Enzymatic oxidation of phenolic compounds in coffee processing wastewater

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ABSTRACT

Peroxidases can be used in the treatment of wastewater containing phenolic compounds. The effluent from the wet processing of coffee fruits contains high content of these pollutants and although some studies propose treatments for this wastewater, none targets specifically the removal of these recalcitrant compounds. This study evaluates the potential use of different peroxidase sources in the oxidation of caffeic acid and of total phenolic compounds in coffee processing wastewater (CPW). The identification and quantification of phenolic compounds in CPW was performed and caffeic acid was found to be the major phenolic compound. Some factors, such as reaction time, pH, amount of H_2O_2 and enzyme were evaluated, in order to determine the optimum conditions for the enzyme performance for maximum oxidation of caffeic acid. The turnip peroxidase (TPE) proved efficient in the removal of caffeic acid, reaching an oxidation of 51.05% in just 15 minutes of reaction. However, in the bioremediation of the CPW, the horseradish peroxidase (HRP) was more efficient with 32.70% ± 0.16 of oxidation, followed by TPE with 18.25% ± 0.11. The treatment proposed in this work has potential as a complementary technology, since the efficiency of the existing process is intimately conditioned to the presence of these pollutants.

Key words | environmental biocatalysis, enzymatic oxidation, peroxidase, phenolic compound, wastewater

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INTRODUCTION

The presence of different aromatic compounds in wastewater from several industries is a matter of great environmental concern (Ashraf & Husain 2010). Phenols and their derivatives occur in the wastewater of many industries, such as coal conversion, oil refining, manufacturing of plastics, paper, pulp, resins, textiles, iron, steel production (Ashraf & Husain 2009) and notably in coffee processing wastewater (CPW) (Fia *et al.* 2010).

These substances, once released into the environment, may accumulate in groundwater, surface water and soil (Coniglio *et al.* 2008). The phenolic compounds are highly recalcitrant, feature a high degree of toxicity, toxic to the microorganisms in the biological treatment, beyond bioaccumulate in various food chains even at low concentrations (Rodrigues *et al.* 2010). As a result, the release of these compounds in rivers and sloughs without prior treatment may be responsible for the toxification of aquatic systems (Diao *et al.* 2011). The CONAMA Resolution No. 430 from 05/13/2011 states that any effluents from polluting sources can only be released directly or indirectly into water bodies if their maximum phenol concentration is 0.5 mg L^{-1}.

Furthermore, these compounds of recalcitrant nature are potential interferents in commercially important bioprocessing technologies such as the production of biofuels. In such technology, a pretreatment step of lignocellulose is required to deconstruct the complex cell wall structure and expose cellulose to cellulolytic enzymes. On the other hand, lignocellulose pretreatment has the detrimental effect of also releasing a wide range of compounds, such as phenolics, acetate, and furan aldehydes, which are inhibitory to fermenting microorganisms and cellulolytic enzymes. Therefore, removal of these inhibitors is essential for the production of biofuels from lignocellulosic biomass. Several chemical and physical methods are employed but feature some drawbacks. Biological methods such as biological abatement (or bioabatement) stand out as being

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potentially effective and efficient in terms of cost, by employing microorganisms which metabolize inhibitors (Ximenes *et al.* 2010, 2011; Cao *et al.* 2013). Furthermore, studies have confirmed that the addition of hemicellulase in the bioabated biomass enhanced cellulose conversion (Cao *et al.* 2015).

Coffee is one of the popular beverages most consumed worldwide, Brazil being the largest producer and exporter of the product, and the second largest consumer, with currently a coffee crop estimated at 2.3 million hectares (Esquivel & Jiménez 2012; Brasil 2013).

Coffee is processed either by wet or dry method. The wet method of processing results in a coffee of superior quality, compared to the dry method. Wet processing of coffee uses a lot of water at different stages of its processing. The resultant effluent is rich in total suspended and dissolved solids, containing a wide variety of organic compounds, such as caffeine, sugars and phenolic compounds, unsuitable for direct disposal in soil or waterways (Gonçalves *et al.* 2008). There is little information about wastewater from the coffee wet processing, and little is known about the physical, chemical and biological status. If the wastewater emanating from these operations is discharged into natural water bodies without treatment, in addition to the negative effects of phenol on ecosystems and other natural resources, this pollutant can enter into the food chains through agricultural products or drinking water (Coniglio *et al.* 2008).

Pulping water can be reused for depulping, however it results in an increase in organic matter and decreasing pH, which can come to deplete the life-supporting oxygen from the water bodies (Haddis & Devi 2008).

The removal of phenolics from wastewater can be achieved either by physicochemical or biological processes. Some of the physicochemical processes employed for the removal of phenolic compounds from industrial wastewater include extraction, adsorption on activated carbon, steam distillation, chemical oxidation, electrochemical techniques, irradiation, etc. (Coniglio *et al.* 2008). However, these methods have their own drawbacks, such as high cost, incomplete purification, formation of hazardous by-products, low efficiency and applicability to a limited concentration range (Husain & Husain 2008; Husain *et al.* 2009; Diao *et al.* 2011). Nevertheless, these methods are not suitable for treating moderate to high concentrations of phenols (Diao *et al.* 2011). The biological treatment of aromatic compounds also has several limitations, such as sludge formation, slow reaction and formation of side products, which at times are even more toxic (Ashraf & Husain 2010).

For these reasons, more attention has been paid to the development of alternative and/or complementary technologies for the removal of toxic organic pollutants in wastewater (Hejri & Saboora 2009). It was also found from previous studies that only a small amount of work has been done to analyze various aspects of coffee processing, as well as the impact of such effluent on the surrounding environment and human health. Thus, it is necessary to develop economically viable and eco-friendly technologies for handing such type of wastewaters (Haddis & Devi 2008).

The enzymatic method has some advantages over conventional methods of treatment, which include: applicability over a broad range of pH, temperature, salinity and contaminant concentration, action on recalcitrant materials, simplicity in controlling the process and minimal environmental impact (Bódalo *et al.* 2006b; Ashraf & Husain 2010). The application of oxidoreductive enzymes, such as peroxidases, to catalyse the oxidation of aromatic compounds from wastewater has been widely investigated (Akhtar & Husain 2006; Quintanilla-Guerrero *et al.* 2008; Ashraf & Husain 2009, 2010; Al-Ansari *et al.* 2010; Diao *et al.* 2011). In the presence of hydrogen peroxide (H_2O_2), which acts as an electron acceptor, peroxidases (H_2O_2 donor oxidoreductase, E.C.1.11.1.7) are able to catalyze the oxidative polymerization of phenolic compounds to form insoluble polymers (Quintanilla-Guerrero *et al.* 2008).

The involvement of peroxidases in the oxidative polymerization of phenols was previously demonstrated by several authors (Buchanan & Nicell 1997; Wright & Nicell 1999). Most reports on the detoxification of wastewater contaminated with phenols, cresols and chlorophenols have used horseradish peroxidase (Husain *et al.* 2009), which is usually cultivated and harvested in cold climate countries (Maciel *et al.* 2006). Due to the widespread use of peroxidases, mainly as an environmental biocatalyst, there is a growing interest in new sources of this enzyme (Silva *et al.* 2012b). Recently, peroxidases from other sources, such as soybean (Al-Ansari *et al.* 2010), turnip (Quintanilla-Guerrero *et al.* 2008) and bitter gourd (Akhtar & Husain 2006) have been suggested as alternatives to horseradish.

Studies involving the enzymatic removal of phenolic compounds from real effluents are limited, mainly for CPW. In this context, the objectives of this study included the identification and quantification of phenolic compounds from CPW, the evaluation of different peroxidase sources in the oxidation of caffeic acid, a phenolic compound, and in the total phenolic oxidation of CPW.

MATERIALS AND METHODS

Chemicals and reagents

Catalase (EC 1.11.1.6 ≥ 10.000 units per mg protein) and horseradish peroxidase (EC 1.11.1.7) were purchased from Sigma-Aldrich. Soybean peroxidase (EC 1.11.1.7) was kindly provided by Bio-Research Products. Acetic acid and methanol were purchased from Merck (Darmstadt, Germany).

The phenolic standards used were: caffeic acid, gallic acid, gallocatechin, 3,4-dihydroxybenzene, catechin, chlorogenic acid, epigallocatechin, vanillic acid, epicatechin, syringic acid, *p*-coumaric acid, ferulic acid, *m*-coumaric acid, *o*-coumaric acid, resveratrol, ellagic acid, salicylic acid, all purchased from Sigma-Aldrich (St Louis, MO, USA). Milli-Q system and nylon membrane were obtained from Millipore (Billerica, MA, USA).

Horseradish peroxidase (HRP) and soybean peroxidase (SBP commercial enzyme) were used in aqueous solution (30 mg of the commercial enzyme in 5 mL of 0.1 mol L^{-1} phosphate buffer, pH 7.0 and 26 mg of the commercial enzyme in 20 mL of 0.1 mol L^{-1} citrate phosphate buffer, pH 6.0, respectively). After the preparation, the enzymes were stored at an average temperature of 4 °C.

Coffee processing wastewater

Collection

The CPW was obtained on a farm located in Bom Sucesso, Minas Gerais. 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.

Identification and quantification of phenolic compounds from CPW

A high-performance liquid chromatography (HPLC) system was used for separation, identification and quantification of the phenolic compounds present in the wastewater from the coffee fruits processing. Chromatographic analyses were performed using an Agilent HPLC equipment model 1100, equipped with a binary pump, an auto injector and a detector with diode array at a wavelength of 280 nm. The wastewater and the standards were separated on an Ascentis C_{18} column (25 cm × 4.6 mm, 5 μm), attached to an Ascentis C_{18} pre-column (2 cm × 4.0 mm, 5 μm). The mobile phase was composed of the following solutions: 2% acetic acid (A) and methanol:water:acetic acid (70:28:2 v/v/v) (B). Analyses were performed in a total time of 50 min at 15 °C, in a gradient-type system: 100% solvent A from 0.01 to 5 min, 70% solvent A from 5 to 25 min, 60% solvent A from 25 to 43 min, 55% solvent A from 43 to 50 min. Solvent A was increased to 100%, seeking to maintain a balanced column. The flux used in all tests was 1 mL min^{-1} and the injection volume was 20 μL.

The stock standard solutions were prepared in methanol in a concentration range from 0.1 to 127.72 mg L^{-1}. Acetic acid and methanol were used to prepare the mobile phase and ultrapure water was obtained by the Milli-Q system.

The wastewater was initially injected into the pre-column to remove impurities that could damage the chromatographic column. The wastewater and the standards were filtered on a 0.45 μm nylon membrane and then injected directly into the chromatographic system in three replications. The phenolic compounds in the wastewater were identified by comparison with the retention times of the standards. Quantification was performed by the construction of analytical curves.

Obtention of the enzymatic extract

The enzyme was extracted from turnip roots purchased from the local market. The roots (with peel) were washed in water and cut into small uniform pieces. Turnip roots (30 g) were homogenized in a blender with 100 mL of 0.05 mol L^{-1} phosphate buffer, pH 6.5, containing 0.2 mol L^{-1} NaCl for 30 s. The homogenate was filtered in organza cloth and centrifuged at 10,000 × *g* for 15 min, at 4 °C (Fatibello Filho & Vieira 2002). The obtained solution was subjected to precipitation by adding cold acetone until reaching 65% (v/v). After a rest from 12 to 14 h, at −18 °C, the homogenate was centrifuged at 11,000 × *g* for 15 min, at 4 °C. The precipitate obtained after the removal of the acetone by a treatment in the fridge for 72 h was redissolved in phosphate buffer, pH 7.0 and then used for the studies on phenolic compound oxidation (Silva *et al.* 2012a).

To obtain the soybean seed hull extracts, the seeds were immersed in distilled water for 24 h and hull removal was performed manually. The procedure for the extraction of peroxidase was similarly to that for the turnip roots, differing only in the amount of the plant tissue used, which was 15 g of seed hull.

Enzymatic activity

The enzymatic activity was determined according to Khan & Robinson (1994; with modifications) by using the following reaction medium: 1.5 mL of 1% guaiacol (v/v) (Vetec, PA, 97%, v/v), 0.4 mL of 12.25 mmol L^{-1} H_2O_2 (Vetec, PA), 0.1 mL enzyme (kept in an ice bath) and 1.2 mL of 0.1 mol L^{-1} phosphate buffer pH 7.0 for turnip peroxidase (TPE) and HRP, and 1.2 mL of 0.1 mol L^{-1} citrate phosphate buffer pH 6.0 for the peroxidase extracted from soybean seed hulls (SBP) and commercial soybean peroxidase (SBP, commercial enzyme). The reaction was carried out for 3 min at 30 °C in a Spectrovision spectrophotometer coupled to a thermostatic bath.

One unit of peroxidase activity (U) represents the formation of 1 μmol tetraguaiacol per minute of reaction in the assay conditions and it was calculated by using data relative to the linear portion of the curve (Khan & Robinson 1994).

Reaction time

The first part of this study was to optimize the reaction conditions for the removal of caffeic acid in the presence of the turnip peroxidase. A caffeic acid solution (1 mmol L^{-1}) 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, according to the results of the HPLC analysis in the present study, and the concentration utilized was based on the concentration of total phenols found in CPW.

Initially, the enzymatic reactions were conducted in phosphate buffer, 0.1 mol L^{-1}, pH 7.0 (1.2 mL), containing (1) 5 mmol L^{-1} H_2O_2 (0.4 mL) and (2) caffeic acid at a concentration of 1 mmol L^{-1} (1.5 mL) and 0.1 mL enzymatic solution (16.83 U mL^{-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 withdrawn at 5 min intervals up to 180 min, 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) (Diao *et al.* 2011). The same procedure to stop the enzymatic reaction and to remove the insoluble product were done in the following assays.

The insoluble product was removed by centrifugation at 3,000 × *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 standard (AOAC 2012). The calculation to determine the percentage of removed caffeic acid is given according to Equation (1):

$$\text{Oxidation of caffeic acid (\%)} = \frac{[C]\,\text{initial} - [C]\,\text{final}}{[C]\,\text{initial}} \times 100 \quad (1)$$

where [*C*]initial = initial concentration of caffeic acid, and [*C*]final = final concentration of caffeic acid.

Three repetitions were carried out for each treatment.

Effect of pH

The caffeic acid solutions 1 mmol L^{-1} (1.5 mL) were incubated with TPE (16.83 U mL^{-1}) in the buffers of different pH values (2 to 10.6) in the presence of 5 mmol L^{-1} H_2O_2 (0.4 mL) for 30 min at 30 °C. The buffers used were citrate phosphate buffer (0.1 mol L^{-1}, pH 2.0 to 7.0), phosphate buffer (0.1 mol L^{-1}, pH 7.0), Tris-HCl buffer (0.1 mol L^{-1}, pH 7.0 to 9.0) and glycine-NaOH buffer (0.1 mol L^{-1}, pH 10.0 and 10.6). The controls were carried out in the absence of enzymes.

Effect of H_2O_2

The caffeic acid solutions 1 mmol L^{-1} (1.5 mL) were incubated with TPE (5.41 U mL^{-1}) with H_2O_2 concentrations from 1 to 10 mmol L^{-1} (0.4 mL) in a 0.1 mol L^{-1} Tris-HCl buffer, pH 8.0 for 30 min at 30 °C. The controls were carried out in the absence of enzymes. This selected peroxide concentration range was to ensure an excess of this reagent, since according to the classical peroxidases mechanism a minimum hydrogen peroxide:phenol molar ratio is of 1:2 for complete conversion.

Effect of enzyme concentration

The caffeic acid solutions 1 mmol L^{-1} (1.5 mL) were incubated with increasing concentrations of TPE (0.0673 U mL^{-1} to 31.25 U mL^{-1}) in the presence of 7 mmol L^{-1} H_2O_2 (0.4 mL) in a 0.1 mol L^{-1} Tris-HCl buffer, pH 8.0 for 30 min at 30 °C.

Caffeic acid conversion by different sources of peroxidase

After optimization of the reaction conditions for the oxidation of caffeic acid by TPE, the assays were carried out proposing the evaluation of the potential of different peroxidase

sources: SBP, SBP (commercial enzyme) and HRP on the conversion of caffeic acid, for comparison purposes.

The enzymatic reactions were conducted in a 0.1 mol L^{-1} Tris-HCl buffer, pH 8.0 (1.2 mL), containing: (1) 7 mmol L^{-1} (0.4 mL) H_2O_2, and (2) caffeic acid solution at a concentration of 1 mmol L^{-1} (1.5 mL) and 0.1 mL of peroxidase different sources: peroxidase extracted from soybean seed hulls – SBP (29.93 U mL^{-1}), commercial soybean peroxidase – SBP commercial enzyme (29.57 U mL^{-1}) and horseradish peroxidase – HRP (29.20 U mL^{-1}) for 30 min at 30 °C.

General procedure for the bioremediation of CPW

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 standard (AOAC 2012).

The enzymatic reactions were conducted in a 0.1 mol L^{-1} Tris-HCl buffer, pH 8.0 (1.2 mL), containing: (1) 7 mmol L^{-1} (0.4 mL) H_2O_2, and (2) CPW (1.5 mL) and 0.1 mL of peroxidase from different sources: TPE (31.25 U mL^{-1}), SBP (29.93 U mL^{-1}), SBP commercial enzyme (29.57 U mL^{-1}) and HRP (29.20 U mL^{-1}) at 30 °C. The reaction mixture was stirred continuously for 30 min. The controls were carried out in the absence of enzymes and H_2O_2.

RESULTS AND DISCUSSION

Identification and quantification of phenolic compounds in CPW

The wet method of coffee fruits processing generates large volumes of wastewater rich in various organic and inorganic compounds of high-polluting power. There is little information on the constitution of these effluents, especially in relation to phenolic compounds. In order to identify and quantify the phenolic compounds present in this wastewater (CPW), a sample of the effluent was analyzed by HPLC.

The following phenolic compounds were identified in CPW, in mg L^{-1}: caffeic acid (40.08), catechin (8.51) and gallocatechin (0.55), the last two are tannin monomers (Figure 1). It is possible to verify the presence of a few other peaks, which were not identified, but which are possibly other phenolic compounds. Caffeic acid was the major phenolic compound found in CPW and was, therefore, chosen for the optimization reactions.

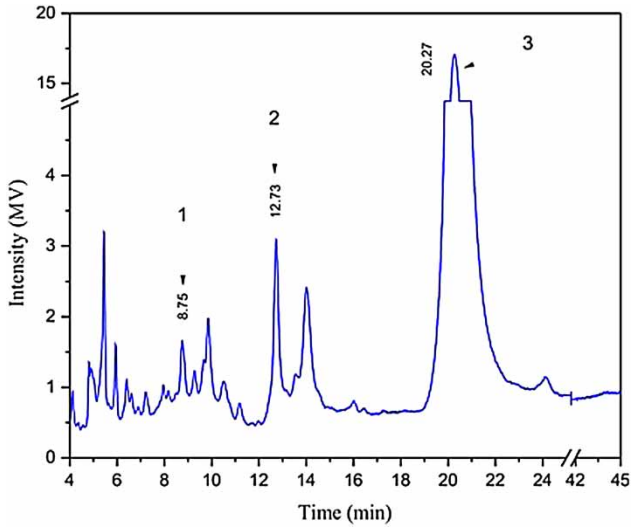

Figure 1 | Chromatogram of phenolic compounds of wastewater from coffee fruits processing. Identification of peaks: 1 – gallocatechin, 2 – catechin and 3 – caffeic acid.

Reaction time

According to Figure 2, in only 15 min the maximum removal of caffeic acid occurred (51.07%) and a further increase in the reaction time had no significant effect on the oxidation. The drastic slowdown may be mainly attributed to the simultaneous decrease of all reacting species or enzyme inactivation (Miland *et al.* 1996). However, peroxidases are also quickly inactivated through some mechanisms. In treating wastewater, it should be recognized that certain other

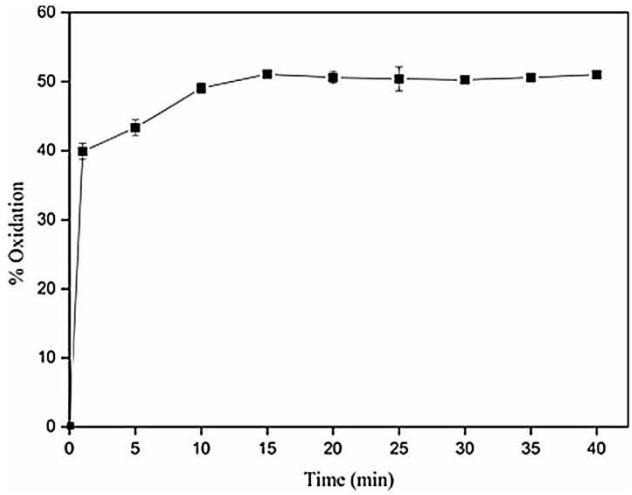

Figure 2 | Reaction progress on the oxidation of caffeic acid. Reaction conditions: enzyme load – 16.83 U mL^{-1}; concentration in the reaction medium – 1 mmol L^{-1} caffeic acid and 5 mmol L^{-1} H_2O_2.

pollutants may compete for the oxidants (O_2 or H_2O_2) (Steevensz *et al.* 2009).

It is generally observed that peroxidases are faster in oxidizing substrates with predetermined enzyme concentrations (Steevensz *et al.* 2009), as occurred in the present study. The enzymatic oxidation of phenolic compounds in a short period of time makes this method attractive, since conventional treatments require long periods of time (Duarte-Vázquez *et al.* 2003).

The incubation of TPE 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 removal of these compounds is governed only by the peroxidase activity, which was identified by electrophoresis in the crude extract (data not shown). Subsequent experiments were performed for 30 min reaction time.

Bódalo *et al.* (2006b) evaluated the removal of 4-chlorophenol (2 mmol L^{-1}) by soybean peroxidase and H_2O_2 (2 mmol L^{-1}) in a discontinuous tank reactor. The conversion percentage of the phenolic compound, which was also evaluated, exhibited fast kinetics. In only 10 minutes, a conversion of about 99% was attained for an enzyme concentration of 10^{-4} mg mL^{-1}.

Effect of pH

In Figure 3 the effect of different pH values is demonstrated on the oxidation of caffeic acid by TPE. Higher rates of removal were observed at pH 8 and 9. The use of pH values close to neutrality has been recommended for the treatment of phenolic effluents. However, at a pH below 5.0 and above 9.0, a decline in the conversion of caffeic acid was observed. At low pH values, the decrease in caffeic acid conversion might be attributed to the detachment of the heme prosthetic group from the polypeptide chain, which resulted in the loss of enzyme activity (Deepa & Arumughan 2002).

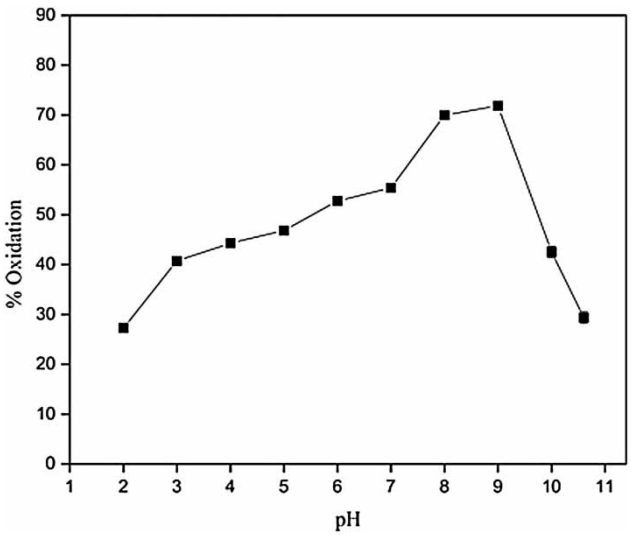

Figure 3 | Effect of pH on the oxidation of caffeic acid at a concentration of 1 mmol L^{-1} in the reaction medium by turnip peroxidase at 16.83 U mL^{-1}, at 30 °C for 30 min.

The effects of pH on the catalytic efficiencies of different enzymatic coupling reaction systems had been studied extensively and was frequently in the range from 5 to 9 (Coniglio *et al.* 2008). Ashraf & Husain (2009) evaluated the oxidation of α-naphthol (0.5 mmol L^{-1}) by white radish peroxidase. The phenolic compound was oxidized at a pH range from 5.0 to 8.0. Karim & Husain (2009) evaluated the oxidation of bisphenol A (0.5 mmol L^{-1}) by bitter gourd peroxidase and also found that, in a pH range from 5.0 to 8.0, it achieved a maximum percentage at pH 7.0.

Máximo *et al.* (2012) determined the maximum conversion in the concentration of 4-chlorophenol obtained by soybean peroxidase, horseradish peroxidase and artichoke peroxidase as a function of pH. In the case of soybean peroxidase and horseradish peroxidase, the maximum conversion was reached with pH 7.0, while for artichoke peroxidase it was obtained at pH 6.5.

According to Yamada *et al.* (2010), the optimum pH value depends on experimental parameters, such as the kind of peroxidase and the kind and concentration of phenolic compounds, the position of the substituent groups of some phenols and the additives used to protect the enzymatic activity.

To evaluate determined parameters, such as enzyme concentration, pH and H_2O_2, only the parameter under study was modified and all others kept constant. Thus, in the study of the effect of pH on the oxidation of caffeic acid, the time, enzyme concentration and H_2O_2 was kept constant, and only the pH was varied from 2 to 10.6. From these considerations, the results obtained are related to the enzyme performance using caffeic acid as substrate. As well as in the study of pH, all the other optima's conditions evaluated are a function the type of employed peroxidase. Steevensz *et al.* (2009) evaluated the conversion of phenol using two different sources of peroxidase and observed difference in percentage of removal depending on the type of enzyme used.

Effect of H_2O_2

Hydrogen peroxide (H_2O_2) acts as a co-substrate to activate the enzymatic action of the peroxidase radical. However,

the excess of this reagent in the reaction inhibits the enzyme activity and, when present in small quantities, it limits the reaction rate (Silva *et al.* 2012a).

Based on the known mechanism of peroxidases, a minimum hydrogen peroxide: 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 (Yu *et al.* 1994). The radicals diffused from the active sites of peroxidase will link up non-enzymatically to form these dimers. If these dimers 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. H_2O_2 is consumed in these series of reactions, since peroxidase can also treat these intermediates (Yamada *et al.* 2010). Therefore, as discussed above, this implies that the demand of H_2O_2 will be higher than the stoichiometry of the mechanism of peroxidases suggests (Steevensz *et al.* 2009).

It was observed that for H_2O_2 concentrations in the range from 3 to 10 mmol L^{-1} in the reaction medium of oxidation (peroxide:phenol ratio 7:1), there was no significant influence on the performance of the enzyme (Figure 4). Therefore, the ability of peroxidase to oxidize phenolic compounds was only affected at H_2O_2 concentrations below 3 mmol L^{-1}. Controls done in the absence of enzymes to assess the influence of a possible chemical oxidation showed no conversion percentage.

Effect of enzyme concentration

Since the biocatalyst has a finite lifetime and the conversion is found to be dependent on the contact time, normally removal of phenol is dependent on the amount of catalyst added (Alemzadeh & Nejati 2009). The conversion of caffeic acid as a function of enzyme concentration is shown in Figure 5. The increase in the enzyme load is accompanied by an increase in the oxidation of up to 14.43 U mL^{-1} (76% phenolic oxidation). When the enzyme concentration increased approximately twice (31.25 U mL^{-1}), no influence on the removal of the phenolic compound was observed. From these results, it was concluded that high concentrations of enzyme have no significant effects on the conversion efficiency.

This observation is in agreement with the outcome presented by Karim & Husain (2009). The authors observed that the oxidation of bisphenol A was increased with increasing concentrations of bitter gourd peroxidase and its maximum removal was 67% by 0.32 U mL^{-1} of peroxidase, and, from this concentration, no increase in the oxidation of bisphenol A was observed.

Al-Ansari *et al.* (2010) also studied the enzymatic oxidation of phenol by soybean peroxidase and, within the enzyme concentrations evaluated by the authors (2 to 14 U mL^{-1}), the minimum soybean peroxidase concentration required to achieve 95% oxidation of phenol was 14 U mL^{-1}.

Similar results were observed by Kalaiarasan & Palvannan (2014), who studied the effect of enzyme concentration

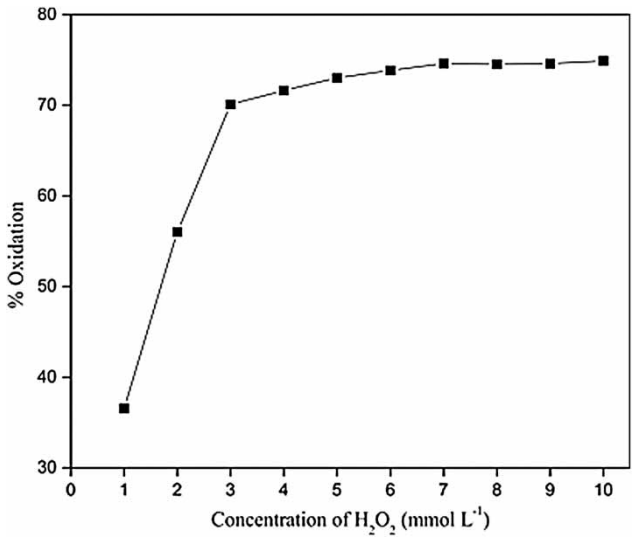

Figure 4 | Effect of the concentration of H_2O_2 on the oxidation of caffeic acid at a concentration of 1 mmol L^{-1} in the reaction medium by turnip peroxidase at 5.41 U mL^{-1}, pH 8.0, at 30 °C for 30 min.

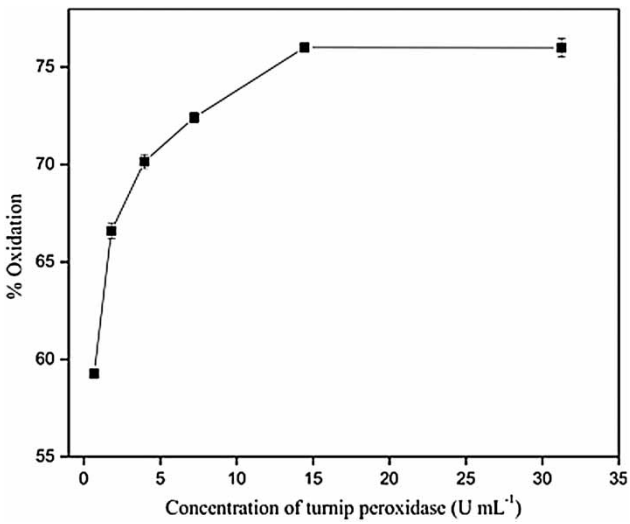

Figure 5 | Effect of the concentration of turnip peroxidase on the oxidation of caffeic acid at a concentration of 1 mmol L^{-1} in the reaction medium, pH 8.0, at 30 °C for 30 min.

on phenol oxidation. Different concentrations of horseradish peroxidase, from 0.1 to 1 U mL^{-1}, were used to compare the efficiency of the stabilized enzyme. It was found that, for 0.1 mmol L^{-1} of phenol concentration, increasing enzyme concentrations from 0.1 to 0.9 U mL^{-1} resulted in the gradual increase in phenol oxidation. A further increase in enzyme concentration has no significant effect on phenol oxidation.

Caffeic acid conversion by different sources of peroxidase

A study was conducted to compare the efficiency of the conversion by different sources of peroxidase. The comparison highlights the advantages that some enzymes have over others in regard to substrates and their reaction environment (Steevensz *et al.* 2009). The conversion of caffeic acid catalyzed by HRP (29.20 U mL^{-1}), SBP commercial enzyme (29.57 U mL^{-1}), SBP (29.93 U mL^{-1}) and TPE (31.25 U mL^{-1}) is shown in Figure 6. The following oxidation percentages were obtained: 75.99% ± 0.48 by TPE, followed by HRP (67.55% ± 0.10), SBP commercial enzyme 501 (64.74% ± 0.34) and SBP (64.28% ± 0.32). The data showed that there was no pronounced effect of the use of different enzymes sources on the efficiency of caffeic acid conversion, which is of great convenience and encourages the use of other biocatalysts in the same treatment.

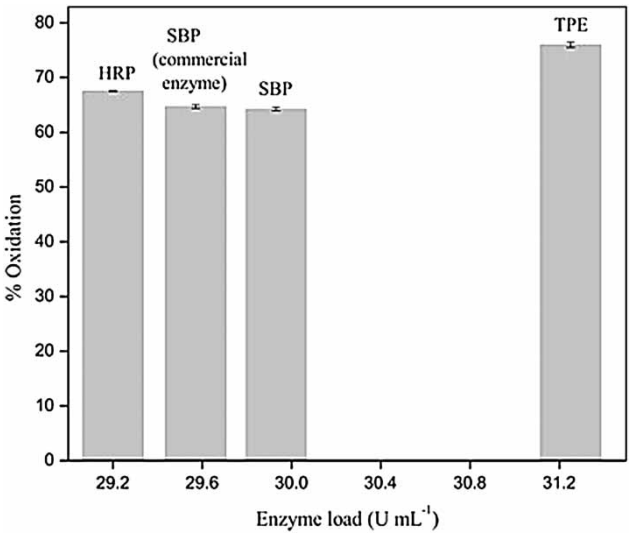

Figure 6 | Oxidation of caffeic acid at a concentration of 1 mmol L^{-1} by different sources of peroxidase: horseradish peroxidase (HRP – 29.20 U mL^{-1}), commercial soybean peroxidase (SBP commercial enzyme – 29.57 U mL^{-1}), peroxidase extracted from soybean seed hulls (SBP – 29.93 U mL^{-1}) and turnip peroxidase (TPE – 31.25 U mL^{-1}), pH 8.0, at 30 °C for 30 min with 7 mmol L^{-1} H_2O_2.

The efficiency of peroxidases on catalysis is strongly dependent on the chemical nature of substrates (substrate specificity) and on the botanical source of peroxidase (Diao *et al.* 2011). Although SBP (commercial enzyme) has roughly presented the same conversion of caffeic acid compared with SBP, the latter in turn has the advantage that it is an enzyme extracted from soybean seed hulls, an abundant and cheap residue from soybean-processing industries, which represents a cost reduction, when compared to commercial enzymes normally used, such as HRP and SBP (commercial enzyme) (Silva *et al.* 2013).

TPE presented a higher potential for caffeic acid oxidation when compared to HRP and SBP (commercial enzyme), without the need for purification. The elimination of purification steps decreases the cost to obtain the enzyme, enabling it to be used as an economically viable alternative in the treatment of wastewaters (Silva *et al.* 2012b).

Bódalo *et al.* (2006a), comparing the action of commercial soybean peroxidase and horseradish peroxidase in the oxidation of phenol, reported that, by employing a concentration of 1,800 U mL^{-1} for both enzymes, the oxidation efficiency was 41% and 74% for horseradish peroxidase and soybean peroxidase, respectively. Moreover, they observed that soybean peroxidase is slower than horseradish peroxidase, but it is less sensitive to inactivation and/or inhibition effect.

Wastewater bioremediation by peroxidase for different sources

Research on enzymatic treatments with real industrial phenolic wastewater matrices has been relatively sparse (Al-Ansari *et al.* 2010). The wastewater from coffee fruits processing has a high organic load, with chemical oxygen demand of approximately 7,000 mg L^{-1} and a large variety of organic compounds such as caffeine, sugars and phenolic compounds unsuitable for direct disposal in soils or watercourses (Gonçalves *et al.* 2008).

Campos *et al.* (2010) noted the considerable presence of phenolic compounds (954.2 mg L^{-1}) in CPW, which exceed the CONAMA Resolution 430/2011, whose maximum concentration is 0.5 mg L^{-1} for release. These compounds are considered toxic, acting as inhibitors of the biological activities of microorganisms, and plant and animal organisms (fish, shellfish, etc.). In excess, they can also interfere with the biological treatment of CPW, both aerobic and anaerobic bacteria, and inhibit other microorganisms. These compounds in addition act as inhibitors of biological activities, can also be potent inhibitors and/or deactivators of

some enzymes, encouraging studies which seek to minimize the effect of these substances. A promising method, it is the biological abatement (or bioabatement) that employs some fungus, such as *Coniochaeta ligniaria* NRRL30616 which possess the ability to metabolize these inhibitors due to its tolerance (Cao *et al.* 2013, 2015). Therefore, it is necessary to treat these effluents before disposal, and the treatment through enzymatic catalysis has shown great potential.

The best reaction conditions found for the conversion of caffeic acid by TPE (pH 8.0 and 7 mmol L^{-1} peroxide for 30 min), were used in the bioremediation of CPW. The CPW used in this study showed a concentration of 233.56 mg $L^{-1} \pm 5.07$ total phenols, which exceeds the CONAMA Resolution 430/2011. A study was also conducted to compare the efficiency of the oxidation for different sources of peroxidase (Figure 7). The highest oxidation (32.70%) was obtained by HRP, followed by TPE (18.25%), SBP (14.33%) and SBP commercial enzyme (13.56%). The controls made without hydrogen peroxide did not cause any conversion, confirming that peroxidases are activated only in the presence of peroxide. The controls performed in the absence of enzyme showed a slight removal of the phenolic compounds from CPW (12.62%) by a non-enzymatic mechanism.

In natural environments, disadvantages that may hinder or diminish the catalytic potential of enzymatic catalysts may depend on both the pollutants to be transformed and the enzymes. In a polluted site, mixtures or composed

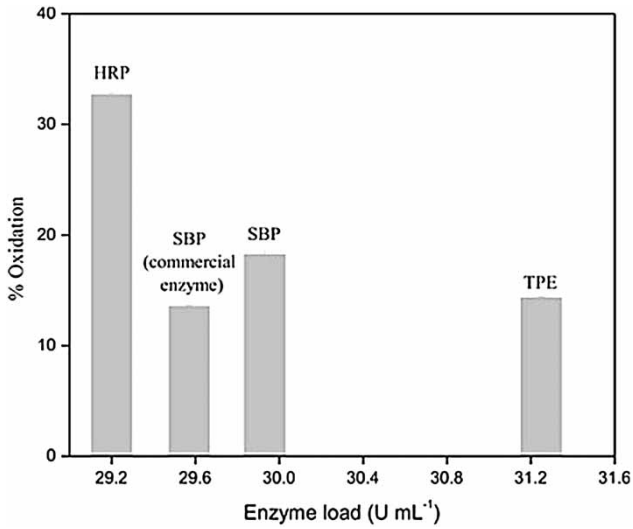

Figure 7 | Bioremediation of coffee processing wastewater by different sources of peroxidase: horseradish peroxidase (HRP – 29.20 U mL^{-1}), commercial soybean peroxidase (SBP commercial enzyme – 29.57 U mL^{-1}), peroxidase extracted from soybean seed hulls (SBP – 29.93 U mL^{-1}), turnip peroxidase (TPE – 31.25 U mL^{-1}), pH 8.0, at 30 °C for 30 min with 7 mmol L^{-1} H_2O_2.

combinations of many organic (and inorganic) contaminants, rather than a single pollutant, are present, and the complexity of the pollution may entail possible negative or positive synergistic effects on the enzyme efficiency. Enzymes may reduce or even lose their activity upon pollutant transformation or they may present a low stability and survival under very often harsh environmental conditions (Rao *et al.* 2010).

The data presented in this study suggest that the degradation mechanism of phenolic compounds for CPW has not been predominantly enzymatic. However, these results encourage the use of peroxidases in the treatment of wastewater containing phenolic compounds associated with conventional treatment processes, in order to make it more amenable to biological treatment.

The efficiency of the enzymatic treatment could have been higher; however, it should be noted that other compounds present in wastewaters could interfere with the peroxidase-catalyzed conversion process and therefore further research is required to optimize the process for these conditions (Duarte-Vázquez *et al.* 2003).

Despite the scarcity of studies related to coffee processing wastewater, some researchers have reported the possibility of the presence of some trace elements in these effluents, such as cadmium, arsenic, zinc, copper, iron, among others. The cadmium forms part of the machinery enamel and red paint, while the arsenic is used as an additive in metal alloys. The zinc can be derived from fertilizer or chemical formulations used to control pests such as the zinc sulphate used in coffee plants before the harvest. The presence of Cu can probably be due to the wear of the machinery, which is made mainly from copper. Some authors have reported high concentrations of iron in pulping water as a result of corrosion and wear of the machinery, which is made of iron and copper (He *et al.* 2005; Siu *et al.* 2007). These substances and others present in these effluents can negatively influence the performance of the enzymes.

Industrial effluents contain various complex mixtures of phenols and other aromatic compounds (Ashraf & Husain 2009). One of the approaches carried out to improve the conversion of certain phenolic compounds is the enzymatic oxidation of the compound in question, in the presence of another phenolic compound, which is easily oxidized by the enzyme (Roper *et al.* 1995). Peroxidases have the ability to co-precipitate certain phenols which are difficult to remove, including certain non-substrates of peroxidases along with the more easily removable compounds by inducing the formation of mixed polymers that behave similarly to the polymeric products of easily removable compounds. This is of great importance, since many industrial

effluents contain a variety of phenolic contaminants; some of them are more amenable to enzymatic treatment than others (Akhtar & Husain 2006).

CONCLUSIONS

The CPW showed a high content of phenolic compounds (233.56 mg L^{-1}) that exceeds the maximum permissible concentration for disposal into receiving water bodies. The major phenolic compound found was caffeic acid, followed by catechin and gallocatechin. The high concentration of these pollutants justifies the need for treatment of this effluent.

In this study, the enzymes SBP, TPE, as well as the commercial enzymes for SBP and HRP, are able to catalyze the conversion of caffeic acid. Nevertheless, the efficiency of TPE is associated with reaction time, pH, H_2O_2 and enzyme concentrations used. The conversion of phenolic compounds in a short period of time (30 min) makes this method attractive, since conventional treatments require extended periods of time.

The data showed no significant influence on the efficiency of the conversion of caffeic acid and phenolic compounds in coffee processing wastewater, when different sources of enzyme were studied. The highest conversion percentages obtained were 75.99% for caffeic acid and 32.70% for CPW. The need of the removal of phenolic compounds from CPW previous to biological treatment due to the inhibiting effect of these upon the microorganisms becomes the promising process.

Since very little work concerning such effluents has been done, this result encourages the use of the proposed treatment for remediation of effluents containing phenolic compounds, as an efficient and sustainable technology, and can be used as a pretreatment to conventional biological treatments.

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