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Modeling of *Xanthophyllomyces dendrorhous* Growth on Glucose and Overflow Metabolism in Batch and Fed-Batch Cultures for Astaxanthin Production

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ABSTRACT: An astaxanthin-producing yeast *Xanthophyllomyces dendrorhous* ENM5 was cultivated in a liquid medium containing 50 g/L glucose as the major carbon source in stirred fermentors (1.5-L working volume) in fully aerobic conditions. Ethanol was produced during the exponential growth phase as a result of overflow metabolism or fermentative catabolism of glucose by yeast cells. After accumulating to a peak of 3.5 g/L, the ethanol was consumed by yeast cells as a carbon source when glucose in the culture was nearly exhausted. High initial glucose concentrations and ethanol accumulation in the culture had inhibitory effects on cell growth. Astaxanthin production was partially associated with cell growth. Based on these culture characteristics, we constructed a modified Monod kinetic model incorporating substrate (glucose) and product (ethanol) inhibition to describe the relationship of cell growth rate with glucose and ethanol concentrations. This kinetic model, coupled with the Luedeking–Piret equation for the astaxanthin production, gave satisfactory prediction of the biomass production, glucose consumption, ethanol formation and consumption, and astaxanthin production in batch cultures over 25–75 g/L glucose concentration ranges. The model was also applied to fed-batch cultures to predict the optimum feeding scheme (feeding glucose and corn steep liquor) for astaxanthin production, leading to a high volumetric yield (28.6 mg/L) and a high productivity (5.36 mg/L/day).

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KEYWORDS: kinetic model; overflow metabolism; ethanol inhibition; diauxic growth; fed-batch culture

Introduction

Astaxanthin (3,3′-dihidroxy-β,β-carotene-4,4′-dione) is a carotenoid pigment which confers a characteristic coloration to some birds, crustaceans and salmons. It has been increasingly used as a feed and food pigment in the aquaculture industry, and also has been evaluated as a potential functional food and pharmaceutical component because of its excellent antioxidant activity (Andrewes et al., 1976; Johnson and Schroeder, 1995). *Xanthophyllomyces dendrorhous* (formerly *Phaffia rhodozyma*) is a high astaxanthin-producing yeast and has been widely explored as a promising producer of dietary astaxanthin through fermentation technology (Guerin et al., 2003; Johnson, 2003).

The culture of *X. dendrorhous* yeast for astaxanthin production is an aerobic process and glucose is the most common and favorable carbon source. The complete aerobic catabolism of glucose in many organisms usually proceeds through three phases including glycolysis fermenting glucose to pyruvate, the tricarboxylic acid (TCA) cycle converting pyruvate to CO_2 and NADH, and the electron transport chain for ATP production. This is the most efficient metabolic route for biomass and energy production. In certain yeast and bacterial species such as baker's yeast *Saccaromyces cerevisiae* and related species (Fiechter and Seghezzi, 1992; Hoek et al., 1998) and *Escherichia coli* (Vemuri et al., 2006), however, fermentative catabolism of glucose leading to ethanol or other partially oxidized products such as acetic acid can take place even under aerobic conditions. This phenomenon, originally referred to as Crabtree effect, is now recognized as a case of overflow metabolism, which is mainly attributed to the limited respiratory capacity of the microorganisms. The alcoholic fermentation of glucose is usually more pronounced at relatively high glucose concentrations and high specific

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growth rates. Therefore, to eliminate the alcoholic fermentation at high sugar concentrations, industrial fermentation for baker's yeast production is usually operated in fed-batch mode.

X. dendrorhous is a Crabtree-positive yeast with overflow metabolism, generating ethanol even when ample oxygen is supplied to the culture (Liu and Wu, 2007; Reynders et al., 1997; Yamane et al., 1997). The fermentative production of ethanol is undesirable as it decreases the biomass yield on sugar and may also cause inhibitory effect on cell growth and astaxanthin biosynthesis. To minimize overflow metabolism and to maximize process efficiency and the astaxanthin productivity, we need to determine the optimal glucose concentration and cell growth rate, and the feeding scheme for fed-batch culture processes. Mathematical models describing the quantitative relationships for growth kinetics and for substrate and product stoichiometry are most useful for rational optimization and effective control of fermentation processes. To the best of our knowledge, however, no such mathematical models have been reported for the *X. dendrorhous* cultures.

This study was to construct a kinetic model for cell growth in batch cultures of *X. dendrorhous* yeast using glucose as the main substrate, taking into account of overflow metabolism and growth inhibition by ethanol and high concentrations of glucose that are commonly observed in yeast culture, and to analyze and quantify the kinetic and stoichiometric relationships of biomass, substrate and products. Model parameters and stoichiometric relationships of biomass, substrate, and products were derived by simulating experimental data obtained from stirred-tank fermentors with a constant and adequate dissolved oxygen (DO) concentration. These model and relationships were applied to predict cell growth, substrate consumption, ethanol formation, and astaxanthin production in batch cultures at various glucose concentrations, and to predict the optimal fed-batch culture process for high astaxanthin production, and to analyze the physiological characteristics of the yeast culture.

Materials and Methods

Microorganism and Culture Media

X. dendrorhous strain ENM5 was used in this study, which is an astaxanthin-hyperproducing strain derived from ATCC 24230 by NTG mutagenesis (Schroeder and Johnson, 1995). The yeast strain was stored in frozen vials at -80°C. The liquid medium for routine maintenance of the yeast culture and the fermentation experiments was composed of 50 g glucose, 3 g $(NH_4)_2SO_4$, 1.5 g KH_2PO_4, 1.5 g $MgSO_4 \cdot 7H_2O$, 1.5 g yeast extract and 10.0 g corn steep liquor (per liter), at pH 5.0. The inoculum for the fermentor and shake-flask cultures in the following experiments (6% v/v for all) was prepared by growing the cells in 250-mL Erlenmeyer flasks (filling volume, 50 mL) for 2 days, on an orbital shaker (250 rpm, 20°C). All culture experiments in the shake flasks or fermentors were run for an overall period of 5 days (120 h).

Batch and Fed-Batch Culture Experiments

The culture experiments were carried out in two identical 2-L stirred fermentors (Biostat, B. Braun, Germany) with a working volume of 1–1.5 L (initial filling volume 1.0-L). The air flow rate into the fermentors was fixed at 1,000 mL/min (1.0 vvm). The fermentor was controlled at pH 5.0 (with 2 M NaOH and 2 M HCl) and temperature 20°C. DO was controlled at $40 \pm 4\%$ air saturation (with the stirring speed varying from 300 to 1,200 rpm), which was well above the critical DO (~10%) of this yeast (Liu et al., 2006). Batch culture experiments were carried out at various glucose concentrations (25, 50, 75, and 100 g/L) and with all other nutrients at fixed concentrations. In fed-batch culture experiments, the initial glucose concentration was 25 g/L, and glucose and corn steep liquor were fed to the fermentors (from a liquid reservoir containing 250 g/L glucose and 50 g/L corn steep liquor) during the culture period according to selected feeding schemes as shown later. Feeding was set to start when glucose concentration in the culture was lower than 1 g/L during both constant and exponential fed-batch culture. The selection of glucose and corn steep liquor but not other medium components for the feeding was based on our previous study (Liu and Wu, 2007).

The effects of ethanol on cell growth and astaxanthin production were also examined in shake-flask culture by external feeding of ethanol (2.5 and 5.0 g/L). The shake-flask culture was carried out with 250-mL Erlenmeyer flasks, each filled with 25 mL medium, at 250 rpm and 20°C. The medium pH was adjusted to 5.0 and buffered with 0.1 M potassium hydrogen phthalate.

Analytical Methods

Yeast biomass was separated from the liquid medium by centrifugation, rinsed twice with double distilled water, and then dried at 105°C overnight to constant dry weight as the dry cell weight (DCW). Carotenoid pigment was extracted from the dry yeast biomass (disrupted with DMSO at 55°C) with hexane/ethyl acetate at 50:50 (v/v), and astaxanthin content in the extract was analyzed by HPLC as described in detail elsewhere (Liu and Wu, 2006). Glucose in the culture medium was analyzed by a YSI 2700 Biochemistry Analyzer (YSI, Inc., Yellow Spring, OH), and ethanol (and acetic acid) by GC using an AT-Aquawax-DA column (0.32 mm × 30 m) (Alltech Associates, Inc., Deerfield, IL). At all experimental conditions in this study, acetic acid accumulated in the yeast cultures was no more than 0.15 g/L, which was not shown in the results or considered in the model development. The oxygen uptake rate (OUR) was calculated with the following equation:

$$\mathrm{OUR} = K_L a(C^* - C_{O_2}) - \frac{dC_{O_2}}{dt}$$

where $K_L a$ is the oxygen transfer coefficient, determined by sulfite oxidation method (Linek and Vacek, 1981), C^* and C_{O_2} are the saturated and actual DO in the culture broth, respectively.

Model Development

Previous studies (Johnson and An, 1991; Liu and Wu, 2007) have shown the following characteristics of *X. dendrorhous* yeast cultures, (1) ethanol production due to overflow metabolism, that is, glucose uptake rate exceeding the maximum respiratory capacity of the cells, (2) the utilization of ethanol as glucose becomes exhausted, and (3) the inhibition of cell growth by ethanol and glucose at high concentrations. This behavior in *X. dendrorhous* is very similar to that in *S. cerevisiae* (Daran-Lapujade et al., 2004; Postma et al., 1989). The respiratory capacity of yeast cells is represented by a bottleneck. Overflow metabolism occurs when glucose flux (uptake) exceeds the respiratory bottleneck of yeast cells, generating ethanol (in the early stage of batch culture when glucose concentration is high). The ethanol generated from overflow metabolism is utilized as a carbon source when glucose becomes exhausted in late stage of batch culture (Fiechter and Seghezzi, 1992; Pham et al., 1998). Considering these characteristics and based on previously established models in *S. cerevisiae* yeast cells, a model was proposed to depict the overflow metabolism of glucose with the reuse of ethanol in *X. dendrorhous* yeast cultures in this study.

As both glucose and ethanol can be utilized for biomass production, the net cell growth rate can be represented by

$$\frac{dX}{dt} = X(\mu_G + \mu_E - k_d) \tag{1}$$

where μ_G and μ_E are the specific growth rates on glucose and ethanol, respectively, and k_d is the first-order rate constant for cell death due to endogenous metabolism.

The most common unstructured and non-segregated kinetic model for microbial growth is the Monod equation, which relates the specific growth rate to a single limiting substrate in a saturation form (Ge and Bai, 2006; Shuler and Kargi, 2002; Tang et al., 2007). This is partially true for *X. dendrorhous* yeast culture in which glucose is the limiting substrate and all other nutrients including nitrogen, phosphorus and minerals are abundant or in excess as found from our previous study (Liu and Wu, 2007). In addition, all experiments in the fermentors were carried out in fully aerobic conditions with the DO concentration fixed at a level well above the critical DO of this yeast (40% vs. 10%). Therefore, oxygen was not included as a limiting substrate in the model expressions. This assumption was also confirmed by fermentation experiments performed at DO levels lower and higher than 40%, that is, 20% and 60% air saturation (data not shown). However, the basic Monod model is not adequate for the *X. dendrorhous* yeast cultures characterized by substrate and product inhibition, and overflow metabolism. Considering these characteristics of *X. dendrorhous* yeast cultures, we propose a modified Monod model with glucose and ethanol inhibition for the specific growth rates

$$\mu_G = \left(\frac{\mu_{m,G} C_G}{K_{S,G} + C_G}\right)\left(\frac{K_{1,G}}{K_{1,G} + C_G}\right)\left(\frac{K_{1,E}}{K_{1,E} + C_E}\right) \tag{2}$$

$$\mu_E = \left(\frac{\mu_{m,E} C_E}{K_{S,E} + C_E}\right)\left(\frac{C_E}{C_E + K_C C_G}\right)\left(\frac{K_{1,E}}{K_{1,E} + C_E}\right) \tag{3}$$

where $\mu_{m,G}$ and $\mu_{m,E}$ are the maximum specific growth rates on glucose and ethanol, respectively, C_G and C_E are the glucose and ethanol concentrations, respectively. Table I provides a complete list of all symbols and definitions in the model equations in this section. In the above equations, μ_G is affected by substrate (glucose) and product (ethanol) inhibition, and μ_E affected by glucose competitive inhibition and substrate (ethanol) inhibition.

Table I. Definitions of model parameters and parameter values derived by simulating with batch culture data at 50 g/L initial glucose in stirred fermentors.

Symbol	Definition	Unit	Value[a]
$\mu_{m,G}$	Maximum specific growth rate on glucose	h^{-1}	0.129 ± 0.005
$\mu_{m,E}$	Maximum specific growth rate on ethanol	h^{-1}	0.0184 ± 0.0013
$K_{S,G}$	Monod glucose saturation constant	g/L	0.0872 ± 0.0042
$K_{S,E}$	Monod ethanol saturation constant	g/L	1.42 ± 0.26
$K_{I,G}$	Glucose inhibition constant	g/L	178 ± 12
$K_{I,E}$	Ethanol inhibition constant	g/L	7.64 ± 1.05
K_C	Glucose competitive inhibition constant	—	1.95 ± 0.12
k_d	Rate constant for endogenous metabolism	h^{-1}	0.00114 ± 0.00011
K	Ethanol generation coefficient	—	0.273 ± 0.010
$Y_{X/G}$	Biomass yield coefficient on glucose	g cell/g glucose	0.372 ± 0.018
$Y_{X/E}$	Biomass yield coefficient on ethanol	g cell/g ethanol	0.324 ± 0.022
$Y_{E/G}$	Ethanol formation coefficient on glucose	g ethanol/g glucose	0.511
α	Astaxanthin growth-associated constant	mg/g cell	0.342 ± 0.011
β	Astaxanthin non-growth-associated constant	mg/g cell/ h	0.00698 ± 0.00025

[a]All standard errors except $Y_{X/E}$ and $Y_{E/G}$ (measurement errors) were computed using Monte–Carlo method by incorporating the random variation of experimental data from batch culture.

For the mass balance of carbon source, we only consider ethanol as a metabolic product of glucose, and neglect other metabolites such as acetate (<0.15 g/L) and astaxanthin (<0.1 g/L) because their contents were much lower than ethanol in the yeast culture. Therefore, glucose is mainly used for the production of biomass and ethanol, and also for maintenance energy. The energy requirement for cellular maintenance is very small relative to that for growth, and is neglected in the mass balance calculations (Meyer and Du Preez, 1994). Then, the glucose consumption rate can be represented by

$$-\frac{dC_G}{dt} = \frac{\nu_E}{Y_{E/G}} + \frac{X\mu_G}{Y_{X/G}} \quad (4)$$

where ν_E is the rate of ethanol formation from glucose utilization (Table I for all other symbols in the equation). In this equation, the first term refers to glucose used for ethanol formation and the second for cell growth. Similarly, the change of ethanol concentration equals the amount produced minus that consumed by the yeast cells

$$\frac{dC_E}{dt} = \nu_E - \frac{X\mu_E}{Y_{X/E}} \quad (5)$$

Ethanol production from fermentative catabolism of glucose occurs in the rapid or exponential growth phase, and is growth-associated (Ge and Bai, 2006; Shuler and Kargi, 2002). Therefore, ethanol formation is related to the yeast growth on glucose by

$$\nu_E = KX\mu_G \quad (6)$$

where K is the ethanol formation coefficient.

Astaxanthin production in *X. dendrorhous* yeast cultures is partially growth associated, occurring in both growth and stationary phases (Liu and Wu, 2007), and can thus be related to cell growth and biomass concentration by the Luedeking–Piret equation (Luedeking and Piret, 1959),

$$\frac{dP}{dt} = \alpha\frac{dX}{dt} + \beta X \quad (7)$$

where α and β are coefficients for growth-associated and non-growth-associated product formation, respectively.

In the above we have constructed four differential equations, cell growth (1), substrate consumption (4, 5) and astaxanthin production (7), and can determine the corresponding equation constants (or parameters) by simulating with experimental data. As these equations are over-determined (excessive) and nonlinear, only the optimum values can be sought in given ranges. The initial parameter values and ranges in this study were selected from the typical results from *X. dendrorhous* and *S. cerevisiae* yeasts (Ge and Bai, 2006; Meyer and Du Preez, 1994; Shuler and Kargi, 2002; Yamane et al., 1997) as few of these parameters have been quantified for *X. dendrorhous* yeast cultures. The parameters were computed with the Levenberg–Marquardt and Universal Global Optimization algorithm using the mathematical Software 1stOpt (7D-Soft High Technology, Inc., Beijing, China). This software is widely used and works well for multivariable simulation, which can minimize the initial value problems (Wei et al., 2007). Matlab 7.0 (''ode 45'' solver) was used for numerical integration of the differential equations for biomass, glucose, ethanol and astaxanthin changes. The 95% confidence intervals (or standard errors) of parameters were computed by Monte–Carlo analysis using the commercial software Prism 4.0 (GraphPad Software, Inc., San Diego, CA) for statistical assessment of the parameter reliability (Poscheta et al., 2003; Tang et al., 2007).

With a view that the major physiological characteristics considered in the model development are independent of the process mode, the model parameters may also be applicable for fed-batch culture within the glucose concentration range. Therefore, the model was applied to fed-batch culture together with the following material balance equations for a fed-batch process

$$\text{Biomass}: \frac{dVX}{dt} = XV(\mu_G + \mu_E - k_d) \quad (8)$$

$$\text{Glucose}: \frac{dVC_G}{dt} = FS_R - \frac{V\nu_E}{Y_{E/G}} - \frac{VX\mu_G}{Y_{X/G}} \quad (9)$$

$$\text{Ethanol}: \frac{dVC_E}{dt} = V\nu_E - \frac{VX\mu_E}{Y_{X/E}} \quad (10)$$

$$\text{Product}: \frac{dVP}{dt} = \alpha VX(\mu_G + \mu_E - k_e) + \beta VX \quad (11)$$

$$\text{Feeding rate}: \frac{dV}{dt} = F \quad (12)$$

where F is the volumetric feeding rate, and S_R the glucose concentration in the feed. The initial values were, volume $V_0 = 1$ L, $S_R = 250$ g/L, and $X_0 = 0.6$ g/L; the boundary conditions were, culture period 120 h, total feeding volume 0.3 L, $F < 0.050$ L/h; the step length for integration was 1 h. With the objective (optimization target) set for maximum volumetric astaxanthin yield (P), the optimum feeding scheme or profile can be derived with the above Equations (8)–(12) using the Matlab optimization tools (''ode 45'' solver and ''fminsearch'' function).

Results

Growth and Metabolism Characteristics of *X. dendrorhous* in Batch Culture

Figure 1 shows the batch culture time courses of cell growth, ethanol production and consumption and astaxanthin

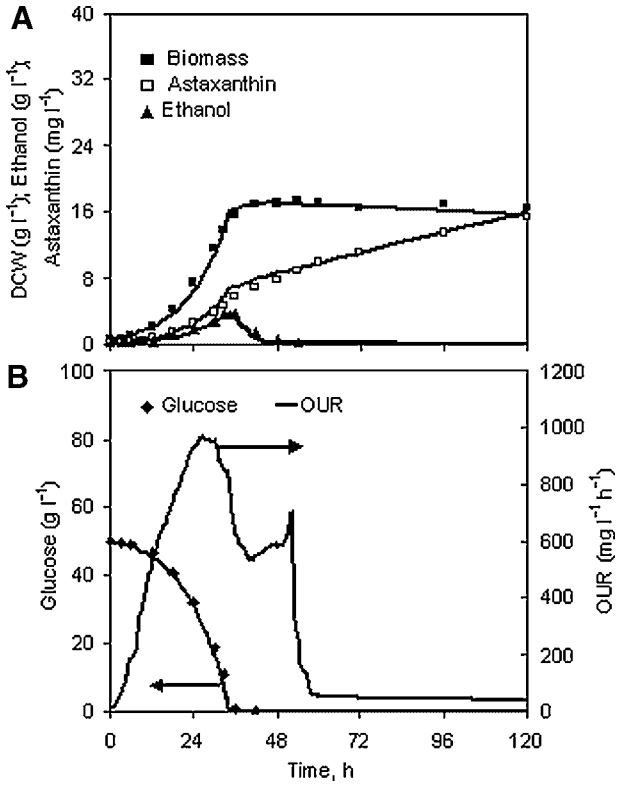

Figure 1. Batch culture time courses of *X. dendrorhous* with 50 g/L initial glucose (**A**) biomass, ethanol and astaxanthin, and (**B**) glucose consumption and oxygen uptake rate (OUR) (symbols for experimental data points, and lines for model simulation results, except for OUR which was recorded from the culture fermentor; experimental data representing the mean of triplicate runs and maximum SE < 10% of the mean).

production (Fig. 1A), and glucose consumption and OUR (Fig. 1B) of *X. dendrorhous* in a stirred fermentor with 50 g/L initial glucose. The yeast culture exhibited an exponential growth period in the initial 36 h, a short slow-growth period to reach a maximum biomass density at about 42 h, and a stationary phase in the remaining culture period (72 h) with nearly a constant biomass density. Astaxanthin production started in the early exponential growth phase and continued throughout the whole culture period, confirming that the astaxanthin biosynthesis was partially associated with cell growth in this yeast culture. The biomass and volumetric astaxanthin yields obtained from this 5-day batch culture were 16.8 g/L and 15.3 mg/L, respectively.

The glucose concentration in culture decreased rapidly during the exponential growth phase, and was completely exhausted at 36 h post-inoculation. In parallel with the rapid cell growth and glucose consumption, ethanol accumulated to a peak level of 3.5 g/L at 36 h; then the ethanol level decreased rapidly to zero at 48 h. The beginning of ethanol consumption (36 h) was just at the time of glucose depletion; the period of ethanol level decrease mostly corresponded to the period of slow cell growth (36–42 h). This time course comparison suggests that the ethanol initially produced in the yeast culture was later utilized by the yeast cells for growth as the major carbon source glucose was used up. Moreover, the OUR time course consists of two peaks (very clearly), a large and broad peak corresponding to the period of glucose consumption and a small and narrow peak corresponding to the period of ethanol consumption. This OUR time course shows more clearly than the biomass time course for a diauxic growth pattern of the yeast culture, with the primary growth phase on glucose, and the secondary growth phase on ethanol as the carbon-energy source.

Effects of External Ethanol

To detect and confirm the double roles of ethanol as an inhibitory metabolite and a useful nutrient for cell growth, we tested the effects of externally supplied ethanol on the yeast cultures (in shake flasks). As shown in Figure 2, both cell growth and glucose consumption were suppressed (slower) in the earlier culture period of 0–72 h with the addition of ethanol, more seriously at a higher concentration. As shown in Table II, both the maximum specific growth rate and the maximum cell density during the exponential growth phase were decreased with the addition of ethanol. However, the maximum biomass density attained in the whole culture period increased slightly with the addition of ethanol. These results confirmed that ethanol inhibited the yeast cell growth during the early growth phase on glucose, but was used later as a carbon source for cell growth. Indeed, the ethanol added to culture was completely consumed as there was no detectable ethanol in the culture at the end of culture period (data not shown). Moreover, the addition of ethanol also resulted in a lower astaxanthin yield (Table II), indicative of the negative effect of ethanol accumulation on astaxanthin biosynthesis in the yeast culture.

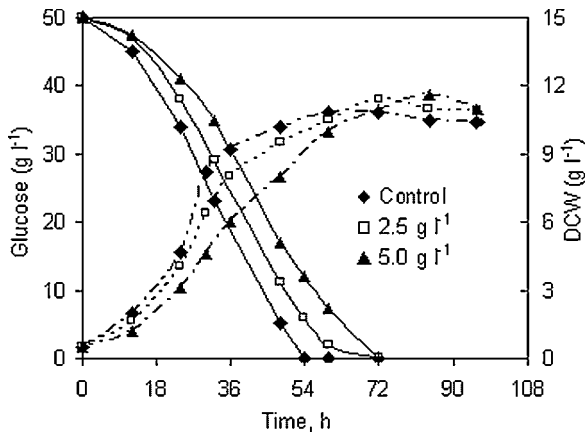

Figure 2. Effects of external ethanol addition on cell growth (dashed lines) and glucose consumption (solid lines) (experimental data representing the mean of triplicate measurements and maximum SE < 10% of the mean).

Table II. Effects of external ethanol addition on cell growth and astaxanthin production (DCW_{36h}, dry cell weight at 36 h culture time; DCW_{max}, maximum dry cell weight; error ranges = SE, n = 3).

Ethanol	μ_{max} (h^{-1})	DCW_{36h} (g/L)	DCW_{max} (g/L)	Astaxanthin yield (mg/L)
Control	0.095 ± 0.003	8.8 ± 0.3	10.8 ± 0.4	9.0 ± 0.7
2.5 g/L	0.078 ± 0.002	8.0 ± 0.2	11.4 ± 0.4	8.6 ± 0.4
5.0 g/L	0.068 ± 0.002	6.6 ± 0.4	11.5 ± 0.5	8.4 ± 0.5

Simulation of Model Parameters With Batch Culture Data at 50 g/L Glucose

The above kinetic model and stoichiometric Equations (1)–(7) contain a total of 14 constants or model parameters. Of these, the biomass yield coefficient on glucose and ethanol ($Y_{X/G}$ and $Y_{X/E}$) were calculated directly from the experimental data during batch culture, and ethanol yield on glucose ($Y_{E/G} = 0.511$ g ethanol/g glucose) was obtained from the stoichiometric equation, $C_6H_{12}O_6 \rightarrow 2C_2H_5OH + 2CO_2$ (Shuler and Kargi, 2002). The remaining 11 model parameters were derived by simulating the model equations with the batch culture time courses in stirred fermentors at 50 g/L glucose (Fig. 1), using mathematical software 1stOpt. The values of all parameters computed from the simulation are shown in Table I. The model simulation achieved a fairly large coefficient of determination $R^2 = 0.944$, which is indicative of the adequacy of model fit to experimental data. The 95% confidence intervals of model parameters as shown in Table I are fairly narrow, implying that the model parameters have a high degree of statistical reliability. Figure 1 (lines) shows the simulated time courses by the model equations with these parameter, which fit closely with the experimental data points.

The above kinetic model was modified from the basic Monod model by including substrate and product inhibition. For direct comparison of the merits and adequacy of the modified model versus the basic Monod model, we also computed the time courses of culture at 50 g/L initial glucose with the Monod equation (Fig. 3). The simulated biomass and glucose concentrations as well as the astaxanthin yield exhibited large deviations from the experimental data. Not surprisingly, the Monod model gave much higher rates of cell growth and glucose consumption because of the omission of substrate and product inhibition. The coefficient of determination for the simulation with the basic Monod equation $R^2 = 0.787$, was also much smaller than that with the modified Monod model ($R^2 = 0.944$).

Application of Kinetic Model to Batch Cultures

With the parameter values (Table I) determined at 50 g/L initial glucose, the model equations were applied to estimate the time courses of batch cultures at other glucose concentrations. Figure 4 shows the time courses of the yeast cultures at 25, 75, and 100 g/L initial glucose concentrations predicted with the model equations and the experimental

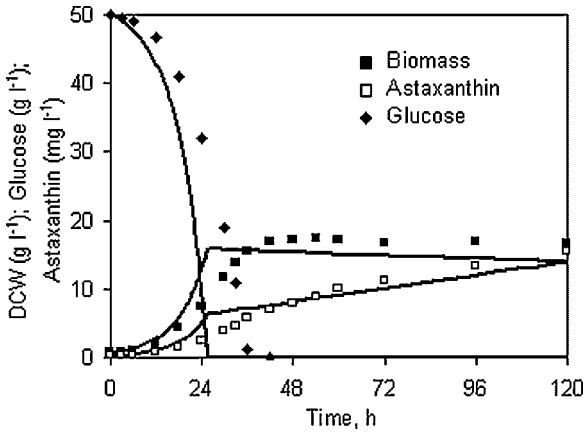

Figure 3. Simulation of batch culture time courses (at 50 g/L initial glucose) with the basic Monod model without glucose and ethanol inhibition (symbols for experimental data points, and lines for model simulation results; experimental data representing the mean of two repeated runs and maximum SE < 10% of the mean).

data from stirred fermentors. At 25 and 75 g/L initial glucose concentrations, the model simulation provided very good and close fit with the experimental data for the four culture time courses including glucose consumption, cell growth, ethanol formation and consumption, and astaxanthin production. At 100 g/L glucose concentration, the model simulation provided close fit with the biomass and astaxanthin time courses, but showed relatively large deviations with the glucose and ethanol time courses.

Modeling and Optimization of Fed-Batch Culture

The kinetic model has proved adequate for describing the relationships between biomass, substrate and products in batch cultures at glucose concentrations up to 75 g/L. Considering that the major physiological characteristics considered in the model development are independent of the process mode, the model parameters may also be applicable for fed-batch culture within the glucose concentration range. Then the optimum feeding scheme (Fig. 5A) for maximum volumetric astaxanthin yield (P) was derived with the above equations using the Matlab optimization tools (''ode 45'' solver and ''fminsearch'' function). The optimum fed-batch culture process consisted of three stages, an initial batch process with $F = 0$ between 0 and 26 h, followed by a fed-batch period with exponential feeding between 26 and 42 h, and finally a batch process with $F = 0$ between 42 and 120 h.

A fed-batch culture experiment was performed with the model-predicted optimum feeding strategy, and the experimental data of biomass and astaxanthin were plotted in the same graph with model-predicted results (Fig. 5B). The close fit of model predication with the experimental data validated the robustness and applicability of the kinetic model for both batch and fed-batch cultures. In addition,

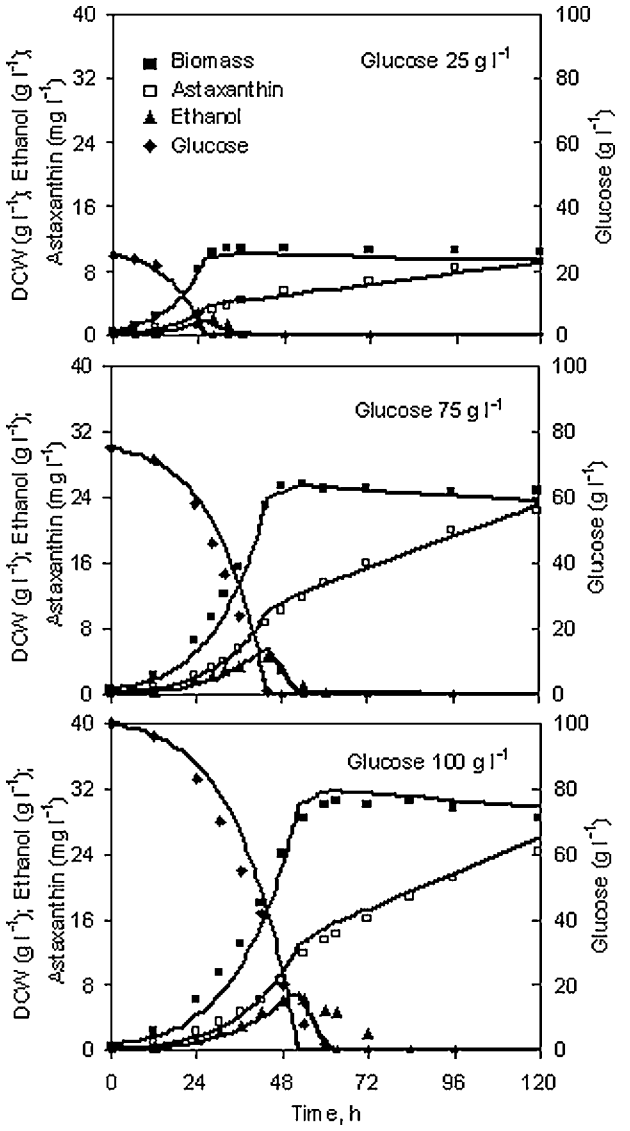

Figure 4. Time courses of cell growth, glucose consumption, and ethanol concentration and astaxanthin production in batch cultures of *X. dendrorhous* with 25, 75, or 100 g/L initial glucose (symbols for experimental data points, and lines for model simulation results; experimental data representing the mean of two repeated runs and maximum SE < 10% of the mean).

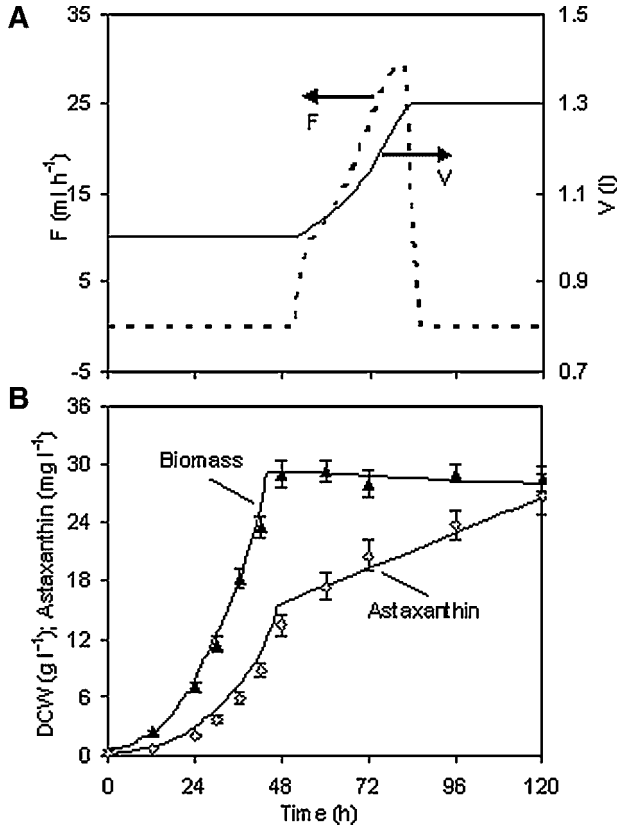

Figure 5. Simulation and optimization of fed-batch culture: (**A**) optimal feeding scheme predicted with the kinetic model; (**B**) time courses of biomass and astaxanthin production from experimental data (symbols) and model prediction (solid line) with the optimal feeding scheme shown in (A) (experimental data representing the mean of two repeated runs, error ranges = SE).

Table III shows the experimental results of maximum biomass and astaxanthin yields attained with various feeding schemes in fed-batch cultures. The highest biomass and astaxanthin yields were achieved with the optimal feeding scheme derived from the kinetic model (29.2 g/L and 26.8 mg/L, respectively), thus confirming the efficiency of feeding optimization.

Discussion

The modified Monod kinetic model with substrate and product inhibition has shown to be fairly adequate for describing the relationship of *X. dendrorhous* growth with glucose consumption, and ethanol production in batch cultures. The close agreement between the model-predicted values and the experimental results serves to justify the basic assumptions for the model construction and also the accuracy of the model parameters derived by the simulation

Table III. Biomass and astaxanthin production in fed-batch culture with various feeding schemes (experimental data representing the mean of two repeated runs, error ranges = SE).

Feeding scheme	DCW_{max} (g/L)	Astaxanthin yield (mg/L)	Astaxanthin productivity (mg/L/day)
Constant feeding			
0.009 L/h	24.9 ± 0.9	21.8 ± 1.6	4.36 ± 0.32
0.015 L/h	27.2 ± 1.2	24.2 ± 1.5	4.84 ± 0.30
0.021 L/h	26.1 ± 1.5	23.7 ± 1.2	4.74 ± 0.24
Exponential feeding			
$\mu_{set} = 0.05$	25.5 ± 1.4	22.3 ± 1.8	4.46 ± 0.36
$\mu_{set} = 0.07$	27.4 ± 1.1	24.6 ± 1.7	4.92 ± 0.34
$\mu_{set} = 0.09$	26.5 ± 1.5	24.3 ± 1.3	4.86 ± 0.26
Optimal feeding	29.2 ± 1.3	26.8 ± 1.7	5.36 ± 0.34

methods. The model showed the best and closest fit with experimental data at initial glucose concentrations in the range of 25–75 g/L, but larger deviations at much higher initial glucose concentrations. The model slightly underestimated the biomass at lower glucose concentrations, for example, 25 g/L, and notably overestimated the biomass at higher glucose, for example, 100 g/L. The biomass underestimated at lower glucose levels by the model may be attributed to the use of other carbon sources such as corn steep liquor available in the culture for growth. The notable overestimation of biomass at higher glucose levels may be due to the accumulation of other inhibitory metabolites in addition to ethanol, such as acetic acid in the culture at high glucose concentrations as reported previously in batch and fed-batch cultures of *X. dendrorhous* (Liu and Wu, 2007; Meyer and Du Preez, 1994). The inhibition of growth may also lead to slower consumption of ethanol than that predicted by the model. The extremely high glucose concentration could also induce changes of other physiological characteristics of the yeast culture which cannot be predicted by a simple kinetic model. Nevertheless, the applicable range of 25–75 g/L glucose by the model covers the most common and favorable glucose concentrations for the yeast cultures and any other concentration beyond this range is rarely used in practice. Therefore, the modified kinetic model will be very useful for the operation and optimization of the yeast fermentation processes in industry. The model was also successfully applied to fed-batch culture to predict the optimal feeding scheme for astaxanthin production. The volumetric productivity of astaxanthin (5.36 mg/L/day) attained with this optimum feeding scheme was the highest ever reported so far with this yeast cultures in various operation processes such as batch, fed-batch and perfusion processes (Liu and Wu, 2007; Yamane et al., 1997), and also higher than those with the hyperproducing alga *Haematococcus pluvialis* cultures (Zhang et al., 1999).

The quantitative parameters in the kinetic models and the stoichiometric equations provide useful information of the culture characteristics. The much higher specific growth rate on glucose $\mu_{max,G}$(0.129 h^{-1}) than on ethanol $\mu_{max,E}$(0.0184 h^{-1}) implies that glucose is a more favorable substrate for *X. dendrorhous* growth. The value of $\mu_{max,G}$ is very close to those reported by others for this yeast culture (Meyer and Du Preez, 1994; Yamane et al., 1997). The Monod saturation constant on glucose $K_{S,G}$ (0.0872 g/L) is quite small, implying that the specific growth rate was close to its maximum in most of the culture period when glucose was still available (Ge and Bai, 2006; Shuler and Kargi, 2002). The Monod saturation constant on ethanol ($K_{S,E}$) derived from our simulation studies is notably higher than that for *S. cerevisiae*, which is about the same order of magnitude (around 0.1 g/L) as that on glucose (Pham et al., 1998). An explanation for this is that the *X. dendrorhous* yeast may have a lower preference for ethanol as a carbon source than *S. cerevisiae*. An overestimation of the parameter could also arise from the imperfect model formulation for μ_E (Eq. 3), especially the inclusion of a substrate multiplier K_C in the denominator of the second term, which tends to give a higher $K_{S,E}$ value. Both the inhibition constants of glucose $K_{I,G}$ (178 g/L) and ethanol $K_{I,E}$ (7.64 g/L) fall within the ranges reported from previous studies as shown in Table IV, suggesting that both inhibition effects are significant. The greater competitive inhibition constant K_C (1.95) implies that glucose is a more favorable and competitive substrate than ethanol for cell growth. In other words, the uptake of ethanol by the yeast cells will be inhibited when glucose is still available in the culture medium. The diauxic growth observed in the culture at 50 g/L glucose (Fig. 1) also proves the sequence of substrate consumption from glucose to ethanol. According to Yamane et al. (1997), the chemical formula of *X. dendrorhous* yeast cells in batch cultures at 50 g/L glucose was estimated as $CH_{1.44}O_{0.50}N_{0.10}$. With this, we can arrive at the following stoichiometric equations for the batch culture with 50 g/L glucose based on our modeling study and mass balance

$$\text{Primary growth}: C_6H_{12}O_6 + 0.29NH_3 + 2.58O_2 = 2.92CH_{1.44}O_{0.50}N_{0.10} + 0.40C_2H_6O + 3.08CO_2 + 3.14H_2O$$

$$\text{Secondary growth}: C_2H_6O + 0.065NH_3 + 2.33O_2 = 0.65CH_{1.44}O_{0.50}N_{0.10} + 1.35CO_2 + 2.63H_2O$$

The inhibitory effects and inhibition mechanisms of ethanol on cell growth in yeast cultures have been examined in numerous studies. Aiba et al. (1968) suggest that the inhibitory effect of ethanol on *S. cerevisiae* growth may be associated with the decrease in the proton motive force across the cell membrane. Osman and Ingram (1985) report that ethanol can increase membrane destabilization, which leads to loss of the essential cofactors and coenzymes and the activities of many enzymes for glycolysis in *Zymomonas mobilis*. Fiechter and Seghezzi (1992) show that the inhibition of respiratory catabolism is enhanced by increasing ethanol concentration from both exogenous and endogenous sources. Therefore, ethanol production should be eliminated or minimized during yeast cultivation in order to achieve high biomass yields.

In conclusion, a modified Monod model with glucose and ethanol inhibition was developed for the *X. dendrorhous* yeast culture characterized by overflow metabolism. The

Table IV. Comparison of kinetic model parameters in different yeast cultures (unit, g/L; n.a. not available or not applicable)

Microorganisms	$K_{S,G}$	$K_{S,E}$	$K_{I,G}$	$K_{I,E}$	References
S. cerevisiae	3.3	n.a.	n.a.	5.2	Hoppe and Hansford (1982)
S. cerevisiae	0.12	0.1	n.a.	10	Pham et al. (1998)
S. cerevisiae	0.565	n.a.	283.7	n.a.	Krishnan et al. (1999)
S. cerevisiae	0.155	n.a.	160.7	n.a.	Ge and Bai (2006)
Z. mobilis	1.45	n.a.	200	n.a.	Leksawasdi et al. (2001)
X. dendrorhous	0.0872	1.42	178	7.64	This study

modeling study is helpful for gaining a better understanding of the culture factors and conditions for the respiratory and fermentative catabolism of carbon sources. The kinetic model is quite simple and straightforward, but can adequately describe the relationship of cell growth kinetics with glucose consumption and ethanol formation in the yeast cultures. Besides the kinetic model equation and constants, we have derived the major stoichiometric relationships and parameters for biomass, substrate, and product. This kinetic model, together with the stoichiometric relationships, will be useful for rational design, optimal control, and efficient operation of the yeast culture process for improved astaxanthin production.

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References

Aiba S, Shoda M, Nagatani M. 1968. Kinetics of product inhibition in alcohol fermentation. Biotechnol Bioeng 10:845–864.

Andrewes AG, Phaff HJ, Starr MP. 1976. Carotenoids of *Phaffia rhodozyma*, a red-pigmented fermenting yeast. Phytochemistry 15:1003–1007.

Daran-Lapujade P, Jansen MLA, Daran JM, van Gulik W, de Winde JH, Pronk JT. 2004. Role of transcriptional regulation in controlling fluxes in central carbon metabolism of *Saccharomyces cerevisiae*. J Biol Chem 279:9125–9138.

Fiechter A, Seghezzi W. 1992. Regulation of glucose metabolism in growing yeast cells. J Biotechnol 27:27–45.

Ge XM, Bai FW. 2006. Intrinsic kinetics of continuous growth and ethanol production of a flocculating fusant yeast strain SPSC01. J Biotechnol 124:363–372.

Guerin M, Huntley ME, Olaizola M. 2003. Haematococcus astaxanthin: Applications for human health and nutrition. Trends Biotechnol 21: 210–216.

Hoek PV, Dijken JPV, Pronk JT. 1998. Effect of specific growth rate on fermentative capacity of baker's yeast. Appl Environ Microbiol 64: 4226–4233.

Hoppe GK, Hansford GS. 1982. Ethanol inhibition of continuous anaerobic yeast culture. Biotechnol Lett 4:39–44.

Johnson EA. 2003. Phaffia rhodozyma: Colorful odyssey. Int Microbiol 6:169–174.

Johnson EA, An GH. 1991. Astaxanthin from microbial sources. Crit Rev Biotechnol 11:297–326.

Johnson EA, Schroeder WA. 1995. Microbial carotenoids production. Adv Biochem Eng 53:119–178.

Krishnan MS, Ho NWY, Tsao GT. 1999. Fermentation kinetics of ethanol production from glucose and xylose by recombinant *Saccharomyces* 1400 (pLNH33). Appl Biochem Biotechnol 77–79:373–388.

Leksawasdi N, Joachimsthal EL, Rogers PL. 2001. Mathematical modeling of ethanol production from glucose/xylose mixtures by recombinant *Zymomonas mobilis*. Biotechnol Lett 23:1087–1093.

Linek V, Vacek V. 1981. Chemical engineering use of catalyzed sulphite oxidation kinetics for the determination of mass transfer characteristics of gas–liquid contactors. Chem Eng Sci 36:1747–1768.

Liu YS, Wu JY. 2006. Hydrogen peroxide-induced astaxanthin biosynthesis and catalase activity in *Xanthophyllomyces dendrorhous*. Appl Microbiol Biot 73:663–668.

Liu YS, Wu JY. 2007. Perfusion culture process plus H_2O_2 stimulation for efficient astaxanthin production by *Xanthophyllomyces dendrorhous*. Biotechnol Bioeng 95:568–573.

Liu YS, Wu JY, Ho KP. 2006. Characterization of oxygen transfer conditions and their effects on Phaffia rhodozyma growth and carotenoid production in shake-flask cultures. Biochem Eng J 27:331–335.

Luedeking R, Piret EL. 1959. A kinetic study of the lactic acid fermentation. Batch process at controlled pH. J Biochem Microbiol Technol Eng 1:393–412.

Meyer PS, Du Preez JC. 1994. Effect of culture conditions on astaxanthin production by a mutant of *Phaffia rhodozyma* in batch and chemostat culture. Appl Microbiol Biotechnol 40:780–785.

Osman A, Ingram O. 1985. Mechanism of ethanol inhibition of fermentation in *Zymomonas mobilis* CP4. J Bacteriol 164:173–180.

Pham HTB, Larsson G, Enfors SO. 1998. Growth and energy metabolism in aerobic fed-batch cultures of *Saccharomyces cerevisiae*: Simulation and model verification. Biotechnol Bioeng 60:474–482.

Poscheta F, Geeraerda AH, Scheerlinckb N, Nicola BM, Van Impe JF. 2003. Monte Carlo analysis as a tool to incorporate variation on experimental data in predictive microbiology. Food Microbiol 20:285–295.

Postma E, Verduyn C, Scheffers WA, Dijken JPV. 1989. Enzymic analysis of the Crabtree effect in glucose-limited chemostat cultures of *Saccharomyces cereviciae*. Appl Environ Microbiol 55:468–477.

Reynders MB, Rawlings DE, Harrison STL. 1997. Demonstration of the Crabtree effect in *Phaffia rhodozyma* during continuous and fed-batch cultivation. Biotechnol Lett 19:549–552.

Schroeder WA, Johnson EA. 1995. Carotenoids protect *Phaffia rhodozyma* against singlet oxygen damage. J Ind Microbiol 270:502–507.

Shuler ML, Kargi F. 2002. Bioprocess engineering. NJ: Prentice Hall PTR.

Tang YJ, Meadows AL, Keasling JD. 2007. A kinetic model describing *Shewanella oneidensis* MR-1 growth, substrate consumption, and product secretion. Biotechnol Bioeng 96:125–133.

Vemuri GN, Altman E, Sangurdekar DP, Khodursky AB, Eiteman MA. 2006. Overflow metabolism in *Escherichia coli* during steady-state growth: Transcriptional regulation and effect of the redox ratio. Appl Environ Microbiol 72:3653–3661.

Wei LY, Liu SJ, Niu CG. 2007. Application of envelope forecast method in traffic flow prediction. Microcomputer Inf 23:226–228.

Yamane Y, Higashida K, Nakashimada Y, Kakizono T, Nishio N. 1997. Influence of oxygen and glucose on primary metabolism and astaxanthin production by *Phaffia rhodozyma* in batch and fed-batch cultures: Kinetic and stoichiometric analysis. Appl Environ Microbiol 63:4471–4478.

Zhang XW, Gong XD, Chen F. 1999. Kinetic models for astaxanthin production by high cell density mixotrophic culture of the microalga *Haematococcus pluvialis*. J Ind Microbiol Biotechnol 23:691–696.