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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.

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:

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

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

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,