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ORIGINAL PAPER

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# A growth kinetic model of *Kluyveromyces marxianus* cultures on cheese whey as substrate

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**Abstract** This work presents a multi-route, non-structured kinetic model for determination of microbial growth and substrate consumption in an experimental batch bioreactor in which β-galactosidase is produced by *Kluyveromyces marxianus* growing on cheese whey. The main metabolic routes for lactose, and oxygen consumption, cell growth, and ethanol production are derived based on experimental data. When these individual rates are combined into a single growth rate, by rewriting the model equations, the model re-interpretation has a complexity similar to that of the usual variations of the Monod kinetic model, available in the literature. Furthermore, the proposed model is in good agreement with the experimental data for different growth temperatures, being acceptable for dynamic simulations, processes optimization, and implementations of model-based control technologies.

**Keywords** Kinetic model · *Kluyveromyces marxianus* · Bioreactor modeling · Oxygen transport modeling

## Introduction

Applications of biochemical processes are increasing in importance for process engineering. Bioprocesses present several advantages over conventional chemical processes. For example, they can cause less environmental damage, can be carried out under cheaper and more secure operating conditions (near atmospheric pressure and temperatures), and they save more energy. However, the two major drawbacks of working with biochemical processes are: (1) it is difficult to obtain reliable and efficient measures of the key process variables (sensor technology): (2) it is difficult to formalize biological knowledge about the process using practical mathematical models (process modeling).

The usual approach for mathematical modeling of bioreactors considers isothermal systems and is based on a single growth rate with variants of Monod kinetics [11] to represent the cellular growth rate. However, this approach is not able to adequately represent the process dynamics and, consequently, its dynamic simulating results have limited practical value. Another approach is that of structured models, in which metabolic changes during cultivation are taken into account [10, 12]. Frequently, this method results in detailed modeling of the regulating processes but it requires a larger number of measured variables. A third possibility is that represented by structured unsegregated cybernetic modeling [4], which is an intermediate approach between structured and non-structured models. In this kind of modeling, the values of the growth rates of different metabolic pathways are calculated applying a modified Monod equation rate in which the growth rate is proportional to the concentration of a key enzyme controlling the single metabolic pathway [4]. However, this kind of enzyme-coupled model has a larger number of estimated parameters.

In this work, the proposed model, instead of considering a single growth rate, evaluates the main metabolic routes for the variables of interest (lactose, oxygen, cells, and ethanol) and , yielding a multi-route, non-structured kinetic model. The production or consumption rates for each route are derived based on the observed experimental data [14]. Modeling the mass transport phenomena also presents a non-conventional

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approach to describe oxygen transport inside the reactor. This is a second-order model that is able to represent accurately the dissolved oxygen concentration using the same estimated parameter, commonly denoted by $k_l a$, the oxygen gas-liquid mass transfer coefficient, as in the usual first-order transport equation.

The experimental data to validate the model was obtained from a 2-l batch bioreactor used to produce $\beta$-galactosidase (lactase) by the yeast *Kluyveromyces marxianus* when grown on cheese whey. $\beta$-Galactosidase is intensively used in the food industry, and cheese whey is an abundant residue of the dairy industry (83% of the total milk volume used), making this system for transforming the residue into a product of higher aggregated value of great interest.

## Materials and methods

### Yeast strain, media, bioreactor, and culture conditions

*Kluyveromyces marxianus* CBS 6556 was kindly provided by the Biotechnological Development Center (Joinville, SC, Brazil) and maintained on glycerol. Prior to use, fresh cultures were grown on agar slants, of the same medium composition as the liquid cultures. Isolated yeast colonies were aseptically transferred to a 300-ml shake-flask containing 50 ml of culture medium, and incubated on an orbital shaker at 200 rpm, for 15–20 h. Pre-inoculate temperature was the same as that for the subsequent experiments (see "Modeling").

The basic medium contained 70 g reconstituted sweet cheese whey powder $l^{-1}$ (71.0 g lactose $l^{-1}$, 11.0 g protein $l^{-1}$, 0.7 g fat $l^{-1}$, 3.0 g water $l^{-1}$, 7.2 g ash $l^{-1}$; ELEGÊ Laticínios, Lageado, RS, Brazil); and 5 g yeast extract $l^{-1}$; the pH was kept at 5.5. For bioreactor cultures, 1 ml of the antifoaming agent polyoxyethylene-polyoxypropylene co-polymers (Mazu DF 800 S, Mazer Chemicals, Manchester, UK) $l^{-1}$ was used. To avoid protein precipitation during the sterilization process (121 °C, 15 min), cheese whey proteins were hydrolyzed with a commercial protease (Alcalase 2.4 l, 1 ml $l^{-1}$, Novo Nordisk A/S, Copenhagen, Denmark), at pH 8.5 and 55 °C for 3 h.

The experimental system consisted of a glass bioreactor coupled with a control unit (BIOSTAT B, Braun Biotech International, Germany), connected to a remote PC-computer for data acquisition, monitoring, and control. The bioreactor (total working volume 2 l) was fitted with a polaragraphic oxygen probe (Mettler-Toledo, Brazil), a pH sensor (Mettler-Toledo), and a PT-100 temperature sensor. In order to avoid gas stripping of water, the bioreactor was equipped with a large condenser cooled by a flow of 2 l chilled water $min^{-1}$. The agitation system consisted of two six-bladed, Rushton-type impellers, and the agitation speed was set at 600 rpm, with an airflow rate of 6 l air $min^{-1}$. Four cultures were run for 30 h at 30, 34, 38, and 42 °C, respectively. These operating conditions were chosen based on previous knowledge of the experimental system [14].

### Cell concentration

Cell concentration was estimated by measuring turbidity in a spectrophotometer at 620 nm, and relating the readings to a calibration curve of g dry weight biomass $l^{-1}$. The cells were harvested at 6,000 *g*, 3 min, washed twice with cold distilled water, and dried at 90 °C to a constant weight.

### Analytical procedures

Lactose was assayed by the phenol-sulfuric acid method described by Barale [1].

Ethanol was assayed by gas chromatography (Varian Star 3400 CX, USA) with a 50-m×0.25 mm (internal diameter) capillary column LM-100 (L and M, Brazil). The chromatograph was programmed as follows: injector and detector at 250 °C, column at 50 °C for 2 min, followed by heating to 200 °C at a rate of 5 °C $min^{-1}$, for 32 min. To quantify the amount of ethanol, a calibration curve was constructed by adding a solution of cheese whey (7% w/v), varying ethanol concentration, and a fixed amount of *n*-propanol as internal standard.

$\beta$-Galactosidase activity was assayed by the ONPG (*o*-nitrophenol-*p*-D-galactopyranoside) method as described by Lederberg [9].

### Modeling

The proposed kinetic model is based on the following assumptions: (1) the biotic phase is unique (the model developed is non-structured); (2) the elemental composition of the biomass does not change significantly; (3) lactose is metabolized both aerobically and anaerobically, with different rates and efficiencies; (4) ethanol is metabolized only aerobically; (5) there is no intracellular carbohydrate storage [4]; (6) the nitrogen source is in excess, therefore not a substrate-limited parameter; (7) the main products are biomass, $CO_2$, $H_2O$, and ethanol, the latter being the only significant metabolic by-product.

Three main metabolic routes for the culture of *K. marxianus* on cheese whey were considered, (Eqs. 1, 2, and 3):

Fermentation:

$$S + a\ NH_3 \rightarrow b\ X + c\ Et + d\ CO_2 + e\ H_2O \tag{1}$$

Respiration I (oxidation of lactose):

$$S + f\ O_2 + g\ NH_3 \rightarrow h\ X + i\ CO_2 + j\ H_2O \tag{2}$$

Respiration II (oxidation of ethanol):

$$Et + k\ O_2 + l\ NH_3 \rightarrow m\ X + n\ CO_2 + o\ H_2O \tag{3}$$

where $X$ represents viable cells, $S$ represents lactose ($C_{12}H_{22}O_{11}$), $O_2$ represents oxygen, $Et$ represents ethanol ($C_2H_6O$) and $a$, $b$, $c$, $d$, $e$, $f$, $g$, $h$, $i$, $j$, $k$, $l$, $m$, $n$, and $o$ are the unknown stoichiometric coefficients, used to define the main yield coefficients (on a mass basis) for the system:

$$Y_{X/S}^{ferm} = b\frac{\text{molecular weight of biomass}}{\text{molecular weight of Lactose}} \tag{4}$$

$$Y_{X/E}^{ferm} = \frac{b}{c}\frac{\text{molecular weight of biomass}}{\text{molecular weight of ethanol}} = \frac{Y_{X/S}^{ferm}}{\phi_{X/S}^{ferm}} \tag{5}$$

$$Y_{X/S}^{oxid} = h\frac{\text{molecular weight of biomass}}{\text{molecular weight of lactose}} \tag{6}$$

$$Y_{X/O}^{S} = \frac{h}{f}\frac{\text{molecular weight of biomass}}{\text{molecular weight of oxygen}} = \frac{Y_{X/S}^{oxid}}{\phi_{X/S}^{oxid}} \tag{7}$$

$$Y_{X/E}^{oxid} = m\frac{\text{molecular weight of biomass}}{\text{molecular weight of ethanol}} \tag{8}$$

$$Y_{X/O}^{Et} = \frac{m}{k}\frac{\text{molecular weight of biomass}}{\text{molecular weight of oxygen}} = \frac{Y_{X/E}^{oxid}}{\phi_{X/E}^{oxid}} \tag{9}$$

where $\phi_{X/S}^{ferm}$, $\phi_{X/S}^{oxid}$ and $\phi_{X/E}^{oxid}$ are stoichiometric ratios relative to the cell concentration. If the cell composition is unknown, all yield coefficients have to be estimated, otherwise only three yield coefficients (say, $Y_{X/S}^{ferm}$, $Y_{X/S}^{oxid}$ and $Y_{X/E}^{oxid}$) need to be estimated and the remaining can be obtained by simple stoichiometric analysis.

The substrate-limited specific growth rates, (expressed as g $l^{-1}$ $h^{-1}$), for the metabolic routes are given by:

Fermentation route (Eq. 1):

$$r_{ferm} = \frac{\mu_{1\max} \cdot S}{k_1 + S} X \tag{10}$$

First respiration route (Eq. 2):

$$r_{oxid}^{S} = \frac{\mu_{2\max}.S.C_L}{(k_3 + S).(k_{ox1} + C_L)}.X \tag{11}$$

Second respiration route (Eq. 3):

$$r_{oxid}^{Et} = \frac{\mu_{3\max}.Et.C_L}{(k_2 + Et).(k_{ox2} + C_L)}.X \tag{12}$$

where $X$ is the concentration of viable cells (g $l^{-1}$); $S$ is the concentration of lactose (g $l^{-1}$); $C_L$ is the concentration of dissolved oxygen in the liquid phase (g $l^{-1}$); $Et$ is the concentration of ethanol (g $l^{-1}$); $\mu_{1\max}$, $\mu_{2\max}$, $\mu_{3\max}$, $k_1$, $k_2$, $k_3$, $k_{ox1}$, $k_{ox2}$ are the kinetic model parameters. Schepers et al. [15] also included in their specific growth rate model the effects of nitrogen substrate and pH. In this work, pH was kept constant and the nitrogen source was added in excess relative to the carbon source.

Based on the above kinetic model and considering that the bioreactor operates in an aerated and well-mixed isothermal batch mode, the following mass balances for the components can be written:

Biomass:

$$\frac{dX}{dt} = r_{ferm} + r_{oxid}^{S} + r_{oxid}^{Et} - r_d \tag{13}$$

Substrate:

$$\frac{dS}{dt} = -\frac{1}{Y_{X/S}^{ferm}}.r_{ferm} - \frac{1}{Y_{X/S}^{oxid}}.r_{oxid}^{S} \tag{14}$$

Oxygen in the liquid phase:

$$\frac{dC_L}{dt} = k_l a.(C_{Le} - C_L) - \left[\frac{\phi_{X/S}^{oxid}}{Y_{X/S}^{oxid}}.r_{oxid}^{S} + \frac{\phi_{X/E}^{oxid}}{Y_{X/E}^{oxid}}.r_{oxid}^{Et}\right] \tag{15}$$

Oxygen in the gas phase:

$$\frac{dC_G}{dt} = \frac{F_{ar}}{V_G}.(C_{Go} - C_G) - k_l a.(C_{Le} - C_L).\frac{V_L}{V_G} \tag{16}$$

Ethanol

$$\frac{dEt}{dt} = \frac{\phi_{X/S}^{ferm}}{Y_{X/S}^{ferm}}.r_{ferm} - \frac{1}{Y_{X/E}^{oxid}}.r_{oxid}^{Et} \tag{17}$$

where $C_{Le}$ is the liquid-phase oxygen concentration in equilibrium with the gas ( g $l^{-1}$); $C_G$ is the concentration of oxygen in the gas phase (g $l^{-1}$); $k_l a$ is the gas-liquid mass transfer coefficient of the oxygen ($h^{-1}$); $r_d$ is the specific cell-death rate (g $l^{-1}$ $h^{-1}$); $F_{ar}$ is the air flow rate injected in the bioreactor (l $h^{-1}$); $V_G$ is the internal volume of gas in the bioreactor (l); $V_L$ is the volume of liquid of the bioreactor (L); $C_{Go}$ is the oxygen concentration in the air feeding flow (g $l^{-1}$).

The liquid-phase oxygen concentration in equilibrium with the gas-phase is written as:

$$C_{Le} = K.C_G \tag{18}$$

where $K$ is the equilibrium coefficient (dimensionless), given by:

$$K = \frac{C_m}{C_{Go}} \tag{19}$$

where $C_m$ is the saturating concentration of oxygen in the liquid phase (g $l^{-1}$) which is a function of temperature and pressure. The $C_m$ equation was obtained by regression from experimental data reported in the literature [13] for water at the atmospheric pressure, resulting in:

$$C_m = 3.62\ 10^{-6}\ T^2 - 3.312\ 10^{-4}\ T + 0.01466217 \tag{20}$$

The oxygen concentration of the feed gas was obtained by the following equation, assuming an ideal gas:

$$C_{Go} = \frac{P\ \bar{M}\ x_{O_2}}{R(T + 273.15)} \tag{21}$$

where $T$ is the bioreactor temperature (°C); $x_{O_2}$ is the molar fraction of oxygen in the feed gas (air) (dimensionless); $P$ is the bioreactor pressure (bar); $\bar{M}$ is the oxygen molecular weight of oxygen (g g $mol^{-1}$); $R$ is the universal constant of gases (bar l g $mol^{-1}$ $K^{-1}$).

## Results and discussion

In order to determine $k_l a$, experiments without cells were also carried out in the bioreactor. For this situation, the model equations that describe oxygen transport in an aqueous medium can be written as in Eq. 16, above, and Eq. 22:

$$\frac{dC_L}{dt} = k_l a(C_{Le} - C_L) \tag{22}$$

Equations 16 and 22 constitute a two-layers model: (1) oxygen is transferred from the gas phase to the gas-liquid interface and, (2) from this interface to the liquid phase. The good agreement with experimental data of the two-layers (second-order) model can be observed in Fig. 1, where the fitting of the Eqs. 16 and 22 is compared with the classical one-layer (first-order) model, given by Eq. 23:

$$\frac{dC_L}{dt} = \bar{k}_l a(C_m - C_L) \tag{23}$$

where $\bar{k}_l a$ is the oxygen mass transfer coefficient for this model ($h^{-1}$).

Despite their different complexities, both models have only one parameter to be estimated, $k_l a$. However, the two-layers model can describe the system dynamics more appropriately, as shown in Fig. 1.

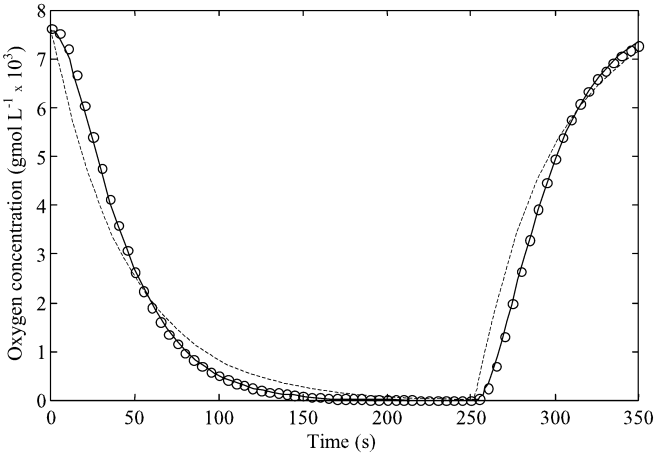


**Fig. 1** Liquid-phase oxygen concentration. Classical first order model (—) versus second order (—) and experimental data (○) for an agitation speed of 600 rpm. The experiments were carried out by first replacing air with nitrogen and then re-injecting air until equilibrium was reached. Results represent the mean of three experiments

The oxygen mass transfer coefficient estimated for an agitation speed of 600 rpm was $k_1a = 143.28\ h^{-1}$. This estimate was obtained from data derived from several tests in aqueous media without reaction (Eqs. 16 and 22) assuming similar solubility between this medium and the fermentation medium.

Regarding the kinetic model, note that, if the cell balance is rewritten as Eq. 24, the modeling approach considered in this work has a single growth rate interpretation. For a list of variants of the Monod model using a single specific growth rate, refer to Bastin and Dochain [2].

$$\frac{dX}{dt} = \mu.X \tag{24}$$

$$\text{where } \mu = \frac{\mu_{1\max}.S}{k_1 + S} + \frac{\mu_{2\max}.S.C_L}{(k_3 + S).(k_{ox1} + C_L)} + \frac{\mu_{3\max}.Et.C_L}{(k_2 + Et).(k_{ox2} + C_L)} \tag{25}$$

Due to the lack of experimental data and their significance, and the unavailability of $CO_2$ and glycerol concentration measurements, the parameter $k_3$ was assumed to be equal to $k_1$, and $k_{ox2} = k_{ox1}$. Also, the specific cell-death rate was considered negligible during the fermentation period ($r_d \approx 0$). The remaining model parameters, $\mu_{1max}$, $\mu_{2max}$, $\mu_{3max}$, $k_1$, $k_2$, $k_{ox1}$, and the yield coefficients were estimated for each operating condition by fitting the process model, described by the set of ordinary differential equations 13, 14, 15, 16, and 17, and the constitutive equations 10, 11, and 12 and 18, 19, 20, and 21 to the experimental data.

The main factors that influence these parameters are temperature and pH. In this work, the pH was fixed at the optimal value of 5.5, determined by laboratory assays [14], and the effect of the temperature was studied. The objective was to obtain a parsimonious model in which some parameters are fixed and others are temperature-dependent.

The resulting set of differential-algebraic equations 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, and 21 was solved with a standard BDF (backward-differentiation formula) method of variable order and variable step size [3]. The model parameters were estimated by the least-square technique using the Gauss-Newton method with the BFGS (Broyden, Fletcher, Goldfarb, and Shanno) updating scheme for the Hessian matrix [5].

The estimates for the kinetic parameters and the yield coefficients are presented in Table 1. Note that the kinetic parameter $k_1$ and $k_{ox1}$ are independent of temperature, and the yield coefficient $Y^{oxid}_{X/E}$ varies only slightly among the different temperatures.

The yield coefficients (or the stoichiometric coefficients) vary with temperature because the reaction scheme of Eqs. 1, 2, and 3 is just a simplification of the true metabolic routes. A rigorous stoichiometric analysis of the main metabolic route for a similar system can be found in Krzystek and Ledakowicz [8]. Moreover,

**Table 1** Estimated parameters for each operating condition

| Parameter | T = 30 °C | T = 34 °C | T = 38°C | T = 42 °C |
|---|---|---|---|---|
| $\mu_{1max}$ ($h^{-1}$) | 0.55 | 0.60 | 0.60 | 0.30 |
| $\mu_{2max}$ ($h^{-1}$) | 0.15 | 0.24 | 0.06 | 0.18 |
| $\mu_{3max}$ ($h^{-1}$) | 0.11 | 0.22 | 0.16 | 0.07 |
| $k_1$ ($g\ l^{-1}$) | 20.00 | 20.00 | 20.00 | 20.00 |
| $k_2$ ($g\ l^{-1}$) | 4.60 | 4.05 | 4.26 | 4.82 |
| $k_{ox1}$ ($g\ l^{-1}$) | $1.0\ 10^{-4}$ | $1.0\ 10^{-4}$ | $1.0\ 10^{-4}$ | $1.0\ 10^{-4}$ |
| $Y^{ferm}_{X/S}$ | 0.25 | 0.31 | 0.41 | 0.23 |
| $Y^{oxid}_{X/S}$ | 0.60 | 0.60 | 0.38 | 0.49 |
| $Y^{oxid}_{X/E}$ | 0.15 | 0.15 | 0.15 | 0.15 |
| $\phi^{ferm}_{X/S}$ | 0.27 | 0.30 | 0.35 | 0.24 |
| $\phi^{oxid}_{X/S}$ | 0.37 | 0.40 | 0.24 | 0.28 |
| $\phi^{oxid}_{X/E}$ | 0.93 | 1.00 | 0.93 | 0.91 |

the metabolic paths do not occur simultaneously but depend on the specific operating condition. It is possible to encompass this non-simultaneity, even using the reaction scheme of Eqs. 1, 2 and 3, if some enzyme-activity-related parameters are incorporated into the reaction rates (Eqs. 10, 11, and 12) as in Di Serio et al. [4]. However, as already mentioned, this kind of enzyme-coupled model requires a larger number of estimated parameters.

In Figs. 2, 3, 4, and 5, comparisons between the simulations and the experimental data are presented for each fermentation. In those figures, the oxygen concentration is replaced by its saturation percentage ($pO_2$). Use of this variable allows normalization of the data between 0 and 100, the zero value corresponding to the absence of dissolved oxygen and its maximum corresponding to the saturating concentration for the fermentation temperature.

It is observed that, at the end of the fermentations, the oxygen data do not agree with the simulation predictions. This could be explained by the consumption of glycerol through an oxidative pathway after the primary sources of carbon (lactose and ethanol) have been

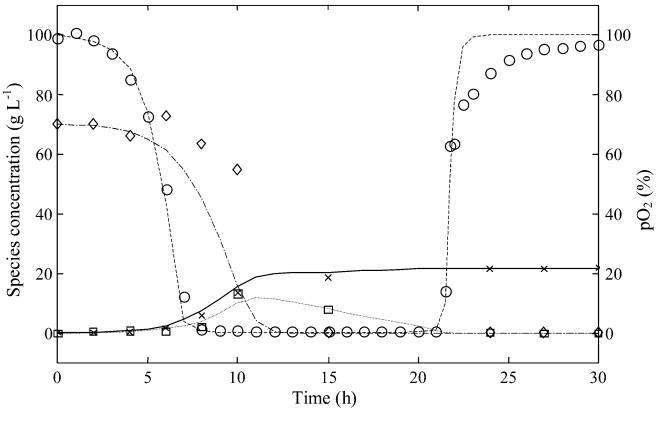


**Fig. 2** Experimental data (*symbols*) and model fittings (*lines*) for batch cultures at 30 °C: (x,—) biomass concentration ($X$); (◇,-·-·) substrate concentration ($S$); (□,····) ethanol concentration ($Et$); and (○,—) dissolved oxygen concentration ($pO_2$)

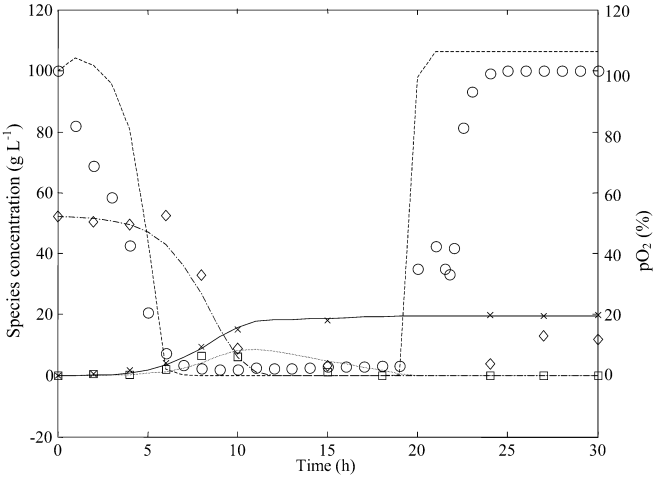


**Fig. 3** Experimental data (*symbols*) and model fittings (*lines*) for batch cultures at 34 °C: (x,—) biomass concentration (*X*); (◇,-·-·) substrate concentration (*S*); (□,····) ethanol concentration (*Et*); and (○,—) dissolved oxygen concentration ($pO_2$)

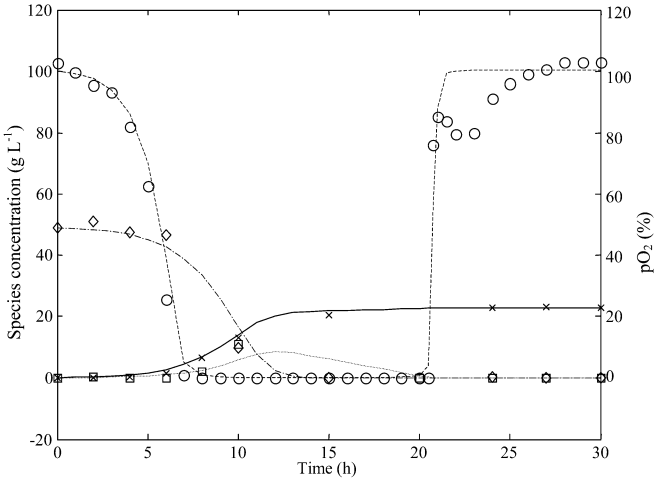


**Fig. 4** Experimental data (*symbols*) and model fittings (*lines*) for batch cultures at 38 °C: (x,—) biomass concentration (X); (◇,-·-·) substrate concentration (*S*); (□,····) ethanol concentration (*Et*); and (○,—) dissolved oxygen concentration ($pO_2$)

exhausted. As the production and consumption of glycerol are not present in the model—because this species was not measured in the experiments—this disagreement is expected. Except for this behavior, and the low-confidence lactose data, the agreement between the experimental data and the simulations is satisfactory.

From the results presented, it can be concluded that the multi-route, non-structured kinetic model for microbial growth, substrate consumption, and product formation is a good alternative to describe an experimental batch bioreactor in which $\beta$-galactosidase is produced by the yeast *Kluyveromyces marxianus* grown on a medium composed of cheese whey. The model also considers thermal effects on its parameters, which are largely neglected in the scientific literature, but can be very useful to determine the optimal conditions to carry

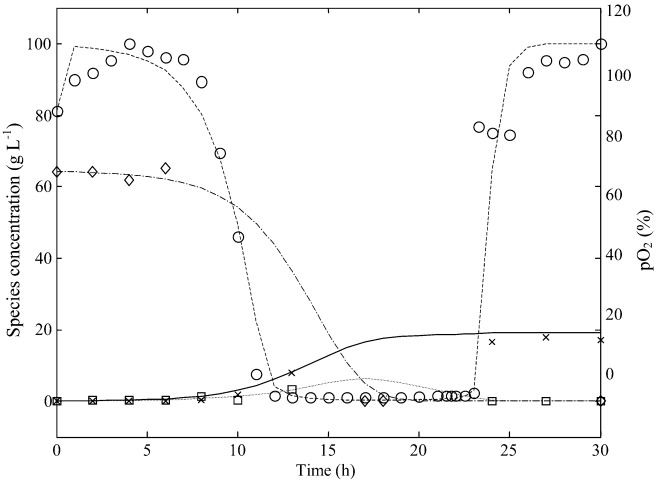


**Fig. 5** Experimental data (*symbols*) and model fittings (*lines*) for batch cultures at 42 °C: (x,—) biomass concentration (*X*); (◇,-·-·) substrate concentration (*S*); (□,····) ethanol concentration (*Et*); and (○,—) dissolved oxygen concentration ($pO_2$)

out specific runs. The second-order model for oxygen transport was able to represent accurately the dissolved oxygen concentration using the same estimated parameter, commonly denoted by $k_1a$, as in the usual first-order transport equation.

The perspectives for future work include studying the influence of pH on the model parameters, generalization of the multi-isothermal model to a non-isothermal model by using temperature-dependent parameters, optimization of the operating conditions, development of software sensors, and implementation of model-based control technologies.

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