MULTIPOINT IMMOBILIZATION AND STABILIZATION OF AMINED PEROXIDASES FROM SOYBEAN HULL AND CHAYOTE EMPLOYING BACTERIAL CELLULOSE AS SUPPORT

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Peroxidases are homoproteins that catalyze redox processes, generating free radicals and polyaromatic products insoluble in water, facilitating their removal from the aqueous medium. The objective of this research was to extract the peroxidases of soybean and chayote, immobilize these enzymes on a highly activated bacterial cellulose (BC) support and use the derivatives obtained for discoloration of bromophenol blue. The amination of soluble peroxidases was realized in ethylenediamine buffer, at pH 4.75, and 10 and 50 mM carbodiimide. Amined peroxidases of 10 and 50 mM were covalently immobilized on the BC-glyoxyl support, with recovered activity of 82% for the derivative BC-Gly-S-NH_2 50 mM and 92% for the derivative BC-Gly-Ch-NH_2 50 mM. Total discoloration of bromophenol blue was obtained in 60 min, using the four amino derivatives. The derivatives were reused for five cycles and they maintained an average of 40% of their catalytic properties, suggesting that these products are suitable as a low-cost alternative for wastewater treatment and other industrial processes.

Keywords: soybean hull, chayote, peroxidases extraction, bacterial cellulose, immobilization, discoloration of the bromophenol dye

INTRODUCTION

Peroxidases are enzymes known as EC 1.11.1.7, present in both animals and plants.[1–4] The peroxidases are very useful in various industrial and analytical applications due to their capacity to reduce peroxides with electron donor substrates. Chayote (*Sechium edule* L.) is a species native to Central America, specifically to Southern Mexico and Guatemala. It grows at a wide range of altitudes from the sea level to over 2.000 meters above the sea level. Chayote may be a great alternative to obtain peroxidases, because it is cheaper and the peroxidases extraction is easier when compared with that from horseradish.[5]

Industrial processes generate large amounts of toxic compounds, which are released into wastewater, affecting the flora and fauna of the regions involved. Aromatic compounds, such as phenols and derivatives, belong to the largest class of contaminants in the wastewater discharged from various chemical and food industries. One method for their removal is by polymerization using redox enzymes in the presence of H_2O_2, which acts as an electron acceptor.[2,5–8]

The use of enzymes immobilized on various supports has been described in the literature due to some operational advantages of this method, such as possibility of reuse in batch or continuous reactions, controlled hydrolysis and easy separation of the product.[9–18] However, there is no ideal method for enzymatic immobilization. Covalent multipoint bonds can increase the stiffness of the immobilized enzyme molecule and still induce an increased resistance to small conformational changes caused by high temperatures, organic solvents and denaturing agents, among others.[19] Some strategies allow for three different routes of multipoint protein immobilization, through amino-terminal residues, lysine residues and carboxylic groups. These chemical changes may be accomplished on different types of supports, not only on agarose.[17,18,20,21]

An alternative support for enzymatic immobilization may be biopolymers, such as cellulose. Bacteria, such as *Gluconacetobacter xylinus*, are very attractive from a commercial point of view due to their high ability to produce cellulose. Bacteria produce cellulose as an extracellular component for mechanical and chemical protection, as well as to facilitate the adhesion of host cells and tissues.[22–25] Due to the large use of peroxidases in several industrial sectors, there is a growing interest in new sources of these enzymes. Considering this, the objective of this study was to evaluate the potential of peroxidase extracted from soybean hull and chayote for the discoloration of the bromophenol blue dye using bacterial cellulose as an alternative support.

EXPERIMENTAL

Reagents and materials

Soybean tegument was acquired from the São Paulo State University (UNESP), School of Pharmaceutical Sciences (FCF), UNISOJA, Araraquara-SP, Brasil, and commercial chayote was used to extract the peroxidase enzyme. Bacterial cellulose (BC) was manufactured by the Laboratory of Photonic Materials, Chemistry Institute of UNESP, Araraquara-SP, Brasil, to be used as an alternative support.

2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS), ethylenediamine (EDA), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDAC), guaiacol, H_2O_2, glycidol, sodium borohydride and sodium periodate were obtained from Sigma-Aldrich (St. Louis, United States). Low-molecular-weight standards were purchased from GE Healthcare. All reagents were of analytical grade.

Peroxidase extraction and treatment with kaolin

The chayote was washed, peeled and chopped into cubes of 2 cm. An amount of 100 g of chayote and 50 mL of distilled water were added in a blender and shredded for 2 min. The obtained extract was filtered twice using a gauze. It was then centrifuged at 7.552 g for 20 min at 4 °C for the removal of interferents. Kaolin was added to the centrifuged crude extract in the ratio of 4% (w/v). The suspension was then centrifuged at 7.552 g for 20 min at 4 °C. The activity and concentration of total proteins in the crude extract were measured after this procedure.

Addition of amino groups to peroxidase (amination)

For the amination process, 5 mL of the extract treated with kaolin and precipitated with acetone (0.143 $mgmL^{-1}$) was mixed with 5 mL of 1M EDA, pH 4.75, containing 10 mM and/or 50 mM EDAC. The mixture was gently stirred on a roller table at 25 °C during 2 h and then dialyzed for 17 h at 4 °C. The aminated enzyme was used in the immobilization process on bacterial cellulose activated with glyoxyl.[21]

Determination of enzymatic activity

The enzymatic activity of the free and immobilized peroxidases was continuously measured by following the oxidation rate of $ABTS^{2+}$ [2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)] to $ABTS^{\bullet+}$, using a spectrophotometer (λ = 420 nm; FEMTO 700S). The activity of the free enzyme was quantified in 50 mM acetate buffer (pH 4.0), containing ABTS 5 mM, 25 µL of 100 mM H_2O_2, 10 µL of enzyme extract, to a final volume of 2.5 mL at 25 °C. The activity of the derivative was performed by incubating 10 mg of derivative under conditions identical to the free enzyme assay. The molar extinction coefficient used was 31.100 $M^{-1}cm^{-1}$ for the radical cation of $ABTS^{+}$.[26] Activity was expressed in units (U), where a unit was defined as the amount of enzyme that oxidizes 1 µmol of ABTS per min. The specific activity of peroxidases was calculated as shown in Equation (1):[27,28]

$$SA = \frac{U_0}{P} \quad (1)$$

where SA is specific activity (Umg^{-1}), U_0 – total enzyme activity of free enzyme (U), P – protein mass (g).

Preparation of supports and activation

Bacterial cellulose (BC) was functionalized by the same method as that used for agarose supports.[29] BC presents reactive free hydroxyls that can be exploited for the immobilization of enzymes, producing BC-glyoxyl supports.[29,30] An amount of 20 g of hydrated BC was added to 6 mL of ultrapure water (milli-Q®). A cold solution of 9.52 mL of 1.7 N NaOH, containing

0.2714 g of $NaBH_4$ (sodium borohydride), was slowly added to 6.86 mL of glycidol (2,3-epoxy propanol) maintained in an ice bath. This suspension was kept for 18 h under slow stirring at 25 °C. The suspension was filtered under vacuum and washed with distilled water thoroughly. Thus, the activated matrix started to present glyceryl active groups in its structure. The matrix was subjected to oxidation in the presence of 100 mM sodium periodate ($NaIO_4$) under gentle stirring during 90 min, forming glyoxyl groups (aldehydes). After this, the functionalized residue was filtered under vacuum and washed with distilled water.

Preparation of enzymatic derivative

The aminated peroxidases were placed in contact with the BC-glyoxyl supports. Then, 4 mL of the aminated enzymatic solutions were diluted in 16 mL of 0.1 M sodium bicarbonate buffer (pH 10). At that time, 1 mL of this solution was used as enzymatic control and the rest (19 mL) was added to 2 g of support. The suspension was maintained at 25 °C under mild and constant stirring. Subsequently, the suspension was divided into two aliquots. The first one was used for determination of the enzymatic activity of peroxidase in the entire suspension (supernatant and derivative). The second one was centrifuged, and used for determining the enzymatic activity of peroxidase in the supernatant for monitoring the enzymatic activities. After the immobilization process, sodium borohydride (1 $mgmL^{-1}$) was added to the derivative and maintained for 30 min under gentle stirring to reduce the remaining activated groups on the support and the Schiff bases formed upon binding of the enzyme. Then, the obtained derivative was washed with distilled water and stored at 4 °C. In order to determine the success of the enzymatic immobilization, the following parameters were calculated: immobilization yield and recovered activity. The calculations were performed according to Equations (2) and (3), respectively.[31]

$$\mathbf{IY} = \left(\frac{U_0 - U_f}{U_0}\right) * \mathbf{100\%} \quad (2)$$

where IY – immobilization yield (%), U_0 – total enzyme activity of free enzyme (U), U_f – total enzyme activity of supernatant after immobilization (U);

$$\mathbf{RA} = \frac{(U_d)}{(U_0)} * \mathbf{100\%} \quad (3)$$

where RA – recovered activity (%), U_d – total enzyme activity of derivative (U), U_0 – total enzyme activity of free enzyme (U). All experiments were performed in triplicate.

Inactivation temperature

Temperatures between 45 °C and 65 °C were tested for both the soluble enzymes and the derivatives, until 90% inactivation of the enzyme activity was recorded. The samples were prepared by suspending 0.3 g of derivate in 3.0 mL of 0.1 M sodium phosphate buffer and 2.0 mL of enzyme extracts. The samples were placed in different baths for an interval of time. 10 µL of suspensions were used to measure activity by the method described for enzymatic activity. These activities were compared with initial activity at 25 °C and expressed as percentage.

Optimum pH of the extract and derivatives

The optimal conditions of pH were determined using 0.1 M sodium citrate buffer (pH 3.0, 4.0, 5.0 and 6.0), 0.1M sodium phosphate (pH 7.0 and 8.0), and 0.1M sodium bicarbonate (pH 9.0 and 10.0).

Bromophenol blue discoloration

BC-glyoxyl-peroxidases were tested in bromophenol blue discoloration. For this purpose, 0.3 g of each derivative was placed in a reactor, 4.0 mL of 0.01 mM bromophenol blue and 100 µL of 100mM H_2O_2 were added to it and kept under gentle stirring for 60 min. Readings by the spectrophotometer (λ = 590 nm; FEMTO 700S) were taken after 10 min. All experiments were performed in triplicate.

RESULTS AND DISCUSSION

The concentration of total proteins present in the extracts of soybean and chayote were of 0.235 $mgmL^{-1}$ and 0.290 $mgmL^{-1}$, respectively. The specific activity of peroxidase was 86.1 Umg^{-1} and 9.0 Umg^{-1} in the soybean and chayote, respectively. The activities of aminated peroxidases of chayote and soybean are shown in Table 1 in comparison with the initial activity of the native peroxidase.

As illustrated in Table 1, there was a decrease of the enzymatic activity in aminated chayote peroxidase – probably, some irreversible changes or degradation may have occurred in the enzyme structure. Another possibility is a modification in the amine group surface. As has been reported in the literature, a decrease in enzymatic stabilization was noted when formaldehyde concentration was increased.[32] Thus, the authors suggested a modification in the amine group that may be important to an additional stabilization. In another paper report in the literature,[33] the authors improved the stabilization of chemically aminated enzymes *via* multipoint covalent attachment on glyoxyl supports. The results showed a decrease of 50% in enzymatic activity for the non-modified enzyme and of 40% for the aminated enzyme. The authors suggested the possibility of chemical modification that may be related to the higher multipoint covalent attachment.

The electrophoresis of the extracts from the soybean tegument and chayote reveals different peroxidase activities, as demonstrated by PAGE

(Fig. 1). It is possible to observe that the chayote peroxidase showed at least one activity band, while the soybean peroxidase presents three activity bands. This can suggest different molecular weight of the same enzymes. The activity bands were revealed with guaiacol in the presence of hydrogen peroxide.[34,35] Using denaturing electrophoresis (SDS-PAGE), it was possible to observe multiple protein bands.

The inactivation of native and aminated soybean peroxidase enzyme at 60 °C occurred similarly, while 75% of activity was maintained after 120 h of treatment. These data are important are they indicate that these enzymes can bear a high temperature during a long period of time. The aminated peroxidase with 50 mM EDAC showed only 10% of activity after 120 h (Fig. 2). These results suggest that high amination can alter the three-dimensional enzyme structure, as demonstrated before.[21,32]

Using a temperature at 60 °C during 120 h, the native enzyme exhibited 75% of activity compared to its initial level. The enzyme aminated using 10 mM EDAC showed 22% of activity, while that aminated with 50 mM EDAC lost all activity. The chayote peroxidase was less stable at high temperature, when compared with the soybean peroxidase. This could be explained by the fact that the soybean tegument can resist high temperature in its natural environment. Our research group has demonstrated similar results. A 75% soybean peroxidase activity was recorded after 120 h at 60 °C. The results demonstrate that the soybean tegument enzyme can resist a high temperature.[36] Figure 3 exhibits the chayote activity in its native form, and when aminated with 10 mM and 50 mM EDAC. The multipoint immobilization of soybean tegument and chayote peroxidases on the bacterial cellulose support functionalized with glyoxyl groups was performed following the protocols reported in the literature.[11]

Table 1
Specific activity (%) recorded after amination of soybean tegument and chayote peroxidases

Peroxidases	Native	Aminated EDAC 10 mM	Aminated EDAC 50 mM
Soybean tegument	100%	88%	100%
Chayote	100%	88%	52%

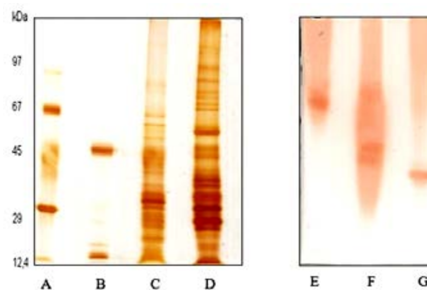

Figure 1: SDS-PAGE of (A) standard molecular weight, (B) commercial horseradish peroxidase, (C) soybean tegument peroxidase, (D) chayote peroxidase; PAGE revealed with guaiacol in the presence of H_2O_2: (E) commercial horseradish peroxidase, (F) soybean tegument peroxidase, (G) chayote peroxidase

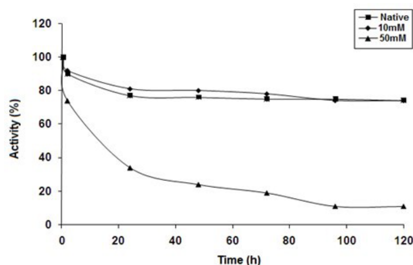

Figure 2: Relative percentage of residual activity of soluble soybean peroxidases: ● native; ■ aminated with 10 mM EDAC and ▲ aminated with 50 mM EDAC, at 60 °C

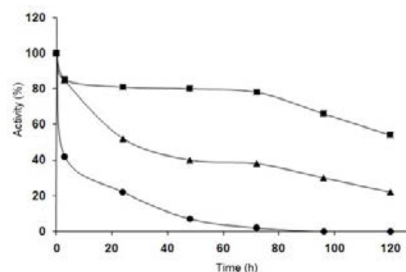

Figure 3: Relative percentage of residual activity of soluble chayote peroxidase: ● native, ■ aminated with 10 mM EDAC, ▲ aminated with 50 mM EDAC, at 45 °C

The multipoint covalent immobilization of peroxidases on the BC-glyoxyl support at pH 10.0 occurred through the aminated areas of the three-dimensional structure. These amines were deprotonated, allowing the nucleophilic attack of the amino groups over the aldehydes from the support, and forming the derivatives: BC-glyoxyl-S-NH_2 10 mM, BC-glyoxyl-S-NH_2 50 mM, BC-glyoxyl-Ch-NH_2 10 mM and BC-glyoxyl-Ch-NH_2 50 mM.

The immobilization occurs through the surface regions of the enzyme that have higher density of primary amine groups, rather than through the more reactive amino group, as occurs in other techniques of immobilization.[36] For the immobilization to occur, it is necessary to react the primary amine groups of the protein with the pre-activated agarose support, so that the surface presents a mono-layer from aldehydes slightly apart from the wall of the support and fully exposed to the reaction.[29] At first, the formation of links similar to the Schiff base (amine group) occurs between the ε-NH_2 from lysine and the aldehyde of the support groups. The lysine amino acids are normally abundant on the protein surface when exposed to the environment and when they are deprotonated, they become very reactive, as the nucleophiles. This first interaction between the enzyme and the support occurs through two points and the enzyme should self-orient towards the support to a richer region of reactive amino groups.[34] This interaction leads to the formation of a weak and irreversible bond. In the next step, the Schiff base reduces linkages to secondary amine with sodium borohydride. This reduction allows, in addition to stabilizing the enzyme-support link, to convert all the remaining aldehyde groups to hydroxyl groups, giving rise to an inert and hydrophilic matrix.[9] According to Guisan,[11] when the remaining reactive groups are not blocked, a number of unwanted or uncontrolled reactions may occur, which destabilize the protein, leading to the inactivation of the enzyme.[30] The results showed that the peroxidases aminated with 50 mM EDAC presented a higher yield of immobilization than those aminated with 10 mM EDAC. This may be explained by a higher concentration of reactive amine groups of the enzymes to form links with the carboxyl groups of BC support. These data were obtained after three hours of the immobilization process (Table 2). Similar values of immobilization and activity were presented in other studies on peroxidase immobilization.[18,36,37]

The recovered activity of BC-Gly-S-NH_2 50 mM was 57% higher than that of BC-Gly-S-NH_2 10 mM. However, the activity of BC-Gly-Ch-NH_2 50 mM was 30% higher than that of the secondary BC-Gly-Ch-NH_2 10 mM. This could be due to the amination process from the glutamate and aspartate groups of native enzymes, which may increase the number of amines on the surface of the enzyme, thereby increasing the number of peptide bonds between the enzyme and the BC-Gly support (Table 2). Similar results were observed when histidine chains were added to the enzymes.[7]

Also, it was observed that the derivative BC-Gly-S-NH_2 10 mM showed higher stability at 60 °C for a period of 120 h, maintaining 82% of its initial activity, while the derivative BC-Gly-S-NH_2 50 mM kept only 57% of its initial activity (Fig. 4). This is probably caused by the formation of a high number of covalent bonds, which decreased the stability owing to some changes in the enzymes' structure. Similar results have been reported in the literature[36] with regard to enzymes extracted and immobilized in corncob powder. The authors suggested that the formation of covalent bonds was able to modify the structure of the enzymes, making them more rigid.

Table 2
Enzymatic activity in the process of immobilization of chayote and soybean tegument aminated peroxidases on BC-Gly support

Support	Immobilization (%)	Activity in suspension (%)	Recovered activity (%)
BC-Gly-S-NH_2 10 mM	47	82	25
BC-Gly-S-NH_2 50 mM	92	71	82
BC-Gly-Ch-NH_2 10 mM	35	91	62
BC-Gly-Ch-NH_2 50 mM	69	73	92

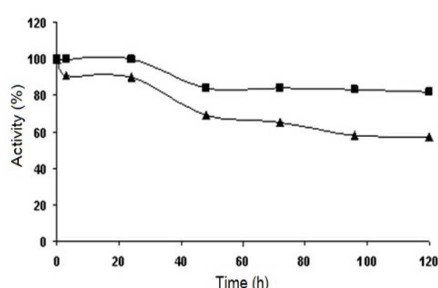

Figure 4: Relative percentage of residual activity of soluble soybean tegument peroxidase: ■ aminated with 10 mM EDAC, ▲ aminated with 50 mM EDAC, at 60 °C

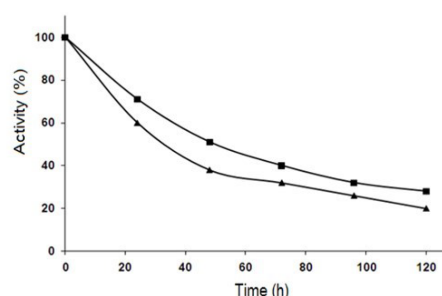

Figure 5: Relative percentage activity of residual of soluble chayote peroxidase: ■ aminated with EDAC 10 mM, ▲ aminated with EDAC 50 mM, at 45 °C

Table 3
Relative percentage of residual activity for native and aminated peroxidases, as well as for those immobilized on BC-Gly, at different pH after 120 h of treatment

Sample	Relative activity (%)			
	pH 3	pH 5	pH 7	pH 10
Soybean-Native	76	67	100	100
Chayote-Native	18	64	17	82
Soybean-Aminated 10 mM	76	90	95	92
Chayote-Aminated 10 mM	17	42	66	63
Soybean-Aminated 50 mM	67	100	100	95
Chayote-Aminated 50 mM	8	77	58	49
BC-Gly-S-NH_2 10 mM	67	56	89	74
BC-Gly-Ch-NH_2 10 mM	40	63	99	82
BC-Gly-S-NH_2 50 mM	70	89	82	76
BC-Gly-Ch-NH_2 50 mM	28	161	286	155

The derivative BC-Gly-Ch-NH_2 10 mM was more stable at 45 °C during a period of 120 h, maintaining 28% of the initial activity, while BC-Gly-Ch-NH_2 50 mM kept only 20% of its activity. These results show an enhanced stability in the free aminated enzyme (Fig. 5).

Both soybean tegument and chayote peroxidases with a higher degree of amination linked to the support with high affinity and velocity, forming a larger number of covalent bonds. However, the stability was lower, probably because of the conformational native spatial changes, leading to a more rigid structure, which may have compromised the catalytic sites (Figs. 4 and 5).

The inactivation of native peroxidases, the aminated ones and the derivatives was carried out for a period of 120 h at different pH values. It was possible to observe that the four derivatives maintained great performance at pH 7.0. Two amino derivatives with 50 mM carbodiimide exhibited greater stability and, consequently, increased activity.

The derivative BC-Gly-Ch-NH_2 50 mM showed increased activity by more than 100% from pH 5.0 to pH 10.0, probably due a better arrangement of the catalytic sites of the enzyme on the support. The lowest performance of this derivative was recorded at pH 3.0 (Table 3).

Table 4 exhibits the bromophenol blue discoloration results, using the stabilized derivatives under gentle stirring during 60 min. A discoloration of 100% was observed in the first cycle in the case of the four derivatives. The stabilized derivatives were reused in five cycles, and the results showed that for three cycles, BC-Gly-S-NH_2 50 mM achieved 100% discoloration, while the other derivatives reached about 50%. After the third cycle, the derivatives decreased their discoloration activity, probably because of reduced enzymatic ability for discoloration, this loss occurs because the polymer formed in the discoloration process is adsorbed to the enzyme.[7,38] The derivatives obtained in this study have the same capacity to discolor bromophenol blue by oxide-reduction.[39–41] Similar values were obtained for chayote peroxidase enzyme applied

in the removal of polyazo textile dyes, which demonstrates the ability and velocity that these enzymes to act in the oxidation of phenolic groups.[18,37] The enzymatic treatment provides a high degree of specificity, operating under mild conditions and at high reaction rate.

Table 4
Bromophenol blue (0.01 mM) discoloration by BC-Gly-peroxidases aminated with 10 mM and 50 mM in H_2O_2 during five cycles of 60 min

Derivative	Cycles				
	1°	2°	3°	4°	5°
BC-Gly-S-NH_2 10 mM	100	94	77	68	32
BC-Gly-S-NH_2 50 mM	100	100	100	93	74
BC-Gly-Ch-NH_2 10 mM	100	79	50	34	21
BC-Gly-Ch-NH_2 50 mM	100	93	78	48	33

Peroxidases are used for the treatment of aromatic compound contaminants originating from different sources and present in waters. Especially, horseradish peroxidase (HRP) has been used for the removal of phenolic contaminants, and also, has been found capable of removing aromatic anilines, such as hydroxyquinoline and carcinogenic benzidines and naphthylamines.[8,42–44] The results obtained in this paper may suggest the possibility of using the obtained BC-Gly-peroxidase derivatives, with soybean tegument aminated enzyme, in the treatment of polluted waters, for at least three cycles of treatment, thus reducing the costs of enzyme application.

CONCLUSION

Soybean tegument and chayote enzymes were multipoint immobilized on bacterial cellulose support activated by the glyoxyl method. Although the supports were activated differently, the peroxidases enriched with amino groups allowed their multipunctual immobilization on the BC-glyoxyl supports.

The most stabilizing factor was achieved when the enzyme aminated with 10 mM EDAC was immobilized on the support through new amine groups, and after this, was incubated at pH 10.0 to optimize the multipoint covalent linking between all the amine residues of this enzyme region and all the glyoxyl groups of the support.

Both aminated derivatives of stabilized peroxidase exhibited similar catalytic properties, when compared to those of the corresponding native soluble enzymes. Therefore, the two derivatives BC-glyoxyl peroxidases, from aminated soybean and chayote enzyme, showed optimum activity in the bromophenol blue discoloration process, demonstrating that it is possible to use such derivatives in the treatment of contaminated wastewater or water containing phenols or phenolic groups.

The findings of this study indicate that the peroxidase enzyme from both soybean teguments and chayote, immobilized on bacterial cellulose, can be an excellent alternative for horseradish peroxidase (HRP), considering that the derivatives obtained exhibited excellent thermal stability and efficiency at different pH values.

List of abbreviations

BC: bacterial cellulose; ABTS: 2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid); EDA: ethylenediamine; EDAC: 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide; HRP: horseradish peroxidase

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