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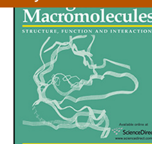

Immobilized soybean hull peroxidase for the oxidation of phenolic compounds in coffee processing wastewater

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ABSTRACT

Chitosan beads were prepared, using glutaraldehyde as a crosslinking agent for the immobilization of soybean hull peroxidase (SBP). The activity of free and immobilized SBP was studied. The optimum pH was 6.0 for both the free and immobilized enzyme; however, enzyme activity became more dependent on the temperature after immobilization. This study evaluated the potential use of immobilized and free enzyme in the oxidation of caffeic acid, of synthetic phenolic solution (SPS) and of total phenolic compounds in coffee processing wastewater (CPW). Some factors, such as reaction time, amount of H_2O_2 and caffeic acid were evaluated, in order to determine the optimum conditions for enzyme performance. Both enzymes showed a potential in the removal of caffeic acid, SPS and CPW, and immobilized SBP had the highest oxidation performance. The immobilized enzyme showed a potential of 50% in the oxidation of caffeic acid after 4 consecutive cycles.

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1. Introduction

In recent decades, phenols and their derivatives have been introduced into the environment from a variety of sources. They are widely used in the manufacturing process of plastics, dyes, drugs, pesticides, papers, petroleum refineries, and textiles [1,2], and frequently appear in coffee processing wastewater (CPW) [3].

The wastewater produced from wet coffee processing, originated from coffee fruits, is rich in a wide variety of organic compounds, such as caffeine, sugars and phenolic compounds, unsuitable for direct disposal into the soil or waterways [4]. In wastewater, phenols (phenol or a mixture of phenols) range from 10 to 17,000 mg L^{-1}; however, according to CONAMA Resolution No 430 from 05/13/2011 [5], the concentration of phenols should be lower than 0.5 mg L^{-1}. Therefore, the treatment of industrial effluents containing aromatic compounds becomes necessary prior to their final discharge into the environment [6,7].

The reported methods for removing phenolics include microbial degradation, incineration, adsorption, membrane separation, solvent extraction and advanced oxidation processes, which have drawbacks such as high energy requirements and/or applicability only in certain concentration ranges, high cost, low efficiency, incomplete removal and formation of hazardous by-products [8–10]. For the enzymatic degradation of phenolic compounds, the use of several peroxidases and/or phenoloxidases (laccases and tyrosinases) have been explored [6,11]. Peroxidases are able to catalyze the oxidative polymerization of phenolic compounds to form insoluble polymers [6,7,12]. One of the significant drawbacks of this method is the relatively short catalytic lifetime of the enzyme, which is attributed to the inactivation of the catalyst by the polymerization process [13]. The drawback could be overcome by the use of enzymes in the immobilized form, which can be used as catalysts with a long lifetime [14]. Enzyme immobilization can be defined as the attachment of soluble enzymes to different types of support, resulting in the reduction or loss of the mobility of the enzyme [15].

Immobilized enzymes offer more advantages, when compared to free enzymes, like enhanced stability against various denaturing conditions, higher catalytic activity, easier product and enzyme recovery, continuous operation of enzymatic processes, reusability and reduced susceptibility to microbial contamination [1,16]. However, limitations in applications of immobilized enzymes include high cost and low yield [12].

Support materials play an important role in the usefulness of an immobilized enzyme, since it should be low-cost and provide

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an adequate large surface area, together with the least diffusion limitation in the transport of substrate and product [17]. Natural polymers used as carrier materials in immobilization technology, such as alginate, carrageenan, agarose, chitin, and chitosan, along with their application in the treatment of various pollutants, have the advantages of being nontoxic, biocompatible and biodegradable [14,18].

Chitosan has been widely used for a variety of purposes. Commercially available chitosan is mainly obtained from chitin, an abundant carbohydrate polymer [19]. Chitosan is an attractive material with unique properties of non-toxicity, film and fiber forming properties, adsorption of metal ions, coagulation of suspensions or solutes, and distinctive biological activities, due to reactive amino and hydroxyl groups, which are potentially capable of being crosslinked with different substances (e.g. Glutaraldehyde) [20–24]. Chitosan is known as an ideal support material for enzyme immobilization,like improved mechanical strength, resistance to chemical degradation, avoiding the disturbance of metal ions to the enzyme, anti-bacterial properties and low cost [25].

The extraction of enzymes from agro-industrial residues is an alternative for reducing costs in biocatalyst production. Soybean seed hulls have been identified as a rich source of peroxidases and, as a soybean-processing industry by-product, they are a low-cost alternative [26]. Peroxidase extracted from soybean hulls (SBP) has been immobilized on a type of support such as: activated carbon [27]; entrapped within hybrid (silica sol-gel/alginate) particles [28]; macroporous glycidylmethacrylates with different surface characteristics [29]; aldehyde glass through their amine groups [30], and is used in the oxidation of organic compounds. Therefore, the study of the immobilization of SBP in chitosan beads crosslinked with glutaraldehyde becomes interesting, since immobilization reports on this type of support were not found, and there are no studies of its application in the oxidation of phenolic compounds present in CPW.

Given the above, the objective of this study was to find a simple and efficient method to stabilize the peroxidase extracted from soybean hulls (SBP) and investigate the application of the biocatalyst obtained in the oxidation of caffeic acid, one of the most abundant phenolic compounds in CPW [31]. SBP was immobilized on chitosan beads crosslinked with glutaraldehyde, and the performance and reusability of the immobilized enzyme were investigated in the process of caffeic acid removal. Moreover, the major points of this study are: (a) enzymatic oxidation of the standard solution containing phenolic compounds frequently found at CPW and the real effluent by peroxidase extracted from an agricultural by-product (SBP); (b) There are few studies on the treatment of wastewater from coffee processing. Among them, the biological treatment in which a major obstacle is the presence of phenolic compounds that are highly toxic to microbial decomposers of organic matter and the use of chitosan beads as a potential support for SBP.

Thus, this study encourages the use of the proposed treatment for the remediation of effluents containing phenolic compounds, as an efficient and sustainable technology, and can be used as a pretreatment to conventional biological treatments.

2. Material and methods

2.1. Collection of coffee processing wastewater

CPW was obtained on a farm located in Bom Sucesso, Minas Gerais, Brazil. This water was collected in a storage tank on the farm, where all the processing effluent is mixed. After collecting the samples, the effluent was stored in amber bottles at 4 °C.

2.2. Obtention of the enzymatic extract

In order to obtain soybean seed hull extracts, the seeds were immersed in distilled water for 24 h and hull removal was carried out manually. Hulls (15 g) were homogenized in a blender for 30 s, with 100 mL of 0.05 mol L^{-1} phosphate buffer at pH 6.5, containing 0.2 mol L^{-1} NaCl. The homogenate was filtered in organza cloth and centrifuged at 10,000 × g for 15 min, at 4 °C [32]. The obtained solution was subjected to precipitation, with the addition of cold acetone until reaching 65% (v/v). After a rest from 12 to 14 h, at −18 °C, the homogenate was centrifuged at 11,000 × g for 15 min, at 4 °C. The precipitate containing peroxidase was submitted to acetone removal by evaporation in an ice bath for 24 h. This precipitate was resuspended in a 0.1 mol L^{-1} phosphate buffer, pH 7.0, used to measure enzyme activity, during enzyme immobilization, and then used for studies on phenolic compound oxidation.

2.3. 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 solution. The chitosan solution was dropped into an aqueous 2 mol L^{-1} NaOH solution, where chitosan precipitated immediately to form gelatinous beads [33]. The chitosan beads were thoroughly washed with distilled water until neutrality was reached. The beads had an average diameter of 0,4428 ± 0,0104 mm [34].

2.4. Crosslinking of chitosan beads by glutaraldehyde treatment

The crosslinking of chitosan beads with glutaraldehyde was carried out by the 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 °C. The beads were washed with distilled water to remove excess glutaraldehyde. The beads had an average diameter of 0.4173 ± 0.0093 mm [34].

2.5. Immobilization of soybean hull peroxidase

The crosslinked chitosan beads were used as supports for the immobilization of soybean hull peroxidase; 120 beads were added (or 5.04 g ± 0.015 hydrated weight of the beads) in 10 mL of SBP enzyme and were subjected to slight stirring in a water bath at 20 °C during 4 h. Total protein determination was carried out according to Bradford (1976) [35] and the enzymatic activity, by the method proposed by Khan and Robinson (1994) [36], with modifications described in the item below. After the determination of total protein and enzymatic activity, coupled protein (CP) and immobilization yield (IY) were estimated according to equations (1) and (2), respectively:

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

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

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

2.6. Activity measurement of free and immobilized soybean hull peroxidase

The activities of the free and immobilized enzyme were tested according to the methodology described by Khan and Robinson (1994) [36], 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 monitored during 3 min at 30 °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 kept under stirring, in a water bath. 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 Eq. (3).

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

2.7. 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 (0.1 mol L^{-1}, pH 2.0 to 6.0) and Tris-HCl (0.1 mol L^{-1}, pH 7.0 to 9.0). The immobilized enzyme was incubated for 10 min in the buffers before the activity measurement.

2.8. Influence of temperature

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

2.9. Oxidation of caffeic acid

2.9.1. Reaction time

The first part of this study was to optimize the reaction conditions for the oxidation of caffeic acid. The caffeic acid solution (1.0 mmol L^{-1}) (Sigma–Aldrich) was prepared in 2% ethanol/water. The choice of this phenolic was based on the fact that caffeic acid is one of the major phenolic compounds found in CPW by our research group [31].

Initially, the enzymatic reactions were conducted in a citrate phosphate buffer, 0.1 mol L^{-1}, pH 6.0 (1.2 mL), containing: (1) 7 mmol L^{-1} H_2O_2 (0.4 mL), (2) caffeic acid at a concentration of 1.0 mmol L^{-1} (1.5 mL) and 0.1 mL free enzyme (31.01U mL^{-1} or specific activity 34.15 U mg^{-1}) and, for the immobilized enzyme, 0.012 g ± 0.003 (0.041 U $bead^{-1}$ or specific activity 2.51 U mg^{-1}) and incubated at 30 °C, to estimate the optimum contact time. Controls were carried out in the absence of H_2O_2. The reaction mixture was stirred continuously. Aliquots of the reaction mixture were removed at intervals, and the enzymatic reaction was stopped by adding 0.1 mL catalase solution (1.2 mg of the commercial enzyme in 1.0 mL of 0.1 mol L^{-1} phosphate buffer, pH 7.0) [37,with modifications]

The insoluble product was removed by centrifugation at 3000 × g, for 10 min at 25 °C. The residual concentration of caffeic acid was measured by the colorimetric method of Folin and Denis, using caffeic acid as a standard [38].

The same procedure was performed with the chitosan beads without enzyme to evaluate the adsorption material. Three replicates were carried out for each treatment. Subsequent experiments were performed by varying the concentrations of the H_2O_2 dose (from 1.0 to 10 mmol L^{-1}) and the caffeic acid concentration (from 0.125 to 4 mmol L^{-1}) to understand the optimum conditions for caffeic acid removal.

2.9.2. Reusability of immobilized soybean peroxidase

The immobilized SBP was studied by repeated use for oxidation of caffeic acid. (1.5 mL of caffeic acid concentration 1.0 mmol L^{-1}; H_2O_2 dose 0.4 mL 3.0 mmol L^{-1}; citrate phosphate buffer, 0.1 mol L^{-1}, pH 6.0, 0.012 g ± 0.003 of immobilized peroxidase). The reaction mixture was incubated in a water bath with mild agitation during 90 min. At the end of each cycle, the same immobilized enzyme was washed with 0.1 mol L^{-1} citrate phosphate buffer, pH 6.0, and the procedure was repeated with a fresh aliquot of substrate. The analysis was carried out in three replications.

2.10. General procedure for the oxidation of CPW and synthetic phenolic solution (SPS)

A solution containing a number of phenolic compounds was prepared from the dilution of standards (caffeic acid, chlorogenic acid, ferulic acid, catechin and epicatechin) in 2% ethanol/water, so that the final concentration of each standard was 1 mmol L^{-1}. The phenolic compounds investigated are the major compounds found in CPW [39].

Wastewater samples were previously centrifuged at 10,000 × g for 10 min to remove interfering impurities. The supernatant was analyzed in order to determine the initial concentration of phenolic compounds by the colorimetric method of Folin and Denis, using caffeic acid as a standard [38].

The enzymatic reactions were conducted in a 0.1 mol L^{-1} citrate phosphate buffer, pH 6.0 (1.2 mL), containing: (1) 3 mmol L^{-1} (0.4 mL) of H_2O_2, (2) CPW or SPS (1.5 mL), 0.1 mL of free enzyme (34.15 U mg^{-1}) and 0.012 g ± 0.003 of immobilized peroxidase (2.51 U mg^{-1}). The enzymatic reaction was stopped by adding 0.1 mL stock catalase solution (1.2 mg of the commercial enzyme in 1.0 mL of 0.1 mol L^{-1} phosphate buffer, pH 7.0). The controls were carried out in the absence of enzyme and H_2O_2 and were performed with chitosan beads without the enzyme to evaluate the adsorption material. The residual concentration of phenolic compounds was measured according to Section 2.9.1.

3. Results and discussion

3.1. Characterization of chitosan beads and immobilization of peroxidase

The chains in the chitosan molecule have abundant free amino groups, which can interact with glutaraldehyde, a very reactive substance [40]. In this study, chitosan beads were prepared and treated with glutaraldehyde 2.5% (w/v). The importance of glutaraldehyde is due to its reliability and ease of use, and availability of amino groups for the glutaraldehyde reaction, not only with enzymes, but also with chitosan [17].

The reaction between the amino and an aldehyde group to produce a Schiff base is a well-known reaction occurring under mild reaction conditions. SBP could also be immobilized on chitosan beads under rather mild conditions (Fig. 1). Multipoint covalent attachment of enzymes on highly activated supports causes a rigidification in the structure of the immobilized enzyme. This rigidification reduces any conformational changes involved in enzyme inactivation and increases enzyme stability [17,41].

The characterization of chitosan beads used in this study was previously performed by our research group [34]. In that study, the morphology and structure of materials were examined by degree of deacetylation (DD), X-ray diffraction and scanning electron microscopy. 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 crosslinked with glutaraldehyde did not

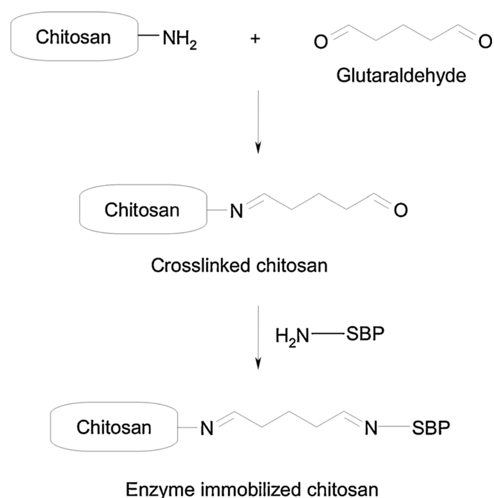


Fig. 1. Scheme of SBP immobilization on the chitosan beads.

present protonatable amino groups, measured by the potentiometric titration method [42]. This result suggests that glutaraldehyde is binded to chitosan amino groups.

The immobilization was performed by adding the free SBP (activity 31.01 U mL^{-1}) to crosslinked chitosan beads. After a coupling time of 4 h [34] the immobilization yield and coupled protein were determined (YI = 51.20% and CP = 65.35%). The maximum amount of SBP immobilized in the beads was 5.93 mg of protein. In higher protein quantities, saturation occurs and any excess SBP will be lost in the filtrate and washing buffer solutions. The specific activity of the immobilized SBP under optimum conditions was determined as 2.51 U mg^{-1}. The results indicate that chitosan beads are efficient for covalent attachment on SBP immobilization.

3.2. Influence of pH on enzymatic activity and thermal stability

The results of pH on the activity of the free and immobilized enzyme are presented in Fig. 2a. The stability of both enzymes shows some similarities. The free and immobilized SBP presented a high activity at pH 6.0 and, in acid and basic regions, a decrease in activity was observed. This activity decrease probably occurs due to ionic changes in the enzyme, which alter the form of the enzyme and, consequently, the active site.

The microenvironment of the immobilized enzyme and the bulk solution usually have an unequal partitioning of H+ and OH^- concentrations due to electrostatic interactions with the matrix, which often leads to displacements in the pH activity profile [43]. However, in the present study, the optimal pH of the free and immobilized enzyme was the same.

The study on the influence of temperature over the activity of the free and immobilized enzyme after 1 h of incubation is shown in Fig. 2b. The maximum enzyme performance was observed in the range from 20–30 °C for the immobilized enzyme. On the other hand, for the free enzyme, the maximum activity was observed at the range from 40–60 °C, resulting in a higher stability, when compared with the immobilized enzyme. At 50 °C and 60 °C, the free peroxidase displayed a relative activity of 92.60% and 96.30%, respectively, whereas the activity of the immobilized enzyme significantly dropped at these temperatures (76.90% and 47.71% relative activity), indicating that the enzyme activity became more dependent on the temperature after immobilization.

As a consequence of enzyme immobilization, some properties of the enzyme molecule, such as its catalytic activity or thermal stability, become altered with respect to those of its soluble counterpart [44].

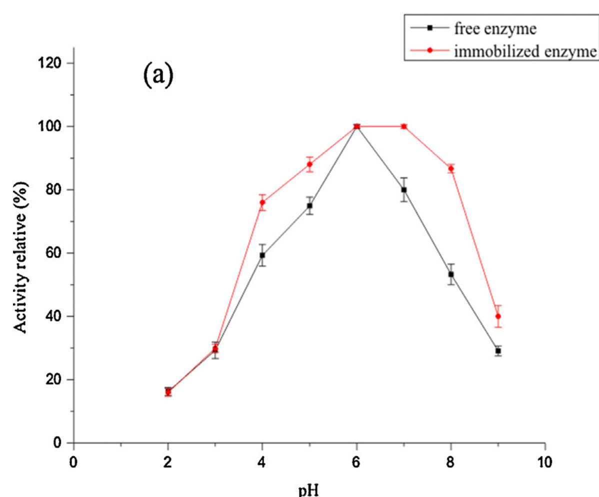
The enzyme active site may be distorted or destroyed when a support has a large expansion coefficient, leading to contraction or expansion during temperature changes [45]. The decrease in thermal stability after immobilization can be assigned to a possible distortion in the conformation of the enzyme active site, due to the decrease in chitosan beads when subjected to high temperatures, as experimentally observed.

According Costa et (2001) [46], 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.

3.3. Oxidation of caffeic acid

3.3.1. Reaction time

Initially experiments were performed in order to assess the optimum contact time required for caffeic acid oxidation. The conversion of caffeic acid against time was studied for both immobilized and free enzyme (Fig. 3). According to Fig. 3, the maximum caffeic acid removal (53.50%) catalyzed by free enzyme was observed in only 30 min. After 30 min of reaction, phenol removal became negligible.



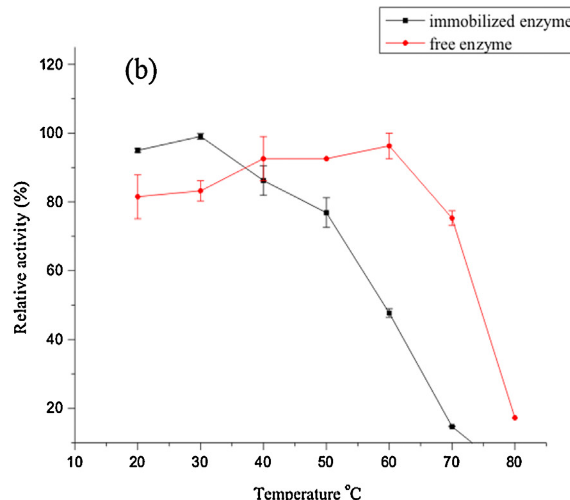


Fig. 2. (a) Activity of soybean peroxidase at various values of pH, at 30 °C; (b) activity of soybean peroxidase at various temperatures, at pH 6.0.

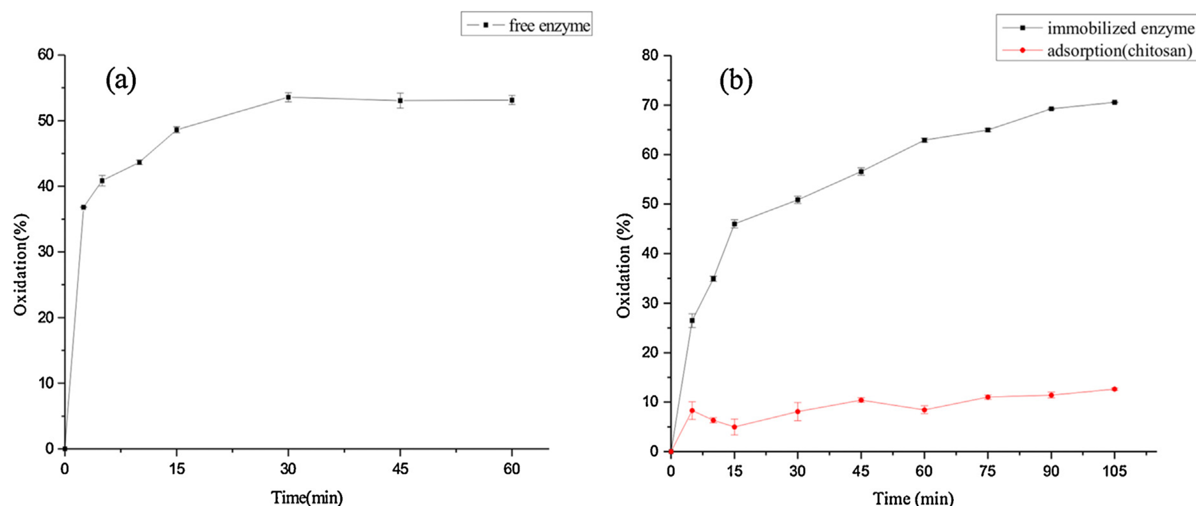


Fig. 3. Reaction progress on the oxidation of caffeic acid. Reaction conditions at 30 °C: concentration in the reaction medium: 1 mmol L^{-1} caffeic acid and 7 mmol L^{-1} H_2O_2; (a) free enzyme; (b) immobilized enzyme and adsorption of chitosan.

Similar results were found by Torres et al. (2014) [31], which obtained 51.07% of removal of caffeic acid in aqueous solution with turnip peroxidase under similar conditions, in only 15 min.

The immobilized enzyme caused a higher oxidation of caffeic acid (70.54% ± 1.45 after 105 min of incubation), where two phenomena were observed: removal due to adsorption on the support (12.60% ± 1.12) and caffeic acid oxidation, due to the action of the enzyme (57.94% ± 0.46). The increase in reaction time for the immobilized enzyme can be explained, since the enzyme is confined, and the contact with the substrate may occur more slowly than when the enzyme is in solution in the reaction medium. Alemzadeh and Nejati (2009) [47], in the removal of phenols by immobilized horseradish peroxidase in porous calcium alginate, obtained a higher removal (approximately 50%) at 100 min of reaction.

The incubation of free soybean peroxidase with caffeic acid without H_2O_2 (control sample) did not result in a change in the concentration of the initial phenolic compound, suggesting that the oxidation is governed only by the activity of peroxidase. In subsequent analyses, the reaction time was set at 45 min for the free enzyme, and 90 min for the immobilized enzyme.

3.3.2. Effect of H_2O_2

Hydrogen peroxide acts as a co-substrate to activate the enzymatic action of the peroxidase radical. However, the excess of this reagent in the reaction inhibits enzyme activity and, when present in small amounts, it limits the reaction rate [48–50]. An increasing in the percentage of phenol removal could be obtained by choosing an appropriate hydrogen peroxide concentration. It has also been described [51] that the optimum peroxide concentration totally depends on the initial phenol concentration and differ from case to case [47].

It was observed that, for H_2O_2 concentrations at the range from 1 to 10 mmol L^{-1} (Fig. 4), both enzymes show similar behaviors. The amount of caffeic acid removed was sharply increased with an increase in the dose of H_2O_2 up to an optimal point of 3 mmol L^{-1} (76.50% ± 0.379, 57.60% ± 0.754 immobilized and free enzyme, respectively).

It appears that hydrogen peroxide is a limiting factor in this range. A reason for this phenomenon would be that an excess amount of hydrogen peroxide results in higher concentrations of intermediate products which inhibit the activity of the enzyme, and/or that enzyme is inactivated by an excess of hydrogen peroxide. The deviation of the aforementioned ratio might be the result of the polymer produced in the catalytic process, larger than the dimer [52].

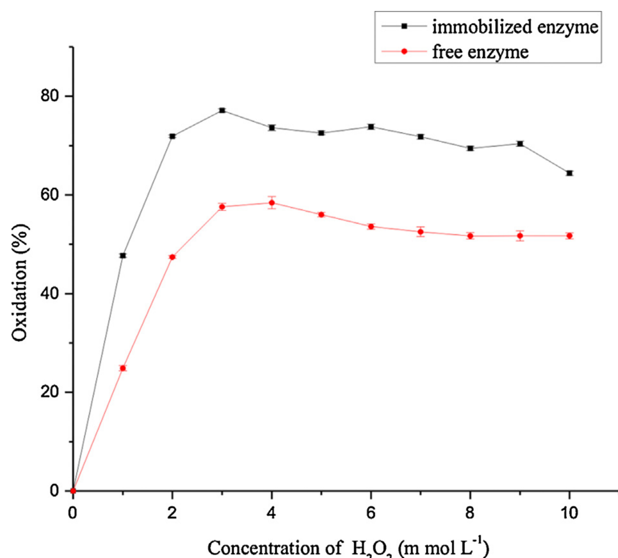


Fig. 4. Effect of the concentration of H_2O_2 on the oxidation of caffeic acid at a concentration of 1 mmol L^{-1} in the reaction medium by free and immobilized enzyme pH 6.0, at 30 °C for 90 min (immobilized enzyme) and at 40 °C for 45 min (free enzyme).

3.3.3. Effect of the concentration of caffeic acid

The effect of substrate concentration was investigated and the obtained data are presented in Fig. 5. The free and immobilized enzyme showed similar profiles. The increase in the concentration of caffeic acid until 1 mmol L^{-1} (78.20% ± 0.422, 60.20% ± 0.955 immobilized and free enzyme, respectively) provides an increase in enzyme performance. From the obtained data, it is possible to suggest that the optimum H_2O_2/caffeic acid molar ratio is 3:1. Controls without enzyme on beads showed a lower adsorption percentage, 10.56 ± 0.768, in these conditions.

Based on the known mechanism of peroxidases, a minimum H_2O_2/phenol molar ratio of 1:2 is required for complete conversion. However, it has been demonstrated that some of the dimers produced in the initial radical coupling stages are also substrates of the enzyme [53]. The radicals diffused from active peroxidase sites will link up non-enzymatically to form these dimers. If these dimers

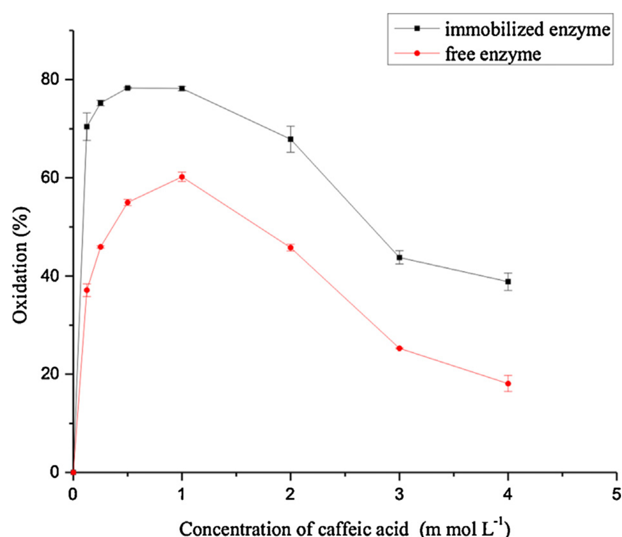


Fig. 5. Effect of the concentration of caffeic acid at a concentration of 3 mmol L^{-1} H_2O_2 in the reaction medium by free and immobilized enzyme pH 6.0, at 30 °C for 90 min (immobilized enzyme) and at 40 °C for 45 min (free enzyme).

are soluble, then they will also be substrates of peroxidase and undergo radicalization by peroxidase, forming higher oligomers. This process will continue until the generated oligomers reach their solubility limit and precipitate in the solutions [54]. As discussed above, this implies that the hydrogen peroxide demand would be increased.

3.3.4. Reusability of immobilized soybean peroxidase

Reusability is one of the significant indices to evaluate the application of immobilized enzymes in industries [55]. The immobilized enzyme could be easily removed and assessed for its remained catalytic activity. To demonstrate the reusability of the immobilized enzyme, beads were separated after 90 min of reaction and then rinsed thoroughly with 0.1 mol L^{-1} citrate phosphate buffer, pH 6.0. The beads were used for subsequent batches. After the test was repeated 4 times, the efficiency in phenol removal was reduced to half its initial value (Fig. 6).

This result might be explained by the plugging of chitosan accumulation of radicals and dimer in the interior environment of each bead; another possible negative effect that should not be overlooked is that of the formed polymer which, in the present study, instead of adsorbing the enzyme and hindering the access of the substrate to the enzyme active site, was adsorbed over the immobilized enzyme particles, with a similar effect [56].

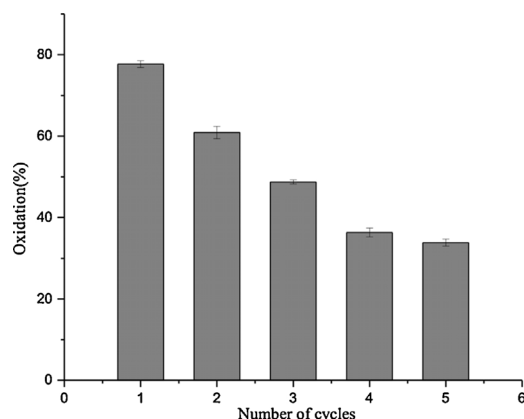


Fig. 6. Oxidation of 3 mmol L^{-1} caffeic acid with repeated applications of immobilized soybean peroxidase pH 6.0, at 30 °C for 90 min.

Table 1
Oxidation (%) of coffee processing wastewater (CPW) and synthetic phenolic solution (SPS).

	CPW	SPS
Immobilized enzyme + adsorption	32.69 ± 0.64	79.18 ± 0.62
Free enzyme	19.38 ± 1.53	61.51 ± 0.89
Adsorption on chitosan[a]	12.46 ± 1.04	11.06 ± 0.77
Chemical oxidation[b]	Nd[c]	Nd[c]

[a] Absence of enzyme and H_2O_2.
[b] Absence of enzyme.
[c] Nd = not detected.

3.4. General procedure for the oxidation of CPW and synthetic phenolic solution (SPS)

The best reaction conditions found for the oxidation of caffeic acid by immobilized and free enzyme, were used in the oxidation of CPW and SPS. The CPW used in this study showed a concentration of 218.21 mg L^{-1} total phenols, which exceeds the CONAMA Resolution 430/2011 [5]. The mixture of phenolic compounds showed a concentration of 219.54 mg L^{-1} total phenols, very close to the concentration of CPW. A study was also conducted to compare the efficiency of the oxidation percentage for immobilized and free enzyme (Table 1).

The mixtures of oxidation rates of phenolic compounds by SPS were 79.18 ± 0.62 and 61.51 ± 0.89 for immobilized and free enzyme, respectively. Peroxidases have the ability to co-precipitate certain phenols, including certain non-substrates, which would otherwise be difficult to remove by inducing the formation of mixed polymers easily removable [49,57]. This is of great importance, since many industrial effluents contain a variety of phenolic contaminants; some of them are more amenable to enzymatic treatments than others. In this study, the controls made with hydrogen peroxide and without the free enzyme did not cause any oxidation percentage, while the controls without the enzyme on chitosan beads showed a lower adsorption percentage, 11.06 ± 0.77.

Phenol oxidation of CPW in an aqueous solution catalyzed by the free enzyme (31.01 U mL^{-1}) was 19.38% ± 1.53. Similar results were found by Torres et al. (2014) [31], who obtained 18.25% enzymatic oxidation of CPW in aqueous solution with turnip peroxidase under similar conditions. Phenol oxidation of CPW obtained by the immobilized enzyme was 32.69% ± 0.64, and the immobilized enzyme had the higher oxidation performance, which could be explained by the adsorption of phenolic compounds on the chitosan support (12.46 ± 1.04).

The efficiency of the enzymatic treatment was lower in the case of the real effluent, which can be explained by the influence of other substances involved in the reaction medium, since the effluent is rich in organic (sugars and proteins) and inorganic compounds, besides a large amount of settleable solids [4]. The complexity of the effluent from coffee processing can have directly influenced the enzyme-substrate specificity, product formation or even caused enzyme inactivation.

4. Conclusions

Soybean peroxidase was successfully immobilized onto chitosan with glutaraldehyde. Both the free and immobilized enzyme showed similarities in some conditions such as pH, amount of H_2O_2 and caffeic acid; however, the reaction time of the immobilized enzyme was two times higher than that of the free enzyme.

The enzymatic efficiency in the removal of phenolic compounds in the synthetic solution was similar, when compared to a caffeic acid standard. This suggests that the presence of other phenolic compounds in the reaction medium did not influence the oxidation potential. However, the enzyme performance in a real sample was limited, due to the high complexity of the coffee processing effluent.

Enzyme immobilization provided an increase in the oxidation of caffeic acid, SPS and CPW, besides enabling the recovery of the enzyme from the reaction medium and its reuse. These results are significant, with the possibility of application of immobilized enzymes in industrial sectors, leading to a reduction in operating costs.

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