Research Article

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Performance of different immobilized-cell systems to efficiently produce ethanol from whey: fluidized batch, packed-bed and fluidized continuous bioreactors

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Abstract

BACKGROUND: The bioconversion of whey into ethanol by immobilized *Kluyveromyces marxianus* in packed-bed and fluidized bioreactors is described. Both batch and continuous cultures were analyzed using three different strains of *K. marxianus* and the effect of the operating mode, temperature, and dilution rates (D) were investigated.

RESULTS: All immobilized strains of *K. marxianus* (CBS 6556, CCT 4086, and CCT 2653) produced similar high yields of ethanol (0.44 ± 0.01 g EtOH g^{-1} sugar). Significant variations of conversion efficiencies (66.1 to 83.3%) and ethanol productivities (0.78 to 0.96 g L^{-1} h^{-1}) were observed in the experiments with strain *K. marxianus* CBS 6556 at different temperatures. High yields of ethanol were obtained in fluidized and packed-bed bioreactors continuous cultures at different D (0.1 to 0.3 h^{-1}), with the highest productivity (3.5 g L^{-1} h^{-1}) observed for D = 0.3 h^{-1} in the fluidized bioreactor (87% of the maximal theoretical conversion), whereas the highest ethanol concentration in the streaming effluent (28 g L^{-1}) was obtained for D = 0.1 h^{-1}. Electronic micrographs of the gel beads showed efficient cell immobilization.

CONCLUSION: Batch and continuous cultivations of immobilized *K. marxianus* in fluidized and packed-bed bioreactors enable high yields and productivities of ethanol from whey.

Keywords: biofuel; bioreactors; fermentation; immobilization; yeast

INTRODUCTION

Cell-immobilized bioreactors have contributed to bioprocesses optimization due to their unique characteristics of high volumetric productivity, small operational volumes, cell protection against inhibitory products, and shorter reaction times when compared with free-cell bioreactors.[1,2] Ethanol production in cell-immobilized bioreactors has been studied using different entrapment supports, such as cellulose beads,[3] agar,[4] delignified cellulosic materials,[5] alginate,[6–8] sorghum bagasse,[9] and olive pits,[10] among others. However, alginate gels are the most widely used because they are non-toxic, inexpensive, and easy to prepare.[2,11,12]

Several studies have investigated the production of ethanol in batch cultivations and found that the cost of using this process is quite high when compared with continuous operation. In continuous cultivation, such factors as cell concentration and product formation rate can be controlled and maintained at desired levels.[13] Different approaches have been described for continuous ethanol production using immobilized cell bioreactor systems, with packed bed bioreactors been the most extensively studied among them. However, only a few studies have reported the use of fluidized bed bioreactors for these purposes. Continuous fermentation in a packed bed bioreactor with Ca-alginate immobilized cells has been reported with various degrees of success, and, at present, it still lacks commercial viability.[8] On the other hand, some agroindustrial residues have also been studied as support for cell immobilization for the production of ethanol, among them sorgum bagasse, in a continuous packed bed column reactors operating at different dilution rates,[9] and olive pits as supporting particles for cell attachment operated at different hydraulic residence time and sugar concentrations.[10,14] However, these bioreactors configurations where cells are not entrapped often release cells to the medium, losing productivity.

The use of industrial by-products to obtain ethanol is being extensively studied in order to increase the production of this important biofuel, while reducing costs and environmental impacts. Whey, a by-product of the dairy industry, is an inexpensive and abundant substrate, rich in nutrients that could be used for ethanol production. Whey is a significant source of environmental pollution, posing a major disposal problem due to its high volumes of production and organic load. At present, a mass fraction of

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approximately 50% of the total worldwide production of whey is disposed of in wastewater treatment plants, or sub-used in farms.[15–18]

Although several yeasts have shown the ability to metabolize the lactose present in whey, strains belonging to the genus *Kluyveromyces* have been the best, characterized on their abilities to use lactose as a source of energy, with strains of *Kluyveromyces marxianus* being explored and studied due to their potential bioconversion of whey into ethanol.[19,20]

The aims of this research were to investigate several aspects of the bioconversion of whey into ethanol by Ca-alginate immobilized strains of *K. marxianus* and the influences of operating mode (batch or continuous cultivation, packed or fluidized bed bioreactors), temperature, and dilution rates on this process. Electronic microscopic scanning of beads was also carried out in order to investigate the cell distribution and colonization of supports.

MATERIALS AND METHODS

Microorganisms

Three strains of *Kluyveromyces marxianus* were used in this work. *K. marxianus* CBS6556 was obtained from Centraalbüreau vor Schimmel-Cultures (Amsterdam, The Nederlands); *K. marxianus* CCT 4086 and *K. marxianus* var. *lactis* CCT 2653 were provided by The Tropical Culture Collection of André Tosello Foundation (Campinas, Brazil). The strains were maintained on agar slants at 4 °C, as described elsewhere.[21]

Immobilization technique

For the immobilization technique, the yeasts were grown in 2 L flasks containing 800 mL of YEP-lactose medium (in g L^{-1}: yeast extract, 10; bactopeptone, 20; lactose, 20), 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. A sterile solution of a mass fraction of 4% sodium alginate was added to the cell suspension (20 mg dry biomass mL^{-1} alginate solution) and 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 mol L^{-1} $CaCl_2$ sterile solution at 35 °C, and gently agitated for 30 min to stabilize the system. The beads formed were washed three times with distilled water at 4 °C. Average alginate beads of 3.8 mm diameter were obtained. Yeast growth was determined by absorbance at 600 nm and correlated with dry cell weight (g L^{-1}) using a calibration curve.

Bioreactor cultivations

The medium used in the bioreactor experiments was reconstituted whey (70 g L^{-1} of whey powder; Elegê Laticínios S.A., Teotônia, Brazil), which has the equivalent of 60 g L^{-1} of lactose, 9 g L^{-1} of protein, and 1 g L^{-1} of minerals. To avoid protein precipitation during the sterilization process (121 °C, 15 min), whey proteins were hydrolyzed with a commercial protease (Alcalase 2.4 L, 2.4 UA-A g^{-1}, Novozymes, Araucária, Brazil) at pH 8.5, 55 °C, for 3 h. Bioreactor experiments were performed in glass column bioreactors with a total volume of 370 mL, filled with 85 mL of alginate beads and 250 mL of fermentation medium. Figure 1 depicts the bioreactor with its constructive details. Temperature

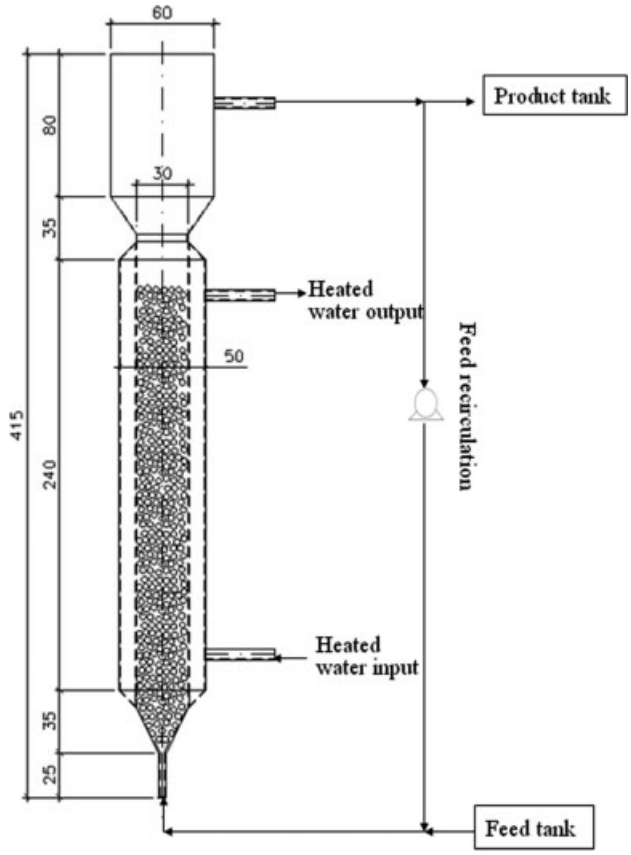

Figure 1. Schematic diagram of the continuous fluidized bed bioreactor.

was kept constant in the water jacket coupled to a thermostat bath.

Batch fluidized bed fermentations were carried out for 24 h at 30 °C with medium recirculation through the column by a peristaltic pump fluidizing the system (upward flow). Initially, the three strains of *K. marxianus* were grown at 30 °C to evaluate whether they differed in their ability to metabolize lactose and convert this sugar into ethanol. Then, *K. marxianus* CBS 6556 was chosen to test the influence of temperature (30, 35, and 40 °C) on ethanol production.

Continuous fluidized bed and packed bed fermentations were performed using three dilution rates (*D*) (0.1, 0.2, and 0.3 h^{-1}) at 30 °C for 128 h with *K. marxianus* CBS 6556. The fluidization was carried out by medium recirculation through the bioreactor using a peristaltic pump at a flow rate of 150 mL min^{-1}. Cultures were started in batch mode as a cell-adaptation stage and in order to accumulate cells in the Ca-alginate beads; then, feeding started at the 10th hour for the fluidized bed bioreactor, and at the 24th hour for the packed bed bioreactor. All experiments were carried out in duplicate.

Analytical determinations

Sampling was from the top of bioreactors in batch fermentations, and as the out stream of the continuous bioreactors. Samples were centrifuged at 3000 *g* for 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 estimated as dry weight as described before. Lactose concentration was determined by the dinitrosalicyclic acid (DNS)

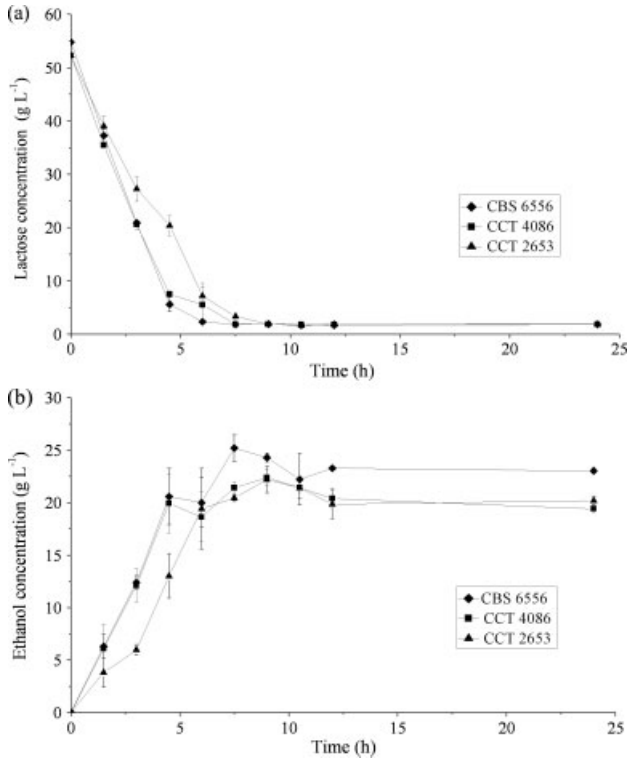

Figure 2. Kinetics of lactose consumption (a), and ethanol production (b) of the three strains of *Kluyveromyces marxianus* in batch fluidized bioreactor at 30 °C.

method for reducing sugars described by Chapplin and Kennedy.[22] Ethanol concentration was determined by gas chromatography using a gas chromatograph (GC-14B Shimadzu, Tokyo, Japan) with a FID detector using a capillary column DB-1 of 30 m length and 0.25 mm internal diameter. The column temperature was constant at 40 °C, and the temperature of both injector and detector was 250 °C. To quantify the amount of ethanol, a calibration curve was constructed, varying ethanol concentration and using a fixed amount of *n*-propanol as internal standard.

Electronic microscopic scanning of beads with immobilized cells

The distribution and colonization of Ca-alginate beads by yeast cells was investigated by scanning electron microscopy (SEM), using an electronic microscope (JSM-6060, Tokyo, Japan). Bead samples were collected at 0 and 128 h of fermentation from bioreactors. The spheres were dipped into liquid nitrogen for 15 min and immediately freeze-dried for 48 h. The samples were fixed on aluminum stubs and coated with gold before being analyzed.

RESULTS AND DISCUSSION

Immobilized batch fluidized bed bioreactors with three different strains of *K. marxianus*

This set of experiments was carried out in order to determine the bioconversion of whey into ethanol by the three strains of *K. marxianus* immobilized in Ca-alginate beads. This was done in order to compare the widely used CBS 6556 with other strains, CCT 2653 and 4086, which had not been tested in this type of bioprocess.

Table 1. Ethanol yields ($Y_{EtOH/S}$), efficiency of conversion (η), and productivity (Q_P) of three strains of *Kluyveromyces marxianus* in fluidized batch fermentation

Yeast	$Y_{EtOH/S}$ (g EtOH g^{-1} sugar)	Conversion efficiency (%)	Q_P (g L^{-1} h^{-1})
CBS 6556	0.45	83.3	0.96
CCT 4086	0.43	79.1	0.81
CCT 2653	0.45	83.3	0.84

The profiles of lactose consumption and ethanol production for the three strains in batch fluidized bed bioreactor are presented in Fig. 2. All strains completely depleted lactose after only 9 h of cultivation, with strain CCT 2653 showing a slower sugar consumption rate than the other two (Fig. 2(a)). The highest ethanol concentration was produced by strain CBS 6556 (Fig. 2(b)), reaching 25.2 g L^{-1} after only 7.5 h of fermentation. This strain also showed the highest ethanol productivity (Q_P) at 0.96 g L^{-1} h^{-1}, compared with 0.81 g L^{-1} h^{-1} for CCT 4086 and 0.84 g L^{-1} h^{-1} for CCT 2653 (Table 1). Nevertheless, high yields of ethanol ($Y_{EtOH/S}$) were obtained for all three strains, varying from 0.43 to 0.45 g EtOH g^{-1} sugar, with conversion efficiencies (η) ranging from 79.1% to 83.3% of the maximal theoretical conversion (Table 1). These results compare very well with others reported in the literature. Lins and Leão[12] studied ethanol production from skimmed milk bioconversion with *K. marxianus* CBS 6164 immobilized in 2% Ca-alginate at 30 °C in batch cultivations, and obtained a conversion efficiency of 70%. Gunasekaran and Kamini,[11] used 3.5% Ca-alginate gel to immobilize *K. fragilis* NRRL 665 to produce ethanol in batch cultivation with synthetic medium containing 200 g L^{-1} of lactose, and reported a productivity of 0.88 g L^{-1} h^{-1} and a yield of 0.44 g EtOH g^{-1} sugar. Guo *et al*.[23] co-immobilized *K. marxianus* and *S. cerevisiae* in Ca-alginate gel beads to obtain ethanol from whey (100 g L^{-1} of lactose) and obtained a maximum conversion of 79.9% with ethanol productivity of 0.88 g L^{-1} h^{-1}. All these works were conducted in shaker flasks, while in our research a more realistic representation of a production system, the bioreactor, was used.

Compared with cultures of free suspended cells, the entrapped systems used in this research performed much better. Cultures of free cells of *K. marxianus* MTCC 1288 on whey (35 g L^{-1}) in shaker flask produced only 2.10 g L^{-1} of ethanol over 22 h of cultivation.[24] For free suspended cells of *K. marxianus* NRRL 1195 growing on whey (70 g L^{-1}), the best yields of ethanol were 0.36 g g^{-1} after 48 h of cultivation.[25]

Effects of temperature on ethanol production in batch fluidized bed bioreactors

K. marxianus CBS 6556 gave the best results in the first set of experiments and was, therefore, chosen for the cultivations to test the effects of temperature in the batch fluidized bed bioreactors system. Three fermentation temperatures were tested in order to determine the highest ethanol production. This was an important investigation, since some authors reported that *K. marxianus* strains were capable of growing and producing ethanol at elevated temperatures, using different carbon sources, such as molasses,[6] sucrose,[26] glucose,[27,28] and lactose.[29,30] Fig. 3 shows the profile of lactose consumption and ethanol production at 30, 35, and 40 °C in the batch fluidized bed bioreactor. Although the kinetics of lactose consumption was somewhat slower at

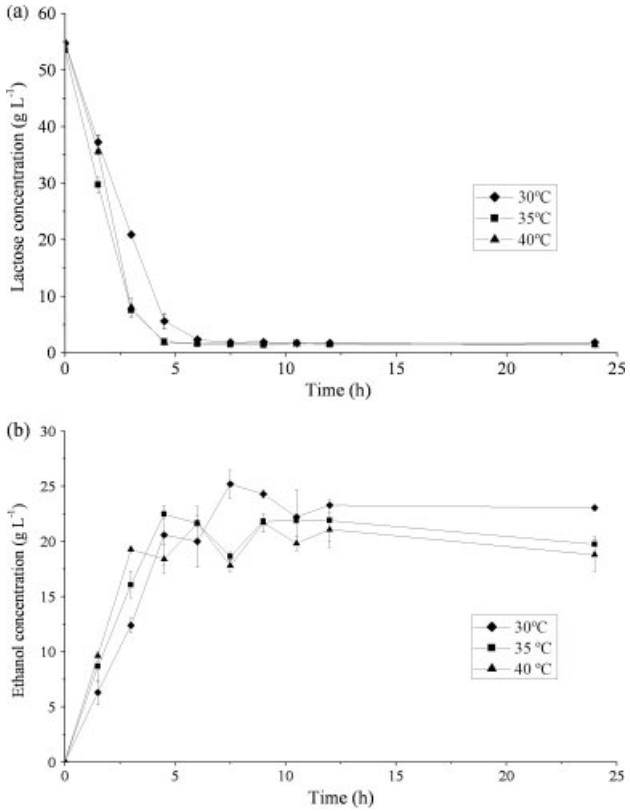

Figure 3. Kinetics of lactose consumption (a), and ethanol production (b) by *Kluyveromyces marxianus* CBS 6556 in the batch fluidized bed bioreactor as a function of temperature.

Table 2. Ethanol yields ($Y_{EtOH/S}$), efficiency of conversion (η), and productivity (Q_P) of three strains of *Kluyveromyces marxianus* in fluidized batch fermentation as a function of temperature

Temperature (°C)	$Y_{EtOH/S}$ (g EtOH g^{-1} sugar)	Conversion efficiency (%)	Q_P (g L^{-1} h^{-1})
30	0.45	83.3	0.96
35	0.41	76.3	0.82
40	0.36	66.1	0.78

30 °C, at this temperature the highest ethanol yields ($Y_{EtOH/S}$) and productivity (Q_P) were obtained, as is shown in Table 2. This was also true for the conversion efficiency (η), reaching 83.3% of the maximum theoretical conversion at 30 °C, compared with 76.3% and 66.1% for 35 and 40 °C, respectively.

Kourkoutas *et al*.[5] investigated the influence of temperature (37 °C, 45 °C, and 50 °C) on cultures of immobilized *K. marxianus* IMB3 in delignified cellulosic material growing in synthetic medium and lactose, and reported a significant decrease in ethanol production above 45 °C. The maximum conversion efficiency reported by these authors was approximately 27%. Limtong *et al*.[31] investigated the effects of temperature (30 to 45 °C) on ethanol production from sucrose by free cells of several strains of *K. marxianus* (DMKU 3–1042, DMKU 3–118, DMKU 3-p106, and DMKU 3-p1042), and reported best ethanol productivities at 30 and 37 °C (0.99 and 1.06 g L^{-1} h^{-1}), representing around 60 and 77% of the theoretical maximum conversion in shake flask, which dropped to only 57% in bioreactor cultures at 37 °C. Banat and Marchant[32] investigated ethanol production by free suspended cells of several *Kluyveromyces* strains (IBM1, IBM2, IBM3, IBM4, and IBM5) at 45 °C, using synthetic medium with different carbon sources (lactose, whey permeate, cellobiose, and xylose) and observed the highest ethanol concentration of 17 g L^{-1} for whey permeate (40 g lactose L^{-1}), representing 83% of the maximum theoretical conversion by strain IBM2. Finally, Nolan *et al*.[28] studied ethanol production by Ca-alginate immobilized *K. marxianus* IMB3 at 45 °C in synthetic medium containing glucose as carbon source and reported an efficiency conversion of 70 to 80% of the maximum theoretical.

Continuous fluidized and packed beds bioreactor cultivations

Cell immobilization is a promising technology to improve the operation of continuous culture (CC) bioreactors, since the biocatalyst can be kept at high concentrations in the system. This set of experiments tested the effects of different dilution rates (0.1, 0.2, and 0.3 h^{-1}) in both fluidized and packed bed bioreactors with *K. marxianus* 6556 at 30 °C. Figure 4 shows the kinetics of these experiments. The rate of lactose utilization and the mean ethanol concentration decreased inversely with D, with the fluidized bed bioreactors performing better than packed bed bioreactors, as is shown in Table 3. The better performance of the fluidized bed bioreactor reflects the better mass transfer mechanisms of this system over the packed bed configuration, since homogenization of the medium is provided by recirculation. Lactose was totally consumed at $D = 0.1$ h^{-1} in the fluidized bed bioreactor. Nevertheless, true steady-state was achieved for all tested dilution rates (Fig. 4). These are very good results when compared with other research using the *Kluyveromyces* yeasts on whey, in continuous cultivation. *K. marxianus* DSMZ-7239 immobilized in olive pits was grown using whey (50 g L^{-1} of total sugar) in a packed bed bioreactor, with ethanol production varying from 10.5 to 19.8 g L^{-1} and sugar consumption from 63 to 70% when increasing the hydraulic residence time (HRT = 1/D) from 17.6 to 50 h.[14] In contrast, in this work, the bioreactors operated with a much shorter HRT, which varied from 3.3 to 10 h. Work with other yeasts and systems can also be compared. Immobilized *S. cerevisiae* growing on glucose and sucrose from sorgum bagasse varied its ethanol production from 62 to 38 g L^{-1} when increasing D from 0.2 to 0.4 h^{-1}.[9] The experimental validation of the mathematical model for predicting sugar and ethanol concentrations during the continuous fermentation of whey by *Candida pseudotropicalis* showed the highest ethanol production of 58 g L^{-1} at the highest hydraulic residence time of 42 h, with 150 g L^{-1} of lactose in the feeding medium.[13] Finally, Nigam[33] investigated the ethanol production from pineapple cannery waste (82.3 g L^{-1} of total sugars) with *S. cerevisiae* ATCC 24 553 immobilized in k-carrageenan in a packed-bed tapered glass column reactor at 30 °C and observed the highest sugar consumption and ethanol production of 37 g L^{-1} for the lowest tested D (0.2 h^{-1}), with performances sharply decreasing up to $D = 2.5$ h^{-1}.

Figure 4(c) depicts the concentration of free cells in the fluidized bed bioreactor showing that it decreased inversely with D. These free cells appeared with time during the continuous culture as a consequence of cell growth and the complete colonization of the Ca-alginate spheres (cell leaking to the medium). No significant mechanical disruption of these spheres was observed. These results reflect the fact that there is a cell washout at dilution rates higher than the critical dilution rate (where $D > \mu_m$), that for *K. marxianus* growing under anaerobiosis, has been reported to vary from 0.16 to 0.25 h^{-1}.[24,34] At $D = 0.3$ h^{-1} the free suspended

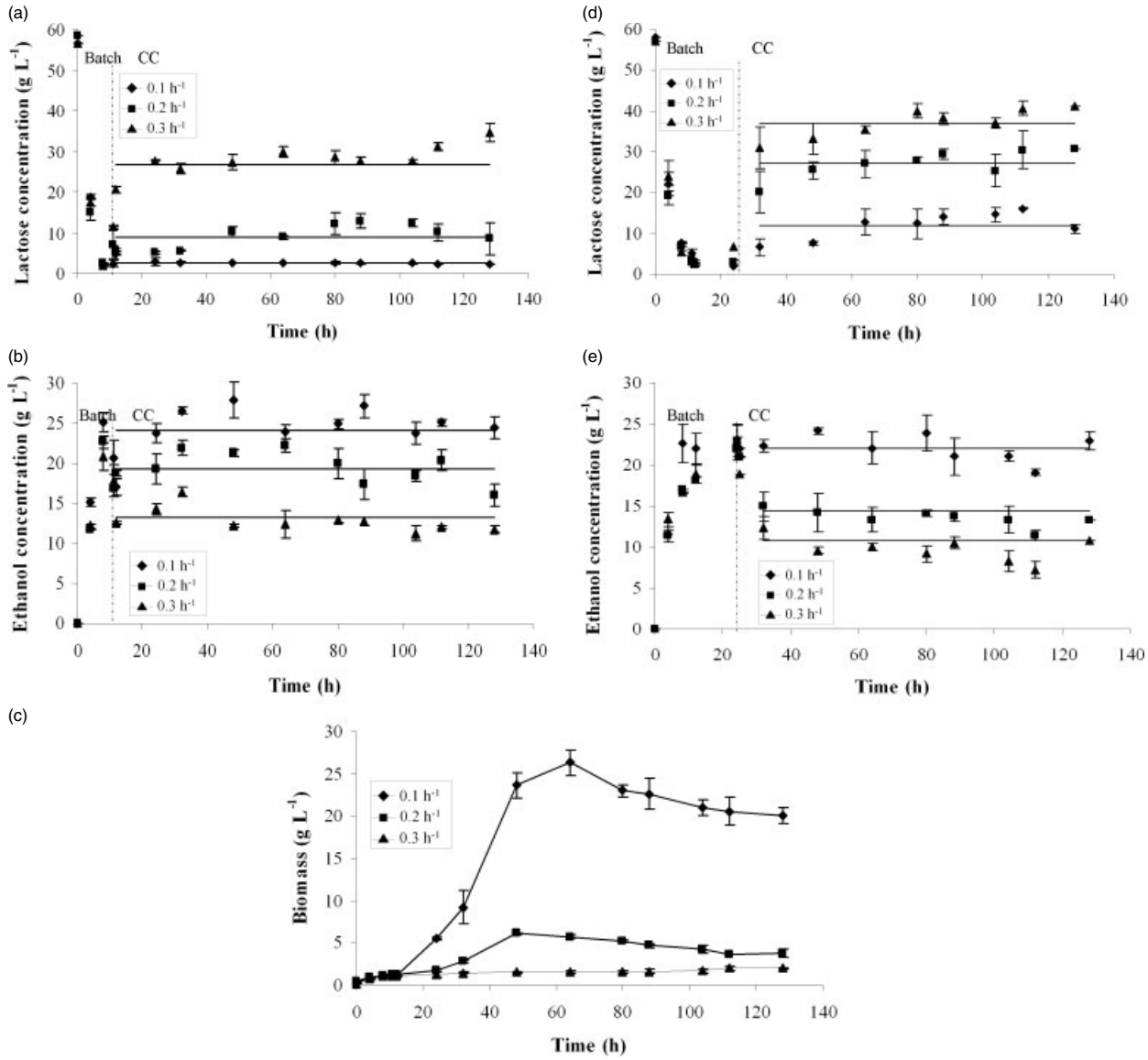

Figure 4. Kinetics of lactose consumption (a), ethanol production (b), and free biomass (c) in the continuous fluidized bed bioreactor at three dilution rates; kinetics of lactose consumption (d) and ethanol production (e) in the continuous packed bed bioreactor at three dilution rates.

Table 3. Comparison of lactose utilization and ethanol production as functions of dilution rates in continuous fluidized and packed bed bioreactors

	Lactose consumption (%)		Ethanol production (g L^{-1})	
D (h^{-1})	Fluidized	Packed	Fluidized	Packed
0.1	95.6	78.4	25.3	22.1
0.2	83.8	51.5	19.7	13.3
0.3	48.8	33.5	12.9	9.3

cells in the fluidized bioreactor was negligible, compared with slower feedings, with approximately 25 and 5 g L^{-1} at D of 0.1 and 0.2 h^{-1}, respectively. Comparatively, Ozmihci and Kargi[14] reported concentrations of free suspended cells varying from 7.8 g L^{-1} to 4 g L^{-1} when decreasing the HRT from 50 to 17.6 h in packed bed bioreactors with immobilized *K. marxianus* DSMZ-7239 growing on whey. Some other work also showed similar profiles of reduction of free suspended cells in continuous cultivations with increasing dilution rates, all attributing this fact to the washout of cells from the system.[13,33,35] As can be seem in the electronic micrograph (Fig. 5), *K. marxianus* CBS 6556 entirely colonized the Ca-alginate matrix, saturating the beads at the end of the runs, suggesting a vigorous growth under the conditions of this work and further supporting the findings of leakage of free suspended cells into the liquid medium. Table 4 summarizes the findings of this research compared with other reports discussed above.

Figure 6 shows the variation of ethanol productivity (Q_P), yields ($Y_{EtOH/S}$), and concentration with dilution rate. Ethanol productivity increased with dilution rate in both fluidized and packed bed bioreactors, with the first system showing higher productivities, probably due to better homogenization of the fermentation medium. The highest ethanol productivity (3.5 g L^{-1} h^{-1}, D = 0.3 h^{-1}; fluidized bed bioreactor) reached 87% of theoretical conversion. The yields of ethanol ($Y_{EtOH/S}$) were also affected

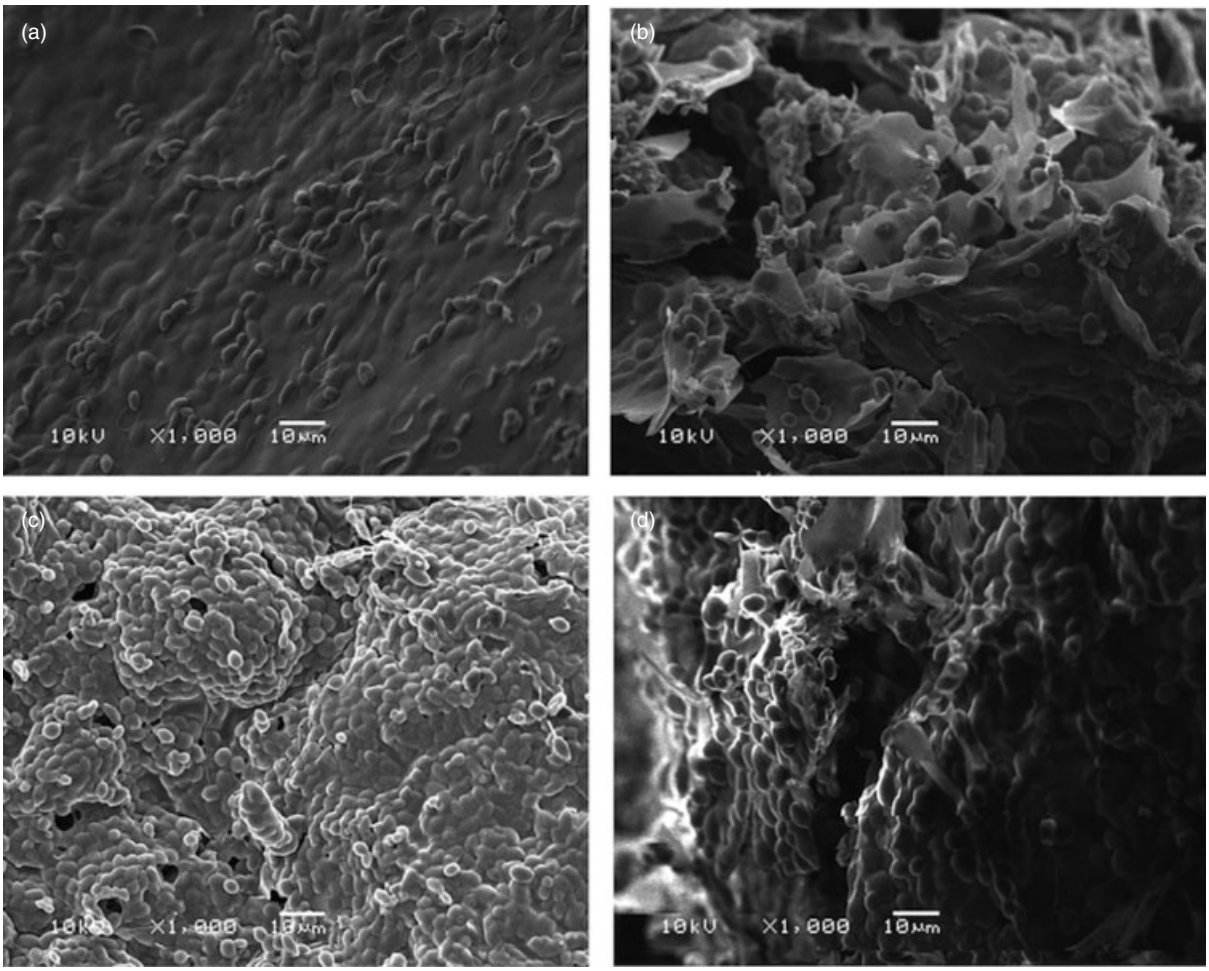

Figure 5. Scanning electron micrography showing the colonization profile of Ca-alginate beads by *K. marxianus* CBS 6556: (a) outer surface of beads at time 0 h; (b) inner surface of beads at time 0 h; (c) outer surface of beads at cultivation time 128 h; (d) inner surface of beads at cultivation time 128 h. Magnification 1000×.

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

Bioreactor operational system	Substrate	$Y_{EtOH/S}$ (g EtOH g^{-1} sugar)	Q_P (g L^{-1} h^{-1})	Conversion efficiency (%)	Reference
Batch	Cheese whey	0.45	0.96	83.3	This work
	Synthetic medium with lactose	0.44	0.88	61.9	11
	Cheese whey	–	0.88	79.9	23
	Skim milk	–	–	70	12
Continuous (HRT)*					
3.3 to 10	Cheese whey	0.40 to 0.47	3.5	74.6 to 87	This work
18 to 42 h	Cheese whey	0.44 to 0.47	–	75 to 98.3	13
17.6 to 64.4	Cheese whey	0.32 to 0.54	0.28 to 0.58	–	14

* Hydraulic residence time (HRT = 1/D).

by the bioreactor system and the dilution rates (Fig. 6), ranging from 0.40 and 0.48 g EtOH g^{-1} sugar. Ghaly and El-Taweel[13] reported similar ethanol yields (0.44 to 0.47 g EtOH g^{-1} sugar) in continuous cultivations using different concentrations of lactose and HRTs using *Candida pseudotropicalis* ATCC 8619. Although not directly comparable with results in this research, other yeasts and substrates were also investigated by several authors in packed bed immobilized continuous cultures and results showed the same profile of ethanol productivities and yields depending on the dilution rates.[8,27,36]

CONCLUSIONS

Immobilized cell bioreactors hold a good potential for ethanol production from whey, since the biocatalyst can be managed to high densities, increasing the overall system productivity.

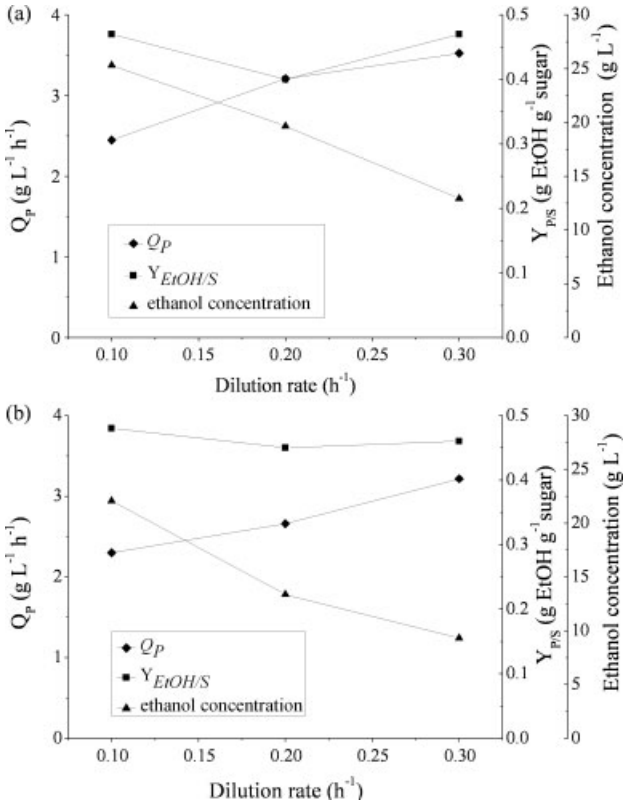

Figure 6. Variations of ethanol productivity (Q_P), yields ($Y_{EtOH/S}$), and concentration (g L^{-1}) as a function of dilution rates (D) in continuous fluidized bed bioreactor (A) and continuous packed bed bioreactor (B).

Comparisons of batch and continuous cultivations of Ca-alginate immobilized cells of *K. marxianus* strains were tested at different temperatures and dilution rates with fluidized and packed bed bioreactor forms, and high yields and productivities of ethanol were obtained. Fluidized bed bioreactors could be operated at dilution rates higher than the critical dilution value for *K. marxianus* CBS 6556 when growing in anaerobiosis, without any noticeable mechanical disruptions of Ca-alginate beads. Scanning electron microscopy of the support showed that cell immobilization in the alginate gel was very effective. Further research is to be undertaken in order to scale-up this process, specially aiming at increased sugar concentration feedings in order to improve the final ethanol concentration.

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