EVALUATING *Solieria filiformis* IOTA-CARRAGEENAN HYDROGEL FOR EFFICIENT ALCALASE IMMOBILIZATION

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ABSTRACT

Proteases are highly sought-after enzymes with a wide range of industrial applications. They are used in various industries, including the production of bioactive compounds, cleaning products, and particularly in the food industry. These enzymes, which break down peptide bonds, can help reduce allergenicity in dairy products by breaking down casein. However, their poor stability and high cost often limit the industrial application of enzymes. Immobilization offers a solution by enhancing enzyme stability and facilitating reuse. This study investigates the immobilization of alcalase from *Bacillus licheniformis* on iota-carrageenan hydrogel beads (CHB). Iota-carrageenan was extracted from *Solieria filiformis* seaweed, formed into hydrogel beads, and chemically characterized. The enzyme was then adsorbed onto these beads, and the total protein content and enzymatic activity were evaluated. Results demonstrated that carrageenan beads, effectively immobilize alcalase, reducing the supernatant enzymatic activity and producing a biocatalyst with 8.4 ± 0.4 U/g proteolytic activity, which indicates that the enzyme remains active after the immobilization. Thus, this work advances using natural polymers like carrageenan for enzyme immobilization, promoting green chemistry in the food industry.

Keywords: Alcalase, Carrageenan, Enzyme immobilization, Hydrogel beads.

1 INTRODUCTION

The dairy sector is crucial to the food industry and represents high production rates in many countries. For example, Brazil is the 5th largest milk producer globally, generating 24.5 million tons of milk in 2023. Proteases are hydrolase enzymes that can act on the hydrolysis of peptide bonds and are used in various industrial sectors, especially in the dairy sector of the food industry. Thus, being used to enhance the flavor, texture, and solubility of certain foods, in the synthesis of bioactive compounds, such as antioxidants, and, especially, in the production of cheese. Also, the application of protease has led to the development of hypoallergenic dairy products, due to its proteolytical potential over casein, a protein commonly found in dairy products, which may cause allergenicity[1]. However, industrially, despite its high potential, the poor stability and high cost of enzymes hinder their application. Thus, immobilization is presented as an alternative to fully exploit their economic potential, as it facilitates separation processes, allows its reutilization, and improves its overall stability[2]. A commonly applied immobilization method is adsorption, which consists of electrostatic linkages formed between the enzyme and the support surface, resulting in an inexpensive and reversible method. However, many synthetic supports may be toxic, non-biodegradable, and non-biocompatible. Therefore, using natural polymers may be advantageous, especially for the food and pharmaceutical industries. In this context, carrageenan, a low-cost polymer extracted from red seaweed suitable for producing hydrogel beads for the adsorption of enzymes with affinity by the substrate, such as proteases, also provides environmentally friendly support.

In this work, the immobilization of alcalase from *Bacillus licheniformis* was performed in iota-carrageenan beads. Firstly, the iota-carrageenan extraction from locally produced red seaweeds (*Solieria filiformis*), followed by the hydrogel beads, was formed to produce the support, followed by its chemical characterization. Then, the enzyme was adsorbed in the hydrogel surface to produce the immobilized biocatalyst. The total protein content and enzymatic activity of the supernatant were used as parameters to evaluate the immobilization process, and the final activity of the derivate was also assessed. Thus, the present work aims to improve the overall understanding of the immobilization protease enzymes in natural polymers such as carrageenan, enhancing its industrial potential and synthesizing environmentally friendly biocatalysts, improving the application of green chemical methods in the food industry.

2 MATERIAL & METHODS

2.1 Materials

Alcalase (EC 3.4.21.62) from *B. licheniformis* (2.4 U/g) was obtained from Sigma-Aldritch (St. Louis, USA). *Solieria filiformis* seaweed was gently furnished by the Algae Producers Association from Flecheiras and Guajiru, cultivated on beaches at Trairi – CE, Brazil. The macroalgae were rinsed and stored at an ultra-freezer (-80 ºC) before the carrageenan extraction.

2.2 Iota-carrageenan extraction and production of the carrageenan hydrogel beads (CHB)

The polymer extraction was performed as an adapted methodology from Farias *et al*.[3]. Thus, the dry macroalgae was milled and suspended in a 0.1M KOH solution in the proportion of 1% (w/v) at 25 ºC for 24h. The carrageenan in the biomass was extracted in deionized water (80 ºC) for 3h. The supernatant was precipitated by ethanol 1:4 (v/v) and then kept for 24h at 4ºC. The precipitated biopolymer was retrieved by centrifugation (3000 rpm/20 min), then freeze-dried.

The hydrogel beads were produced by dropping a 1% (w/v) iota-carrageenan solution in a gelling solution of 5% (w/v) of NaCl, $CaCl_2$, or $AlCl_3$. The beads were soaked in the gelling solution for 2h, then rinsed with distilled water, as adapted methodology from Wahba *et al.*[4]. The Fourier-transform infrared spectroscopy (FT-IR) spectra of the CHB were obtained for evaluation of the organic groups present in the produced support in a Cary 630 FT-IR infrared spectrophotometer (Agilent Technologies, USA) at 400-4000 cm^{-1} and 2 cm^{-1} resolution.

2.4 Alcalase immobilization and determination of enzymatic activity

Alcalase enzymes were immobilized as an adapted methodology from De Oliveira *et al.*[5]. Thus, 1 g of the iota-carrageenan hydrogel beads (CHB) were added to 50 mL-Falcon tubes containing 40 mL of TRIS-HCl solution (pH 8.0). Then 1 mg of alcalase per gram of support was added to the medium. The solution was incubated under stirring (100 rpm) at 25 ºC for 120 min. After separating the supernatant, the total adsorbed protein in the CHB was determined by calculating the difference between the initial protein mass and the residual mass after a certain period of contact with the CHB. The Bradford method (1976) was applied and compared to a standard curve of bovine serum albumin (BSA, Sigma-Aldritch Chemical Co.) to evaluate the protein concentration in the medium throughout the adsorption essay. The FT-IR analysis of the derivate was also performed.

The enzymatic activity of the free enzyme and the derivate was determined using a methodology adapted from Dos Santos et al.[1]. Azocasein was used as a substrate, and trichloroacetic acid was used as a precipitating agent. After removing the precipitate by centrifugation (3000 g for 20 minutes), a 1:1 ratio of 1M NaOH solution was added to the supernatant, and the reading was performed on a spectrophotometer at 440 nm. One unit of proteolytic activity was defined as the enzyme required to produce a 0.01 absorbance change compared to the reaction blank.

3 RESULTS & DISCUSSION

Figure 1 presents a conceptual diagram of iota-carrageenan hydrogel beads (CHB) production for alcalase immobilization. The carrageenan extraction and ethanol precipitation yielded 31 ± 4% freeze-dried carrageenan. After that, the CHB production was performed using a saline solution containing different cations (Na^+, Ca^{2+}, and Al^{3+}), which may interact with the SO_3^- portion of the carrageenan monomer, forming gels with different strengths. These interactions may also vary between the carrageenan classifications (*kappa*, *iota,* and *lambda*), which present different conformations and numbers of ester-sulfate groups per monomer, resulting in different interactions between the polymer and the added cations[6].

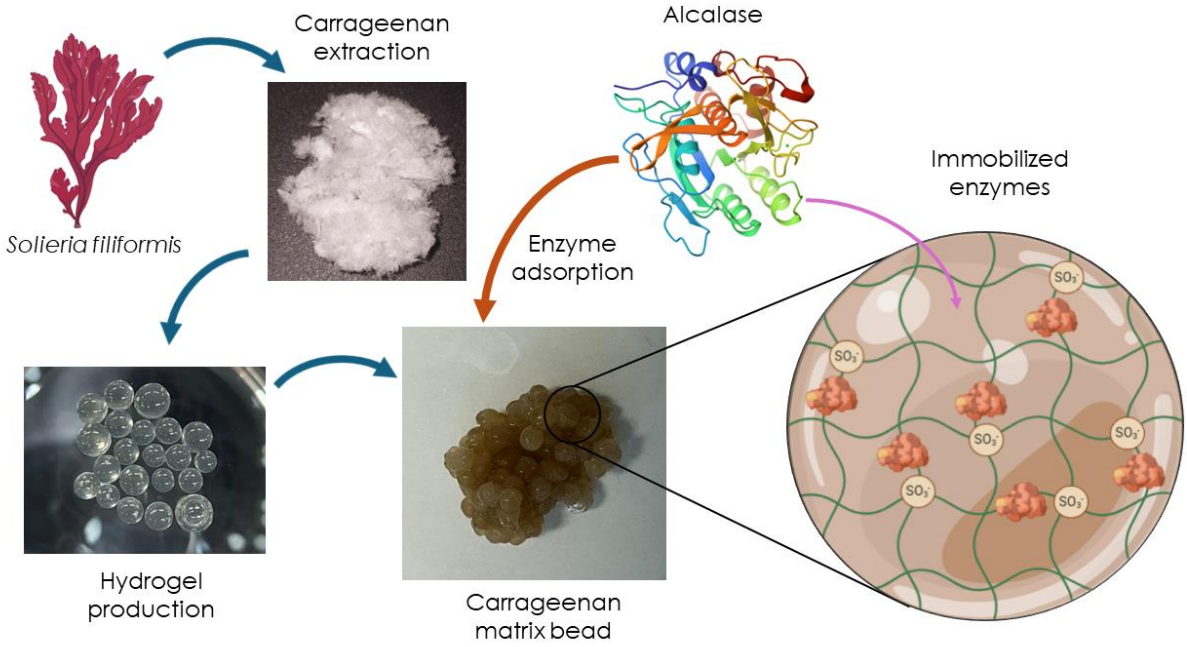

Figure 1 Conceptual diagram of the carrageenan extraction and production of CHB for alcalase immobilization

The beads formed in $AlCl_3$ presented higher durability than the ones formed in $CaCl_2$ and NaCl, maintaining their shape for at least 24 hours in an aqueous solution after being rinsed. This may be explained by the Al^{3+} being a trivalent cation, which increases the interaction between carrageenan monomers, resulting in a firm and resistant hydrogel[6]. Then, the CHB was applied for alcalase immobilization, as presented in Figure 2A, which presents the reduction in the enzyme concentration in the media, indicating the potential of the CHB for protein adsorption. It is also observed that the enzyme immobilization occurs mainly in the first 15 minutes of the process, which is indicative of the affinity of the protease to the CHB. After 30 minutes, the absence of a free enzyme in the medium is observed, similar to the previous report for the same enzyme[2]. Figure 1B presents the reduction of the enzymatic activity in the supernatant, which has reached zero after 15 minutes of immobilization essay, which is also when the total protein of the medium reaches its minimum, thus indicating the alcalase immobilization. Also, the final derivate enzymatic activity was 8.4 ± 0.4 U/g, suggesting that the immobilization did not result in the enzyme inactivation, thus presenting a high potential for industrial applications.

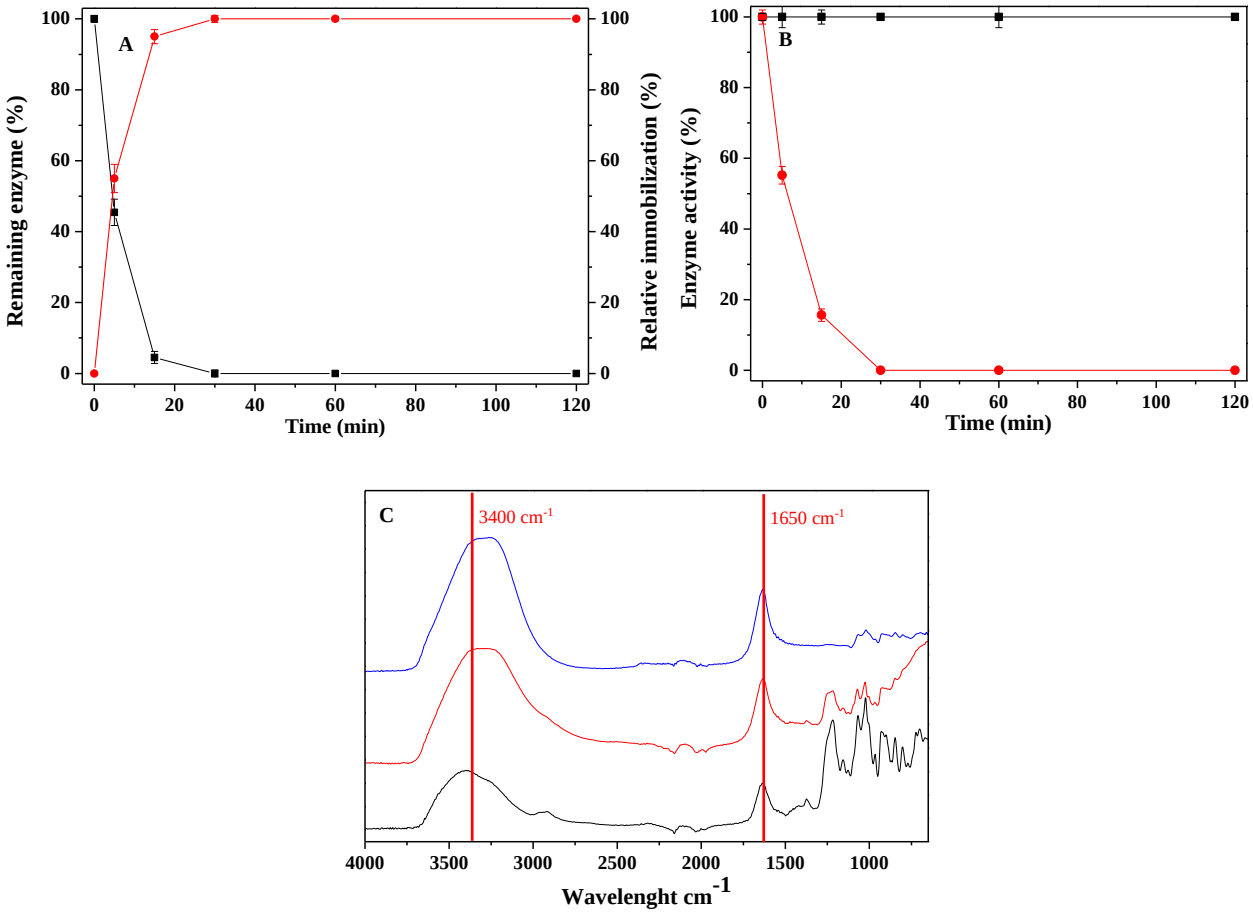

Figure 2 (A) Immobilization profile of alcalase in CHB for 120 minutes at 20 ºC and pH 8.0: (■) free enzyme (●) immobilized enzyme. (B) Proteolytic activity profile to azocasein at 30 ºC: (■) free enzyme (●) supernatant activity (C) FT-IR spectra of the (black line) carrageenan, (red line) CHB, and (blue line) CHB after enzyme immobilization.

Figure 1C indicates the organic groups in the iota-carrageenan (black line), CHB (red line), and CHB after enzyme immobilization (blue line). The bands between 1000-1200 cm^{-1} indicate the presence of C-O and C-O-C linkages, typical of alcohols and esters organic groups in the carrageenan structure. The bands between 1200-1300 cm^{-1} indicate the presence of O-SO_3^- in the molecule, which is the linkage group for the gel formation. Between 2800-3000 cm^{-1} it is observed the presence of -CH_2-, present in carbon chains, and at 3000-3600 cm^{-1} the presence of -OH, typical of alcohols in the carrageenan molecule. This is indicative of the preservation of carrageenan chemical groups after the hydrogel production. In the protease immobilized in the CHB, the increase in band intensity in 1650 cm^{-1}, representing C=O and C=N bonds, may indicate the presence of amide groups in the enzyme. The increase in signal intensity at 3400 cm^{-1} also may indicate the interaction of the enzyme with the -NH support and arguments, amines related to peptide bonds. Thus, indicating the presence of the enzyme in the support.

4 CONCLUSION

This study demonstrated the feasibility of immobilizing alcalase from Bacillus licheniformis on carrageenan hydrogel beads extracted from the red seaweed *Solieria filiformis*. The immobilization of alcalase resulted in a significant reduction of the medium enzyme activity, indicating efficient adsorption. The derivated has presented high proteolytic activity, indicating that the enzyme was not deactivated. FT-IR analysis confirmed the presence of typical carrageenan organic groups, with no significant alterations after immobilization, preserving the biopolymer's characteristics. Thus, the use of carrageenan as a support for enzyme immobilization may be applied to enhance the reusability of the enzyme, representing an economically viable approach aligned with the principles of green chemistry.

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