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Optimized Conditions for Preparing a Heterogeneous Biocatalyst via Cross-Linked Enzyme Aggregates (CLEAs) of β-Glucosidase from *Aspergillus niger*

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Abstract: This study mainly aims to find the optimal conditions for immobilizing a non-commercial β-glucosidase from *Aspergillus niger* via cross-linked enzyme aggregates (CLEAs) by investigating the effect of cross-linking agent (glutaraldehyde) concentration and soy protein isolate/enzyme ratio (or spacer/enzyme ratio) on the catalytic performance of β-glucosidase through the central composite rotatable design (CCRD). The influence of certain parameters such as pH and temperature on the hydrolytic activity of the resulting heterogeneous biocatalyst was assessed and compared with those of a soluble enzyme. The catalytic performance of both the soluble and immobilized enzyme was assessed by hydrolyzing *ρ*-nitrophenyl-β-D-glucopyranoside (*ρ*-NPG) at pH 4.5 and 50 °C. It was found that there was a maximum recovered activity of around 33% (corresponding to hydrolytic activity of 0.48 U/mL) in a spacer/enzyme ratio of 4.69 (mg/mg) using 25.5 mM glutaraldehyde. The optimal temperature and pH conditions for the soluble enzyme were 60 °C and 4.5, respectively, while those for CLEAs of β-glucosidase were between 50 and 65 °C and pH 3.5 and 4.0. These results reveal that the immobilized enzyme is more stable in a wider pH and temperature range than its soluble form. Furthermore, an improvement was observed in thermal stability after immobilization. After 150 days at 4 °C, the heterogeneous biocatalyst retained 80% of its original activity, while the soluble enzyme retained only 10%. The heterogeneous biocatalyst preparation was also characterized by TG/DTG and FT-IR analyses that confirmed the introduction of carbon chains via cross-linking. Therefore, the immobilized biocatalyst prepared in this study has improved enzyme stabilization, and it is an interesting approach to preparing heterogeneous biocatalysts for industrial applications.

Keywords: β-glucosidase; immobilization; CLEAs technique; stabilization

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1. Introduction

Enzymes are biological catalysts having a wide range of industrial applications, as they can be employed more efficiently and sustainably than traditional chemical processes. However, their free forms (crude enzyme preparations, either soluble or powder enzyme extracts) have a few drawbacks if employed on an industrial scale, such as low physical and chemical stabilities and difficult separation from the reaction mixture for reusability purposes [1]. In order to overcome such drawbacks, different immobilization techniques have been proposed, since proper immobilization approaches can improve the thermal and operational stability of the resulting biocatalysts [2–6]. Moreover, a proper immobilization technique can improve the selectivity or specificity and reduce the inhibition effects and costs in downstream processes, in addition to increasing the flexibility of reactor configuration and design parameters [7,8]. In this context, several immobilization techniques have been used for preparing highly active and stable industrial biocatalysts, such as CLEAs (cross-linked enzyme aggregates) due to the fact that they are considered a cost-effective immobilization protocol requiring no supports, in addition to providing a platform for

enzyme cascade reactions using multiple enzymes [8,9]. Moreover, this promising immobilization protocol offers a simultaneous purification of enzymes through a simple step that is well capable of reducing the total costs to prepare the heterogeneous biocatalyst [10–12].

CLEAs can be prepared through a couple of steps. Initially, enzymes undergo aggregation/precipitation induced by precipitating agents such as salts, water-miscible organic solvents or non-ionic polymers, among others. Afterward, the formed aggregates are cross-linked amino groups of lysine residues, and sulfhydryl groups of cysteine, phenolic OH groups of tyrosine, or imidazole groups of histidine present on the enzyme surface; they react with carbonyl groups at the extremity of a bifunctional reagent, i.e., glutaraldehyde in the majority of previous studies reported in the literature [9,11,13]. The CLEAs technique is an irreversible immobilization method to link enzymes together and form a complex three-dimensional structure composed of intermolecular cross-links between the enzyme and bi- or multifunctional reagents in order to make them insoluble in the reaction medium [14,15]. Given that the enzyme has a low surface density of amino groups, enzyme co-precipitation using an inert protein rich in lysine residues, such as bovine serum albumin (BSA), should contribute to form CLEAs having greater activity recovery and operational stability [11,16]. Although it is a widely explored technique involving several enzyme complexes, such as α-L-arabinosidase [17], invertase [18], naringinase [19], β-amylase [20], lipases [13,21], lipase and protease [22], glucose oxidase and catalase [23], there are few studies on the use of the CLEAs technique in β-glucosidase immobilization.

β-glucosidases (β-D-glucoside glucohydrolases, EC 3.2.1.21) are enzymes capable of hydrolyzing β-1,4 glycosidic bonds present in aryl-, amino-, alkyl-β-D-glucosides and cyanogenic glycosides, and oligo- and disaccharides [24]. It is also able to catalyze several reactions of industrial interest in a variety of segments, and it has attracted considerable attention in recent years due to its important roles in several biotechnological processes, such as the hydrolysis of exogenous glycolipids and glycosides and isoflavonoids glycosides, cell wall catabolism of cello-oligosaccharides, defense mechanisms, activation of conjugated phytohormones, the release of flavor compounds in plants, the release of aromatic compounds from flavor precursors used in beverage industries, the production of second-generation bioethanol from agricultural wastes, and so on [20,25–28]. In a study conducted by Zong et al. [26], β-glucosidase from plum seeds was immobilized via the CLEAs technique using a mixture of ethanol and isopropanol as precipitating agents and glutaraldehyde (20 mM) as a cross-linking agent. Ahumada et al. [17] co-immobilized β-glucosidase and arabinosidase from a commercial enzyme preparation (Rapidase®AR2000) via CLEAs technique to assess the effect of BSA (spacer) and glutaraldehyde concentrations on the enzyme immobilization process and the catalytic performance of the heterogeneous biocatalyst. These authors observed that the prepared CLEAs were more stable than the soluble enzyme. On the other hand, studies on the preparation of immobilized microbial β-glucosidase from *Aspergillus niger* via the CLEAs technique still are scarce in the literature.

Thus, this novel study mainly consists in preparing a heterogeneous biocatalyst through immobilizing β-glucosidase from *Aspergillus niger* using the CLEAs technique. For such a purpose, β-glucosidase was produced via solid-state fermentation using wheat bran as a substrate as it is an eco-friendly and economic alternative to produce industrial enzymes [29]. Enzyme precipitation of a crude extract obtained by solid-state fermentation was performed under optimal experimental conditions determined through a previous study using *n*-propanol as a precipitant agent [30]. The effect of relevant factors, such as spacer/enzyme ratio and glutaraldehyde (cross-linking agent) concentrations, was evaluated using the central composite rotatable design (CCRD), given that it is a well-known, robust and cost-effective statistical approach [31]. A low-cost spacer from soy protein isolate was used due to its cost-effectiveness and satisfactory performance in producing heterogeneous biocatalysts with enhanced catalytic performances [32]. The effect of certain factors (pH, and temperature) on the immobilized enzyme performance was evaluated and compared with its soluble form so as to enhance enzyme immobilization and consolidate the results of utilizing β-glucosidase in industrial bioprocesses. Thermogravimetric

(TG/DTG) and Fourier transform infrared (FT-IR) analyses were also conducted in order to characterize the resulting heterogeneous biocatalyst.

2. Results and Discussion

In this study, a heterogeneous biocatalyst of industrial interest was prepared via the CLEAs technique using β-glucosidase from *A. niger.* The effect of relevant factors such as spacer/enzyme ratio and cross-linking agent (glutaraldehyde) concentration on the catalytic performance of the enzyme was evaluated using a statistical tool (CCRD), as shown in Table 1. These parameters were selected due to their great relevance to the catalytic performance and stability of several industrial enzymes [8,9,12]. The levels of each parameter were selected based on preliminary studies conducted in our lab (data not shown). These tests were performed randomly so as to avoid system errors under fixed experimental conditions (50 °C, pH 4.5, 1 h of cross-linking time). Under such experimental conditions, recovered activity percentage values (response) ranged from 8.1% (Test #1) to 33.1% (Test #8)—see Table 1.

Table 1. CCRD experimental design of CLEAs of β-glucosidase from *A. niger* obtained after 60 min of reaction using glutaraldehyde as cross-linking agent. The response variable was the recovered activity percentage of the enzyme.

Tests	Coded Variables (Real Variables)		Response	
	Spacer/Enzyme Ratio (mg/mg)	Cross-Linking Agent (mM)	Recovered Activity (%)	Deviation (%)
1	−1 (1.37)	−1 (8.12)	8.1	−162.5
2	+1 (8.00)	−1 (8.12)	9.1	−34.1
3	−1 (1.37)	+1 (42.88)	21.5	−68.6
4	+1 (8.00)	+1 (42.88)	21.7	−25.3
5	−1.41 (0.00)	0 (25.50)	27.7	−38.3
6	+1.41 (9.37)	0 (25.50)	27.2	6.1
7	0 (4.69)	−1.41 (1.00)	9.1	33.6
8	0 (4.69)	+1.41 (50.00)	33.1	17.9
9	0 (4.69)	0 (25.50)	32.2	0.8
10	0 (4.69)	0 (25.50)	30.8	−3.7
11	0 (4.69)	0 (25.50)	32.8	2.7

Center points showed a slight variation, which indicates good process reproducibility. The CCRD analysis has revealed that the quadratic term of spacer/enzyme ratio ($x_1{}^2$) and linear (x_2) and quadratic terms of the cross-linking agent ($x_2{}^2$) were statistically significant at 10%, once the *p*-value < 0.1 (Table 2). Thus, non-significant terms were removed from the model and recalculated. Coded coefficients were then recalculated and used to write Equation (1).

$$\text{Recovered activity } (\%) = 31.93 - 4.45x_1^2 + 7.49x_2 - 7.71x_2^2 \quad (1)$$

Table 2. ANOVA of the CCRD using statistically significant regression coefficients at 10% significance.

Source of Variation	Coefficients	Degrees of Freedom	Mean Square	F-Calculated	*p*-Value
Regression	816.62	3	272.21	10.65	0.005319
Residual	179.00	7	25.57		
Lack of Fit	176.89				
Pure error	2.11				
Total	995.62	10			
R^2 = 82.02% $F_{tab.}$ = 3.07					

The ANOVA (Table 2) was performed to identify statistically significant coefficients and higher $F_{calc.}$ (10.65) than $F_{tab.}$ (3.07) and a coefficient of determination (R^2) of 0.82

was found. Therefore, it is possible to use the obtained model equation to generate the response surface and analyze the studied variables (spacer/enzyme ratio and cross-linking agent concentration).

The model was used to generate the response surface (Figure 1A) and contour curves (Figure 1B). It was observed that the formation of an optimal region in which the highest values of β-glucosidase recovered activity was obtained using a spacer/enzyme ratio of 4.69 (mg/mg) at 25.5 mM of cross-linking agent concentration. Some factors may be accountable for affecting the performance of the CLEAs, among which cross-linking agent concentration stands out, as it is related to particle size and affects mass transfer (substrate or pH gradients) [9]. Moreover, cross-linking agent concentration is affected by the number of cross-links, i.e., the higher the concentration, the greater the number of links, therefore, leading to reduced enzyme release [5,12]. However, high concentration increases enzyme rigidity and causes catalytic activity loss. A combination of spacer/enzyme ratio (4.69) and glutaraldehyde (25.5 mM) provided optimal recovered activity percentage, as it increased the density of groups available to create cross-links using glutaraldehyde, thus preserving groups of β-glucosidase side chains which are important for its catalytic activity [33–35]. At the lowest glutaraldehyde concentrations (1.0 mM), there was no binding and the enzyme was leached, which explains such a low recovered activity value (only 9.1%—see Test #7 in Table 1). Furthermore, a high glutaraldehyde concentration (42.8 mM) increases enzyme rigidity and reduces its catalytic activity, which is in agreement with previous reports [2,12].

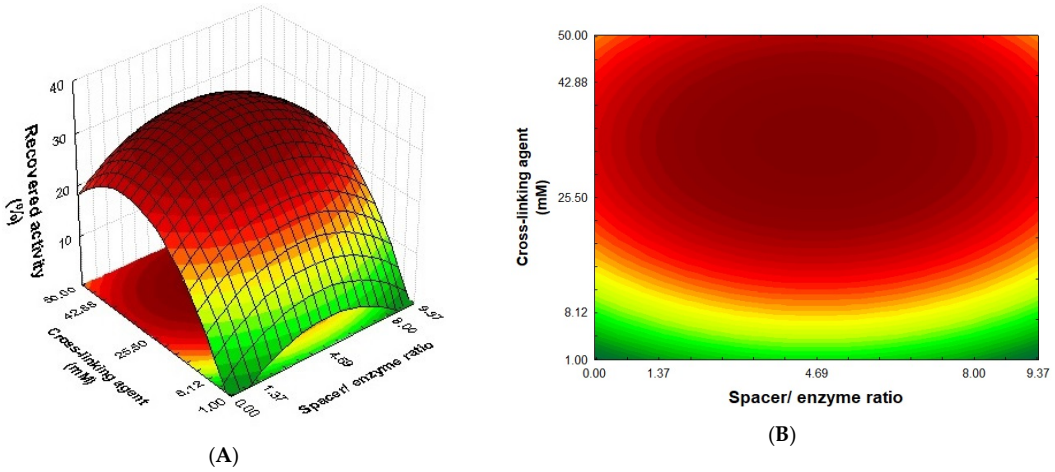

Figure 1. β-glucosidase recovered activity as a function of cross-linking agent (glutaraldehyde) and spacer/enzyme ratio after 60 min of reaction time: (**A**) response surface and (**B**) contour curve.

The model was further validated by performing five experiments under optimal conditions, i.e., a spacer/enzyme ratio of 4.69 at 25.5 mM of cross-linking agent concentration, thus reaching β-glucosidase recovered activity of 31.12 ± 1.85% and demonstrating that the process has good reproducibility, as shown in Figure 2.

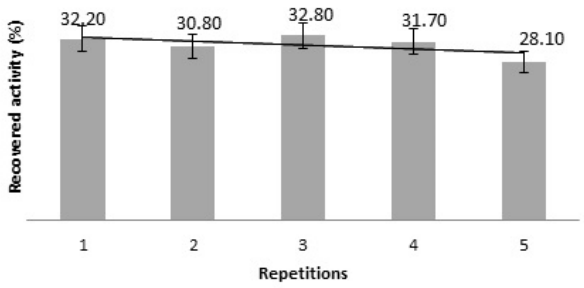

Figure 2. Optimized model validated for β-glucosidase immobilization via the CLEAs technique.

2.1. Study on the Effect of Cross-Linking Time

Once having optimized the parameters of cross-linking agent concentration and spacer/enzyme ratio, the effect of cross-linking time on the catalytic performance of the heterogeneous biocatalyst under optimal conditions determined by the response surface methodology was investigated. These results are illustrated in Figure 3.

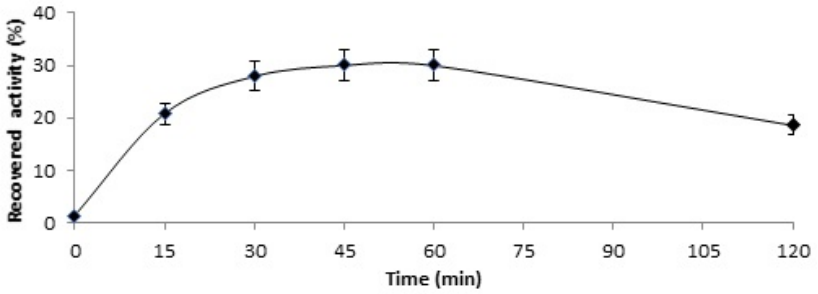

Figure 3. Effect of cross-linking time on β-glucosidase activity immobilized via the CLEAs technique (100% Relative activity corresponds to a hydrolytic activity of 1.48 U/mL).

According to the results, a gradual increase in the recovered activity percentage by increasing cross-linking time was observed. This phenomenon is possibly due to insufficient cross-links as reaction time was reduced, thus evidencing the leaching of free enzyme molecules while washing protein aggregates [34,35]. The maximum recovered activity value around 33% was found at a cross-linking time ranging between 45 min and 60 min, followed by a slight decrease after 120 min. For longer reaction times, an excessive number of cross-links can provide a more rigid heterogeneous biocatalyst having smaller pores in addition to hindering substrate access to its microenvironment [9]. In fact, further tests using immobilized β-glucosidase were performed using the biocatalyst prepared after 60 min of cross-linking time.

2.2. Biochemical Characterization

2.2.1. Optimum Temperature and pH Activity and Stability of Soluble and Immobilized β-Glucosidase

The highest catalytic activity was achieved at optimal temperature and pH conditions, i.e., 60 °C and pH 4.5 for soluble β-glucosidase (Figure 4A), and at temperatures ranging from 50 to 65 °C and pH from 3.5 to 4.0 for the CLEAs (Figure 4B). The heterogeneous biocatalyst used herein was the same as the one prepared in a spacer/enzyme ratio of 4.69, 25.5 mM of glutaraldehyde at 60 min of cross-linking time, i.e., conditions determined through the proposed CCRD.

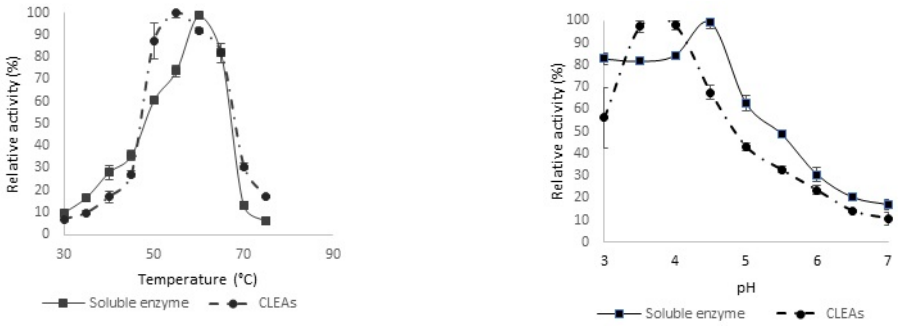

Figure 4. Hydrolytic activity profiles of soluble and immobilized β-glucosidase as a function of optimal temperature (**A**) and pH (**B**) conditions.

Optimal temperature shifts (Figure 4A), however, can be attributed to the lower flexibility of the enzyme's molecular structure within the structure of the CLEAs promoted by glutaraldehyde cross-linking, which makes the lateral bonds accountable for conferring conformational stability to the most stable and well-protected enzyme [36,37]. There were shifts in optimal pH conditions for the CLEAs activity (Figure 4B), probably due to a negatively charged microenvironment whose heterogeneity in size was comparable to that of soy protein isolate [38], thus leading to many different electrical charges in the internal microenvironment of the CLEAs. This might also explain the thermal stability of the CLEAs in comparison with that of the soluble enzyme (Figure 5A,B).

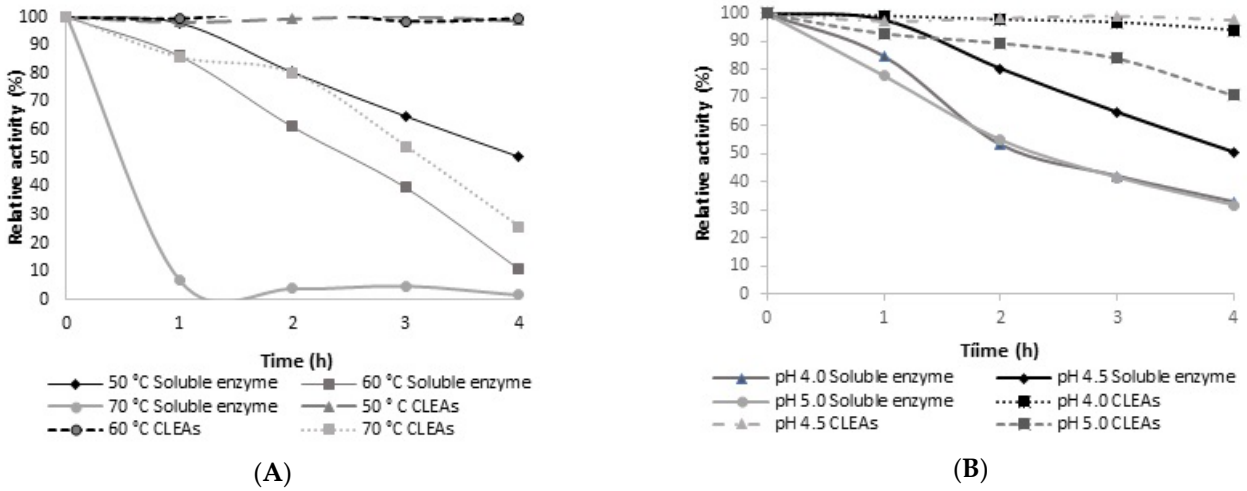

Figure 5. Hydrolytic activity profiles of soluble and immobilized β-glucosidase as a function of temperature (**A**) and pH (**B**) stability.

These results are in agreement with previous reports. Deng et al. [37] conducted assays using commercial β-glucosidase immobilized by CLEAs and found an increase by 0.5 of optimum pH for enzyme activity and a shift to a more alkaline range, i.e., from 5.0 to 5.5. In another study, Ahumada et al. [17] studied the co-immobilization of β-glucosidase and arabinosidase by the CLEAs technique and reported no changes in the biochemical profile of the enzyme, in addition to finding that the optimal temperature is 50 °C and pH 4.5 regarding enzyme activity using bovine serum albumin as a spacer. Different enzyme immobilization techniques possibly modify the biochemical profile of enzymes, and systems ought to be characterized thereof. Other authors also found no significant changes in the biochemical profile of β-glucosidase immobilized on various solid supports, such as chitosan and polyacrylamide [36], sodium alginate [39], silica gel and sol-gel [28].

The immobilized enzyme showed greater stability than the soluble enzyme at all studied temperatures. At 50 °C, the soluble enzyme retained around 50% of its original activity after 4 h of incubation. At 60 °C, the soluble enzyme maintained only 10% of its original activity after 4 h of incubation and it was completely inactivated after 1 h of the experiment at 70 °C. Nevertheless, the CLEAs were stable throughout the period tested at temperatures of 50 °C and 60 °C and maintained 100% of their activity. Moreover, it retained 54% of its initial activity after 3 h of incubation at 70 °C, as can be seen in Figure 5A. A thermal protective effect after enzyme immobilization is expected since there is an increase in rigidity and alteration in the enzyme's structural flexibility [36].

The CLEAs were also more stable at diverse pH conditions if compared to the soluble enzyme (Figure 5B). At pH 4.0 and after 4 h of incubation, the soluble enzyme maintained 50% of its initial activity, while the CLEAs presented only a 30% of loss of activity under such conditions. At pH values of 4.5 and 5.0, the soluble enzyme showed a retention of 30% of its original activity, while the CLEAs retained almost all of their original activity.

2.2.2. Storage Stability Tests

β-glucosidase immobilization via the CLEAs technique provided a derivative with greater storage stability if compared to the soluble enzyme, as can be seen in Figure 6.

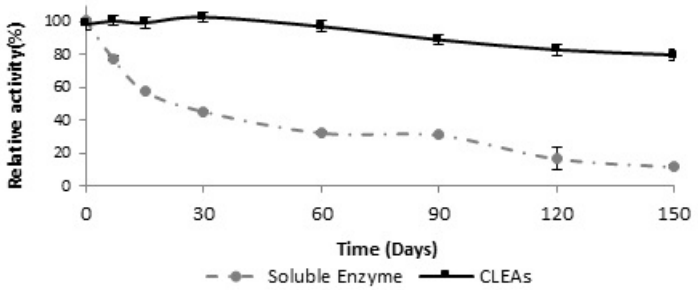

Figure 6. Storage stability of soluble and immobilized enzyme via the CLEAs technique after 150 days at 4 °C.

After 15 days of storage under refrigeration, the soluble enzyme lost 40% of its original activity, while the CLEAs still maintained 100% of their initial activity. After 150 days of storage, the soluble enzyme only retained 10% of its initial activity, while the CLEAs still maintained about 80% of their activity. During the immobilization process, enzyme crosslinking allows the enzyme to become more rigid and confined within a porous solid, in addition to offering protection against attacks from other proteolytic enzymes present in the fermented extract, thus preventing its degradation or decomposition [5,11,14] and improving storage stability. The profile of resulting CLEAs is more robust from an industrial process standpoint. Although there are mild pH and temperature fluctuations, there would be no yield alterations in enzymatic hydrolysis effectiveness.

2.3. Characterization of Biocatalysts by TGA and FT-IR Analyses

Fourier Transform Infrared spectroscopy (FT-IR) and thermogravimetric (TGA) analyses were performed for soluble and immobilized enzymes in order to confirm the structural changes occurring after the glutaraldehyde cross-linking step under optimal experimental conditions, as aforementioned. TGA was performed to assess whether modifications provided greater thermal stability to the heterogeneous biocatalyst prepared via the CLEAs technique.

The thermal stability of soluble and immobilized enzymes using TGA/DTG analysis was analyzed under a nitrogen atmosphere. Figure S1A,B shows that both enzyme forms (soluble—Figure S1A) and immobilized—Figure S1B) exhibited two main stage mass losses at temperatures ranging between 25 °C and 600 °C. It is observed that mass loss curves are very similar and there are two main thermal events occurring in the two samples by a loss of water through physical adsorption on the protein structure from the initial temperature to 100 °C, in addition to a thermal decomposition [40]. However, greater mass loss was found in CLEAs (39%—see Figure S1B) at around 300 °C if compared to the soluble enzyme (32%—see Figure S1A). These results may be due to the great compaction of the free enzyme chains, which in turn leads to greater thermal stability. The introduction of carbon chains after cross-linking using glutaraldehyde prevented such compaction and resulted in the formation of aggregates with enhanced porosity and, consequently, less stability in this temperature range [41]. The addition of functional groups can lead to a reduction in the effective compaction of these macromolecules, thus reducing their thermal stability in the temperature range evaluated in this study (from 25 °C and 600 °C), as they have a more porous structure [42], which explains the results obtained herein.

In this study, FT–IR analysis for soluble (Figure S2A) and immobilized β-glucosidase (Figure S2B) was performed to obtain the structural information after cross-linking using glutaraldehyde as a cross-linking agent. According to Figure S2A, an intense absorption band in the range of 3600–3000 cm^{-1} attributed to O–H and N–H stretching in the enzyme

structure can be observed [43,44]. After cross-linking using glutaraldehyde, there was a reduction in the intensity of these bands (see Figure S2B), as this bifunctional agent interacts with different nucleophilic groups of the enzyme, such as O–H and N–H [45]. On the other hand, the band referring to the stretching of C–H (asymmetric) into $–CH_2$ (methyl groups), i.e., between 2923 and 2946 cm^{-1}, is intensified after biocatalyst preparation due to the cross-linking of nucleophilic groups described above, which confirms a chemical modification on the enzyme surface by glutaraldehyde molecules [2,41]. In cross-linked samples, the band referring to C–H (symmetric) ranges between 2870 and 2892 cm^{-1}, which is also intensified as carbon chains are introduced via glutaraldehyde cross-linking [46].

3. Materials and Methods

3.1. Materials

Aspergillus niger LBA 02 was obtained from the Culture Collection of Food and Biochemistry Laboratory, University of Campinas, Campinas-SP, Brazil and wheat bran (Nattuday, Formiga, MG, Brazil) was purchased from a local store. The glutaraldehyde solution (25% solution) and *n*-propanol were acquired from Dinâmica Química Contenporânea Ltd. (São Paulo, SP, Brazil). Bovine serum albumin (BSA) and *ρ*-nitrophenyl β-D-glucopyranoside (*rho*-NPG) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Soy protein isolate (N4Natural, Santo André, SP, Brazil) was acquired from a local store. All other reagents and organic solvents were of analytical grade and acquired from Dinâmica and Synth® Ltd. (São Paulo, SP, Brazil).

3.2. β-Glucosidase Production via Solid-State Fermentation

The enzyme was produced by solid-state fermentation according to Angelotti et al. [29], but with a few modifications. *Aspergillus niger* strain belonging to the culture collection of the Biochemistry and Food Laboratory, Faculty of Food Engineering, State University of Campinas, Brazil, was kindly donated and used as a source of β-glucosidase. A culture medium of potato dextrose agar was placed in slant tubes for fungi growing, and tubes were covered with a protective layer of Vaseline during storage. The culture medium for enzyme production is composed of dry wheat bran (10 g) and 10 mL of distilled water, which was then poured into 500 mL Erlenmeyer flasks and sterilized through autoclaving (20 min, 121 °C); 10^5 spores/g of culture medium were inoculated in the flasks. Erlenmeyer flasks were incubated for 5 days at 30 °C.

3.3. Enzyme Extraction

Enzyme extraction was performed by adding 50 mL sodium acetate buffer 0.01 M pH 4.5 into the Erlenmeyer flasks containing the fermented medium. The flasks were shaken at 150 rpm for 20 min. The resulting suspension was then filtered with filter paper. The filtered solution was used as raw enzyme extract.

3.4. Determination of β-Glucosidase Activity

The β-glucosidase activity was determined in accordance with Matsuura et al. (1995), but with a few modifications [47]; 300 µL aliquots of a 5 mM solution of *ρ*-nitrophenyl β-D-glucopyranoside (*ρ*-NPG) in 0.05 M sodium acetate buffer at pH 4.5 were pre-incubated at 50 °C for 5 min, and 300 µL of enzyme extract was added afterward. Then, the mixture was incubated at 50 °C for 15 min and the reaction was stopped by adding 300 µL of a 0.5 M solution of sodium carbonate (pH 12). Synthetic substrate hydrolysis (*ρ*-NPG) was estimated by measuring absorbance at 410 nm using a UV/Vis spectrophotometer and a quartz cuvette. The amount of *ρ*-nitrophenol release was determined based on the standard curve of *ρ*-nitrophenol ranging from 5 to 300 µmol. A unit of enzyme activity was defined as the amount of enzyme required to release 1 µmol of *ρ*-nitrophenol per minute of reaction in the experimental conditions described previously.

3.5. Determination of Protein Concentration

Total protein concentration was determined through Bradford's protein assay [48] using BSA as the standard.

3.6. Immobilization of β-Glucosidase via CLEAs: Optimization by CCRD

The CLEAs were prepared according to Gupta and Raghava [49] with a few modifications. *n*-propanol was added to several protein solutions prepared in 100 mM sodium acetate buffer at pH 4.5 using a fixed volume ratio of 1:4 (protein-to-organic solvent). Under such experimental conditions, the complete precipitation of all initial proteins was determined by Bradford's method (see Section 3.5). After 30 min of precipitation in an ice bath, amounts of glutaraldehyde 25% (*v*/*v*) having final concentrations ranging from 8.0 to 50 mM (depending on the CCRD test), were added to the precipitated enzyme (cross-linking step). The aggregate suspension was incubated under gentle stirring (150 rpm) in a laboratory shaker at 4 °C for 1 h. After incubation, the suspension was centrifuged (14,000× *g* for 5 min at 4 °C) and the precipitate was washed three times using 50 mM sodium acetate buffer pH 4.5 and re-suspended in the same buffer.

The effect of spacer/enzyme ratio (x_1) and cross-linking agent concentration (x_2) on the recovered activity percentage values of the immobilized enzyme was evaluated using a 2^2 central composite rotatable design (CCRD). Table 3 shows the CCRD with three center points and four axial points, totaling 11 tests and real and coded values for independent variables.

Table 3. Independent variables and their respective levels and real values used in the proposed CCRD approach.

	Independent Variables	**Levels**				
		−1.41	**−1**	**0**	**+1**	**+1.41**
x_1	Spacer/enzyme ratio (mg/mg)	0.00	1.37	4.69	8.00	9.37
x_2	Cross-linking agent (mM)	1.00	8.12	25.50	42.88	50.00

The recovered activity percentage, determined as shown in Equation (2), of the CLEAs, obtained after 60 min of cross-linking time was used as a response in the experimental design. Results were analyzed at 10% statistical significance using independent variables coded to fit a second-order polynomial. The model was further validated by performing five experiments under optimal conditions.

$$\text{Recovered activity } (\%) = \left(\frac{\text{Hydrolytic activity of immobilized enzyme}}{\text{Hydrolytic activity of soluble enzyme}} \right) \times 100 \quad (2)$$

Effect of Cross-Linking Time

After optimizing the parameters evaluated in the experimental design, the effect of the cross-linking time (15–120 min) was assessed in order to prepare β-glucosidase CLEAs. The overall yield of immobilization was calculated by the ratio of enzyme activity of derivatives and the soluble enzyme under the same conditions described in Section 3.4.

3.7. Catalytic Properties of Soluble and Immobilized β-Glucosidase in Hydrolysis Reaction

3.7.1. Determination of Optimum Temperature

The effect of temperature on enzyme activity was determined for soluble and immobilized enzymes at temperatures ranging from 30 °C to 75 °C. Soluble and immobilized enzymes were incubated for 15 min in a 5 mM solution of ρ-NPG and 0.05 M sodium acetate buffer at pH 4.5. The reaction was stopped by adding 0.5 M sodium carbonate and enzyme activity was determined according to Section 3.4. Experimental results of the effect of temperature on enzyme activity were plotted in graphs in which the highest activity for

each biocatalyst (soluble or immobilized β-glucosidase) was considered as 100%. All tests were performed in triplicate.

3.7.2. Determination of Optimum pH

The effect of pH on enzyme activity was determined at pH values ranging between 2.0 and 7.0 for both soluble and immobilized β-glucosidase (pH 3.0 at 5.0–100 mM sodium acetate buffer, pH 5.5 at 7.0–100 mM sodium phosphate buffer). Several *ρ*-NPG solutions at a fixed concentration (5 mM) were prepared and immersed in several buffer solutions (100 mM) at optimum activity temperature (55 °C for both soluble and immobilized β-glucosidase samples) after 15 min. Enzyme activity was determined as described in Section 3.4. Experimental results were also plotted in graphs and the maximum activity for each biocatalyst (soluble or immobilized β-glucosidase samples) was considered 100%.

3.7.3. pH and Thermal Stability Tests

Thermal stability tests were performed at the optimum pH activity of derivatives. In the absence of a substrate, both biocatalysts (soluble and immobilized enzyme) were immersed in a buffer solution (pH 4.5–100 mM sodium acetate buffer for a maximum period of 4 h) at temperatures ranging from 50 to 70 °C under static conditions. Samples were periodically collected (intervals of 1 h) to determine the residual catalytic activity of biocatalysts on *ρ*-NPG hydrolysis, as described in Section 3.4. The initial hydrolytic activity of each biocatalyst (3.33 U/mL for the soluble enzyme and 1.2 U/mL for the immobilized enzyme) was considered a control (100%).

pH stability tests were performed at optimum activity temperature (55 °C). In the absence of a substrate, soluble and immobilized enzymes were incubated for a maximum period of 4 h in solutions at pH values ranging from 4.0 to 5.0 under static conditions (sodium acetate buffer, 100 mM). In this set of experiments, samples were also periodically collected (intervals of 1 h) to determine the residual catalytic activity of biocatalysts, also on *ρ*-NPG hydrolysis (see Section 3.4). The initial hydrolytic activity of each biocatalyst (3.37 U/mL for the soluble enzyme and 1.02 U/mL for the immobilized enzyme) was also considered a control (100%).

3.7.4. Storage Stability Tests

Storage stability tests were also conducted for both biocatalyst forms (soluble and immobilized enzyme) for a maximum period of 5 months by immersion in a sodium acetate buffer pH 4.5 (100 mM) at 4 °C in a freezer under static conditions. In this study, samples were also collected every 15 days and the residual catalytic activity of biocatalysts was determined with *ρ*-NPG hydrolysis (see Section 3.4). The initial hydrolytic activity of each biocatalyst (1.15 U/mL for the soluble enzyme and 1.12 U/mL for the immobilized enzyme) was also considered a control (100%).

3.8. Characterization of Biocatalysts by TG/DTG and FT-IR Analyses

Thermogravimetric curves (TG/DTG) were obtained for the lyophilized soluble and immobilized enzyme using SII TG/DTA7300 Exstar in a temperature range of 25 °C to 600 °C at a heating rate of 10 °C/min under an inert atmosphere using nitrogen at a constant flow rate of 50 mL·min^{-1}. A Fourier Transform Infrared Spectroscopy (FT-IR) analysis was performed using Shimadzu equipment, model Affinity-1, coupled with a Pike Miracle ATR sampling accessory with ZnSe crystal plates at wavelengths ranging between 400 and 4000 cm^{-1} and 4 cm^{-1} of resolution.

4. Conclusions

The present results reveal that the model fits well with the data on the process of β-glucosidase immobilization via the CLEAs technique due to being statistically significant according to the experimental design. The spacer/enzyme ratio and cross-linking agent concentration were significant at the studied confidence level. Optimal conditions for

CLEAs production were the pinnacle of this study, as values of spacer/enzyme ratio of 4.69 and cross-linking agent of 25.5 mM were found. The generated model allowed plotting its response surface and explained 82% of the results. Furthermore, the heterogeneous biocatalyst is more robust than the soluble enzyme, whose optimal activity was achieved in a wider temperature range than the soluble enzyme (from 60 °C to 50–65 °C) and in a slightly more acidic pH range (from pH 4.5 to pH 3.5–4.0), thus offering greater stability in adverse temperature and pH conditions during a period of 4 h. TG/DTG and FT-IR analyses evidenced the effectiveness of cross-linking reaction and the insertion of carbon chains. The heterogeneous biocatalyst prepared in this study showed longer shelf life than that of the soluble enzyme, and residual activity of 80% was achieved after 150 days of storage at 4 °C if compared to 10% of residual activity reached by the soluble enzyme under the same conditions. These findings reveal that immobilized β-glucosidase via the CLEAs technique can be effectively used in industrial processes due to the enhanced pH activity and thermal stability, in addition to the improved storage time. Furthermore, this study offers new possibilities for further industrial applications of the heterogeneous biocatalyst prepared in this study in batch or continuous processes.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/catal13010062/s1, Figure S1: TG (blue curves) and DTG (red curves) analyses for soluble (A) and immobilized (B) β-glucosidase; Figure S2: FT-IR analysis for (A) and immobilized (B) β-glucosidase.

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