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Maltose Production Using Starch from Cassava Bagasse Catalyzed by Cross-Linked β-Amylase Aggregates

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Abstract: Barley β-amylase was immobilized using different techniques. The highest global yield was obtained using the cross-linked enzyme aggregates (CLEA) technique, employing bovine serum albumin (BSA) or soy protein isolate (SPI) as feeder proteins to reduce diffusion problems. The CLEAs produced using BSA or SPI showed 82.7 ± 5.8 and 53.3 ± 2.4% global yield, respectively, and a stabilization effect was observed upon immobilization at neutral pH value, e.g., after 12 h at 55 °C, the free β-amylase is fully inactivated, while CLEAs retained 25 and 15% of activity (using BSA and SPI, respectively). CLEA using SPI was selected because of its easier recovery, being chosen to convert the residual starch contained in cassava bagasse into maltose. This biocatalyst permitted to reach almost 70% of maltose conversion in 4 h using 30.0 g/L bagasse starch solution (Dextrose Equivalent of 15.88) and 1.2 U of biocatalyst per gram of starch at pH 7.0 and 40 °C. After 4 reuses (batches of 12 h) the CLEA using SPI maintained 25.50 ± 0.01% of conversion due to the difficulty of recovering.

Keywords: barley β-amylase; cross-linked enzyme aggregates (CLEA); cofeeders; enzyme immobilization; cassava bagasse

1. Introduction

Cassava (*Manihot esculenta* Cranz) is produced worldwide and plays an important role in food security in tropical areas [1]. One of the most important cassava industrial products is starch [2], which is usually extracted by a mechanical shear. The mechanical extraction of starch from 1 ton of cassava roots results in 254.7 kg of starch (~10% moisture) and 928.6 kg bagasse (85% moisture) [3]. Sriroth et al. [4] reported that the mechanical extraction of starch from cassava is limited, removing only partially the starch existing in the root fibers. The bagasse waste can contain up to 60 wt % of starch (dried basis) and is usually considered as a residue and disposed to the environment as landfill without any treatment [2], although in some instances it is used in animal feed mixtures or as additive to enrich fertilizers [2,4,5]. An alternative application of the residual starch from cassava waste could be the conversion into added-value products such as maltodextrins, glucose and maltose

syrups using amylolytic enzymes. Maltose is widely used in the food industry, particularly in the brewing industry [6].

The starch chain can be hydrolyzed into dextrins by several groups of amylases [7,8]. α-Amylases cleave the starch molecule into smaller oligosaccharides, being used to improve the gelatinization process, in enzymatic starch extraction, to decrease medium viscosity and in saccharification processes (combined or not with other amylases) [7,8]; β-amylases (EC 3.2.1.2) are responsible for partial hydrolysis of starch non-reducing ends into maltose molecules [7–9]. There are several different sources of β-amylase, i.e., *Bacillus* spp, *Bacillus cereus*, soybean, sweet potato and barley [7,8]. A commercial β-amylase used for maltose production is that from barley [7]. Barley β-amylase has a monomeric structure with a molecular mass of 56–60 kDa [10], high activity in a pH ranging from 4.0 to 7.0 (optimum pH of 4.35 [11]). However, it is unstable at temperatures over 55 °C [12,13]. The use of a high temperature is desirable in maltose syrup production to improve operational aspects like viscosity of gelatinized starch solutions and highly concentrated sugar solutions and to avoid microbial growth [7]. Moreover, it looks convenient for the economy of the process to develop strategies that may permit the easy reuse of the barley β-amylase in several reactions cycles.

The enzyme immobilization may be a solution for these enzyme problems and even other enzyme limitations [14–17]: it allows a simple biocatalysts recovery and reuse [18], and becomes a powerful tool to improve enzyme features [19,20], such as stability [21,22], selectivity, specificity [23] or even purity [24]. These advantages have, as main costs, the immobilization process and immobilization carrier prices.

Chitosan is a prominent and versatile support, allowing different immobilization strategies [25–28]. It is obtained by deacetylation of chitin, a component of the exoskeleton of crustaceans and other arthropods [26]. That way, a support containing primary amino groups is easily obtained from a residue. This support may be used to directly ionically exchange enzymes [29], enabling a reversible immobilization that may not have high stabilizing effects on the protein [30]. The reversibility of the immobilization enables the reuse of the support, but may also raise a problem: the release of the enzyme during operation. To solve this, covalent glutaraldehyde immobilization may be a good alternative, this is a very used reactive even though the exact mechanism is unknown [31–33]. Amino-glutaraldehyde supports are heterofunctional ones [34] and they are very versatile for enzyme immobilization [35], enabling the use of glutaraldehyde preactivated supports [36] or the treatment of the previously adsorbed enzymes to obtain enzyme-support bonds [37]. Recently, the effect of the pH on the first immobilization step has been also showed [38,39]. Chitosan may be also utilized to encapsulate the enzyme [40,41]. Encapsulation has always the risks of enzyme release, as the pores need to be very small to relay trap the enzyme [42–44], but using chitosan some ion exchange of the enzyme on the polymer may be expected to reduce the enzyme release and crosslinking with glutaraldehyde may further reduce this enzyme leakage.

Professor Sheldon's group proposed a strategy that avoided the use of supports to immobilize enzymes: the cross-linked enzyme aggregates (CLEAs) [45–47]. The preparation of CLEAs is a very simple method that consists of two steps: (i) aggregation/precipitation of proteins induced by precipitant agents (salts, water-miscible organic solvents, non-ionic polymers, etc.); and (ii) chemical crosslinking of the formed aggregates with a bifunctional reagent (e.g., glutaraldehyde) via reaction with amino groups of the lysine residues that are presented on the surface of the enzyme [48,49]. However, if the enzyme has low surface density of amino groups, the immobilization could be inefficient, due to the low cross-linking bonds. Some authors suggest the amination of the enzyme (genetically or chemically) [50,51], or the coaggregation of enzyme and aminated polymers [52,53]. Yet, the first solution is complex and the second one produces a highly hydrophilic and cationic micro-environment around the enzyme that may be undesired. An alternative with a great success is the use of a feeder protein rich in lysine residues, such as bovine serum albumin (BSA), which can contribute to the formation of CLEAs with high global yield and excellent operational stability [48,49,54–60]. A cheaper alternative to BSA is soy protein isolate (SPI). This is an industrial

residue from extraction of soybean oil [61], which has about 90% of heterogenic protein content, mainly composed by 7S (β-conglycinin) and 11S (glycinin) storage proteins [62], of molecular sizes of 150–175 kDa [63] and 340–375 kDa [64], respectively. SPI is about 250 times cheaper than BSA (R$34.90/kg and R$9803.00/kg, respectively; values from Estação dos Grãos Ltd. (Perdizes, Brazil) and Sigma-Aldrich (St. Louis, MO, USA) in March 2018), what could turn CLEA technology economically viable when a feeder protein is necessary. The feeder could also reduce other CLEAs problems, due to the high volumetric activity in many instances diffusion limitations become very high and the feeder protein will "dilute" the enzyme [18].

Several researchers immobilized β-amylases from different sources using carrier-bound methods, stabilizing the enzyme [65–67] but recovering low activity [65–69], attributed mainly to substrate diffusional problems. As far as we know, there are no reports about the immobilization of β-amylase by the CLEA technique.

This study shows an option for the use of cassava bagasse as starch source for maltose production and explores the barley β-amylase immobilization by different techniques, including the CLEA methodology using two different feeder proteins, bovine serum albumin (BSA), which is used as feeder protein in many other studies [48,54–60], and soy protein isolate (SPI). The best biocatalyst obtained in this study will be tested in the maltose production using commercial and residual cassava starch.

2. Results and Discussion

2.1. β-Amylase Immobilization

The enzyme was first immobilized using chitosan as a carrier using the different strategies described in the Methods section. Immobilization via ion exchange presented low immobilization and global yields (less than 25%, Table 1); however, high expressed activity was achieved (around 100%). The treatment of the already ionically exchanged enzyme with glutaraldehyde drives to full enzyme inactivation. Immobilization on glutaraldehyde activated chitosan permitted the full enzyme immobilization, but the expressed activity and global yield were very low (just over 6%). Thus, these strategies seem unsuitable to immobilize this enzyme; as an alternative, we tried to trap the enzyme on chitosan beads at a lower concentration, to have larger pores. The immobilization is not really a trapping, as the polymer will ionically immobilize the enzyme and we treat the immobilized enzyme with glutaraldehyde; in any case the expressed activity was even lower than in the other cases, perhaps the enzyme may be released, perhaps the polymer may block the active center of the enzyme, or the glutaraldehyde inactive the enzyme.

Table 1. Carrier-bond immobilization using chitosan 2% (for adsorption and covalent attachment), 1% (for encapsulation) and free-carrier immobilization (CLEA) without feeder protein. It was supplied 0.53 mg of protein per gram of carrier in each immobilization method using chitosan.

Immobilization Method	Immobilization Yield (%)	Expressed Activity (%)	Global Yield (%)
Adsorption	21.4 ± 3.4	108.9 ± 17.6	23.3 ± 3.8
Adsorption followed by crosslinking [1]	21.4 ± 3.4	0.0 ± 0.0	0.0 ± 0.0
Covalent attachment [2]	100.0 ± 0.0	6.7 ± 0.8	6.7 ± 0.8
Encapsulation [3]	–	–	1.1 ± 0.5
CLEA [4]	–	–	34.2 ± 3.3
CLEA [5]	–	–	25.8 ± 1.2

[1] Using 0.15% glutaraldehyde solution as crosslinker; [2] activated with 0.80% glutaraldehyde solution; [3] using 0.10% glutaraldehyde solution as crosslinker; [4] using 0.30% glutaraldehyde solution as crosslinker; [5] using 0.60% glutaraldehyde solution as crosslinker.

For this reason we tried the immobilization of β-amylase using CLEA technology. The results (Table 1) were better than when using chitosan (near to 30% of global yield). Thus, CLEAs technology appeared to be more suitable to immobilize this β-amylase, with the advantage of saving the support.

Aiming to improve the CLEA global yield, we tried to optimize some immobilization aspects of this biocatalyst.

2.2. Optimization of β-Amylase Immobilization Using CLEAs Technology

To optimize the CLEA, we decided to use feeder proteins. As explained in introduction, this may have a double positive effect: to improve the crosslinking step and to reduce the diffusion problems by diluting the enzyme into inert protein [46,48,49]. Thus, the effects of the feeder proteins (BSA or SPI) and glutaraldehyde concentrations for the same β-amylase concentration on the global yield were studied (Section 3.4). More than 80% of global yield was achieved using BSA as feeder at condition 5 (CLEA-β-BSA-5) and more than 50% using SPI as feeder at condition 12 (CLEA-β-SPI-12), as presented in Table 2. Using a feeder protein, the final activity of the biocatalyst was higher than using just the enzyme.

Table 2. Global yields for CLEAs of β-amylase prepared with different concentrations of feeder protein and glutaraldehyde.

Assay	Feeder Protein	Global Yield (%)
1	None	34.2 ± 3.3
2		25.8 ± 1.2
3	BSA	54.5 ± 0.9
4		43.2 ± 0.4
5		82.7 ± 5.8
6		53.1 ± 0.1
7		33.6 ± 0.5
8		0
9		0
10		0
11	SPI	45.2 ± 2.1
12		53.3 ± 2.4
13		48.0 ± 2.3
14		47.5 ± 1.5
15		38.2 ± 5.2
16		31.0 ± 1.8
17		28.4 ± 2.9
18		24.4 ± 0.5
19		28.6 ± 0.0

The CLEAs obtained using SPI (CLEA-β-SPI) were noticeably larger than the CLEAs produced using BSA or without feeder protein, helping in the separation of the biocatalyst from the viscous medium when submitted to centrifugation. This larger size may be the cause of the lower expressed activity of CLEA-β-SPI-12 compared to CLEA-β-BSA-5.

Figure 1 shows images of CLEA-β-SPI-12 using scanning electron microscopy, where it is possible to see the heterogeneity in the particle size (lower than 70 μm) of the prepared biocatalyst.

The activities obtained using both feeder proteins in the CLEA preparation were better than those reported in several studies employing β-amylases from different sources immobilized on preexisting supports. Vretblad and Axen [66] reported a global yield of 35% for barley β-amylase covalently attached on Sepharose activated with 4,4′-methylene dianiline. Tavano et al. [69] reported global yield of 21% for sweet potato β-amylase immobilized on glyoxyl-agarose support. These results are much closer to those obtained in our work for barley β-amylase ionically adsorbed on chitosan (Table 1).

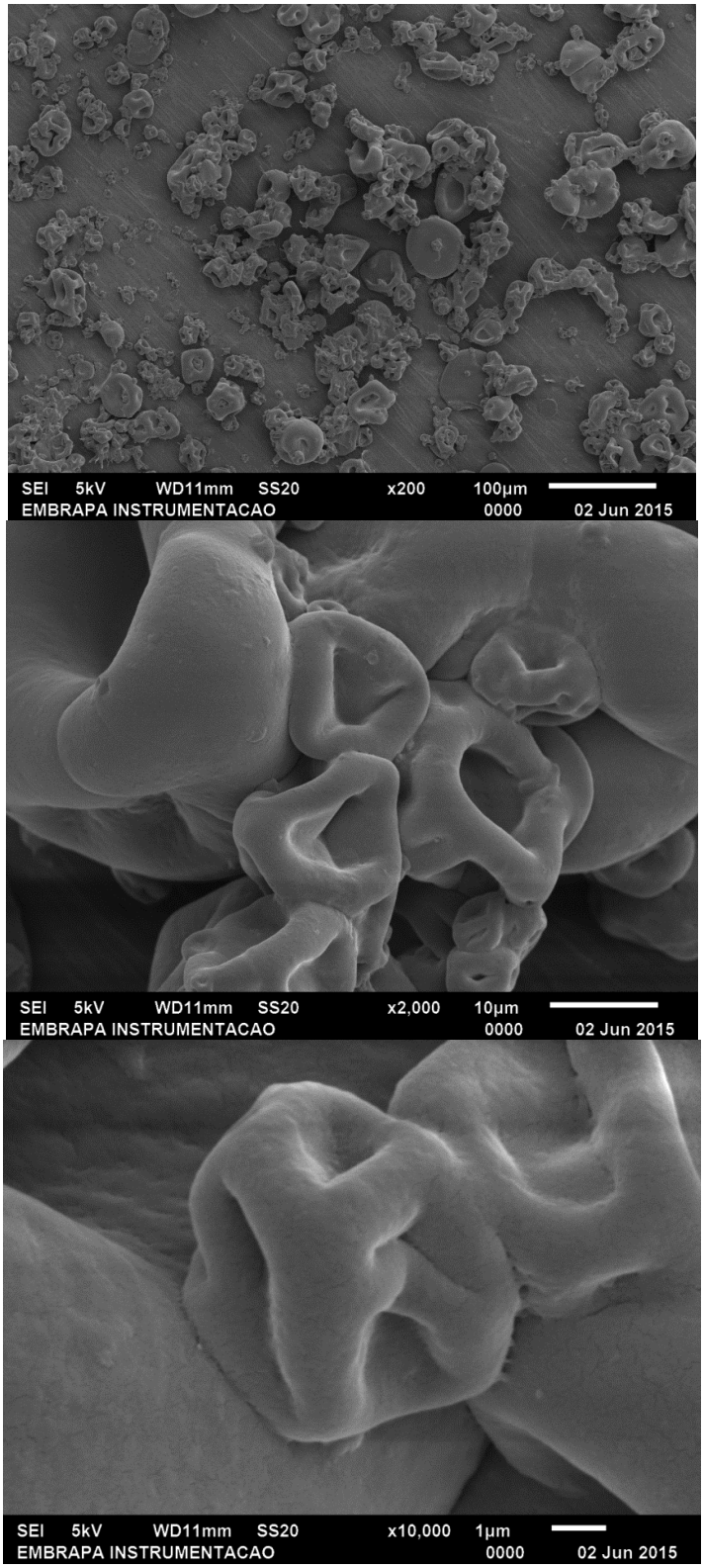


Figure 1. Images of CLEA-β-SPI-12 using scanning electron microscopy at different amplifications.

2.3. Effects of pH and Temperature on the Activity and the Stability of β-Amylase Preparations

The study of the effect of the reaction pH and temperature on the enzyme activity of β-amylase was performed using free enzyme, CLEA-β-BSA-5 and CLEA-β-SPI-12 to investigate the influence of the enzyme immobilization technique on enzyme response to the medium [68–71] (Figure 2).

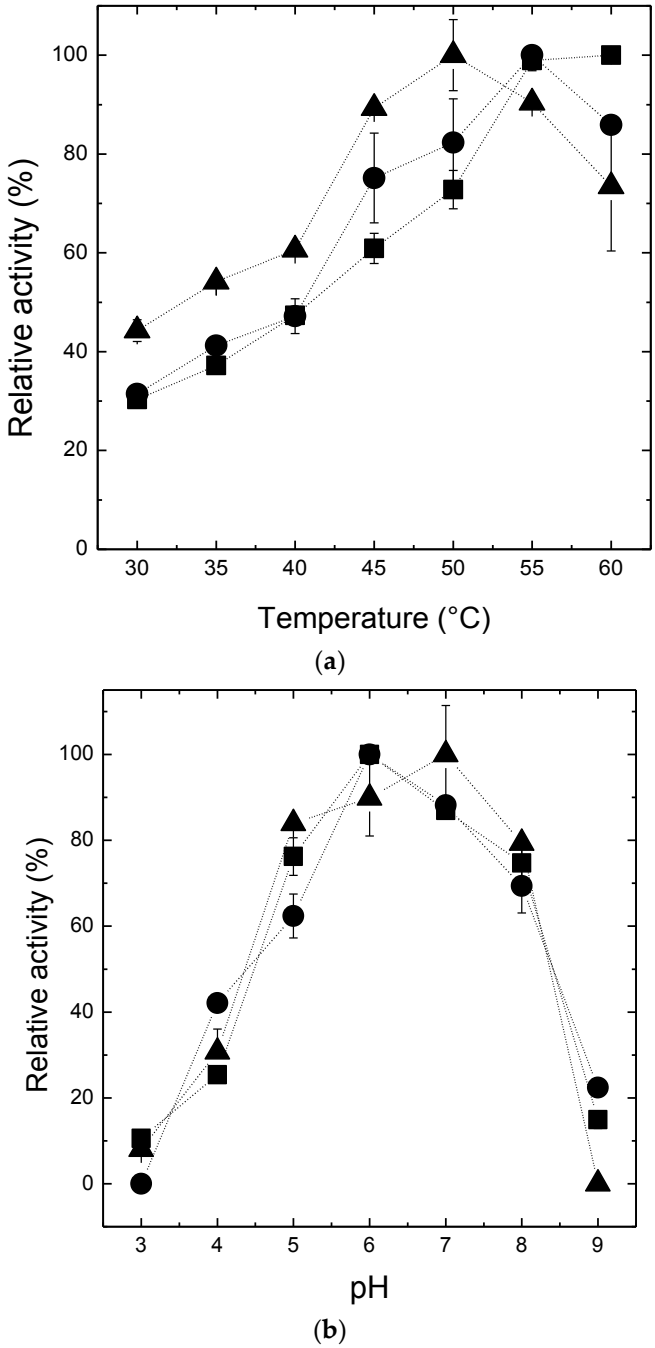


Figure 2. Temperature activity profile (**a**), using 1% (*w*/*v*) soluble starch solution at pH 4.8 (standard condition for enzymatic assay as described in the Section 3.2) and pH activity profile (**b**), using 1% (*w*/*v*) soluble starch solution at 20 °C, for free β-amylase (squares), CLEA-β-BSA-5 (circles) and CLEA-β-SPI-12 (triangles). The maximum activities were taken as 100%.

The effect of the temperature on the enzyme activity was similar for all β-amylase preparations (Figure 2a), expressing maximum activities in the range 50–55 °C. In the case of CLEA-β-SPI-12,

a small shift to lower temperature (approximately 5 °C) of the maximum activity temperature was observed. It was expected that optimal pH and temperature of enzymes immobilized as CLEAs were shifted because covalent bonds established between amino groups of the enzyme and glutaraldehyde, changing the microenvironment and the conformational flexibility of the enzyme [49]. Talekar et al. [72] reported a shift to higher temperature (around 15 °C) for β-amylase from *Bacillus amyloliquefaciens* after immobilization by CLEA technique. However, Mafra et al. [58] reported the same maximum activity temperature for soluble and CLEAs of catalase. The effect of the pH on enzyme activity (Figure 2b) was similar for all enzymes, expressing maximum activity in the pH range 6.0–7.0. Talekar et al. [72] reported a pH shift from 6.0 to 7.0 for α-amylase from *B. amyloliquefaciens* immobilized as CLEA, while Mafra et al. (2016) also reported a pH shift from 7.0 to 7.4 for catalase immobilized as CLEA with BSA as feeder protein. In our work, the pH shift from 6.0 to 7.0 for β-amylase immobilized as CLEA with SPI as feeder protein was not different statistically, considering the standard deviations (as provided by the F test).

Figure 3a shows the residual activity after 12 h incubation at different pH values and 25 °C of free β-amylase, CLEA-β-BSA-5 and CLEA-β-SPI-12. In almost all cases, the free enzyme exhibited a slightly higher residual activity, followed by CLEA-β-BSA-5. The highest activity retained for each sample was observed at pH 7.0 (96.0 ± 0.6%), 6.0 (88.2 ± 3.5%) and 7.0 (64.2 ± 22.9%), for free β-amylase, CLEA-β-BSA-5 and CLEA-β-SPI-12, respectively. Figure 3a shows that at pH 5.0 (very similar to pH 4.8 used in optimal temperature assay), the stability of free β-amylase is higher than those of CLEA-β-BSA-5 and CLEA-β-SPI-12 (residual activities of 94.2 ± 0.0, 75.7 ± 3.2 and 45.1 ± 2.2% respectively). This enzyme inactivation (especially high for CLEA-β-SPI-12) at acid pH value can explain the results observed in the effect of temperature on enzyme activity.

The temperature stability test was performed at the optimal pH for the stability of each β-amylase, pH 6.0 for CLEA-β-BSA-5 and pH 7.0 for free β-amylase and CLEA-β-SPI-12. Figure 3b shows that at 40 °C free β-amylase and CLEA-β-SPI-12 did not present significant thermal deactivation after 12 h (109. 2 ± 3.2 and 96.1 ± 5.5% of residual activity respectively). At 55 °C the CLEAs retained some activity when the free enzyme was fully inactivated. The thermal stabilization observed at 55 °C was reported for several enzymes immobilized as CLEAs by Sheldon [48] and Talekar et al. [49].

Although CLEA-β-BSA-5 presented higher global yield and pH stability than CLEA-β-SPI-12, its stability at high temperatures was high. Also, the CLEA-β-BSA-5 was more difficult to manipulate and separate from the very viscous reactional medium, containing a high sugar concentration. Thus, the CLEA-β-SPI-12 was chosen to be compared with free β-amylase on the maltose production assay.

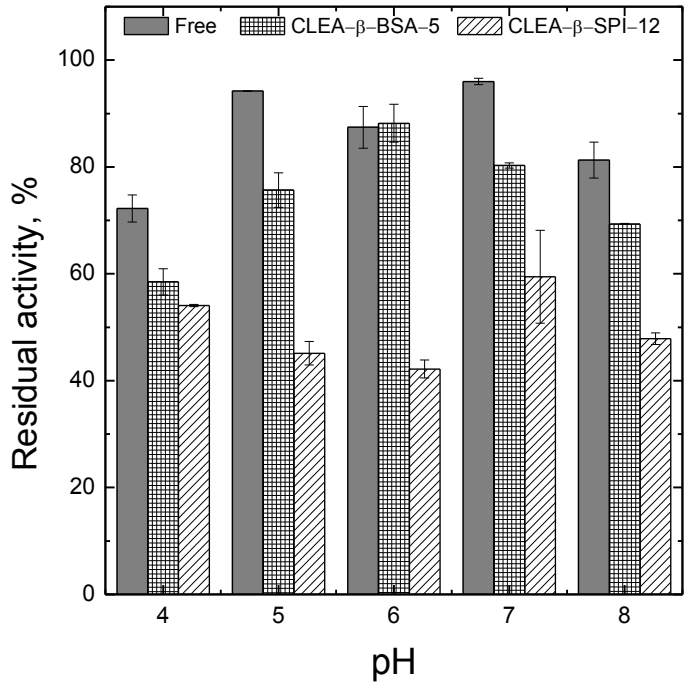
2.4. Maltose Production

Maltose production was performed using soluble and residual cassava bagasse (enzymatically extracted) starches with free and CLEA-β-SPI-12 under the respective pH and temperature conditions where the biocatalysts exhibited optimal stabilities.

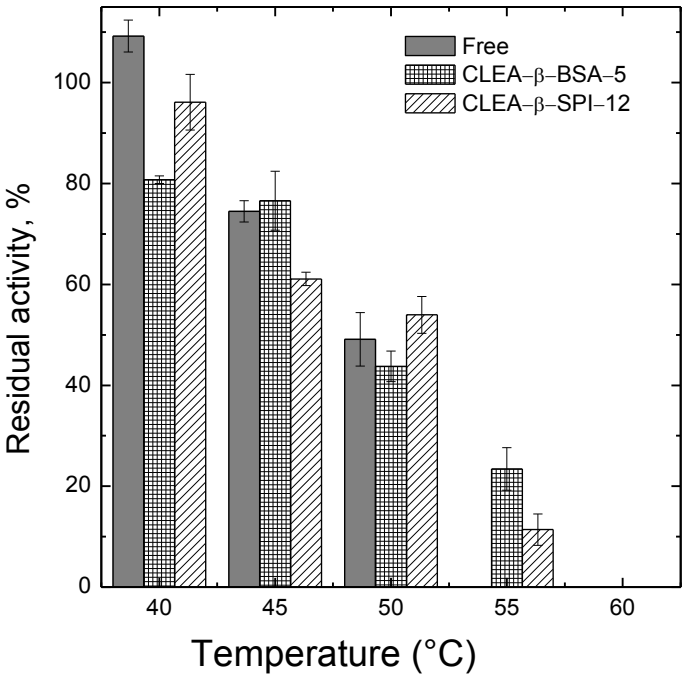
The residual starch solution was obtained via enzymatic extraction of cassava bagasse with starch content of 45.7 ± 0.8% (d.m.). The high starch content in cassava bagasse from industrial mechanical extraction can be related, as explained in introduction, to the limitations of shear and broke the cassava root fibers to extract the starch [4] and this content can vary depending on cassava crop and processing conditions [2].

The maltose conversion by free β-amylase and CLEA-β-SPI-12 were compared using soluble starch solution containing 30.0 g/L of commercial starch (dextrose equivalent, DE ≈ 0) and 36.0 U/L, the equivalent of 1.2 U/g (enzymatic unit per gram of starch), for each enzyme preparation. Figure 4a shows that maltose conversion using free enzyme was faster than using CLEA-β-SPI-12. After 45 min, the free enzyme achieved 44.5% of conversion, while CLEA-β-SPI-12 presented the same conversion after 9 h. The maximum conversions were 52.3 ± 2.3 (after 6 h) and 46.4 ± 5.4% (after 12 h) for free β-amylase and CLEA-β-SPI-12, respectively. The productivities and specific productivities of both soluble starch conversions were 2.7 ± 0.1 and 1.2 ± 0.1 kg/m^3 h, and 75.9 ± 3.3 and 33.7 ± 3.9 mg/Uh

using free β-amylase and CLEA-β-SPI-12, respectively. Yoshigi et al. [73] reported 57.7% of starch conversion using free barley β-amylase and 5.0 g/L soluble starch solution (50 mM acetate buffer, pH 5.5) incubated at temperature of 37 °C. This could be due to the high size of the starch used, as well as the low DE, probably causing diffusional limitations in the CLEA-β-SPI-12.



(**a**)



(**b**)

Figure 3. Residual activities of free β-amylase, CLEA-β-BSA-5 and CLEA-β-SPI-12 for pH stability (**a**) after 12 h of incubation at 25 °C and temperature stability and, (**b**) after 12 h of incubation at pH 6.0 (CLEA-β-BSA-5) and pH 7.0 (free β-amylase and CLEA-β-SPI-12). 100% is the initial activity.

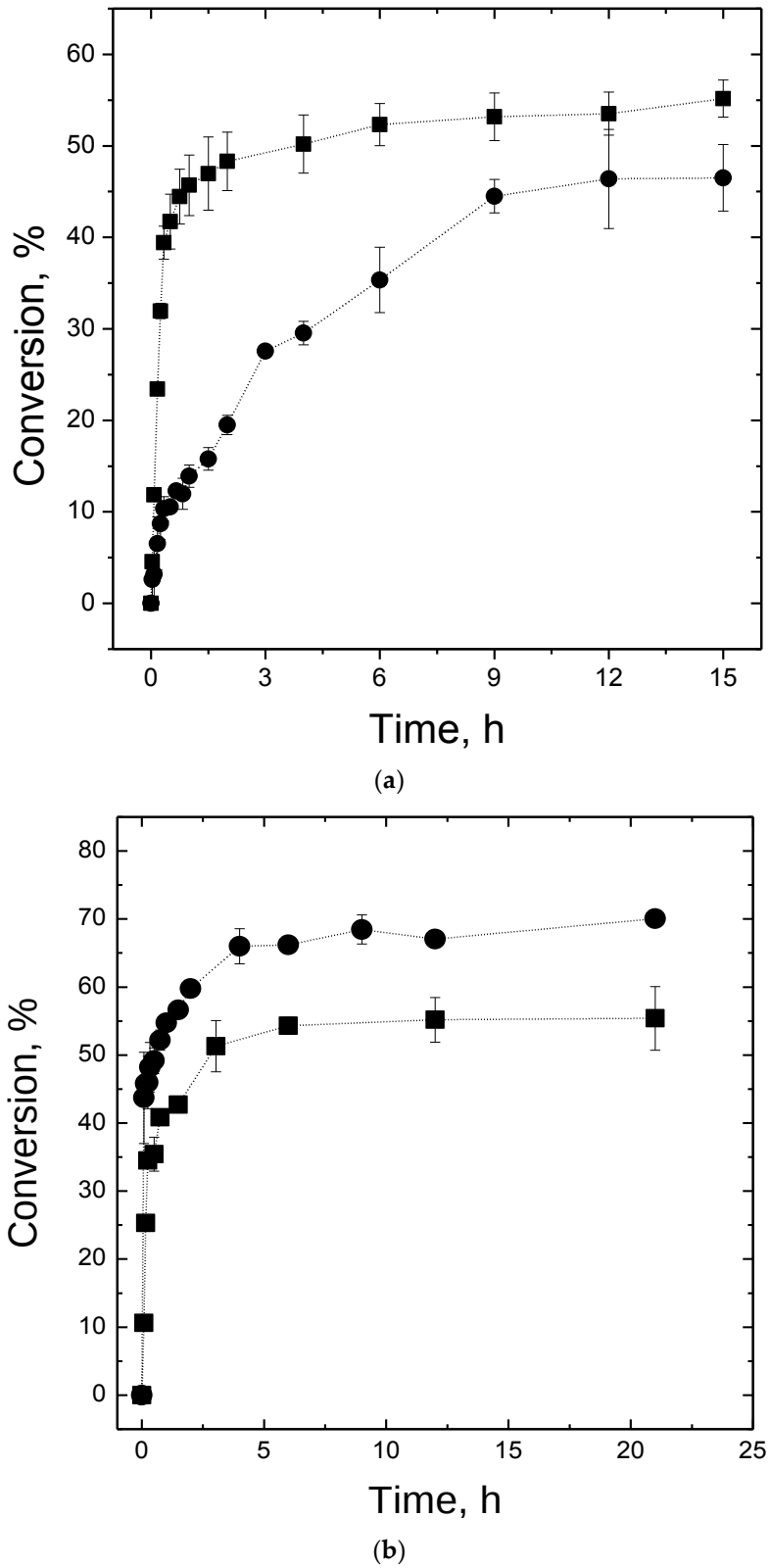


Figure 4. Comparative soluble starch (30 g/L) conversion using free β-amylase (squares) and CLEA-β-PS-12 (circles) at pH 7.0 and 40 °C; (**a**) comparative starch conversion using cassava bagasse starch solutions with free β-amylase (squares) and CLEA-β-PS-12 (circles), using 1.2 U/g of starch, pH 7.0 and 40 °C (**b**).

The assay using cassava bagasse starch solutions 30.0 g/L (DE of 7.95 and 15.88) with free enzyme and CLEA-β-PS-12 (using 36 U/L) reached maximum conversion of 54.3 ± 1.2% and 66.0 ± 2.6% at 6 h and 4 h, respectively (Figure 4b). With both catalyst a higher maltose yield and activity was detected using this product (starch partially hydrolyzed extracted by α-amylase action), very likely due to the very low DE using the commercial starch (near 0) compared to the high one of these home-obtained products (DE 8-16), offering more non-reducing ends to the enzyme action. Moreover, the lower size of the starch extracted from bagasse can reduce the diffusion limitations found using commercial starch, greatly improving the performance of the immobilized enzyme, that now overpass the free enzyme. The productivity and specific productivity of cassava bagasse starch conversions using free β-amylase and CLEA-β-PS-12 were 2.8 ± 0.1 and 5.2 ± 0.2 kg/m3h, and 78.8 ± 1.8 and 143.7 ± 5.6 mg/Uh, respectively. The CLEA-β-PS-12 productivity and specific productivity using cassava bagasse starch solution with DE of 15.88 was 4.3 times higher than using soluble starch (DE near 0).

Gaouar et al. [74] used in this reaction a highly concentrated liquefied starch, 340 ± 25 g/L (DE unknown) and free Maltogenase 4000 L (from *Bacillus subtilis*) as biocatalyst, at pH values between 5.0 to 5.5 in a 1.0 L jacketed Pyrex cell with magnetic stirring, achieving 65.4% of maltose production, practically the same as using cassava bagasse residual starch with DE of 15.88 and CLEA-β-SPI-12. Shiraishi et al. [75] also achieved comparable starch conversion, using 150, 200 and 300 g/L of liquefied white potato starch (DE = 5.6) with 1.59 g/L of free soybean β-amylase in a baffled flask with magnetic stirring at temperature of 40 °C and pH 4.8, achieving a value of maltose content of 58.2% for all the tests.

CLEA-β-SPI-12 was utilized for four successive cycles of reaction (12 h reaction for each cycle) (Figure 5); the continuous decrease on yields becomes evident. The reasons for this may be the difficulty to capture the CLEAs in this very viscous media, even though the size of the biocatalyst was quite large, as enzyme inactivation under these conditions may be discarded in these very short reaction courses. The breakage of the CLEA particles during use will further increase this difficulty in the recovery, and that occurs even using a vortex reactor. In fact, Talekar et al. [72] also reported a reduction in the activity of CLEA of α-amylase to around 65% of its original activity after four cycles of 30 min reaction at 60 °C. They attributed the loss of activity to the substantial change in the CLEA morphology after four batches as shown by SEM images. In fact, in our work, the weight of CLEA obtained after the four reuses almost fit with the decreased activity. An alternative to improve the CLEA recovery may be to use magnetic nanoparticles trapped in the CLEAs to facilitate the capture [71,76–80]. Another possibility may be the trapping of the CLEAs in some larger and more rigid solids, e.g., lentikats [81,82] or silica [83,84].

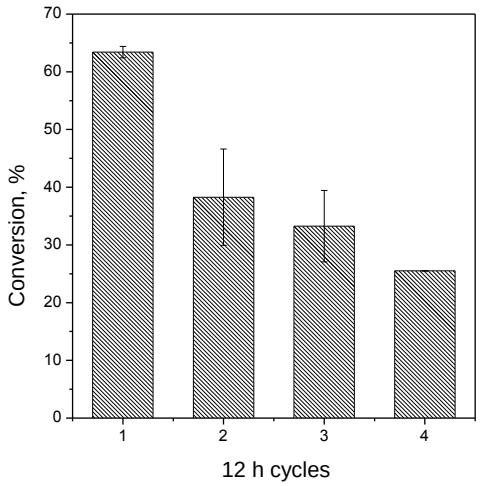


Figure 5. Reuse assays converting starch to maltose at 40 °C, pH 7.0 in a vortex flow reactor stirred at 900 rpm, using cassava bagasse starch solution (8.2 g/L) and CLEA-β-PS-12 (36.0 U/L).

3. Materials and Methods

3.1. Materials

Cassava bagasse was supplied by Tereos Syral (Palmital, Brazil), soluble starch was purchased from Sigma-Aldrich (St. Louis, MO, USA). The α-amylases Termamyl 120 L from *Bacillus licheniformis* and BAN 480 LS from *Bacillus amyloliquefaciens* were supplied by LNF Latino Americana (Bento Gonçalves, Brazil). Crude extract of barley β-amylase (type II-B) and bovine serum albumin (BSA, 96% purity) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Soy protein isolate (SPI, 90% purity) was purchased from Doremus Ingredientes (Guarulhos, Brazil). Other reagents were of analytical grade.

3.2. Enzymatic Activity Assay

The activity of β-amylase was determinate according to Bernfeld [85] by measuring the initial velocity of maltose production. A solution of 1% (*w*/*v*) soluble starch in 16 mM sodium acetate buffer (pH 4.8) was used as substrate. Free or immobilized β-amylase was added to the reaction medium, which was thermostatically equilibrated at 20 °C in a batch reactor. Periodically, sample aliquots were withdrawn until 12 min and the maltose content was quantified by the dinitrosalicylic acid method [86] using pure commercial maltose as standard. An enzymatic unit of β-amylase (U) was defined as the amount of enzyme required to release 1.0 mg of maltose per minute under the assay conditions.

3.3. β-Amylase Immobilization Using CLEA Technique

A set of experiments were carried out using 0.5 mL of BSA or SPI solution (feeder protein), with concentrations values described in Table 3, and 0.5 mL of 100 mM sodium phosphate buffer at pH 7.0 containing 2.0 mg of protein/mL of β-amylase. Then, 1.0 mL of 5.0 M ammonium sulfate solution was added to precipitate the proteins; after 5 min stirring 24.0 or 48.0 µL of 25% (*w*/*w*) glutaraldehyde aqueous solution were added, reaching a glutaraldehyde final concentration of 30.0 or 60.0 mM, respectively. The protein suspension was kept under 200 rpm stirring at 4 °C for 3 h. Finally, the CLEA of β-amylase was separated from the supernatant by centrifugation at 21,000× g and 4 °C, washed with 100 mM sodium phosphate buffer pH 7.0, centrifuged again and resuspended in 1.0 mL of 100 mM sodium phosphate buffer pH 7.0 for β-amylase activity measurement.

The global yield was calculated according to Equation (1):

$$\text{Global yeld } (\%) = \frac{\text{CLEA activity}}{\text{Initial activity}} \times 100, \quad (1)$$

where the CLEA activity is the observed activity in the CLEA after centrifugation, washing (2 times with 1.0 mL of 100 mM phosphate buffer pH 7.0) and resuspension in the same washing buffer; initial activity is the total activity offered to the immobilization.

3.4. Immobilizations on Chitosan Based Supports by Adsorption, Covalent Attachment and Encapsulation

β-amylase was immobilized on chitosan according to Vieira et al. [87], with chitosan-based matrices of 2.0% (for adsorption and covalent attachment) and 1.0% (for encapsulation) (*w*/*w*), aiming to achieve bigger pore sizes. The amount of enzyme supplied for immobilization was 0.53 mg of protein per gram of chitosan. Table 4 shows the conditions for chitosan-based immobilizations.

Chitosan powder was solubilized in 2.0% (*v*/*v*) acetic acid solution and coagulated using 0.5 M potassium hydroxide solution (volume ratio chitosan:KOH solutions of 2:3). The suspension was stirred at 50 °C for 30 min to form flakes. The flakes were filtrated and washed with Milli-Q water and 5 mM sodium phosphate buffer pH 7.0.

Table 3. Conditions of preparation of CLEAs of β-amylase using bovine serum albumin (BSA) and soy protein isolate (SPI) as cofeeder and glutaraldehyde as crosslinker.

Assay [1]	Feeder Protein	Cofeeder Concentration [2] (mg/mL)	Glutaraldehyde Concentration [3] (mM)	Glutaraldehyde/Total Protein [4] Ratio (mM/mg)
1	None	0	30	60.0
2		0	60	120.0
3	BSA	160	30	0.74
4		120	30	0.98
5		80	30	1.46
6		60	30	1.93
7		40	30	2.86
8		120	60	1.97
9		80	60	2.93
10		40	60	5.71
11	SPI	80	30	1.46
12		60	30	1.93
13		40	30	2.86
14		30	30	3.75
15		20	30	5.45
16		60	60	3.87
17		40	60	5.71
18		30	60	7.50
19		20	60	10.91

[1] All assays were carried out at 4 °C and amonium sulfate solution (5 M) was used to precipitate the proteins. [2] Feeder protein concentration in water solution; [3] Glutaraldehyde concentration in protein solution (2 mg/mL), prepared in 100 mM sodium phosphate buffer pH 7.0.; [4] Total amount of enzyme and cofeeder (BSA or SPI).

Table 4. Conditions for adsorption, covalent attachment and encapsulation in chitosan using 0.53 mg of protein (crude enzyme) per gram of chitosan.

Immobilization Method	Adsorption	Adsorption Followed by Crosslinking	Covalent Attachment	Encapsulation
Carrier	Chitosan 2 wt %	Chitosan 2 wt %	Chitosan 2 wt %	Chitosan 1 wt %
Glutaraldehyde concentration	0	0.15%	0.80%	0.10%
Temperature (°C)	25	25	25	0 (Ice bath)
Stirring	100 rpm	100 rpm	100 rpm	50 rpm

The β-amylase adsorption was performed in a shaker at 25 °C and 100 rpm, suspending 1 g of chitosan flakes in 5 mL of enzyme solution (0.53 mg of β-amylase/g of chitosan), prepared in 5 mM sodium phosphate buffer pH 7.0. The immobilization was monitored by measuring the enzyme activity in the supernatant and suspension, using an identical enzyme solution without chitosan as blank reference. At the end, the immobilization yield was determined (Equation (2)), then the flakes were filtrated, washed with distilled water and the expressed activity (Equation (3)) and the global yield (Equation (4)) were calculated. We also determined the global yield of the adsorbed enzyme after treatment with 0.15% (*v*/*v*) glutaraldehyde solution under mild stirring, at room temperature for 1 h at pH 7.0.

For covalent immobilization, firstly the chitosan flakes were activated with 0.8% (*v*/*v*) glutaraldehyde solution at pH 7.0 under mild stirring for 30 min, followed by washing with Milli-Q water and 100 mM sodium phosphate buffer at pH 7.0. The immobilization of β-amylase on activated chitosan was carried out in 100 mM sodium phosphate buffer pH 7.0 (1:5 solid/liquid ratio, 0.53 mg of β-amylase/g of chitosan, as in the adsorption method) at 25 °C and 100 rpm stirring, monitoring the supernatant, suspension and blank reference. The immobilization yield, expressed activity and the global yield were calculated as described above.

For the encapsulation into chitosan, chitosan powder was solubilized in 2.0% (*v*/*v*) acetic acid solution and homogenized under stirring at room temperature for 1 h. Then the pH was adjusted

to 4.35 using sodium hydroxide solution and the β-amylase was added. This solution was added dropwise into a 100 mM Tris-HCl buffer pH 8.0 (in ice bath) in a volume ratio chitosan solution:buffer of 1:10, under mild stirring for 30 min. After, glutaraldehyde 25% (*w*/*w*) was added to achieve a final concentration of 0.1% (*v*/*v*) and the reaction occurred under mild stirring at room temperature for 2 h. The chitosan gels were filtrated, washed with distilled water and 100 mM sodium phosphate buffer pH 7.0, and the global yield was calculated by Equation (4).

The immobilization yield and expressed activity for adsorption and covalent attachment on chitosan were calculated according to Equations (2) and (3), respectively:

$$\text{Immobilization yield } (\%) = \frac{\text{Immobilized activity}}{\text{Initial activity}} \times 100, \quad (2)$$

$$\text{Expressed activity } (\%) = \frac{\text{Derivative activity}}{\text{Immobilized activity}} \times 100, \quad (3)$$

where the immobilized activity is the initial activity offered to the immobilization minus the activity in the final supernatant. Derivative activity is the activity measured in the immobilized enzyme.

The global yield was calculated according Equation (4):

$$\text{Global yield } (\%) = \frac{\text{Derivative activity}}{\text{Initial activity}} \times 100, \quad (4)$$

3.5. Effect of pH and Temperature on Activity and Stability of β-Amylase Preparations

The enzymatic activity of immobilized or free enzyme was determined at different pH values and 20 °C as described in the Section 3.2, using different buffers (16 mM): sodium acetate buffer at pH values from 3.0 to 5.0, sodium phosphate buffer at pH values from 6.0 to 8.0 or sodium carbonate buffer at pH 9.0.

To determinate the optimum activity temperature of free or immobilized β-amylase, the enzymatic activity was measured using 16 mM sodium acetate buffer at pH 4.8 in a temperature range from 30 to 60 °C.

For the stability assays, free and CLEAs of β-amylase samples were incubated at the indicated temperature and pH values for 12 h under 200 rpm stirring. The pH stability assay was studied at 25 °C and pH values from 4.0 to 8.0, using the same buffers of the pH effect on activity assay. The temperature stability assay was performed incubating the samples in the buffer of highest stability for each sample (free or CLEAs β-amylase) and temperatures from 40 to 60 °C. The relative residual activity was calculated as the ratio between the activity after the incubation and the initial activity.

3.6. Cassava Bagasse Compositional Analysis

Starch was extracted from cassava bagasse using 0.625 mL of α-amylase (Termamyl 120 L) per g of bagasse (dry mass) in 50.0 mL of water, at 90 °C, with 540 rpm mechanic stirring for 2 h [88]. Then, the reactional medium was filtrated to remove the solid fraction. The liquid fraction, containing mainly maltodextrins, was submitted to acid hydrolysis (1.0 mL of 72% (*v*/*v*) sulfuric acid to 22.5 mL of maltodextrin solution) in an autoclave for 30 min to produce glucose monomers [89]. The acid hydrolysis in an autoclave can also generate a small amount of hydroxymethylfurfural (HMF) molecules. Glucose was quantified by liquid chromatography in a Shimadzu chromatograph equipped with a refractive index detector (RID) at 50 °C, using a BioRad Aminex HPX-87H column (300 × 7.8 mm) set to 65 °C and 5 mM sulfuric acid solution as eluent with a flow of 0.6 mL/min. HMF was quantified in a Shimadzu Chromatograph equipped with a UV-Visible detector (set to 274 nm) using a SunFire C18 column (4.6 × 150 mm) at 40 °C and acetonitrile-water solution (1:8 *v*/*v*), containing 1% (*v*/*v*) acetic acid, as mobile phase at 0.8 mL/min flow rate. The amount of glucose and HMF was used to calculate the total amount of extractable starch from cassava bagasse.

3.7. Residual Starch Extraction

The residual starch contained in cassava bagasse was gelatinized in 50 mM sodium phosphate buffer pH 7.0 (28.0 g of starch/L) under stirring in boiling water bath for 10 min. The bagasse suspension was cooled to 60 °C and the pH was adjusted to 6.0 using diluted HCl solution. Then 12.6 mL of α-amylase (BAN 480 LS) per kg of starch was added to the bagasse suspension, the medium was stirred for 5 min, and the reaction was stopped by adding a 50% (v/v) HCl solution to the reactional medium (1:333 volume ratio). The medium was filtrated at 60 °C, the starch concentration and dextrose equivalent (DE = 100 × amount of reducing sugar expressed as glucose/solid dry mass) were quantified and, if necessary, the soluble starch was diluted or concentrated. DE is a measure of polymerization degree of the starch chains, using glucose as pattern. When the DE value is near to zero the polymerization degree is very high, the starch molecules have high molecular weight. When the DE value is near to 100 the polymerization degree is very low, the starch molecules were practically converted into glucose [7].

The amount of reducing sugar was determinate by DNS method [86] using glucose as standard.

3.8. Maltose Production

The enzymatic maltose production was performed in a batch Taylor vortex flow reactor (VFR) [90] with radius ratio (η = Ri/Re) of 0.24, aspect ratio (Γ = L/d) of 6.32 and inner cylinder rotation rate (ω) of 900 rpm, using 50.0 mL of commercial starch solution or residual starch solution extracted from cassava bagasse and 36.0 U/L of free or CLEA of β-amylase, at pH and temperature values of maximum stability (determined as described before). The starch conversion was followed by the increase of maltose concentration during the reaction time. Samples of the reactional medium were treated with 1.0 M HCl (50:3 v/v) to inactivate the enzymes in the sample, the maltose concentrations were determinate by HPLC (Shimadzu Chromatograph, using a BioRad Aminex HPX-87H column and 5 mM sulfuric acid solution at 0.6 mL/min as eluent, as described above for glucose quantification) and the starch conversion was calculated using 0.957 g of starch/g of maltose as conversion factor.

3.9. Reuse Assays

The reuse assays were performed using the same conditions for maltose production catalyzed by CLEA of β-amylase. Each batch was carried out for 12 h reaction, being the maltose conversion determined in the end of each batch. Between each reactional cycle the biocatalyst was separated by centrifugation (15,370× g at 4 °C for 10 min) and washed with distilled water.

3.10. Protein Assay

The protein concentrations of β-amylase extracts were determined according to the Bradford's method [91] using BSA as the standard protein.

4. Conclusions

This study showed that barley β-amylase can be immobilized by CLEA technology and can achieve a higher global yield than other strategies used to immobilize this β-amylases. Soy protein isolated could be used as feeder protein for barley β-amylase CLEA, creating an immobilized enzyme cheaper and easier to manipulate than using bovine serum albumin as feeder protein. The use of soy protein isolated as an alternative as feeder protein could reduce the cost of the CLEAs biocatalyst production when a feeder is required. The bagasse-starch conversion using CLEA-β-SPI-12 reached maximum starch conversion even slightly better that that obtained by using free β-amylase, and achieved high starch conversion similar to the best results reported in the literature in a short period of time. This study also shows that the cassava bagasse could be used as starch source for maltose production, giving this residue a new and more profitable use. CLEAs recovering in this viscous medium become a problem that should be attacked using different strategies, e.g., use of magnetic

nanoparticles, trapping of the CLEAs in larger and more rigid solids, easier to recover in this very viscous medium.

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