construct in the YPB-G strain. This strain normally secretes 96% of the α-amylase and glucoamylase into the medium [9]. Here, the secretion is again described by active transport with saturation, assuming that accumulation within the cell is negligible and that 100% of the α-amylase and glucoamylase are secreted. In other words, intracellular α-amylase (A_o) and glucoamylase (G_o) activities are assumed to be equal to the extracellular α-amylase (A) and glucoamylase (G) activities, respectively. The enzyme production and secretion rates are also considered to be equal to each other since enzyme accumulation within the cell is not observed throughout the batch cultivation (unpublished data).

$$\frac{dA}{dt} = \frac{k_a A}{K_a + A}x \quad (\text{U ml}^{-1}\ \text{h}^{-1}) \qquad (8)$$

$$\frac{dG}{dt} = \frac{k_g G}{K_g + G}x \quad (\text{U ml}^{-1}\ \text{h}^{-1}) \qquad (9)$$

Calculation procedure

The target function(s) selected in the optimization methodology to obtain the parameter values were related to the sum of the least squares between experimental and simulated data. In other words, the objective function, J,

$$J = \sum (y_i - y_\theta(x_i))^2$$

was optimized, where y_i values are the experimental and $y_\theta(x_i)$ values are the simulated values with data points x_i and the parameter set θ. Since the model equations are nonlinear in parameters, the optimization of J was accomplished using the steepest descent method. Due to the nature of the steepest descent algorithm, a set of initial guesses was necessary. Various initial points were used to ensure that the optimal parameter values reached at the end of the optimization procedure were the global ones. The results obtained indicated that the global optima for the described model equations were reached. Although it is possible to convert the nonlinear least squares problem for this model to a linear one by considering the reciprocals, it was found to be numerically unstable, yielding a higher χ^2 than the steepest descent method, for the original variables.

The intracellular substrate concentration at the beginning of the exponential phase of growth was treated by using two different assumptions: (1) intracellular substrate concentration was assumed to be equal to the extracellular (measured) substrate concentration, (2) transport of the substrate into the cell was assumed to take some time. Starting at the beginning of the exponential growth phase allowed the assumption that the cells took inside only a certain fraction of the extracellular substrate. When the first assumption was used, an overshoot was observed in the estimation of the biomass concentration at the stationary phase, and the intracellular substrate concentration exceeded the extracellular substrate concentration in the course of time (Figure 2a and b). Therefore, a parametric study was conducted for testing the second assumption, initial values for the ratio of intracellular to extracellular substrate concentration were optimized, and the ratio for a good fit of the data was found to occur at 0.90.

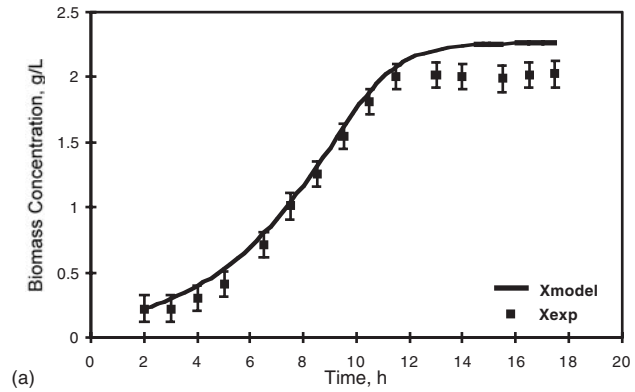
Results and discussion

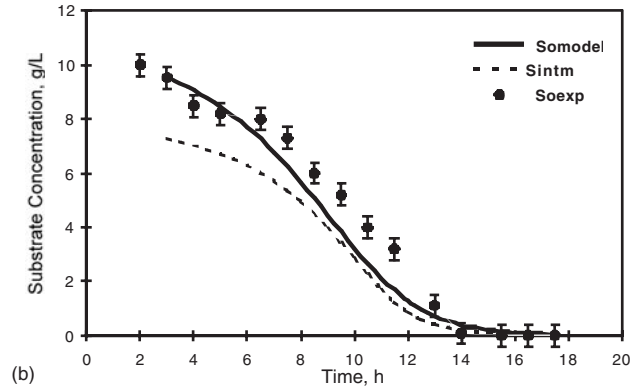
Experimental data obtained under controlled conditions in the New Brunswick Bioflo III bioreactor operated at 50 g l^{-1} initial glucose concentration were used in the model calibration procedure. Model parameters obtained with the optimization routine described above are presented in Table 2. A sixth-order Runge Kutta integrator was used to simulate the cultivation pattern (Figure 2). The model correctly represents the general behavior of standard cultivations,

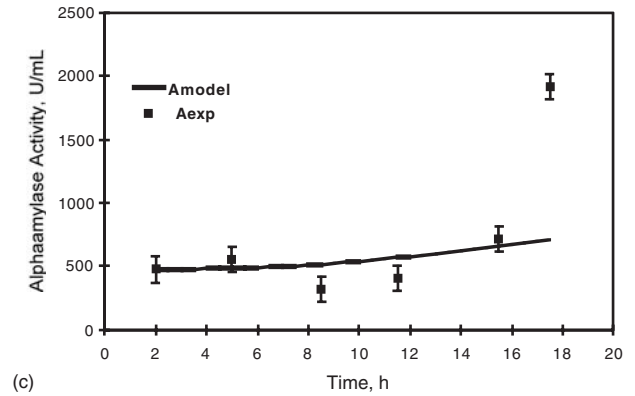
Table 2 Optimal parameter set obtained on the basis of experimental data with 50 g l^{-1} initial glucose in a New Brunswick bioreactor

Parameter	Value
μ_{max}'	0.308 (h^{-1})
K_s	1.369 (g l^{-1})
K	0.044 (l g^{-1})
k_{so}	4.41(g g^{-1} h^{-1})
K_{so}	13.29 (g l^{-1})
k_a	12.98 (U mg^{-1} h^{-1})
K_a	150 (U ml^{-1})
k_g	16.84 (U mg^{-1} h^{-1})
K_g	29.490 (U ml^{-1})

showing only slight differences between simulated and experimental results. The entire model was then validated against shake-flask experiments that were not used in parameter optimization, and gave satisfactory results (Figure 4). It will be noted in Figure 4c that there is a difference of about 1200 U ml^{-1} between the experimental and predicted activities of α-amylase at around 18 h of cultivation time. Similarly, a difference of about 1800 U ml^{-1} is observed in the glucoamylase activity around 8–10 h of batch cultivation. One possible explanation for this discrepancy may be that the pH is allowed to follow its natural course in shake-flask experiments, while the model parameters are optimized on the basis of controlled bioreactor data displaying smooth increases in α-amylase and glucoamylase activities. Another explanation may be the insufficient level of glucose toward the end of the batch cultivation, which might contribute to triggering of enzyme production by inducing the PGK promoter in the plasmid.







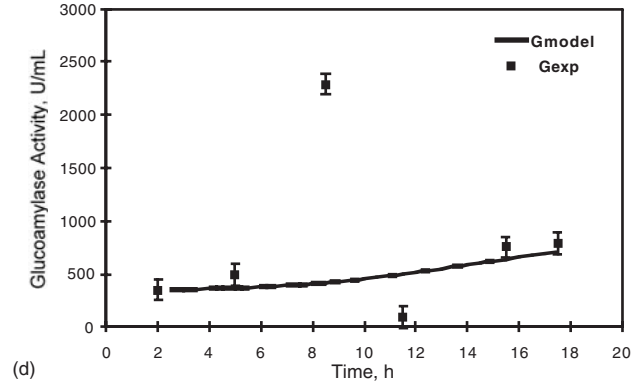


Figure 4 Time course of (a) biomass formation, (b) substrate utilization, (c) α-amylase and (d) glucoamylase activities for YPB-G grown on YPG containing 10 g l^{-1} glucose in shake flask. Duplicated experimental data are averaged. Model is validated against the shake flask experiments that have not been used for the estimation of model parameters. Xmodel, Somodel, Sintm, Amodel and Gmodel represent the model simulations for biomass, extracellular and intracellular glucose concentrations and extracellular α-amylase and glucoamylase activities, respectively. Xexp, Soexp, Aexp and Gexp represent the experimental values of biomass, extracellular glucose concentrations and extracellular α-amylase and glucoamylase activities, respectively.

Table 3 Sensitivity of model predictions to parameter value

		1% change in								
		μ_{max}'	K_s	K	k_s	K_s	k_a	K_a	k_g	K_g
$\delta\chi^2$ (%)	A	8.9	0.7	–	–	–	8.5	3.2	–	–
	G	2.2	0.1	–	–	–	–	–	2.6	0.3
	X	10.7	0.9	–	–	–	–	–	–	–
	S	9.6	0.5	–	8.4	3.2	–	–	–	–

– no change.

In order to determine the sensitivity of model predictions to the values of the parameters used for simulating cell growth and enzyme secretion, the value of each parameter was increased by 1% as suggested by Copella and Dhurjati [7]. The resulting relative absolute change in χ^2 is reported in Table 3. Changes in χ^2 significantly below the median indicate that the model results are not sensitive to that parameter value in comparison to the others, and thus those parameters are not needed by the model. Absolute changes are compared since the direction of the changes in χ^2 do not have an impact on model sensitivity [7].

The rate of ethanol production is written as,

$$\frac{dP}{dt} = \frac{d}{dt}\left(\frac{1}{K}\left(1 - \frac{(K_s + S)}{\mu_{max'} Sx}\frac{dx}{dt}\right)\right) \quad \text{elsewhere} \tag{10}$$

$$\frac{dP}{dt} = 0 \quad \text{at the stationary phase} \tag{11}$$

Since at the stationary phase, dx/dt vanishes and

$$P = \frac{1}{K}. \tag{12}$$

The reader is cautioned that the model in its present form considered the effect of ethanol concentration on growth only at the stationary phase when ethanol concentration value was at its

maximum. Hence, the model parameters were optimized and the sensitivity analysis was performed accordingly. Changes in the parameters μ_{max}', K_s and K were expected to affect the χ^2 values of all the fits, since they were used in modeling the dynamics of x and all other observables were modeled through x, while 1% change in other parameters would affect only the relevant observable. The results showed that the parameter K did not play an important role in the model, since the product ethanol reached its highest values only at the stationary phase of growth at high initial glucose concentrations. As a general tendency, model equations showed greater sensitivity to the parameters in the numerator, i.e., μ_{max}', k_g, k_a and k_s, rather than those in the denominator, i.e., K_s, K_g, K_a and K_s; this is reasonable since the parameters in the numerator act as coefficients while those in the denominator are additive quantities.

As seen from Table 3, K does not play an ''active'' role in the modeling of the system. It is, however, affected by the system and the K value determined predicts the reciprocal of the final quantity of the ethanol product (Eq. (12)). It can be concluded that K is a ''passive'' parameter, which gives the final quantity of product in the system. Indeed, the parameter K was found to be 0.044 l g^{-1}, and the final product (ethanol) concentration was 25 g l^{-1} for the experiment performed in a batch bioreactor with an initial glucose concentration of 50 g l^{-1} (Table 1).

The present study provides a description of biomass and extracellular enzyme production in batch cultures of recombinant *S. cerevisiae* YPB-G grown in YPG medium via a simple structured model based on transport and excretion phenomena, and the use of the intracellular substrate concentration (S); it is shown that the model is useful in explaining enzyme production processes and also avoiding a sophisticated structured model.

This work constitutes a first stage in the modeling of recombinant *S. cerevisiae* strains in starch-containing media. It is important to know the behavior of these strains on the glucose substrate, since the enzymes excreted by the recombinant strain first degrade starch into glucose for subsequent utilization by the cells. In order to model the entire system starting from starch as substrate, the next step should be the development of a scheme for correlating the conversion of starch into glucose. The present model can also be used for operation in the optimal range of substrate concentrations and for designing strategies of substrate feeding to improve enzyme production and in turn ethanol production.

Nomenclature

x	biomass concentration (g l^{-1})
S	substrate concentration (g l^{-1})
P	ethanol concentration (g l^{-1})
μ_{max}	maximum specific growth rate (h^{-1}) (experimentally observed)
μ_{max}'	maximum specific growth rate (h^{-1}) (actual)
K_s	saturation constant (g l^{-1})
K	model parameter (l g^{-1})
S_T	total substrate (g l^{-1})
S_o	extracellular substrate (g l^{-1})
S_i	intracellular substrate (g g^{-1})
$Y_{x/s}$	biomass yield on substrate (g g^{-1})
k_{so}	model parameter (g g^{-1} h^{-1})
K_{so}	model parameter (g l^{-1})
A	extracellular activity of α-amylase (U ml^{-1})
G	extracellular activity of glucoamylase (U ml^{-1})
A_o	intracellular activity of α-amylase (U ml^{-1})
G_o	intracellular activity of glucoamylase (U ml^{-1})
k_a	model parameter (U mg^{-1} h^{-1})
K_a	model parameter (U ml^{-1})
k_g	model parameter (U mg^{-1} h^{-1})
K_g	model parameter (U ml^{-1})

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