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EFFICIENT IMMOBILIZATION OF ALCALASE USING I-CARRAGEENAN HYDROGEL BEADS AS A SUPPORT

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Abstract

Enzymes are efficient industrially applicable biocatalysts; however, their lower stability and reuse potential in their soluble form have hindered their industrial potential. The immobilization of enzymes may enhance their industrial potential, improving their scalability. The application of biopolymers as support for enzyme immobilization has been investigated as an environmentally friendly alternative to traditional commercial supports. In this context, this study investigates the immobilization of Alcalase, a protease from *Bacillus licheniformis*, onto carrageenan hydrogel beads (CHB) extracted from the red algae *Solieria filiformis* cultivated in Northeast Brazil. The hydrogel beads were produced using $AlCl_3$ as a gelling agent. The optimal pH for the immobilization (7.0, 8.0, and 9.0) was investigated, and the produced biocatalyst was characterized by Fourier-transform infrared spectroscopy (FT-IR). The results showed that the optimal pH for immobilization was 8.0, yielding a biocatalyst with high enzymatic activity (10.4 U/g) and recovered activity (89.7%). FT-IR analysis revealed the ι-carrageenan as the main monomer present in the hydrogel; also, the presence of Alcalase hydrogel matrix was confirmed, additionally, the low alterations in bands present in the support may indicate immobilization immobilization through adsorption. Finally, SEM analysis has indicated CHB as a microporous material (50-200 µm); enzyme clusters were observed on the derivate surface after the immobilization essays. These results highlight the potential of carrageenan as a cost-effective and sustainable support for enzyme immobilization in various industrial processes.

Keywords: Adsorption; Alacase; Enzyme immobilization; Hydrogel beads; ι-Carrageenan.

1. Introduction

Enzymes are used industrially as environmentally friendly and efficient biocatalysts in comparison to traditional chemical catalysts due to their selectivity, effectiveness, and environmental sustainability. Among the primary enzymes used industrially are lipases, amylases, and proteases, categorized based on the catalyzed reaction they perform, which are applicable in the food, pharmaceutical, cosmetic, and cleaning product sectors. However, their high cost and complex recovering methods from the reaction medium, in its soluble form, have proven to be bottlenecks for their large-scale use.

An alternative approach involves immobilizing enzymes by integrating them into insoluble solid materials, resulting in heterogeneous catalysts. This process transforms soluble enzymes into insoluble forms, facilitating their recovery from the reaction medium [1]. The immobilization mechanism may differ depending on the enzyme and chosen support, including functionalization to form covalent bonds, encapsulation, and adsorption. Among these, adsorption stands out due to its high efficiency without the use of reagents to achieve functionalization, resulting from electrostatic interaction between the support and the enzyme. This process is also reversible, allowing enzyme desorption and reuse of the support. [2].

Various materials may be applied for enzyme immobilization, including biopolymers such as agarose, chitosan, and carrageenan. Carrageenan, in particular, has presented high potential for producing effective biocatalysts throughout a wide range of pH and temperatures [3]. In this context, this study aims to investigate the optimal conditions for immobilizing the enzyme Alcalase, protease from *Bacillus licheniformis*, onto carrageenan hydrogel derived from the macroalga *Solieria filiformis*.

2. Material and methods

2.1 Materials

Alcalase from *Bacillus licheniformis* (2.4 U/g) (EC 3.4.21.62), azocasein, and Tris(hydroxymethyl)aminomethane were obtained from Sigma-Aldrich (St. Louis, MO, USA). $AlCl_3$ and HCl were purchased from Dinâmica Química Contemporânea LTDA® (Indaiatuba, SP, Brazil). Trichloroacetic acid was obtained from Neon Comercial® (Suzano, SP, Brazil). All reagents used were of analytical grade.

The macroalga *Solieria filiformis*, cultivated on the beaches of Trairi - CE, Brazil, was kindly provided by the association of algae producers from Guajiru and Flecheiras. The algae were washed to remove impurities and stored in an ultra-freezer (-80 ºC).

2.2 Production of Carrageenan Hydrogel

The dried *S. filiformis* macroalga was ground and suspended (1% w/v) in a KOH solution (0.1 mol/L) under agitation for 24 hours. After filtration, carrageenan was extracted using deionized water (80 ºC) for 3 hours and precipitated with ethanol (1:4 v/v) for 24 hours at 4 ºC, followed by lyophilization. Subsequently, hydrogel beads were produced by dripping a 1% (w/v) carrageenan solution into a 5% (w/v) AlCl3 solution, which was applied as the gelling agent. The beads were immersed in the gelling solution for 2 hours and then washed with distilled water.

2.3 Alcalase Immobilization Assays

For immobilization, 1 g of carrageenan hydrogel beads (CHB) was added to 50 mL tubes containing 40 mL of 200 mM TRIS-HCl buffer (pH 7.0, 8.0, and 9.0). An enzyme load of 1 mg of enzyme per gram of support was used. The system was kept under agitation at 25 ºC for 120 minutes [4].

2.4 Analytical Methods

The enzyme immobilization yield was evaluated by total protein content analysis, using Bradford methodology (1976). The enzymatic activity of free and immobilized enzymes was determined by the hydrolysis of azocasein (0.5% w/v), using trichloroacetic acid as the precipitating agent (1:1 v/v). The reaction occurred at 32 ºC for 30 minutes. After removing the non-hydrolyzed residue, a 1M NaOH solution was added to the supernatant and measured in a spectrophotometer at 440 nm. One proteolytic unit (U) was defined as the amount of enzyme that increases by 0.01 in absorbance.[5].

The evaluation of the obtained carrageenan monomer and the presence of the enzyme in the carrageenan hydrogel was conducted using Fourier Transform Infrared Spectroscopy (FT-IR). The analysis was performed on a Cary 630 spectrophotometer (Agilent Technologies) in the range of 650-4000 cm-1, with a resolution of 1 cm^{-1} and 32 scans.

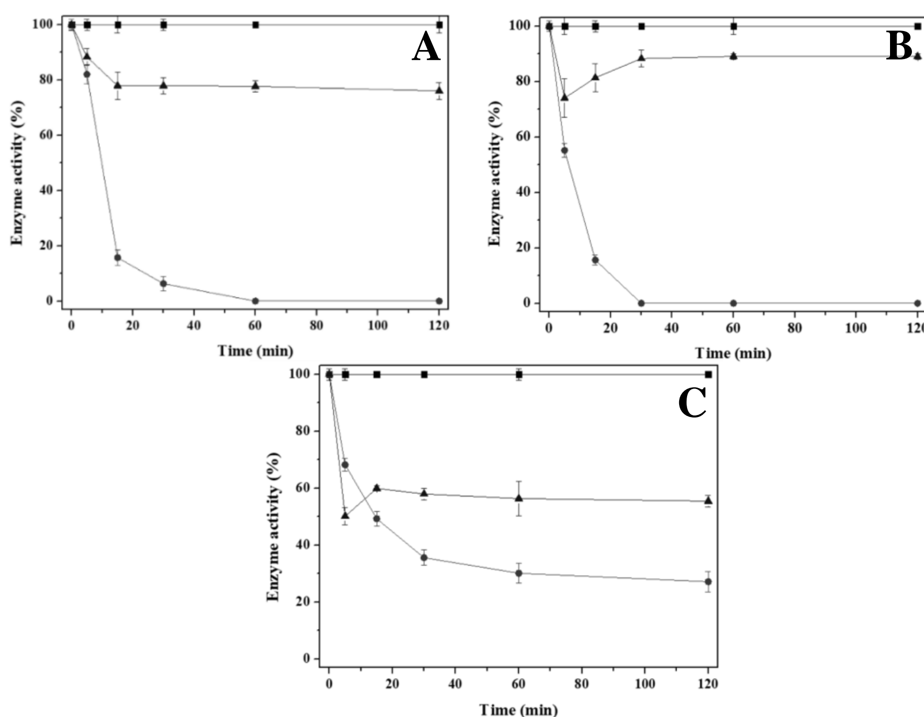
For morphological analysis scanning electron microscopy (SEM) was performed (FEG Quanta 450). The samples were prepared using carbon tape and covered in silver using Quorum QT15ES Metallizer. The SEM chamber was at 10 Pa with an incident electron beam of 20 kV.

3. Results and discussion

Alcalase enzyme has a broad range of industrial applications. Thus, studies to optimize its use, such as producing supports that enhance its stability, and allow its reuse, are important to enhance its industrial potential. Additionally, alternative supports have gained prominence recently due to their sustainability and low cost, as commercial supports tend to be more expensive. Therefore, carrageenan extraction yielded 31 ± 4% (w/w) of lyophilized carrageenan, which was used to produce the beads.

The pH of the immobilization medium was evaluated to optimize the immobilization conditions (Fig. 1). It was observed that the enzyme remained stable in its soluble form, probably not performing autohydrolysis at any of the studied pH levels. In Figures 1A and 1B, the complete immobilization of the enzyme can be seen up to 60 minutes of the assay. This likely occurs due to the presence of arginine and lysine residues in the Alcalase enzyme. [5], which are positively charged at pH levels below 10, likely promoting their adsorption onto carrageenan supports negatively charged by ions SO_3^-.

Fig. 1. Immobilization profile of Alcalase enzyme in TRIS-HCl Buffer (200 mM), at 250 ºC for 120 min, at pH (A) 7.0, (B) 8.0, and (C) 9.0: (■) Free enzyme, (●) supernatant, and (▲) suspension activity.



However, Fig. 1A (pH 7.0) presents a lower suspension activity than that observed in Fig. 1B (pH 8.0). This occurs due to the presence of histidine in the Alcalase enzyme, which has an isoelectric point at pH 7.6 and is thus positively charged at pH 7.0, promoting more significant adsorption. On the other hand, since histidine is part of the catalytic triad present in the active site of Alcalase, probably interfering with the enzyme's activity, as the final activity of the produced biocatalyst at pH 7.0 (5.7 ± 0.3 U/g), lower than that produced at pH 8.0 (10.4 ± 0.5 U/g) (Table 1).

Table 1. Influence of immobilization pH on the final activity of the biocatalyst produced with CHB.

pH	Immobilization yield (%)	Theoretical activity (U/g)	Derivate activity (U/g)	Recovered activity (%)	Activity yield ($U/mg_{adsorbed\ enzyme}$)
7.0	100%^{a}	7.6 ± 0.1^{a}	5.7 ± 0.3^{a}	75.5 ± 4.7%^{a}	5.7 ± 0.3^{a}
8.0	100%^{a}	11.7 ± 0.4^{b}	10.4 ± 0.5^{b}	89.7± 5.6%^{b}	10.4 ± 0.4^{b}
9.0	48.2 ± 4.1%^{b}	12.1 ± 0.4^{b}	7.0 ± 0.9^{c}	58.1± 8.1%^{c}	14.6 ± 2.0^{c}

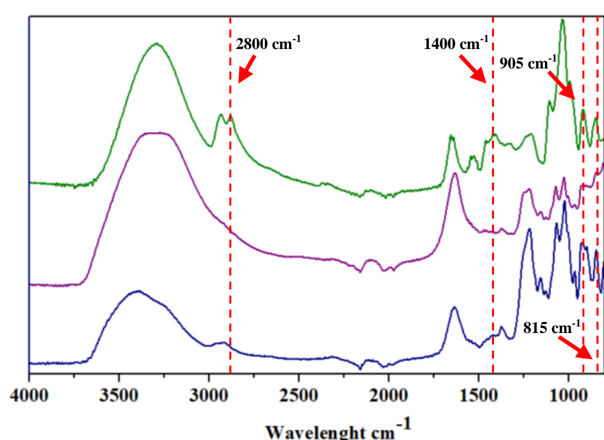
Superscript letters (a, b, and c) within the same row indicate significant differences ($p < 0.05$) according to Tukey's test.

At pH 9.0 (Fig. 1C), there is a reduction in suspension activity and a lower immobilization yield (48.2 ± 4.1%), indicating a lower efficiency of the support at this pH in comparison to pH 7.0 and 8.0. Suggesting that the pH interferes with the carrageenan support, affecting intermolecular forces, increasing water absorption, and reducing adsorption efficiency. This is corroborated by the increase in the average diameter of the beads at the end of the assay at pH 9.0 (173.85%), in comparison to 7.0 (45.5%) and 8.0 (83.2%). Thus, pH 8.0 was selected as the optimal pH for Alcalase immobilization due to the higher activity of the

derivative (10.4 U/g) and recovered activity (89.7%) compared to pH 7.0 and 9.0.

Then, the organic groups present in the support before and after immobilization were analyzed by FT-IR to evaluate the interactions between carrageenan and Alcalase (Fig. 2).

Fig. 2. FT-IR spectrum of carrageenan (■), CHB (■), and the produced biocatalyst (■).

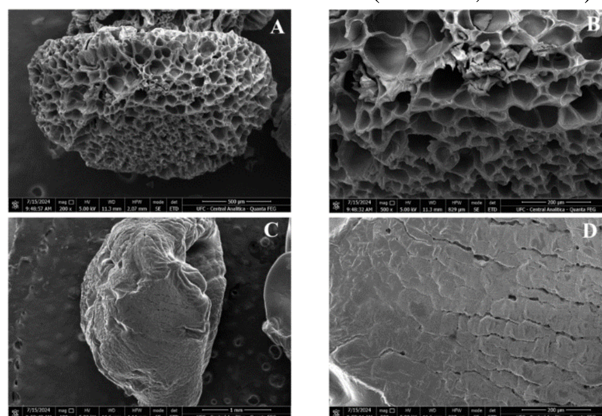


Bands at 905 and 815 cm^{-1}, are representative of carrageenan isomers containing 2 SO_3^- groups, indicating the presence of ι-carrageenan. Additionally, bands at 1400 and 2800 cm^{-1} present in the produced biocatalyst represent the presence of the Alcalase enzyme in the hydrogel, indicating enzyme immobilization. This result presents a biocatalyst produced without significant alterations in the structure of ι-carrageenan, suggesting immobilization by adsorption.

Also, SEM (Fig. 3) presented CHB as a microporous material with an initial porosity of 50-200 µm (Fig. 3A and 3B). After enzyme immobilization, it is possible to observe the decreased porosity caused by the formation of enzyme clusters after the immobilization essays (Fig. 3C and 3D).

Thus, this study demonstrated that using ι-carrageenan for Alcalase immobilization at pH 8.0 results in a highly active biocatalyst (10.4 U/g). Also, the immobilization resulted in high recovered enzymatic activity (89.7%), indicating its potential for industrial applications. Thus, it enhances the application of sustainable and cost-effective alternatives to produce biocatalysts for industrial applications.

Fig. 3. SEM images of CHB (A: 200x, B: 500x) and Alcalase immobilized in CHB (C: 100x, D: 200x).



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