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Original Research Article

Catalytic stability of turnip peroxidase in free and immobilized form on chitosan beads

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Keywords

Discoloration, Enzyme, Glutaraldehyde, Remazol Brilliant Blue R, Storage

A B S T R A C T

Chitosan beads were prepared, using glutaraldehyde as a cross linking agent for the immobilization of turnip peroxidase (TP). The morphology and structure of materials were examined by X-ray diffraction and scanning electron microscopy. The activity of free and immobilized TP was studied. The optimum pH was 7.0 for both free and immobilized enzyme and both were active in the temperature range between 30 ^{o}C and 50 ^{o}C. It was found that storage stability of the immobilized enzyme was better than that of the free enzyme. Both free and immobilized enzymes were used in the color removal of the dye Remazol Brilliant Blue R (RBBR). In discoloration experiments with immobilized TP, two phenomena were observed: discoloration, due to adsorption on the support (60.45%) and dye degradation, due to the enzyme action (27.50%). The free enzyme removed 62.86% of the color. The immobilized enzyme showed a potential of 61.17% for the removal of the dye color after 6 consecutive cycles.

Introduction

Enzymes found in nature have been exploited in industry due to their inherent catalytic properties in complex chemical processes under mild experimental and environmental conditions. Biocatalysts have been successfully exploited for the synthesis of various complex drug intermediates, especially chemicals, and even commodity chemicals in pharmaceutical, chemical, and food industries, due to their ability to catalyze reactions with high speed and specificity under a variety of conditions, as well as their potential as a greener alternative to chemical catalysts (Husain, 2006; Singh *et al.*, 2013).

Due to their substrate specificity and catalytic properties, biocatalysts have shown potential in wastewater treatment, biotransformations, as well as biosensor constructs. These enzymes could be exploited for the detoxification and

remediation of various aromatic pollutants such as phenols, aromatic amines, 2,4,6-trinitrotoluene and dyes, present in wastewater/industrial effluents coming out from several industries, such as textile dyes (Kulshrestha and Husain, 2006).

Peroxidases (donor H_2O_2 oxidoreductase, E.C.1.11.1.7) are enzymes that catalyze the reduction of peroxides, such as hydrogen peroxide and the oxidation of a variety of organic and inorganic compounds (Hamid and Khalilur, 2009).

Peroxidase has been used in biotechnology and several other areas of science for establishing clinical diagnoses, in the evaluation of pathological processes, food quality analysis, construction of biosensors for qualitative and quantitative analysis of pharmaceutical and cosmetic formulas, and in paper and cellulose manufacturing (Maciel *et al.,* 2006, 2007). This great diversity of applications is due to the wide substrate specificity of peroxidase catalysis (Hiner *et al.,* 2001). Furthermore, due to the oxidative nature of peroxidases, there are several areas where it could replace current chemical oxidant techniques (Hamid and Khalilur, 2009).

For environmental purposes, peroxidases have been employed for detoxification and removal of various organic pollutants, like phenols, aromatic amines and dyes from polluted wastewater (Regalado *et al.,* 2004). However, a major obstacle in the commercial application of peroxidases for environmental purposes is their limited stability and reusability, which means that a continuous supply of a large amount of fresh enzyme is required (Akhtar and Husain, 2006). The stability and catalytic ability of soluble enzymes dramatically decrease with effluent complexity (Zille *et al.,* 2003). The industrial application of enzymes is often hampered by their lack of long-term operational stability and shelf-storage life, their cumbersome recovery and reuse. In many cases, a simple way to avoid at least some of these drawbacks is to immobilize enzymes, and a major challenge in industrial biocatalysis is the development of stable, robust and preferably insoluble biocatalysts (Satar and Husain, 2011; Singh *et al.,* 2013). Immobilized enzymes improve thermostability, operational stability, recovery, reusability, high purity and high product yields in their industrial applications (Kennedy and White, 1985). To make enzymes cost-effective, long-lived, and highly active, various supports have been used to immobilize enzymes (Wang *et al.,* 2009).

Natural polymers used as carrier materials in immobilization technology, such as alginate, carrageenan, agarose, chitin, and chitosan, have the advantages of being nontoxic, biocompatible and biodegradable (Chun *et al.,* 1996). Chitosan is a deacetylated product of a natural polymer of chitin; it is a polycationic polysaccharide with abundant amino groups, widely used for numerous applications in industrial and biomedical areas, which include: wastewater treatment, chromatographic support, enzyme immobilization and carriers for controlled drug delivery (Franca *et al.,* 2008; Jiang *et al.,* 2005; Kosaraju *et al.,* 2006; Zhai *et al.,* 2013).

Chitosan is known as an ideal support material for enzyme immobilization, since its chemical structure, containing reactive amino and hydroxyl groups, makes chitosan easily modified, and also because of its many characteristics, like improved mechanical strength, resistance to chemical degradation, avoiding the disturbance of metal ions to the enzyme, anti-bacterial properties and low cost (Duran *et al.,* 2002;

Juang *et al.,* 2002; Li *et al.,* 2009). Immobilization can be carried out by using glutaraldehyde to form the Schiff's base (Jiang *et al.,* 2005). Glutaraldehyde is used for immobilization because, in addition to activating the beads, glutaraldehyde also stabilizes enzymes due to the multipoint attachment, as well as due to its polymeric nature. Furthermore, glutaraldehyde provides a long lash, attaching the protein to the matrix and allowing a greater flexibility for conformational changes required for the activity (Singh *et al.,* 2013).

The properties of the immobilized turnip peroxidase (TP) on chitosan beads were investigated. Experiments were carried out to determine the characteristics of the support and the effect of immobilization on enzyme activity. The best conditions for peroxidase immobilization were defined and some properties of the immobilized system were compared to the free enzyme, including optimum pH and temperature, storage stability, application in dye removal and reusability.

Materials and Methods

Dye

The textile dye Remazol Brilliant Blue R (RBBR), which presents an anthraquinone as chromophore, was kindly provided by DyStar (Porto – Portugal) and used as received, without further purification. The dye solution used for degradation experiments was prepared with distilled water.

Obtention of the enzymatic extract

The enzyme was extracted from turnip roots purchased from the local market. The roots (with peel) were washed in water and cut into small uniform pieces. Turnip roots (30 g) were homogenized in a blender with 100 mL of 0.05 mol L^{-1} pH 6.5 phosphate buffer for 30 s. The homogenate was filtered in organza cloth and centrifuged at 10000 × g for 15 min, at 4 ^{o}C (Silva *et al.,* 2012). The obtained solution was subjected to precipitation, by adding cold acetone until reaching 65% (v/v). After a rest from 12 to 14 h, at -18 ^{o}C, the homogenate was centrifuged at 11000 × g for 15 min, at 4 ^{o}C. The supernatant was collected and acetone was subsequently recovered by distillation in a rotary evaporator, at controlled temperature of 56 ^{o}C. The precipitate containing the peroxidase was submitted to the removal of acetone by evaporation in an ice bath for 24 h and was used in the testing storage assays, in the assays of enzyme immobilization, and then used in the dye removal studies.

Preparation of chitosan beads

1.5 g chitosan (low molecular weight, obtained from Sigma) was dissolved in 40 mL of 2.0% aqueous acetic acid. The chitosan solution was dropped into an aqueous 2 mol L^{-1} NaOH solution, where chitosan precipitated immediately to form gelatinous beads (Goy *et al.,* 2004). The chitosan beads were thoroughly washed with distilled water until neutrality was reached.

Cross linking of chitosan beads with glutaraldehyde treatment

The cross linking of chitosan beads with glutaraldehyde was carried out by immersion of 17.60 g hydrated chitosan (1 g dry weight of chitosan) in 15 mL of 2.5% glutaraldehyde solution, stirring for 24 h at 25 ^{o}C. The beads were washed with distilled water to remove excess glutaraldehyde.

Characterization of chitosan beads

Diameter and density of the chitosan beads were determined by picnometry, and bead porosity was determined using the

gravimetric method in three replications (Gonçalves *et al.*, 1997).

The chitosan degree of deacetylation was obtained by potentiometric titration in three replications (Broussignac, 1972). The structure of the materials was analysed by X-ray diffraction (XRD) spectra, data were collected using an X'Pert Pro Multi-purpose X-ray diffraction (MPD) system employing Cu Kα radiation (λ= 0.154 nm), and operated at 40 mA and 45 kV. The morphology of the materials was obtained using a SEM LEO 440 equipment provided with an OXFORD detector, and the electron beam operated at 15 k.

Immobilization of turnip peroxidase

The cross linked chitosan beads were used as supports for the immobilization of turnip peroxidase; 120 beads were added (or 5.04 g ± 0.015 hydrated weight of the beads) in 10 mL of TP precipitate dissolved in 0.1 mol L^{-1} phosphate buffer pH 7.0. The assembly was subjected to slight stirring in a water bath at 20 °C, and aliquots of supernatant were removed at 0, 1, 2, 3, 4, 5 and 6 h. The total protein determination was carried out according to Bradford (1976) and the enzymatic activity, by the method proposed by Khan and Robinson (1994). After the determination of total protein and enzymatic activity, the coupled protein (CP) and immobilization yield (IY) were estimated according to equations (1) and (2), respectively:

$$\text{Coupled protein (\%)} = \frac{\text{amount coupled protein}}{\text{amount introduced protein}} \times 100 \quad (1)$$

$$\text{Immobilization yield (\%)} = \frac{At_0 - At_t}{At_0} \times 100 \quad (2)$$

At_t = enzymatic activity of the supernatant after the incubation period and At_0 = enzyme activity of the supernatant before incubation

Activity measurement of free and immobilized turnip peroxidase

The activities of the free and immobilized enzyme were tested according to the methodology described by Khan and Robinson (1994), with modifications; the following reaction media were used: 1.5 mL guaiacol (Vetec; 97%, v/v) 1% (v/v); 0.4 mL H_2O_2 (Vetec, PA) 0.3% (v/v); 0.1 mL free enzyme (or approximately 4 mg immobilized enzyme, the equivalent weight of a bead) and 1.2 mL of 0.1 mol L^{-1} phosphate buffer pH 7.0. The reaction was monitorized during 3 min at 30 ^{o}C, using a Spectrovision spectrophotometer coupled to a thermostatic bath.

Tests for the immobilized enzyme were performed in the same conditions used for the free enzyme, except that the reaction medium was maintained with stirring and interrupted by removal of the chitosan beads from the reaction mixture. The absorbance of the mixture was determined, and the enzymatic activity of immobilized peroxidase was calculated.

One unit of peroxidase activity represents the formation of 1 µmol tetraguaiacol during 1 min in the assay conditions and it was calculated using data relative to the linear portion of the curve.

The relative activity was calculated using equation 3.

$$\text{Relative activity (\%)} = (\text{Activity/Maximum activity}) \times 100 \quad (3)$$

Influence of pH

The optimum pH was determined by varying the pH of the buffer solutions from 2.0 to 9.0 in intervals of one pH unit. Reaction rates of free and immobilized enzyme preparations were investigated using the

buffers citrate buffer (0.1 mol L^{-1}, pH 2.0 to 6.0), phosphate (0.1 mol L^{-1}, pH 7.0) and Tris-HCl buffer (0.1 mol L^{-1}, pH 8.0 and 9.0). The immobilized enzyme was incubated for 10 min in the buffers before the activity measurement.

Influence of temperature

The thermal stability of peroxidase was evaluated by incubating the free and immobilized enzyme for 1, 2, 3 and 4 h, and by varying the temperature from 20 ^{o}C to 90 ^{o}C at intervals of 10 ^{o}C. After the thermal treatment, the samples were cooled in an ice bath, and the residual activity was determined.

Storage stability of free and immobilized turnip peroxidase

The activity of free TP was investigated subjecting the precipitate to the four forms of storage, in three replicates : A) 0.2 g of TP precipitate stored in eppendorf at -20 ^{o}C in the freezer; B) 0.2 g of TP precipitate stored in the refrigerator at 3 ^{o}C ; C) 1.5 mL of enzyme extract stored in eppendorf in the freezer at -20 ^{o}C (prepared from 4 g of TP precipitate resuspended in 30 mLof 0.1 mol L^{-1} phosphate buffer pH 7.0) and D) 1.5 mL of enzyme extract stored in the refrigerator in eppendorf at 3 ^{o}C (the same preparation which was performed in C).

The activity of the stored immobilized enzyme was measured in two different ways: A) beads stored in the refrigerator at 3 ^{o}C; B) beads stored in the freezer at -20 ^{o}C. From 10 to 10 days, the activity of free and immobilized enzyme was measured for each form of storage for a period of 170 days.

Discoloration assay

Based on the methodology described by Silva *et al.* (2012) with modifications, the enzymatic oxidation reactions of the textile dyes were conducted at 30 ^{o}C in a 1.2 mL phosphate buffer (0.1 mol L^{-1}, pH 7.0) containing 0.4 mL H_2O_2 (100 μmol L^{-1}), 1.5 mL Remazol Brilliant Blue R (RBBR) (50 mg L^{-1}) , and 0.1 mL free enzyme (23.80 U mL^{-1} or specific activity 63.13 U mg^{-1}) and, for the immobilized enzyme, 0.012 g ± 0.003 (0.036 U $bead^{-1}$ or specific activity 5.61 U mg^{-1}).

The reaction mixture was incubated in a water bath with mild stirring. The consumption of RBBR was monitored at 596 nm, which corresponds to the maximum absorption wavelength of this dye. The amount of oxidized dye was estimated according to the equation 4.

$$\frac{absorbance_{initial} - absorbance_{final}}{absorbance_{initial}} \times (100) \quad (4)$$

The same procedure was performed with the chitosan beads without enzyme to evaluate the adsorption material; the analysis was carried out in three replications.

Reusability of immobilized turnip peroxidase on dye discoloration

Repeated application of immobilized TP was studied by the repeated use of immobilized peroxidase beads for RBBR removal [1.5 mL dye concentration 50 mg L^{-1}; H_2O_2 dose 0.4 mL; 1.2 mL of 0.1 mol L^{-1} phosphate buffer pH 7.0, 0.012 g ± 0.003 of immobilized peroxidase (0.036 U $bead^{-1}$)]. The reaction mixture was incubated in a water bath with mild agitation during 80 min. At the end of each cycle, the same immobilized enzyme was washed with 0.1 mol L^{-1} phosphate buffer pH 7.0, and the procedure was repeated with a fresh aliquot of substrate. The analysis was carried out in three replications.

Operational stability

The residual activity of the immobilized

enzyme was determined under standard assay conditions. Several consecutive operating cycles were performed using guaiacol and H_2O_2 as substrates, in order to evaluate assess the operating stability of the immobilized TP. At the end of each cycle, the same immobilized enzyme was washed with 0.1 mol L^{-1} phosphate buffer pH 7.0, and the procedure was repeated with a fresh aliquot of substrate. The analysis was carried out in three replications.

Results and Discussion

Turnip peroxidase characterization

Due to the widespread use of peroxidases, many works have reported the importance of peroxidases production and its application, however, high costs associated with biocatalyst production and application still hinder their use on a large scale with environmental purposes and there is a growing interest in new sources of this enzyme and processes for obtaining it at a low cost. The novelty in this work consists at obtaining an alternative and low-cost plant peroxidase from turnip and in the immobilization of the obtained enzyme onto chitosaneous material.

It is important to emphasize that immobilized enzyme preparations, in most cases, require the use of commercially available enzymes, which makes the application of immobilized enzymes an unfavorable process from an economical point of view (Singh *et al.,* 2013). In other works developed by our research group (Silva *et al.,* 2012) we developed a low-cost process for obtaining turnip peroxidase in which no purification was performed to the crude extract. With this purpose in this work we investigated the immobilization of the biocatalyst obtained to improve important parameters like thermostability, operational stability, recovery, reusability and others. Another aspect also investigated in this work was the storage stability of the immobilized and free enzyme. Thus, the strong point of this manuscript is the optimization of a low cost process to obtain the enzyme.

After the precipitation of TP, the mass yield was 5.25 % ± 0.17. The enzymatic TP extract was subjected to electrophoresis to estimate the molecular weight and a denatured broad peroxidase band was detected, with an approximate molecular weight of 39.50 kDa. Similar results were obtained by Quintanilla-Guerrero *et al.* (2008), who found an approximate molecular weight of 40.0 kDa for purified turnip peroxidase. Also, Duarte Vazquez *et al.* (2000) observed that the molecular weight of denatured peroxidases were 39.2 to 42.5 kDa for the isoenzyme.

Characterization of chitosan beads

In this study, the immobilization of TP onto chitosan beads with 2.5% glutaraldehyde solution was investigated. Glutaraldehyde activation of solid support media has been extensively studied for biomolecule immobilization since it is simple, efficient and inexpensive (Goddard and Hotchkiss, 2007). Glutaraldehyde is used to activate aminated supports and immobilize the enzyme by physical adsorption, followed by covalent binding, immobilizing the enzyme on a glutaraldehyde pre-activated support (Aybastier *et al.,* 2011; Betancor *et al.,* 2006). The importance of glutaraldehyde activation is due to its reliability and ease of use, but more importantly, due to the availability of amino groups for glutaraldehyde reaction, not only with enzymes, but also with chitosan (Krajewska, 2004).

During the reaction, the aldehyde group on

the surface of chitosan beads interacted with the amino group of the enzyme to form an imino group (–CH=N–). Multipoint covalent attachment of enzymes on highly activated supports promotes a rigidification of the structure of the immobilized enzyme. This rigidification reduces any conformational changes involved in enzyme inactivation and increases enzyme stability (Mateo *et al.,* 2007).

The properties of the immobilized enzyme are significantly determined by the immobilization procedure; thus, it is important to discuss the main factors that influence TP immobilization.

Therefore the immobilization process was realized in a neutral pH. In these conditions the covalent immobilization on chitosan beads activated with glutaraldehyde is more efficient, due to amino groups available in the structure of the chitosan reacting with this agent under mild conditions (Jiang *et al.,* 2005).

Recent studies show that chitosan can be used to prepare beads for different purposes. Its versatility allows the preparation of polymer beads of different shapes and sizes, including different products and derivatives. It has been shown that supports with different particle sizes have an effect of the amount of immobilized enzyme, immobilization yield, immobilized enzyme activity and specific activity of the immobilized enzyme. The use of porous chitosan particles presents numerous advantages in the enzyme immobilization process (Aybastier *et al.,* 2011; Azevedo *et al.,* 2007).

Table 1 shows the results of the physical characterization of chitosan beads activated with glutaraldehyde. It can be observed that the activated beads showed a decrease in particle porosity and diameter, when compared to non-activated particles. This decrease was probably due to the crosslinking of polymeric chitosan chains with the glutaraldehyde that approached the chains and, therefore, decreased the pore sizes of the matrix (Adriano *et al.,* 2005). On the other hand, particle density did not change after activation.

The degree of deacetylation (DD) is defined as the average number of amino groups in relation to the amide groups of the polymer chain (Raymond *et al.,* 1993). The DD calculated in the chitosan powder and in the beads without crosslinking by this method was 77.02% ± 1.17 and 80.7% ± 1.35. The beads cross linked with glutaraldehyde did not present protonatable amino groups, measured by the potentiometric titration method. This result suggests that glutaraldehyde bound to the chitosan amino groups.

The structure of the materials was studied by X-ray diffractometry. All materials showed diffraction peaks at $2\theta = 20°$ related to chitosan (Fig. 1). In Fig. 1A and 1B, in addition to the peak at $2\theta = 20°$, a halo diffraction was observed at $2\theta = 10°$, also referring to chitosan; these peaks are in agreement with the results described in the literature (Liu and Bai, 2005; Martins *et al.,* 2004). According to Uragami and Tokura (2006), strong intra-and intermolecular interactions, due to hydrogen bonds between the amine groups, alcohol and amide groups present in the molecule of chitosan, make this material present certain crystallinity. In Fig. 1C, it was possible to observe the complete disappearance of the diffraction peak at $2\theta = 10°$ together with a significant decrease in the crystallinity of the material after activation with glutaraldehyde.

The immobilization of peroxidase on the

surface of chitosan beads activated with glutaraldehyde was analyzed by scanning electron microscope (SEM). The results obtained by SEM showed the formation of small granules of irregular sizes for the chitosan powder (Fig. 2, A and B). Following the procedure for obtaining the chitosan beads, it is noted that the bead without treatment with glutaraldehyde (Fig. 2, C and D) showed roughness on its surface, while the beads crosslinked with glutaraldehyde had a significant change in the surface (Fig. 2, E and F), making it more uniform. In the reaction of chitosan with glutaraldehyde, there was a nucleophilic attack of the amino groups of chitosan to the carbonyl groups of glutaraldehyde, causing it to resist extremes of pH and temperature (increased physical and chemical stability of chitosan) (Kosaraju *et al.*, 2006; Krishna *et al.*, 2011). It was found, however, that crosslinking did not generate pores in the surface, practically preserving the original dimensions of the beads before crosslinking. Changes in the topology of the beads (Fig. 2, G and H) confirm the immobilization of the enzyme on the surface of chitosan beads.

Immobilization of turnip peroxidase

Turnip peroxidase was immobilized on chitosan beads for different times, ranging from 1 h to 6 h. The effect of time on TP immobilization in terms of *coupled protein* (CP) and *immobilization yield* (IY) is shown in Fig. 3. The coupling time of 4 h gives the highest coupled protein and immobilization yield (CP = 50.48%, and YI = 51.23%). However, prolonging the reaction time up to 6 h, decreases in immobilization yield and coupled protein were observed.

The effect of different concentrations of the free enzyme was investigated in the immobilization process by *coupled protein* (CP) and *immobilization yield* (IY). A decrease in immobilization yield and coupled protein was observed (Table 2), due to the increasing concentration of free enzyme; however, the immobilized enzyme activity increased, and it may be due to saturation of the binding sites of the support, causing a partial or total blockage of the pore entrance (Adriano *et al.*, 2005).

Effect of pH on the activity of turnip peroxidase

The results of pH on the activity of free and immobilized enzyme are presented in Fig. 4. The stabilities of both enzymes show some similarities. In acidic pH, TP presented a decrease in activity. This activity decrease might have occurred mainly by ionic alterations of the enzyme, which alter the form of the enzyme and, consequently, the active site. The activity decrease can also be observed at pH 8 and 9.

Both free and immobilized enzyme presented an optimum pH of 7.0; however, the immobilized peroxidase had broader pH stability than the free enzyme. It suggested that the immobilized enzyme was less sensitive to pH changes than the free enzyme, owing to the protection of the enzyme by immobilization. However, it has been demonstrated that the entrapment of enzymes in gel beads provides a microenvironment for enzyme, which plays an important role in the protonation state of the protein molecule (Matto and Husain, 2007; Matto *et al.*, 2009).

The procedure of enzyme immobilization on insoluble carriers has a variety of effects on protein conformation, as well as on the ionization state of the enzyme and its environment, and it is not uncommon to result in changes in the relationship between pH and enzyme stability, and activity (Costa *et al.*, 2001).

Effect of temperature on activity of turnip peroxidase

The study of the effect of temperature on the activity of the free and immobilized enzyme after 1 h, 2 h, 3 h and 4 h of incubation is shown in Table 3. The thermal stability of the free and immobilized enzyme after 1 hour of incubation follow the same profile, both showed a high activity at 30 ^{o}C and, at 60 ^{o}C, a decrease in the reaction rate was observed . After 70 ^{o}C, the free and immobilized enzymes are completely denatured.

After 4 h of incubation at 50 ^{o}C and 60 ^{o}C, the free enzyme displayed a relative activity of 82.65% and 60.47% respectively, whereas the activity of the immobilized enzyme dropped significantly at these temperatures (53.84% and 19.23% relative activity), indicating that the enzyme activity became more dependent on the temperature after immobilization. The enzyme thermal stability is one of the important criteria for its long-term and commercial application: the higher stability and enzymatic activity, the better enzyme application capacity in diverse methods, such as application as biocatalysts (Costa *et al.,* 2001; Maciel *et al.,* 2007). According to the results obtained in a temperature range of 30 ^{o}C to 50 ^{o}C, the free and immobilized enzymes did not present a significant decrease in the enzymatic activity, which favors its use in industrial processes.

Increased operational stability of immobilized enzymes is essential in order to achieve the cost benefits already mentioned. Enzyme stability can be controlled by assaying the activity decay over time until half-life activity is reached (Cardoso and Emery, 1978). This is a useful means of control when thermal inactivation takes place according to first-order kinetics. Table 3 presents the half-life data at each temperature, the free enzyme unstable at high temperature, with a half-life approximately 3 times greater than the immobilized enzyme at 50^{o}C and 60^{o}C, however the immobilized enzyme presents a remarkable increase in thermostability at 20^{o}C and 30^{o}C.

Storage stability of turnip peroxidase

The enzymatic activities for the four storage forms of the free TP ((A) precipitated enzyme at -20 ^{o}C, (B) precipitated enzyme at 3 ^{o}C, (C), enzyme extract at -20 ^{o}C and (D) enzyme extract at 3 ^{o}C) are shown in Table 4. The enzymatic activity was maintained at an average of 80% within 90 days, regardless of the storage form. After 170 days of storage, form (A) showed the highest enzyme activity with a loss of 33.4%, while form (B) presented a loss of 52.4%, which can be attributed to fungal growth, which occurred after 80 days of storage. The obtained data suggests, therefore, that the storage form B was not adequate.

Mohamed *et al.* (2012) studied the storage of miswak peroxidase, measuring peroxidase activity in liquid and powder (lyophilization) forms during 8 weeks at 4 °C, with a relative activity of 70% and 95%, respectively. At the end of the storage period, the results were similar to those found in the present study, considering enzyme powder the best form of storage.

For enzyme immobilization, another important factor that needs to be taken into account is the storage stability of the immobilized enzyme. The enzymatic activities for the two storage forms of the immobilized enzyme (E) beads stored in the refrigerator at 3 ^{o}C; (F) beads stored in the freezer at -20 ^{o}C) are shown in Table 4. The

enzymatic activity of storage form (E) was kept around 85% after 80 days and, at the end of storage, showed a loss in activity of 68%. The storage of the immobilized enzyme in the freezer showed excellent results, since it was observed that it maintained its activity by 100% up to 130 days, with a loss of only 9% at the end of storage.

The immobilized TP retained about 91% of its original activity after 170 days at -20^{o}C (F), while the free enzyme maintained about 66% of its original activity over the same period of time (A). The reason might be attributed to possible distortion effects imposed by the aqueous medium. The charged residues could be neutralized by the interaction with the substrate or less exposed after immobilization, thus leading to an increase in the resistance of the immobilized TP to conformational changes in solution (Zhai *et al.*, 2013).

The operational stability of an enzyme can be quantified by its half-life, or the length of time after which 50% of its original activity has degraded. A first-order model was used to determine the half-life time of the free and immobilized enzymes with the data presented in Table 4. The half-life activity of 4 forms the free enzyme storage were determined with a mean of 213 days, form (A) showing the longer half-life. The immobilized enzyme (F - half-life time 1732.0 days) exhibited a seven fold longer half-life activity in comparison (A- half-life time 247.5 days) to the free enzyme. For both free and immobilized enzyme, the best way to store was keeping the enzyme at -20 ^{o}C.

Discoloration of RBBR by the free and immobilized enzyme

The dye Remazol Brilliant Blue R (RBBR) has been used as a model substance in several studies on dye degradation and different kinds of physical, chemical and biological processes have been tested for its removal. RBBR is an industrially important dye that is frequently used as a starting material in the production of polymeric dyes, is a derivative anthracene and represents an important class of toxic and recalcitrant organopollutants (Fontenot *et al.*, 2002).

The discoloration of the dye in aqueous solution catalyzed by the free TP (23.80 U mL^{-1}) was 62.86% ± 1.8 after 40 min in the presence of the enzyme (Fig. 5A). Similar results were found by Silva *et al.* (2012), who obtained 66.00% ± 3.8 of RBBR discoloration in aqueous solution with turnip peroxidase under similar conditions. The removal of RBBR catalyzed by the immobilized enzyme was 87.95% ± 0.56 after 140 min of incubation (Fig.5B). The greater discoloration performance of the immobilized enzyme, in comparison to the free enzyme, could be explained by a high dye adsorption on the chitosan support. Two phenomena were observed: discoloration, due to adsorption on the support (60.45%) and dye degradation, due to the enzyme action (27.50%).

Chitosan is well known as an excellent biosorbent of heavy metal ions, dyes and proteins. It contains a large number of –NH_2 and –OH groups and is able to remove this stuff in near–neutral solutions, through various types of interaction mechanisms, such as electrostatic attractions, chelation, among others. This explains the high adsorption of the dye in chitosan beads (Crini, 2005; Liu *et al.*, 2013).

Similar behaviors were observed by Zille *et al.* (2003), who investigated the removal of the dye Reactive Black 5, catalyzed by free

and immobilized laccase. In this study, a dye removal of 79% was observed, due to adsorption on the alumina support, and 4% due to the enzyme action.

Reuse of immobilized turnip peroxidase in the discoloration of RBBR

Reusability is one of the significant indices to evaluate the application of immobilized enzymes in industries (Akhtar and Husain, 2006). Besides enhanced stability, enzymes can acquire additional advantageous properties via immobilization: immobilized enzymes can be used repeatedly or continuously in a variety of reactors; can be easily separated from soluble reaction products and untreated substrate and they involve reduced operational cost (Krajewska, 2004; Matto and Husain, 2009).

The immobilized enzyme could be easily removed and assessed for its remained catalytic activity. In this study, after 6 times of the repeated test, the dye removal efficiency was reduced to 38.83% of the initial value (Fig. 6 A). This result might be explained by the plugging of the chitosan pore through the adsorption of the dye in the interior environment of each bead, reaching the active site of the enzyme, resulting in the loss of the enzymatic activity.

Operating stability

The operational stability of immobilized TP is shown in Fig. 6B. After 5 consecutive operations, the immobilized enzyme could retain 55.25% ± 1.01 residual activity and at the end of 8 cycles, the enzyme still retained 43.18% ± 0.43 residual activity. This loss in activity may be due to the adsorption of tetraguaiacol (product formed in the reaction medium), because it causes the beads to brown after subsequent cycles.

Monier *et al.* (2010) observed that, after 6 consecutive operations, the immobilized horseradish peroxidase (HRP) on modified chitosan beads could retain 65.8% residual activity. Arslan (2011) also obtained better results, using a different material in the immobilization, the immobilized HRP on the activated poly (ethylene terephthalate) fibers retained a relative activity of 69.6% after 5 cycles. These responses can be better attributed to the use of different carriers and purified enzymes.

Table.1 Physical characterization of chitosan beads

Chitosan beads	Density (g mL^{-1})	Bead diameter (mm)	Porosity
Non-activated beads	1.06 ± 0.06	0.44 ± 0.01	1.04 ± 0.04
Activated glutaraldehyde	1.07 ± 0.06	0.42 ± 0.01	0.93 ± 0.00

Data are the average of three replicates.

Table.2 Effect of different concentrations of the free turnip peroxidase on the *coupled protein* (CP) and *immobilization yield* (IY)

Enzyme (U mL^{-1})	IY (%)	CP (%)	Enzyme activity U $beads^{-1}$
4.60	84.33	92.01	0.005
8.47	79.20	85.32	0.015
17.31	70.83	72.00	0.026
34.61	58.33	53.91	0.045

U = 1 µmol tetraguaiacol min^{-1} of reaction.

Table.3 Influence of temperature and incubation time on the stability of turnip peroxidase

	Relative Activity (%)									
Time	1 hour		2 hours		3 hours		4 hous		**half-life (t1/2)(hours)**	
Temperature (^{o}C)	Free Enzyme	Immobilized Enzyme	Free Enzyme	Immobilized Enzyme	Free Enzyme	Immobilized Enzyme	Free Enzyme	Immobilized Enzyme	Free Enzyme	Immobilized Enzyme
20 ^{o}C	91.27	92.31	83.88	92.31	87.58	92.31	87.58	92.31	**10.50**	**38.50**
30^{o}C	98.77	100	82.65	88.46	81.42	84.61	81.42	84.61	**11.55**	**14.40**
40^{o}C	92.50	88.46	91.27	88.46	91.27	80.77	91.27	80.77	**38.50**	**14.40**
50^{o}C	92.50	80.77	91.27	73.08	82.65	53.85	82.65	53.85	**16.50**	**5.25**
60^{o}C	71.56	50	66.63	26.92	60.47	19.23	60.47	19.23	**6.07**	**1.62**
70^{o}C	0.236	0	0	0	0	0	0	0	-	0
80^{o}C	0	0	0	0	0	0	0	0	-	0
90^{o}C	0	0	0	0	0	0	0	0	-	0

Data are the average of three replicates.

Table.4 Storage stability of turnip peroxidase (TP)

	Relative Activity (%)					
	Free Enzyme				Immobilized enzyme	
Storage (Days)	A*	B*	C*	D*	E*	F*
0	100.0	100.0	100.0	100.0	100.0	100.0
10	100.0	100.0	100.0	95.81	100.0	100.0
20	100.00	90.46	100.0	100.0	99.96	100.0
30	96.29	100.0	100.0	91.63	98.35	100.0
40	92.58	100.0	100.0	87.44	97.01	100.0
50	88.87	90.46	100.0	87.44	92.53	100.0
60	88.87	95.23	93.30	87.44	92.27	100.0
70	88.87	85.69	93.30	79.13	92.01	100.0
80	81.48	95.23	86.61	79.13	85.28	100.0
90	85.17	85.69	79.91	74.98	63.57	100.0
100	77.78	85.69	79.91	74.98	59.68	100.0
110	77.78	76.19	66.62	74.98	51.68	100.0
120	77.78	71.44	66.62	74.98	48.22	100.0
130	77.78	76.19	66.62	74.98	45.53	99.33
140	66.65	66.67	59.97	66.67	42.37	97.26
150	66.65	61.90	59.97	62.51	38.94	94.51
160	59.26	66.67	59.97	62.51	36.72	91.38
170	66.65	47.63	59.97	54.14	31.77	91.14
half-life (t1/2)(days)	**247.5**	**203.8**	**177.7**	**223.5**	**93.64**	**1732.0**

* The enzymatic activities for the four storage forms of the free TP (A) precipitated enzyme at -20 ^{o}C (B) precipitated enzyme at 3 ^{o}C (C) enzyme extract at -20 ^{o}C and (D) enzyme extract at 3 ^{o}C); the enzymatic activities for the two storage forms of the immobilized enzyme (E) beads stored in the refrigerator at 3 ^{o}C (F) beads stored in the freezer at -20 ^{o}C. Data are the average of three replicate.

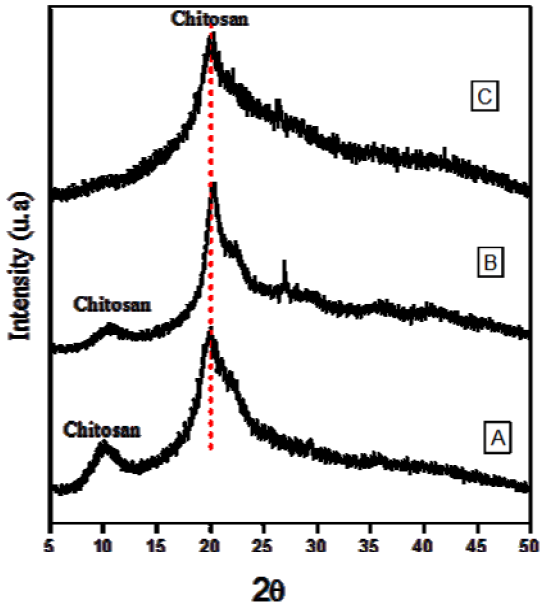

Fig.1 X-ray diffraction. A) powder chitosan; B) chitosan beads without crosslinking; C) beads crosslinked with 2.5% glutaraldehyde

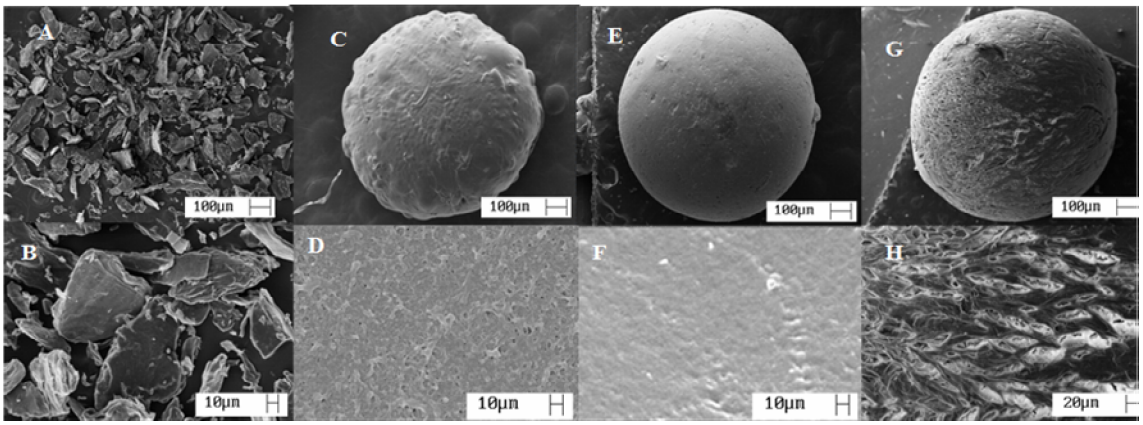

Fig.2 Scanning electron microscope (SEM) images. A) chitosan powder (100 µm) ; B) chitosan powder (10 µm); C) beads without cross-linking (100 µm); D) beads without cross-linking (10 µm); E) chitosan beads crosslinked with 2.5% glutaraldehyde (100 µm); F) beads cross-linking with 2.5% glutaraldehyde (10 µm); G) chitosan beads after immobilization of peroxidase (100 µm); H) chitosan beads after immobilization of peroxidase (20 µm)

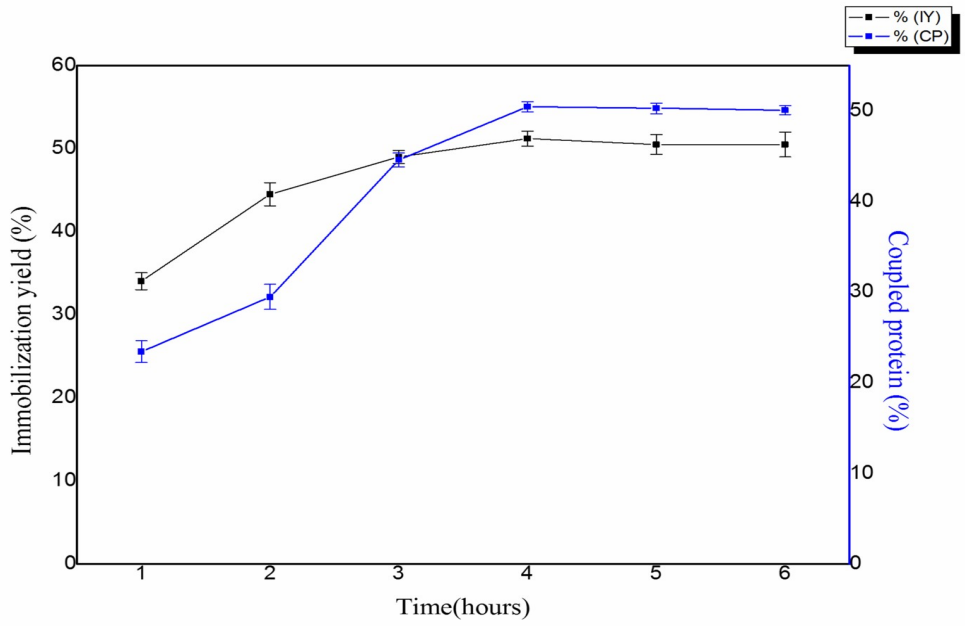

Fig.3 Effect of time on immobilization of turnip peroxidase at 20 ^{o}C

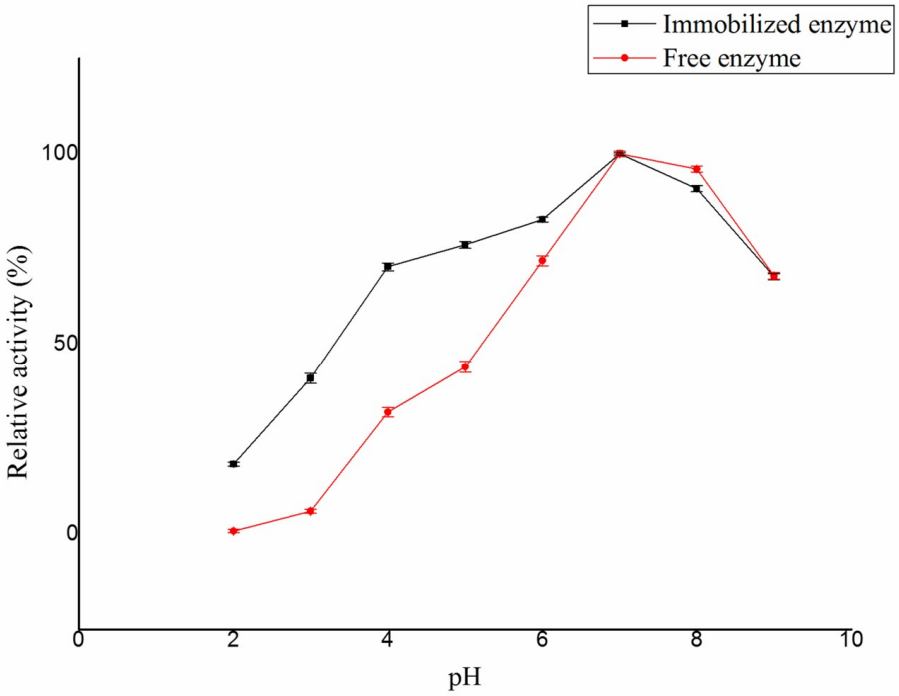

Fig.4 Effect of pH on the activity of turnip peroxidase

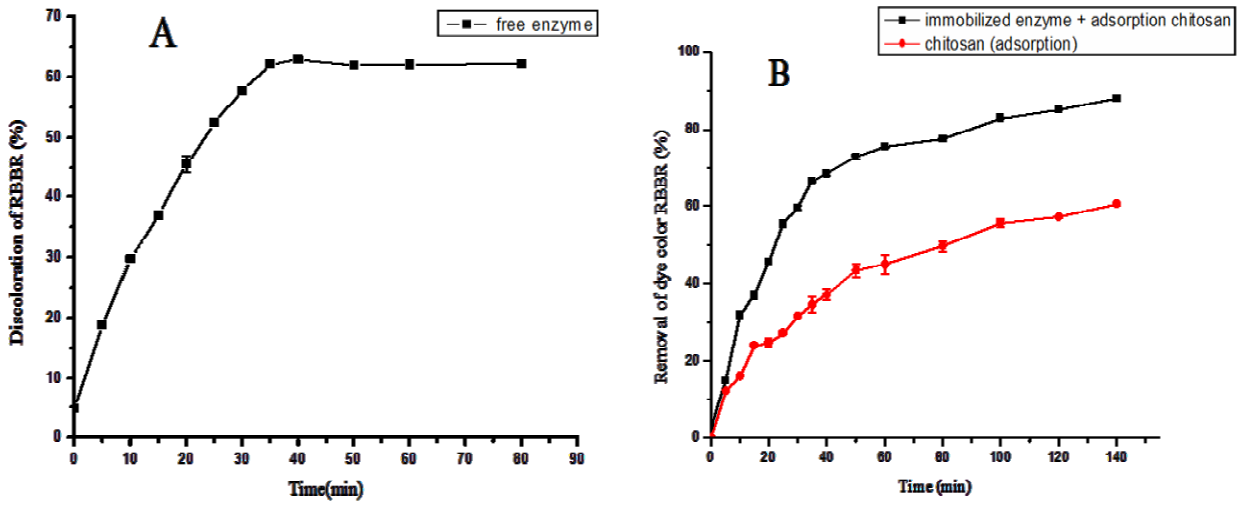

Fig.5 Reaction progress on the discoloration of Remazol Brilliant Blue R. A) free turnip peroxidase; B) immobilized turnip peroxidase and adsorption on chitosan. Data are the average of three replicates

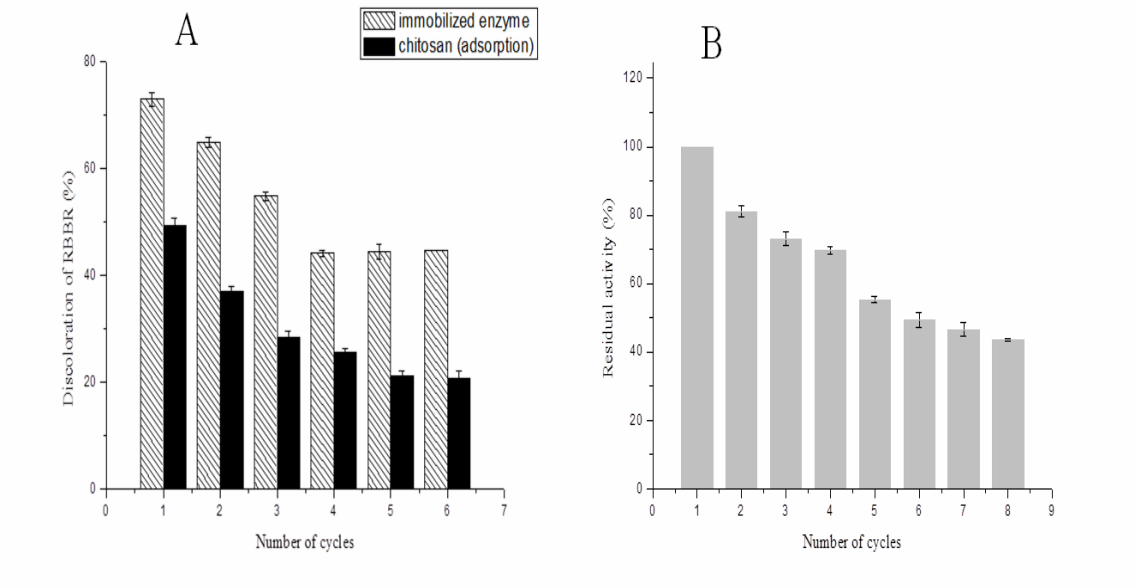

Fig.6 A) Removal of 50 mg L^{-1} Remazol Brilliant Blue R with repeated applications of immobilized turnip peroxidase; **B)** Operating stability of the immobilized turnip peroxidase. Data are the average of three replicates

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