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Improving the Performance of a Continuous Process for the Production of Ethanol from Starch

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Abstract In a previous work, a continuous simultaneous saccharification and fermentation process to produce ethanol from cassava starch was studied, using a set of fixed-bed reactors. The biocatalyst consisted of glucoamylase immobilized in silica particles and co-immobilized with *S. cerevisiae* in pectin gel. Using 3.8 U $mL^{-1}_{reactor}$ and 0.05 $g_{wet\ yeast}$ $mL^{-1}_{reactor}$ at start-up, starch hydrolysis was the rate-limiting step. Maximum ethanol productivity was 5.8 $g_{ethanol}\ L^{-1}\ h^{-1}$, with 94.0% conversion of total reducing sugars (TRS) and 83.0% of the ethanol theoretical yield. In this work, the molar mass of the substrate and the biocatalyst particle size were reduced in an attempt to improve the bioreactor performance. The diameters of silica and pectin gel particles were reduced from 100 μm and 3–4 mm, respectively, to 60 μm and 1–1.5 mm, and the degree of substrate prehydrolysis by α-amylase was increased. The bioreactor performance was assessed for different loads of immobilized glucoamylase (2.1, 2.8, and 3.8 U $mL^{-1}_{reactor}$), for the same initial cell concentration (0.05 $g_{wet\ yeast.}mL^{-1}_{reactor}$). Feeding with 154.0 g L^{-1} of TRS and using 3.8 U $mL^{-1}_{reactor}$, fermentation became the rate-limiting step. Productivity reached 11.7 g $L^{-1}\ h^{-1}$, with 97.0% of TRS conversion and 92.0% of the ethanol theoretical yield. The reactor was operated during 275 h without any indication of destabilization.

Keywords Ethanol · Cassava starch · *Saccharomyces cerevisiae* · Glucoamylase · Packed-bed reactor · SSF · Mass transport effects

Introduction

Sugarcane juice in Brazil and starch from corn in USA are the main raw materials to produce ethanol nowadays, but the increasing world demand for biofuels makes other sources of biomass eligible [1]. The tuberous root of cassava, a plant native from South America, contains a high concentration of carbohydrates (about 80%) and gives the highest

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yield of bioenergy per cultivated area per day among crop plants, except possibly for sugarcane. Furthermore, cassava is probably the only crop whose production is feasible in all ecological zones because it adapts well to a variety of soil and climatic conditions, is drought tolerant, and grows on depleted and marginal soil. Cassava, therefore, may be cultivated in soils and climates not favorable to sugarcane, what might allow sugarcane distilleries to extend the use of the industrial plant from December to March, the raining season, out of the sugarcane crop period. Besides, culture rotating would improve the use of the soil, which might be managed in a more sustainable manner. Of course, cassava is also an important source of starch for feeding, and the food versus fuel question demands a more extended analysis; however, in spite of that discussion, several crop residues contain large amounts of starch, including the ones coming from the cassava processing. Therefore, studying the production of ethanol from cassava starch is certainly justifiable.

The global cassava production in 2006 was 208 million ton [2], 55% in Africa and 32% in Asia. Brazil (13%) and Thailand (12%) are important producers, and bioethanol is becoming a new market for this crop. For instance, in Thailand, 12 cassava ethanol plants, with total output of 3.4 million liters per day, are expected to be running in 2008 [3].

Starch cannot be directly fermentable to ethanol by *Saccharomyces cerevisiase*, the main microorganism used in the industry. A saccharification step before the fermentation reaction is required, using the enzymes α-amylase and glucoamylase to catalyze the hydrolysis reaction producing glucose. Although the use of recombinant yeast strains that express the enzymes required for the direct conversion of starch into ethanol is a possible solution [4, 5], the industrial current process employs the enzymatic step [6]. The robustness and high yields of strains of *S. cerevisiase* that are available in the industry, after decades of classical genetic selection, is a fact that still hinders the use of recombinant microorganisms for large-scale production of ethanol. Therefore, when using starch as raw material, the time and energy required for its prehydrolysis and the price of enzymes represent additional costs when compared to directly fermentable raw materials.

In the Melle–Boinot process, the leading technology to produce ethanol from sugarcane that has been used in Brazil since the 1960s [1], centrifugation is a necessary step for the separation and recycle of the yeast cream. The costs of this unit operation have a significant impact on the production of ethanol via fermentation. The use of immobilized microorganisms avoids this step, thus reducing total costs.

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 the sequential hydrolysis and fermentation, are the following: reduction of the number of reactor vessels that are required (lower capital costs), increased rate of hydrolysis due the lower inhibition by product, and the reduction of fermentation time [6]. The SSF process is already used in the conventional dry-grind corn process [7]. Immobilization of the enzyme and of the microorganism allows their reutilization, what may turn economically viable the use of high concentrations of biocatalysts in the reactor, reducing reaction times. This technique has been extensively studied in order to reduce process costs [8–13].

The production of ethanol from liquefied cassava root flour syrup, using microorganism and enzyme co-immobilized in pectin gel, was studied in a previous work [14]. Glucoamylase was first covalently immobilized in controlled porosity silica (CPS). After that, the derivative CPS-enzyme was co-immobilized with *Saccharomyces cerevisiae* in pectin gel. With this technique, the economical operation of the process is feasible even at 30°C, an adequate temperature for the yeast, due to the high loads of enzyme in the reactor. A higher enzyme concentration in the reactor allows reaching high rates of hydrolysis,

counterbalancing the fact that the optimal operation temperature for glucoamylase is 60°C. In [14], the performances of the SSF in batch mode and in continuous mode were compared, using similar operational conditions (enzyme load, pH, temperature, and initial substrate and initial yeast concentrations). For the continuous experiments, a setup of three fixed beds in series was used in order to allow the purge of CO_2 after each stage. The SSF continuous process showed stable operation, reaching a productivity of 5.9 g L^{-1} h^{-1} for a feeding of 163 g L^{-1} of total reducing sugars (TRS), with 97% of TRS conversion and 65.4 g L^{-1} of ethanol. In the continuous experiment, hydrolysis was the rate-limiting step. Nevertheless, in batch mode, the fermentation was the rate-limiting step, and a productivity of 8.3 g L^{-1} h^{-1} was reached, with 99.8% of TRS conversion and yield of 0.47 (91% of the theoretical).

A possible explanation for these results could be the presence of severe external and/or internal diffusion effects, hold-up of gas, and channeling problems in the bed. Higher external diffusion effects may be due to the lower fluid velocities in the continuous experiments. A higher intraparticle mass transport resistance could be explained by the modification of the internal structure of the gel caused by yeast growth during long operation times. If these hypotheses are true, the decrease of the molar mass of the substrate and of the particle size of the silica and pectin gel particles would improve the performance of the system. This work intends to verify these hypotheses. With this purpose, the degree of prehydrolysis of the starch by soluble α-amylase was increased, using higher enzyme concentrations and reaction times, until no significant change in the distribution of the molecular size of the oligosaccharides could be observed; that is, the starch was converted to limit dextrins. The enzyme α-amylase hydrolyses very efficiently the internal α,1–4 linkages in the starch molecule. However, it cannot hydrolyze neither α,1–6 linkages nor the α,1–4 ones that are close to α,1–6 linkages. Therefore, when all the amylopectin molecules are hydrolyzed to this point, the final product will be a pool of small oligosaccharides (limit dextrins) that cannot be attacked by α-amylase, together with maltose and glucose.

The enzyme was immobilized in silica with smaller particle sizes than the ones used in the previous work (from 100 to 60 μm). Smaller pectin gel particles were produced (from 3–4 to 1–1.5 mm) and used in the present work as well. A new set of continuous runs in a packed-bed reactor with enzyme and yeast co-immobilized was then performed using different enzyme loads.

Materials and Methods

Materials Cassava flour, from peeled, dried, and milled root, was purchased from "Ricieri Pechatt and 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^{-1} 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^{-1}, was donated by NOVO Industri do Brasil. Commercial *S. cerevisiae* (60% of moisture) was from Fleischmann SA. Controlled porosity silica (CPS) was supplied by Corning Glass Works (Corning, NY, USA), with average pore size of 37.5 nm and internal porosity of 56.6% (diameter below 60 μm). Citric pectin type 8002 was supplied by Braspectina S.A. All other reagents used were laboratory grade from different commercial sources.

Enzyme immobilization Silica was silanized with a 5% *v*/*v* γ-aminopropyltrietoxysilane solution, pH 3.3, 75°C, for 3 h at a liquid/solid ratio of 5 mL g^{-1}. The silica was then washed with water, dried at 105°C for 15 h, and activated with glutaraldehyde (2.5%

hydrogenophosphate buffer, 0.1 M, pH 7.0) for 1 h at 20–25°C, 5 mL g^{-1}. Then, the enzyme was immobilized by contacting an enzyme solution (0.06 M acetate buffer, pH 4.2) with the activated silica, in a liquid/solid ratio of 7.5 mL g^{-1}, under stirring during the three stages.

Coimmobilization Six grams of pectin was dissolved in 78 mL of distilled water with subsequent addition of 6 mL sodium acetate buffer (1 M, pH 4.2) and 10 g of wet yeast. Silica containing the immobilized enzyme (206.5 U $g^{-1}_{\text{dried silica}}$ or 272.2 U $g^{-1}_{\text{dried silica}}$) was then mixed with the suspension at the ratio 1.5 g of wet silica–enzyme/20 g yeast–pectin suspension. One gram of dried silica corresponded to 1.5 g of wet silica. Spherical particles (Φ 1–1.5 mm) were produced after dropping the suspension in a 0.2 M $CaCl_2$ solution, using a pneumatic extruder (Fig. 1). The particles were cured in a refrigerator for 18–20 h.

Cassava root flour syrup The hydrolysis with α-amylase had two steps. In the first prehydrolysis step (PHS), a suspension of flour (approximately 300 g L^{-1}) in 0.01 M NaOH/0.01 M $CaCl_2$, pH 6.0–6.5 was heated under stirring at 65°C. α-amylase (0.5 g per liter of suspension) was added at 90°C for 30 min, followed by filtration (number 20 mesh). The filtrate was submitted to a second hydrolysis using different enzyme concentrations (1.5 or 3.0 $g_{\alpha\text{-amylase}}$ L^{-1}) and reaction times (30, 60, or 90 min), followed by 20 min of autoclave (120°C) and paper-filtration while hot, using Celite® as filtration aid. For the fermentation, the following nutrients were used: $NaH_2PO_4{\cdot}H_2O$ (1.0 g L^{-1}), $MgSO_4{\cdot}7H_2O$ (0.25 g L^{-1}), yeast extract (0.5 g L^{-1}), $CaCl_2$ (2.0 g L^{-1}), and urea (1.5 g L^{-1}).

Experimental assays All experiments were performed at 30°C, initial pH 4.0. In continuous tests, a jacketed packed-bed glass reactor was used with a volume of 100 mL and internal diameter of 5 cm. The escape of the formed CO_2 in a water column containing $CuSO_2$ allowed control of the internal pressure of the reactor. Figure 2 is a scheme of the experimental apparatus.

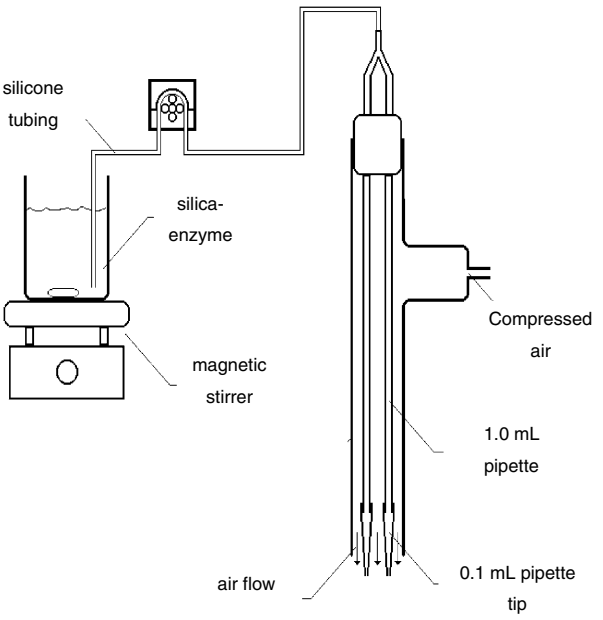

Fig. 1 Pneumatic extruder for biocatalyst conformation

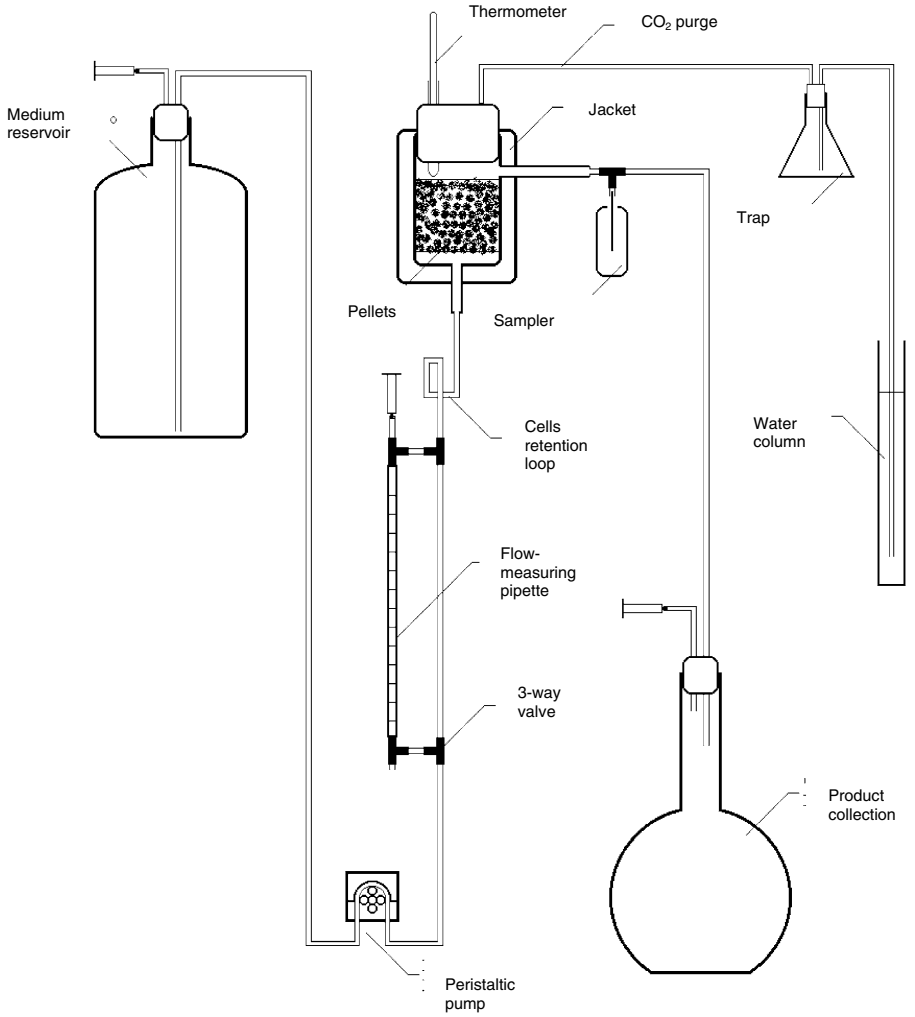

Fig. 2 Schematic representation of the apparatus used in the continuous experiments

Analysis

TRS and Glucose Liquefied starch concentration was determined by measuring 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 citrate buffer 0.1 M, pH 4.2, at 45°C, for 30 min, followed by enzyme inactivation in boiling water for 5 min [14]. TRS (g of glucose/L) was calculated according to Eq. 1:

$$\mathrm{TRS} = 0.977\,\frac{G_\mathrm{m} - G_\mathrm{i}}{0.9} + G_\mathrm{i} \tag{1}$$

where G_m = glucose concentration in the sample after enzymatic hydrolysis; G_i = glucose concentration in the sample before enzymatic hydrolysis; 0.977=empirical conversion factor to convert starch to glucose, obtained by enzymatic hydrolysis of liquefied starch,

corresponding to 90% yield of the enzymatic hydrolysis, as determined by Schmidell and Fernandes using soluble starch of analytical grade [15] and 0.9=stoichiometric glucose/starch conversion factor.

Glucose and TRS were measured using a glucose-oxydase kit (CELM S.A.)

Ethanol Ethanol was determined using a Waters HPLC, ion exchange Shodex ® column at 80°C, refraction index detection at 34°C, elution with Milli-Q water, flow rate 1.0 mL min^{-1}

Cellular Viability and Concentration This was determined by counting cells in nine square cells of a Neubauer chamber after dying them with methylene blue. Viable cells were not colored, and dead cells were blue. Free yeast concentration was also obtained by filtering a known volume of cells suspension and drying the wet mass until constant weight. 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 according to Eq. 2:

$$X_g = \frac{N_{viable}}{4 \times 10^{-6}} \frac{\left(20 + \frac{M_{gs}}{\rho_g}\right)}{M_{gs}} \times 10 \quad (2)$$

where X_g = cell concentration in the gel (viable cells g^{-1}_{gel}); N_{viable} = average number of viable cells in one square cell 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 one square cell of the chamber (mL); and 10=dilution factor of the sample in methylene blue solution.

Beads Diameter Beads density was determined using a pycnometer. The particles were first dried by contacting them with an absorbent paper, and the beads diameter was determined measuring the volume of 500 particles.

Soluble and Immobilized Enzyme Activity This was determined at standard conditions [16]: 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. The amount of immobilized enzyme was calculated by the difference between the offered load and the remaining enzyme in the supernatant, after the immobilization procedure.

TRS Conversion (X_{TRS}), Ethanol Yield (η_{Et}) and Productivity (Pr_{Et})

X_{TRS} was calculated from Eq. 3, for batch or continuous reactors, respectively:

$$X_{TRS} = \left[\frac{\text{TRS (initial or feed)} - \text{TRS (final or outlet)}}{\text{TRS (final or outlet)}}\right] \times 100\% \quad (3)$$

The metabolic ethanol yield (η_{Et}) is provided by Eq. 4, for batch or continuous reactors:

$$\eta_{Et} = \frac{1}{0.51}\left[\frac{\text{Et (final or outlet)}}{\text{TRS (initial or feed)} - \text{TRS (final or outlet)}}\right] \times 100\% \quad (4)$$

where 0.51=theoretical glucose-ethanol yield

Productivity (Pr_{Et}) was calculated by Eq. 5, for batch or continuous reactors:

$$Pr_{Et} = \left[\frac{\text{Et (final or outlet)}}{\text{(time of batch) or (residence time)}}\right] \times 100\% \quad (5)$$

Results and Discussion

Starch Syrup Pretreatment: Increasing the Degree of Hydrolysis with α-Amylase

To decrease the molar mass of the substrate, the liquefaction reaction occurred in two steps. In the first one, 0.5 g of α-amylase/L of suspension was used. After filtrating to separate most of the insoluble fibers, a second step of hydrolysis employed 1.5 or 3.0 g of enzyme for 30, 60, or 90 minutes.

From the chromatogram shown in Fig. 3, it can be seen that a significant reduction in the molar mass of the substrate is obtained in the second step of the liquefaction reaction, using the lower enzyme concentration and reaction time, 1.5 g L^{-1} and 30 min, respectively. Increasing these variables did not lead to a significant decrease of the molar mass of the starch, indicating that limit dextrins were already obtained for the first condition tested. Therefore, in all the continuous assays, the hydrolysis reaction with α-amylase proceeded in two stages, using 0.5 g L^{-1} and 1.5 g L^{-1} of enzyme, respectively, 30 min of reaction time in each stage, at 90°C.

Preparation of the Biocatalyst Aiming at Increasing Enzyme Concentrations in the Reactor

Enzyme concentrations of 2.1, 2.8, and 3.8 U $mL^{-1}{}_{reactor}$ were tested, always using 0.05 g of wet yeast/L of reactor. In the coimmobilization assays, to produce 100 g of biocatalyst, 10 g of wet yeast, 6 g of pectin, and different amounts of silica containing immobilized glucoamylase (acetate buffer was added to complete 100 g of suspension) were used.

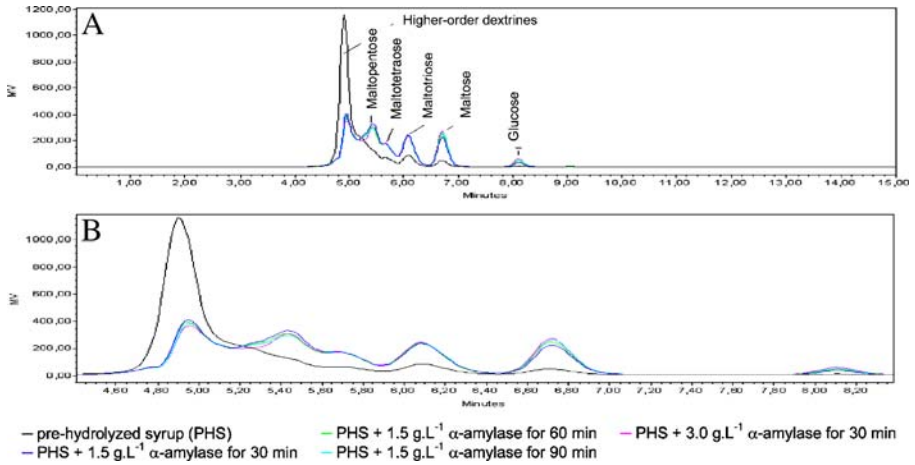

Fig. 3 Chromatograms for different substrate prehydrolysis conditions. **a** Overview, **b** detail

Higher proportions of silica in the co-immobilized biocatalyst make the extrusion procedure more difficult. Therefore, in order to increase the enzyme concentration in the reactor, different silica derivatives were prepared, containing different ratios of immobilized glucoamylase/g of dried silica.

Table 1 shows results of the enzyme immobilization assays.

Results in Table 1 indicate that, up to 160 U/g of dried silica, an increase of the offered enzyme load/g of support led to higher immobilization yields and more active enzyme derivatives (silica containing immobilized glucoamylase). However, when 325 U/g of dried silica were offered, the less active derivative of all was obtained. A possible explanation for this behavior follows.

The support activation reaction forms Schiff's bases between aldehyde groups of glutaraldehyde and amine groups of the support (amine groups generated by the reaction of silanol groups of silica with γ-aminopropyltrietoxysilane). Glutaraldehyde has two aldehyde groups, and the remaining one in each molecule reacts with the terminal amine group of the enzyme, making the desired crosslinking. During the enzyme immobilization step, glucoamylase has to diffuse through the pores of the activated silica to react with the aldehyde groups. For glutaraldehyde, being a very reactive crosslinking agent, when a high load of enzyme was offered, all the available aldehyde groups located closer to the external surface of the silica bead reacted with the amine groups of the enzyme before other enzyme molecules could reach the aldehyde groups generated deeper in the silica pores. This high number of enzyme molecules, linked to the support close to the pore mouth, may have hindered the access of enzyme molecules to the aldehyde groups located deeper within the pore. In consequence, a less active enzyme derivative was obtained when a very high enzyme load was offered. This hypothesis is supported by studies showing that the higher the enzyme concentrations in the immobilization solution, the more enzyme molecules are immobilized in the more external layers of the support, leading to more heterogeneous distributions of the immobilized enzyme [17]. Nevertheless, another possible explanation would be the presence of some additive(s) in the enzyme solution that can react with the aldeyde groups of the support. The increase of the enzyme concentration in the immobilization solution would increase also the concentration of these other molecules. If these additives can diffuse into the support faster than glucoamylase can, they will occupy most of the aldehyde sites in the support, preventing the enzyme immobilization.

Hence, in order to obtain a higher enzyme load in the reactor, in the third continuous run of this work, the co-immobilized pectin biocatalyst had a higher mass of silica than in the two first ones, which might result in more fragile biocatalysts. Nevertheless, even in this case, no mechanical rupture of the gel particle was observed during 245 h of operation of the reactor.

Table 1 Immobilization assays of glucoamylase in silica modified with γ-APTS and activated with glutaraldehyde.

Run no.	Offered enzyme (U $g^{-1}_{dried\ silica}$)	Immobilization yield (%)	Immobilized enzyme activity (U.$g^{-1}_{dried\ silica}$)	Co-immobilized enzyme concentration (U $mL_{reactor}$)
1	138	64.0	87.9	2.1
2	160	72.5	116	2.8
3	325	19.0	62.3	3.8

Table 2 Glucose (G), total reducing sugars (TRS), ethanol (Et) and glycerol (Gly) concentrations, and TRS conversion (X_{TRS}) for different residence times (θ), using glucoamylase and *S. cerevisae* co-immobilized in pectin gel.

θ (h)	Assay duration (h)	G (g L^{-1})	TRS (g L^{-1})	Et (g L^{-1})	Gly (g L^{-1})	X_{TRS} (%)
10.4	56.5	0.0	1.1	44.1	2.9	99.0
8.05	100.4	0.0	1.4	45.4	2.7	99.0
6.08	203.5	0.1	3.1	44.6	3.1	97.0
5.07	235.5	0.2	3.7	44.3	3.0	97.0
4.04	262.5	0.7	7.0	44.1	2.2	94.0
3.06	278	1.5	12.7	41.1	2.2	89.0
1.86	293.5	4.2	52.7	31.2	1.9	55.0
1.04	299.8	4.7	80.7	17.3	1.2	32.0

Enzyme concentration=2.1 U $mL^{-1}{}_{reactor}$. Continuous run 1. Feed: TRS, 118.0 g L^{-1}. G, 2.0 g L^{-1}

Performance of the Continuous Reactor System for Different Enzyme Loads in the Reactor

Three samples were taken at every residence time. After changing the flow rate, samples were taken after five residence times. Tables 2, 3, and 4 show the results for three continuous runs.

Results in Table 2 indicate that, using 2.1 U $mL^{-1}{}_{reactor}$ of immobilized glucoamylase, hydrolysis is still the rate-limiting step: Glucose concentrations were always lower than those of TRS, and for residence times above 3 h, the amount of free glucose became negligible. Hence, there was no accumulation of the intermediate product (glucose). All of the glucose was metabolized by the yeast into ethanol, and saccharification was the controlling step.

Even for the lowest enzyme concentration that was tested in the present work (run 1, results in Table 2), the performance of the reactor was higher than the one achieved in [14], using 3.8 U $mL^{-1}{}_{reactor}$. A TRS conversion of 97.0% (with η_{Et}=76.0%) was reached with residence time of 5.1 h, leading to an ethanol productivity of 8.7 g L^{-1} h^{-1}, higher than the one obtained in our previous work, 5.9 g L^{-1} h^{-1}, for the same TRS conversion. The increase in the glycerol concentration seems to follow the increase of ethanol concentration, indicating that the metabolic routes for the two products are paired.

Table 3 Glucose (G), total reducing sugars (TRS), ethanol (Et) and glycerol (Gly) concentrations, and TRS conversion (X_{TRS}) for different residence times (θ), using glucoamylase and *S. cerevisae* co-immobilized in pectin gel.

θ (h)	Assay duration (h)	G (g L^{-1})	TRS (g L^{-1})	Et (g L^{-1})	Gly (g L^{-1})	X_{TRS} (%)
10.0	68	0.0	0.1	57.5	3.9	100.0
8.0	109	0.1	0.2	56.7	4.1	100.0
6.0	190	0.1	0.5	55.2	4.6	100.0
5.0	227	1.2	4.0	53.1	4.6	97.0
4.0	248	6.3	15.0	48.0	4.3	89.0
3.0	268	7.4	33.0	42.0	3.7	75.0
2.0	288	7.7	70.0	27.1	2.0	48.0
1.0	316	12.0	84.0	17.5	1.2	38.0

Enzyme concentration=2.8 U $mL^{-1}{}_{reactor}$. Continuous run 2. Feed: TRS, 136.0 g L^{-1}; G. 2.8 g L^{-1}

Table 4 Glucose (G), total reducing sugars (TRS), ethanol (Et) and glycerol (Gly) concentrations, and TRS conversion (X_{TRS}) for different residence times (θ), using glucoamylase and *S. cerevisae* co-immobilized in pectin gel.

θ (h)	Assay duration (h)	G (g L^{-1})	TRS (g L^{-1})	Et (g L^{-1})	Gly (g L^{-1})	X_{TRS} (%)
8	55	0.1	0.3	72.7	4.9	100.0
6	159	4.2	5.0	70.3	5.2	97.0
4	185	22.0	25.0	58.7	4.8	84.0
3	209	25.3	48.6	45.1	3.8	68.0
2	230	15.0	69.7	36.7	2.9	55.0
1	238	8.4	97.4	22.3	1.7	37.0
6	275	3.1	8.7	70.0	4.4	94.0

Enzyme concentration=3.8 U $mL^{-1}{}_{reactor}$. Continuous run 3. Feed: TRS=154 g L^{-1}; G=2.8 g L^{-1}

A higher enzyme load, 2.8 U $mL^{-1}{}_{reactor}$, was then tested. The TRS in the feed (TRS concentration at the reactor inlet), 136.0 g L^{-1}, was also higher than in run 1, 118.0 g L^{-1}. Table 3 shows the obtained results.

As expected, results shown in Table 3 indicated some improvement in the performance of the process after the increase of enzyme concentration. The feeding TRS was higher in run 2 than in run 1, and the same TRS conversion, 97.0%, was also achieved with 5 h of residence time, leading to a higher ethanol productivity, 10.6 g L^{-1} h^{-1}, with η_{Et}=79.0%. However, glucose concentration in this condition was still lower than the TRS concentration, indicating that the hydrolysis was still the rate-limiting step. The highest ethanol concentration reached in run 2 was higher than the one obtained in run 1. A correspondent increase in the glycerol concentration, from 3.0 g L^{-1} in run 1 to 4.5 g L^{-1} in run 2, was also observed, in agreement with the previous hypotheses that the two metabolic routes of the yeast are paired.

In order to operate at the same enzyme load previously used [14], in run 3, 3.8 U $mL^{-1}{}_{reactor}$ was used. In this run, after analyzing the system for residence times between 8 and 1 h, in a reactor overall journey of 238 h, the flow rate was set back to 16.7 mL h^{-1} (residence time of 6 h). The performance of the packed-bed reactor at this condition (θ=6 h) was evaluated again, after a reactor continuous operation of 275 h. Table 4 and Fig. 3 show the results.

Results in Table 4 show clearly that in this operational condition, the hydrolysis is no longer the rate-limiting step: Above θ=4 h, the conversion of the limit dextrins and maltose into glucose approaches 100.0% (i.e., TRS ≅ G), and for residence times (θ) up to 6 h, there is accumulation of glucose. In this run, another increase in the highest ethanol concentration was achieved, which reached 72.7 g L^{-1}. In agreement, the correspondent glycerol concentration increased to 4.9 g L^{-1}.

Table 4 provides a positive indication of the reactor operational stability. Indeed, after 275 h of operation, the feed flow rate was adjusted to a residence time of 6 h, the same condition that was employed at 159 h. The ethanol concentration measured at the reactor outlet returned to the same value, and the TRS conversion was only 3.0% lower.

Figure 4 shows the calculated values of productivities for different residence times, for the three continuous runs. For residence times higher than 3 h, higher enzyme concentrations in the reactor imply higher ethanol productivity for all the residence times tested. Of course, the optimal operational condition must take into account both productivity and starch conversion. A good combination of these indices was achieved for θ=6 h in run 3 (feed, TRS=154.0 g L^{-1}): Ethanol productivity reached 11.7 g L^{-1} h^{-1},

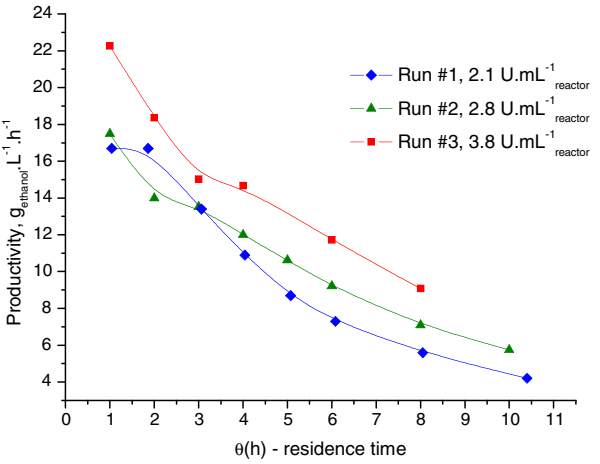

Fig. 4 Ethanol productivities for different glucoamylase loads in the packed-bed reactor. Ethanol productivities at different residence times, for runs 1, 2, and 3

with 97.0% of TRS conversion, H_{Et}=92.3 and ethanol concentration of 70.3 g L^{-1} at the reactor outlet. This was a significant improvement, when compared with the previous work (productivity of 5.9 g L^{-1} h^{-1}).

Krishnan et al. [18, 19] also studied the SSF process to produce ethanol from a solution of 15% dry-milled corn starch previously liquefied with α-amylase in a fluidized bed reactor. The authors used immobilized glucoamylase co-immobilized with *Zymomonas mobilis* in κ-carrageenan beads, a biocatalyst very similar to the one used in this work. They achieved a ethanol productivity of 9.1 g L^{-1} h^{-1}, 89.3% of TRS conversion, and 36.44 g L^{-1} of ethanol.

Table 5 shows initial and final pH, cell concentrations, cell viability, and mass of biocatalyst in the three continuous runs. It can be observed that the values of these variables were very similar in the three runs. The increase of the mass of particles was higher than the one that would be expected due to the growth of the yeast only. Therefore, some swelling of the particles may have occurred.

Comparing the Performances of SSF Processes to Produce Ethanol from Liquefied Cassava Syrup

Figure 5a (previous work [14]) and b(this work) shows glucose, TRS, and ethanol concentrations at different residence times, using the same enzyme and yeast concentration

Table 5 Initial and final cell concentrations (X_g), cell viability (Vb_g), and mass (M_g) of the biocatalyst particles (co-immobilized biocatalyst) and initial and final pH, cell concentrations (X_{ef}), and cell viability (Vb_{ef}) in the effluent.

Run		X_g (viable cell g^{-1})	X_{ef} (viable cell mL^{-1})	Vb_g (%)	Vb_{ef} (%)	M_g (g)	pH effluent
1	Initial	8.3×10^8	–	91.6	–	50.0	4.2
	Final	1.5×10^9	2.0×10^7	96.8	91.2	61.7	4.6
2	Initial	2.2×10^8	–	83.3	–	50.0	4.2
	Final	1.3×10^9	3.2×10^7	85.0	93.6	63.7	3.9
3	Initial	9.3×10^8	–	94.7	–	50.0	4.3
	Final	1.4×10^9	2.8×10^7	82.0	86.3	62.1	4.0

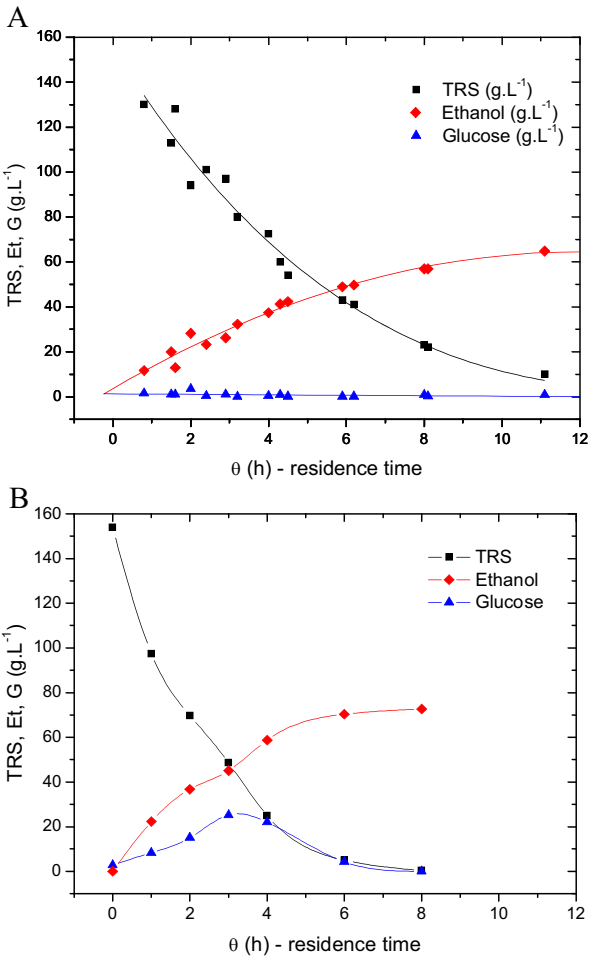

Fig. 5 Glucose (G), total reducing sugars (TRS) and ethanol (Et) concentration for different residence times (θ), using glucoamylase and *S. cerevisae* co-immobilized in pectin gel. Enzyme concentration=3.8 U $mL^{-1}_{reactor}$. **a** Previous work [14], **b** this work (with smaller biocatalyst particles), continuous run 3

in the reactor, but for different sizes of the biocatalyst and mass molar of the substrate. It can be seen that, using smaller particles diameters, there is glucose available to the yeast for all the residence times (Fig. 5b).

Table 6 shows parameters of three SSF processes to produce ethanol from liquefied cassava syrup: a batch process, using soluble yeast and enzyme [20]; a continuous, packed-bed reactor, using enzyme immobilized in silica particles with diameter of 100 μm and co-immobilized with yeast in 4 mm pectin gel particles [14]; and the process studied in this work, a continuous packed bed reactor using enzyme immobilized in silica particles with diameter of 60 μm and co-immobilized with yeast in 1–1.5 mm pectin gel particles, and feeding with substrate submitted to a two-stage pretreatment with α-amylase.

Aboutboul [20] studied the influence of different glucoamylase and *S. cerevisiae* concentrations in the performance of the SSF process to produce ethanol from cassava flour pre-hydrolyzed with α-amylase (a substrate similar to the one used in our work with co-immobilized biocatalyst [14]). The condition that led to the highest productivity in [20] was used in this study to compare this SSF process (soluble biocatalysts) with the continuous process using enzyme and yeast co-immobilized in pectin. The improvement in the

Table 6 Comparison of SSF processes to produce ethanol from liquefied cassava syrup.

Operational condition	Process 1 Batch, soluble [19]	Process 2 Packed-bed [14]	Process 3 Packed-bed (this work)
Syrup pretreatment	0.5 g L^{-1} α-amylase, 30 min, 90°C	0.5 g L^{-1} α-amylase, 30 min, 90°C (PHS)	(PHS)+1.5 g L^{-1} α-amylase, 30 min, 90°C
Glucoamylase (U L^{-1})	240	3,800	3,800
X_i (g L^{-1})	9	50	50
Biocatalyst dimensions	–	ϕ pectin=4.0 mm ϕ silica=60 μm	ϕ pectin=1.0–1.5 mm ϕ silica=60 μm
Reaction time (1) or residence time (2, 3) (h)	12	12	8
Initial (1) or feed (2,3) TRS (g L^{-1})	155	163	154
Reaction time (1) or residence time (2, 3) for TRS = G	12	12	4
Et (g L^{-1})	57.9	64.8	70.3
Pr_{Et} (g_{Et} L^{-1} h^{-1})	4.82	5.9	11.7
X_{TRS} (%)	98	97	97
η_{et} (%)	74	81	92
$Pr_{Et/U}$ ($g_{Et}.U^{-1}$ h^{-1})	0.020	0.0015	0.0031

Process 1: Batch process using soluble enzyme and yeast [19]. Process 2: Packed-bed using yeast and enzyme co-immobilized in pectin gel, Giordano et al. [14]. Process 3: this work

performance of the continuous process with the decrease of the particle diameters and substrate molar mass is very clear, indicating the presence of important mass transfer effects in the continuous process.

From the results shown in Table 6, a rough analysis of possible savings in glucoamylase costs can be draw. The co-immobilized SSF (process 3 in Table 6) was operated steadily at least for 275 h, thus producing 0.85 g ethanol/U of glucoamylase. During the same period, approximately 23 batches could be run, providing 0.24 g ethanol/U of glucoamylase (process 1 in Table 6). This is a very crude comparison, since the enzyme life in the continuous process is higher than 275 h, and labor costs for the batch process would be higher because they would include cleaning and loading times. On the other hand, immobilization costs were not taken into account in this study nor were the surplus costs of α-amylase for the second prehydrolysis. Anyway, these figures, showing a 3.5-fold reduction of the glucoamylase demand when using the SSF process, are a positive indication of its feasibility. Of course, a deeper evaluation of process costs would demand assessing the deactivation patterns of the continuous process in long-term runs.

The improvement in the process performance observed in this work shows the importance of promoting a more drastic pretreatment of starch with α-amylase, providing a substrate with lower molar mass for the SSF reactor. With this substrate, a medium with lower viscosity is attained, and the substrates diffusivities increase. Thus, mass transfer resistances through the extra-particle film and through the biocatalyst pores are reduced.

The influence of this pretreatment on the intrinsic rates of hydrolysis by glucoamylase cannot be discarded either. This enzyme attacks the non-reducing ends of the substrate molecule, and the rates of hydrolysis decrease with the oligosaccharide chain length [21, 22]. However, a more extensive action of α-amylase during the pretreatment (as shown in

Fig. 3) would result in more cleaving sites for glucoamylase per mass of substrate (i.e., more non-reducing oligosaccharide molecules' ends available). In other words, the effective substrate concentration (concentration of non-reducing end sides) would be higher at the inlet of the catalytic bed, causing an increase of the reaction rates—if substrate inhibition effects are not significant. These are opposite trends, and a study of the influence of the degree of prehydrolysis on the kinetics of free (soluble) glucoamylase would be interesting to clarify this point. This is out of the scope of this work, however. Nevertheless, our results clearly point out that the overall effect of a more drastic prehydrolysis with α-amylase was favorable.

On the other hand, reducing the size of the biocatalyst (both the silica particles and the pectin gel beads) facilitates the mass transport of the substrate, thus increasing the apparent reaction rates. Possibly, a set of repeated batches using the same operational conditions would help in clarifying the relative importance between internal and external mass transport resistances.

Conclusions

A significant improvement in the performance of a continuous SSF process to produce ethanol from liquefied cassava syrup in a packed-bed reactor, using glucoamylase immobilized in silica and co-immobilized with *S. cerevisiae* in pectin gel, was achieved by reducing the biocatalyst size and by promoting a more drastic prehydrolysis of the substrate, cassava starch, with α-amylase. The reactor was tested during 245 h and operated stably during all this period.

In a previous work, using a less severe liquefaction step with α-amylase and particles diameters of 4 mm and 100 μm, for pectin gel and silica, respectively, the SSF process reached 5.9 g L^{-1} h^{-1}, for a feeding of 163.0 g/L (TRS), with 97.0% of TRS conversion, ethanol yield of 81.0%, and 65.4 g/L of ethanol. In this work, using a second liquefaction step and smaller pectin and silica particle diameters, the process reached ethanol productivity of 11.7 g L^{-1} h^{-1}, TRS conversion of 97.0%, ethanol yield of 92.0%, and ethanol concentration of 70.3 g/L. The comparison with a SSF process that used the yeast and enzyme in soluble form showed that this process is very promising. Nevertheless, longer reactor operating times are necessary to evaluate its deactivation dynamics, thus providing the basis for a more realistic economic analysis of the process.

Nomenclature

Et	ethanol concentration (g L^{-1})
G	glucose concentration (g L^{-1})
N_{viable}	average viable cell number in a square of the Neubauer chamber
M_g	mass of the gel particles
PHS	α-amylase pre-hydrolyzed syrup
Pr_{Et}	ethanol productivity ($g_{ethanol}$ L^{-1} h^{-1})
$Pr_{Et/U}$	ethanol productivity per unit of enzyme ($g_{ethanol}$/h/glucoamylase concentration)
Q_{feed}	feed volumetric flow rate (ml/h)
U	unit of enzyme
TRS	total reducing sugar, expressed as glucose concentration (g L^{-1})
Vb_{ef}	cell viability in the effluent of the reactor
Vb_g	cell viability in the pectin gel

X_g	cell concentration in the gel (viable cells/g gel)
X_{ef}	cell concentration in the effluent (viable cells/mL)
X_i	initial cell concentration in the reactor (wet cells/L)
X_{TRS}	TRS conversion
η_{Et}	ethanol yield (%)—percentage of the theoretical yield
ϕ	particle diameter
θ	residence time (h) = reactor volume/Q_{feed}

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