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Kinetics of Bioethanol Production from Lactose Converted by *Kluyveromyces Marxianus*

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A kinetic model for ethanol fermentation of lactose using yeast *Kluyveromyces marxianus* DSMZ 5422 is proposed. The model consists of a set of differential equations which account for substrate consumption, ethanol production and biomass production. In the model, it is assumed that alcoholic fermentation is inhibited by ethanol itself and that a different metabolic pathway is set at certain ethanol concentrations. Furthermore, lactose consumption is hypothesized to be associated to biomass growth. The model proposed is able to correctly describe lactose consumption and ethanol production. Also the main trend of biomass variation is satisfactorily correlated. The model with the set of the regression parameters is validated for its predictive ability in a larger scale batch reactor experiment.

1. Introduction

Cheese whey disposal is a difficult task due to the high content of organic pollutant (BOD 40-50 g L^{-1} and COD 60-80 g L^{-1}) in whey. A possible approach to recover some value from cheese whey before disposal is separation and recovery of proteins and the subsequent the biotransformation of the residual lactose content into ethanol by means of alcoholic fermentation. There exist numerous yeasts strains that assimilate lactose aerobically. However, only few yeasts strains are able to convert lactose to ethanol in fermentation processes. *Kluyveromyces lactis*, *Kluyveromyces marxianus* and *Candida pseudotropicalis* are some of these strains (Breunig, 2006). In fact, *Kluyveromyces marxianus,* is one of the lactose assimilating yeasts that has shown both a good ability to ferment lactose and a certain resistance to temperature variations. From an overview of the literature reported by Guimarães *et al.* (2010), it appears that lactose fermentation with *Kluyveromyces marxianus* determines final ethanol concentrations that are generally lower than 5%, unless fed lactose concentrations are higher than 10%. Therefore, in view of a possible industrial use of *Kluyveromyces marxianus,* the low values of final ethanol concentrations make it necessary to optimize the lactose to ethanol conversion stage as much as possible. In this respect, process scale up requires the availability of a reliable mathematical model. Experimental results available in the literature seem to indicate that, in order to describe adequately the batch and continuous fermentation with *Kluyveromyces marxianus*, mathematical models should be able to reproduce some common features found in the observation of batch experimental results reported in the literature. A part from ethanol inhibition, which is expected in fermentation processes (Zafar and Owais, 2006), many authors report substrate inhibition at substrate concentrations higher than about 125 g L^{-1} with batch, (Parrondo *et al.*, 2009) fed batch (Ozmihci and Kargi, 2007a) and continuous operation, (Ozmihci and Kargi, 2007b);. Limitation to high substrate operation, however, is not significant working with lactose concentrations close to values found in fresh whey (about 40 g L^{-1}) and therefore often it was not observed because not much higher substrate concentration were used in the experiments. Another specific feature found in batch fermentation with *Kluyveromyces marxianus* regards the microbial growth that is not simply associated to lactose consumption. In fact, experiments showed that often microbial growth stops or significantly reduces at intermediate values of lactose conversion, generally corresponding to the attainment of the final value of ethanol concentration such as 30 g L^{-1} after about 24 h (Parrondo *et al.*, 2009) or 25 g L^{-1} after about 18 h (Sansonetti *et al.* 2009). Similar results were also observed by Joshi *et al.* (2011) who carried out experiments with *Kluyveromyces marxianus* DSMZ 5422 in incubated flasks at

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37°C, using 50, 100 and 150 g L^{-1} and two different volumes of inoculum suspensions, 1 and 5 %. Batch experiments were carried out for times up to 60 h. In all these experiments the final ethanol concentration appears to be around 40 g L^{-1}, independent of the initial lactose concentration and biomass inoculum. In all the cases reported, the final ethanol concentration is found to be reached for batch times between 10 and 20 h, while an almost complete lactose conversion required at least times between 30 and 50 h.
In the present work a simple unstructured model will be presented accounting for the above reported conversion features. The objective is to find a single set of model parameters to describe all the experiments of Joshi *et al.* (2011). Furthermore the effectiveness of the model to scale up operation will be verified by using the same set of parameters to predict new experimental results found on a laboratory scale batch fermenter.

2. Model description

The proposed model consists of a set of three differential equations which describe the variation over time of substrate of ethanol and microorganism. It has been assumed that the ethanol production as observed, is limited to the attainment of a threshold value of the ethanol, concentration. Therefore two different kinetics occur with time. Namely an alcoholic fermentation at low ethanol concentration and an non-alcoholic fermentation at high ethanol concentrations are respectively assumed. With reference to the transition between the two regimes, the switch to non-alcoholic fermentation is described by an activation factor with an Arrhenius type of expression:

$$\alpha = \exp\left[\left(E - E_{max}\right) K_{IE}\right] \tag{1}$$

where E is the ethanol concentration, E_{max} is the maximum attainable ethanol concentration and K_{IE} is a constant which determines how close to E_{max} the inhibition effect become significant. The lactose consumption is assumed to be described by a saturation kind of kinetics of the Monod type in which the frequency factor changes according to the ethanol concentration:

$$\frac{dL}{dt} = \frac{\left[K_1(1-\alpha) + K_2\alpha\right]L}{K_M + L} X \tag{2}$$

where L is the lactose concentration, t is time, X is the biomass concentration, K_1 is the first order kinetic constant for lactose consumption during alcoholic fermentation, K_2 is the first order kinetic constant for lactose consumption during non-alcoholic fermentation and K_M the Monod constant. Ethanol production is due to alcoholic fermentation only and it is assumed to be proportional to lactose consumption during that phase:

$$\frac{dE}{dt} = \frac{Y_E K_1 L}{K_M + L} X\left(1-\alpha\right) \tag{3}$$

where Y_E is the yield of ethanol from lactose due to alcoholic fermentation. The biomass growth it is considered to be proportional to lactose consumption during the whole process:

$$\frac{dX}{dt} = \frac{Y_X\left[K_1(1-\alpha) + K_2\alpha\right]L}{K_M + L} X \tag{4}$$

where Y_X is the is the yield of biomass from lactose.

3. Materials and procedures

3.1 Yeast

The microorganism used in this work was a *Kluyveromyces marxianus* DSMZ 5422 that was obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ). This microorganism has an optimal thermal tolerance and a good capacity of lactose fermentation. It had been maintained frozen at -80 °C in 20% glycerol.

3.2 Batch fermentation

The inoculum of the microorganism was prepared according to Kargi and Ozmihci (2007) by using an inoculum medium containing lactose (50 g L^{-1}), yeast extract (5 g L^{-1}), peptone (5 g L^{-1}), NH_4Cl (2 g L^{-1}), KH_2PO_4 (1 g L^{-1}), $MgSO_4 \cdot 7H_2O$ (0.3 g L^{-1}), Na-thioglycolate (200 mg L^{-1}). The culture medium for the

fermentation experiment was prepared by dissolving spray dried cheese whey powder in sterile distilled water. Two different cheese whey powder concentrations of 50 and 100 g L^{-1} were tested.
The batch fermentation was carried out in a bench top fermenter BIOSTAT® B plus within a 2 L jacketed jar. The cheese whey powder solution was autoclaved at 121 °C for 15 min prior to the experiment. The sterile medium was centrifuged at 9000 rpm to remove coagulated protein. The pH of the medium was adjusted to pH 5 using 0.1 M H_2SO_4 solution. The batch experiments were started using 5% (v/v) inoculum for 48 hrs.
The samples were removed from the reactor periodically and centrifuged at 6500 rpm to remove solids from the liquid media. Total reducing sugar concentrations were measured by using the phenol-acid method (Dubois *et al.*, 1956). Ethanol concentration was measured using dichromate method (Williams and Resse, 1950).

4. Results and discussion

The model in Section 2 was integrated in an Excel spread sheet by the application of the Euler method. It was fitted on the data reported by Joshi *et al.* (2011) by minimizing, with the internal Excel Solver add-in (generalized reduced gradient method), the sum of square errors calculated between experiments and model prediction for ethanol, lactose and biomass The errors of this latter parameter were multiplied by 10 to account for the difference in the magnitude order between biomass concentration and those of lactose and ethanol. Actually this difference of magnitude order is larger than one, however, a lesser weight of the error on the biomass is left because of the much higher scatter in the experimental error experienced with this parameter. On the other hand, biomass appears in the differential mass balance equations of all the three species considered and, therefore, the search of the best model parameter values for biomass is in any case sensitive to the experimental values of all species. The Monod constant K_M had been experimentally determined by Joshi (2011) to be 610 g L^{-1}. This value is in agreement with other evaluations found in the literature (Ozmihci and Kargi, 2007a) and therefore it was not considered as a parameter to be optimized. Also the parameter K_{IE} was not included in the regression process but it was set to the value of 0.05 g^{-1} L which was a reasonable compromise to ensure a smooth and numerically stable, but rapid, change between alcoholic and non-alcoholic fermentation.
Two different cases were considered for the search of the optimized model parameters:

I) In the first case the parameter K_2 was forcedly set to be equal to K_1. This assumption corresponds to the hypothesis that the stop in ethanol fermentation does not bring any change in the biomass growth and lactose consumption.
II) In the second case K_2 was left free to change and therefore it is assumed that the onset of non-alcoholic fermentation determines a change in the lactose consumption and a proportional change in the biomass growth.

The best fitting model parameters for the two cases are reported in Table 1. Comparison between model and experiments is reported in Figures 1 and 2 for cases I) and II) respectively.
Inspection of Figure 1 indicates that case I) of the model optimization is able to satisfactorily describe ethanol production, but not lactose consumption and biomass growth. A part from not being able to describe the trends of both these two species, the model appears inadequate also in terms of conversion times and in terms of final lactose and biomass concentration. It has to be noted that without constrains on Y_E the best fitting procedure produced an unrealistic value of this parameter of 1.4. Therefore the regression aof parameter was repeated by limiting the maximum value of the ethanol yield to 1.
Inspection of Figure 2 shows a much better ability of the model optimized in case II) to reproduce the biomass growth. In particular the adoption of the reaction rate change in the switch between alcoholic and non-alcoholic fermentation produces a significant improvement of the ability of the model to describe the

Table 1: Model parameters after regression on experimental data

Calculation case	K_1 (h^{-1})	K_2 (h^{-1})	Y_E (-)	Y_X (-)	K_{IE} (g^{-1} L)	E_{max} (g L^{-1})	K_M (g L^{-1})
I)	150	150*	1.0†	0.013	0.05**	34	610‡
II)	100	10	0.7	0.030	0.05**	37	610‡

**) According to case I) hypothesis $K_2 = K_1$.*
***) Defined according to numerical stability considerations.*
†) For case I) the further limit $Y_E \leq 1$ was required.
‡) Not obtained with the regression procedure. Evaluated experimentally by Joshi (2011).

The predictive ability of the model with the sets of regressed parameters was assessed on the results of

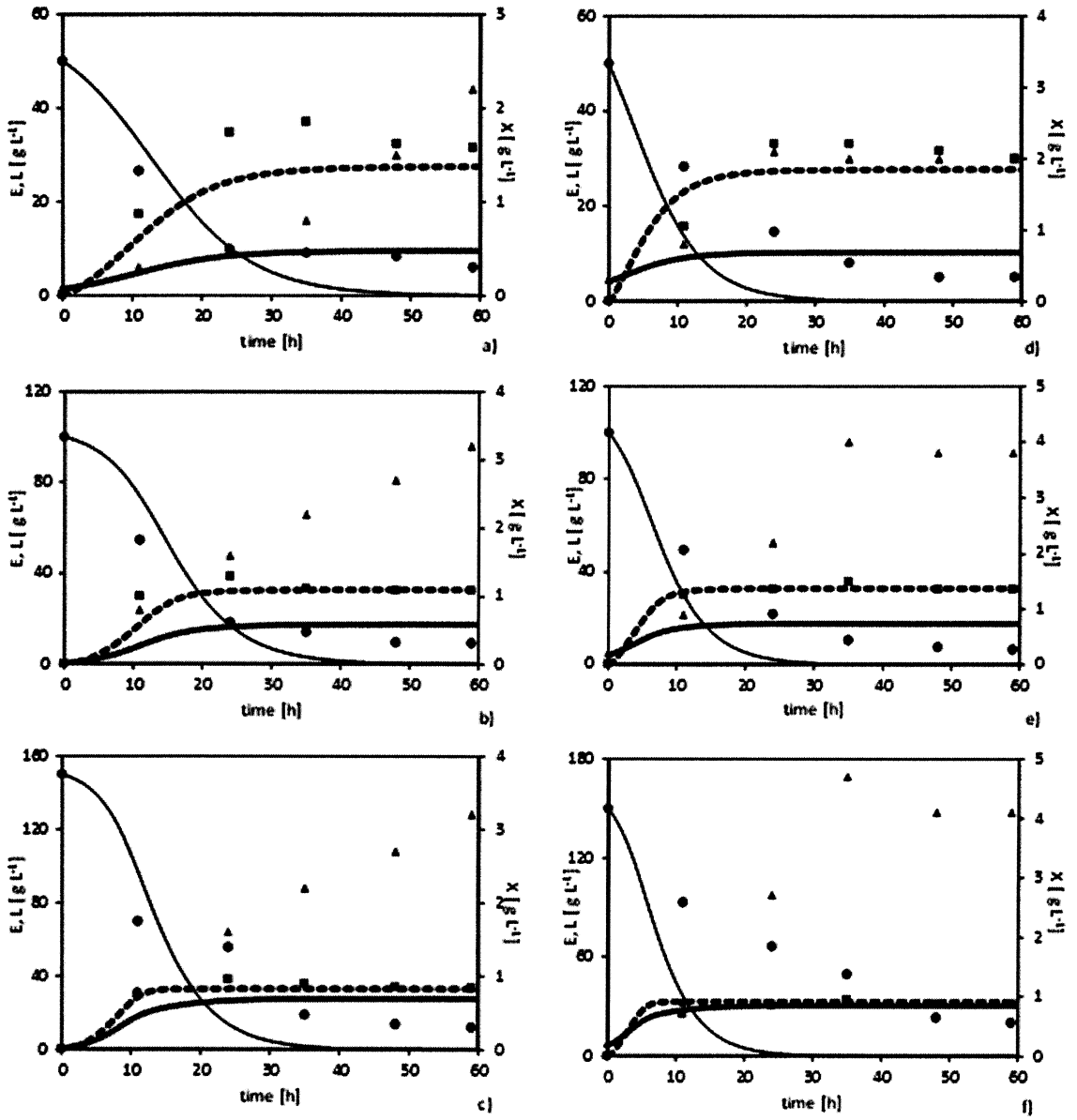

Figure 1: Model regression on experimental data by Joshi et al. (2011) with constrains of case I) (K_2=K_1). Data were obtained at different initial inoculum amounts, i, and substrate concentrations, s: a) s=50 g L^{-1}, i=1 %; b) s=100 g L^{-1}, i=1 %; c)s=150 g L^{-1}, i=1 %; d)s=50 g L^{-1}, i=5 %; e)s=100 g L^{-1}, i=5 %; f)s=150 g L^{-1}, i=5 %. Experiments:●,lactose; ■, ethanol; ▲, biomass. Model: ———, *lactose;* − − −,*ethanol;* ·····, *biomass.*

the batch fermentation of cheese whey for initial substrate concentration of 50 and 100g L^{-1}. Experimental and model results are both reported in Figure 3. In these experiments, batch cheese whey fermentation was carried out in a more controlled environment especially for parameters such as the dissolved oxygen concentration (pO_2, the level was left in the range 0.1-0.2%). Also the rate and extent of ethanol formation or sugar utilization was found increased with increasing cheese whey concentrations. Substrate conversions of 92 and 80% for 50 and 100 g L^{-1} initial cheese whey concentrations, respectively, indicate that, with increasing concentration of cheese whey, lactose conversion tends to decrease. Final ethanol concentrations in both the experiments did not show significant differences and were close to the value of about 40 g L^{-1} already found in flask fermentation. As shown in Figure 3 using the second set of parameters, the model is able to predict satisfactorily ethanol and lactose concentrations at least in the

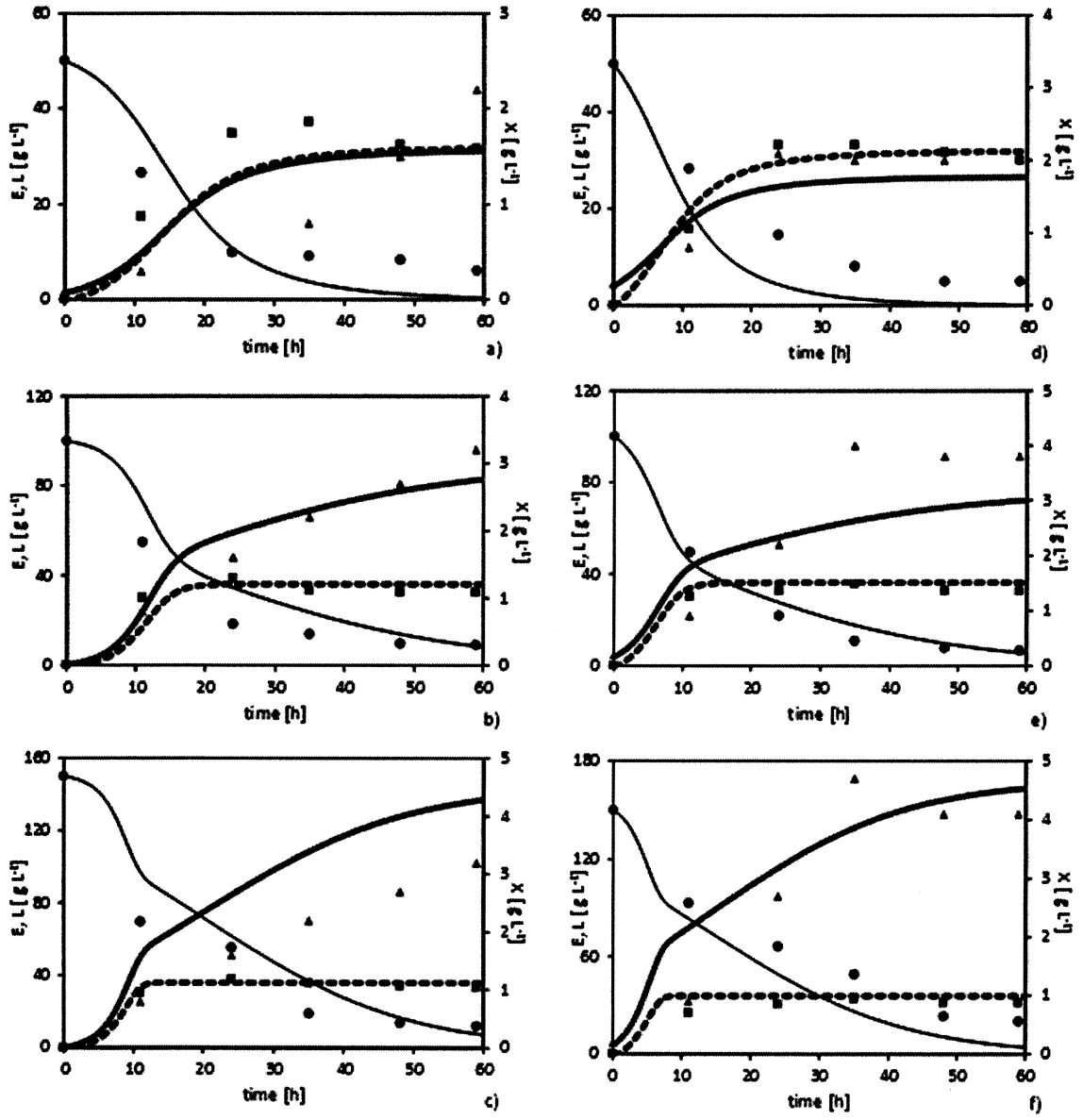

Figure 2: Model regression on experimental data by Joshi et al. (2011) for case II) (no constrains on K_2).Data were obtained at different initial inoculums amounts, i, and substrate concentrations, s: a) s=50 g L^{-1}, i=1 %; b) s=100 g L^{-1}, i=1 %; c)s=150 g L^{-1}, i=1 %; d)s=50 g L^{-1}, i=5 %; e)s=100 g L^{-1}, i=5 %; f)s=150 g L^{-1}, i=5 %. Experiments:●,lactose; ■, ethanol; ▲, biomass. Model: ———, lactose; – – –,ethanol; ·····, biomass.

concentrations in both the experiments did not show significant differences and were close to the value of about 40 g L^{-1} already found in flask fermentation. As shown in Figure 3 using the second set of parameters, the model is able to predict satisfactorily ethanol and lactose concentrations at least in the basic features of their change. No reliable data, instead, is available on biomass concentration that is shown only in the model predictions.

5. Conclusions

A simple kinetic model accounting for a first alcoholic fermentation stage inhibited by ethanol and a further non-alcoholic fermentation stage was proposed to describe lactose conversion operated by *Kluyveromyces marxianus* DSMZ 5422. Lactose consumption is growth associated, independent of the kind of fermentation. Ethanol production, instead is growth associated only during alcoholic fermentation.

Table 2: Initial conditions and main experimental results for a batch reactor.

Inoculum volume (%)	Initial lactose ($g\ L^{-1}$)	Final ethanol (%v/v)	Lactose conversion (%)	Ethanol yield ($g_{EtOH}/g_{lactose}$)
5	37	2.8	91.9	0.59
5	75	3.0	80.0	0.31

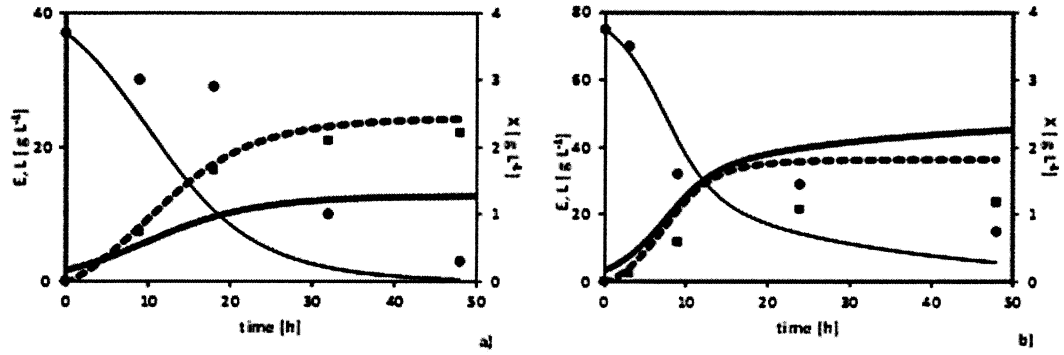

Figure 3: Batch experimental data and model predictions with regressed parameter for case II). Initial lactose concentration: a) 37 g L^{-1}; b) 75 g L^{-1}. Experiments: ●,lactose; ■,ethanol. Model: ———, lactose; – – –,ethanol; ·····, biomass.

The model with a single lactose kinetics is not able to satisfactorily describe experimental results. Instead, assuming different lactose consumption rates in the two fermentation stages, it is possible to fit all experimental data proposed by Joshi *et al.* (2011) and satisfactorily predict experimental results obtained on a bench top fermenter. Further studies are required to better understand what happens in the microbial system when the limiting ethanol concentration is reached. Nevertheless, conversion of lactose in non-alcoholic fermentation considerably reduces the overall ethanol yield of the process. Therefore, an accurate ethanol concentration control is required to enhance the ethanol production of the process.

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