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Continuous Production of Ethanol from Starch Using Glucoamylase and Yeast Co-Immobilized in Pectin Gel

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Abstract This work presents a continuous simultaneous saccharification and fermentation (SSF) process to produce ethanol from starch using glucoamylase and *Saccharomyces cerevisiae* co-immobilized in pectin gel. The enzyme was immobilized on macroporous silica, after silanization and activation of the support with glutaraldehyde. The silica–enzyme derivative was co-immobilized with yeast in pectin gel. This biocatalyst was used to produce ethanol from liquefied manioc root flour syrup, in three fixed bed reactors. The initial reactor yeast load was 0.05 g wet yeast/ml of reactor (0.1 g wet yeast/g gel), used in all SSF experiments. The enzyme concentration in the reactor was defined by running SSF batch assays, using different amount of silica–enzyme derivative, co-immobilized with yeast in pectin gel. The chosen reactor enzyme concentration, 3.77 U/ml, allowed fermentation to be the rate-limiting step in the batch experiment. In this condition, using initial substrate concentration of 166.0 g/l of total reducing sugars (TRS), 1 ml gel/1 ml of medium, ethanol productivity of 8.3 g/l/h was achieved, for total conversion of starch to ethanol and 91% of the theoretical yield. In the continuous runs, feeding 163.0 g/l of TRS and using the same enzyme and yeast concentrations used in the batch run, ethanol productivity was 5.9 g ethanol/l/h, with 97% of substrate conversion and 81% of the ethanol theoretical yield. Diffusion effects in the extra-biocatalyst film seemed to be reduced when operating at superficial velocities above 3.7×10^{-4} cm/s.

Keywords Ethanol · Cassava starch · *Saccharomices cerevisiae* · Glucoamylase · Packed-bed reactor · Simultaneous saccharification and fermentation

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Nomenclature

TRS	total reducing sugar, expressed as glucose concentration (g/l)
Et	ethanol concentration (g/l)
G	glucose concentration (g/l)
X_g	cell concentration in the gel (viable cells/g gel)
N_{viable}	average viable cell number in a square of the Neubauer chamber
M_{gs}	mass of the gel particles sample
Q_{feed}	feed volumetric flow rate (ml/h)
Q_r	recycle volumetric flow rate (ml/h)
RR1	recycle ratio in the first reactor
V_s	superficial velocity flux (cm/s)
U	unit of free enzyme
x_s	liquified starch (TRS) conversion
θ	residence time (h)=reactor volume/Q_{feed}
Pr_{et}	ethanol productivity (%)
η_{et}	ethanol yield (%)
theoretical yield	theoretical conversion of glucose to ethanol=0.51

Introduction

Brazil has been the major ethanol producer since the 1970s, with the implementation of the PROALCOHOL Program, using sugar cane as raw material [1]. In the last years, using the corn dry-grind process, USA has steadily increased its ethanol production, reaching in 2005, a figure similar to the one obtained in Brazil, around 4.2 billion gallons of ethanol per year [2].

The most important sources of biomass to produce bioethanol are clearly defined in the two more important producers. However, the increasing world demand for biofuels makes all the possible biomass eligible as raw materials. In Brazil, sugar cane distilleries only operate from March to November, the sugar cane crop period, and therefore, the option of a different feedstock that could extend the use of the industrial plant would be very attractive. Besides, culture rotating would improve the use of the soil that would be managed in a more sustainable manner.

Cassava, casava, or manioc (*Manihot esculenta*) is a plant native to South America that is extensively cultivated as an annual crop in tropical and subtropical regions for its edible starchy tuberous root, a major source of carbohydrate. It is a shrub with an average height of 1 m, and has a palmate leaf formation. Cassava roots contain a high concentration of carbohydrates (about 80%), mainly starch, significant amounts of calcium (50 mg/100 g), phosphorus (40 mg/100 g), and vitamin C (25 mg/100 g). Cassava gives the highest yield of food energy per cultivated area per day among crop plants, except possibly for sugarcane. Although the manioc roots are poor in protein and other nutrients, the leaves are a good source of protein if supplemented with the amino acid methionine. Whereas other crops such as yam, maize, banana, and plantain, cowpea, or sorghum and millet are eco-regionally specific, cassava is probably the only crop whose production cuts across all ecological zones.

The world cassava production in 2005 was 208 million tons, 55% in Africa, 32% in Asia (12% in Thailand), and 13% in Brazil. The global trade in cassava products in 2005 was 6.2 million tons. Dried cassava roots are used as raw material for compound animal feed, while

cassava starch is used for manufacture of paper, textiles, adhesives, and alcohol [3]. In Thailand, the construction of 12 cassava ethanol plants, with the total output of 3.4 million l/day by the next 2 years has just been approved. An assessment of the net energy and supply potentials to evaluate the utilization of cassava for fuel ethanol (CFE) in Thailand was performed [4]. This study showed that the CFE system is energy efficient, with positive net energy value and net renewable energy value of 8.80 and 9.15 MJ/l, respectively.

Centrifugation is a necessary step for the separation and recycle of the yeast cream in the Melle-Boinot process, the leading technology to produce ethanol from sugar cane that has been used in Brazil since the 1960s [1]. Centrifugation costs have a significant role in the production of ethanol via fermentation, and the use of immobilized microorganisms in the process avoids this step, thus reducing total costs. On using starch as raw material, the time and energy required for hydrolysis and the price of enzymes represent additional costs when compared to directly fermentable raw materials. Simultaneous saccharification and fermentation (SSF) involves the hydrolysis of the polysaccharides into glucose and its conversion to ethanol in the same vessel. Some advantages of this method, compared to separate hydrolysis and fermentation, are: the cost saving resulting from the reduction of the number of reactor vessels that are required (lower capital costs), the increased rate of hydrolysis due the lower inhibition by product, and the reduction of fermentation time [5]. The SSF process is already used in the conventional dry-grind corn process [6]. Immobilization of the enzyme and of the microorganism allows their reutilization, what may turn economically viable the use of high concentrations of the biocatalysts in the reactor, reducing reaction times. This technique has been extensively studied to reduce process costs [7–9]. Using immobilized glucoamylase and *Zymomonas mobilis*, the SSF process was compared to a separated hydrolysis and fermentation (SHF) process for the production of ethanol from starch in a fluidized bed reactor (FBR) [10]. This work showed that the SHF led to higher productivities than the SSF process, due to the low activity of the enzyme at 35°C, operation temperature for the SSF process. Economic analysis of the ethanol production in FBR reactors showed that there is operation and cost savings when this reactor configuration is used to produce ethanol from starch [11].

The fabrication of ethanol from liquefied manioc root flour syrup is studied here, using microorganism and enzyme co-immobilized in pectin gel. This process would be complementary to the fermentation of sugar cane molasses in sugar mills. In this process, glucoamylase (or amyloglucosidase) is first covalently immobilized in controlled porosity silica (CPS). After that, the derivative CPS-enzyme is co-immobilized with *Saccharomyces cerevisiae* in pectin gel. This technique makes operation at 30°C (optimum fermentation temperature) economically feasible because the immobilization of high loads of enzyme allows reaching high rates of hydrolysis in the reactor, even at such a low temperature. This strategy counterbalances the fact that the optimal operation temperature for glucoamylase is 60°C.

Pectin pellets are formed by the action of bivalent cations, such as calcium, that allow the formation of crossed bonds between the polymeric pectin precursors. The presence of calcium in the medium is essential to maintain gel integrity, thus avoiding the leaching of this cation [9].

Batch and continuous experiments were run. The continuous set-up consisted of three fixed-bed reactors in series, to allow the escape of the produced CO_2 in each reactor. Various process features were studied: determination, in batch experiments, of the most adequate rate between concentrations of enzyme and yeast in the reactor, investigation of the performance of continuous SSF in a packed-bed reactor, to analyze the influence of the superficial velocity on the performance of the continuous SSF process.

Materials and Methods

Materials Manioc root flour, from peeled, dried, and milled root, was purchased from "Ricieri Pechatt & Filhos", Araras, SP, Brazil; α-amylase P-500 (EC 3.2.1.1), was supplied by Pfizer S. A.; glucoamylase 200 L (EC 3.2.1.3), with 190 U/ml of activity (where 1 U is the quantity of enzyme that produces 1 g of glucose per hour, from 4% soluble starch at 60°C and pH 4.2) and 128 mg protein/ml, was donated by NOVO Industri do Brasil; commercial *S. cerevisiae* (60% of moisture), from Fleischmann SA; controlled pore silica (CPS) was supplied by Corning Glass Works (Corning, NY), with average pore size of 37.5 nm and internal porosity of 56.6% (diameter below 100 μm); citric pectin type 8002 supplied by Braspectina S.A. All the other reagents used were laboratory grade from different commercial sources.

Enzyme Immobilization Silica was silanized with a 5% *v*/*v* aminopropyltrietoxysilane solution, pH 3.3, 75°C, for 3 h at a liquid/solid ratio of 3 ml/g. The silica was then washed with water, dried at 105°C for 15 h, and activated with glutaraldehyde (2.5% hydrogenphosphate buffer, 0.1 M, pH 7.0) for 1 h at 20–25°C at a liquid/solid ratio of 5 ml/g, under stirring at the three stages.

Co-immobilization Pectin, 6 g, was dissolved in 78 ml distilled water, with subsequent addition of 6 ml sodium acetate buffer (1 M, pH 4.2) and 10 g wet yeast. Silica containing immobilized enzyme (206.5 U/g dried silica or 272.2 U/g dried silica) was then mixed with the suspension at the ratio of 1.5 g of wet silica–enzyme/20 g yeast–pectin suspension. One gram of dried silica corresponded to 1.5 of wet silica. Finally, the suspension was dropped in a 0.2 M $CaCl_2$ solution, and 18.5 g of 4 mm spherical particles were formed after curing in a refrigerator, for 18 to 20 h.

Manioc Root Flour Syrup A suspension of flour (approximately 300 g/l) in a 0.01-M NaOH/0.01 M $CaCl_2$ solution was prepared, adjusting the pH set between 6.0 and 6.5. Thereafter, the suspension was heated under stirring, and at 65°C, 0.3 g alpha-amylase per liter of suspension was added. This was kept at 90°C for 10 min, then boiled for 5 min, and vacuum filtered in syrup paper. For the fermentation, $NaH_2PO_4{\cdot}H_2O$ (1.0 g/l), $MgSO_4{\cdot}7H_2O$ (0.25 g/l), yeast extract (0.5 g/l), $CaCl_2$ (2.0 g/l), and urea (1.5 g/l) were dissolved and added to the liquefied starch solution.

Experimental Assays All experiments were performed at 30°C, initial pH of 4.0. Batch tests were carried out in glass flasks with 3 cm of internal diameter, in a reciprocal shaker, at 150 rpm. Each flask contained a known volume of substrate (liquefied cassava starch syrup) and an equal volume of biocatalyst beads. In continuous tests, a setup of three glass reactors was used with volumes of 35, 30, and 25 ml or 110, 104, and 100 ml, internal diameter of 5 cm, with or without recycle, and with exhaustion of the CO_2 formed in the first two stages in a water column. This allowed control of the reactors' internal pressure as shown in Fig. 1.

Analysis

TRS and Glucose Liquefied starch concentration was determined by dosing total reducing sugars (TRS), expressed in terms of glucose concentration, after enzymatic hydrolysis of the liquefied starch present in the sample, using glucoamylase, diluted 1:200 in acetate or

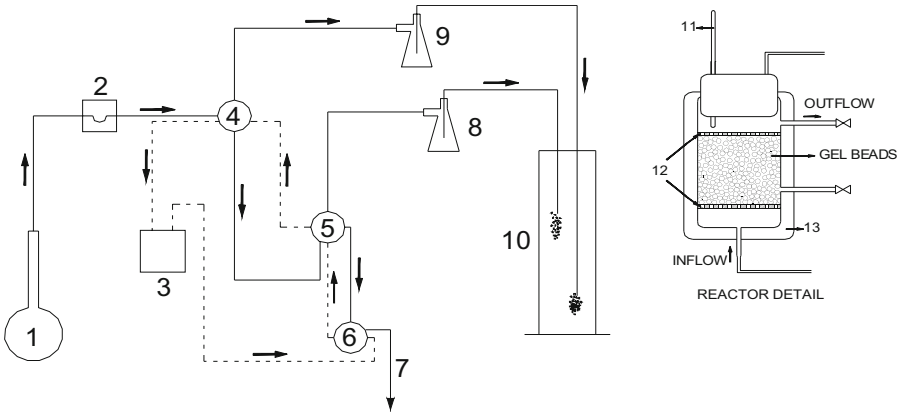

Fig. 1 Reactor system for continuous runs: *1* Feed flask, *2* pump, *3* bath; *4*, *5*, *6* reactors 1, 2, and 3, *7* effluent, *8*, *9* security flasks, *10* water/$CuSO_4$ column. A reactor detail: *11* thermometer, *12* stainless steel screen, *13* jacket. Water recirculation (*dashed line*); substrate (*line*)

citrate buffer 0.1 M, pH 4.2, at 45°C, for 30 min, followed by enzyme inactivation in boiling water, for 5 min [12].

TRS (grams of glucose/liter)=$(0.977\times(G_m-G_i)/0.9)+G_i$($G_m$=glucose concentration in the sample after enzymatic hydrolysis; G_i = glucose concentration in the sample before enzymatic hydrolysis; 0.977=empiric factor to convert in starch the glucose obtained by enzymatic hydrolysis of the liquefied starch=0.9/enzymatic hydrolysis yield, determined by Schmidell and Fernandes, 1977, using soluble starch, analytical grade [11]; 0.9= stoichiometric glucose/starch conversion factor). Glucose and TRS were dosed using a glucose-oxidase Kit (CELM S.A.)

Ethanol This is determined by oxidation with $K_2Cr_2O_7$, followed by titration with Fe $(NH_4)_2(SO_4)_2{\cdot}6H_2O$–Mohr salt [13] and using a Waters high performance liquid chromatography (HPLC), an ion exchange Shodex® column, at 80°C, refraction index detection, at 34°C, elution with Milli-Q water, flow rate of 1.0 ml/min. All samples were analyzed using the titration method. HPLC was used to confirm the results obtained with the titration method. The two methods led to very similar results.

Cellular Viability and Concentration Determined by counting cells in nine squares of a Neubauer chamber after dying them with methylene blue solution. Viable cells were not colored, and dead cells were blue; free yeast concentration was also obtained by filtering a known volume of cell suspension and drying the wet mass until the constant weight was achieved; viability and concentration of immobilized yeast were determined as already described, after dissolution of the pectin gel [1.0 g of cured pellets was dissolved in 20 ml of 5% ethylenediaminetetraacetic acid (EDTA) solution at constant agitation]. The cell concentration was calculated as:

$$X_g = \left(N_{viable}/4\times10^{-6}\right)\times\left(\left(20+\left(M_{gs}/\rho_g\right)\right)/M_{gs}\right)\times 10,$$

where:

X_g	—cell concentration in the gel (viable cells/gram gel)
N_{viable}	—average viable cell number in one square of the Neubauer chamber
20	—volume of EDTA solution (ml)
M_{gs}	—mass of the gel particles sample
ρ_g	—gel particles density
4×10^{-6}	—volume of the one square of the chamber (ml)
10	—sample dilution factor in methylene blue solution

Bead density and diameter Bead density was determined before the beginning of the run and immediately after finishing it, using a pycnometer. After finishing the run, the liquid and the particles were collected and separated. The liquid volume was measured. The particles were first dried by contacting them with an absorbent paper, following the same procedure used before the beginning of the run. The pectin bead diameter was determined measuring the volume of 500 particles, using a pycnometer.

Soluble and Immobilized Enzyme Activity Soluble and immobilized enzyme activity was determined in standard conditions [14]: a unity (U) is the amount of enzyme that liberates 1 g of glucose in 1 h at 60°C, pH 4.2, from a 4% soluble starch solution. Amount of immobilized enzyme was calculated by the difference between the offered load and the remaining enzyme in the supernatant, after the immobilization procedure.

Starch Conversion (X_s) and Ethanol Yield (η_{et}) X_s was calculated as (TRS(*i* or feed)−TRS(*f* or outlet))/TRS(*i* or feed); η_{et} was calculated as (Et(*f* or outlet)/(TRS(*i* or feed)−TRS(*f* or outlet))×0.51).

Results and Discussion

Batch Experiments: Selecting Enzyme Concentration for the Continuous Experiments

The initial yeast concentration in the reactor, for all the SSF assays was 50 g wet yeast/l of reactor (0.1 g wet yeast/g gel). In previous continuous tests using the same equipment 50 g wet yeast/1 of reactor allowed obtaining increasing ethanol profile from the first to the third reactor, in the conversion of sucrose from molasses in ethanol (results not shown). In the present system, the liquefied starch has to be hydrolyzed inside the particle before the fermentation step, and therefore, the substrate availability to the microorganism is different. Nevertheless, as it was intended to operate the SSF process with the fermentation being the rate-limiting step, it was believed that the ethanol profile obtained with sucrose might be reproduced with hydrolyzed starch, using the same yeast concentration in the reactor. This yeast concentration in the reactor was then chosen and fixed to study the SSF process. The next step was to choose an enzyme concentration in the reactor that allowed keeping glucose available in the reaction medium, with the total hydrolysis time lower than the total fermentation time. In this way, fermentation would be the rate-limiting step. The experiments for choosing the enzyme concentration were performed in batch mode. Therefore, batch SSF tests were run, keeping the same yeast concentration—50 g wet yeast/l of reactor (0.1 g wet yeast/g gel) and varying the amount of enzyme. Figure 2a,b shows

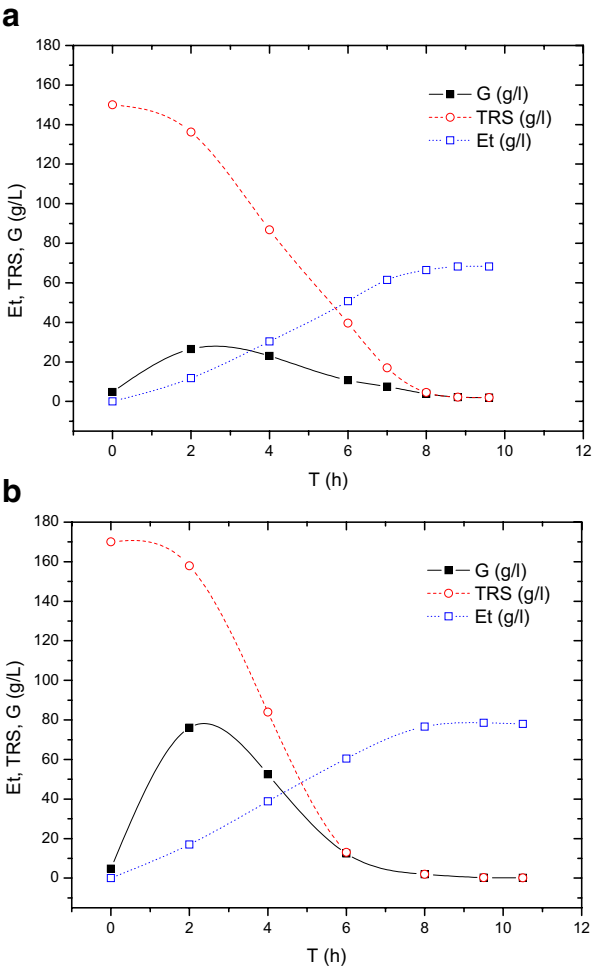

Fig. 2 Total reducing sugar (TRS), ethanol (Et), and glucose (*G*) concentrations with time (*T*), for operation of SSF process in discontinuous runs, under two different enzyme concentration in the reactor: **a** 2,860 U glucoamylase/l reactor; TRS_i=150.0 g/l; G_i=5.3 g/l; **b** 3,770 U glucoamylase/l reactor; TRS_i=166.0 g/l; G_i=4.8 g/l. Yeast concentration**:** 50 g wet yeast/l reactor; gel volume/medium volume=1.0. Batch runs

the obtained results for the two assayed conditions, 2,860 and 3,770 U/l, respectively. Observing the results showed in Fig. 2a, it can be seen that the hydrolysis is the rate-limiting step for the lower enzyme concentration tested. Figure 2b shows the results for the higher enzyme concentration, 3,770 U/l of reactor. Using this condition, the time for total hydrolysis of the liquefied starch was 6 h (TRS and glucose concentrations were equal), and the total fermentation time was 9.5 h. The maximum glucose concentration was 76.0 g/l, and maximum ethanol concentration was 78.5 g/l, which were reached, respectively, around 2 and 9.5 h.

It can be observed from the results presented in Fig. 2b that the system is well-balanced, using 3770 U/l and 50 g of wet yeast/l of reactor. There is always glucose available for the microorganism, the time for total hydrolysis is lower than for total fermentation, and therefore, starch hydrolysis is not the rate-limiting step. Productivity was 8.3 g/l/h, with 99.8% of conversion and yield of 0.47 (91% of the theoretical). This condition was then chosen to run the continuous SSF experiments.

Performance of the Continuous Reactor System

The three SSF continuous experiments were operated using 3,770 U/l of enzyme and 50 g of wet yeast/l of reactor, as determined in the batch runs. The first continuous run was performed with a total reactor volume of 90 ml (the volume was delimited by two stainless sieve), during 226 h, feeding 163.0 g/l of TRS. Flow rates changed from 8.1 to 44.0 ml/h. Sampling was made three residence times after changing the flow rate. Table 1 shows results of ethanol production and yeast concentration in the outlet of each reactor. Table 2 shows the concentrations of total reducing sugars and glucose.

Table 1 shows that the maximum ethanol concentration in this test was 67.7 g/l, in the outlet of the third reactor, for a flow rate of 8.1 ml/h. In this condition, the TRS and glucose concentrations were 3.3 and 0.8 g/l, respectively. The system is stable for all flow rates. The lowest flow rate, 8.1 ml/h, was more difficult to control, which was what led to some more pronounced oscillation of the variable values in this condition. It was expected in the continuous run that similar results than the ones obtained in the batch run will be achieved. As in batch operation, the cycle time is considerably longer than the reaction time, if the continuous process had reached a similar performance than the one obtained with the batch run; in the continuous operation, we could save cycle time (times to clean, to fill ant to empty the reactor). Therefore, from the industrial point of view, the continuous process would lead to higher productivities.

It is clear, however, that the operation of the SSF process in continuous reactors led to a worse performance than the one obtained in the batch experiments, with the same enzyme and yeast concentrations in the reactor and similar total reducing sugar concentration (initial, in the batch run and in the feed, for the continuous run). The concentration of free glucose in the effluent was lower than 1 g/l for all the tested flow rates, except for the two

Table 1 SSF continuous run 1.

T (h)	Q_{feed} (ml/h)	Et_1 (g/l)	Et_2 (g/l)	Et_3 (g/l)	Xef_1 cel/ml×10^7	Xef_2 cel/ml×10^7	Xef_3 cel/ml×10^7
23.0	8.1	52.3	63.1	63.1			
42.5	8.1	48.8	63.3	67.7			
54.0	8.1	47.9	58.7	66.0			
68.0	8.1	41.3	57.0	64.8	6.4	6.2	5.8
95.0	11.0	31.6	48.5	57.1			
114.0	11.0	32.8	49.3	57.0			
122.0	11.0	32.5	49.2	56.8			2.1
144.0	14.5	23.5	42.1	49.5			
152.0	14.5	23.5	42.0	50.1			
176.0	14.5	22.5	43.0	49.8		5.3	4.1
199.0	22.0	13.7	27.8	36.4			
203.5	22.0	13.7	25.0	38.2	3.8		
207.5	22.0	11.5	26.2	37.6			
222.0	44.0	11.7	20.8	28.0			
224.0	44.0	12.1	19.8	28.8			
226.0	44.0	11.3	19.4	28.0			

Ethanol concentration (Et) and effluent cell concentration (X_{ef}) in the outlet of reactors 1, 2, and 3, for different feed flow rates (Q_{feed}). TRS_{feed}=163 g/l; G_{feed}=3.9 g/l; enzyme concentration=3.77 U/ml reactor; initial yeast concentration=50.0 g wet yeast/ml of reactor; pH in the outlet of reactor 3=3.6

T Assay time.

Table 2 SSF continuous run 1.

T (h)	Q_{feed} (ml/h)	TRS_1 (g/l)	TRS_2 (g/l)	TRS_3 (g/l)	G_1 (g/l)	G_2 (g/l)	G_3 (g/l)
23	8.1	43.4	6.3	1	1.8	2.6	0.5
42.5	8.1	50.5	13.8	3.3	0.9	1	0.8
54	8.1	49.6	16.9	3.9	0.8	1.3	0.9
68.5	8.1	60	23	10	0.8	0.9	0.9
95.5	11	81	42.8	19.5	0.1	0.2	0.4
114.5	11	80.5	43.1	22.3	0.3	0.2	0.2
122.5	11	78.5	43	24.1	0.1	0.2	0.3
144	14.5	96.5	53.1	41.4	0.7	0.1	0.2
152	14.5	101	53.3	38.7	0.3	0.1	0.2
176	14.5	105.5	55.7	42.8	0.5	0.3	0.2
199	22	130.7	102	71.9	1	1	0.3
203.5	22	124.2	92.7	72.5	1	1	0.7
207.5	22	129.6	96.2	73.1	1	1	0.5
222	44	132	113.3	93.3	2.7	2.3	7.6
224	44	129	113.1	94.8	2.1	1	2.8
226	44	129.1	112.7	93.9	0	0	0

Total reducing sugar concentration (TRS) and glucose concentration (G), in the outlet of reactors 1, 2, and 3, for different feed flow rates (Q_{feed}).

T Assay time

first samples at 44 ml/h. Therefore, in this first continuous test, starch hydrolysis was the rate-limiting step.

Table 3 shows initial and final values for mass and volume of beads and initial and final cell concentration and cell viability of the yeast in the beads. Table 3 shows an increase in the mass and volume of beads after running the experiments for 226 h. All the detected bead alterations are more pronounced in the first reactor, where the reaction rates are the highest. The final bead density in the first reactor, 0.96 g/ml, decreased when compared to the initial bead density, 1.07 g/ml. A great amount of gas could be seen in the first reactor. The reactor system tested in this work (three reactors in series, with escape of CO_2 between two consecutives stages) had been designed to avoid the gas hold-up in the reactors. However, to keep the reactors under pressure (there were no pumps between the reactors), the CO_2 escaped in a water column (containing $CuSO_4$ for avoiding bed contamination), and some gas hold-up was still observed, mainly in the first reactor. Therefore, the designed

Table 3 SSF continuous run 1.

Reactor	Vr (ml)	M_{gi} (g)	M_{gf} (g)	V_{gf} (ml)	ρ_{gf} (g/ml)	ε_f	X_{gf} (cel/g gel)	Vb (%)
1	35	17.5	26.0	27.0	0.96	0.23	1.37×10^9	89
2	30	15	21.8	20.5	1.06	0.34	1.0×10^9	56
3	25	12.5	13.8	13.0	1.07	0.48	8.40×10^8	50

Bed characterization in the reactors 1, 2, and 3. Xg, Mg, Vg and ρg stand for cell concentration, mass, volume, and density of the beads, respectively.

ε Reactor porosity, Vr reactor volume, Vb viability. Subscripts: i initial, f final

ρg_i=1.07 g/ml; $Xg_i=1.8\times10^9$ viable cell/g gel; Vb_i=98%; ε_i=0.5

system only diminished the problem. The final cell concentration in the beads in each reactor decreased when compared to the initial one, the decrease being more pronounced in the third reactor. However, as the mass of beads increased, the total number of viable cells present in each reactor after 226 h ($X_{gf} \times M_{gf}$) was kept almost constant in the first reactor—from the initial 3.1×10^{10} to the final 3.2×10^{10} and decreased in the second and third ones—from the initial 2.6×10^{10} and 2.2×10^{10} to 1.2×10^{10} and 5.7×10^{9} in reactors 2 and 3, respectively. This was already expected, as the cells in the first reactor are living in better nutrition conditions than the ones in the second and third reactors. In the first reactor, the concentration of sugar is higher, and ethanol concentration is lower than in reactors 2 and 3. Some gel disruption was also observed, being always more significant in the first reactor and still more when the system was operated with recycle in the first reactor (runs 2 and 3). The leaching of calcium and the high production of CO_2 in the first reactor may be responsible for this problem. Gel disruption may be minimized by using particle size around 1 mm and adding 2–6 g/l of calcium chloride to the medium [9].

Figure 3 shows the results of ethanol, TRS, and glucose as function of the residence time, which was calculated considering the first reactor volume, the sum of reactors 1 and 2 volumes and the sum of the volumes of the three reactors. As it can be seen in Fig. 3, the concentration values obtained for residence times calculated considering only the first reactor operating with the lower flow rates shows a good agreement with the values obtained for the same residence time, calculated considering the sum of the volumes of the three reactors, operating with the higher flow rates.

Table 4 shows the calculated values of conversion, productivity, and yield for each residence time tested: the productivity for the maximum conversion reached in the continuous SSF (97%) was 5.9 g/l/h, with yield of 81%, for a residence time of 11.1 h, calculated using the average values of the four determined TRS and ethanol concentrations for this residence time. In the discontinuous SSF reactor, for the same enzyme and yeast concentrations, the results were: total conversion of TRS in glucose in 6 h and 99.8% of glucose/ethanol conversion in 9 h, with high concentrations of free glucose in the medium.

Possible causes for the worse performance of the continuous process may be the presence of severe external and/or internal diffusion effects, hold-up gas and channeling prob-

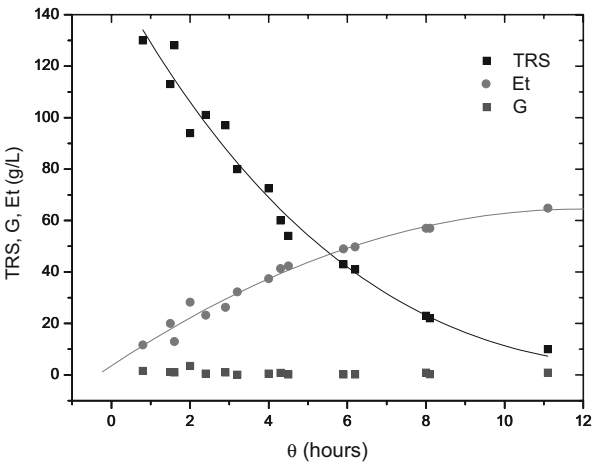

Fig. 3 SSF continuous run 1. Ethanol (Et), total reducing sugar (TRS), and glucose (*G*) concentrations with residence time (θ). θ values are related to: reactor 1 volume (35 ml); (reactor 1 + reactor 2) volume=65 ml; (reactor 1 + reactor 2 + reactor 3) volume=90 ml. TRS_{feed}= 163.0 g/l

Table 4 Ethanol concentration (Et), substrate conversion (x_s), ethanol productivity (Pr_{et}), and ethanol yield (η_{et}) for several residence time in the reactor (θ).

θ (h)	Et (g/l)	x_s	Pr_e (gEt/l h)	η_{et} (%)
0.8	11.7	0.2	14.6	69.0
1.5	20.0	0.31	13.3	77.0
1.6	13.0	0.21	8.1	74.0
2.0	28.3	0.42	14.1	81.0
2.4	23.2	0.38	9.7	73.0
2.9	26.3	0.40	9.1	79.0
3.2	32.3	0.51	10.1	76.0
4.0	37.4	0.56	9.4	80.0
4.3	41.3	0.63	9.6	79.0
4.5	42.4	0.67	9.4	76.0
5.9	49.0	0.74	8.3	79.0
6.2	49.5	0.75	8.0	79.0
8.0	57.0	0.86	7.1	80.0
8.1	57.0	0.86	7.0	80.0
11.1	65.4	0.97	5.9	81.0

TRS_{feed}=163 g/l. SSF continuous run 1.

lems in the bed. The decrease of the bead density in the first reactor, determined after finishing the run, indicates retention of the produced CO_2. It could be seen that some particles were floating in the first reactor in the beginning of the run, when there was space for that (the bed porosity decreased from 0.5, initial, to 0.23, final, in the first reactor). So, the exhaustion system proposed did not allow the complete escape of the large amount of gas produced in the first reactor, which was what implied a decrease in the real volume of the first reactor. The comparison between the performance of the batch and the continuous run is made considering around 9 h for the batch run and 226 h for the continuous one. It is a reasonable hypothesis that there might have been a preferential yeast growth near the silica, as glucose concentration is higher there. The considerable quantitative growth and the fact that yeast grows in clusters [15], near the substrate [16, 17], caused an increase in the tortuosity of the substrate course inside the gel. The access of the substrate to the enzyme in the interior of the silica might also be restrained, consequently lowering the hydrolysis rate in the continuous system. Therefore, as the continuous assay went by, a different distribution of the microorganism was established, which implied higher restrictions for the starch diffusion inside the biocatalyst. Gonçalves et al. [18] have modeled the hydrolysis of maltotriose catalyzed by glucoamylase immobilized in silica and co-immobilized in pectin gel, using a bi-disperse model. This study allowed the authors to estimate the maltotriose diffusivity in silica. Nevertheless, the presence of yeast may lead to different results. Finally, in the discontinuous run, the beads and medium were mixed at 150 rpm (in fact, oscillations/m, it was a reciprocal shaker), in glass flasks with a diameter of 3 cm, in what may lead to fluid velocities higher than the superficial flow rates of the fluid in the continuous test (run 1). In consequence, the external mass transport coefficient for the starch diffusion from the bulk to the particle surface may be lower in the continuous run, and the rate-limiting step in the process would be the diffusion of the liquefied starch from the bulk to the particle surface. If this last phenomenon was significant, increasing the superficial velocity would lead to an improvement in reactor productivity of the continuous run.

Influence of Superficial Velocity on the Performance of the Continuous SSF Reactor

To verify the possibility of limitation of the hydrolysis rate in the system by the external mass transport resistance, the bed height of the gel containing enzyme and co-immobilized yeast was increased. This was done to increase the total volume of the system (from 90 to 314 ml), without changing the flow area, and consequently, enabling higher superficial rates in the system for the same residence time. The use of recycle in the first reactor enabled obtaining even higher rates in this stage, where most of the conversion took place. TRS concentration in the feed decreases when operating the process with recycles. That implies a decrease in the driving force of starch diffusion through the external film and in the biocatalyst pores. Inherent reaction rates would also decrease. However, if the external diffusions effects were very high, the increase in the substrate mass transport coefficient may compensate the decrease of concentrations, thus, leading to an improvement in the reactor performance.

These effects were studied running two new continuous assays. In continuous run 2, the system was operated with a total volume of 314 ml (110, 104, and 100 ml, for reactors 1, 2, and 3, respectively), keeping constant the feed flow rate, 55 ml/h, with and without recycle in the first reactor. Only one recycle flow rate was tested, 67.2 ml/h. In continuous run 3, only the first reactor was operated, changing the feed and recycle flow rates. Table 5 shows operational conditions and respective average values of ethanol, TRS, and glucose concentrations obtained for related steady states, in the two new tests. Figure 4 shows ethanol production vs residence time for operational conditions operated without recycle, with superficial velocities up to 3.1×10^{-4} cm/s and above 3.7×10^{-4} cm/s.

Results in Table 5 show a worse performance of the system with recycle (always in the first reactor). All values of ethanol concentration obtained with recycle decreased when compared to the ones obtained without recycle. Therefore, the increase in the external mass transport coefficient was not sufficient to compensate for the decrease in the TRS concentration in the first reactor due to dilution. The concentration of free glucose in the effluent is still very low for both cases, with and without recycling. Only

Table 5 Ethanol (Et), total reduction sugar (TRS), and glucose (*G*) concentrations, for different operation conditions.

Run	Q_{feed} (ml/h)	RR1	Θ (h)	Et (g/l)	TRS (g/l)	*G* (g/l)
2	55	1.22	2	24.2	84.4	0
2	55	0	2	33.8	69.1	1.7
2	55	1.22	3.9	44.8	41.9	0,7
2	55	0	3.9	46.4	41.2	0
2	55	1.22	5.7	55.9	20.5	0.9
2	55	0	5.7	55.4	21.8	0.3
3	13.2	0	8.3	56.3	18.8	0.5
3	18.5	0	5.9	51.4	28.7	0
3	26.1	0	4.2	47.4	37.9	0
3	54.2	0	2	33.2	66	0
3	54.2	0.26	2	29.5	79.4	0
3	54.2	1.05	2	24.4	92.3	0

Q_f Feed flow, *RR1* recycle ratio=recycle flow=Q_r/Q_{feed}, θ residence time=reactor volume/Q_{feed}, TRS_{feed2}, TRS_{feed3}=feed TRS in the runs 2 and 3=144.0 and 151.0 g/l, respectively

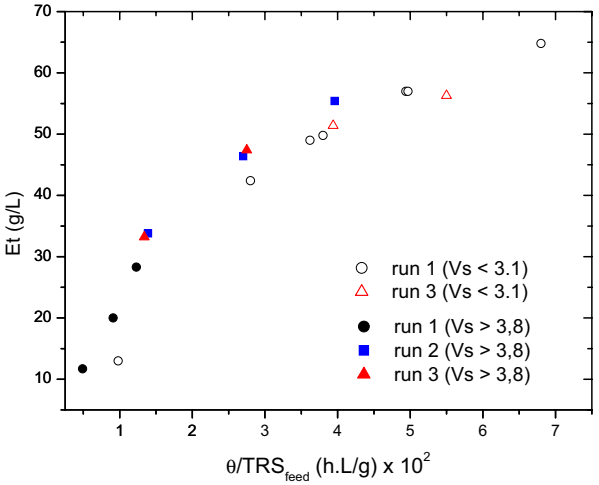

Fig. 4 Ethanol (Et) concentration with residence time/feed total reducing sugar (θ/TRS$_{feed}$), for two superficial velocities ranges: Vs≤3.1×10^{-4} cm/s and Vs≥3.8×10^{-4} cm/s, for the three SSF continuous runs

for the residence time of 2 h, without recycle, glucose concentration is higher than 1.0 g/l. However, the superficial velocities in runs 2 and 3 are higher than in run 1, and the comparison of the ethanol concentrations obtained for similar residence times in runs 1, 2, and 3 shows that the highest ethanol productivities are achieved for the highest volumetric flow rates.

Aiming at analyzing the influence of the superficial flow rates in the ethanol concentration, all the obtained results in the three continuous run, without recycle, are compared with respect to the respective superficial rates, as shown in Fig. 4. As the TRS feed used in the three assays were not exactly the same, the residence times are divided by the corresponding TRS feed concentration.

Analyzing the results in Fig. 4, it can be seen that the assays performed with velocities higher than 3.8×10^{-4} cm/s have better performance than the ones with velocities lower than 3.1×10^{-4} cm/s. Ethanol concentration values were higher for velocities equal to or bigger than 3.7×10^{-4} cm/s, indicating that limitation by external transport really exists. Despite this improvement, the results of the continuous system are still far from the batch process. The hydrolysis rate in the continuous process is lower when compared to the discontinuous tests, with no free glucose present in all operational conditions, including the ones with superficial velocities higher than 3.7×10^{-4} cm/s. The results of the assays with recycle in the first reactor (and consequently with increasing flow rates in this stage) support this conclusion.

Table 5 indicates a negative influence of the recycle on the performance of the reactor in this operational range (superficial velocities above 3.7×10^{-4} cm/s). Therefore, other phenomena may be contributing for the inferior performance of the continuous system: gas hold-up, channeling, and the intra-gel mass transport resistance due to yeast growth inside the particle during the long operation of the continuous runs.

Sun et al. [8] and Krishnan et al. [10] also found that starch hydrolysis was the rate-limiting step, when operating a SSF-fluidized bed reactor to produce ethanol from soluble starch and liquefied corn starch, respectively. The authors used immobilized glucoamylase co-immobilized with *Z. mobilis* in κ-carrageenan beads, a biocatalyst very similar to the one used in this work. Krishnan et al. [10], by testing the activity of the immobilized enzyme after deactivation of the microorganism using ethanol 75%, concluded that microorganism

growth was not responsible for the decrease of the starch hydrolysis rate in the continuous process. Mattos et al. [19] also found similar values when the glucose diffusivity in pectin gel was measured with and without immobilized yeast, supporting that conclusion. In that work, the authors deactivated the microorganism with formaldehyde 18.5%. However, living cells may lead to different results. The operation of the discontinuous process using repeated batch assays would allow the cell growth in the co-immobilized biocatalyst and could help to elucidate this point. Using a solution of 15% dry-milled corn starch previously liquefied by α-amylase, Krishnan et al. [10] achieved productivity of 9.1 g/l/h, with 89.3% of conversion, ethanol concentration of 36.44 g/l, without pH control, and operating at 35°C. In this work, a lower productivity, 5.9 g/l/h, but a higher ethanol concentration and conversion were reached, using similar starch concentrations in the feed, at 30°C and without pH control. They also compare the SSF process to the SHF process and found much better results for operating the hydrolysis and the fermentation steps separately. However, it is easier to operate packed beds than fluidized beds, and the SSF process demands less energy than the SHF one. Besides, the results obtained in this work with the SSF, operated in a packed bed system can still be improved. A small increase in the temperature, in the range between 30 and 35°C, may modify not only the relation between the hydrolysis/fermentation velocities but also the reaction/diffusion rates, thus improving the performance of the SSF packed bed reactor. The use of smaller bead diameters may be an option to change bed porosity, decrease diffusion effects, and consequently, improve the performance of the packed-bed reactor tested in this work. Therefore, further studies are still needed to allow a better comparison of the presented system with other possible ones.

Conclusions

A packed-bed reactor using glucoamylase immobilized in silica and co-immobilized with *S. cerevisiae* in pectin gel was stably operated during 226 h to produce ethanol from liquefied dry-milled manioc root starch.

Ethanol productivity of this SSF process reached 5.9 g/l/h, for a feeding of 163 g/l TRS concentration, with 97% of conversion and 65.4 g/l of ethanol. Our results showed that the presence of recycle in the first reactor led to a worse performance of the system. A better performance can be achieved operating the reactor with higher superficial velocities, indicating that operation of the packed bed using bead diameter smaller than 4.0 mm may lead to a better performance of the studied process.

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