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Dynamics of ethanol production from whey and whey permeate by immobilized strains of *Kluyveromyces marxianus* in batch and continuous bioreactors

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ABSTRACT

We investigated the bioconversion of whey and whey permeate into ethanol by *Kluyveromyces marxianus* immobilized in Ca-alginate, in both batch and continuous cultivations. Different strains of *K. marxianus* and cultivation media were tested in batch mode and the effects of dilution rate (D) and substrate concentration were investigated in continuous bioreactors. In shaker cultivations, the highest ethanol yield (0.51 g g^{-1}) and ethanol productivities (0.77–1.15 g L^{-1} h^{-1}) were obtained by strains CBS 6556, CCT 4086, and CCT 2653 in raw (not supplemented) whey permeate. These strains were immobilized in Ca-alginate beads and cultivated in batch fluidized-bed bioreactors, where the highest ethanol productivity (2.53 g L^{-1} h^{-1}) was observed for strain CCT 4086. The effects of D (0.1–0.3 h^{-1}) and whey permeate concentration (C_{WP}, 60–180 g L^{-1}) were also investigated in continuous fluidized-bed bioreactors using *K. marxianus* CCT 4086, and the highest ethanol productivity (6.01 g L^{-1} h^{-1}) was achieved at D of 0.3 h^{-1} and C_{WP} of 150 g L^{-1}, whereas the highest ethanol yield (0.51 g g^{-1}) and concentration (42.8 g L^{-1}) were observed for D 0.1 h^{-1} and C_{WP} of 90 g L^{-1}. Two continuous fluidized-bed bioreactors operated in sequence were tested, showing increased ethanol productivities and concentrations to 6.97 g L^{-1} h^{-1} and 70.4 g L^{-1}, respectively. Continuous immobilized-cell bioreactor showed promising results to improve the performance of ethanol production from whey fermentation processes.

1. Introduction

The evolution of ethanol fermentation process, and the growing concern with environmentally sound technologies are stimulating the research on new strategies for energy generation, particularly with respect to renewable, alternative sources such as agricultural crops, lignocellulosic biomass, and waste materials [1–3]. High ethanol productivity from low cost feedstock, in addition to lower investment and operational costs, are aspects of interest in this kind of bioprocess. Continuous fermentation (CF) offers several advantages compared to batch mode, such as the improvement on ethanol yield and the reduction of fermentation time and product losses. In CF, product formation rate can be controlled and maintained at desired levels [4–6]. Cell immobilization techniques can improve CF by enhancing ethanol productivity and protecting cells from inhibitory products and environmental variations, resulting on smaller bioreactor volumes and lower operational costs [5,7–9].

Several approaches for ethanol production in continuous cell-immobilized bioreactors have been tested, using different supports such as cellulose beads [10], k-carrageenan [11], calcium alginate [12,13], sorghum bagasse [14], sugarcane bagasse chips [6], among others. However, these researches were mainly conducted using *Saccharomyces cerevisiae* as biocatalyst and molasses or glucose as substrates. Only recently, studies on ethanol production using whey or whey permeate as carbon sources in continuous cell immobilized systems were reported [15–17]. Although yeasts showing the ability to metabolize the lactose present in whey and whey permeate are rather rare, strains belonging to the genus *Kluyveromyces* have been well characterized on their abilities of

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using lactose as a source of energy, with strains of *Kluyveromyces marxianus* being studied because of their potential bioconversion of this sugar into ethanol [18–20].

Whey and whey permeate are by-products of the dairy industry, which are inexpensive and abundant, rich in nutrients that could be used as substrates for ethanol production. Whey permeate contains approximately 70% of whey total solids, having the same disposal problems of the whey. Presently, about 50% of the total worldwide production of whey is disposed in wastewater treatment plants or sub-utilized in farms, whereas 10% being transformed into whey protein concentrates, with whey permeate as a remaining by-product [8,20–22]. Direct fermentation of whey and whey permeate is not economically feasible because the low ethanol concentration and high distillation costs of diluted fermentation broths [15,23]. However, dried or evaporated whey permeate might be an attractive raw material for ethanol fermentation because of the advantages of its utilization, such as the high concentrations of lactose and other nutrients, yielding high ethanol productions [23].

The aims of this research were to investigate the use of *K. marxianus* to convert whey or whey permeate into ethanol using continuous fluidized-bed Ca-alginate immobilized-cell bioreactors. To optimize this bioprocess, a two-stage continuous fluidized-bed bioreactors operating in sequence, were tested. Screening of *K. marxianus* strains and media were also evaluated in shaker flask and in batch fluidized-bed immobilized-cells bioreactors.

2. Materials and methods

2.1. Microorganisms

Six strains of *K. marxianus* were used in this work. *K. marxianus* CBS 6556 was obtained from Centraalbüreau vor Schimmel-Cultures (Amsterdam, The Nederlands); *K. marxianus* CCT 4086 and *Kluyveromyces marxianus* var. *lactis* CCT 2653 were provided by Tropical Culture Collection of André Tosello Foundation (Campinas, Brazil); and *K. marxianus* UFMG 95 302.2, *K. marxianus* UFMG 95 205.3, and *K. marxianus* UFMG 95 270.1 were supplied by Laboratory of Taxonomy, Biodiversity and Biotechnology of Fungi from Department of Microbiology, Federal University of Minas Gerais, Brazil. It is important to note that the last three strains were recently isolated from natural environments and have never been tested in bioprocesses before. The strains were maintained on agar slants at 4 °C, as reported elsewhere [24].

2.2. Experimental system

The experiments were carried out in three steps. At first, a screening among six strains of *K. marxianus* and six different media were performed in rotary shaker to evaluate the lactose bioconversion into ethanol. In the second set of experiments, batch fluidized-bed bioreactors with cells entrapped in 4% (mass fraction) Ca-alginate beads were studied with the strains that showed the highest bioconversion capacity in the media previously tested in the first step. Finally, fermentations were carried out in continuous fluidized-bed bioreactors using the best strain, immobilized in 4% Ca-alginate, under three different dilution rates and five whey permeate concentrations. A two-stage bioreactors operated in sequence, where the feeding flow of the second stage was the effluent of the first, was investigated based on the better results of ethanol yield and ethanol productivity attained in the third step.

Results were evaluated by analysis of variance (ANOVA), Tukey test, or multiple regression using Statistica 10.0 software (StatSoft, USA).

2.3. Shaker flask cultivation

Inocula were prepared by transferring isolated yeast colonies to a 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 optical density (OD, 600 nm) of 1, which corresponded to 1.4 g L^{-1} for strains of *K. marxianus* CBS 6556, CCT 4086, CCT 2653 and UFMG 95 270.1, 1.5 g L^{-1} for *K. marxianus* UFMG 95 302.2 and 1.6 g L^{-1} for *K. marxianus* UFMG 95 205.3.

Supplementation of the main carbon sources (whey and whey permeate) was tested, totalizing 6 culture media compositions: 1) whey (W); 2) whey permeate (WP); 3) whey added of 3 g L^{-1} raw yeast extract (WY); 4) whey added of 3 g L^{-1} raw yeast extract and 5 g L^{-1} bactopeptone (WYP); 5) whey permeate added of 3 g L^{-1} raw yeast extract (WPY); and 6) whey permeate added of 3 g L^{-1} raw yeast extract, and 5 g L^{-1} bactopeptone (WPYP). Reconstituted whey (70 g L^{-1} of whey powder; Elegê Laticínios S.A., Teotônia, Brazil) was used for experiments, which has the equivalent of 60 g L^{-1} of lactose, 9 g L^{-1} of protein, and 1 g L^{-1} of minerals. Whey proteins were hydrolyzed using a commercial protease (Alcalase 2.4 L, 2.4 UA-A/g, Novozymes, Araucária, Brazil) at pH 8.5, 55 °C for 3 h, in order to avoid protein precipitation during the sterilization process (121 °C, 15 min). Reconstituted whey permeate (Sooro, PR, Brazil) was used at concentration of 60 g L^{-1}, corresponding to 59 g L^{-1} of lactose, 1 g L^{-1} of protein, and 1.8 g L^{-1} of minerals.

The fermentations were performed in conical flasks of 250 mL containing 144 mL of cultivation medium and 16 mL of inoculum, totalizing 160 mL of fermentation medium at 150 rpm and 30 °C.

2.4. Immobilization technique

Immobilization techniques followed procedures previously optimized and described in earlier works of the group [17]. The diffusivity coefficients for lactose and ethanol, under the conditions used in this work, were determined to be 4.84×10^{-10} m^2 s^{-1} and 1.46×10^{-10} m^2 s^{-1}, respectively [25]. Yeasts were grown in 2 L 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 30 °C, in an orbital shaker at 180 rpm for 15 h in order to obtain exponential-phase cells. At the end of cultivation, 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 dropped 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 agitated for 30 min to stabilize the system. Average alginate beads of 3.8 mm of diameter were obtained. The beads were washed thrice with distilled water at 4 °C and kept in peptone water with 0.1 M $CaCl_2$ overnight. Then, the beads were washed thrice with sterile distilled water at 4 °C and transferred into the bioreactors.

2.5. Bioreactor cultivations

Bioreactor experiments were performed in glass column bioreactors (fluidized section column, 30 mm internal diameter, and 180 mm height), described elsewhere [17] using reconstituted whey permeate as fermentation medium. The bioreactors were filled with 85 mL of alginate beads and 250 mL of fermentation medium. Temperature was controlled at 30 °C by circulating water from a thermostat bath in the bioreactor jacket. The growth

medium was recirculated through the column by a peristaltic pump, promoting the fluidization of alginate beds (upward flow).

Batch cultivations were carried out in duplicate to evaluate the strains ability of lactose consumption and ethanol production.

Continuous fluidized-bed fermentations were performed at 30 °C for 128 h under 3 different dilution rates (0.1 h^{-1}, 0.2 h^{-1}, and 0.3 h^{-1}) and 5 concentrations of whey permeate (60 g L^{-1}, 90 g L^{-1}, 120 g L^{-1}, 150 g L^{-1}, and 180 g L^{-1}) according to a hexagonal experimental design (Table 1). The fluidization was carried out by medium recirculation through the bioreactor using a peristaltic pump. Cultures were started in batch mode in order to allow for cell accumulation in the system and then feeding was started at the 11th hour. The experimental results were approximated by a quadratic polynomial equation (Equation (1)):

$$Y = \beta_0 + \beta_1 \cdot D + \beta_2 \cdot C_{WP} + \beta_{11} \cdot D^2 + \beta_{22} \cdot C_{WP}^2 + \beta_{12} \cdot D \cdot C_{WP} \quad (1)$$

where D and C_{WP} are the regression variables (dilution rate and whey permeate concentration) and Y represents the dependent variables, in this case, ethanol yield, productivity, or residual sugar. The symbols β_0, β_1, β_2, β_{11}, β_{22} and β_{12} are the regression coefficients of the model.

Continuous fluidized-bed bioreactors in sequence were carried out at 30 °C for 128 h under D of 0.1 h^{-1}. The culture was started in batch mode and then feeding was started at the 11th hour for both bioreactors. The first bioreactor was fed with C_{WP} of 150 g L^{-1}, and its effluent flow was the feeding of the second vessel. This experiment system was performed in duplicates.

2.6. Analytical determinations

Samples were collected from the top of bioreactors in batch fermentations or from the out stream of the continuous bioreactors, centrifuged (3000 *g*, 15 min) and the supernatant was analyzed for sugar and ethanol concentrations. The concentration of suspended cells that were freed from the alginate spheres was determined by absorbance at 600 nm and correlated with dry cell weight (g L^{-1}). Lactose, galactose, glucose, and ethanol concentrations were determined by HPLC (Shimadzu, Japan) with refractive index detector and Bio-Rad HPX-87H column (300 mm × 7.8 mm) using 5 mM sulfuric acid as eluant at 45 °C, flow rate of 0.6 mL min^{-1} and sample volumes of 20 μL.

3. Results and discussion

3.1. Screening of K. marxianus strains and fermentation media

This set of experiments was performed to determine the effect of medium supplementation on the capacity of lactose bioconversion to ethanol by six different strains of *K. marxianus*. The strains *K. marxianus* UFMG 95 302.2, UFMG 95 205.3 and UFMG 95 270.1 were recently isolated from natural environments and have never been tested in bioprocesses before.

Table 1
Hexagonal experimental design used to study the effect of dilution rate (D) and whey permeate concentration (C_{WP}) on continuous fermentation.

Assay	Coded variables		Uncoded variables	
	x_1	x_2	D (h^{-1})	C_{WP} (g L^{-1})
1	1	−1	0.3	90
2	−1	−1	0.1	90
3	1	1	0.3	150
4	−1	1	0.1	150
5	0	−1.5	0.2	60
6	0	1.5	0.2	180
7	0	0	0.2	120
8	0	0	0.2	120
9	0	0	0.2	120

The lactose metabolism and bioconversion to ethanol differed substantially among the strains. The ethanol yields (Table 2) were dependent on strains and media supplementation. The highest ethanol yields ($Y_{EtOH/S}$) were observed for commercial strains of *K. marxianus* (CBS 6556, CCT 4086, and CCT 2653) in all fermentation media, ranging from 0.34 g g^{-1} to 0.51 g g^{-1}, whereas, low ethanol yields were found for the strains UFMG 95 302.2, UFMG 95 205.3, and UFMG 95 270.1 (0.17–0.38 g g^{-1}). The lowest ethanol yields ($Y_{EtOH/S}$) were observed for strains UFMG 95 205.3 and UFMG 95 270.1 where both bactopeptone and yeast extract were used (Table 2). This phenomenon occurred probably due to the substrate imbalance or inhibition, because whey is already rich in nutrients and the addition of nitrogen sources, such as raw yeast extract and bactopeptone, could lead to cell metabolism impairment, affecting product formation. *K. marxianus* NRRL-1195 also showed repression of ethanol fermentation when nitrogen and phosphorus sources were added in whey, on a rotatory shaker at 28 °C, 150 rpm for 72 h [26]. Surprisingly, for all tested strains (Table 2), the yields of ethanol were slightly higher in cultivations using WP than for W medium, reaching 0.51 g g^{-1} for strains CBS 6556, CCT 4086, and CCT 2653, with high conversion efficiencies (η) of 95% of the theoretical yield. The values obtained when using W medium were 0.50 g g^{-1}, 0.48 g g^{-1} and 0.49 g g^{-1} respectively (conversion efficiencies of 94%, 89%, and 91%), suggesting good prospects of application of the whey permeate without supplementations as substrate in this bioprocess. These results compare well with those reported in the literature. Conversion efficiencies of 97% and 83% were obtained using Scotta and whey media, respectively, in orbital shaker cultures of *K. marxianus* at 37 °C, 150 rpm for 18 h [27]. Ethanol yields ranging from 0.51 g g^{-1} to 0.52 g g^{-1} were obtained for *K. marxianus* UFV-3 under hypoxic and anoxic conditions, respectively, in whey permeate (lactose concentration of 50 g L^{-1}) [28]. Ethanol yield of 0.51 g g^{-1} was obtained using raw whey in batch fermentations using *K. marxianus* DSMZ 7239 at 30 °C and 100 rpm [16]. The ethanol yields obtained in this work using strains CBS 6556, CCT 4086, and CCT 2653 in WP are higher when compared to other researches using the *Kluyveromyces* yeasts. Ethanol concentration of 20.2 g L^{-1}, corresponding 74% of the theoretical yield, was observed for *K. marxianus* NCYC 179 in whey permeate (lactose concentration of 50 g L^{-1}) at 30 °C, 200 rpm for 24 h [29]. Several *Kluyveromyces* strains (IBM1, IBM2, IBM3, IBM4, and IBM5) were investigated for ethanol production at 45 °C (200 rpm for 140 h), and the highest ethanol concentration (17 g L^{-1}) was reported for strain IBM2 in whey permeate (lactose concentration of 40 g L^{-1}), representing 83% of the theoretical yield [30].

The highest ethanol productivities (Q_P) were obtained for strains *K. marxianus* CBS 6556, CCT 4086, and CCT 2653, whereas the lower productions were found for strains *K. marxianus* UFMG 95 302.2, UFMG 95 205.3, and UFMG 95 270.1, confirming their slower metabolism and lack of adaptation to laboratory cultivation. The best productivity obtained in this work is similar to the highest ethanol productivities reported in the literature. Silva et al. [31] showed a productivity of 1.2 g L^{-1} h^{-1} in a study using recombinant *S. cerevisiae* and deproteinized concentrate cheese whey in rotary shaker at 30 °C and 150 rpm. Dragone et al. [23] investigated ethanol production from deproteinized cheese whey powder using *Kluyveromyces fragilis* in various lactose concentration, temperature and inocula concentrations, and attained ethanol productivities ranging from 0.23 g L^{-1} h^{-1} to 1.27 g L^{-1} h^{-1}. A low ethanol productivity (0.2 g L^{-1} h^{-1}) was found using *K. marxianus* MTCC 1288 on whey (50 g L^{-1}) in shaker flask at 34 °C [32], which is

Table 2
Ethanol yields ($Y_{EtOH/S}$ (g g^{-1})) and ethanol productivity (Q_P (g L^{-1} h^{-1})) of strains of *Kluyveromyces marxianus* and varying fermentation media in orbital shaker cultures at 30 °C and 150 rpm.

Strains	Fermentation media											
	W		WY		WYP		WP		WPY		WPYP	
	$Y_{EtOH/S}$	Q_P	$Y_{EtOH/S}$	Q_P	$Y_{EtOH/S}$	Q_P	$Y_{EtOH/S}$	Q_P	$Y_{EtOH/S}$	Q_P	$Y_{EtOH/S}$	Q_P
KM CBS 6556	0.50 ± 0.02	1.00 ± 0.09	0.39 ± 0.00	0.65 ± 0.16	0.38 ± 0.03	0.58 ± 0.01	0.51 ± 0.01	1.13 ± 0.04	0.47 ± 0.02	1.10 ± 0.06	0.50 ± 0.00	1.02 ± 0.14
KM CCT 4086	0.48 ± 0.08	0.65 ± 0.11	0.40 ± 0.01	1.63 ± 0.03	0.43 ± 0.01	0.72 ± 0.05	0.51 ± 0.03	1.14 ± 0.03	0.50 ± 0.01	1.15 ± 0.00	0.49 ± 0.03	1.02 ± 0.04
KM CCT 2653	0.49 ± 0.02	1.00 ± 0.08	0.35 ± 0.05	0.69 ± 0.07	0.34 ± 0.01	0.68 ± 0.02	0.51 ± 0.03	0.77 ± 0.02	0.51 ± 0.02	0.79 ± 0.04	0.48 ± 0.06	0.66 ± 0.00
KM UFMG 95 302.2	0.30 ± 0.08	0.08 ± 0.00	0.19 ± 0.01	0.09 ± 0.01	0.21 ± 0.03	0.13 ± 0.02	0.38 ± 0.16	0.18 ± 0.08	0.28 ± 0.04	0.18 ± 0.05	0.30 ± 0.04	0.13 ± 0.02
KM UFMG 95 205.3	0.32 ± 0.00	0.11 ± 0.01	0.18 ± 0.03	0.13 ± 0.03	0.19 ± 0.09	0.12 ± 0.00	0.37 ± 0.01	0.15 ± 0.03	0.25 ± 0.03	0.12 ± 0.02	0.35 ± 0.04	0.17 ± 0.05
KM UFMG 95 270.1	0.30 ± 0.07	0.12 ± 0.01	0.18 ± 0.01	0.11 ± 0.01	0.17 ± 0.01	0.12 ± 0.02	0.39 ± 0.07	0.11 ± 0.03	0.25 ± 0.04	0.14 ± 0.02	0.34 ± 0.02	0.21 ± 0.00

W: whey; WY: whey supplemented with raw yeast extract; WYP: whey supplemented with raw yeast extract and bactopeptone; WP: whey permeate; WPY: whey permeate supplemented with raw yeast extract; WPYP: whey permeate supplemented with raw yeast extract and bactopeptone.

similar to the productivities observed for strains UFMG 95 302.2, 95 205.3 and 95 270.1 in this work.

The marked differences in the results for growth and product formation can be evidenced by the kinetic profiles of sugar consumption of the traditional and recently isolated strains of *K. marxianus*. In Fig. 1 is presented the mean sugar consumption of the two yeast groups, showing that *K. marxianus* CBS 6556, CCT 4086, and CCT 2653 depleted lactose in 24 h of fermentation, whereas for the strains UFMG 95 302.2, UFMG 95 205.3, and UFMG 95 270.1 (isolated yeasts) the lactose was not entirely consumed. This marked difference might have occurred due to a low ethanol tolerance of the UFMG strains, which showed growth in batch shaker cultures containing up to 10 g L^{-1} of alcohol (results not shown), being repressed above this threshold. Another hypothesis could be the low lactose affinity for the lactose-permease enzymes of these strains, coded by the LAC12 gene [18,19], which has been reported for some strains of *K. marxianus* [33,34].

3.2. Immobilized batch fluidized bed bioreactors

Strains *K. marxianus* CBS 6556, CCT 4086, and CCT 2653, showing the best results on WP, were chosen for the batch fluidized-bed bioreactor systems. These experiments were carried out at 30 °C for 24 h in order to evaluate the capacity of the yeasts to convert WP lactose into ethanol when immobilized in Ca-alginate beads.

The profiles of lactose and ethanol concentration are presented in Fig. 2. The strains CBS 6556 and CCT 4086 completely depleted lactose after 12 h of cultivation, whereas strain CCT 2653 showed slower sugar consumption rate, consuming 93% of initial lactose in 24 h (Fig. 2A). High ethanol yields ($Y_{EtOH/S}$) were observed for the first two strains, 0.45 g g^{-1} and 0.47 g g^{-1}, respectively, with yield efficiencies (η) of 84% and 89% of the theoretical value (Table 3), while strain CCT 2653 produced ethanol to yields of 0.33 g g^{-1}. The highest ethanol concentration and productivity were achieved for strain CCT 4086 (Fig. 2B), 28.0 g L^{-1} and 2.53 g L^{-1} h^{-1}, compared to 1.96 g L^{-1} h^{-1} for CBS 6556 and 0.75 g L^{-1} h^{-1} for CCT 2653 (Table 3). Physiological differences between CBS 6556 and CCT 4086, including sugar consumption profiles, have been reported by Ref. [35]. The more contrasting differences of physiology of CCT 2653 compared with the other two strains (CBS 6556 and CCT

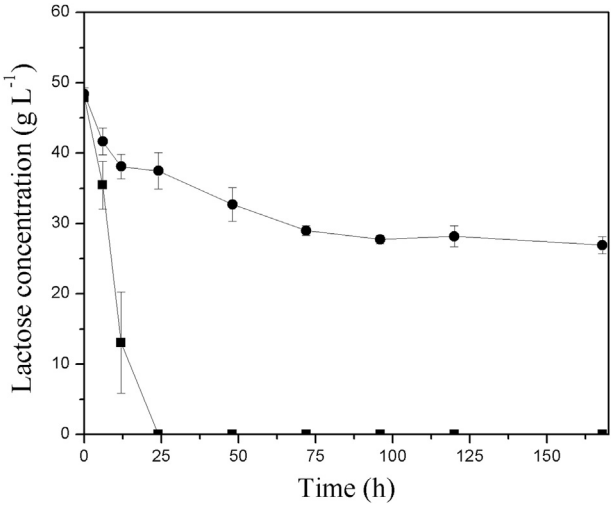

Fig. 1. Profile of lactose consumption from whey under the experimental conditions of this work for the 2 groups of yeasts: group 1 (commonly used strains): *K. marxianus* CBS 6556, CCT 4086 and CCT 2653 (-■-) and group 2 (newly isolated): *K. marxianus* UFMG 95 302.2, 95 205.3 and 95 270.1 (-●-).

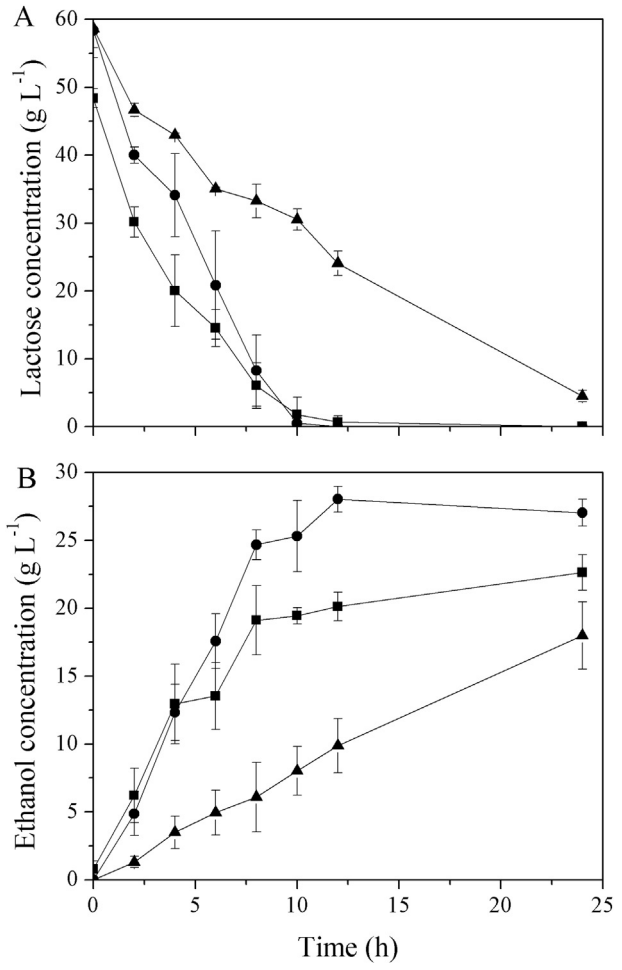

Fig. 2. Kinetics of lactose consumption (A), and ethanol production (B) of the three strains of *Kluyveromyces marxianus* in batch fluidized bed bioreactor at 30 °C. *K. marxianus* CBS 6556 (-■-), *K. marxianus* CCT 4086 (-●-) and *K. marxianus* CCT 2653 (-▲-).

4086) might be explained by its taxonomy, which classifies it as *K. marxianus* var. *lactis*, instead of var. *marxianus*. The physiological characteristics of the *K. lactis* group are generally associated with low ethanol productions [20]. The expression of genes involved in the lactose fermentation by *K. lactis*, such as *LAC4* and those involved in Leloir pathway (*RAG6*, *GAL7* and *GAL10*), and the production of enzymes such as β-galactosidase and pyruvate decarboxylase, were observed in physiology studies comparing *K. marxianus* and *K. lactis* [36].

In this work, it was obtained the highest ethanol yields compared to the literature, concerning similar bioreactor systems and yeasts (Table 4), indicating the improvement in the fermentation conditions. Diverse factors could explain these results, ranging from physiological characteristics of strains, to diffusivity phenomena, to aspects of bioreactor geometry and operation.

Table 3
Ethanol yields ($Y_{EtOH/S}$), yield efficiency (η), and ethanol productivity (Q_P) of 3 best strains of *Kluyveromyces marxianus* under fluidized batch bioreactor cultivations.

Yeast	$Y_{EtOH/S}$ (g g^{-1})	η (%)	Q_P (g L^{-1} h^{-1})
CBS 6556	0.45 ± 0.00	83.6 ± 0.31	1.96 ± 0.06
CCT 4086	0.47 ± 0.05	89.2 ± 9.08	2.53 ± 0.26
CCT 2653	0.33 ± 0.04	61.8 ± 7.82	0.75 ± 0.15

Table 4
Comparison of results obtained in this work with other reports in the literature for ethanol yields, ethanol productivities, and conversion efficiencies.

Yeast	Substrate	$Y_{EtOH/S}$ (g g^{-1})	Q_P (g L^{-1} h^{-1})	η (%)	Reference
K. marxianus					
CBS 6556	Whey permeate	0.45	1.96	83.6	This work
CCT 4086		0.47	2.53	89.2	
CCT 2653		0.33	0.75	61.8	
K. marxianus					
NCYC 179	Whey permeate	0.42	–	78.0	[29]
K. marxianus					
TY-3	Whey	0.34	0.31	63.0	[37]
K. fragilis					
NRRL 665	Synthetic medium	0.44	0.76	83.0	[38]
K. marxianus					
CBS 6556	Whey	0.45	0.96	83.3	[17]
CCT 4086		0.43	0.81	79.1	
CCT 2653		0.45	0.84	83.3	

K. marxianus species are characterized by substantial degree of intraspecific polymorphism (genetic and physiological), which results in a high metabolic diversity [19]. In this work we carried out cultivations in fully controlled bioreactors, whereas in others researches (Table 4), the experimental system consisted of shaker flasks. The column bioreactor design in this work allows for a high volume of spheres (compared to medium volume) than that possible in a shaker flask [37,38], and the fluidization of the system can contribute to homogeneous conditions, hence improving the mass transfer phenomena.

3.3. Continuous fluidized bed bioreactor cultivations

In this set of experiments, the effects of different D and C_{WP} on ethanol production during continuous cultivation of *K. marxianus* CCT 4086 were tested following a hexagonal experimental design (Table 1), and results are presented in Table 5 and Fig. 3, respectively. Ethanol yield increased inversely with the D and C_{WP} (Fig. 3A). The highest ethanol yield, 0.51 g g^{-1}, was achieved with D of 0.1 h^{-1} and C_{WP} of 90 g L^{-1}, with ethanol production of 42.8 g L^{-1} (calculated data). The lowest ethanol yields were observed for the highest D and C_{WP} (0.32 g g^{-1} and 0.34 g g^{-1}, respectively). This behavior is suggesting that catabolite repression is in place when sugar feeding is above D of 0.2 h^{-1}, somewhat a low value for

Table 5
Regression coefficients of the variables and the regression parameters for ethanol yield, ethanol productivity, and residual sugar by *Kluyveromyces marxianus* CCT 4086 under continuous fluidized bed bioreactor cultivations.

	Ethanol yield		Ethanol productivity		Residual sugar	
	Coefficient	p	Coefficient	p	Coefficient	p
β_0	0.749	<0.001	–	–	−72.637	0.0001
β_1	−1.628	0.029	16.004	0.002	164.971	0.009
β_{11}	2.468	0.120	–	0.002	–	–
β_2	−0.001	0.003	0.056	0.010	0.742	<0.0001
β_{22}	–	–	−0.001	0.011	–	–
β_{12}	–	–	−0.084	–	–	–
Regression						
p-Value	0.002		0.001		<0.0001	
F	24.950		52.284		117.885	
R^2	0.937		0.981		0.975	
LOF*	0.577		0.876		0.388	

*p-value of lack of fit.

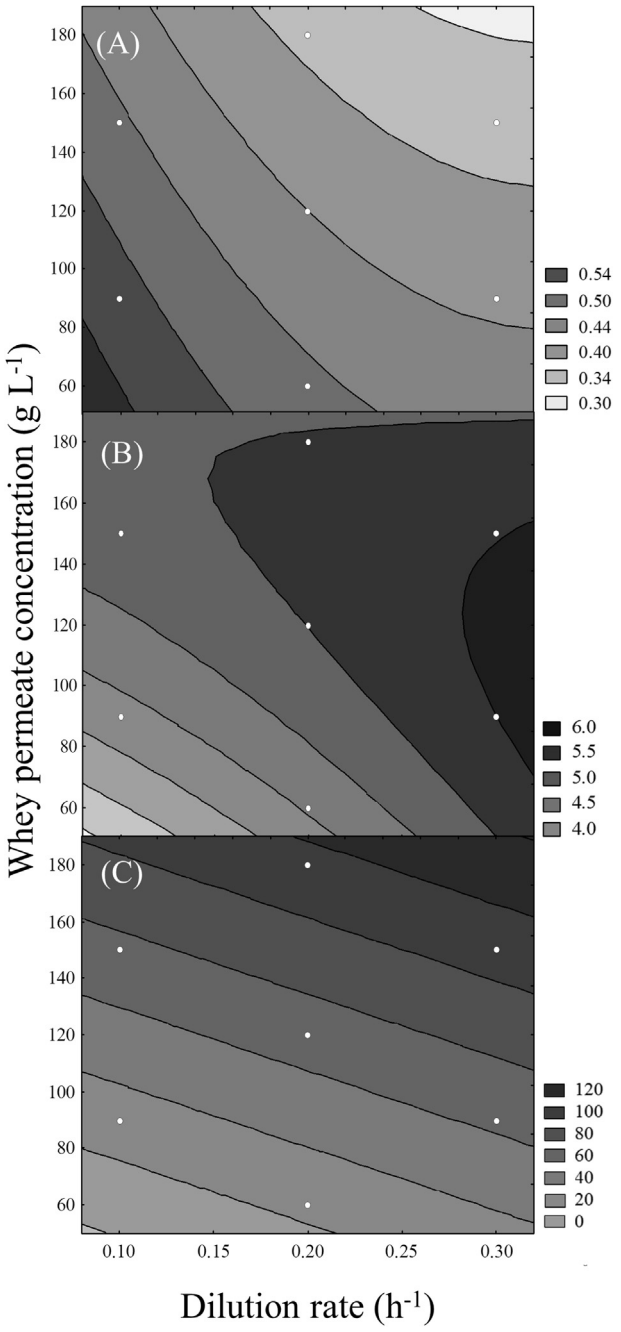

Fig. 3. Contour surface of ethanol yield (A), ethanol productivity (B) and residual sugar (C) from whey permeate fermentation by *Kluyveromyces marxianus* CCT 4086 on continuous fluidized-bed bioreactor at 30 °C as function of substrate concentration and dilution rate.

Kluyveromyces yeasts. It is known that the metabolism of *K. marxianus* is regulated by the amount of available sugar. At high medium sugar content, high maintenance requirements are necessary because of factors such as osmotic pressure, demanding higher retention time or low dilution rates to allow proper bioconversion of sugar into final products [15]. A similar behavior was observed by other researches. For instance, *K. marxianus* DSMZ-7239, immobilized in olive pits, cultivated in a continuous packed-bed bioreactor, showed ethanol yields of 0.32 g g^{-1} and 0.54 g g^{-1} at D of 0.057 h^{-1} and 0.02 h^{-1}, respectively [39]. When increased whey concentration (from 50 to 200 g L^{-1}) was used, there was a decrease in ethanol yields, from 0.52 g g^{-1} to 0.17 g g^{-1} at D of 0.02 h^{-1} [15]. Continuous cultivation of *Candida pseudotropicalis* ATCC 8619 in 50 g L^{-1} of whey also showed an increase in ethanol yield from 0.25 g g^{-1} to 0.37 g g^{-1} with decreased D from 0.05 h^{-1} to 0.02 h^{-1} [4]. Contrasting with our results, ethanol yield was not affected by D (0.5–1.25 h^{-1}) and sugar concentration (50–150 g L^{-1} of glucose) during continuous ethanol production by *Kluyveromyces* sp. IIPE453 immobilized on bagasse chips in a packed bed bioreactor [6].

Fig. 3B shows the ethanol productivity (Q_P) as function of C_{WP} and D. Ethanol productivity increased proportionally with dilution rate and whey permeate concentration up to approximately 150 g L^{-1}. The highest ethanol productivities (6.01 g L^{-1} h^{-1} and 5.96 g L^{-1} h^{-1}) were obtained at the highest D (0.3 h^{-1}), however, these conditions presented low ethanol yields (0.34 g g^{-1} and 0.38 g g^{-1}). The highest ethanol yield (0.51 g g^{-1}) was obtained under conditions of the lowest C_{WP} and D (90 g L^{-1} and 0.1 h^{-1}), reaching 95% of maximum theoretical yield, and 93% of lactose consumption. Comparatively, Christensen et al. [16] reported that an increase of ethanol productivity from 2.5 g L^{-1} h^{-1} to 4.5 g L^{-1} h^{-1} was possible varying the D from 0.04 h^{-1} to 0.2 h^{-1} in the continuous cultivation of non-sterilized whey using *K. marxianus* DSMZ 7239 immobilized in Ca-alginate. In a previous work of our group [17], continuous cultures of *K. marxianus* CBS 6556 immobilized in Ca-alginate showed maxima ethanol productivities of 3.2 g L^{-1} h^{-1} and 3.5 g L^{-1} h^{-1} at the highest D (0.3 h^{-1}) for packed and fluidized bed operations, respectively. Ozmihci and Kargi [39] reported ethanol productivities varying from 0.28 g L^{-1} h^{-1} to 0.58 g L^{-1} h^{-1} depending of D (0.015–0.06 h^{-1}) in packed bed column continuous cultures of *K. marxianus* immobilized in olive pits using whey as substrate. Finally, ethanol productivities by *K. marxianus* IMB3 varied from 2.5 g L^{-1} h^{-1} to 5.5 g L^{-1} h^{-1} proportionally with D from 0.05 h^{-1} to 0.2 h^{-1} on continuous fermentation using synthetic medium and glucose as carbon source [40]. The highest ethanol productivities in this work were possible due to the optimization of fermentation conditions obtained in the hexagonal experimental design, which coupled high substrate concentration with relatively high dilution rates. This is an important difference when compared with other researches, in which the influences of substrate concentration and of dilution rate were analyzed separately. Moreover, the fluidized bed system in this work might have probably improved the mass transfer mechanisms when compared with packed bed bioreactors used in some researches and, consequently, higher values of fermentative parameters were attained.

Fig. 3C shows the residual sugar as function of whey permeate concentration and dilution rate. As expected, residual sugar was affected by increasing whey permeate concentration and dilution rates (Table 5). The lowest residual sugar of 6.3 g L^{-1} was obtained with D of 0.1 h^{-1} and 90 g L^{-1} of C_{WP}, up to a maximum of 92.6 g L^{-1} for D of 0.2 h^{-1} and C_{WP} of 180 g L^{-1}. Table 7 presents a comparison of results among several researches operated under similar conditions. High substrate concentrations can lead to inhibitory effects of cell growth and reduce fermentation rates, often related to changes

Table 6
Ethanol concentration, lactose consumption, ethanol yields ($Y_{EtOH/S}$), and ethanol productivity (Q_P) obtained in the continuous fluidized bed bioreactor cultivations operated in sequence.

Bioreactor	Lactose consumption (g L^{-1})	Ethanol (g L^{-1})	$Y_{EtOH/S}$ (g g^{-1})	Q_P (g L^{-1} h^{-1})
1st stage	89.0	52.4	0.47	5.26
2nd stage	58.0	18.0	–	–
Overall	147.0	70.4	0.48	6.97

Table 7
Literature data on residual sugar during continuous cultivation.

Yeast	Substrate ($g\ L^{-1}$)	D (h^{-1})	Residual sugar ($g\ L^{-1}$)	Reference
K. marxianus CCT 4086	Whey permeate (60–180)	0.1–0.3	6.3–92.6	This work
K. marxianus IMB3	Glucose (75)	0.05–0.15	48.0–56.0	[42]
K. marxianus IIPE453	Glucose (50–150)	0.5	6.8–69.0	[6]
C. pseudotropicalis ATCC 8619	Lactose (50–150)	0.02	1.0–25.5	[4]
K. marxianus DSMZ 7239	Whey (50)	0.057–0.02	15.0–18.5	[39]

in sugar-carriers affinities, osmotic sensitivity, and low tolerances to high ethanol concentration [20,28]. Generally, initial lactose concentrations above 100 $g\ L^{-1}$ have been reported to result in high residual sugar concentrations and inhibitory effects for *K. marxianus* strains [15,28,32,41].

3.4. Sequential continuous fluidized bed bioreactor cultivations

From the previous set of experiments, it was clear the physiological limitations of *K. marxianus* towards lactose concentration, even at relatively low dilution rates. Therefore, in order to improve lactose bioconversion into ethanol, it was performed a sequential two-bioreactors cultivation in which the feeding of the second tank was provided by the effluent of the first. This was carried out based on the satisfactory results reached in previously hexagonal experimental design at conditions of 150 $g\ L^{-1}$ of C_{WP} and 0.1 h^{-1} of D ($Y_{EtOH/S}$ = 0.47 $g\ g^{-1}$ and Q_P = 5.33 $g\ L^{-1}\ h^{-1}$). Notwithstanding, at these conditions the residual sugar was of 53.3 $g\ L^{-1}$. Thus, this remaining sugar could be used as the feeding stream in a second bioreactor in order to exhaust the residual sugar, and with this, improve the overall yields of conversion. Results are presented in Table 6, showing that *K. marxianus* CCT 4086 was able to metabolize the lactose in the second stage, even in the presence of ethanol concentrations of 52.4 $g\ L^{-1}$. The overall ethanol yield of 0.48 $g\ g^{-1}$ and productivity of 6.97 $g\ L^{-1}\ h^{-1}$ were achieved, with only 3 $g\ L^{-1}$ of remaining sugar at the second bioreactor downstream. Ethanol concentration obtained in this system was 24% higher than the one-stage bioreactor (at D of 0.1 h^{-1} and 150 $g\ L^{-1}$ of C_{WP}) reaching 70.4 $g\ L^{-1}$. *K. marxianus* CCT 4086 showed a high ethanol tolerance, indicating that this strain is not inhibited by the product. Ethanol productivity, obtained in the two-stages continuous bioreactors, is one of the highest so far reported in the literature using *K. marxianus* and whey as substrate on continuous systems [15–17].

4. Conclusions

Screening of strains of *K. marxianus* and media based on residual whey and whey permeate demonstrated the ability to use this system to produce ethanol. Media supplementation was tested and results showed that this is not necessary, allowing the direct utilization of these by-products, with cost savings from an industrial perspective. Batch fluidized bed bioreactors of Ca-alginate immobilized-cells showed to reduce fermentation time and improved ethanol yields, compared to shaker cultivations. The continuous culture of immobilized-cells considerably enhanced ethanol productivities and yields. A two-stage sequential continuous culture was employed to improve sugar consumption, further improving the overall ethanol productivity.

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