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Bromophenol blue discoloration using peroxidase immobilized on highly activated corncob powder

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ABSTRACT

The aim of the present study was to evaluate the efficacy of peroxidase immobilized on corncob powder for the discoloration of dye. Peroxidase was extracted from soybean seed coat, followed by amination of the surface of the tertiary structure. The aminated peroxidase was immobilized on highly activated corncob powder and employed for the discoloration of bromophenol blue. Amination was performed with 10 or 50 mmol.L^{-1} carbodiimide and 1 mol.L^{-1} ethylenediamine. The amount of protein in the extract was 0.235 ± 0.011 mg.mL^{-1} and specific peroxidase activity was 86.06 ± 1.52 µmol min^{-1}. mg^{-1}, using 1 mmol.L^{-1} ABTS as substrate. Ten mmol.L^{-1} and 50 mmol.L^{-1} aminated peroxidase retained 88 and 100% of the initial activity. Following covalent immobilization on a corncob powder-glyoxyl support, 10 and 50 mmol.L^{-1} aminated peroxidase retained 74 and 86% of activity, respectively. Derivatives were used for the discoloration of 0.02 mmol.L^{-1} bromophenol blue solution. After 30 min, 93 and 89% discoloration was achieved with the 10 mmol.L^{-1} and 50 mmol.L^{-1} derivatives, respectively. Moreover, these derivatives retained 60% of the catalytic properties when used three times. Peroxidase extracted from soybean seed coat immobilized on a low-cost corncob powder support exhibited improved thermal stability.

Keywords: Peroxidases. Multipoint immobilization of enzymes. Aminated enzymes. Corncob powder.

INTRODUCTION

Peroxidases (phenolic donor:hydrogen-peroxide oxidoreductase) are classified as EC 1.11.1.7 and are found both in animals and plants (Duarte-Vázquez et al., 2003; Scott., 1975). In plants, these enzymes play roles in the defense against pathogens and mechanical stress, lignin biosynthesis and degradation, the removal of hydrogen peroxide and the oxidation of toxic xenobiotics (O'Brien, 2000). Vegetable peroxidases, such as horseradish peroxidase, have approximately 300 amino acid residues, a molecular mass of 44 kDa and the heme group is not covalently bound to the protein structure. In contrast, mammalian heme peroxidases are larger (576 to 783 amino acid residues) and are covalently bound to the protein structure (O'Brien, 2000).

Soybean peroxidase has a molecular mass of 37 kDa (Gillikin & Graham, 1991). Peroxidase activity in the soybean seed coat varies among cultivars and the measurement of this activity serves as an alternative or complementary method for the identification of different varieties. The reduction of peroxides at the expense of electron-donating substrates makes peroxidases useful in different industrial and analytical applications (Dunford & Stillman, 1976; Regalado et al., 2004). Soybean peroxidase is a substantially more thermostable enzyme than horseradish peroxidase (McEldoon & Dordick, 1996) and has been used since the 1990s in the fields of bioscience, catalysis, bioremediation and clinical diagnosis. In bioremediation, most studies report the use of horseradish peroxidase for the detoxification of water contaminated by phenolic and chlorinated compounds (Ferrer et al., 1991; Nakamoto & Machida, 1992; Rojas-Melgarejo et al., 2004). However, soybean peroxidase has become a viable alternative in this process (Kennedy & Warith, 2002; Kinsley & Nicell, 2000; Wright & Nicell, 1999).

The immobilization of enzymes on different supports offers operational advantages, such as reusability, the fast interruption of hydrolysis, controlled formation and easy separation of the product (Arslan et al., 2000; Goulart et al., 2008; Guisán, 2006; Marques et al., 2011; Mateo et al., 2004; Paula et al., 2007; Paula et al., 2008; Tavano et al., 2008; Vieira & Fatibello, 2000). Lignocellulosic fibers, such as those found in corncobs, have accessible hydroxyl groups that can react with other polar functional groups, such as those found in phenolic resins. The free reactive hydroxyl groups in corncob fiber allow the addition of spacer arms that act as chemical links for the immobilization of enzymes, thereby producing a corncob powder-glyoxyl support (Guisán, 1988; Mateo et al., 2006).

The tertiary structure of enzymes can be subjected to chemical modifications in its surface carboxyl groups (carboxyl-terminus, aspartic acid and glutamic acid) with 1-ethyl-3-(dimethylaminopropyl) carbodiimide (EDAC) in the presence of ethylenediamine (EDA), thereby promoting stabilization. This reaction produces an amide between the

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activated carboxyl group of the protein and the amino group of EDA, forming a free primary amino group (see Figure 1, adaptation from López- Gallego et al., 2005). This new amino group has a pKa at about 9.2 and is more reactive than amino groups of lysine residues on the protein surface. Furthermore, the degree of modification can be controlled by the EDAC concentration used in the reaction (López-Gallego et al., 2005; Ó'Fágáin, 2003).

There is a growing interest in new sources of peroxidases due to the wide use of these enzymes in different industrial sectors. The aim of the present study was to evaluate the efficacy of peroxidase extracted from soybean seed coat and immobilized on corncob powder for the discoloration of the dye bromophenol blue.

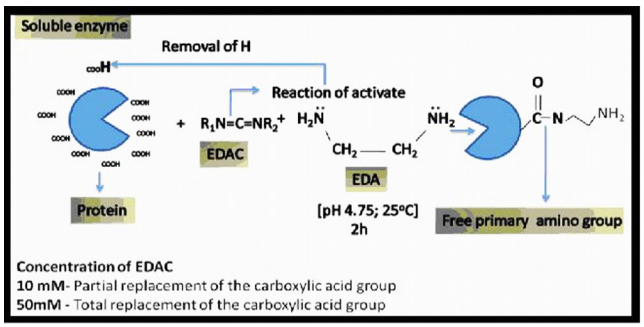

Figure 1: Chemical amination of protein carboxyl groups activated with EDAC in presence of EDA

MATERIALS AND METHODS

Materials

Peroxidase was extracted from soybean seed coat. Corncob powder was used as a low-cost support for the immobilization of peroxidase.

Methods

Extraction and dosage of enzyme protein

Peroxidase was extracted by adding 10 mL of Milli-Q water per gram of soybean seed coat and milling in a blender for 5 minutes. The extract was then filtered through gauze and centrifuged at 7552 *g* for 20 min at 4 ºC. The protein concentration in the extract was determined using Bradford's method (Bradford, 1976).

Enzyme amination and determination of specific activity

Amination of the native soluble peroxidase enzyme was performed with 1 mol.L^{-1} ethylenediamine (EDA) buffer (Sigma), pH 4.75, and solid carbodiimide (Sigma) for a final concentration of either 10 mmol.L^{-1} or 50 mmol.L^{-1} (Fernández- Lafuente et al., 1995). Specific activity of the native enzyme was determined using 1 mmol.L^{-1} 2,2'-azinobis(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS; Sigma) prepared in 0.1 mol.L^{-1} sodium acetate buffer, pH 4.0, and 25 µL of 100 mmol.L^{-1} H_2O_2 in the same buffer as substrate. The reaction was monitored in a spectrophotometer at λ = 430 nm (Muñoz et al., 1997; Re et al., 1999).

Preparation and activation of support

The procedure for the functionalization of corncob powder (CP) was similar to that described for an agarose support (Guisán, 1988), with modifications. Corncob was milled until obtaining a powder. One 1 L of 2 mol.L^{-1} NaOH solution was added to each 100 g of CP, with mild agitation for 24 hours at room temperature. In a vacuum, CP was then filtered through paper and washed thoroughly with distilled water.

One aliquot of CP was added to water Milli-Q, 1.7 mol.L^{-1} NaOH containing 28.5 mg.mL^{-1} $NaBH_4$ and glycidol (2,3-epoxypropanol) for a final concentration of 2.0 M. This suspension was kept under mild agitation for 18 h at a temperature of 4 ºC. In a vacuum, the suspension was filtered through paper and washed thoroughly with Milli-Q water. Thus, the activated matrix had active glyceryl groups in its structure. The matrices were then subjected to oxidation with 100 mmol.L^{-1}sodium periodate ($NaIO_4$) under mild agitation for 90 minutes, reacting to form glyoxyl groups (aldehydes). In a vacuum, the functionalized support was filtered through paper and washed with Milli-Q water.

Preparation of derivatives

Aminated peroxidase (4 mL containing 0.12 mg.mL^{-1}) was diluted in 16 mL of 0.1 mol.L^{-1} sodium bicarbonate buffer, pH 10. One mL of this solution was the enzyme control and the remaining solution was added to 2 g of the CP-glyoxyl support. The suspension was maintained under mild agitation at 25 ºC. At regular time intervals, two aliquots were removed: one for the analysis of enzyme activity in the suspension and one for centrifugation followed by the analysis of enzyme activity in the supernatant. After the immobilization procedure, the suspension was kept under mild agitation with 1mg.mL^{-1} sodium borohydride for 30 min to reduce the remaining activated groups in the support. The derivative obtained was washed with Milli-Q water and stored at 4 ºC.

Bromophenol blue discoloration

Discoloration of bromophenol blue was conducted by assaying 0.3 g of the CP-glyoxyl-aminated peroxidase in 4.0 mL of 0.02 mM bromophenol blue with 100 µL of 100 mmol.L^{-1} H_2O_2 under mild agitation for 60 minutes. Discoloration was monitored in a spectrophotometer at λ = 590 nm.

Statistical analysis

All experiments were performed at least in triplicate. Values are presented as means and standard deviations do not exceed 5%.

RESULTS

Total protein concentration in the soybean seed coat extract was 0.235 ± 0.011 mg.mL^{-1} and specific peroxidase activity was 86.06 ± 1.72 µmol min^{-1}.mg^{-1} in 100 mmol.L^{-1}

sodium acetate buffer, pH 4.0. The 10 mmol.L^{-1} and 50 mmol.L^{-1} aminated enzymes exhibited 88% and 100% of the native peroxidase activity, respectively (Table 1). Inactivation of both the native enzyme and 10 mmol.L^{-1} aminated peroxidase at 60 ºC was similar (75% of initial activity) after 120 h of experimentation. Amination strongly altered the stability of the 50 mmol.L^{-1} aminated enzyme (10% of initial activity) after 120 hours (Figure 2).

Table 1: Percentage of specific activity recovered after amination of peroxidase extracted from soybean seed coat.

Peroxidase	Specific Activity (μmol min^{-1}.mg^{-1})	%
Native	86.06±1.72	100
10 mmol.L^{-1} aminated	75.73±2.27	88
50 mmol.L^{-1} aminated	85.76±1.47	100

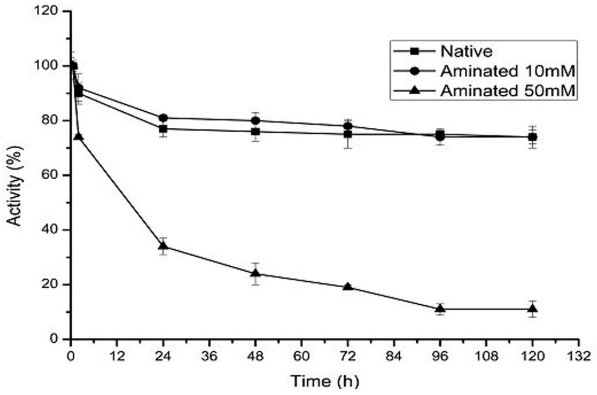

Figure 2: Inactivation profile of soybean soluble peroxidase (native; 10 mM and 50 mM of aminated peroxidase) at 60 ºC

The results of the multipoint immobilization procedures on the highly activated CP support were obtained using protocols similar to those described by Guisán (1988). The protein concentration that allowed the maximum recovery of activity was 0.235 mg per gram of activated support. Multipoint covalent immobilization on the CP-glyoxyl support at pH 10.0 was performed using the three-dimensional aminated surface area of peroxidase.

At pH 10.0, these amines were deprotonated, which allowed the nucleophilic attack of the amino groups on the aldehydes of the support, thereby forming the CP-glyoxyl-10 mmol.L^{-1} and CP-glyoxyl-50 mmol.L^{-1} aminated peroxidase derivatives (CP-G-P10 and CP-G-P50, respectively). Greater immobilization yields (during three hours of procedure) were found in comparison to the recovered activity (Table 2), which is considered normal, since it demonstrates the activity of the derivative before reduction with sodium borohydride. During reduction with sodium borohydride, these molecules are removed and what remains is the derivative with only the covalently bound enzyme and slightly lesser but real activity.

The CP-G-P10 derivative demonstrated better stability at 60 °C for 120 h than the CP-G-P50 derivative (92% and 66% of initial activity, respectively). Soybean peroxidase with a greater degree of amination is connected to the support with very high affinity and velocity, forming more covalent linkages in comparison to less aminated peroxidase. When a higher number of linkages are formed, lesser stability occurs due to small changes in the enzyme structure, leading to a slightly more rigid structure and hindering the linkage of the substrate with the catalytic center (Figure 3).

Table 2: Enzyme activity of aminated peroxidase during and after immobilization procedure

Derivative	Immobilized (%)	Suspension (%)	Recovered (%)
CP-G-P10	74.3±2.2	76.5±3.1	57.7±1.1
CP-G-P50	86.4±1.8	70.1±4.4	69.2±3.2

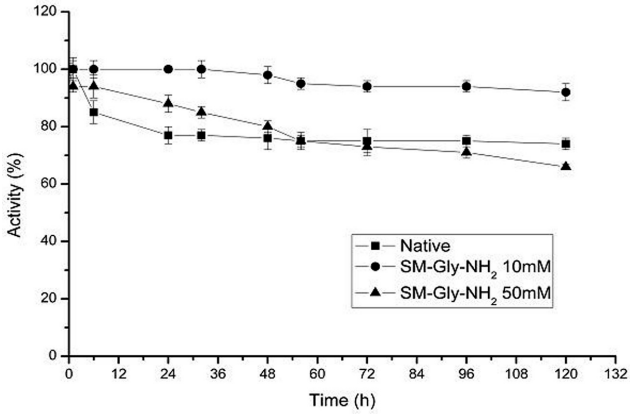

Figure 3: Stability of CP-glyoxyl-peroxidase derivatives at 60 °C

Table 3 displays the results of the pH stability of the native peroxidase, aminated peroxidase and derivatives after 120 hours of treatment. CP-G-P10 had the best performance, as the residual activity of this derivative was approximately the same as the initial activity at pH 3.0 to 10.0. CP-G-P50 retained activity above 90% at both pH 3.0 and 10.0, but activity was around 80% at pH 5.0 and 7.0.

Figure 4 displays the reuse capacity of the CP-G-P10 and CP-G-P50 derivatives in four cycles of the discoloration of 0.02 mM of bromophenol blue solution in the presence of H_2O_2 under mild agitation for 60 min (Figure 4).

Table 3: Percentage of inactivation of native, aminated and immobilized soybean peroxidase at different pH values after 120 hours of treatment

Form of enzyme	Activity (%)			
	pH 3.0	pH 5.0	pH 7.0	pH 10.0
Native	76.1±1.2	67.2±0.9	100.7±0.7	100.2±1.1
10 mmol.L-1 aminated	76.8±0.9	90.4±2.1	95.6±3.3	92.2±1.4
CP-G-P10	96.5±3.1	95.0±1.7	100.9±1.1	100.5±0.8
50 mmol.L-1 aminated	67.9±3.3	100.3±0.3	100.5±2.1	95.4±3.1
CP-G-P50	96.3±4.1	82.1±2.9	81.3±3.8	91.8±4.0

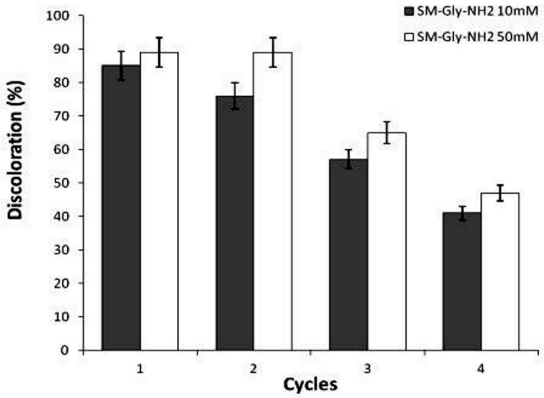

Figure 4: Reuse capacity of 10 mM and 50 mM CP-glyoxyl-aminated peroxidase derivatives in 4 cycles for 60 min in discoloration of 0.02 mM of bromophenol blue solution in presence of H2O2

DISCUSSION

In the present study, peroxidase was isolated from soybean seed coat and immobilized on corncob powder. There is no consensus on the ideal immobilization method. Multipoint covalent bonds can increase the rigidity of the immobilized enzyme and induce greater resistance to small conformational changes caused by high temperatures, organic solvents, denaturing agents and other factors (Tardioli et al., 2003). Studies on the multipoint immobilization of proteins using three different residues (amino-terminus, lysine residues and carboxyl groups) began with Spanish researcher José Manuel Guisán in 1988, using an agarose support. However, these possible chemical transformations are not unique to agarose and can be accomplished with other supports as well (Vieira & Fatibello, 2000; Guisán et al., 1997; López-Gallego, 2005). Immobilization on a glyoxyl-agarose support occurs through regions of the enzyme surface with a greater density of primary amino groups rather than through the more reactive amino group, as in most other immobilization techniques (Mateo et al., 2006).

Immobilization constitutes a reaction between primary amino groups of the protein and the previously activated support, with a monolayer of aldehydes on the surface slightly away from the support wall and fully exposed to the reaction medium (Guisán, 1988). First, the formation of a Schiff base linkage (imino groups) occurs between the ε-NH_2 of lysine and aldehyde groups of the support. Lysine is usually abundant on the protein surface exposed to the medium and becomes a very reactive nucleophile when deprotonated. The reaction takes place within a narrow pH range near or above the pK of the ε-NH_2 groups (pH 10.5). The first interaction between the enzyme and support occurs at a minimum of two points. Thus, the enzyme must be oriented toward the richer region in reactive amino groups of the support (Guisán, 1988). The result is a weak, reversible bond. The next step is to reduce the Schiff bases to secondary amino bonds with sodium borohydride. This reduction stabilizes the enzyme-support link and converts all remaining aldehyde groups to hydroxyl groups, giving rise to an inert, hydrophilic matrix (Blanco & Guisán, 1989). According to Guisán, the remaining reactive groups may lead to a number of unwanted or uncontrolled reactions in such a way that destabilizes the protein, leading to the inactivation of the enzyme (Mateo et al., 2004).

In the present study, 10 and 50 mmol.L^{-1} aminated peroxidase respectively retained 88 and 100% of the initial activity of the native peroxidase (Table 1). Inactivation of native peroxidase and 10 mmol.L^{-1} aminated peroxidase was similar at a temperature of 60 ºC, maintaining 75% of the initial activity after 120 hours of experimentation. In contrast, 50 mmol.L^{-1} aminated peroxidase exhibited only 10% of the initial activity under the same conditions, demonstrating that a higher degree of amination strongly alters enzyme stability.

Yields of 74% and 86% were found regarding immobilization of the CP-G-P10 and CP-G-P50 derivatives, although recovered activity was 57% and 69% following three hours of immobilization, respectively, demonstrating 21% greater activity with the latter derivative (Table 2). The two derivatives had the same capacity and tendency to discolor bromophenol blue through redox processes (Ferrer et al., 1991; Davis & Burns., 1990) and maintained activity above 60% in the three first cycles, with a decrease in discoloration capacity thereafter. This loss occurs because the polymer formed in the discoloration process is adsorbed by the enzyme (Nakamoto & Machida, 1992; Rojas-Melgarejo, 2004). Similar values have been obtained with chayote peroxidase (which also has polyazo nature) used to remove textile dyes, demonstrating the capacity of these enzymes to act on the oxidation of phenolic groups (Villegas-Rosas et al., 2003).

Industrial activities lead to the disposal of large amounts of toxic compounds that can affect the flora and fauna. Aromatic compounds, such as phenol and its derivatives, are the largest class of contaminants in wastewater from different chemical and food industries (Duarte-Vázquez et al., 2003; Nakamoto & Machida, 1992; Klibanov & Scott, 1983; Nicell et al., 1993). Polymerization using redox enzymes in the presence of H_2O_2 acting as an electron acceptor is one of the methods employed for the removal of these contaminants. Peroxidases can catalyze the oxidative polymerization of phenols, anilines and other aromatics, transforming these compounds into insoluble oligomers, which can be removed using simple sedimentation or filtration systems (Dunford & Stillman, 1976; Klibanov & Scott, 1983).

The present findings demonstrate that peroxidase from soybean seed coat immobilized on corncob powder is catalytically active and efficient for the discoloration of bromophenol blue.

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RESUMO

Descoloração de azul de bromofenol utilizando peroxidase imobilizada em pó de sabugo de milho altamente ativado

Nesta pesquisa a enzima peroxidase foi extraída do tegumento de sementes de soja, e a superfície da estrutura terciária foi aminada. A peroxidase aminada foi imobilizada em suporte pó de sabugo de milho altamente ativado e utilizado na descoloração de azul de bromofenol. A aminação da peroxidase foi realizada com carbodiimida em concentrações de 10 e 50 mmol.L^{-1}, e 1 mol.L^{-1} de etilenodiamina. A quantidade de proteínas no extrato foi de 0,235 ± 0,011 mg.mL^{-1}, e a atividade específica da peroxidase foi 86,06 ± 1,52 µmol min^{-1}.mg^{-1}, usando 1 mmol.L^{-1} de ABTS como substrato. A peroxidase aminada a 10 mmol.L^{-1} reteve 88% e a aminada a 50 mmol.L^{-1} reteve 100% da atividade inicial. As peroxidases aminadas a 10 ou 50 mmol.L^{-1} foram covalentemente imobilizadas em suporte glioxil-pó de sabugo de milho com atividade recuperada de 74% e 86%, respectivamente. Os derivados obtidos foram utilizados na descoloração de solução de azul de bromofenol 0,02 mmol.L^{-1}. Após 30 min 93% de descoloração foram alcançados com o derivado glioxil-pó de sabugo de milho com a peroxidase aminada 10 mmol.L^{-1} e 89% com a aminada 50 mmol.L^{-1}. Estes derivados mantiveram 60% das propriedades catalíticas, quando utilizado por três vezes. A peroxidase extraída do tegumento da semente de soja imobilizada em suporte de baixo custo pó de sabugo de milho apresentou melhoria na estabilidade térmica da enzima.

Palavras-chave: Peroxidases. Imobilização multipontual de enzimas. Aminação de enzimas. Pó de sabugo de milho.

REFERENCES

Arslan F, Tümtürk H, Çaykara T, Sen M, Güven O. The effect of gel composition on the adsorption of invertase on poly (acrylamide/maleic acid) hydrogels. Food Chem. 2000;70:33-8.

Blanco RM, Guisán JM. Stabilization of enzymes by multipoint covalent attachment to agarose-aldehide gels. Borohydrite reduction of trypsin-agarose derivates. Enzyme Microb Technol. 1989;11:360-6.

Bradford MM. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 1976;72:248-54.

Davis S, Burns RG. Decolorization of phenolic effluents by soluble and immobilized phenol oxidases. Applied Microbiol Biotechnol.1990;32:721-6.

Duarte-Vázquez MA, Ortega-Tovar MA, García-Almendarez BE and Regalado C. Renoval of aqueous phenolic compounds from a model system by oxidative polymerization with turnip (*Brassica napus* L var purple top white globe) peroxidase. J Chem Technol Biotechnol. 2003;78:42-7.

Dunford HB, Stillman JS. On the function and mechanism of action of peroxidases. Coordin Chem Rev. 1976;19:187-251.

Ferrer I, Dezotti M, Durán N. Decolorization of Kraft effluent by free and immobilized lignin peroxidases and horseradish peroxidase. Biotechnol Lett. 1991;13:577-82.

Fernandez-Lafuente R, Rosell CM, Rodriguez V, Guisan JM. Strategies for enzyme satabilization by intramolecular crosslinking with bifunctional reagents. Enzyme Microb Technol. 1995 17(6):517-50.

Gillikin JW, Graham JS. Purification and developmental analysis of the major anionic peroxidases from the seed coat of *Glysine max*. Plant Physiol. 1991;96:214-20.

Goulart AJ, Benedetti ACEP, Tavano OL, Marques DP, Contiero J, Carmona EC, Monti R. Multipoint immobilization of invertase on agarose: stability and kinetic properties. Curr Trends Biotechnol Pharm. 2008;2:462-70.

Guisán JM. Aldehyde-agarose gels as activated supports for immobilization-stabilization of enzymes. Enzyme Microl Technol. 1988;10:375-82.

Guisán JM, Bastida A, Blanco RM, Fernández-Lafuente R, García-Junceda E. Immobilization of enzymes on glyoxyl agarose – Strategies for enzyme stabilization by multipoint attachment. In: Bickerstaff GF (editor). Immobilization of enzymes and cells. Totowa: Humana Press; 1997. p. 277-87.

Guisán JM. Immobilization of enzymes as the 21st century begins: an already solved problem or still an exciting challenge. In: Guisán JM (editor). Immobilization of Enzymes and Cells. Totowa: Humana Press; 2006. p. 1-13.

Kennedy K, Alemany K, Warith M. Optimization of soybean peroxidase treatment of 2, 4-dichlorephenol. Water Res. 2002;28:149-58.

Kinsley C, Nicell JA. Treatment of aqueous phenol with soybean peroxidase in the presence of polyethylene glycol. Bioresource Technol. 2000;73:139-46.

Klibanov AM, Tu TM , Scott KP. Peroxidase catalyzed removal of phenoes from coal conversion waste waters. Science. 1983;221:259-61.

López-Gallego F, Montes T, Fuentes M, Alonso N, Grazu V, Betancor L, Guisán JM. Improved stabilization of chemically aminated enzymes via multipoint covalent attachment on glyoxyl supports. J Biotechnol. 2005;116:1-10.

Marques D, Pessela BC, Betancor L, Monti R, Carrascosa AV, Rocha-Martin J, Guisán JM, Fernández-Lorente G. Hydrolysis of protein by immobilized and stabilized trypsin. Biotechnol Prog. 2011;27:677-83.

Mateo C, Palomo JM, Fuentes M, Betancor L, Grazu V, López-Gallego F, Pessela BC, Hidalgo A, Fernández-

Lorente G, Fernández-Lafuente R and Guisán JM. Glyoxyl agarose: a fully inert y hydrophilic support for immobilization y high stabilization of proteins. Enzyme Microb Technol.2006;39:274-80.

Mateo C, Monti R, Pessela BC, Fuentes M, Torres R, Guisán JM, Fernández-Lafuente R. Immobilization of lactase from *Kluyveromyces lactis* greatly reduces the inhibition of promoted by glucose: full hydrolysis of lactose in milk. Biotechnol Prog. 2004;20:1259-62.

McEldoon JP, Dordick JS.Unusual thermal stability of soybean peroxidase. Biotechnol Prog. 1996;12:555-8.

Muñoz C, Guillén F, Martínez AT, Martínez MJ. Laccase isoenzymes of *Pleurotus eryngii:* characterization, catalytic properties and participation in activation of molecular oxygen and Mn^{2+} oxidation. Appl Environ Microbiol. 1997;63:2166-74.

Nakamoto S, Machida N. Phenol removal from aqueous solutions by peroxidase-catalyzed reaction using additives. Water Res. 1992;26:49-54.

Nicell JA, Bewtra JK, Biswas N, Taylor E. Reactor development for peroxidases catalyzed polymerization and precipitation of phenols from wastewater. Water Res. 1993;27:1629-39.

O´Brien P. Peroxidases. Chemico-Biological Interactions. 2000;113:139-29.

Ó'Fágáin C. Enzyme stabilization: recent experimental progress. Enzyme Microb Technol. 2003;33:137-49.

Paula FC, Cazetta ML, Monti R, Contiero J. Screening of supports for *Kluyveromyces marxianus* var. *bulgaricus* inulinase immobilization. Curr Trends Biotechnol Pharm. 2007;1:34-40.

Paula FC, Cazetta ML, Monti R, Contiero J. Sucrose hydrolysis by gelatin-immobilized inulinase from *Kluyveromyces marxianus* var.*bulgaricus*. Food Chem. 2008;111:691-95.

Re R, Pellegrini N, Proteggente A, Pannala A, Yang M, Rice-Evan C. Antioxidant activity applying an improved ABTS radical cation decolorization assay. Free Radical Bio Med.1999;26:1231-7.

Regalado C, García Almendárez, BE, Duarte-Vázquez MA. Biotechnological applications of peroxidases. Phytochemistry Rev. 2004;4:243-56.

Rojas-Melgarejo F, Rodríguez-López JN, García-Cánovas F, García-Ruiz PA, Immobilization of horseradish peroxidase on cinnamic carbohydrate esters. Process Biochem. 2004;39:1145-64.

Scott D. Oxidoreductases. In: Reed G (editor). Enzymes in food processing. New York: Academic Press; 1975. P. 219-54.

Tardioli PW, Pedroche J, Giordano RL, Fernández-Lafuente R, Guisán JM. Hydrolysis of proteins by immobilized-stabilized alcalase-glyoxyl agarose. Biotechnol Prog. 2003;19:352-60.

Tavano OL, Pessela BC, Goulart AJ, Fernández-Lafuente R, Guisán JM, Monti R. Stabilization of an amylase from *Neurospora crassa* by immobilization on highly activated supports. Food Biotechnol. 2008;22:262-75.

Vieira IC, Fatibello FO. Biosensor based on paraffin/graphite modified with sweet potato tissue for the determination of hydroquinone in cosmetic cream in organic phase. Talanta. 2000;52:681-9.

Villegas-Rosas MLO, Geissler G, Handal-Silva A, González-Vergara E. Inmovilización de una peroxidasa de chayote [sechium edule (jacq.) sw] y su potencial aplicación en la remoción de sustancias fenólicas en aguas contaminadas. Rev. Int. Contam. Ambient, 2003;19:73-81.

Wright H, Nicell A. Characterization of soybean peroxidase for the treatment of aqueous phenols. Bioresource Technol. 1999;70:69-79.

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