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SCIENTIFIC PAPER

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ALCALASE IMMOBILIZATION ONTO CHITOSAN/GLUTARALDEHYDE/TRIPOLYPHOSPHATE BEADS OBTAINED BY INVERSE EMULSION TECHNIQUE

Article Highlights

- Alcalase was covalently immobilized onto chitosan beads obtained by inverse emulsion technique
- The beads were additionally cross-linked by immersion in tripolyphosphate solution
- The beads had a sufficient amount of attachment points for enzyme immobilization
- The additional cross-linking resulted in smaller beads and higher specific activity of the enzyme
- The immobilized alcalase could potentially be used for several industrial applications

Abstract

Enzymes immobilization can efficiently solve limitations of their large-scale application, such as stability and reusability. In this study, Alcalase® 2.4L (protease from Bacillus licheniformis) was covalently immobilized onto chitosan beads obtained by inverse emulsion technique using 1.5% (m/v) of chitosan and 0.67% (v/v) or 1.0% (v/v) of glutaraldehyde (CTPP (1.5/0.67) and CTPP (1.5/1.0)). Afterward, the beads were additionally crosslinked by immersion into 10 % (m/v) tripolyphosphate solution. The parameters studied were enzyme loading, enzyme coupling yield, bead diameter, SEM, biocatalyst activity, and FTIR. The beads had adequate enzyme loading and enzyme coupling yield (Pg_{max} was 117.1 mg/g dry CTPP 1.5/0.67 and 90.1 mg/g dry CTPP 1.5/1.0, and μ_{max} was 96.7% for both carriers). CTPP (1.5/1.00) beads were smaller (diameter 635.2 ±25.2 mm wet/ 230.4±12.5 mm dry beads) and showed a higher specific activity of 20.1 ± 0.23 IU/$mg_{protein}$. The immobilized Alcalase® 2.4L was tested for hydrolyzing egg white and soy proteins. Alcalase® 2.4L, covalently attached to CTTP (1.5/1.0) chitosan beads, is a promising choice for industrial processes involving egg white protein hydrolysis, as the enzyme achieved a notable hydrolysis rate of 26.34 ± 0.879% after 195 minutes. Additionally, it remained effective through five successive applications under practical conditions (50 °C, pH 8).

Keywords: Alcalase® 2.4L; covalent immobilization; inverse emulsion technique; chitosan beads; tripolyphosphate.

Alcalase® 2.4L, a non-specific serine-type endoprotease from *Bacillus licheniformis*, is a commercial enzymatic preparation consisting of several proteinases (mainly of subtilisin A) with different specificities [1,2]. The enzyme has been used for various purposes such as detergent, dairy, silk, soy processing, brewing industries applications, production of hydrolysates, chemo-enzymatic synthesis, and others [3–5]. Free enzymes have several disadvantages such as high product cost, poor stability, and inability to be used in continuous production [6]. Enzyme immobilization increases its usability and stability and facilitates product separation and catalyst recycling [7–9]. Enzyme immobilization methods include cross-linking, encapsulation, entrapment,

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adsorption, and covalent binding. The choice of immobilization method and support depends on the intended use of the enzyme, the nature of the support, and the simplicity of the method [6]. The covalent binding of enzymes to the support provides improved stability and reduces enzyme leaching [8–11]. Alcalase® 2.4L has been immobilized using different methods and supports, such as glyoxyl- and monoaminoethyl-N-aminoethyl- agarose beads, hollow core-mesoporous shell silica nanospheres unmodified or modified with various metal ions, chitosan beads activated with different agents (such as glutaraldehyde, glyoxyl, and divinyl sulfone), chitosan-coated Fe_3O_4 nanoparticles, sol-gels, silica supports, alginate micron and submicron beads [3–5,12–22]. Chitosan is produced as a by-product through the alkaline deacetylation of chitin, a process in which the amide group in chitin is hydrolyzed to form a primary amine group (R-NH_2), resulting in chitosan. Chitosan has been one of the most promising renewable biopolymers due to its properties such as biocompatibility, biodegradability, non-toxicity, ability to be easily modified, and low cost. Chitosan's lack of toxicity and its rapid degradability make it ideal for various environmental and agricultural applications. It is used in drug delivery within the human gastrointestinal tract, food processing, biomedical fields, cosmetics, enzyme immobilization, as a heterogeneous catalyst, a sorbent for organic and inorganic contaminants, antimicrobial products, and for uranium recovery [23–27]. In this paper, for the first time, Alcalase® 2.4L was covalently immobilized onto different types of chitosan beads obtained by the emulsion technique, crosslinked with glutaraldehyde (GA) and then, additionally cross-linked by immersion into tripolyphosphate (TPP) solution. TPP is a multivalent anion and a non-toxic ionic cross-linker that interacts with the cationic chitosan forming ionically crosslinked networks through electrostatic forces [28]. TPP is especially appealing as an ionic crosslinker due to its stable performance, straightforward process control, and safety benefits [29]. The mechanical strength of chitosan beads crosslinked with only TPP is somewhat limited, but it can be improved by coating them with a negatively charged polymer, to create a polyelectrolyte complex film, or by employing covalent crosslinking [30]. The immobilized enzyme is potentially recycled, can be easily separated without contaminating a final product, can reduce the costs of downstream processing, has better stability, etc. The effects of chitosan bead properties on enzyme binding capacity, immobilization yield, and immobilized enzyme activity were investigated and the results were compared. The Alcalase® 2.4L immobilized to CTTP (1.5/1.0) chitosan bead was subsequently utilized in the industrial-scale hydrolysis of egg white and soy proteins. The degree of hydrolysis achieved was measured and compared to that obtained with the free enzyme.

In the literature, there are no studies of chitosan beads obtained by inverse emulsion technique, firstly cross-linked with GA and afterward immersed into TPP for additional cross-linking. Current enzyme immobilization methods have specific limitations, particularly in terms of stability, reusability, and efficiency when using conventional materials and techniques. Our approach, using novel chitosan beads, offers a potential solution to these limitations. Likewise, there are only a few studies on the use of immobilized Alcalase® 2.4L for the hydrolysis of egg white and soy proteins.

MATERIALS AND METHODS

Materials

Alcalase® 2.4L (protease from *Bacillus licheniformis*, Subtilisin EC 3.4.21.14) was provided by Sigma-Aldrich (*St Louis, MO,* USA). Chitosan (Ch) and tripolyphosphate (TPP) were obtained from Sigma, Japan, while itaconic acid (2-methylidenebutanedioic acid) was purchased from Sigma Aldrich, Germany. Glutaraldehyde (GA) (pentane-1,5-dial) and paraffin oil (light liquid paraffin) were obtained from Centrohem, Serbia. Tween 80 (polyoxyethylene (20) sorbitanmonooleate) used as an emulsifier was obtained from Riedel-de Haën, Germany. Azo-casein and sodium tripolyphosphate (TPP) were purchased from Sigma Chemical Co. (St. Louis, MO). Chicken egg whites obtained from a local supermarket were separated from the yolk and gently stirred without foam formation to provide a homogeneous mixture. Soy protein isolates with a protein content of 90% were received from Sojaprotein, Serbia. All other chemicals used in this research were of analytical grade.

Preparation and activation of chitosan microbeads

Emulsion technique

Chitosan microbeads were prepared by applying the emulsion technique, with the addition of GA, as a crosslinking agent as previously described [5], with some modifications. The aqueous phase was prepared by adding chitosan (1.5% (m/v)) in 5 mL of acetic acid solution (2.0% (v/v)). Water and oil phases (50 mL of paraffin oil) were mixed in a 1:10 ratio using a magnetic stirrer (Heidolph Hei-Connect, Germany), with the addition of 1.0% (v/v) Tween 80 (0.5 mL) as an emulsifier at room temperature and at stirring speed of 750 rpm. After homogenization, GA solution (0.67% or 1.0% (v/v)) was added, drop-by-drop, and stirring was continued under the same conditions for the next 24 h. To remove all residues of the surfactant, the

microbeads were washed out thoroughly with plenty of water, ethanol, and petroleum ether in three cycles, respectively. Afterwards, the obtained beads were submerged in 10% (m/v) TPP solution at room temperature for the next 24 h and subsequently thoroughly washed with water. The microbeads were dried in the oven at 37 °C for 48 h until constant mass was achieved, and kept until further use in a desiccator at 25 °C.

The samples were labeled as CTPP(Ch/GA), where two numbers correspond to the chitosan concentration (m/v%) and the concentration of the GA (v/v%). For example, CTPP (1.5/0.67) – means chitosan beads obtained by inverse emulsion technique using 1.5% (m/v) of chitosan and 0.67% (v/v) of glutaraldehyde, afterward immersed into 10% (m/v) tripolyphosphate solution.

Bead size

Data related to bead size were obtained using an optical microscope Olympus CX41RF, equipped with picture analyzing software ''CellA'' (Olympus, Tokyo, Japan). Microbeads were examined immediately after formation (native microbeads), in a dry and rehydrated state.

Scanning Electron Microscopy

The effect of Alcalase® 2.4L on the surface morphology of chitosan microbeads was examined using a TESCAN Vega TS 5130MM scanning electron microscope (Brno, Czech Republic) at different magnifications. SEM analysis was performed at 10.0 kV.

Immobilization method

The beads were treated with 1% (v/v) GA in 0.1M phosphate buffer at pH 8.0 and 28 °C for 30 min under gentle stirring. The excess of GA was then washed out using distilled water until no absorbance was read at 280 nm, confirming the absence of GA.

Enzyme coupling solution containing different amounts of Alcalase® 2.4L was prepared in 0.1 M phosphate buffer at pH 8.0 and pre-incubated at 28 °C under stirring for 2 h. After this period, activated microbeads (1 g wet weight) were submerged into the enzyme solution for 22 h at 28 °C under gentle stirring (120 rpm). At the end of this period, Ch/GA/TPP-Alcalase® 2.4L conjugate was formed (Fig. 1). Afterwards, sodium borohydride was added (0.5 mg $NaBH_4$/mL of solution) to reduce the Schiff's bases and the remaining aldehyde groups [31]. After 30 min at 4 °C under mechanical stirring, the produced derivative was washed with 0.1 M sodium phosphate buffer, pH 8.0, followed by washing with distilled water after which it was stored at 4 °C in 0.1 M sodium phosphate buffer, pH 8.0 before being used.

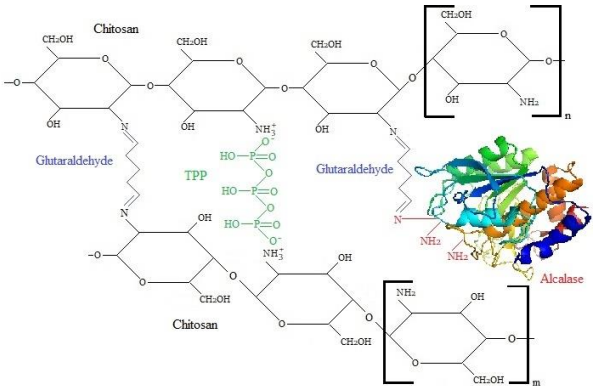

Figure 1. Chemical structure of the Alcalase® 2.4L conjugate formed with chitosan, glutaraldehyde, and tripolyphosphate. The 3-D structure was obtained from Protein Data Bank 1SCB using Pymol.

Samples of the enzyme solution before and after the immobilization, together with the washing solutions, were taken for protein content and enzyme activity determination. Alcalase® 2.4L concentration was determined according to the Bradford method using bovine serum albumin (BSA) as a standard [32]. The amount of bound enzyme was determined indirectly from the difference between the amount of enzyme introduced into the coupling reaction mixture and the amount of enzyme in the filtrate and the washing solutions:

$$\eta_{enz} = \frac{P_1}{P_0} \cdot 100 \quad (1)$$

where P_1 is the immobilized amount of Alcalase® 2.4L and P_0 is the initial amount of Alcalase® 2.4L in the enzyme coupling solution determined by the Bradford method. All experiments were carried out in triplicate. The efficiency of immobilization was evaluated in terms of enzyme coupling yield.

Alcalase activity assay

The Alcalase® 2.4L activity was measured using azo-casein as a substrate [33]. Assay mixture containing 75 µL of the enzyme solution or different mass of immobilized Alcalase® 2.4L (0.01 to 0.2 g) and 125 µL of 2% (m/v) azocasein in 50 mM Tris-HCl (pH 9.0) was incubated for 30 min at 37°C. The reaction was terminated by the addition of 600 µL of 10% (m/v) trichloroacetic acid. To remove the resulting precipitate, the assay tubes were cooled down in an ice bath prior to centrifugation for 10 min at 8000 rpm. Subsequently, 600 µL of supernatant was added to 700 µL 1 M NaOH and the absorbance at 440 nm was measured against a reference tube prepared separately for each sample

by the addition of trichloroacetic acid stop solution immediately after mixing the enzyme solution with the substrate. One unit of Alcalase® 2.4L activity was defined as the amount of enzyme required to produce an increase in absorbance at 440 nm of 1.0 in a 1-cm cuvette, under the above-mentioned assay conditions. All measurements were done in three repetitions.

Fourier transform infrared spectroscopy analysis

Fourier transform infrared spectroscopy (FT-IR) of dry samples was performed using Bomem MB 100 FT-IR spectrophotometer, applying the KBr disc method. Test samples consisted of 1 mg of sample mixed and ground with 50 mg of potassium bromide and compressed into pallets at a pressure of 11 t for about a minute, using a Graseby Specac model: 15.011. The spectra were obtained in the wave number range between 4000 and 400 cm^{-1} at 25 °C and at 4 cm^{-1} spectral resolution. All experiments were carried out in triplicate.

Hydrolysis of egg white protein and soya protein isolate with free alcalase and immobilized alcalase

The activity of free and immobilized alcalase in the industrially feasible reactions was assayed by monitoring the hydrolysis of 1% (w/w) soy protein isolate aqueous solution (2.4 mg/mL, protein content) and 10% (w/w) aqueous solution of pretreated egg white (11.4 mg/mL, protein content determined according to the standard Kjeldahl method, N 9 6.25). The hydrolysis was carried out in a 600 mL mechanically stirred batch reactor with temperature and pH control. Prior to the enzymatic hydrolysis, the egg white protein solution was subjected to thermal pretreatment at a high temperature (75 °C) for half an hour and afterward, the solution was kept out at an ambient temperature to cool. After pH and temperature stabilization (about 20 min) at optimum conditions for protease (50 °C and pH 8.0), the hydrolysis reaction was initiated by adding 2.12 and 0.11 IU of the free enzyme and the equivalent amount of the immobilized enzyme into egg white and soy protein solution, respectively, with stirring at 240 rpm. During the reaction, pH was kept at a constant value by adding 0.2 M NaOH, using the pH-stat method with automatic dosage of the base. The reaction was stopped by heating the mixture at 90 °C for 15 min to inactivate the free enzyme. In the case of the immobilized alcalase, the biocatalyst was removed by filtration, washed with plenty of water, and repeatedly recycled to examine its reusability.

Determination of hydrolysis degree

The progress of the enzymatic hydrolysis was followed by monitoring the hydrolysis degree through the pH-stat method. The hydrolysis degree was calculated as follows [34]:

$$DH(\%) = \frac{h \cdot 100}{h_{tot}} = \frac{N_b \cdot B \cdot 100}{\alpha \cdot m_p \cdot h_{tot}} \quad (2)$$

where h is the number of equivalents of peptide bonds hydrolyzed at the time per weight unit, h_{tot} is the total amount of peptide bonds per weight unit of a protein and can be calculated from its amino acid composition, N_b is the normality of the base, B is the consumption of the base in mL, and α is the degree of dissociation of the α-amino groups (1/α = 1.13 at 50 °C and pH 8.0), and m_p is the mass of protein in g. The degree of conversion (DH) was defined as the ratio of the cleaved peptide bonds to the total amount of peptide bonds.

Statistical analysis

All experiments were performed in triplicate and the data are presented in average of triplicates and standard deviation (SD). Statistical differences were determined by one-way analysis of variance (ANOVA). A Tukey test was applied as a test a posteriori with a level of significance of 95%. All the tests were considered statistically significant at $p < 0.05$. Statistical analysis was performed using the Origin Pro 8 software package.

RESULTS AND DISCUSSION

Alcalase® 2.4L immobilization

As the enzyme loading capacity is an important feature that affects the support price of the final catalysts [35] the total protein loading and the enzyme coupling yields were investigated. The Alcalase® 2.4L concentration was varied in the range of 0.1–1.68 mg/mL and 0.5 g of the wet chitosan beads CTPP (1.5/0.67) or CTPP (1.5/1.0) was immersed in 10 mL of enzyme coupling solution. The results are shown in Fig. 2.

The increase of the initial enzyme concentration in the coupling solution resulted in a linear increase of the enzyme loading on support (Figure 2). The result can be explained by the proportional binding of enzyme molecules to available sites on the support, assuming the support has excess binding sites and is not saturated with the enzyme [36]. The immobilization onto CTPP (1.5/0.67) provides a higher loading of protein than that on CTPP (1.5/1.0). The maximum amount of the Alcalase® 2.4L bound (Pg_{max}) was 117.1 mg/g dry support and 90.1 mg/g dry support, respectively. In the literature, other authors reported lower or comparable Pg_{max} values of Alcalase® 2.4L

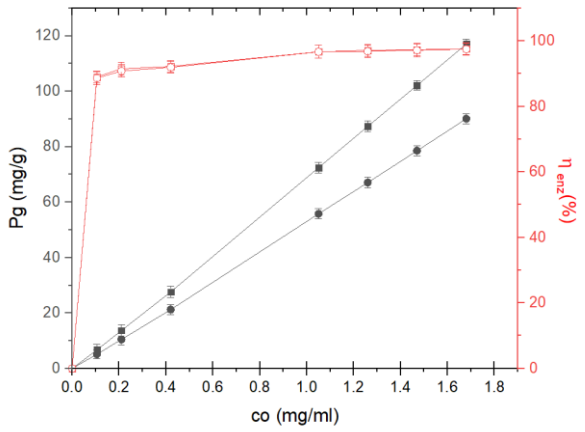

Figure 2. Impact of initial enzyme concentration on Alcalase® 2.4L loading and coupling yield for chitosan/glutaraldehyde/tripolyphosphate beads (CTTP) with different cross-linker ratios (Alcalase 2.4L loading: CTPP (1.5/0.67) (■), CTPP (1.5/1.0) (●) beads and enzyme coupling yield: CTPP (1.5/0.67) (□), CTPP (1.5/1.0) (○) beads). (c_o-initial enzyme concentration in coupling solution, Pg-enzyme loading, η_{enz}-enzyme coupling yield).

immobilized onto different supports. Thus, Zhu *et al.* and Zeng *et al.*, based on metal ion affinity chromatography immobilized Alcalase® 2.4L using triamino-functionalized hollow mesoporous silica spheres modified with various metal ions (Fe^{3+}, Ca^{2+}, Cu^{2+}, Ni^{2+}, Zn^{2+}) and obtained Alcalase® 2.4L loading capacity of 33.7-119.3 mg/g and 124.5 to 227.8 mg/g, respectively. [19,22] Corîci *et al.* immobilized Alcalase® 2.4L in dimethyldimethoxysilane (DMDMOS)-containing sol-gel system and attained the optimum capacity for enzyme loading in the silica matrix of 115 mg of protein per g of dry xerogel [37]. Ferreira *et al.* covalently immobilized Alcalase® 2.4L onto two sets of chemically distinct silica supports presenting terminal amino (S_{APTES}) or hydroxyl groups ($S_{TESPM\text{-}pHEMA}$) and achieved the loading of 2.4–6.3 mg of protein per gram of silica [38]. Zuza *et al.* immobilized Alcalase® 2.4L onto different types of chitosan beads obtained by electrostatic extrusion and inverse emulsion techniques, cross-linked only with glutaraldehyde, achieved the enzyme loading in the range of 116.3 to 340.2 mg/g [5]. Jonovic *et al.* immobilized Alcalase® 2.4L on alginate micron and submicron beads and obtained excellent maximum enzyme bounding of 592.3 mg/g [21]. Therefore, the obtained supports have an adequate content of attachment points for enzyme immobilization.

It can be noticed that the enzyme coupling yield also increased as the initial enzyme concentration was increased to 1.05 mg/mL and leveled off at about 96.7% for both carriers. Therefore, the beads have satisfactory enzyme coupling yield and the enzyme coupling yields were less influenced by the initial Alcalase® 2.4L concentration than the enzyme loading on supports. This can be attributed to the effective use of available binding sites on the support. As long as the support is not saturated and there are sufficient binding sites, the proportion of enzyme-coupled yield remains relatively stable, while enzyme loading increases proportionally with enzyme concentration [36].

As more enzyme was loaded onto the support, the specific activity of the immobilized Alcalase® 2.4L increased, reaching a maximum value A_{max} of 17.3 ± 0.34 IU/$mg_{protein}$ and 20.1 ± 0.23 IU/$mg_{protein}$ for CTPP (1.5/0.67) and CTPP (1.5/1.00), respectively (data not shown). These activities are higher than the activity of Alcalase® 2.4L immobilized to silicas with large pores and $S_{TESPM\text{-}pHEMA}$ (2.2–6.7 IU/$mg_{protein}$), glass sol-gel matrices (3.49-9.91 IU/$mg_{protein}$), chitosan beads obtained by emulsion technique cross-linked with GA S(1.5/0.67) (12.5 IU/$mg_{protein}$) and S(1.5/1.00) (16.3 IU/$mg_{protein}$) and lower than activity of Alcalase® 2.4L immobilized to S(2.0/0.57) (21.5 IU/$mg_{protein}$), EE (23.6 IU/$mg_{protein}$), S_{APTES} (17.9-40.3 IU/$mg_{protein}$) and glyoxyl- and glutaraldehyde-agarose bead (28.4–55.3 IU/$mg_{protein}$) [5,37–39]. Further increase of initial Alcalase® 2.4L concentrations and/or enzyme loading did not result in higher activities for the immobilized enzyme. As enzyme loading increases, the availability of enzyme molecules grows, leading to higher specific activity as more active sites participate in catalysis. As enzyme loading continues to increase, specific activity reaches a maximum due to factors such as saturation of the support's binding capacity, possible enzyme aggregation, and diffusion limitations [36].

Selected CTPP (1.5/0.67) beads in the relaxed polymer state are shown in Fig. 3. Average diameters of chitosan beads were 1008.8 ± 223.3 mm wet/ 288.8±54.2 mm dry CTPP (1.5/0.67) beads and 635.2±25.2 mm wet/ 230.4±12.5 mm dry CTPP (1.5/1.00) beads. Thus, the GA content increase led to a decrease in the size of the beads. The beads were smaller than chitosan beads obtained by inverse emulsion technique using the same chitosan and GA concentration and crosslinked only with glutaraldehyde (S (1.5/0.67), S (1.5/1.00)) [5]. Therefore, additional crosslinking with TPP leads to a bead size decrease. The smaller chitosan beads for the Alcalase® 2.4L immobilization yield higher activity because the surface area per unit mass of smaller beads is larger than that of larger beads. Hence, the enzyme immobilized onto CTPP (1.5/0.67) and CTPP (1.5/1.0) had higher activity (17.3±0.34 IU/$mg_{protein}$ and 20.1±0.23 IU/$mg_{protein}$, respectively) than the enzyme immobilized onto S (1.5/0.67) and S (1.5/1.0) (12.5 IU/$mg_{protein}$ and 16.3 IU/$mg_{protein}$, respectively).

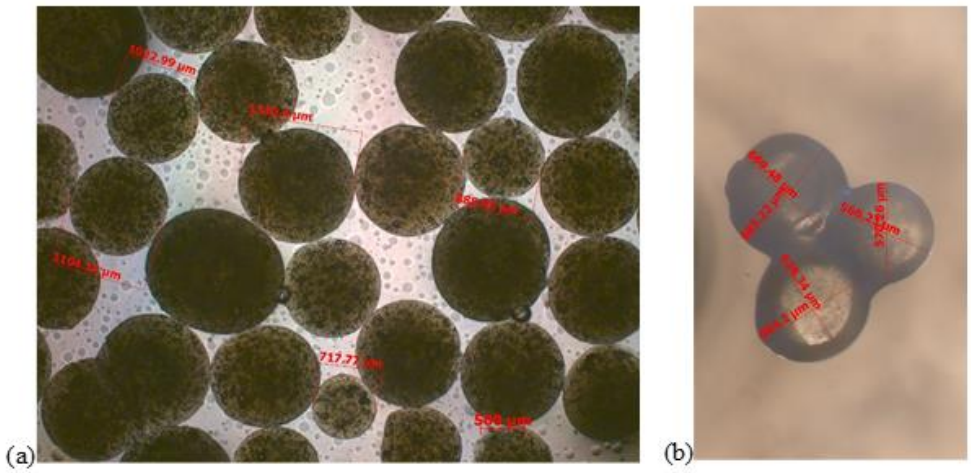

Figure 3. Images obtained by optical microscopy of chitosan/glutaraldehyde/tripolyphosphate beads a) (CTTP) (1.5/0.67) and b) (CTTP) (1.5/1.0) beads in their relaxed state. At a magnification of 10X.

SEM analysis

Figure 4. presents the SEM results of CTPP beads and CTPP beads after Alcalase® 2.4L immobilization. The Figure shows that the surface of the pure beads was smooth and uniform. After GA treatment and the enzyme immobilization, the roughness of the bead surface increased and the bead had rounded structures that may be attributed to protein aggregates after Alcalase® 2.4L immobilization [40,41].

We studied the effects of the mass of the biocatalysts obtained by varying initial enzyme concentration on the activity of the Alcalase® 2.4L – CTPP(1.5/0.67) and -CTPP(1.5/1.0) chitosan beads. The obtained results (Fig. 5) showed that the biocatalyst activity increased with the mass of the immobilized enzyme to some maximal value and then decreased with a further increase in weight probably due to steric hindrance and diffusion limitations. Within the experimental ranges used (0.33-5.32 IU), the highest activity was shown by the biocatalysts obtained in the system with the highest number of enzyme units 5.32 IU in the coupling solution. Alcalase® 2.4L immobilized onto CTPP(1.5/1.0) and CTPP(1.5/0.67) had higher activity than Alcalase® 2.4L immobilized onto S(1.5/0.67) and S(1.5/1.0), as CTPP beads were smaller in size and the contact surface between the substrate and the Alcalase® 2.4L where the reaction takes place was higher [5].

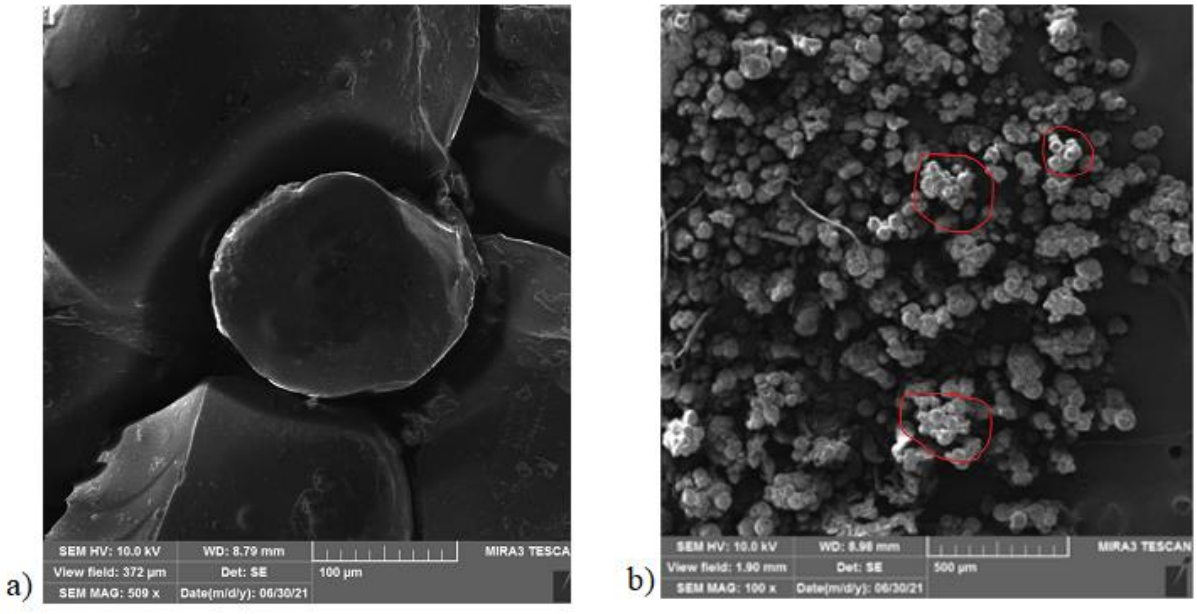

Figure 4. Images obtained by optical microscopy of a) chitosan/glutaraldehyde/tripolyphosphate beads CTPP (1.5/1.0) at a magnification of ×500 and b) chitosan/glutaraldehyde/tripolyphosphate beads CTPP (1.5/1.0) with immobilized Alcalase® 2.4L at a magnification of ×100.

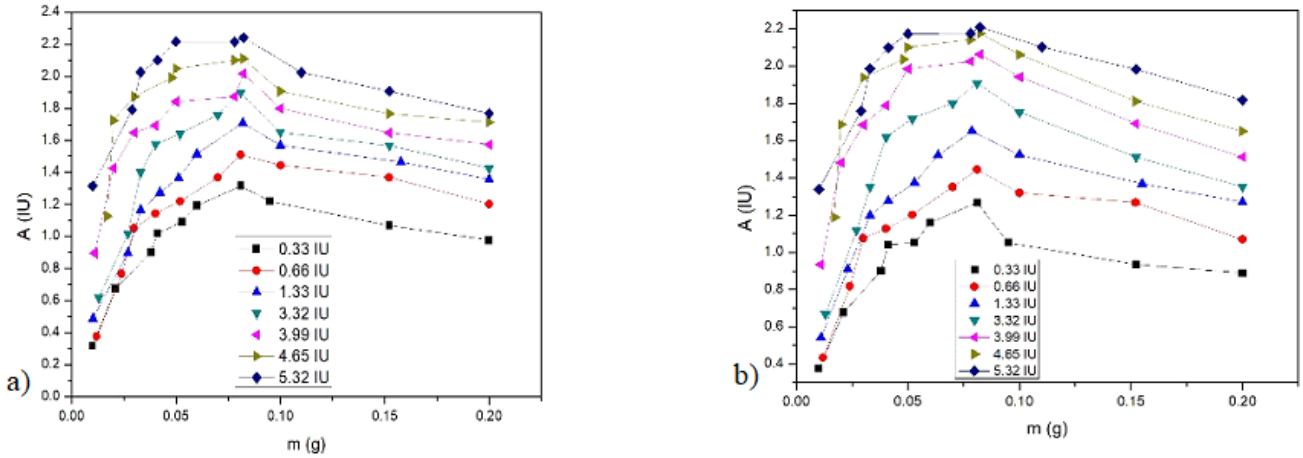

Figure 5. Impact of biocatalyst mass on the activity of Alcalase® 2.4L Immobilized on chitosan/glutaraldehyde/tripolyphosphate beads (CTTP): Comparison Between CTPP (1.5/0.67) and CTPP (1.5/1.0) Cross-Linker Ratios.

Fourier Transform Infrared Spectroscopy analysis

Fourier Transform Infrared Spectroscopy analysis (FT-IR) was performed in the 4000–400 cm^{-1} to confirm relationships between the matrix and enzyme. Figure 6. shows FT-IR spectra of Alcalase® 2.4L (spectrum a), pure CTPP beads spectrum (b); activated CTPP beads spectrum (c); and CTPP beads with immobilized Alcalase® 2.4L spectrum (d). Spectrum (a) shows a strong peak at 1550–1650 cm^{-1} that corresponds to amide I and amide II groups in the protease. The presence of bands in the region around 1350 cm^{-1} indicates the significant CH_2 and CH_3 deformation of the aliphatic amino acids [42]. Spectrum (b), the wide extension peaks observed at 3438 cm^{-1} are attributed to the stretching and bending vibrations of O-H and N-H bonds of chitosan and at 2921 cm^{-1} to CH_3 symmetric stretch. The bands at 1640–1658 cm^{-1} were referenced as amides I and bands in the range between 1390–1380 cm^{-1} correspond to the C-H bending due to the presence of aldehyde. The amide II band was at 1544 cm^{-1}. The bands at 1084 cm^{-1} and 1024 cm^{-1} were groups of C-O-C and C-O (spectrum b) [43–45]. There was no major difference in FTIR profile between pure CTPP beads and activated CTPP beads, indicating that GA and TPP cross-linking slightly hindered chitosan structure. Spectrum (d) shows the characteristic peak for the C=N group at 1664 cm^{-1} which indicates the imine reaction between the aldehyde group of glutaraldehyde and the NH_2 group of the enzyme [18]. It can be noticed that the characteristic peaks of the enzyme at 1110 cm^{-1} (spectrum a) shift the chitosan peak at 1158 cm^{-1} (spectrum b) to 1150 cm^{-1} (spectrum d) probably due to the reaction with GA on the support [46]. A peak at about 541 cm^{-1} indicates characteristic trans-gauche-trans enzyme conformation correlated to the S-S stretching vibrations and is shifted to 548 cm^{-1} upon Alcalase® 2.4L immobilization [5]. The FT-IR data confirmed that the activation of the beads and the subsequent covalent immobilization of the enzyme were carried out successfully. The changes in the FT-IR spectra align with expected chemical interactions, validating the effectiveness of the process. Therefore, we assume that the process of the bead activation with GA and Alcalase® 2.4L covalent immobilization was successful.

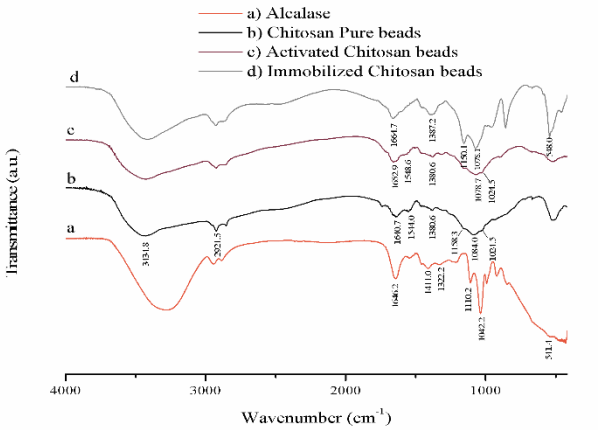

Figure 6. FT-IR spectra of investigated samples: (a) Alcalase® 2.4L (b) pure chitosan/glutaraldehyde/tripolyphosphate beads; (c) activated chitosan/glutaraldehyde/tripolyphosphate beads; and (d) chitosan/glutaraldehyde/tripolyphosphate beads with immobilized Alcalase® 2.4L.

Hydrolysis of egg white and soy proteins

The effectiveness of Alcalase® 2.4L immobilized on CTTP (1.5/1.0) chitosan beads for proteolysis of egg white and soy proteins has also been assessed. Figure 7. illustrates how the degree of hydrolysis (DH) changes over time for the immobilized enzyme at 50 °C. Free Alcalase® 2.4L reached the degrees of hydrolysis off egg white and soy protein of 35.10±1.125 at 75 min and 25.64±0.87 at 120 min [5], while the immobilized Alcalase® 2.4L achieved 18.06±0.963 at 75 min and 11.52±0.987 at 120 min, respectively.

Hydrolysis using free Alcalase® 2.4L proceeded

quickly at the beginning but then slowed down, eventually leveling off after 75 min and 120 min for egg white and soy protein, respectively [5]. In contrast, hydrolysis with immobilized Alcalase® 2.4L was more gradual, reaching a plateau after 150–195 minutes.

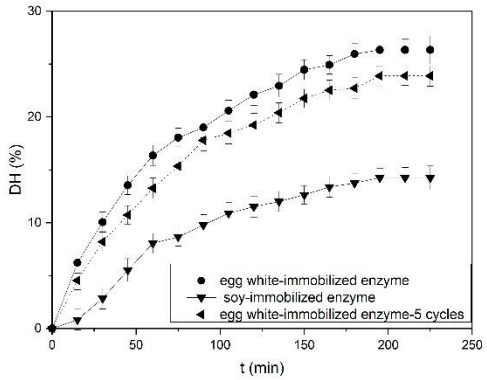

Figure 7. Enzymatic progress curves of hydrolysis of egg white protein and soy protein isolate with alcalase immobilized on CTTP (1.5/1.0) chitosan beads,

The initial rate of free and immobilized enzyme was 0.834 and 0.414, and 0.237 and 0.056 DDH%/min (it was decreased 2.01- and 4.23-fold) for egg white and soy, respectively. Therefore, the immobilization of the enzyme negatively affected the initial rate. Conversely, although the maximum degree of hydrolysis reached with the immobilized enzyme was lower compared to the free enzyme, it is still considered satisfactory (35.09±1.125 and 26.34±0.989, and 25.64±0.875 and 14.25±0.968 for hydrolysis of egg white and soy protein with free and the immobilized, respectively) [5]. Overall, the immobilized enzyme performs significantly better in egg white compared to soy protein hydrolysis, considering both, the degree of hydrolysis and the initial hydrolysis rate. Alcalase® 2.4L immobilized on CTTP (1.5/1.0) chitosan beads exhibited somewhat lower effectiveness compared to Alcalase® 2.4L immobilized on EE chitosan beads, which achieved maximum degrees of hydrolysis of 30.28 ± 1.107% for egg white and 16.38 ± 0.989% for soy protein. Additionally, Alcalase® immobilized on magnetic chitosan nanoparticles reached a maximum degree of hydrolysis of 18.38% for soy protein isolate [5, 18].

The immobilized Alcalase® 2.4L was then used for egg white hydrolysis in five successive cycles. It was obtained that the activity dropped only about 10% after five reaction cycles. Further, the activity progressively decreased, likely due to factors such as product inhibition, denaturation, and enzyme leakage.

CONCLUSION

The paper presents the covalent immobilization of Alcalase® 2.4L onto new chitosan beads obtained by the emulsion technique, crosslinked with glutaraldehyde and for the first time, additionally crosslinked with tripolyphosphate solution. The results showed that the beads had sufficient content of attachment points for the enzyme immobilization as they had satisfactory enzyme loading and coupling yield. It was confirmed that additional crosslinking led to smaller beads and subsequently the immobilized enzyme had higher specific activity. The Alcalase® 2.4L immobilization onto CTPP beads was also confirmed by the FTIR technique. It can be concluded that CTPP beads are promising supports for enzyme immobilization. The Alcalase® 2.4L immobilized onto CTPP beads can be used for egg white proteins hydrolysis in real food systems, as it demonstrated a satisfactory initial rate, a high maximum degree of hydrolysis, and effective reusability over five consecutive cycles.

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NOMENCLATURE

CTPP (1.5/0.67)	chitosan beads obtained by inverse emulsion technique using 1.5% (m/v) of chitosan and 0.67% (v/v) of glutaraldehyde, afterward immersed into 10 % (m/v) tripolyphosphate solution
CTPP (1.5/1.0)	chitosan beads obtained by inverse emulsion technique using 1.5% (m/v) of chitosan and 1.0% (v/v) of glutaraldehyde, afterward immersed into 10 % (m/v) tripolyphosphate solution
Pg	enzyme loading
η	enzyme coupling yield
GA	glutaraldehyde
TPP	tripolyphosphate
Ch	Chitosan
Tween 80	polyoxyethylene (20) sorbitanmonooleate
BSA	bovine serum albumin
S_{APTES}	silica supports presenting terminal amino groups
$S_{TESPM\text{-}pHEMA}$	silica supports presenting terminal hydroxyl group
A	specific activity of the immobilized Alcalase® 2.4L
S (1.5/0.67)	chitosan beads obtained by emulsion technique using 1.5% (m/v) of chitosan and cross-linked with 0.67% (v/v) GA
S (1.5/1.00)	chitosan beads obtained by emulsion technique using 1.5% (m/v) of chitosan and cross-linked with 1.00 % (v/v) GA
EE	chitosan beads obtained by electrostatic extrusion
FT-IR	Fourier Transform Infrared Spectroscopy analysis

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NAUČNI RAD

IMOBILIZACIJA ALKALAZE NA HITOZAN/GLUTARALDEHID/TRIPOLIFOSFAT PERLE DOBIJENE TEHNIKOM INVERZNE EMULZIJE

Imobilizacija enzima može efikasno da reši ograničenja njihove široke primene, kao što su stabilnost i ponovna upotreba. U ovom radu je enzim Alcalase® 2.4L (proteaza Bacillus licheniformis) kovalentno imobilisan na perle hitozana dobijena tehnikom inverzne emulzije korišćenjem 1,5% (m/v) hitozana i 0,67% (v/v) ili 1,0% (v/v)) glutaraldehida (CTPP (1,5/0,67) i CTPP (1,5/1,0)). Nakon toga, perle su dodatno umrežene uranjanjem u 10% (m/v) rastvor tripolifosfata. Proučavani su opterećenje enzima, prinos enzimskog kuplovanja, prečnik perli, SEM, aktivnost biokatalizatora i FTIR. Perle su imale adekvatno punjenje enzima i prinos enzimskog kuplovanja (P_{gmax} je bio 117,1 mg/g suvog CTPP 1,5/0,67 i 90,1 mg/g suvog CTPP 1,5/1,0, a μ_{max} je bio 96,7% za oba nosača). CTPP (1,5/1,00) perle su bile manje (prečnik 635,2 ±25,2 mm vlažne/230,4±12,5 mm suve perle) i pokazale su veću specifičnu aktivnost od 20,1 ± 0,23 IU/mg proteina. Imobilisani enzim Alcalase® 2.4L je testiran na hidrolizu proteina belanaca i soje. Enzim Alcalase® 2,4L, kovalentno vezan za CTTP (1,5/1,0) hitozan perle, je obećavajući izbor za industrijske procese koji uključuju hidrolizu proteina belanaca, pošto je enzim postigao značajan stepen hidrolize od 26,34 ± 0,879% posle 195 minuta. Pored toga, ostao je efikasan kroz pet uzastopnih primena u praktičnim uslovima (50 °C, pH 8).

Ključne reči: Alcalase® 2.4L; kovalentna imobilizacija; tehnika inverzne emulzije; hitozanske perle; tripolifosfat.