Exploring the Efficacy of Trehalose and Polyethylene Glycol in Stabilizing Soluble and Immobilized Alcalase

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ABSTRACT

Enzyme stabilizing agents improve enzymatic activity and stability in solutions containing soluble and immobilized enzymes. These agents are crucial in maintaining the enzyme's active site for catalyzing chemical reactions. This work investigates the effectiveness of two enzyme stabilizing agents, sugar trehalose and polyethylene glycol 6.000, in stabilizing the enzyme alcalase in both its soluble and immobilized forms. First, the effect of the stabilizing agents on soluble alcalase activity was investigated. Then, immobilization of alcalase on Carbopol 996 was conducted in their presence, and the activity of the immobilized enzyme was measured. Casein was used as the substrate to determine enzymatic activity. According to the results, trehalose presented the most prominent effect on stabilizing the soluble since higher activity was observed when compared to polyethylene glycol. However, both stabilizing agents during alcalase immobilization presented no surfactant effect on activity compared to the control (immobilization in the absence of agents).

Keywords: Alcalase 1. Carbopol 996 2. Immobilization 3. Stabilization 4.

1 INTRODUCTION

Alcalase is a proteolytic enzyme produced by *Bacillus leicheniformis* that presents a dual mechanism in biofilm degradation. The first mechanism occurs due to its ability to degrade adhesins on the surface of bacteria, which are structures that enable their adherence to living or non-living surfaces. The second mechanism occurs through the action of the enzyme upon reaching the extracellular polymeric substances present in the composition of biofilms and breaking them down[1].

Alcalase, in addition to its biofilm degradation mechanisms, can break peptide bonds in proteins. Its active site, rich in essential amino acids like lysine, histidine, and arginine, can interact with negatively charged groups in peptide bonds, demonstrating enhanced functionality in alkaline pH. This enzyme's cationic functional groups on its surface can also form stabilizing bonds. These unique properties make alcalase a promising candidate for enzyme immobilization and a valuable biocatalyst to the industry[2]. The surface of alcalase contains essential amino acids such as arginine, lysine, and histidine. These amino acids confer alcalase a cationic character because their side chains can form bonds with protons in an aqueous medium. Additionally, these side groups can also form other bonds that may or may not favor the stability of its structure[2]. Therefore, some strategies to promote stabilization should be investigated.

In this context, Enzyme immobilization, a key strategy in enhancing enzyme activity and stability for commercial use, is gaining prominence[2]. Polyacrylic acid, also known as carbopol, is a biodegradable, biocompatible, non-toxic polymer. It can be used to manufacture pharmaceutical products and support enzyme immobilization. This 'smart' polymer can adjust its viscosity by modifying its pH value, providing stability in enzymatic activity when used as a support[3].

Stabilizing agents, such as polyethylene glycol (PEG)[4] and trehalose[5], enhance enzyme stability. They act as a shield, protecting the enzyme from pH variations, temperature fluctuations, and changes in ionic strength. Under different reaction conditions, these agents also maintain or even enhance enzymatic activity over time. Therefore, this study aims to investigate the effectiveness of stabilizing substances during immobilization of alcalase on carbopol 996.

2 MATERIAL & METHODS

2.1 Materials

In this study, the following materials were used: Carbopol 996 from Ely Martins company; Alcalase (Protease from Bacillus licheniformis, concentration ≥ 2.4 U/g) and casein acquired from Sigma-Aldrich (St. Louis, USA); sodium hydroxide in micro pearls P.A.; trichloroacetic acid P.A from Neon Comercial (Suzano, Brazil). All other chemicals used were of analytical grade and from various brands.

2.2 Methods

2.2.1 Enzymatic Activity

The enzymatic activity of alcalase was determined using a method adapted from the literature[6,1]. Casein solutions 0.5% (w/v) containing TRIS-HCl buffer pH 8.0, 50 mM were incubated at 37°C for 10 minutes. Afterward, the enzyme sample was added, and the mixture was incubated for 20 minutes at 37°C. Next, the reactions were stopped with 5% (w/v) trichloroacetic acid and centrifuged. The supernatant was mixed with 2 M sodium hydroxide, and absorbance was measured at 280 nm in a spectrophotometer.

One unit of enzymatic activity was defined as the amount of enzyme that causes an increase in optical density at 280 nm, corresponding to one micromole of tyrosine per minute under the conditions described in this assay. The activity can be calculated using Equation 1[7].

$$Activity\ (\frac{U}{mL}) = \frac{Abs * Vt * df}{1.34 * 1.0 * t * Vs} \quad (1)$$

Abs = Absorbance; Vt = Total volume (mL); Vs = Sample volume; 1.34 = Millimolar extinction coefficient of tyrosine (F/micromole); 1.0 = Pathlength (cm); t = Reaction time (min); df = Dilution factor.

2.2.2 Effect of stabilizing agents in the activity of the soluble alcalase

The test was conducted in two stages: in the first, 50 mg of Polyethylene glycol Mn = 6,000 were weighed and dispersed in ultra-pure water, together with 150µL of the enzyme alcalase®, adjusting the meniscus to 25 mL. The pH was measured and corrected to 5.5 using a 0.25 M sodium hydroxide solution. After sitting at room temperature without stirring for 30 minutes, the next step was to add ultrapure water to bring the final volume up to 50 mL. After that, a new measurement was taken and the pH was adjusted to 5.5. Then, the solution was transferred to the shaker and stirred at 110 rpm and 37°C, for 30 minutes. The same procedure was carried out using 2.5 g of trehalose sugar. A control experiment was also conducted in the same way but without the presence of stabilizing substances.

2.2.3 Alcalse immobilization on Carbopol 966 in the absence and presence of stabilizing agents.

Alcalase was immobilized into carbopol 966 in the presence and absence of stabilizing agents. For that, the enzyme solution was prepared as described in item 2.2.2. Next, 8.33 g of carbopol base 0.6% w/v was weighed, added to 15 mL of ultrapure water, and stirred at 170 RPM at room temperature until homogenized. The enzyme solution was poured into the carbopol gel, completing the final volume with 50 mL of ultrapure water. During this step, the solution was stirred at 110 RPM at room temperature, and the pH was checked and corrected to 5.5, using 0.25 M sodium hydroxide. The final step to complete the immobilization consisted of transferring the carbopol gel with the enzyme and these stabilizing substances to be shaken in a shaker for 30 minutes at 110 rpm and 37°C temperature[8,9].

3 RESULTS & DISCUSSION

In Figure 1, the results of the activity of soluble and immobilized alcalase against casein as the substrate are shown. It's important to note that using stabilizing agents in a solution containing soluble alcalase effectively increased enzyme activity. This increase was particularly noticeable when compared to the soluble enzyme without stabilizing agents, which had an enzymatic activity of 119 U/mL. Trehalose, one of the stabilizing agents, was significantly more effective in stabilizing enzymatic activity, resulting in 319 U/mL, compared to PEG, which resulted in 251 U/mL. Additionally, the enzyme immobilized in Carbopol 996 remained active and showed an activity of 309 U/mL, a significant increase compared to the non-immobilized soluble enzyme, which had an activity of 119 U/mL. This demonstrates the positive impact of immobilization on enzyme activity.

The ability of trehalose in stabilize enzymes has been reported as it replaces hydrogen bonds of water and stabilizes enzymes. The hydroxyl groups in trehalose can form hydrogen bonds with the enzyme surface groups[5]. On the other hand, polyethylene glycol acts differently, promoting fine-tuning enzyme stabilization through electrostatic bonds. This interaction helps prevent changes in the enzyme structure and aggregation, making polyethylene glycol an effective macromolecular stabilizing agent[4].

Although a stabilizing effect was observed for the soluble enzyme, it was found that stabilizing agents did not have a significant protective effect when the enzyme was immobilized, either in the presence or absence of trehalose and PEG. The protective effect of immobilizing alcalase on carbopol was sufficient to maintain enzyme activity, considering the experimental errors.

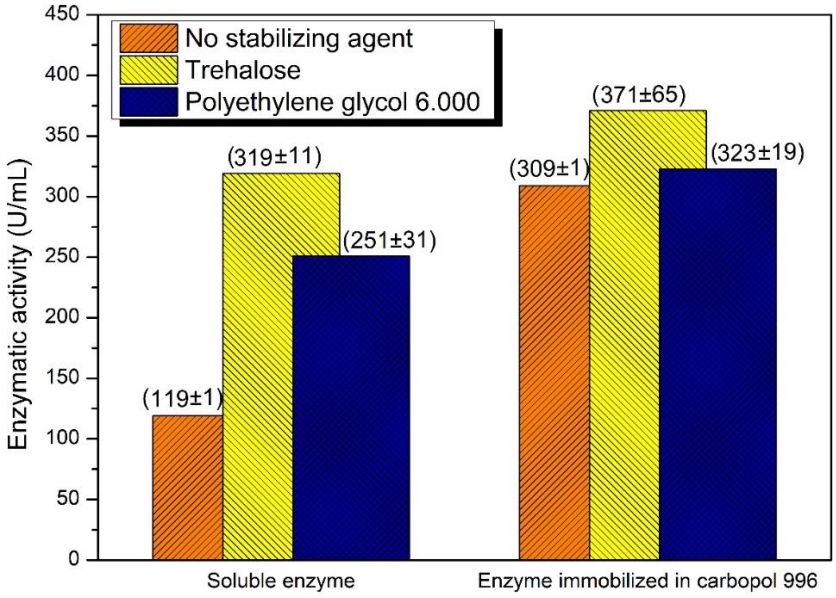

Figure 1 Effect of trehalose and polyethylene glycol as stabilizing agents on the activity of soluble and immobilized alcalase in Carbopol 996 at 37°C and pH 5.5, using 0.5% (w/v) casein as substrate.

4 CONCLUSION

In conclusion, trehalose performed better than PEG as a stabilizing agent for soluble alcalase, demonstrating its efficacy. However, applying these agents in the immobilization process on carbopol 966 did not yield significant protective effects or enhance enzymatic activity compared to the heterogeneous biocatalyst produced without their use. It is important to note that these results do not discount the potential of other stabilizing agents or alternative immobilization methods, and further research is warranted in this area.

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