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Glutaraldehyde Effect in the Immobilization Process of Alpha-Galactosidase from *Aspergillus niger* in the Ion Exchange Resin Duolite A-568

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Alpha-galactosidase has important applications in food processing, medicine and sugar beet industry. However, industrial application of enzymes, such as α-galactosidase, demands for efficient methods of enzyme immobilization. The aim of this work was to study the effect of the cross linking agent glutaraldehyde in the immobilization process of a commercial α-galactosidase StarMax^{TM} from *Aspergillus niger* in the ion exchange resin Duolite A-568. Firstly, four tests were carried out to determine the steps of the immobilization. The results showed better enzymatic activities for the immobilization processes without adding glutaraldehyde and the immobilization process using the support pre-activated with glutaraldehyde 1 % (v/v) followed by the enzymatic immobilization. A Central Composite Design was performed to study the best glutaraldehyde concentration and time of reticulation range in the immobilization process. Best conditions were obtained at low glutaraldehyde concentrations, where time did not have such effect. Finally, the operational stability of the immobilized α-galactosidase without the cross linking process was compared to that using the support pre-activated with glutaraldehyde 1 % (v/v). Glutaraldehyde was able to enhance the operational stability of the immobilized enzyme in 12 %, showing a residual activity of 86 % after 72 h in the reactor against 74 %. When immobilization was performed without the reticulation process, α-galactosidase lost 16 % of its initial activity in the first 2 h in the reactor against only 3 % for the enzyme immobilized in the pre-activated support.

1. Introduction

Alpha-galactosidase (E.C 3.2.1.22) is an enzyme capable to hydrolyze α-1,6 linked galactosyl groups and it has potential applications in food processing, sugar beet industry and even in medicine (Ademark et al., 2001). Even though α-galactosidase has such important applications, the use of enzymes in catalytic industrial process is expensive. So, it requires methods of immobilization, which allow its reutilization and in some instances, improve the enzyme stability.

There are many techniques used to immobilize enzymes and its applications include studies of various supports and variables that affect the immobilization process. Nevertheless, the use of glutaraldehyde is one of the most popular techniques to immobilize enzymes. It is such a simple and in most cases very efficient method and it has been used in many immobilization processes from different enzymes and supports (Li et al., 2012; Podrepšek et al., 2012; Melo et al., 2011). Glutaraldehyde can introduce intermolecular cross linking in proteins or it can improve the attachment of the enzyme molecule in the support (Betancor et al., 2006). This technique may be applied by different ways in the enzyme immobilization process. The support may be previously treated with glutaraldehyde, which consists in firstly activating the support and then proceeding to immobilize the enzyme. Alternatively, the protein molecule can also be added to the support in the first step and then treated with glutaraldehyde. In some cases, both glutaraldehyde and enzyme can be added to the support together.

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However all these protocols seem to follow a similar mechanism, changes in the protein molecule vary depending on the method used, which may affect the enzyme activity (López-Gallego et al., 2005).
The aim of this work was to study the effect of the cross linking agent glutaraldehyde in the immobilization process of a commercial α-galactosidase StarMax™ from *Aspergillus niger* in the ion exchange resin Duolite A-568.

2. Materials

Alpha-galactosidase (E.C 3.2.1.22) was procured from Prozym Biosolutions™ and it has the commercial name of StarMax AGSL. This enzyme was produced by controlled fermentation of *Aspergillus niger*. The product was kept refrigerated (4 ± 2ºC) in closed polyethylene flasks. Duolite A-568 was gently donated by Dow Chemical™ corporation and their characteristics are shown in Table 1. Raffinose D(+) penta-hydrated was used as substrate with an analytical grade of 98% and it was obtained from Vetec™. Glutaraldehyde was procured from Quimibrás™ in solution of 25% and it was used as a cross-linking agent.

Table 1: Duolite A-568 Characteristics (Dow Chemical™)

Matrix	Phenol-formaldehyde
Functional group	Tertiary amino groups
Physical aspects	Grey-green granules
Water retention capacity	62-67 %
Specific mass	1.10-1.14 g/L
Particle size	0.15-0.85 mm
Pore diameter	30-50 nm
Pore volume	0.78-1.00 mL/g
Total ion exchange capacity	1.20 equivalent/L

3. Methods

3.1 Enzyme Assay

Alpha-galactosidase activity was determined by raffinose hydrolysis. The reactions were performed in a batch-reactor containing 50 mL of raffinose solution at 3 g/L in acetate buffer pH 5.0. After reaching the reaction temperature, the enzyme (free or immobilized) was dropped in the reactor and samples of 2 mL were withdrawn at the times 3, 6, 9, 12, 15, 20 and 25 min after reaction has started. Hydrolysis occurred at 40 °C, since the enzyme in both forms showed good activities and good thermal stability at this temperature (data not shown).
For immobilized enzyme, 1 g of Duolite A-568 containing immobilized α-galactosidase was dropped into a stainless steel basket with mesh 100. Then, the basket was put into a batch-reactor. For free enzyme, 2.5 mL of α-galactosidase solution at 10 g/L in acetate buffer pH 5.0 was dropped in the reactor, achieving the concentration of 0.48 g/L of free enzyme in the batch-reactor.
The reaction medium was kept under magnetic stirring and the temperature was controlled by a thermostatic bath. A thermometer was used to check the temperature in the reactor.
Samples were dropped in test tubes and they were kept in a boiling bath for 10 min to inactivate the enzyme.
One unit of enzyme activity was defined as the amount of reducing sugar produced per liter, per minute and per gram of protein, according to the Equation 1. Since α-galactosidase hydrolyses α-1,6 linked galactosyl groups, raffinose hydrolysis by α-galactosidase releases galactose and sucrose as minor sugars. So, galactose is the only reducing sugar in the solution.

$$U = \frac{g_{\text{reducing sugar}}}{L.\min.g_{\text{protein}}} \quad (1)$$

The amount of reducing sugar was measured by its reaction with 3,5 dinitrosalisylic acid, according to the methodology described by Miller (1959).

3.2 Protein determination

Protein content was measured by the method of Lowry (1951). In the free enzyme solution the quantification was performed directly from the α-galactosidase solution at 10 g/L. For immobilized enzyme, the amount of adsorbed protein was determined by the difference between the protein concentration in the solution of α-galactosidase before and after the immobilization procedure has been established.

3.3 Glutaraldehyde effect on the immobilized α-galactosidase activity

A preliminary study was carried out to verify whether the cross linking agent glutaraldehyde could affect the catalytic performance of the enzyme. Four different treatments were done:

1- *Immobilization without glutaraldehyde addition*: 20 mL of enzyme solution at 10 g/L in acetate buffer pH 5.0 was poured into a beaker containing 1 g of Duolite A-568. The content was stirred for 3 h at 120 rpm and 40 °C.
2- *Glutaraldehyde added with enzyme at the same time*: 20 mL of enzyme solution at 10 g/L and glutaraldehyde 1 % (v/v) in acetate buffer pH 5.0 were poured into a beaker containing 1 g of Duolite A-568. The content was stirred for 3 h at 120 rpm and 40 °C.
3- *Glutaraldehyde added before the enzyme immobilization:* Glutaraldehyde 1 % (v/v) in acetate buffer pH 5.0 was poured into a beaker containing 1 g of Duolite A -568. The content was stirred for 3 h at 120 rpm and 40 °C. Afterwards, the resin was filtered and washed with the buffer to remove the excess of glutaraldehyde. Then, the immobilization process described in the first treatment was carried out.
4- *Glutaraldehyde added after the enzyme immobilization:* The immobilization process described in the first treatment was carried out. Then, the resin was filtered and washed with the buffer to remove the excess of enzyme. Afterwards, 20 mL of glutaraldehyde 1 % (v/v) was poured into the beaker containing the resin and it was stirred for 3 h at 120 rpm and 40 °C.

At the end of each treatment, the enzyme activity was measured according to the steps described in the item 3.1.

3.4 Glutaraldehyde concentration and time of reaction in the enzyme activity

A Central Composite Design (CCD) was carried out aiming the optimization of glutaraldehyde concentration and the time of reaction. Therefore, the CCD was performed with 2 variables at 2 levels (2^2) with 3 repetitions at the central point and 4 axial points, resulting 11 experiments. The higher levels (+1) for glutaraldehyde concentration and time of reaction were 5.8 % (v/v) and 5.0 h. The lower levels (-1) were 0.4 % (v/v) and 1.0 h for glutaraldehyde concentration and time of reaction, respectively. The orthogonal alpha used in the CCD was 1.14744.

Glutaraldehyde reaction was carried out pouring 20 mL of glutaraldehyde solution in acetate buffer pH 5.0 in a beaker containing 1 g of Duolite A-568. The content was stirred at 120 rpm and 25 °C for the time specified in the CCD. Afterwards, the resin was filtered and washed with the buffer to remove the excess of glutaraldehyde. The temperature was different from the preliminary tests (item 3.3) since it did not show any effect in the glutaraldehyde activation for the range studied between 25 and 40 °C (data not shown). Then, the immobilization process was performed pouring 20 mL of enzyme solution at 10 g/L in acetate buffer pH 5.0 into a beaker containing 1 g of Duolite A-568. The content was stirred for 3 h at 120 rpm and 40 °C.

Table 2 summarizes the CCD with the real values for glutaraldehyde concentration and reaction times with their related codified values. Since the support activation was concluded, the resin was filtered and washed with acetate buffer pH 5.0 and then the immobilization process was carried out using 20 mL of α-galactosidase solution at 10 g/L (pH 5.0), stirred for 3 h at 120 rpm and 40 °C. Afterwards, Duolite A-568 containing the immobilized enzyme was filtered and washed with the buffer and then α-galactosidase activity was determined according to the item 3.1.

Table 2: Central Composite Design (2^2) with real and codified values for glutaraldehyde concentration (X_1) and reaction time (X_2)

Experiment	Real Value (Codified Value)	
	X_1 Glutaraldehyde % (v/v)	X_2 Reaction time (h)
1	5.8 (+1)	1 (-1)
2	0.4 (-1)	1 (-1)
3	5.8 (+1)	5 (+1)
4	0.4 (-1)	5 (+1)
5	3.1 (0)	0.7 (-α)
6	3.1 (0)	5.3 (+α)
7	0 (-α)	3 (0)
8	6.2 (+α)	3 (0)
9	3.1(0)	3 (0)
10	3.1 (0)	3 (0)
11	3.1 (0)	3 (0)

Data obtained by CCD were statistically analysed using the software STATISTICA™ 7.0. A *t-Student* test was applied to the equation given by the model to determine the significant level of each parameter. Only the ones with *p-value* lower than 10 % were accepted as statistically significant. By this analysis it was possible to obtain the determination coefficient (R^2).

3.5 Glutaraldehyde Effect on the operational stability of immobilized α-galactosidase

Twenty milliliters (20 mL) of glutaraldehyde 1% (v/v) was poured into a beaker containing 1 g of Duolite A-568 and it was stirred for 1 h at 120 rpm and 25 °C. After filtering and washing, enzyme immobilization was carried out using α-galactosidase solution at 10 g/L, 120 rpm and 40 °C.

Afterwards, the resin was added to the basket inside the batch-reactor containing 50 mL of raffinose substrate (3 g/L) under stirring. The resin was withdrawn after 0, 2, 4, 6, 8, 10, 12, 28, 48 and 72 h inside the batch-reactor under operational conditions. For each time specified, a new solution of raffinose 3 g/L was poured into the batch-reactor and the enzyme activity was determined according to the item 3.1. A control test with the immobilized enzyme without glutaraldehyde addition was performed simultaneously. The enzyme activity measured at the time 0 h was determined as U_0 and the activities measured for other specific times (2, 4, 6, 8, 10, 12, 28, 48 and 72 h) were determined as U. The residual activity was determined according to the Equation 2.

$$\text{Residual Activity} = \frac{U}{U_0} \tag{2}$$

4. Results and discussions

4.1 Glutaraldehyde effect on the immobilized α-galactosidase activity

Results of the effect of glutaraldehyde in the immobilized enzyme activity are shown in Table 3. Best activities were observed for the simple immobilization process (without glutaraldehyde addition - 1) and for the one with Duolite pre-activated with glutaraldehyde followed by the enzyme immobilization (glutaraldehyde added before the enzyme immobilization - 3), according to the methods explained in the item 3.3.

When enzyme immobilization was carried out before or at the same time of glutaraldehyde activation, the biocatalyst activity decreased about between 50-64 %. It can be explained by the cross linkings among the amino acids in the protein molecule, which may change the conformational configuration of the polypeptide chain, affecting the active site of the enzyme when the glutaraldehyde reaction occurs during or after immobilization of the biocatalyst on the support.

According to López-Gallego et al. (2005), the pre-activation of the support with glutaraldehyde before the protein immobilization reduces chemical changes in the enzyme only towards the lysine groups involved in the bind. Therefore, less structural changes in the protein may have occurred in the third treatment when compared to the other two treatments with glutaraldehyde. It explains the better activity for the enzyme immobilized after the activation of the support with glutaraldehyde instead of the enzymes immobilized before or at the same time of the reaction with glutaraldehyde.

Table 3: Effect of Glutaraldehyde in the Enzyme Activity

Method	Activity (U)
1. Immobilization without glutaraldehyde addition	0.038 ± 0.003
2. Glutaraldehyde added with enzyme at the same time	0.019 ± 0.001
3. Glutaraldehyde added before the enzyme immobilization	0.035 ± 0.004
4. Glutaraldehyde added after the enzyme immobilization	0.016 ± 0.003

4.2 Glutaraldehyde concentration and time of reaction in the enzyme activity

Table 4 shows the results of the Central Composite Design for the optimization of the glutaraldehyde concentration and the time of reaction.

Analysis of multiple regression performed using STATISTICA 7.0 gave the Equation 3, which correlate the activity observed as function of the variables glutaraldehyde concentration (X_1) and time of reaction (X_2). All the parameters were significant at the level of 10 % of probability by the *t-Student* test.

$$U = 0.0673 + 0.0037X_1 - 0.0037X_1^2 - 0.0096X_2 + 0.0068X_2^2 + 0.0040X_1X_2 \tag{3}$$

Table 4: Results of CCD (2^2) performed for the optimization of glutaraldehyde concentrations and reaction time

Experiment	Real Value (Codified Value)		
	X_1 Glutaraldehyde 1 % (v/v)	X_2 Reaction time (h)	Activity (U)
1	5.8 (+1)	1 (-1)	0.053 ± 0.004
2	0.4 (-1)	1 (-1)	0.082 ± 0.005
3	5.8 (+1)	5 (+1)	0.067 ± 0.002
4	0.4 (-1)	5 (+1)	0.080 ± 0.008
5	3.1 (0)	0.7 (-α)	0.057 ± 0.002
6	3.1 (0)	5.3 (+α)	0.068 ± 0.006
7	0 (-α)	3 (0)	0.086 ± 0.004
8	6.2 (+α)	3 (0)	0.067 ± 0.001
9	3.1(0)	3 (0)	0.068 ± 0.002
10	3.1 (0)	3 (0)	0.065 ± 0.002
11	3.1 (0)	3 (0)	0.069 ± 0.003

The correlation coefficient (R^2) obtained in the statistical analysis was 0.98, which correspond to a good data adjustment. A response surface was created by the Equation 3, and it is shown in Figure 1.
Best activities were obtained for all the time range and for low glutaraldehyde concentrations (lower than 1.5 % (v/v)).

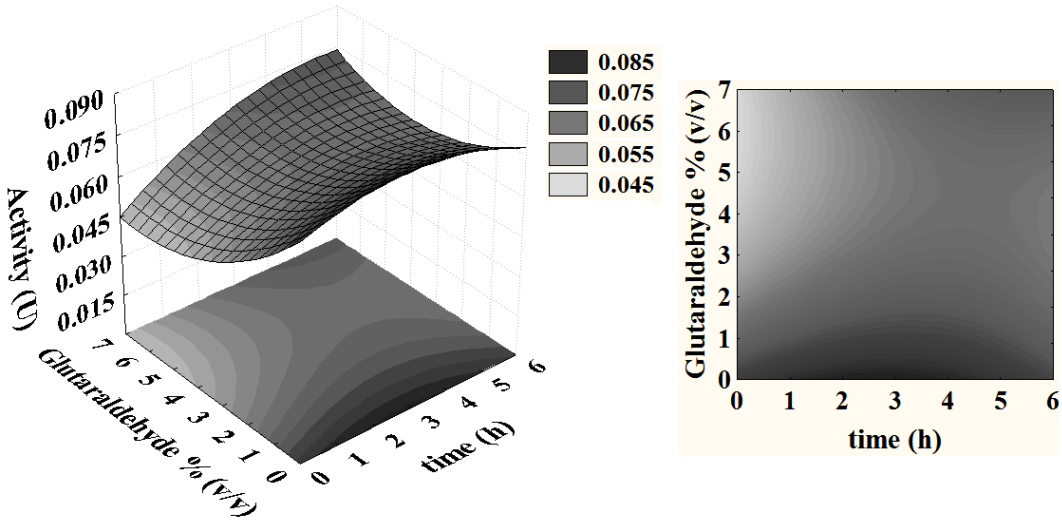

Figure 1: Central Composite Design Response Surface representing immobilized α-galactosidase activity as function of glutaraldehyde concentration and time of reaction in Duolite A-568 pre activation

4.3 Glutaraldehyde Effect on the operational stability of immobilized α-galactosidase

According to the graph shown by Figure 1, support activation was performed using a glutaraldehyde solution 1% (v/v), which is inside the area corresponding to best activities in the graph. Then, the operational stability of the immobilized enzyme was studied to check whether the glutaraldehyde activation is advantageous or not (Figure 2).

The residual activity (U/U_0) of the enzyme without glutaraldehyde addition reduced strongly in the first 2 h in the reactor, losing 16 % of its initial activity, while the enzyme immobilized in the support pre-activated with glutaraldehyde 1 % (v/v) lost only 3 % of its initial activity in this same time in the reactor. After 28 h in the stirred reactor, the residual activity stabilized for both treatments. Enzyme immobilized in pre-activated support showed activities between 82 and 86 % while the other treatment showed activities between 70 and 74 %. Hence, the glutaraldehyde activation enhanced the residual activity of the immobilized enzyme in about 12 % after many cycles of reaction. So, the activation of Duolite A-568 with glutaraldehyde 1 % (v/v) is advantageous since it does not provide significant losses in enzyme activity and it improves the operational stability in about 12 %.

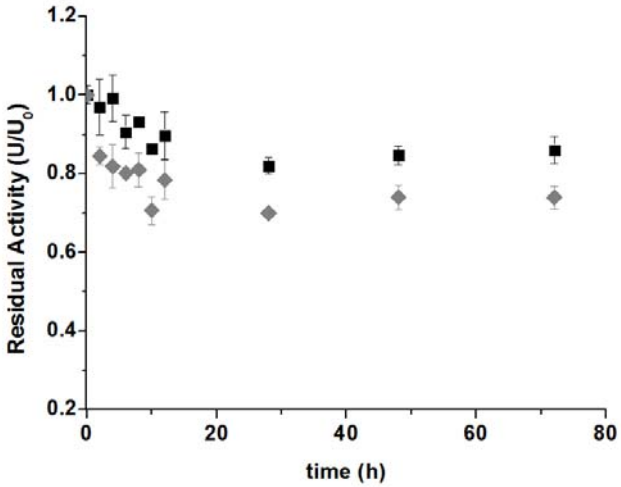

Figure 2: Residual activity (U/U_0) of immobilized α-galactosidase without Duolite A-568 activation with glutaraldehyde (♦) and immobilized α-galactosidase with pre-activated Duolite A-568 with glutaraldehyde 1 % (v/v) (■).

5. Conclusions

Activation of Duolite A-568 with glutaraldehyde 1 % (v/v) is advantageous for immobilization of α-galactosidase since it did not provided significant losses in enzyme activity while it improved the operational stability of the immobilized enzyme in about 12 % even after 72 h in the reactor.

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