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Immobilization of Keratinolytic Metalloprotease from *Chryseobacterium* sp. Strain kr6 on Glutaraldehyde-Activated Chitosan

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Keratinases are exciting keratin-degrading enzymes; however, there have been relatively few studies on their immobilization. A keratinolytic protease from *Chryseobacterium* sp. kr6 was purified and its partial sequence determined using mass spectrometry. No significant homology to other microbial peptides in the NCBI database was observed. Certain parameters for immobilization of the purified keratinase on chitosan beads were investigated. The production of the chitosan beads was optimized using factorial design and surface response techniques. The optimum chitosan bead production for protease immobilization was a 20 g/l chitosan solution in acetic acid [1.5% (v/v)], glutaraldehyde ranging from 34 g to 56 g/l, and an activation time between 6 and 10 h. Under these conditions, above 80% of the enzyme was immobilized on the support. The behavior of the keratinase loading on the chitosan beads surface was well described using the Langmuir model. The maximum capacity of the support (q_m) and dissociation constant (K_d) were estimated as 58.8 U/g and 0.245 U/ml, respectively. The thermal stability of the immobilized enzyme was also improved around 2-fold, when compared with that of the free enzyme, after 30 min at 65°C. In addition, the activity of the immobilized enzyme remained at 63.4% after it was reused five times. Thus, the immobilized enzyme exhibited an improved thermal stability and remained active after several uses.

Keywords: Casein hydrolysis, chitosan, enzyme, immobilization, protease

The effective use of enzymes can sometimes be hampered by certain peculiar properties of enzymatic proteins, such as their non-reusability and high sensitivity to several denaturing agents. However, the use of immobilized enzymes can avoid many of these undesirable constraints, especially in the case of catalysis [2, 16, 25, 35]. Several benefits have already been associated with the use of immobilized enzymes instead of their soluble counterparts, including the reusability of the biocatalysts, reduced production costs by efficient recycling, and process control. In addition, immobilized enzymes can be used as stable and reusable devices for analytic and medical applications, fundamental tools for solid-phase protein chemistry, and effective micro devices for the controlled release of protein drugs [9].

Enzyme immobilization onto insoluble supports has been extensively investigated for over 30 years, resulting in many different methodologies and a wide range of applications [2, 9, 17]. The methods developed for enzyme immobilization can essentially be subdivided into two general classes: (a) chemical methods, where covalent bonds are formed with the enzyme, and (b) physical methods, where weak interactions exist between the support and the enzyme [11]. During the immobilization process, the main task is to select a suitable carrier, appropriate environmental conditions (pH, temperature, and nature of medium), and the best enzyme (source, nature, and purity) to design an immobilized biocatalyst [9].

The natural polymer chitosan is an attractive support for enzyme immobilization, since it is non-toxic, user-friendly, and available in different forms, including powder, gel, fiber, and membrane. This linear polysaccharide consists of β(1→4)-linked 2-amino-2-deoxy-D-glucose (D-glucosamine) and 2-acetamido-2-deoxy-D-glucose (*N*-acetyl-D-glucosamine) units [21, 32]. The effectiveness of chitosan as a support can be associated with certain properties, such as its elevated porosity, high hydrophilicity, large adhesion area, and thus, small mass transfer resistance to enzymes. In addition, chitosan allows easy derivatization, it displays a

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high protein affinity, and is available and inexpensive, since it is derived from chitin, a by-product of the fishing industry [21]. This polymer has also been shown to be an efficient support for the immobilization of such enzymes as glycosidases, proteases, glucose oxidase, and others [12, 32].

Factorial design and response surface techniques are important tools to determine the optimal process conditions and have already been used in several areas of biotechnology, mainly to optimize the production of bioactive molecules [10, 13]. One of the main advantages of experimental design is to reduce the number of required experiments without a significant loss of useful information [3].

Keratinases (E.C. 3.4.99.11) constitute an exciting group of proteases that hydrolyze hard-to-degrade keratin substrates. Most bacterial keratinases are serine-type proteases from *Bacillus* and *Streptomyces*, yet attractive keratinases have also been described from other bacteria [5, 6]. Indeed, *Chryseobacterium* sp. kr6 produces an unusual keratinolytic metalloprotease that belongs to the M14 family of peptidases [28]. Moreover, this same bacterium was also recently found to produce a novel 20 kDa keratinase that could hydrolyze feather, nail, and wool keratins and was obtained with a high purification fold and yield recovery [30]. Yet, despite the growing interest in keratinases, relatively few immobilization studies have been reported [15, 20, 33]. Accordingly, the current study determined the immobilization parameters for the 20 kDa keratinase from *Chryseobacterium* sp. kr6 on glutaradehyde-activated chitosan beads. Additionally, certain properties of the free and immobilized enzyme were compared.

Materials and Methods

Materials

The azocasein, casein, and chitosan flakes with an 85% degree of deacetylation were from Sigma Chemical Co. (St. Louis, MO, USA). The Sephadex G-100 and Q-Sepharose Fast Flow columns were from Pharmacia Biotech (Uppsala, Sweden). The azokeratin was synthesized as described elsewhere [27].

Enzyme Production

The feather-degrading *Chryseobacterium* sp. strain kr6 was maintained on a feather meal agar and the keratinase production carried out using a submerged culture with feather meal as the sole carbon source [27]. The crude enzyme was collected after 48 h of cultivation at 30°C, by centrifugation at 10,000 ×*g*, during 20 min, at 4°C.

Enzyme Purification

The enzyme was purified from the culture supernatant as described elsewhere [30]. The purification protocol consisted of sequential ammonium sulfate precipitation, gel filtration chromatography using a Sephadex G-100, and ion-exchange chromatography using a Q-Sepharose Fast Flow column. The protein concentration was determined using the Folin phenol reagent method [23] with bovine serum albumin as the standard.

Mass Spectrometry and Protein Identification

Samples of the purified enzyme were submitted to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) using 10% polyacrylamide gels. After electrophoresis, the gels were stained with Coomassie Brilliant Blue G-250 and the keratinase band was then excised from the gel and digested with trypsin. The peptide mixture was concentrated and applied to a UPLC system coupled to a Q-TOF micro (Waters) tandem mass spectrometer using nano-ESI. A nanoAcquity UPLC column BHE130 C18 (100 μm × 100 mm, 1.7 μm particle size, Waters) was used. The data were processed using a MASCOT Distiller (Matrix Science, UK), and the software MASCOT 2.2 (Matrix Science, UK) was used to search for peptide homologs in the NCBI database (http://www.ncbi.nlm.nih.gov).

Enzyme Activity

During the purification steps, the enzymatic activity was measured using azokeratin as the substrate [27]. The enzyme sample (100 μl) was mixed with 500 μl of 20 g/l azokeratin in a 50 mmol/l Tris-HCl buffer (pH 8.0) and incubated for 30 min at 45°C, under agitation at 150 rpm. The reaction was stopped by the addition of 600 μl of 100 g/l trichloroacetic acid. The supernatant was collected after centrifugation at 10,000 ×*g* during 10 min and the absorbance measured at 450 nm. The control samples were prepared in a similar manner, except 100 g/l trichloroacetic acid was added prior to the enzyme. One unit of keratinolytic activity was defined as an increase in the A_{450} of 0.01, at pH 8.0 and 45°C.

To optimize the enzyme immobilization on chitosan beads, the enzyme activity was measured using casein as the substrate. The caseinolytic determinations were performed by incubating 100 μl of the enzyme with 500 μl of a casein solution (5 g/l) in a 100 mmol/l Tris-HCl buffer (pH 8.0), for 1 h at 45°C, under agitation at 150 rpm. The reaction was stopped by the addition of 600 μl of 100 g/l trichloroacetic acid. The supernatant was collected after centrifugation at 14,000 ×*g* during 20 min and the absorbance measured at 280 nm. One unit of enzyme activity is defined as the amount of enzyme required to increase one absorbance unit at 280 nm due to 1 μmol of tyrosine produced per minute during casein hydrolysis, under the described conditions [1]. The activity of the immobilized enzyme was analyzed following the same procedure as that used for the corresponding free enzyme with the minor modification of 1 g of the enzyme-immobilized beads and 5 ml of a casein solution in a 100 mmol/l Tris-HCl buffer (pH 8.0). The blanks were also prepared under the same conditions, except 100 g/l trichloroacetic acid was added before the addition of the enzyme.

The protein content of the soluble protease was estimated using the Folin-phenol reagent method [23]. The amount of protein immobilized on the chitosan beads was estimated by subtracting the residual protein (protein left in the protease solution after incubation plus the protein in the washings) from the total protein (enzyme solution used for incubation with the chitosan beads). The assays were conducted in triplicate.

Preparation of Chitosan Beads

The chitosan (2 g) was dissolved in 100 ml of 1.5% (v/v) acetic acid, and heated at 60°C for 1 h at 150 rpm. The resulting viscous solution was submitted to ultrasound for 30 min, and then sprayed drop-wise through a syringe, at constant rate, into a 1 mol/l KOH solution. The resulting beads (2–3 mm diameter) were washed with

Milli Q water to reach a neutral pH and stored at 4°C in a 100 mmol/l Tris-HCl buffer (pH 8.0) until glutaraldehyde activation.

Activation of Chitosan Beads: Second-Order Factorial Design

The chitosan beads were incubated at 30°C and 100 rpm with 50 ml of different glutaraldehyde concentrations (ranging from 10 to 80 g/l) and for different time intervals (from 0.5 h to 10 h). After the activation time for each run, the beads were washed with a 100 mmol/l Tris-HCl buffer (pH 8.0) to remove the excess glutaraldehyde and stored at 4°C in the same buffer until further use. The influence of the glutaraldehyde concentration (GA) and activation time on the enzyme immobilization was then evaluated using a full 2^2 factorial design [3]. In the statistical model, Y is the predicted response: enzyme immobilization (EI); b_0, constant; X_1, glutaraldehyde concentration (GA); X_2, activation time; b_1 and b_2, linear coefficients; b_{11} and b_{22}, quadratic coefficients; b_{12}, interaction coefficient.

The actual levels corresponding to the coded settings, the treatment combinations, and the responses are all shown in Table 1. Runs 1 to 4 correspond to the linear points of the factorial design, which allow the main effect of each independent variable on the evaluated response to be investigated, experiments 5 to 8 correspond to the axial points, and runs 9 to 12 are the central points. The regression model is described by Eq. (1):

$$Y = b_0 + b_1X_1 + b_2X_2 + b_{12}X_1X_2 + b_{11}X_1^2 + b_{22}X_2^2 \quad (1)$$

The regression and graphical analysis of the data were performed using the software Statistica 7.0 (Statsoft, Tulsa, OK, USA). The significance of the regression coefficients and second-order model equation were determined using Student's *t*-test and Fisher's test, respectively.

Fourier Transform Infrared (FTIR)

The infrared spectra were measured using a potassium bromide pellet. Four scans of the samples were taken at a resolution of 2 cm^{-1} using a Mattson 3020 FITR spectrophotometer (Madison, USA).

Enzyme Immobilization

An amount of activated wet beads (1 g; average water content 92%), prepared according the conditions described above, was incubated with 1 ml of the enzyme (enzymatic activity of 30 U/ml) and 3 ml of a 100 mmol/l Tris-HCl buffer (pH 8.0), for 12 h. Following the incubation, the beads were washed with a 100 mmol/l Tris-HCl buffer (pH 8.0) to remove the unbound protease and stored at 4°C until further use. The blanks were prepared under the same conditions, yet without any solid support, to quantify the enzyme denaturation during the reaction. The enzyme activity of the supernatant and chitosan beads was estimated as described above, using casein as the substrate. The enzyme loading on the chitosan beads achieved during the immobilization process was calculated according Eq. (2), in terms of enzyme immobilization (EI):

$$EI = \frac{U_0 - U_{remain}}{U_0} \times 100 \quad (2)$$

where U_0 (U/ml) is the enzyme activity present in the solution used for immobilization, and U_{remain} (U/ml) is the activity remaining in the supernatant at the end of the immobilization procedure.

Table 1. Coded levels and real values (in parentheses) for full factorial design, and partition coefficient.

Run	X_1	X_2	EI (%)
1	-1 (12)	-1 (2.2)	46.8
2	+1 (68)	-1 (2.2)	68.7
3	-1 (12)	+1 (8.6)	64.9
4	+1 (68)	+1 (8.6)	78.2
5	0 (40)	-1.41 (0.5)	39.2
6	0 (40)	+1.41 (10)	93.6
7	-1.41 (10)	0 (5.2)	27.7
8	+1.41 (80)	0 (5.2)	66.6
9	0 (40)	0 (5.2)	84.6
10	0 (40)	0 (5.2)	85.4
11	0 (40)	0 (5.2)	84.3
12	0 (40)	0 (5.2)	83.9

X_1: glutaraldehyde concentration (g/l); X_2: activation time (h).

Loading Capacity of Chitosan Beads

The experiments to investigate the loading capacity of the chitosan beads for protease immobilization consisted of batch reactors with 1 g of previously activated beads (40 g/l glutaraldehyde and 10 h activation time) and 3 ml of purified protease with different levels of activity (ranging from 1.9 to 30 U/ml). After 12 h of incubation at 20°C and 100 rpm, the enzyme activity in both the liquid and solid phases was determined as described above, using casein as the substrate. To detect any enzyme denaturation during the immobilization procedure, blanks were prepared under the same conditions without the chitosan beads. The enzyme activity data obtained at the immobilization equilibrium were used to check the fit of the Langmuir model, allowing the parameters q_m and K_d to be estimated. The kinetic equation for protease immobilization can be expressed by Eq. (3), which is transformed to Eq. (4) at equilibrium:

$$\frac{dq}{dt} = k_1C(q_m - q) - k_2q \quad (3)$$

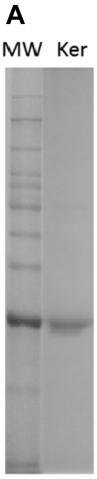
$$q^* = \frac{q_m c^*}{c^* + K_d} \quad (4)$$

Equation 4 was used to determine the maximum amount of protease loaded on the support (q_m) and the dissociation constant (K_d), being q* and c* enzyme activity in the solid and liquid phase at equilibrium, respectively. The parameters were calculated using Statistica 7.0 (Statsoft Inc., USA) by a nonlinear estimation analysis using the Hook–Jeeves and quasi-Newton method.

Stability and Reuse of Immobilized Enzyme

To evaluate the pH and thermal stability of the free and immobilized enzymes, the residual activities were measured after the samples were incubated for 30 min at different temperatures (50, 55, 60, and 65°C) and a pH range from 5 to 10. The free and immobilized enzymes were assayed as described above, using casein as the substrate.

The initial activity of the immobilized enzyme was measured and then compared with the residual activity of the enzyme obtained after its repeated use for five cycles. After each cycle, the immobilized enzyme was filtered, washed with a 100 mmol/l Tris-HCl buffer (pH 8.0), and reintroduced into a fresh reaction medium. The remaining enzyme activity was tested as described above, using casein as the substrate. The thermal stability and reusability results are presented in a normalized form, with the highest values set as 100% activity.



B

Peptide	Sequence	Mass (Da)
P1	SPRVGLAGLAAGVPLTDK	1,720.983
P2	AAPEKLAALVGEYR	1,486.814

Fig. 1. Analysis of the purified keratinase from strain.
(**A**) Polyacrylamide gel showing molecular mass standards (MW) and purified keratinase (Ker). (**B**) Amino acid sequence of tryptic peptides from *Chryseobacterium* sp. kr6 keratinase.

Results and Discussion

Identification of *Chryseobacterium* sp. kr6 Keratinase

Chryseobacterium sp. kr6 produces multiple extracellular proteolytic enzymes during growth on whole feather or feather meal media [29]. To isolate the 20 kDa keratinase, *Chyseobacterium* sp. kr6 was cultivated in a feather meal medium for 48 h and the culture supernatant then submitted to the purification protocol described by Silveira *et al*. [30]. The overall purification factor was about 40-fold, and the final yield was 7%. The molecular mass of the purified extracellular keratinase was approximately 20 kDa by SDS-PAGE (Fig. 1A). This result confirms the purification of the 20 kDa keratinase, as *Chryseobacterium* sp. kr6 also produces two other keratinases of 64 kDa [28] and 38 kDa [4].

The peptides resulting from trypsin digestion of the keratinase band excised from the SDS-PAGE were subjected to an electrospray tandem mass spectrometry analysis. The peptide sequences are presented in Fig. 1B. No significant matches were observed among the amino acid sequences of the keratinase kr6 peptides and nonredundant protein sequences in the NCBI database. For instance, the closest alignment for peptide P1 was with a putative thiolase of *Erwinia tasmaniensis* Et1/99 (GenBank Accession No. YP1906768.1). Similarly, the peptide sequences of the three keratinases from *Chryseobacterium indologenes* TKU014 showed no significant homology to other reported microbial peptides [34].

Immobilization of Keratinase on Chitosan

Modifying the chitosan beads with glutaraldehyde had three objectives: (a) to improve the mobility of the immobilized enzyme by introducing a spacer arm; (b) to reduce the interactions between the protein and the support by distancing the enzyme from the polymer surface; and (c) to introduce carbonyl groups onto the chitosan surface to react with the amino groups of the protease.

Table 2. Main effects and interaction analysis of glutaraldehyde concentration (GA) and activation time (AT) for enzyme immobilization (EI) during protease immobilization on chitosan beads.

Factor	Effect	Std.Err.	*t*-Value	p-Value
Mean	84.5	0.32	266.2	<0.000[a]
GA (Linear)	22.6	0.45	50.2	<0.000[a]
AT (Linear)	26.1	0.45	58.1	<0.000[a]
GA × AT	-4.3	0.63	-6.7	<0.0[a]

[a]Significant factors P<0.05.

The experimental conditions and response obtained from the protease immobilization on the chitosan beads are shown in Table 1. A highest enzyme immobilization (EI) was achieved in experiment 6 (93.6%), which involved a glutaraldehyde concentration of 40 g/l and 10 h of activation time (Table 1), whereas the lowest EI (27.7%) was observed with a glutaraldehyde concentration of 10 g/l and activation time of 5.2 h (run 7). The effects of independent variables on the response are presented in Table 2. Changing the glutaraldehyde concentration from 12 g/l (inferior level) to 68 g/l (superior level) increased the EI, on average, by 22.6%. Moreover, changing the activation time from 2.2 h to 8.6 h increased the EI, on average, by 26.1%. Glutaraldehyde concentrations above 40 g/l did not promote the protease immobilization. When the effects of the variables are interactive, this also needs to be evaluated [3]. In this study, the combined effect of the glutaraldehyde concentration and the activation time resulted in a decrease in the EI, on average, by 4.3%. In general, enzyme immobilization on chitosan is significantly affected by the glutaraldehyde concentration, where the maximum amount depends on the enzyme and the support characteristics [11, 22]. Dwevedi and Kayastha [14] used a response surface methodology and central composite design (CCD) to optimize β-galactosidase immobilization onto chitosan beads. They found that the glutaraldehyde concentration, amount of enzyme, and number of beads significantly affected the enzyme immobilization, whereas the pH did not have a significant effect on the response. After establishing the optimal conditions, the maximum enzyme immobilization they reported was 75%.

In the present study, the cross-linking and activation with glutaraldehyde were confirmed using an FTIR spectra comparison of the plain and activated chitosans (Fig. 2). The plain chitosan showed a typical IR spectrum with major bands at 3,340–3,480 cm^{-1} (OH stretching), 1,640–1,660 cm^{-1} (amide I), and bands of a polysaccharide structure

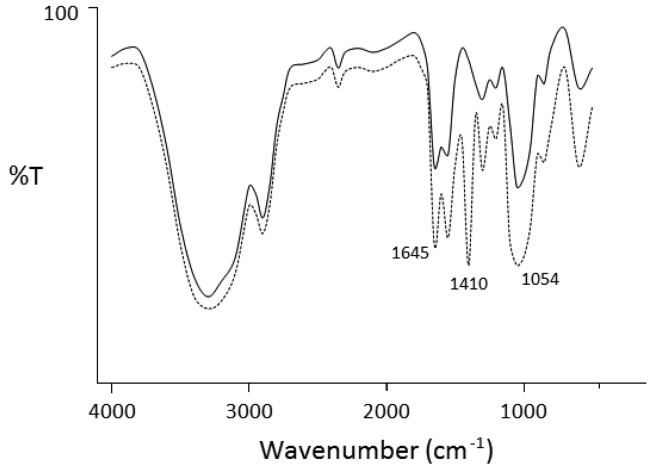


Fig. 2. FTIR spectra of chitosan (—) and glutaraldehyde-activated chitosan (- - -).

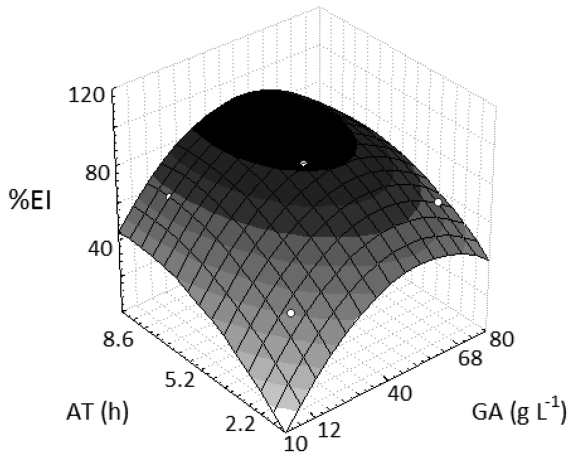


Fig. 3. Response surface for adsorption recovery (EI) as a function of glutaraldehyde concentration (GA) and activation time (AT) for protease immobilization on chitosan beads.

in the range of 800–1,150 cm^{-1} [7]. Meanwhile, the spectrum of the activated chitosan presented a stronger band at 1,410 cm^{-1}, indicating the C-N bond that results from the reaction of the C=O group of glutaraldehyde with the amino group of chitosan through a Schiff base linkage. Additionally, the bands at 1,645 and 1,054 cm^{-1} were attributed to C=N and a polysaccharide structure.

Except for a few published studies, statistical tools are not frequently used for enzyme immobilization. Most reports involving the optimization of conditions for the support production and enzyme immobilization evaluate a single parameter per trial. However, the present study used experimental design and surface response techniques to determine the best conditions for chitosan bead production. The adequacy of the model was also checked by an analysis of variance (ANOVA). The second-order model showed a proper fit to the experimental data, with an r^2 value of 0.898, indicating that the response could be explained by the model. Additionally, Fischer's F-test demonstrated the significance of the regression model, since the calculated F-value ($F_{0.95;5,6} = 4.39$) was higher than the critical F-value. Thus, an equation was established [Eq. (5)] to describe the EI of the 20 kDa keratinase as a function of the independent variables:

$$EI = 84.5 + 11.3X_1 + 13.0X_2 - 2.1X_1X_2 - 16.7X_1^2 - 7.0X_2^2 \quad (5)$$

The model was also used to generate the response surface (Fig. 3). The highest percent of immobilization was achieved at glutaraldehyde concentrations ranging from 34 to 56 g/l and an activation time between 6 and 10 h. Under these conditions, the achievable percent of keratinase immobilization was above 80%. This good loading efficiency for immobilization by covalent binding may have been due to the formation of a stable binding between the carrier and the enzyme through a spacer group (glutaraldehyde).

To investigate the behavior of the chitosan–enzyme system and estimate the maximum load capacity of the chitosan beads for the purified protease from *Chryseobacterium* sp. kr6, experiments using a fixed amount of chitosan beads and variable enzyme concentrations were carried out. The results of the proteolytic activity in both the liquid and solid phases after the immobilization process reached an equilibrium are shown in Fig. 4.

Some parameters of covalent immobilization of lipoprotein lipase onto chitosan beads have also been previously investigated [18], where it was demonstrated that the amount of immobilized lipoprotein lipase increased as the initial enzyme concentration was increased, at low concentration

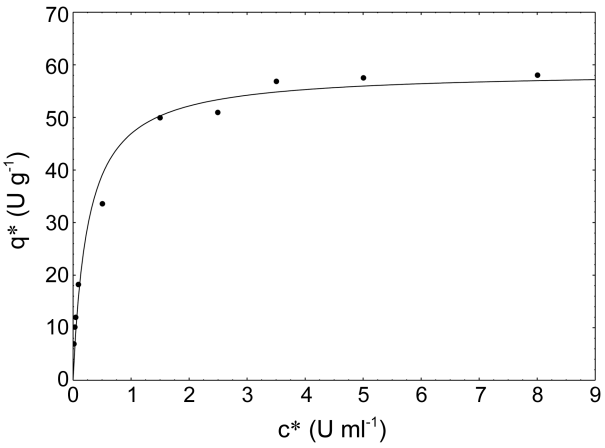


Fig. 4. Binding isotherm of chitosan/protease system.
Immobilization studies were performed at 20°C, at 100 rpm, during 12 h. q* = enzyme activity at equilibrium in solid phase; c* = enzyme activity at equilibrium in liquid phase.

levels below 4.0 mg/ml, and a plateau was obtained at a concentration higher than 5.0 mg/ml. The plot presented in the referred work is close to that obtained in the present study (Fig. 4).

To fit the experimental data, the Langmuir equation was used, resulting in a good agreement ($r^2 = 0.976$). Thus, it was possible to use this model to estimate the parameters q_m and K_d as 58.8 U/g and 0.254 U/ml, respectively.

The Langmuir model is often used to investigate the adsorption behavior of molecules to solid supports, especially to evaluate the protein adsorption to affinity and ion-exchange resins, aiming to optimize the downstream steps [8, 26]. This model assumes the existence of finite sites in the support and, as these sites are occupied, it becomes increasingly difficult for a solute molecule to find an available empty site. Recently, the Langmuir model was used to evaluate the adsorption mechanism of keratinase from *Nocardiopsis* sp. TOA-1 on keratin powder [24]. Those authors mentioned that the keratinase showed a high absorbability on keratin, plus insoluble substrate-hydrolyzing enzymes generally possess a high adsorption capability for insoluble substrates.

Stability and Recycling of Immobilized Keratinase

The immobilized and free keratinases were incubated at 50, 55, 60, and 65°C in a water bath for 30 min, and Fig. 5A shows the residual activity for each temperature. The activity loss was less for the immobilized enzyme than for the free enzyme. After 30 min at 65°C, the free keratinase only exhibited 13% residual enzymatic activity, whereas the immobilized keratinase exhibited around 34% residual activity. The immobilized enzyme also showed a higher stability at different pHs when compared with the free enzyme (Fig. 5B). It seems that immobilization on the chitosan beads affected the conformational flexibility of the enzyme, thereby increasing the rigidity of the enzyme and protecting it from unfolding, so the immobilized enzyme showed a higher stability than the free enzyme [32]. Similarly, chitosan-immobilized procerain B, a 25.7 kDa cysteine proteinase, showed an increased thermal stability in comparison with the free enzyme [31]. However, when compared with keratinase kr6, procerain B is stable at an acidic pH, yet more sensitive at an alkaline pH.

Another important factor for the application of immobilized enzymes is evaluating their capability for reutilization. The results obtained after five cycles of catalysis indicated that it was possible to reutilize the beads containing the immobilized protease from *Chryseobacterium* sp. kr6 at least five times, since the residual activity after the last cycle was 63.4%. Kannan and Jasra [19] previously reported some properties of immobilized alkaline serine endopeptidase from *B. licheniformis* on SBA-15 and MCF by surface covalent binding. The reusability of the immobilized endopeptidase showed 80% residual activity even after 15

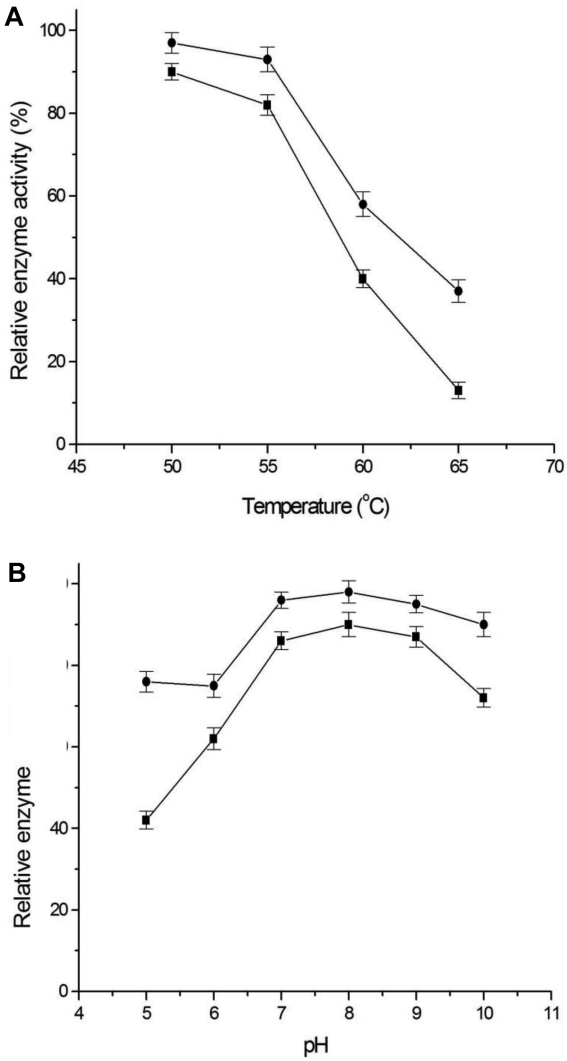


Fig. 5. Effects of temperature (**A**) and pH (**B**) on stability of free (■) and immobilized (●) proteases.
Values are presented as relative to control untreated free enzyme (30 U/ml) or immobilized enzyme (27.5 U/g), which were set as 100%.

cycles, although the activity decreased gradually at temperatures higher than 60°C. A commercial neutral proteinase immobilized on chitosan nanoparticles showed a residual activity of 88% after nine reuses [32], representing an increased operational stability when compared with the free enzyme.

This paper showed that chitosan spheres were suitable for immobilizing a purified protease from *Chryseobacterium* sp. kr6. The optimal conditions for the chitosan bead production were a chitosan concentration of 20 g/l, glutaraldehyde ranging from 34 to 56 g/l, and an activation time between 6 and 10 h. Under these conditions, above 80% of the enzyme was immobilized on the support. As the behavior of the protease immobilization on the chitosan beads fits well with the Langmuir model, the estimated

parameters were a q_m of 58.8 U/g and K_d of 0.254 U/ml. The thermal stability of the immobilized enzyme was improved around 2-fold, when compared with that of the free enzyme, after 30 min at 65°C. Furthermore, 63.4% of the activity of the immobilized enzyme remained after it was reused five times.

Acknowledgments

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Abbreviations

- c* Enzyme activity in liquid phase at equilibrium (U/ml)
- EI Enzyme immobilization (%)
- K_d Dissociation constant for enzyme–chitosan complex (U/ml)
- q_m Maximum loaded capacity (U/g solid support)
- q* Enzyme activity on solid support at equilibrium (U/g)
- r^2 Determination coefficient
- YI Yield of immobilization

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