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Preparation of Crosslinked Enzyme Aggregates of a Thermostable Cyclodextrin Glucosyltransferase from *Thermoanaerobacter* sp. Critical Effect of the Crosslinking Agent

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Abstract: Crosslinked enzyme aggregates (CLEAs) of a thermostable cyclodextrin glucosyltransferase (CGTase) from *Thermoanaerobacter* sp. have been prepared for the production of cyclodextrins (CDs). Different parameters in the precipitation (nature and concentration of precipitant) and crosslinking steps (time of reaction with cross-linker, nature and concentration of the crosslinker) were evaluated on the production of CLEAs of CGTase. Among the seven studied precipitants, acetone with a 75% (*v*/*v*) concentration produced the aggregates of CGTase with higher activity, which retained 97% of the initial activity. Concerning the cross-linker (glutaraldehyde, starch–aldehyde, and pectin–aldehyde), starch–aldehyde produced the most active CLEAs. The use of bovine serum albumin as co-feeder decreased the expressed activity. Addition of polyethylenimine at the end of cross-linking step prevented the leakage of the enzyme and the subsequent Schiff's bases reduction with sodium borohydride permitted to maintain 24% of the initial activity even with the large dextrin as substrate. The optimal conditions for the immobilization process required were defined as 75% (*v*/*v*) acetone as precipitation reagent for 1 h at 20 °C, 20 mM starch–aldehyde as crosslinking reagent for 2 h at 20 °C, treatment with 1 mg/mL of polyethylenimine for 5 min, reduction with 1 mg/mL of sodium borohydride. The CLEAs of CGTase were active catalyst (similarly to the free enzyme) in the production of cyclodextrins at 50 °C and pH 6.0 for 6 h reaction, maintaining intact their structures. Besides this, after five cycles of 3 h the total cyclodextrin yield was 80% of the initial value (first batch, with around 45% CD yield).

Keywords: CGTase; CLEAs; immobilization; crosslinking agent effect; starch; cyclodextrins

1. Introduction

Cyclodextrin glucosyltransferases (CGTase) (EC 2.4.1.19) are extracellular microbial enzymes capable of converting starch and their derivatives into cyclodextrins (CDs), which are non-reducing

cyclic oligosaccharides formed commonly by 6, 7, or 8 glucose units linked by α(1→4) bonds, namely of α-, β-, and γ-CD, respectively [1,2]. Specifically, CGTase from *Thermoanaerobacter* sp. produces mainly α- and β-CD, and small amount of γ-CD [3]. CDs have a truncated cone shape with a hydrophobic inner cavity that allows encapsulation of hydrophobic organic molecules, increasing its solubility in some media and increasing its stability. Due to this inclusion property, CDs have many applications in the food, cosmetics, pharmaceutical, and textile industries [4].

In addition to the cyclization reaction (CD production), this enzyme also catalyzes other two intermolecular transglycosylation reactions (coupling and disproportionation) and the hydrolysis reaction of the starch [3,5]. Although it is mainly used at industrial scale for the production of cyclodextrins, recent studies performed by Yu et al. [6] have demonstrated that CGTase from *Paenibacillus* sp. CGMCC 5316 can be used to eliminate the bitter taste of a natural sweetener known as stevioside by a reaction of transglycosylation using starch as a glucose donor.

Despite the excellent catalytic properties of the enzymes (high specificity and selectivity), their use as industrial catalysts is still limited due to their low operational stability, difficult recovery and reuse [7]. The immobilization of enzymes has been first designed to permit an easy reuse of this expensive catalyst, but it also makes their application feasible in continuous processes, allowing their separation and recycling for long periods of time if the immobilized enzyme is stable enough [7–10]. Moreover, a proper immobilization may improve many other enzyme limitations, like stability, activity, specificity or selectivity, resistance to inhibitors, even purity [11].

A versatile carrier-free immobilization technique for enzyme immobilization has attracted increasing attention [12–14]. This technique called CLEAs (cross-linked enzyme aggregates) consists in the physical formation of enzyme aggregates (induced by salts, organic solvents, non-ionic polymers, etc.) followed by chemical crosslinking with bifunctional (usually glutaraldehyde) or polyfunctional (e.g., aldehyde–dextran) agents [15].

In comparison to enzymes immobilized onto solid supports, CLEAs are cheaper biocatalysts that exhibit higher mass loadings [16,17]. However, some problems of the CLEAs have been reported, such as intraparticle diffusional limitations due to the high enzyme loading and uncontrolled pores and mild physical resistance [18]. Moreover, in some cases the crosslinking step may be problematic, when the enzyme has few Lys residues in its surface [19–23]. Diffusion limitations and crosslinking problems may be decreased if using a feeder rich in amino groups, which decreases the loading of the enzyme and facilitates the crosslinking step. This feeder may be an aminated polymer, like polyethylenimine [24], or a protein rich in amino groups (e.g., bovine serum albumin or soy protein) [23,25]. In some instances, the enzyme has been chemically aminated [26]. As crosslinker, glutaraldehyde is the most utilized molecule [27]. Other alternatives proposed in literature are polyfunctional polymers, like aldehyde–dextran [28], aldehyde–pectin [29], or aldehyde–starch [30]. Advantages of these reagents are a lower chemical modification of internal amino groups of the protein (e.g., groups related to the catalysis located in internal pockets [28]), reduction of diffusional problems by enlarging the pores [30], and decreasing enzyme loading.

CLEAs technology has been successfully applied to several amylolytic enzymes [20,23,29,31,32], but, to the best of our knowledge, there are no papers related to the immobilization of CGTase using this technique. These enzymes have been immobilized and stabilized by conventional adsorption methods (using ion exchange resins, chitosan, silica, among others) or by covalent binding to solid supports such as glyoxyl agarose, Eupergit C, chitosan, etc. [33–38]. Particularly, CGTase from *Thermoanaerobacter* sp. has been only immobilized on solid matrices, such as glutaraldehyde-activated chitosan [38], glutaraldehyde-activated silica [33], octadecyl-Sepabeads [36], glyoxyl-activated agarose [37], glyoxyl-activated silica [36], Eupergit C [34,39], Amberlite IRA-900 [40], CNBr- and 1,6-diaminohexane-activated Sepharose [41].

In this context, this work reports the first preparation of CLEAs of CGTase from *Thermoanerobacter* sp., evaluating different parameters such as precipitating agent nature and its concentration, the best bifunctional or macromolecular crosslinkers and its concentration and the effect of the addition of

some feeders (proteins or PEI). In addition, the applicability and reusability of CLEAs of CGTase were also determined.

2. Results and Discussion

2.1. Selection of Precipitating Agent

The aggregation/precipitation step of CGTase was evaluated using methanol, ethanol, *tert*-butanol, acetone, isopropanol, acetonitrile, and saturated ammonium sulfate solution as precipitants. After precipitation, the precipitate was recovered by centrifugation and re-dissolved in 50 mM sodium citrate buffer pH 6.0. As shown in Figure 1, *tert*-butanol yielded the lowest precipitation yield (60%), whereas acetone allowed a precipitation yield of 98.4%. Consequently, acetone was selected as precipitant of CGTase for CLEA preparation.

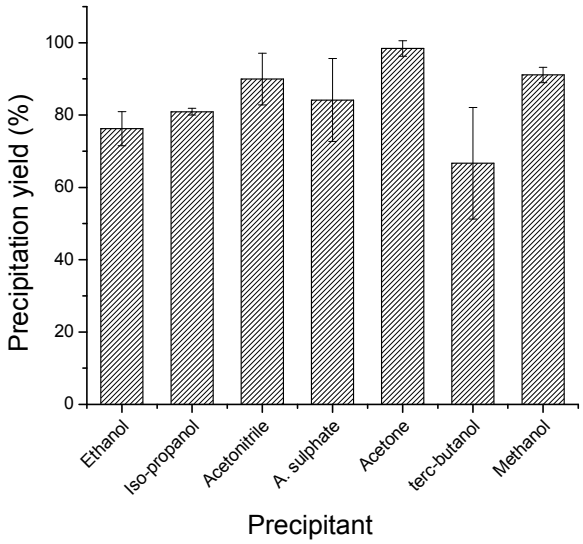

Figure 1. Precipitation of CGTase from *Thermoanaerobacter* sp. with different precipitants. Precipitation conditions: 1 h reaction, 20 °C at 200 rpm stirring, 90% (*v*/*v*) precipitant concentration, enzyme solution prepared in 50 mM citrate buffer pH 6.0. Precipitates were recovered by centrifugation and re-dissolved for enzyme activity measurement. The total initial enzyme activity was taken as 100%.

2.2. Effect of the Precipitant Concentration on the Precipitated Activity of CGTase

Different volumetric concentrations of acetone were evaluated in the precipitation of *Thermoanaerobacter* sp. CGTase. The increase in the volumetric concentration of acetone from 75% to 90% (*v*/*v*) did not affect the activity recovered after re-dissolving the CGTase precipitate, yielding an average CGTase precipitation yield around of 97%. So, a precipitant concentration of 75% was selected for further assays of CLEA preparation (aggregation followed by crosslinking).

2.3. Effect of Glutaraldehyde Concentration and Reaction Time

The concentration of glutaraldehyde is a parameter that must be optimized, because an excess of glutaraldehyde can cause excessive modification of the protein. On the other hand, too low concentrations of glutaraldehyde can promote inefficient cross-linking, leading to loss of enzyme in the reaction medium [42]. The objective is to modify each primary amino group of the enzyme with just one molecule of glutaraldehyde [43].

Figure 2A shows the global activity recoveries in the CLEAs and the activity losses in the first washing (in relation to the total initial activity). The concentration of 10 mM glutaraldehyde was insufficient to properly cross-link the enzyme aggregates, since all enzyme activity was lost during the washing step (data not shown). Higher concentration of glutaraldehyde allowed cross-linking;

however, it is unable to prevent the presence of enzyme activity in the supernatant and produces great losses of activity (more than 90%). Cross-linking with 20 mM glutaraldehyde yielded the highest global activity recovery (around 10%), but it was observed an activity loss above 80%. Even evaluating the crosslinking time at this glutaraldehyde concentration (Figure 2B), the highest global activity recovery was maintained around 10%. This low activity recovery could be associated with diffusional problems of the large substrate within the supramolecular structure of the CLEA [18,30,44], inadequate enzyme crosslinking that permitted the loss of a great percentage of enzyme molecules, or inactivation induced by glutaraldehyde, or a combination, because extensive crosslinking may result in a distortion of the enzyme structure [27,45]. However, the incubation of the free enzyme into 20 mM glutaraldehyde, prepared in 50 mM sodium citrate buffer pH 6.0, showed that glutaraldehyde has a very low deleterious effect on the free enzyme activity (activity loss around 20% after 3 h as shown in Figure 3). Thereby, other strategies were evaluated aiming to increase the global recovery activity of the CGTase CLEAs.

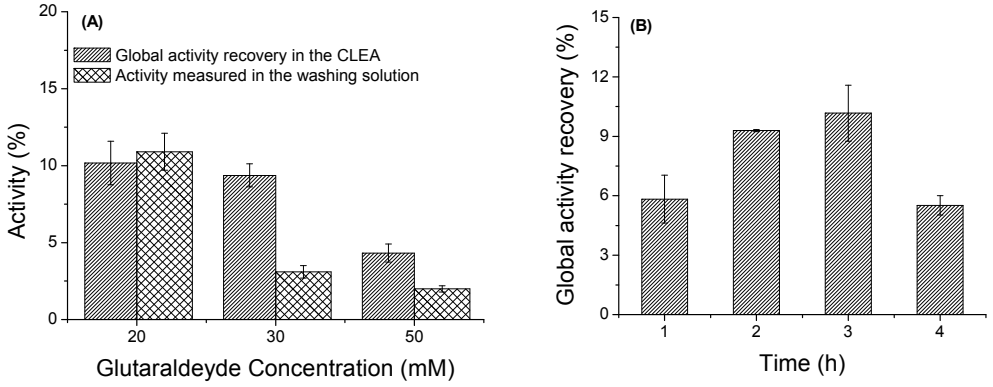

Figure 2. (**A**) Effect of glutaraldehyde concentration on the global activity recovery of CGTase CLEAs (enzyme solution prepared in 50 mM sodium citrate buffer pH 6.0, precipitation with acetone at a volumetric concentration of 75%, 1 h precipitation at 20 °C followed by 3 h crosslinking with glutaraldehyde under 200 rpm stirring); (**B**) influence of crosslinking time with 20 mM glutaraldehyde on the global activity recovery (precipitation and crosslinking conditions at the same conditions above).

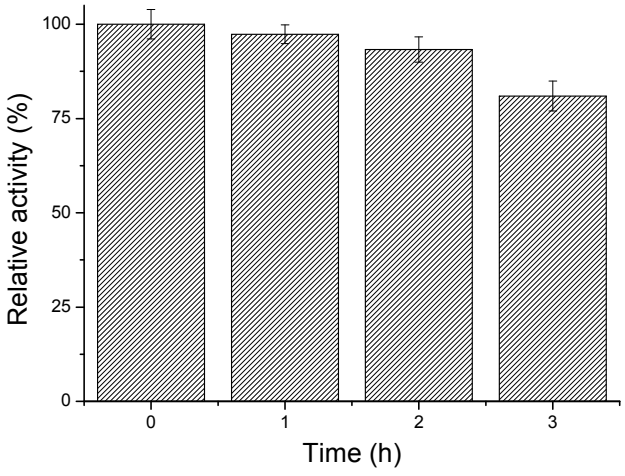

Figure 3. Effect of glutaraldehyde modification on the activity of free CGTase. Enzyme solution was prepared in 50 mM sodium citrate buffer pH 6.0 in the presence of 20 mM glutaraldehyde. Conditions: enzyme concentration of 0.355 mg protein/mL, 20 °C at 200 rpm stirring. The initial activity (11.71 ± 0.46 U/mL) was taken as 100%.

2.4. Strategies to Improve the Global Activity Recovery of CGTase CLEAs

Use of feeder proteins during aggregation may reduce diffusion problems inside the CLEA structures and may facilitate the crosslinking of the enzyme molecules [23,44,46,47]. In addition, it was

previously demonstrated that cross-linking with macromolecular cross-linkers improves the mass transfer in the CLEAs [20,28,30].

According with the considerations mentioned above, in this work, BSA was evaluated as a protein feeder in the preparation of CLEAs of CGTase, using an enzyme/BSA mass ratio of 1:1. In addition, polyaldehydes (starch and pectin oxidized with periodate) were evaluated as macromolecular cross-linkers. It was observed that the addition of BSA in the preparation of CGTase CLEAs using glutaraldehyde as cross-linker (Figure 4) only slightly improved the global activity recovery after CLEA preparation (global activity recovery only increased from 10% to 13%).

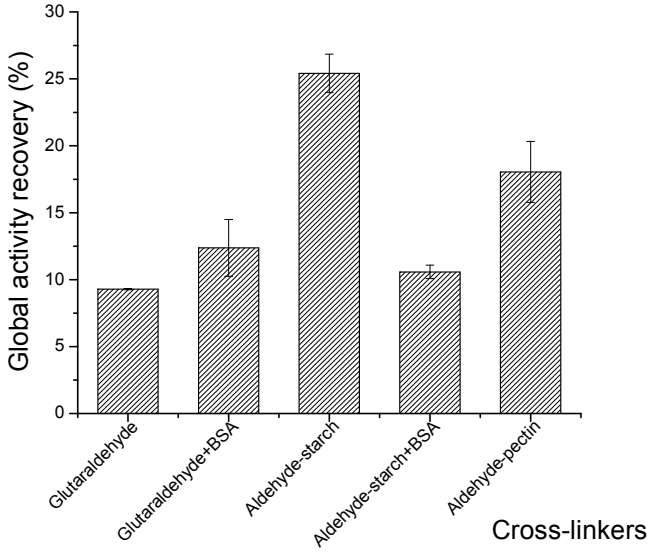

Figure 4. Effect of different cross-linkers on the global activity recovery of CLEAs of CGTase. Reaction conditions: enzyme solution prepared in 50 mM sodium citrate buffer pH 6.0, precipitation with acetone (75% volumetric concentration, 20 °C, 1 h) in the absence or presence of bovine serum albumin (BSA) as co-feeder (enzyme/BSA mass ratio of 1:1); crosslinking at 20 °C for 3 h under 200 rpm stirring.

CLEAs prepared with aldehyde–starch and aldehyde–pectin as cross-linkers in the absence of BSA were approximately 3 and 2 times (respectively) more active than those prepared with glutaraldehyde. However, the crosslinking of the co-aggregates of CGTase and BSA with aldehyde–starch caused a decrease in CLEA activity (by around twofold) in opposition of the effect of BSA using glutaraldehyde. This behavior suggested that an intense crosslinking is the main cause of the low activity recovery, very likely by the decrease in the pore diameters of the CLEA structures, and thereby increasing intraparticle diffusional limitations.

Thus, the highest global activity recovery (25.4 $\pm$ 1.4%) in the CLEAs of CGTase was obtained using aldehyde–starch as cross-linker. Consequently, aldehyde–starch was selected as the cross-linker for further investigation.

The effect of the aldehyde–starch concentration on the CLEA activity was evaluated by varying the concentration of the cross-linker from 10 to 80 mM. In this set of experiments sodium borohydride was added at the end point of the reaction to a final concentration of 1 mg/mL, aiming to reduce the Schiff bases established between aldehyde groups of the cross-linker and amino groups of the proteins to secondary amino bonds, and also to reduce the remnant aldehydes of the cross-linker to inert hydroxyl groups [48].

As shown in Figure 5A, the global activity recoveries of the CGTase CLEAs were very similar at the aldehyde–starch concentrations of 10 and 20 mM, and then started to decrease. Reduced CGTase CLEAs with the highest activity (global activity recovery of 16.3%) were obtained using a concentration of 20 mM aldehyde–starch and 1 mg/mL of sodium borohydride in the cross-linking and reduction steps, respectively.

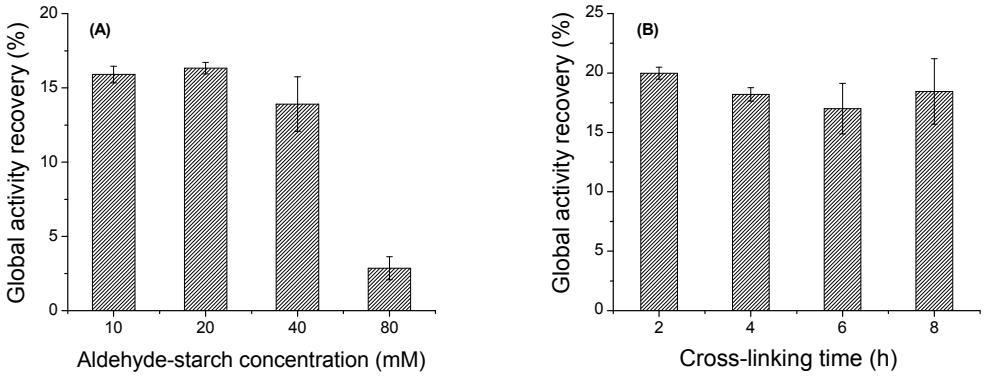

Figure 5. (**A**) Influence of aldehyde–starch concentration and (**B**) effect of the cross-linking time (20 mM aldehyde–starch in the presence of 1 mg/mL maltose) on the global activity recovery of CGTase CLEAs. Reaction conditions: enzyme solution prepared in 50 mM sodium citrate buffer pH 6.0, precipitation with acetone (75% volumetric concentration, 20 °C, 1 h); crosslinking at 20 °C for 3 h under 200 rpm stirring; reduction with sodium borohydride (1 mg/mL, 30 min, 4 °C).

We also evaluated the reduction with sodium borohydride in the presence of β-CD, maltose, and maltotriose, because these compounds (product or inhibitor of the CGTase) might protect the active site of the enzyme against deleterious effect of borohydride [49–51].

The presence of β-CD and maltotriose did not improve the global activity recoveries of the CGTase CLEAs in the reduction step with sodium borohydride. Either in absence and presence of the additives the global activity recovery was around 16%. However, the addition of maltose allowed a slight but significant increase of activity recovery (from 16 ± 0.4 % to 18 ± 0.6 %).

Figure 5B shows that the cross-linking occurred rapidly, thus 2 h of crosslinking time were sufficient to obtain CLEAs with a global activity recovery around 20 ± 0.5%. After this time, the global activity recovery did undergo a small decrease, again suggesting that an intense crosslinking might close the pores inside the CLEA, and this presented a negative effect on the observed enzyme activity.

Although the reduction of CLEAs with sodium borohydride produces secondary amino bonds between aldehyde–starch and protein, the incubation of the CLEAs in 50 mM citrate buffer (pH 6.0) for 1 h led to a partial leaching of the enzyme (~30%). This drawback was overcome by adding polyethylenimine at the end point of the cross-linking step [22]. This treatment only increased the global activity recovery from 18 ± 0.6 to 24 ± 0.4 %, but it allowed the formation of more robust CLEAs, fully preventing the enzyme leakage.

In summary, the most active CLEA of CGTAse (around 24% global activity recovery) was prepared as follows: enzyme solution prepared in 50 mM sodium citrate buffer pH 6.0, protein precipitation with acetone (75% volumetric concentration, 20 °C, 1 h); crosslinking with aldehyde–starch (20 mM, 20 °C, 2 h); subsequent addition of polyethylenimine (1 mg/mL, 20 °C, 5 min) and reduction with sodium borohydride (1 mg/mL, pH 6.0, 4 °C, 30 min, in the presence of 1 mg/mL maltose). All steps were performed under 200 rpm shaking.

It has been reported low activity recoveries (<32%) for covalent immobilization of CGTase from *Thermoanaerobacter* sp. on Eupergit C (10.2%) [34], glyoxyl-activated silica (1.5%) [36], glyoxyl-activated agarose (32%) [37], glutaraldehyde-activated chitosan (6.1%) [38], glutaraldehyde-activated silica (<6%), CNBr-activated Sepharose (4.4%), and 1,6-diaminohexane-activated Sepharose (2.4%) [41]. The main causes for the low recovered activities commonly reported for covalent immobilization of CGTase are probably as follows: (i) intraparticle diffusional limitations due to the large molecular size of substrate (starch) and products (cyclodextrins); (ii) complexity of the CGTase 3D-structure and its catalytic mechanism, because this enzyme has five domains (A to E) and catalyze four possible reactions (hydrolysis, cyclization, coupling, and disproportionation) [5,52]; (iii) the modification of certain Lys residues involved in the catalytic mechanism of the CGTase [41]. Martin et al. [41] reported that some of the Lys residues in the starch-binding site (Lys^{547} and Lys^{549}) display a

high accessibility to the solvent, and are thus possible targets for enzyme immobilization. Also, Wind et al. [52] reported an important stabilizer role of Lys^{47} in the active site of the CGTase from *Thermoanaerobacterium thermosulfurigenes* EM1, which shares an overall identity of 89% with the CGTase from *Thermoanaerobacter* sp. ATCC 53627 [5]. Thus, we might suppose that the involvement of some of these Lys residues in the covalent linkage with the cross-linker (or aldehyde-activated supports) could drastically reduce the catalytic efficiency of the CGTase.

Surprisingly, Matte et al. [33] reported an activity recovery of 73% for covalent immobilization of CGTase from *Thermoanaerobacter* sp. on glutaraldehyde-activated silica. In this case, mesoporous silica microspheres were synthesized using polyethylene glycol 400 as swelling agent, silanized with 3-aminopropyltrimethoxysilane (APTMS), and activated with glutaraldehyde prior to immobilization. Although in our work the activity recovery was smaller, the methodology adopted to immobilize the CGTase does not use solid support and thus, saving on the cost of the support may reduce the impact of the biocatalyst production costs in the overall process.

2.5. Thermal Inactivation Assay

The thermal stabilities of free CGTase and the most active CLEA of CGTase prepared in this paper was evaluated at 60 °C and pH 6.0 (50 mM sodium citrate buffer). Although a high thermostability for *Thermoanaerobacter* sp. CGTase at temperatures below 75 °C [3] has been reported, some inactivation was observed for free CGTase at 60 °C (activity loss of around 20% after 6 h as shown in Figure 6) in absence of substrate. However, no significant loss of activity was observed to the CGTAse CLEA under the same inactivation conditions. That way, the immobilized enzyme was significantly more stable than the free enzyme.

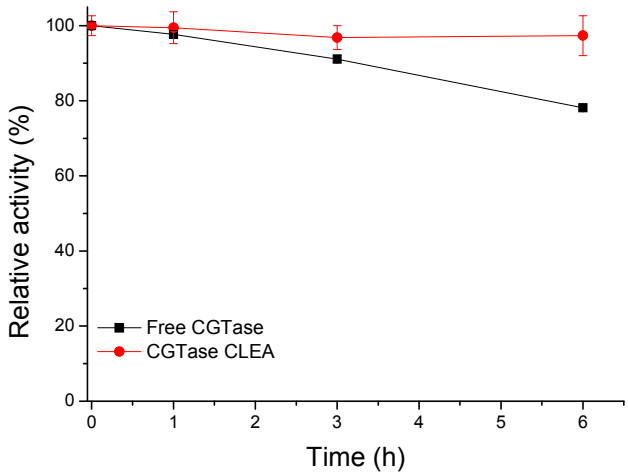

Figure 6. Thermal inactivation of free and immobilized CGTase at 60 °C and pH 6.0 (50 mM citrate buffer). The initial activities of the enzyme solution or suspension (4.72 ± 0.05 U/mL and 1.89 ± 0.05 U/mL, respectively) were taken as 100%.

2.6. Cyclodextrin Production

The most active CLEA of CGTase prepared in this paper was used for cyclodextrin production in a stirred tank-type bench reactor equipped with an impeller without blades (Figure 7). This reactor operating as a vortex flow reactor may avoid damage to shear sensitive catalysts like CLEAs [23]. The temperature was set at 50 °C to avoid thermal inactivation of the free CGTase.

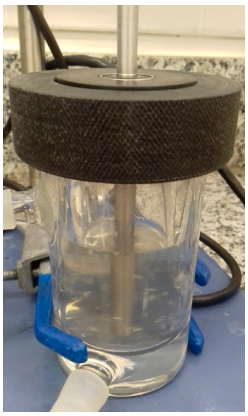

Figure 7. Bench reactor equipped with an impeller without blades used in the production of cyclodextrins from pre-hydrolyzed cassava starch using CGTase CLEA as biocatalyst.

Figure 8 shows the reaction course in the production of CDs (α-, β-, and γ-CD) at 50 °C and pH 6.0, catalyzed by CGTase (free or in the form of CLEAs) using 2.0% (*w*/*v*) pre-hydrolyzed starch (DE 1.4%). It can be observed that CGTase CLEAs produced similar amounts of CDs, but the maximum concentrations of total CDs (around 9.0 g/L) were reached at different reaction times. After 6 h reaction, using free CGTase, the concentrations of α-, β-, and γ-CD reached 3.3, 3.8, and 1.9 g/L (α/β/γ mass ratio of 1.7:2:1), which correspond to a starch-to-CDs yield of 45%. On the other hand, after 3 h reaction, using CLEAs of CGTase, the CD concentrations were 2.7, 4.0 and 1.8 g/L (α/β/γ mass ratio of 1.5:2:1), which correspond to a starch-to-CDs yield of 43%. Similar findings were reported by Martín et al. [34] with CGTase from *Thermoanaerobacter* sp. immobilized on Eupergit C. However, the maximum conversion of 10% (*w*/*v*) soluble potato starch into cyclodextrins (44%) was reached after 48 h reaction at 60 °C and pH 5.5 (10 mM sodium citrate buffer), yielding an α/β/γ mass ratio of 3:5:1. The immobilization may alter the catalytic features of an enzyme, by some enzyme distortion or by the generation of diffusional limitations [11]. CGTase has a very complex catalytic mechanism and tridimensional structure [5,53] that could be changed and/or sterically hindered depending on the immobilization type and thereby altering its normal catalytic action.

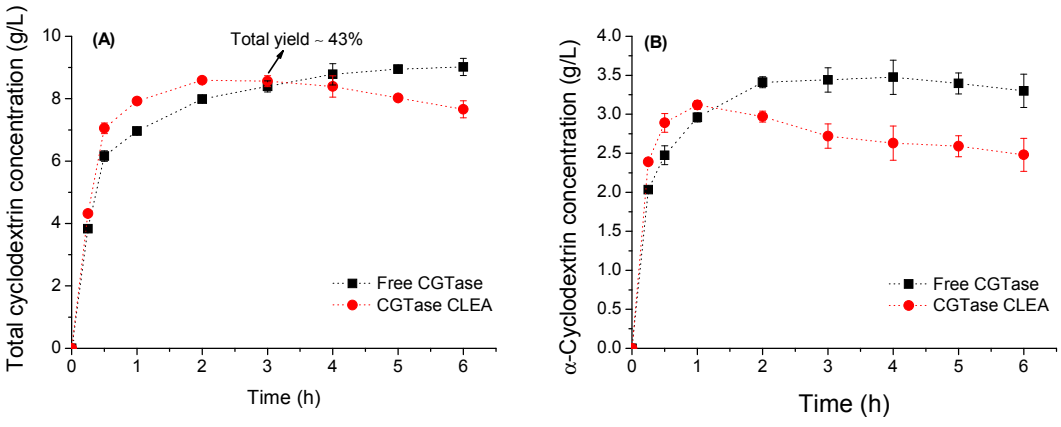

Figure 8. *Cont.*

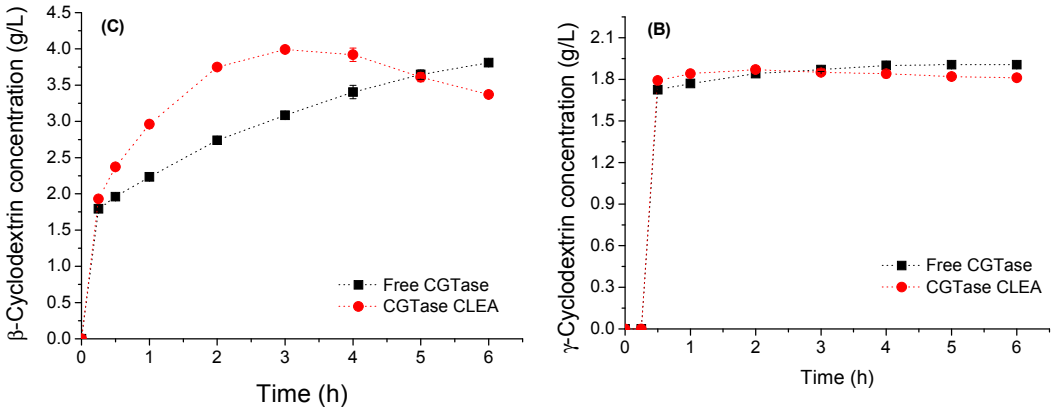

Figure 8. Batch assays of (**A**) total CDs, (**B**) α-CD, (**C**) β-CD, and (**D**) γ-CD production at 50 °C and pH 6.0 for 6 h, catalyzed by free CGTase (5.0 U/g starch) and CLEAs of CGTase (5.0 U/g starch). Substrate: 2.0% (*w*/*v*) pre-hydrolyzed starch (DE 1.4%). One Unit (U) correspond the activity of CGTase measured with 0.5% (*w*/*v*) dextrin as substrate, as described in Section 3.2.

The operational stability of CLEAs of CGTase was evaluated in five reaction cycles of 3 h at 50 °C and pH 6.0 (Figure 9). The total CD yield decreased from 42.6% (first cycle) to 34.6% (fifth cycle), a decrease of 18.8% in the catalytic efficiency of the enzyme. This decrease did not affect similarly to all CDs. The yields of β-CD decreased to 66%, of the initial ones, while for α- and γ-CD the decrease was to 94% of the initial productions. The drop in the β-CD yield was much more pronounced in the second cycle (a decrease of 23%). This suggest the complex catalytic mechanism of this enzyme and revels that an additional problem is that together to the reaction rate, the quality of the product may be different in each reaction cycle. In fact, partial and controlled inactivation of the enzyme under different conditions may be an interesting way to modulate the enzyme selectivity for the different CDs, as each inactivation condition may produce different enzyme changes [54].

The operational stability of CGTase from *Thermoanaerobacter* sp. immobilized on activated supports was previously studied. Martín et al. [34] reported a loss of 60% in the catalytic efficiency of the enzyme immobilized on Eupergit C after 10 cycles of 24 h at 60 °C and pH 5.5 (around 50% loss after five cycles). Schoffer et al. [38] reported a loss of 40% in the production of β-CD from 4% (*w*/*v*) soluble starch after 100 cycles of 15 min at 60 °C and pH 6.0 (around 10% loss after 5 cycles) for the CGTase immobilized on glutaraldehyde-activated chitosan. Matte et al. [33] reported a loss of 40% in the production of β-CD from 4% (*w*/*v*) starch after 15 cycles of 15 min at 80 °C and pH 6.0 (around 5% loss after five cycles) for the enzyme immobilized on glutaraldehyde-activated silica. The operational stability of CLEAs of CGTase (around 20% loss after five cycles of 3 h at 50 °C and pH 6.0) was good compared to the ones with CGTase immobilized on solid supports.

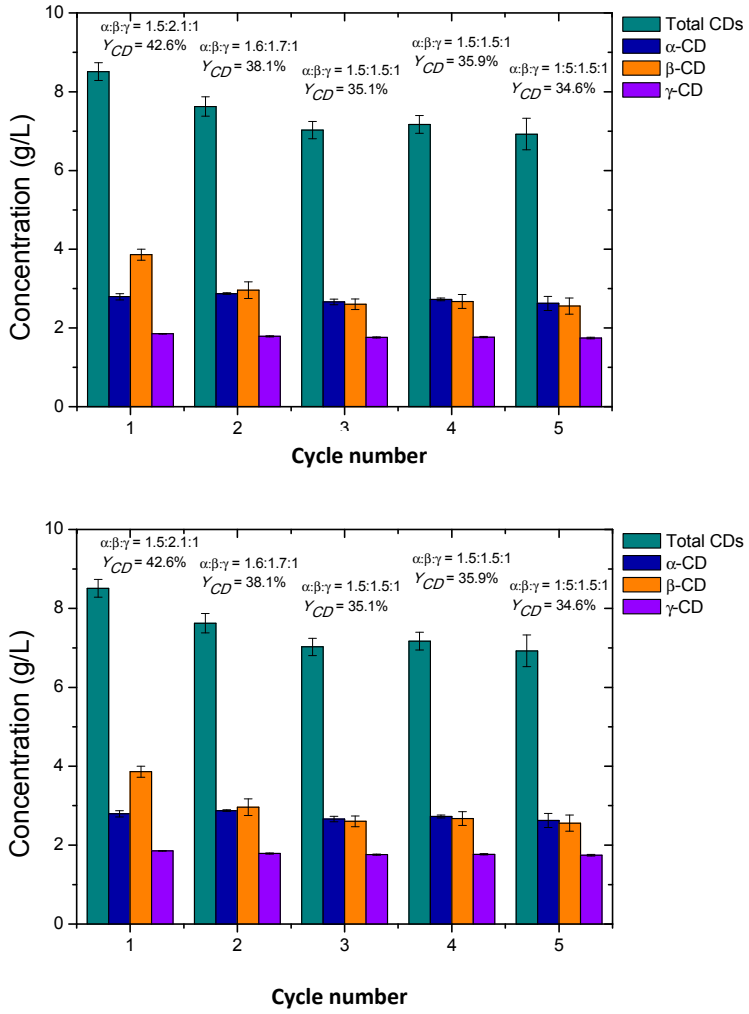

Figure 9. Reusability of CGTase CLEAs in cyclodextrin production at 50 °C and pH 6.0, using an enzyme load of 5.0 U/g starch and 2.0% (*w*/*v*) pre-hydrolyzed starch (DE = 1.43) as substrate. The notes at the top of the columns represent the mass ratios of CDs (α/β/γ) produced and the starch-to-CD yields in each batch. Each cycle takes 3 h.

3. Materials and Methods

3.1. Materials

Thermostable CGTase from *Thermoanaerobacter* sp. (Toruzyme®3.0L, Novozymes, Denmark), containing 7.1 mg protein/mL with a specific activity (initial rate of β-cyclodextrin production) of around 30 U/mg protein and α-amylase from *Bacillus amyloliquefaciens* (BAN®480L) were from Novozymes (Bagsvaerd, Denmark). α-, β-, and γ-Cyclodextrin, dextrin 10, bovine serum albumin (BSA) and polyethylenimine (oligomer mixture, Mn ~423) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Soluble starch, phenolphthalein, and glutaraldehyde (25% *v*/*v*) were obtained from Synth (Diadema, SP, Brazil). The other reagents were of analytical grade.

Protein concentration was determined by Bradford's method [55] using bovine serum albumin (BSA) as standard protein.

3.2. Enzymatic Activity Assay

Enzymatic activity of soluble and immobilized CGTase was determined by measuring the initial reaction rate of β-CD formation using dextrin as substrate. A volume of 55 mL dextrin 10 (0.5% *w*/*v*) in 10 mM citrate buffer (pH 6.0) was added to a batch reactor that was thermostatized at 60 °C, because

the high thermostability of *Thermoanaerobacter* sp. CGTase at temperature $\leq$ 75 °C [3]. One hundred microliters of 50x diluted enzyme solution (soluble) or 300 µL of immobilized enzyme suspension were added to the reaction medium and aliquots of 1 mL were withdrawn at 2-min intervals for 10 min. The enzymatic reaction was stopped by adding 20 µL of 3 M HCl followed by 5 min of boiling. β-CD concentration was determined using the colorimetric method of phenolphthalein, developed in 1981 by Vikmon [56] and modified as described by Tardioli et al. [37]. The assays were carried out by mixing 200 µL of the sample containing β-CD and 1.0 mL of a solution of 0.06 M phenolphthalein, prepared in 120 mM sodium bicarbonate buffer (pH 10.5). This solution was prepared mixing 0.3 mL of phenolphthalein solution (3 mM in 95% ethanol) and 14.7 mL of 120 mM sodium bicarbonate buffer, pH 10.5. The absorbance of the samples was record at 550 nm (Spectrophotometer UV-Visible Ultrospec 200, Pharmacia Biotech, Piscataway, NJ, USA). A blank was used, substituting the sample by distilled water.

The β-CD concentration ($C_{\beta\text{-CD}}$, in mM) was determined from a calibration curve of β-CD ranging from 0 to 1 mM. Equation (1) was fitted to the absorbance data as a function of the β-CD concentrations using the software Origin 8.0:

$$C_{\beta-CD} = 6000\, a\left(1 - \frac{A_{550}}{A_{0/550}}\right)\left(1 + \frac{A_{0/550}}{K_{\beta-CD} a A_{550}}\right), \quad (1)$$

where a is the total concentration of phenolphthalein in the assay cuvette $\left(5 \times 10^{-5}\ \text{M}\right)$; A_{550} and $A_{0/550}$ are the absorbance of samples and blank, respectively; and $K_{\beta-CD}$ ($16193.82 \pm 112.95\ \text{M}^{-1}$) is the equilibrium constant determined by fitting Equation (1) to the experimental data, and 6000 is the sample dilution from assay tube to the cuvette multiplied by the molar to milimolar conversion factor.

One unit (U) was defined as the amount of enzyme that produces 1 µmol of β-CD per minute under the assay conditions.

3.3. CLEAs Preparation

3.3.1. Precipitant Selection

A volume of 900 µL of the precipitating agent (ethanol $\geq$ 99.5%, isopropanol $\geq$ 99.7%, methanol $\geq$ 99.9%, *tert*-butanol $\geq$ 99.0%, acetonitrile $\geq$ 99.8%, acetone $\geq$ 99.9%, or saturated ammonium sulfate solution) was added to 100 µL enzyme solution (3.55 mg protein/mL in 50 mM sodium citrate buffer pH 6.0) into a 2 mL micro-tube to a final concentration of 90% (v/v). After incubation at 20 °C and 150 rpm stirring for 1 h, the suspension was centrifuged at 4 °C and 900 rpm for 10 min. The precipitate was re-dissolved in 1 mL of 50 mM sodium citrate buffer (pH 6.0) and its enzymatic activity and protein concentration were determined using colorimetric phenolphthalein and Bradford's methods, respectively.

The precipitant was chosen based on its precipitation yield in terms of activity (YP_A), calculated as Equation (2):

$$YP_A = \frac{\text{total redissolved activity}}{\text{total initial activity}} \times 100, \quad (2)$$

For the selected precipitant (acetone in this case) it was also evaluated its concentration ranging from 75% to 90% (v/v).

3.3.2. Cross-Linking Using Glutaraldehyde

After enzyme aggregation step, glutaraldehyde (25%, v/v) was added to the mixture to final concentrations of 10, 20, 30, or 50 mM. The stirring at 200 rpm was maintained for 3 h at 20 °C. The mixture was centrifuged at 4000 rpm and the supernatant was separated from the CLEAs. The precipitate was re-suspended in 50 mM sodium citrate buffer pH 6.0 and centrifuged again. This procedure was repeated until disappearance of enzymatic activity in the supernatant.

The effect of the reaction time in the reaction of the enzyme aggregates with glutaraldehyde was studied by ranging the incubation period of the CLEAs at 20 °C from 1 to 4 h and maintaining the concentration of glutaraldehyde previously selected. The CLEA activity was measured using the colorimetric method of phenolphthalein, as described above. The global activity recovery (AR_G) of all CGTase CLEAs was calculated as Equation (3):

$$AR_G = \frac{\text{Total activity of the CLEA}}{\text{Total initial activity}} \times 100, \quad (3)$$

3.4. Evaluation of Macromolecular Cross-Linkers

3.4.1. Preparation of Macromolecular Cross-Linkers

Starch–aldehyde and pectin–aldehyde were prepared following the methodology described by Zhen et al. [30] with some modifications. A mass of 1.5 g of starch was solubilized in 50 mL of distilled water by heating for 3 min in a boiling water bath. Afterwards, the solution was cooled (until 30 °C), the pH was adjusted to 4.0, and 3.85 g of sodium periodate was added for complete oxidation of diol groups to aldehyde (120 min reaction). After, the solution was transferred to a 1 kDa cut-off membrane dialysis and dialyzed against distilled water at room temperature for 24 h under stirring and with six changes of distilled water. Under these conditions, the starch–aldehyde solution had a concentration of 320 mM of aldehyde groups, determined by sodium periodate consumption.

For the preparation of pectin–aldehyde, the pectin (1.5 g) was dissolved in 20% (v/v) ethanol solution, and the other steps were identical as described above.

3.4.2. Cross-Linking Procedure Using Macromolecular Cross-Linkers

The cross-linking with macromolecular cross-linkers (starch–aldehyde and pectin–aldehyde) was carried out at 20 °C by adding an amount of macromolecular cross-likers to the mixture of precipitant and enzyme (75% precipitant volumetric concentration) to a final concentration of 20 mM of aldehyde groups. The CLEA suspension was stirred at 200 rpm for 3 h. After, the CLEAs were recovered by centrifugation and washed with 50 mM citrate buffer (pH 6.0) as described above.

The effect of the concentration of starch–aldehyde on the properties of the final CLEA was studied by ranging its concentration from 10 to 80 mM of aldehyde groups, followed by reduction with sodium borohydride (1 mg/mL) at 4°C and pH 6.0 for 30 min. For the best starch–aldehyde concentration, the reduction step in the presence of additives (β-CD, maltose, and maltotriose) at 1 mg/mL was evaluated. The effect of the reaction time with aldehyde–starch was also evaluated ranging the reaction time from 2 to 8 h maintaining the other parameters previously selected. For the best selected condition, it was evaluated the treatment of the CLEAs with polyethylenimine (PEI). In this case, PEI was added to the CLEA preparation medium to a final concentration of 1 mg/mL after the precipitating and crosslinking steps. After 5 min reaction, the reduction step with sodium borohydride follows as described above.

3.5. Production of Cyclodextrins

CDs were produced from pre-hydrolyzed starch (2.0% w/v, Dextrose Equivalent of 1.4%) at 50°C in a batch reactor stirred with an impeller without blades, operating similarly as a batch Vortex Flow Reactor (VFR) in turbulent regime [57–59] with a cylinder rotation rate (ω) of 900 rpm. This reactor was dimensioned with a radius ratio ($\eta = R_i/R_e$) of 0.24 and aspect ratio ($\Gamma = L/d$) of 6.32 [23].

Initially, a solution of starch (2.0%, w/v, 500 mL), prepared in 50 mM sodium citrate buffer pH 6.0 was gelatinized at 85°C for 10 min. After, the temperature was adjusted to 60°C and 3 µL of α-amylase (BAN®480L, Novozymes Company, Bagsvaerd, Denmark) were added (0.3 mL of α-amylase/kg of starch. After 5 min reaction, the pH was adjusted to 3.0 using 5 M HCl to inactivate the enzyme, and finally adjusted to 6.0 [60]. The total amount of reducing sugar (TRS) was determinate

by DNS method [61] using glucose as standard, and the dextrose equivalent (DE) was calculated using Equation (4):

$$\mathrm{DE} = \frac{\text{mass of TRS}}{\text{mass of starch}} \times 100, \quad (4)$$

The reactions of CD production were carried out for 6 hours at pH 6.0 (50 mM sodium citrate buffer), using an enzyme loading of 5.0 U/g of pre-hydrolyzed starch (30 mL starch solution). The temperature, enzyme loading, and reaction time for the production of CDs were selected based on previous studies with soluble enzyme [60]. Samples were withdrawn at 0.25, 0.5, 1.0, 2.0, 3.0, 4.0, 5.0, and 6.0 h for analysis of the α-, β- and γ-CD concentrations by ELSD-HPLC chromatographic method [62]. The total yield of CD production (Y_{CD}) was calculated following Equation (5):

$$Y_{CD} = \frac{\text{CD concentration } \left(\text{in} \frac{\text{g}}{\text{L}}\right)}{\text{Starch concentration } \left(\text{in} \frac{\text{g}}{\text{L}}\right)} \times 100, \quad (5)$$

3.6. Thermal and Operational Stability

The thermal stability of free and immobilized CGTase was evaluated at 60 °C and pH 6.0 (50 mM sodium citrate buffer) in the absence of substrate. A solution of free CGTase (4.72 ± 0.05 U/mL) or a suspension of CLEAs of CGTase (1.89 ± 0.10 U/mL) was incubated at the temperature above and samples were taken at 2 h intervals within 6 h for activity measurement as described in Section 3.2.

The operational stability of the CLEAs of CGTase was evaluated using a pre-hydrolyzed starch solution (2.0% *w*/*v*, Dextrose Equivalent of 1.4%) in 50 mM citrate buffer pH 6.0. The reactions were performed for 3 h at 50 °C using an enzyme loading of 5.0 U/g pre-hydrolyzed starch (30 mL starch solution). At the end of each batch (five 3-h cycles), the biocatalyst was separated from the reaction medium by centrifugation and washed with distilled water. The CDs were analyzed by ELSD-HPLC method [62].

3.7. Chromatographic Method for Analysis of CDs

The concentrations of α-, β-, and γ-CDs were determined using the chromatographic method described by Rojas et al [62]. Briefly, the CDs were separated through a pyramid NUCLEODUR® C18 column (150 mm × 4.6 mm, 5 µm, Macherey-Nagel, Düren, Germany), at 30 °C, using acetonitrile (solvent B) and water, containing 1% (*v*/*v*) acetic acid (solvent A) at a flow rate of 0.3 mL/min, using a linear gradient, as follows: 0–20 min with 100 to 80% of A and 0 to 20% of B; 20.1–30 min with 100% of A. All analyzes were performed using an Alliance E2695 liquid chromatograph (Waters, MI, USA) equipped with an evaporative light scattering detector (ELSD 2424, Waters, MI, USA).

4. Conclusions

The results presented in this work showed that the free-carrier aggregation/crosslinking is a promising method for immobilizing CGTase when properly performed. It was demonstrated that the type of precipitant, precipitant concentration, cross-linking time, type and concentration of cross-linker greatly influenced the final activity of the CGTase CLEAs. Compared with the traditionally used glutaraldehyde, the preparation of CLEAs using alternative macromolecular cross-linkers allowed increasing the activity recovery. Addition of polyethylenimine after cross-linking step allowed avoiding the lixiviation of enzyme to reaction medium. The application of CGTase CLEAs in cyclization reaction from pre-hydrolyzed starch allowed a yield of CDs around 45%. CLEAs of CGTase might be easily recovered from the reaction medium by gravitational sedimentation or centrifugation and re-used in five 3-h cycles of cyclization (at 50 °C and pH 6.0), retaining around 80% of the initial productions of CDs. The use of a batch reactor operating similarly as a batch vortex flow reactor in turbulent regime avoided loss of biocatalyst by shear, a problem in conventional stirred tank reactors using shear sensitive particles. The system presented in this work, using free-carrier CGTase and vortex flow reactor, may be promising in the production of CDs as alternative to the systems using CGTase

immobilized on shear resistant particles to be used in stirred tank reactors, because saving on the cost of the support may reduce the impact of the biocatalyst production costs in the overall process.

Author Contributions: M.J.R. performed all experimental assays; M.A.-F. helped in the inactivation and reuse assays; R.d.L.C.G., G.M.Z., R.F.-L., and P.W.T. designed and supervised all experiments, as well as wrote/reviewed the manuscript with help of M.J.R. as part of her Doctorate of Science in Chemical Engineering. All authors have given approval to the final version of the manuscript.

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References

1. Szejtli, J. The cyclodextrins and their applications in biotechnology. *Carbohydr. Polym.* **1990**, *12*, 375–392. [CrossRef]
2. Szente, L.; Szejtli, J. Cyclodextrins as food ingredients. *Trends Food Sci. Technol.* **2004**, *15*, 137–142. [CrossRef]
3. Norman, B.E.; Jorgensen, S.T. *Thermoanaerobacter* sp. CGTase: Its properties and application. *J. Jpn. Soc. Starch Sci.* **1992**, *39*, 101–108. [CrossRef]
4. Arun, R.; Kumar, C.K.A.; Sravanthi, V.V.N.S.S. Cyclodextrins as drug carrier molecule: A review. *Sci. Pharm.* **2008**, *76*, 567–598. [CrossRef]
5. Wind, R.D.; Uitdehaag, J.C.M.; Buitelaar, R.M.; Dijkstra, B.W.; Dijkhuizen, L. Engineering of cyclodextrin product specificity and pH optima of the thermostable cyclodextrin glycosyltransferase from *Thermoanaerobacterium thermosulfurigenes* EM1. *J. Biol. Chem.* **1998**, *273*, 5771–5779. [CrossRef] [PubMed]
6. Yu, X.; Yang, J.; Li, B.; Yuan, H. High efficiency transformation of stevioside into a single mono-glycosylated product using a cyclodextrin glucanotransferase from *Paenibacillus* sp. CGMCC 5316. *World J. Microbiol. Biotechnol.* **2015**, *31*, 1983–1991. [CrossRef] [PubMed]
7. DiCosimo, R.; McAuliffe, J.; Poulose, A.J.; Bohlmann, G. Industrial use of immobilized enzymes. *Chem. Soc. Rev.* **2013**, *42*, 6437–6474. [CrossRef]
8. Mateo, C.; Palomo, J.M.; Fernandez-Lorente, G.; Guisan, J.M.; Fernandez-Lafuente, R. Improvement of enzyme activity, stability and selectivity via immobilization techniques. *Enzym. Microb. Technol.* **2007**, *40*, 1451–1463. [CrossRef]
9. Homaei, A.A.; Sariri, R.; Vianello, F.; Stevanato, R. Enzyme immobilization: An update. *J. Chem. Biol.* **2013**, *6*, 185–205. [CrossRef]
10. Adlercreutz, P. Immobilisation and application of lipases in organic media. *Chem. Soc. Rev.* **2013**, *42*, 6406–6436. [CrossRef]
11. Rodrigues, R.C.; Ortiz, C.; Berenguer-Murcia, Á.; Torres, R.; Fernández-Lafuente, R. Modifying enzyme activity and selectivity by immobilization. *Chem. Soc. Rev.* **2013**, *42*, 6290–6307. [CrossRef] [PubMed]
12. Cao, L.; van Rantwijk, F.; Sheldon, R.A. Cross-linked enzyme aggregates: A simple and effective method for the immobilization of penicillin acylase. *Org. Lett.* **2000**, *2*, 1361–1364. [CrossRef] [PubMed]
13. Cui, J.D.; Cui, L.L.; Zhang, S.P.; Zhang, Y.F.; Su, Z.G.; Ma, G.H. Hybrid magnetic cross-linked enzyme aggregates of phenylalanine ammonia lyase from *Rhodotorula glutinis*. *PLoS ONE* **2014**, *9*, 1–8. [CrossRef]
14. Sheldon, R.A.; Schoevaart, R.; Van Langen, L.M. Cross-linked enzyme aggregates (CLEAs): A novel and versatile method for enzyme immobilization (a review). *Biocatal. Biotransform.* **2005**, *23*, 141–147. [CrossRef]
15. Sheldon, R.A. Cross-linked enzyme aggregates as industrial biocatalysts. *Org. Process Res. Dev.* **2011**, *15*, 213–223. [CrossRef]
16. Schoevaart, R.; Wolbers, M.W.; Golubovic, M.; Ottens, M.; Kieboom, A.P.G.; van Rantwijk, F.; van der Wielen, L.A.M.; Sheldon, R.A. Preparation, optimization, and structures of cross-linked enzyme aggregates (CLEAs). *Biotechnol. Bioeng.* **2004**, *87*, 754–762. [CrossRef] [PubMed]
17. Sheldon, R.A.; van Pelt, S. Enzyme immobilization in biocatalysis: Why, what and how. *Chem. Soc. Rev.* **2013**, *42*, 6223–6235. [CrossRef] [PubMed]

18. Garcia-Galan, C.; Berenguer-Murcia, Á.; Fernandez-Lafuente, R.; Rodrigues, R.C. Potential of different enzyme immobilization strategies to improve enzyme performance. *Adv. Synth. Catal.* **2011**, *353*, 2885–2904. [CrossRef]
19. Velasco-Lozano, S.; López-Gallego, F.; Vázquez-Duhalt, R.; Mateos-Díaz, J.C.; Guisán, J.M.; Favela-Torres, E. Carrier-free immobilization of lipase from *Candida rugosa* with polyethyleneimines by carboxyl-activated cross-linking. *Biomacromolecules* **2014**, *15*, 1896–1903. [CrossRef] [PubMed]
20. Amaral-Fonseca, M.; Kopp, W.; Giordano, R.; Fernández-Lafuente, R.; Tardioli, P. Preparation of magnetic cross-linked amyloglucosidase aggregates: Solving some activity problems. *Catalysts* **2018**, *8*, 496. [CrossRef]
21. Guimarães, J.; Giordano, R.; Fernandez-Lafuente, R.; Tardioli, P. Evaluation of strategies to produce highly porous cross-linked aggregates of porcine pancreas lipase with magnetic properties. *Molecules* **2018**, *23*, 2993. [CrossRef] [PubMed]
22. Wilson, L.; Fernández-Lorente, G.; Fernández-Lafuente, R.; Illanes, A.; Guisán, J.M.; Palomo, J.M. CLEAs of lipases and poly-ionic polymers: A simple way of preparing stable biocatalysts with improved properties. *Enzym. Microb. Technol.* **2006**, *39*, 750–755. [CrossRef]
23. Araujo-Silva, R.; Mafra, A.C.O.; Rojas, M.J.; Kopp, W.; Giordano, R.C.; Fernandez-Lafuente, R.; Tardioli, P.W. Maltose production using starch from cassava bagasse catalyzed by cross-linked β-amylase aggregates. *Catalysts* **2018**, *8*, 170. [CrossRef]
24. López-Gallego, F.; Betancor, L.; Hidalgo, A.; Alonso, N.; Fernández-Lafuente, R.; Guisán, J.M. Co-aggregation of enzymes and polyethyleneimine: A simple method to prepare stable and immobilized derivatives of glutaryl acylase. *Biomacromolecules* **2005**, *6*, 1839–1842. [CrossRef] [PubMed]
25. Mafra, A.C.O.; Furlan, F.F.; Badino, A.C.; Tardioli, P.W. Gluconic acid production from sucrose in an airlift reactor using a multi-enzyme system. *Bioprocess Biosyst. Eng.* **2015**, *38*, 671–680. [CrossRef]
26. Galvis, M.; Barbosa, O.; Ruiz, M.; Cruz, J.; Ortiz, C.; Torres, R.; Fernandez-Lafuente, R. Chemical amination of lipase B from *Candida antarctica* is an efficient solution for the preparation of crosslinked enzyme aggregates. *Process Biochem.* **2012**, *47*, 2373–2378. [CrossRef]
27. Barbosa, O.; Ortiz, C.; Berenguer-Murcia, Á.; Torres, R.; Rodrigues, R.C.; Fernandez-Lafuente, R. Glutaraldehyde in bio-catalysts design: A useful crosslinker and a versatile tool in enzyme immobilization. *RSC Adv.* **2014**, *4*, 1583–1600. [CrossRef]
28. Mateo, C.; Palomo, J.M.; van Langen, L.M.; van Rantwijk, F.; Sheldon, R.A. A new, mild cross-linking methodology to prepare cross-linked enzyme aggregates. *Biotechnol. Bioeng.* **2004**, *86*, 273–276. [CrossRef]
29. Talekar, S.; Nadar, S.; Joshi, A.; Joshi, G. Pectin cross-linked enzyme aggregates (pectin-CLEAs) of glucoamylase. *RSC Adv.* **2014**, *4*, 59444–59453. [CrossRef]
30. Zhen, Q.; Wang, M.; Qi, W.; Su, R.; He, Z. Preparation of β-mannanase CLEAs using macromolecular cross-linkers. *Catal. Sci. Technol.* **2013**, *3*, 1937–1941. [CrossRef]
31. Talekar, S.; Waingade, S.; Gaikwad, V.; Patil, S.; Nagavekar, N. Preparation and characterization of cross linked enzyme aggregates (CLEAs) of *Bacillus amyloliquefaciens* alpha amylase. *J. Biochem. Technol.* **2012**, *3*, 349–353.
32. Gupta, K.; Jana, A.K.; Kumar, S.; Maiti, M. Immobilization of amyloglucosidase from SSF of *Aspergillus niger* by crosslinked enzyme aggregate onto magnetic nanoparticles using minimum amount of carrier and characterizations. *J. Mol. Catal. B Enzym.* **2013**, *98*, 30–36. [CrossRef]
33. Matte, C.R.; Nunes, M.R.; Benvenutti, E.V.; da Natividade Schöffer, J.; Ayub, M.A.Z.; Hertz, P.F. Characterization of cyclodextrin glycosyltransferase immobilized on silica microspheres via aminopropyltrimethoxysilane as a "spacer arm". *J. Mol. Catal. B Enzym.* **2012**, *78*, 51–56. [CrossRef]
34. Martín, M.T.; Plou, F.J.; Alcalde, M.; Ballesteros, A. Immobilization on Eupergit C of cyclodextrin glucosyltransferase (CGTase) and properties of the immobilized biocatalyst. *J. Mol. Catal. B Enzym.* **2003**, *21*, 299–308. [CrossRef]
35. Tardioli, P.W.; Zanin, G.M.; de Moraes, F.F.; De Moraes, F.F. Production of cyclodextrins in a fluidized-bed reactor using cyclodextrin-glycosyl-transferase. *Appl. Biochem. Biotechnol.* **2000**, *84–86*, 1003–1019. [CrossRef]
36. Amud, A.E.; Da Silva, G.R.P.; Tardioli, P.W.; Soares, C.M.F.; Moraes, F.F.; Zanin, G.M. Methods and supports for immobilization and stabilization of cyclomaltodextrin glucanotransferase from *Thermoanaerobacter*. *Appl. Biochem. Biotechnol.* **2008**, *146*, 189–201. [CrossRef] [PubMed]

37. Tardioli, P.W.P.W.; Zanin, G.M.G.M.; de Moraes, F.F.F. Characterization of *Thermoanaerobacter* cyclomaltodextrin glucanotransferase immobilized on glyoxyl-agarose. *Enzym. Microb. Technol.* **2006**, *39*, 1270–1278. [CrossRef]
38. da Natividade Schöffer, J.; Klein, M.P.; Rodrigues, R.C.; Hertz, P.F. Continuous production of β-cyclodextrin from starch by highly stable cyclodextrin glycosyltransferase immobilized on chitosan. *Carbohydr. Polym.* **2013**, *98*, 1311–1316. [CrossRef] [PubMed]
39. Martín, M.T.; Angeles Cruces, M.; Alcalde, M.; Plou, F.J.; Bernabé, M.; Ballesteros, A. Synthesis of maltooligosyl fructofuranosides catalyzed by immobilized cyclodextrin glucosyltransferase using starch as donor. *Tetrahedron* **2004**, *60*, 529–534. [CrossRef]
40. Kim, P.S.; Shin, H.D.; Park, J.K.; Lee, Y.H. Immobilization of cyclodextrin glucanotransferase on amberlite IRA-900 for biosynthesis of transglycosylated xylitol. *Biotechnol. Bioprocess Eng.* **2000**, *5*, 174–180. [CrossRef]
41. Martín, M.T.T.; Alcalde, M.; Plou, F.J.F.J.; Ballesteros, A. Covalent immobilization of cyclodextrin glucosyltransferase (CGTase) in activated silica and Sepharose. *Indian J. Biochem. Biophys.* **2002**, *39*, 229–234. [PubMed]
42. Sheldon, R.A. Cross-linked enzyme aggregates (CLEA®s): Stable and recyclable biocatalysts. *Biochem. Soc. Trans.* **2007**, *35*, 1583–1587. [CrossRef] [PubMed]
43. Fernandez-Lafuente, R.; Rosell, C.M.; Rodriguez, V.; Guisan, J.M. Strategies for enzyme stabilization by intramolecular crosslinking with bifunctional reagents. *Enzym. Microb. Technol.* **1995**, *17*, 517–523. [CrossRef]
44. Mafra, A.C.O.; Kopp, W.; Beltrame, M.B.; Giordano, R.L.C.; Ribeiro, M.P.A.; Tardioli, P.W.; de Lima Camargo Giordano, R.; de Arruda Ribeiro, M.P.; Tardioli, P.W. Diffusion effects of bovine serum albumin on cross-linked aggregates of catalase. *J. Mol. Catal. B Enzym.* **2016**, *133*, 107–116. [CrossRef]
45. Migneault, I.; Dartiguenave, C.; Bertrand, M.J.; Waldron, K.C. Glutaraldehyde: Behavior in aqueous solution, reaction with proteins, and application to enzyme crosslinking. *Biotechniques* **2004**, *37*, 790–802. [CrossRef] [PubMed]
46. Cui, J.D.; Jia, S.R. Optimization protocols and improved strategies of cross-linked enzyme aggregates technology: Current development and future challenges. *Crit. Rev. Biotechnol.* **2015**, *35*, 15–28. [CrossRef] [PubMed]
47. Talekar, S.; Joshi, A.; Joshi, G.; Kamat, P.; Haripurkar, R.; Kambale, S. Parameters in preparation and characterization of cross linked enzyme aggregates (CLEAs). *RSC Adv.* **2013**, *3*, 12485–12511. [CrossRef]
48. Fernández-Lafuente, R.; Rodríguez, V.; Mateo, C.; Penzol, G.; Hernández-Justiz, O.; Irazoqui, G.; Villarino, A.; Ovsejevi, K.; Batista, F.; Guisán, J.M. Stabilization of multimeric enzymes via immobilization and post-immobilization techniques. *J. Mol. Catal. B Enzym.* **1999**, *7*, 181–189. [CrossRef]
49. Tardioli, P.W.; Fernández-Lafuente, R.; Guisán, J.M.; Giordano, R.L.C. Design of new immobilized-stabilized carboxypeptidase a derivative for production of aromatic free hydrolysates of proteins. *Biotechnol. Prog.* **2003**, *19*, 565–574. [CrossRef] [PubMed]
50. Blanco, R.M.; Guisán, J.M. Protecting effect of competitive inhibitors during very intense insolubilized enzyme-activated support multipoint attachments: Trypsin (amine)-agarose (aldehyde) system. *Enzym. Microb. Technol.* **1988**, *10*, 227–232. [CrossRef]
51. Borges, D.G.; Baraldo Junior, A.; Farinas, C.S.; Giordano, R.L.C.; Tardioli, P.W. Enhanced saccharification of sugarcane bagasse using soluble cellulase supplemented with immobilized β-glucosidase. *Bioresour. Technol.* **2014**, *167*, 206–213. [CrossRef]
52. Jørgensen, S.T.; Tangney, M.; Starnes, R.L.; Amemiya, K. Cloning and nucleotide sequence of a thermostable cyclodextrin glycosyltransferase gene from *Thermoanaerobacter* sp. ATCC 53627 and its expression in *Escherichia coli*. *Biotechnol. Let.* **1997**, *19*, 1027–1031. [CrossRef]
53. Van Der Veen, B.A.; Van Alebeek, G.J.W.M.; Uitdehaag, J.C.M.; Dijkstra, B.W.; Dijkhuizen, L. The three transglycosylation reactions catalyzed by cyclodextrin glycosyltransferase from *Bacillus circulans* (strain 251) proceed via different kinetic mechanisms. *Eur. J. Biochem.* **2000**, *267*, 658–665. [CrossRef] [PubMed]
54. Sanchez, A.; Cruz, J.; Rueda, N.; Dos Santos, J.C.S.; Torres, R.; Ortiz, C.; Villalonga, R.; Fernandez-Lafuente, R. Inactivation of immobilized trypsin under dissimilar conditions produces trypsin molecules with different structures. *RSC Adv.* **2016**, *6*, 27329–27334. [CrossRef]
55. Bradford, M.M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **1976**, *72*, 248–254. [CrossRef]

56. Vikmon, M. Rapid and simple spectrophotometric method for determination of micro-amounts of cyclodextrins. In *Proceedings of the First International Symposium on Cyclodextrins. Advance in Inclusion Science*; Szejtli, J., Ed.; Springer: Dordrecht, The Netherlands, 1982; Volume 1, pp. 69–74.
57. Giordano, R.C.; Giordano, R.L.C.; Prazeres, D.M.F.; Cooney, C.L. Analysis of a Taylor–Poiseuille vortex flow reactor—I: Flow patterns and mass transfer characteristics. *Chem. Eng. Sci.* **1998**, *53*, 3635–3652. [CrossRef]
58. Giordano, R.L.C.; Giordano, R.C.; Cooney, C.L. Performance of a continuous Taylor–Couette–Poiseuille vortex flow enzymic reactor with suspended particles. *Process Biochem.* **2000**, *35*, 1093–1101. [CrossRef]
59. Resende, M.M.; Sousa, R.; Tardioli, P.W.; Giordano, R.L.C.; Giordano, R.C. Enzymatic tailor-made proteolysis of whey in a vortex flow reactor. *AIChE J.* **2005**, *51*, 314–322. [CrossRef]
60. Rojas, M.J. Recovery of Starch from Cassava Bagasse for Cyclodextrin Production Catalyzed by Cross-Linked CGTase Aggregates. Ph.D. Thesis, Federal University of São Carlos, São Carlos, Brazil, 2017.
61. Miller, G.L. Use of dinitrosalicylic acid reagent for determination of reducing sugar. *Anal. Chem.* **1959**, *31*, 426–428. [CrossRef]
62. Rojas, M.J.; Castral, T.C.; Giordano, R.L.C.; Tardioli, P.W. Development and validation of a simple high performance liquid chromatography—Evaporative light scattering detector method for direct quantification of native cyclodextrins in a cyclization medium. *J. Chromatogr. A* **2015**, *1410*, 140–146. [CrossRef]