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FERMENTATION, CELL CULTURE AND BIOENGINEERING

The modeling of ethanol production by *Kluyveromyces marxianus* using whey as substrate in continuous A-Stat bioreactors

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Abstract We investigated the kinetics of whey bioconversion into ethanol by *Kluyveromyces marxianus* in continuous bioreactors using the "accelerostat technique" (A-stat). Cultivations using free and Ca-alginate immobilized cells were evaluated using two different acceleration rates (a). The kinetic profiles of these systems were modeled using four different unstructured models, differing in the expressions for the specific growth (μ) and substrate consumption rates (r_s), taking into account substrate limitation and product inhibition. Experimental data showed that the dilution rate (D) directly affected cell physiology and metabolism. The specific growth rate followed the dilution rate ($\mu \approx D$) for the lowest acceleration rate ($a = 0.0015$ h^{-2}), condition in which the highest ethanol yield (0.52 g g^{-1}) was obtained. The highest acceleration rate ($a = 0.00667$ h^{-2}) led to a lower ethanol yield (0.40 g g^{-1}) in the system where free cells were used, whereas with immobilized cells ethanol yields increased by 23 % (0.49 g g^{-1}). Among the evaluated models, Monod and Levenspiel combined with Ghose and Tyagi models were found to be more appropriate for describing the kinetics of whey bioconversion into ethanol. These results may be useful in scaling up the process for ethanol production from whey.

Keywords Bioprocess modeling · Ethanol · *Kluyveromyces marxianus* · Continuous fermentation · A-stat control · Whey

List of symbols

a	Acceleration rate (h^{-2})
D	Dilution rate (h^{-1})
D_0	Initial dilution rate (h^{-1})
K_p	Product inhibition for growth (g L^{-1})
K_s	Saturation growth constant (g L^{-1})
m_s	Maintenance energy coefficient (g g^{-1} h^{-1})
P	Product concentration (g L^{-1})
P_{xmax}	Product concentration where microbial growth ceases (g L^{-1})
r_p	Product formation rate (g L^{-1} h^{-1})
r_x	Cell growth rate (g L^{-1} h^{-1})
r_s	Substrate consumption rate (g L^{-1} h^{-1})
S	Substrate concentration (g L^{-1})
S_i	Inlet substrate concentration (g L^{-1})
S_0	Outlet substrate concentration (g L^{-1})
t	Time (h)
X	Cell concentration (g L^{-1})
$Y_{P/S}$	Yield coefficient for product on substrate (g g^{-1})
$Y_{X/S}$	Yield coefficient for cells on substrate (g g^{-1})

Greek symbols

α	Growth-associated constant for product formation (g g^{-1})
β	Non-growth-associated constant for product formation (g g^{-1} h^{-1})
μ	Specific growth rate (h^{-1})
μ_{max}	Maximum specific growth rate (h^{-1})

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Introduction

Ethanol is the main biofuel on the worldwide market, and the knowledge of variables and parameters involved in the process of ethanol production is of foremost importance for the development of new production technologies [5, 43]. Ethanol can be obtained by fermentation of different raw materials, such as agro-industrial residues, and using several microorganisms [7, 14, 21, 32, 53]. Whey, which is a by-product (sometimes a waste material) of dairy industries, is an abundant and inexpensive substrate, rich in nutrients, which could be used for ethanol fermentation because of its unique composition: high lactose content (45–50 g L^{-1}), protein (6–8 g L^{-1}), lipids (4–5 g L^{-1}), and mineral salts (5–7 g L^{-1}) [14, 17, 23, 38, 46].

Different technologies have been studied to improve the ethanol fermentation process. Cell immobilization can contribute to bioprocess optimization because of the unique characteristics of entrapped biocatalysts, such as high productivity, small operational volume of bioreactors, cell protection against inhibitory products, and reduced contamination risks [24, 51]. When immobilization techniques are coupled with continuous operation of bioreactors, the product formation rate can be controlled and maintained at desired levels [15, 25, 51]. The continuous cultivation operated under acceleration stat (A-stat) control has advantages compared to classical continuous systems, because it enables the evaluation of cell physiology under a wide range of dilution rates in a short time [1–3, 39, 50]. This technique, first described by Paalme and Vilu [41], consists in a smooth change of the dilution rate, which increases linearly at a constant acceleration rate, allowing a gradual adaptation of cell metabolism to the changes in growth rates and environmental conditions, thus keeping the system under steady state condition, resulting in an unlimited number of steady state points [22, 40].

Several approaches for ethanol production in continuous cell-immobilized bioreactors have been investigated using different support materials and substrates [25, 27, 33, 35, 49, 54], but only recently researchers have reported the use of whey for ethanol production in immobilized continuous systems [6, 13, 14, 38]. Considering the ever-increasing demand for ethanol production, various mathematical models have been attempted in order to predict the effect of fermentation variables on cell growth, substrate utilization rate, and ethanol production rate [4, 15, 43], thus allowing for better processes design. Mathematical modeling allows studying the optimal operational conditions of a process, therefore being important to achieve efficient configurations for large-scale productions, and helping to understanding the interactions of several process parameters. However, only a few studies have so far been described using mathematical models for continuous ethanol production using whey as substrate [15, 42, 44, 48].

Unstructured phenomenological models are widely applied in fermentation processes because of their manageable complexity and limited number of variables required, giving the most fundamental observation concerning microbial metabolism [4, 28, 44]. The usual approach for the mathematical modeling of bioprocess is based on the Monod kinetics representing the cellular growth rate. Nevertheless, this mathematical model is the simplest type and, quite often, it must be modified and extended in order to adequately represent the process kinetic model. In this way, ethanol kinetic models are generally reported including terms considering the substrate limitation, substrate and/or product inhibition, and of cell death, factors that are known to affect the ethanol productivity [4, 15, 16, 19, 30, 31].

In this context, the aim of this research was to mathematically model the process of continuous ethanol production from whey in continuous A-stat bioreactors, using *K. marxianus* as biocatalyst. Several models were evaluated, taking into account different mathematical expressions and physiological considerations. In order to evaluate the adaptation of cell metabolism, two different acceleration rates were tested and different strategies of cultivation were carried out using free and immobilized cells.

Materials and methods

Yeasts, cell maintenance, and materials

Kluyveromyces marxianus CCT 4086 was provided by the Tropical Culture Collection of André Tosello Foundation (Campinas, Brazil). This strain was chosen because it has shown a high capacity for lactose bioconversion into ethanol as it has been observed in our recent publications [13, 14]. For cell maintenance, the strain was kept frozen at −20 °C in a 60 % cell suspension in glycerol, whereas for immediate use cells were kept on YEP-Lactose agar slants at 4 °C [11].

Unless otherwise indicated, all chemicals used in this research were purchased from Sigma-Aldrich (São Paulo, Brazil, or St. Louis, USA).

Immobilization of cells on Ca-alginate

Immobilization techniques followed procedures previously optimized and described in earlier works of our group [13]. *Kluyveromyces marxianus* CCT 4086 was grown in 2 L conical flasks containing 800 mL of YEP-lactose medium (yeast extract, 10 g L^{-1}; bactopeptone, 20 g L^{-1}; lactose, 20 g L^{-1}), pH 7.0, and incubated in an orbital shaker at 180 rpm, for 15 h at 30 °C in order to obtain

exponential-growth phase cells. At this point, cells were harvested by centrifugation (3000×*g*, 15 min), washed and resuspended in 10 mL of sterile distilled water at 4 °C. The cell suspension was added to a sterile solution of sodium alginate (40 g L^{-1}) to a final biomass concentration of 20 g L^{-1}. The mixture was immediately dripped through a 14 G needle (2.1 mm of diameter) using a peristaltic pump into a flask containing 0.1 M $CaCl_2$ sterile solution at 35 °C, and gently stirred for 30 min to stabilize the system. The beads were washed thrice with distilled water at 4 °C and kept in peptone water with 0.1 M $CaCl_2$ overnight. Finally, the beads were washed thrice with sterile distilled water at 4 °C and transferred into the bioreactors. Average alginate beads of 3.8 mm of diameter were obtained.

A-stat bioreactor cultivations

The medium used in the bioreactor experiments was reconstituted whey (70 g L^{-1} of whey powder; Elegê Laticínios S.A, Teutônia, Brazil), containing the equivalent of 56 g L^{-1} of lactose, 9 g L^{-1} of protein, and 5 g L^{-1} of minerals. Before sterilization of the medium (121 °C, 15 min), whey proteins were hydrolyzed using a commercial protease (Alcalase 2.4L, 2.4 UA-A/g, Novozymes, Araucária, Brazil) at pH 8.5, 55 °C, for 3 h, in order to avoid protein precipitation.

Bioreactor experiments were performed in glass column bioreactors (fluidized section column, 30 mm internal diameter, and 240 mm height), described elsewhere [13]. Cultivations were carried out using two different cell systems: free and immobilized cell cultures.

The inocula of the bioreactors with free cells were prepared by transferring isolated yeast colonies into 250 mL conical flasks containing 50 mL of YEP-lactose medium (yeast extract, 10 g L^{-1}; bactopeptone, 20 g L^{-1}; lactose, 20 g L^{-1}), pH 7.0, and incubated in an orbital shaker at 180 rpm for 12 h at 30 °C. Cell concentration was adjusted for the optical density of one unit (OD, 600 nm), which corresponds to 1.4 g L^{-1} of *K. marxianus* CCT 4086, and then added to the bioreactors as volume fractions of 10 % of the total culture volume of 355 mL.

For the immobilized cell bioreactors, the columns were filled with 85 mL of alginate beads, previously prepared as described above, and with 270 mL of fermentation medium to a final volume of 355 mL. Temperature of both bioreactors systems was controlled at 30 °C by circulating water from a thermostat bath into the bioreactor jacket. The medium was made to recirculate through the column by a peristaltic pump, promoting the homogenization and the fluidization of the system (upward flow).

Cultures were started in batch mode in order to allow for cell accumulation in the system and the feeding was started in the 24th h of cultivation for free cells, and in the 7th h for the immobilized cells culture. A smooth increase in dilution rate was set for two constant acceleration rates ($a = 0.0015$ h^{-2} and $a = 0.00667$ h^{-2}) and controlled by the pumps in the Biostat B (Braun Biotech International, Germany), in a way that the dilution rate (D) changed with time as follows:

$$D = D0 + at \quad (1)$$

where D_0 is the initial dilution rate (h^{-1}) and t is the running time (h).

A-stat systems were performed for 30 or 100 h, according to the different acceleration rates (0.00667 or 0.0015 h^{-2}) and the samples were taken at regular intervals (1 or 4 h).

Washout technique

Bioreactors operated with free cells cultures were prepared as described in the previous section. The feeding started at the end of the batch mode (24th h) and the cell culture was maintained in dilution rate (D) of 0.05 h^{-1} for 48 h to assure the steady state. Then, the dilution rate (D) was abruptly shifted to 0.4 h^{-1}, and the decrease of the biomass concentration was monitored for 4 h. The calculated μ_{max} was obtained by plotting the natural logarithm of the cell concentration ($\ln X$) against time (t), with the washout rate (μ_{max} -D) being calculated from the slope of the curve.

Analytical determinations

Samples were collected from the out stream of the A-stat continuous bioreactors, centrifuged (3000×*g*, 15 min) and the supernatant was analyzed for sugar and ethanol concentrations. Lactose and ethanol concentrations were determined by HPLC (Shimadzu, Japan) equipped with refractive index detector and a Bio-Rad HPX-87H column (300 mm × 7.8 mm) using 5 mM sulfuric acid as eluent at 45 °C, flow rate of 0.6 mL min^{-1} and sample volumes of 20 μL. The concentration of suspended cells was determined by absorbance at 600 nm and correlated with dry cell weight (g L^{-1}). The concentration of cells in the alginate spheres was analyzed dissolving five beads with a diameter of 3.8 mm into 10 mL of sodium citrate buffer 0.1 M (pH 6.2) and vortex stirred by 15 min to accelerate their breakup [12].

Mathematic models

The ethanol bioprocess involves complex interactions of physicochemical, biochemical, and genetic phenomena, thus some simplifications are necessary to describe the kinetics models. In this work, the models were based on the following assumptions: (1) the biotic phase was unique

Table 1 Expression for substrate consumption rate (r_s) and specific growth rate (μ) used in the proposed models

Model	Substrate consumption rate		Specific growth rate	
Mon	$rs = \frac{\mu}{YX/S}X$	(6)	$\mu = \mu\max\left(\frac{S}{Ks+S}\right)$	(8)
Mon-m_s	$rs = (\frac{\mu}{YX/S} + ms)X$	(7)	$\mu = \mu\max\left(\frac{S}{Ks+S}\right)$	(8)
Jrl	$rs = \frac{\mu}{YX/S}X$	(6)	$\mu = \mu\max\left(\frac{S}{Ks+S}\right)\left(\frac{Kp}{Kp+P}\right)$	(9)
Jrl-m_s	$rs = (\frac{\mu}{YX/S} + ms)X$	(7)	$\mu = \mu\max\left(\frac{S}{Ks+S}\right)\left(\frac{Kp}{Kp+P}\right)$	(9)
Hsw	$rs = \frac{\mu}{YX/S}X$	(6)	$\mu = \mu\max\left(\frac{S}{Ks+S}\right)(1 - KpP)$	(10)
Hsw-m_s	$rs = (\frac{\mu}{YX/S} + ms)X$	(7)	$\mu = \mu\max\left(\frac{S}{Ks+S}\right)(1 - KpP)$	(10)
LGT	$rs = \frac{\mu}{YX/S}X$	(6)	$\mu = \mu\max\left(\frac{S}{Ks+S}\right)(1 - \frac{P}{P\max})$	(11)
LGT-m_s	$rs = (\frac{\mu}{YX/S} + ms)X$	(7)	$\mu = \mu\max\left(\frac{S}{Ks+S}\right)(1 - \frac{P}{P\max})$	(11)

(unstructured model); (2) the cultivation medium in the bioreactor was well mixed; (3) the elemental composition of biomass did not significantly change over time; (4) there was no intracellular carbohydrate storage; (5) cells did not lose viability; (6) lactose was anaerobically metabolized; (7) nitrogen was not a substrate-limited parameter; and (8) the main product was the ethanol.

The mathematical models of the lactose bioconversion into ethanol in the continuous bioreactors using free cells were described using the combination of differential equations for cell growth, lactose consumption, and ethanol production. The mass balance was described correlating the cells (X), substrate (S), and product (P) concentrations with the kinetics rates (r_x, r_s, r_p), given by Eqs. (2)–(4):

$$\frac{dX}{dt} = -DX + rx \tag{2}$$

$$\frac{dS}{dt} = D(Si - S0) - rs \tag{3}$$

$$\frac{dP}{dt} = -DP + rp \tag{4}$$

where r_x, r_s, r_p were cell growth, substrate consumption, and ethanol formation rates, respectively. The remaining symbols are as defined in the nomenclature section.

The cell growth (r_x) is given by Eq. (5). The substrate consumption rate was described in two different modes: in the first, as a function of the specific growth rate (μ), according Eq. (6); and then, as a function of both the specific growth rate (μ) and of the maintenance energy coefficient (m_s), as described in Eq. (7).

$$rx = \mu X \tag{5}$$

$$rs = \frac{\mu}{YX/S}X \tag{6}$$

$$rs = \left(\frac{\mu}{YX/S} + ms\right)X. \tag{7}$$

In this work, four different kinetic models for specific growth rate described in the literature where analyzed: Monod (Eq. 8), Jerusalimsky (Eq. 9), Hinshelwood (Eq. 10), and the combination of the Levenspiel model with the Ghose and Tyagi model (Eq. 11).

$$\mu = \mu\max\left(\frac{S}{Ks + S}\right) \tag{8}$$

$$\mu = \mu\max\left(\frac{S}{Ks + S}\right)\left(\frac{Kp}{Kp + P}\right) \tag{9}$$

$$\mu = \mu\max\left(\frac{S}{Ks + S}\right)(1 - KpP) \tag{10}$$

$$\mu = \mu\max\left(\frac{S}{Ks + S}\right)\left(1 - \frac{P}{P\max}\right). \tag{11}$$

The ethanol formation rate (r_p) was described by the Luedeking and Piret expression [29], where α is the growth-associated constant for product formation and β is the non-growth-associated constant for product formation, as shown in Eq. (12).

$$rp = (\alpha\mu + \beta)X \tag{12}$$

where α and β are defined as:

$$\alpha = \frac{YP/S}{YX/S} \tag{13}$$

$$\beta = Y_{P/S}m_s \tag{14}$$

where $Y_{P/S}$ is defined as the yield coefficient for the product on substrate, and $Y_{X/S}$ as the yield coefficient for the cells on substrate.

The eight different models used in this work are the combination between the two different equations of substrate consumption rate (Eqs. (6), (7)) with the four equations proposed for specific growth rate (Eqs. (8)–(11)), as shown in Table 1.

The parameter estimation was carried out from the derivation of experimental data using the software EMSO (Environment for Modeling, Simulation and Optimization) [47], from the Flexible Polyhedron method [34].

Results and discussion

Continuous A-stat bioreactors using free and immobilized cells systems

The accelerostat technique (A-stat) was carried out using two different acceleration rates in order to study the kinetic behavior of *Kluyveromyces marxianus* CCT 4086 for the continuous ethanol production on whey for a wide range of dilution rates in a short time. The highest (0.00667 h^{-2}) and the lowest acceleration rates (0.0015 h^{-2}) were maintained constant up to the point of achieving a final dilution rate of 0.2 h^{-1}. The kinetics profile of biomass, lactose consumption, and ethanol production in the bioreactor with free cell culture are shown in Fig. 1. Similar culture behavior was observed for both acceleration rates tested. A phenomenon not totally elucidated, but most likely linked to a physiological shift was observed, in which a transition phase always occurred for the dilution rates in between 0.1 h^{-1} and 0.13–0.15 h^{-1}. Biomass curves showed a gradual decrease inversely to D, for both acceleration rates (Fig. 1a). The lactose consumption and ethanol production were almost constant until $D = 0.1$ h^{-1}. Thereafter, an abrupt increase in lactose concentration (from 1.9 to 17.2 g L^{-1} for $a = 0.00667$ h^{-2}, and from 0.0 to 20.6 g L^{-1} for $a = 0.0015$ h^{-2}), and a substantial decrease in ethanol concentration (from 20.2 to 12.8 g L^{-1} for $a = 0.00667$ h^{-2}, and from 25.8 to 13.3 g L^{-1} for $a = 0.0015$ h^{-2}) were observed for $D > 0.1$ h^{-1} (Fig. 1b, c). Despite these changes in concentrations, cell washout was not observed, and a second cell metabolism stabilization appears to be reached for D between 0.15 and 0.17 h^{-1}. Although some researchers reported the influence of dilution rate on the cell morphology, suggesting that even small changes in growth rate could result in significant transitions in morphology [18, 36], in this work, no changes in cell morphology were observed for dilution rates between 0.05 and 0.1 h^{-1}. The predominant elongated yeast morphology of *Klyuveromyces marxianus* CCT 4086 was verified. This observation was also reported by O' Shea and Walsh [36] using the same strain *K. marxianus* CCT 4086 (NRRLy2415) and for similar dilution rates.

The A-stat culture operated at the lowest acceleration rate (0.0015 h^{-2}), showed a similar behavior of a classical chemostat steady state, showing agreement between calculated specific growth rate (μ) and dilution rate (D) of the system (average deviation around 12 %, Fig. 2). The largest deviation of the calculated specific growth rate (μ) occurred under $D = 0.1$ h^{-1} (Fig. 2), which matches the abrupt increase in substrate concentration. For the highest

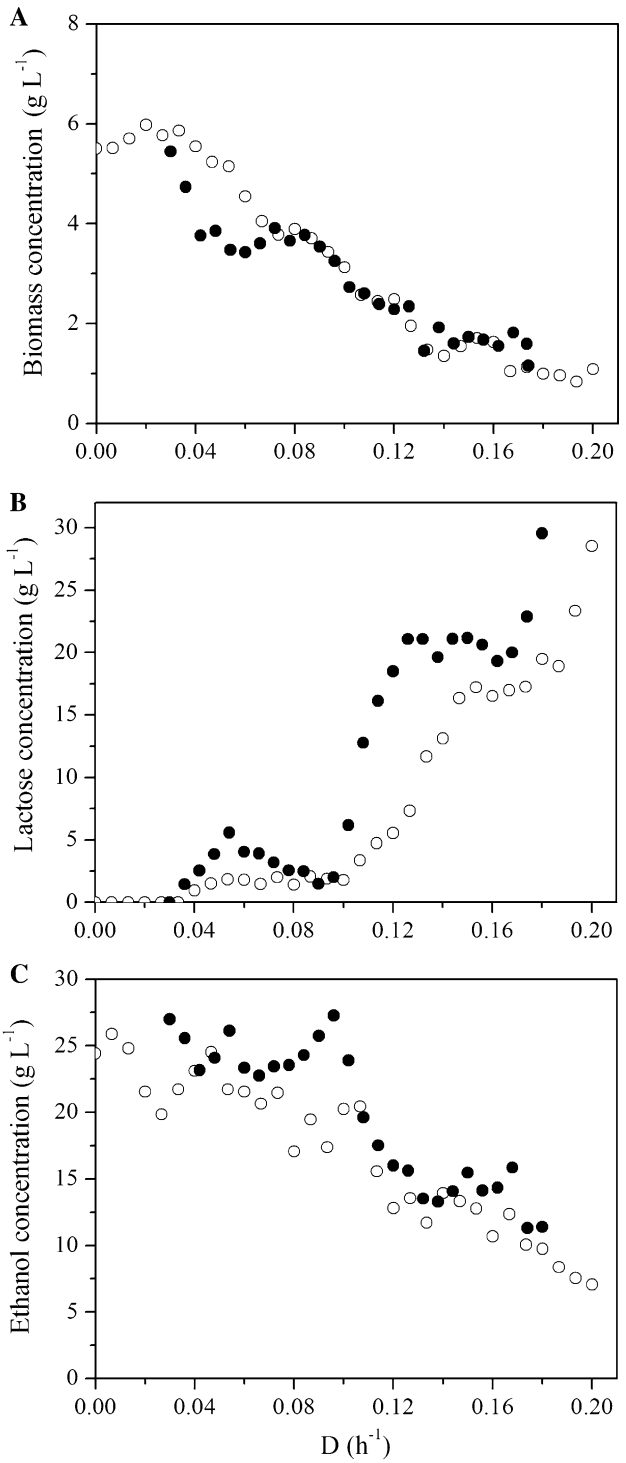

Fig. 1 Continuous culture kinetics of biomass (**a**), lactose consumption (**b**), and ethanol production (**c**) in the A-stat bioreactor using free cells culture of *K. marxianus* CCT 4086, at 30 °C, under different constant acceleration rates: 0.00667 h^{-2} (*open circle*), and 0.0015 h^{-2} (*filled circle*)

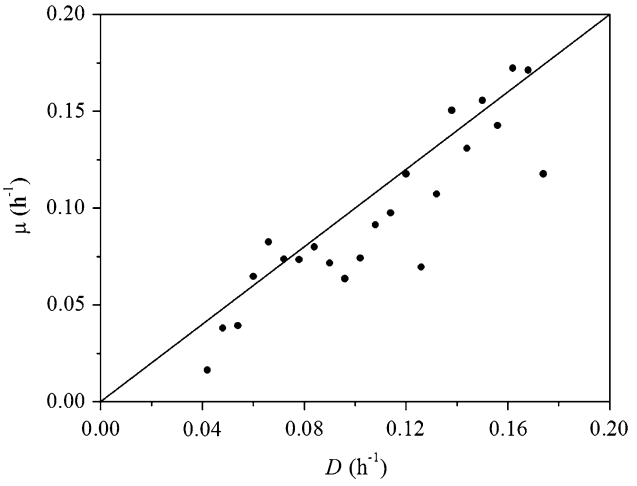

Fig. 2 Comparison between the calculated specific growth rate (μ) from experimental data and the gradual increase of dilution rate (D) in the A-stat system using free cells culture of *K. marxianus* CCT 4086

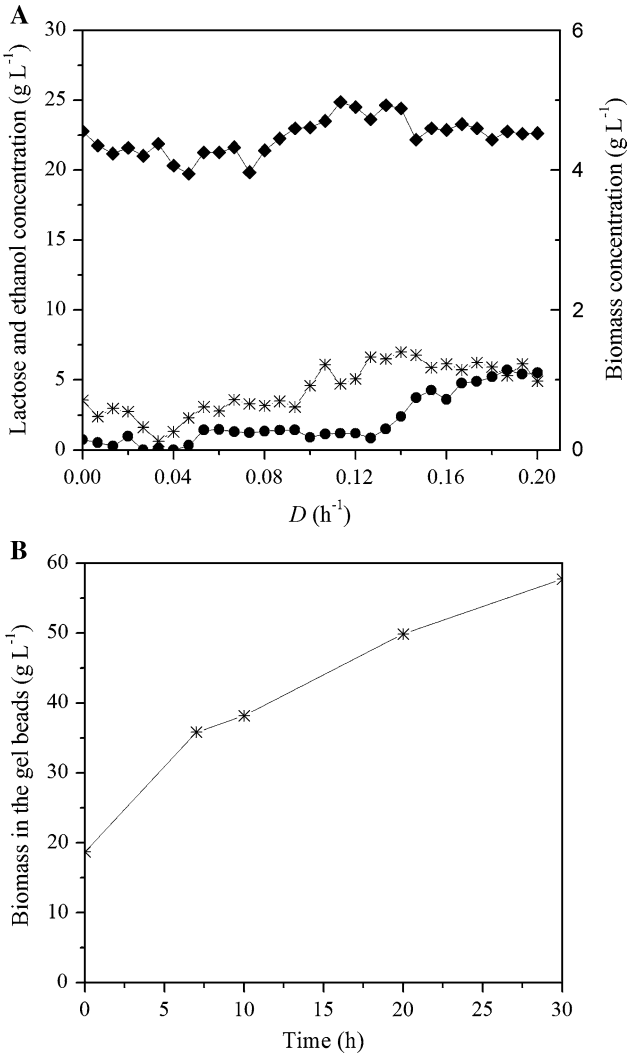

Fig. 3 Continuous culture kinetics profiles of suspended biomass, lactose consumption, and ethanol production **a** in the A-stat bioreactor using immobilized *K. marxianus* CCT 4086, at 30 °C, under constant acceleration rate of 0.00667 h^{-2}, and **b** the biomass in the gel beads. Lactose (*circle*), ethanol (*diamond*), biomass (*asterisk*)

acceleration rate (0.00667 h^{-2}), the equilibrium between D and μ was not reached, because this acceleration rate was higher than the necessary stabilization of the cell metabolism. This behavior showed great influence in the value of the yield coefficient for product on substrate ($Y_{P/S}$), which reached 0.40 g g^{-1} for the highest acceleration rate, and 0.52 g g^{-1} for the lowest acceleration rate. The effect of acceleration rates on the specific growth rate was reported in other researches, showing that lower acceleration rates lead to the approximation of steady state [1, 3, 22, 50]. Therefore, it is clear that the choice of acceleration rate is a critical step in A-stat systems. Some researchers suggested that the acceleration rate depends on maximum specific growth rate (μ_{max}) and it would be best operated in the range of 0.01–0.04 of μ_{max} [3, 22, 39]. Considering the μ_{max} of 0.15 h^{-1} calculated in this work, the acceleration rates used ($a = 0.00667$ and $a = 0.0015$ h^{-2}) are in agreement with these considerations.

The A-stat bioreactor with immobilized cell showed a different kinetic profile when compared with free cell culture (Fig. 3a). For this condition, the lactose and ethanol concentrations were practically constant and the transition phase was not observed. The ethanol concentration was kept at about 22.4 g L^{-1}, and a slow increase in lactose concentration was verified for dilution rates above 0.14 h^{-1}, increasing from 1.5 to 5.5 g L^{-1} in the final dilution rate (0.2 h^{-1}), which is much lower than the concentration observed in the free cells bioreactor (17.2 g L^{-1}). The leakage of cells from beads gradually increased with the dilution rate (Fig. 3a), and this is associated with the increase of cell concentration inside gel spheres (Fig. 3b). The high cell density in the bioreactor is an important characteristic of cell-immobilized systems, leading to several process advantages, such as high productivities, reduction of the contamination risks, and shorter times of cultivation [24]. In this study, these advantages were observed for the cell-immobilized bioreactor, which led to an increase of 23 % in the $Y_{P/S}$ when compared with the free cell culture for the same acceleration rate (0.00667 h^{-2}), reaching 0.49 g g^{-1}. The differences in kinetic profiles for free and immobilized cells bioreactors can be associated with the improvement of the biocatalyst stability, and with the changes in the physicochemical properties of the microenvironment and in the cell membrane, causing changes in permeability, in the membrane composition and the availability of the nutrients [20, 24, 51].

Table 2 Results for the estimation of parameters using the experimental data obtained in the A-stat system ($a = 0.00667\ h^{-2}$)

Model	μ_{max} (h^{-1})	K_s (g L^{-1})	K_p (g L^{-1})	P_{xmax} (g L^{-1})	$Y_{X/S}$ (g g^{-1})	m_s (g $g^{-1}h^{-1}$)	α (g g^{-1})	β (g $g^{-1}h^{-1}$)	R^2 X	R^2 S	R^2 P
Mon	0.12	19.3	–	–	0.019	–	15.5	0.003	0.97	0.94	0.91
Mon-m_s	0.16	23.6	–	–	0.027	0.27	11.0	0.080	0.98	0.94	0.91
Jrl	0.12	11.0	91.7	–	0.025	–	11.8	0.0009	0.97	0.94	0.91
Jrl-m_s	0.16	13.1	78.1	–	0.035	0.28	8.3	0.090	0.97	0.94	0.91
Hsw	0.12	17.4	0.0012	–	0.021	–	13.8	0.005	0.97	0.94	0.91
Hsw-m_s	0.16	17.5	0.0010	–	0.030	0.27	9.1	0.080	0.97	0.94	0.91
LGT	0.12	15.8	–	782.6	0.020	–	12.7	0.016	0.97	0.94	0.91
LGT-m_s	0.25	57.5	–	745.9	0.018	0.28	16.5	0.090	0.98	0.94	0.91

Parameter estimation of the continuous A-stat system

The A-stat system was applied to evaluate the kinetic parameters of fermentation by *K. marxianus* CCT 4086 on whey because it is a powerful technique for the quantitative study of cell physiology, being more informative than the conventional continuous system. The most important aspect of this technique is the need to calculate the macroscopic growth parameters (rates of consumption and formation) in conditions wherein biomass, substrate and product concentrations are constantly changing. When compared to other operational modes, such as batch culture, the values of the calculated parameters are much more precise because the changes in concentrations are much smaller throughout the cultivation [40].

Mathematically modeling this system can contribute to understand the interaction of the variables and parameters involved in the process, being an important tool to improve this bioprocess, from the study of the optimal operational conditions and subsequently achieve efficient configurations for scaling up [8, 9]. In this work, we evaluated four different kinetic models. The models of Jerusalimsky (Eq. 9), Hinshelwood (Eq. 10), and the combination of the Levenspiel model with Ghose and Tyagi model (Eq. 11) take into consideration an additional term compared with the Monod model, which is the product inhibition term, represented by K_p or P_{xmax}. This term is important because of high ethanol concentrations may present a negative effect on the specific growth rate, substrate consumption, and product formation rates [4, 52].

The values of the parameters estimated based on experimental data of the free cell bioreactor using an acceleration rate of 0.00667 h^{-2} are shown in Table 2. A satisfactory coefficient of determination (R^2) was obtained for all sets of the ethanol kinetic models tested, corresponding to 0.98, 0.94 and 0.91 for biomass, lactose, and ethanol, respectively, indicating a suitable fit of the different models predicted from the experimental data. The consideration of maintenance energy coefficient (m_s) term on expressions of substrate consumption rates (r_s) increased the values of parameters such as the maximum specific growth rate (μ_{max}), yield coefficient for cells from substrate ($Y_{X/S}$), and growth-associated constant for product formation (α) of the Luedeking and Piret expression. The m_s is the energy utilized for the cell vital process, and an increase in μ_{max} and $Y_{X/S}$ means that more substrate is being consumed for biomass generation and maintenance and less being converted to ethanol, which also explains the decrease in the α value. The values of K_p and P_{xmax} (Table 2) suggest that product inhibition was not in place, because the expression that represents this effect (second term of the μ expressions) approached the value of 1, which indicates a negligible effect. Consequently, this resulted in the similarity among the curves of the different predicted models (Fig. 4).

Although the coefficients of determination (R^2) for $a = 0.0015\ h^{-2}$ (Table 3) were lower than for $a = 0.00667\ h^{-2}$, an ideal cell metabolism was observed for the first condition, considering the goodness fit of μ with D. Furthermore, the values of $Y_{P/S}$ calculated from different kinetic models showed a satisfactory approximation of the value experimentally determined, differing by an order of 1.8 to 4.8 % of the data value. The estimated μ_{max} values from the models using the lowest acceleration rate (Table 3) were higher than the expected calculated value from the washout technique (0.15 h^{-1}). Other authors also reported higher μ_{max} obtained using the A-stat technique as compared with values obtained in the washout [2, 40], and these differences might be a consequence of the difficulty to fit model curves in the region of metabolic imbalance. Again, for the acceleration rate of $a = 0.0015\ h^{-2}$, the values of K_p and P_{xmax} (Table 3), indicate that there was no cell growth inhibition by product, resulting in convergent curves for all different models evaluated (Fig. 5). The kinetic models allowed the observation that the substrate consumption rate (r_s) and product formation rate (r_p) increased gradually until D reached 0.1 h^{-1}, and after this, in the transition region (0.1 $h^{-1} < D < 0.15\ h^{-1}$), a smaller increase of r_p was obtained. This behavior might indicate

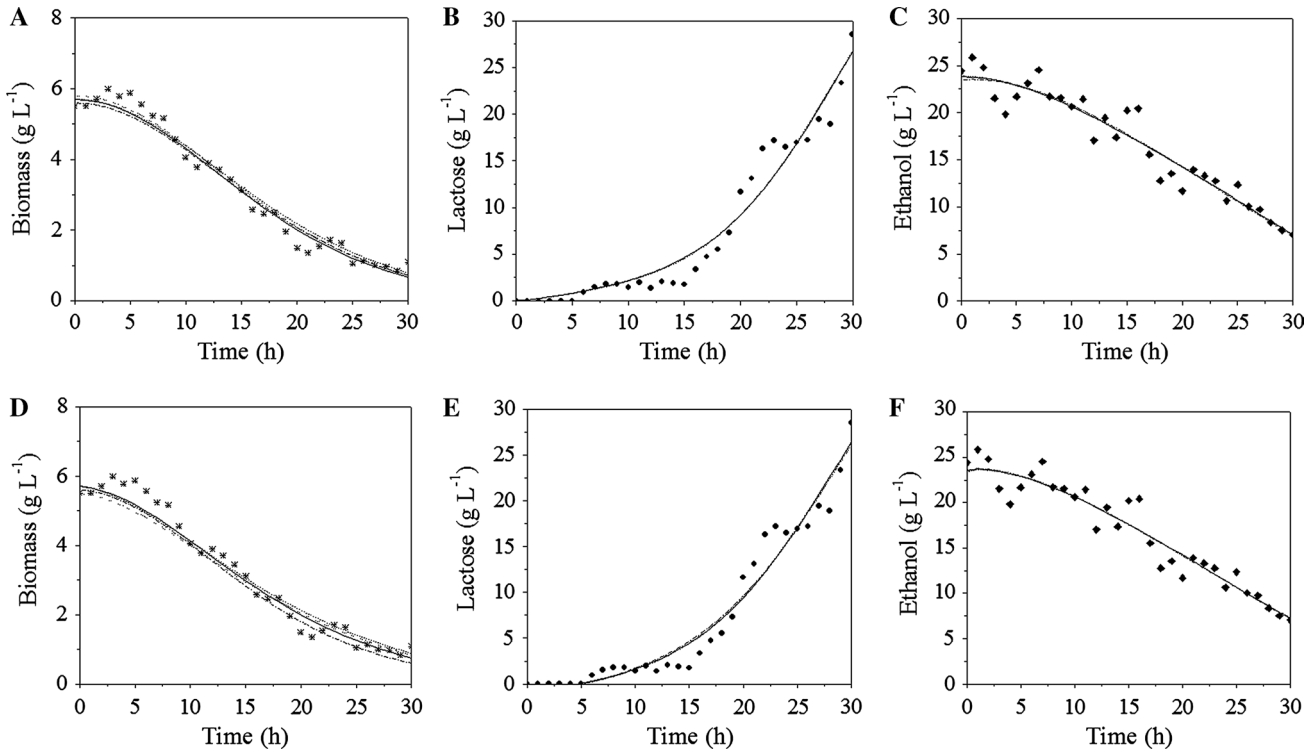

Fig. 4 Comparison of the experimental data with the models prediction curves of biomass (**a**), lactose (**b**), and ethanol (**c**), not taking into account the m_s in the r_s expression (Mon, Jrl, Hsw, LGT), and the same variables (**d**, **e**, **f**) considering m_s in r_s (Mon-m_s, Jrl-m_s, Hsw-m_s, LGT-m_s), in the A-stat system ($a = 0.00667\ h^{-2}$). Experimental data: lactose (*circle*); ethanol (*diamond*); biomass (*asterisk*). Models: Mon, Mon-m_s (——);Jrl, Jrl-m_s (- - - - -); Hsw, Hsw-m_s (··· ···); and LGT, LGT-m_s (--- · ---)

Table 3 Results for the estimation of parameters using the experimental data obtained in the A-stat system ($a = 0.0015\ h^{-2}$)

Model	μ_{max} (h^{-1})	K_s (g L^{-1})	K_p (g L^{-1})	P_{xmax} (g L^{-1})	$Y_{X/S}$ (g g^{-1})	m_s (g $g^{-1}h^{-1}$)	α (g g^{-1})	β (g $g^{-1}h^{-1}$)	R^2 X	R^2 S	R^2 P
Mon	0.30	23.3	–	–	0.05	–	9.7	0.014	0.75	0.80	0.81
Mon-m_s	0.26	18.5	–	–	0.06	0.015	8.2	0.015	0.78	0.79	0.80
Jrl	0.27	14.6	119.6	–	0.07	–	7.3	0.040	0.74	0.78	0.82
Jrl-m_s	0.28	15.5	106.4	–	0.05	0.0001	9.9	0.004	0.73	0.78	0.80
Hsw	0.35	27.4	0.0053	–	0.07	–	6.9	0.033	0.81	0.78	0.80
Hsw-m_s	0.25	15.8	0.0026	–	0.04	0.42	14.6	0.20	0.50	0.78	0.79
LGT	0.26	16.7	–	999.1	0.08	–	6.3	0.02	0.84	0.78	0.80
LGT-m_s	0.25	16.4	–	996.4	0.06	0.02	8.4	0.003	0.76	0.77	0.79

that occurs a change in the carbon flux (metabolism imbalance region), in which the substrate was mainly metabolized to keep cell vital process maintenance to overcome the stressing environmental condition, in detriment of ethanol formation, as it was described above.

Among the evaluated models, the Monod (Mon-m_s, Eq. (8)) and the Levenspiel combined with Ghose and Tyagi models (Eq. (11)) produced the best representations of the biological phenomenon. A good agreement of the estimated parameters with experimental data and a considerable fitting quality was obtained for these two models. The parameter values are consistent with other reports using different strains of *Kluyveromyces marxianus* grown in whey. For instance, K_s values of 16.0, 20, and 22.6 g L^{-1} were observed for *K. marxianus* MTCC 1288, *K. marxianus* CBS 6556, and *K. marxianus* CBS 397 strains, respectively, using unstructured modified Monod model [55], multi-route unstructured model [28], and a biochemically structured model [43]. The K_s values in this work indicate that the cell growth was not limited by

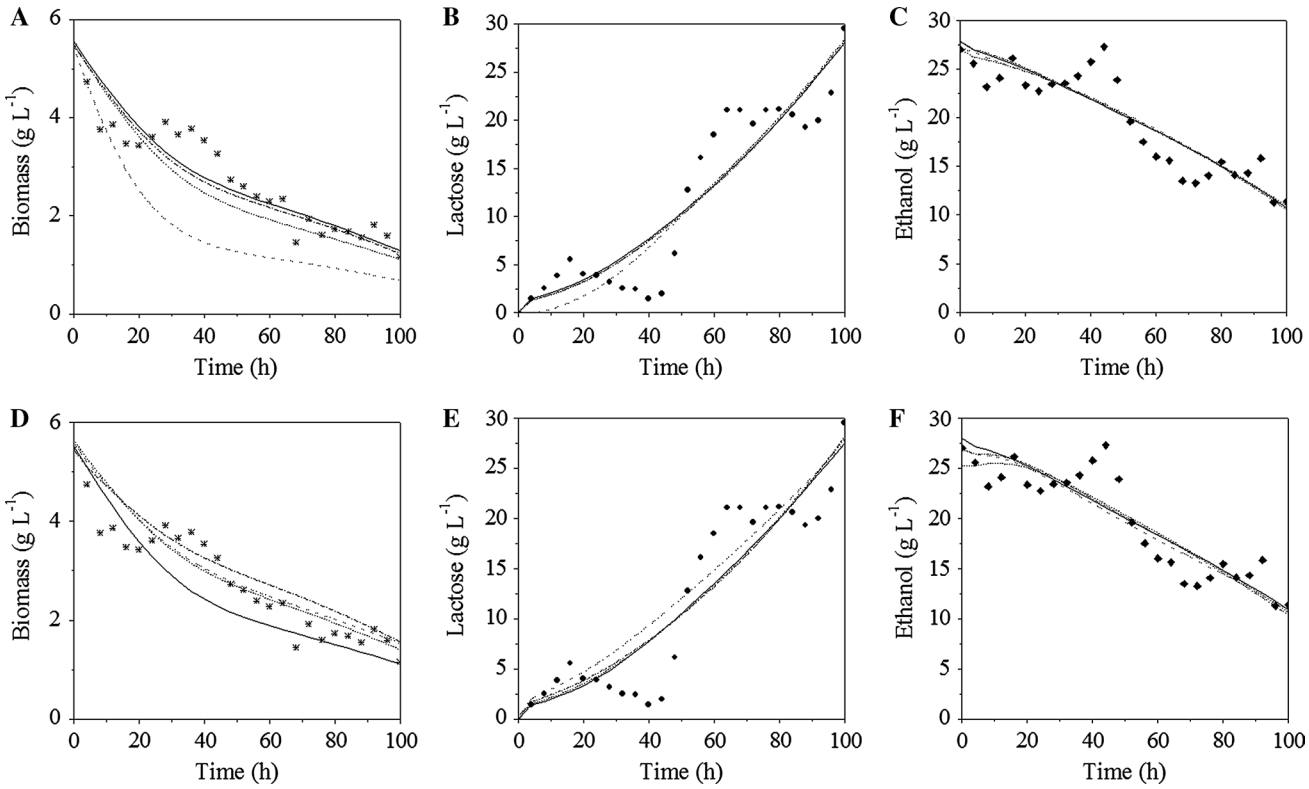

Fig. 5 Comparison of the experimental data with the models prediction curves of biomass (**a**), lactose (**b**), and ethanol (**c**) not taking into account the m_s in r_s expression (Mon, Jrl, Hsw, LGT), and the same variables (**d**, **e**, **f**) considering m_s in r_s (Mon-m_s, Jrl-m_s, Hsw-m_s, LGT-m_s), in the A-stat system ($a = 0.0015\ h^{-2}$). Experimental data: lactose (*circle*), ethanol (*diamond*), biomass (*asterisk*). Models: Mon, Mon-m_s (——); Jrl, Jrl-m_s (------); Hsw, Hsw-m_s (··· ···); and LGT, LGT-m_s (-·-·- -·-·-)

substrate concentration, constant defined as the sugar concentration when μ is half of μ_{max}. The μ_{max} value obtained here is consistent with values reported in the literature, varying from 0.14 to 0.4 h^{-1} depending on the different models used and strain of *Kluyveromyces*. For instance, the Riccati kinetic equation produced a μ_{max} of 0.14 h^{-1} for *K. fragilis* CECT 1123; the modified Monod model a μ_{max} of 0.4 h^{-1} for *K. marxianus* MTCC 1288; and the structured biochemically model, a μ_{max} of 0.17 h^{-1} for *K. marxianus* CBS 397 [42, 43, 45, 55]. However, lower values were reported in the studies by Ghaly and El-Taweel [15], and by Ozmihci and Kargi [37], who showed a lower μ_{max}, ranging from 0.051 to 0.094 h^{-1}, respectively, both applying the modified Monod model. These differences might be attributed to the substantial degree of intraspecific polymorphism in *K. marxianus* strains, which can result in different metabolic diversities [10, 26].

Although the consistence of values obtained for the parameters in describing the biological phenomenon, especially when using the two best models, modeling difficulties were found to describe the metabolic imbalance region, thus, improvements are needed in order to describe the cell metabolic imbalance, a phenomenon not yet totally understood by us. Almost certainly, proteomics and metabolomics of cells along the entire time of cultures might be necessary to fully understand the transition in physiology obtained under the conditions of this work. It would also be interesting to compare results obtained for *K. marxianus* with those obtained for other yeast species growing under similar conditions.

Conclusions

The A-stat technique proved to be a powerful tool for the physiological study of *K. marxianus* in continuous cultures for ethanol production. This strategy allowed the knowledge of the cellular metabolism regarding sugar consumption, product formation, and cell growth on a wide range of dilution rates, accurately predicting the cell behavior on different environmental changes. The acceleration rates directly influenced the cell adaptation and the equilibrium between μ and D was observed for the lowest acceleration, condition in which the highest ethanol yield was obtained. The cell immobilization technique improved the efficiency of the system, increasing the ethanol yield. The lowest

acceleration rate led to a better description of biological phenomenon using the models. The predictive curves of the different models showed a considerable fit for the experimental curves of biomass, substrate and product concentrations, but improvements on the models are needed in order to precisely describe the metabolic imbalance region with better accuracy.

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