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Covalent immobilization of laccase in green coconut fiber and use in clarification of apple juice

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ABSTRACT

Waste materials from agroindustry constituted from lignocellulose have been used in the immobilization process of technological interest enzymes to reduce costs, increasing the value of such products. In the present study, green coconut husk was used to obtain fibers (CF) that were treated through thermal decompression in combination with either acid or alkaline medium. A solid support for enzyme immobilization was prepared using the pretreated CF activated with glyoxyl or glutaraldehyde and was used to immobilize the laccase enzyme (EC 1.10.3.2) produced by *Trametes versicolor*. Immobilized enzyme retained up to 59 ± 1% of the initial activity and showed maximum immobilization profile of 98 ± 1%. The thermal stability was higher when laccase was immobilized on alkaline pretreated support with increments of 6.8-fold (laccase-glutaraldehyde-FC) up to 16.5-fold (laccase-glyoxyl-FC) of the soluble enzyme. The laccase-glutaraldehyde-CF achieved excellent results in the clarification of apple juice, reducing 61 ± 1% of the original juice color and 29 ± 1% of its turbidity, retaining up to 100% of the initial activity after a 10-times reuse assay. This study is pioneer in the utilization of low-cost support for laccase immobilization with application in the juice fruits clarification.

1. Introduction

The use of alternative supports could help to reduce the cost of the enzyme immobilization process, thereby allowing application on an industrial scale. Such supports are agroindustry waste products constituted mainly of lignocellulose, which have desirable physical and mechanical properties [1–11]. In many Brazil regions the coconut fiber (CF) is a very abundant product and it is a potential support for enzyme immobilization since derivatives with excellent properties have been produced [1–11]. The use of CF for such a purpose increases the product value and help to improve the cost-benefit ratio of the enzyme immobilization process. The utilization of free enzymes in industrial processes has some limitations as poor stability, non-reusability and high cost. In this sense immobilized enzymes exhibit advantages for industrial application as improvement of pH and thermal stability, easy separation of the product and derivative reuse [12–14].

The laccase enzyme (EC 1.10.3.2) has applications in many industrial sectors, including the food industry, in the enhancement of the organoleptic characteristics of beverages and in the treatment of waste products generated by this sector [15]. Laccase is an oxidoreductase that catalyzes the oxidation of phenolic compounds with the concomitant reduction of oxygen to water [16]. This enzyme has considerable industrial potential, as it requires only "air" to work, using the oxygen as co-substrate and releasing water and the oxidized product, allowing it to be classified as a "green catalyst" [17].

The haze degree on fruit juice depends on the fruit physicochemical properties. The presence of phenolic compounds in fruit juices is associated with antioxidant activity and health benefits [18]. However, these compounds can interact with proteins, leading to an increase in turbidity during storage and an unpleasant taste [19–21]. Thus the selective removal of some of these compounds could be desirable in industrialized products. Laccase has been used in the clarification of fruit juices to polymerize some oligomers that remain in suspension [22]. Both free and immobilized enzymes have been used successfully in the clarification of apple juice, pomegranate juice, wine and tea [22–26]. This paper

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describes the covalent immobilization of laccase on CF and the use of the derivative in the clarification of apple juice.

2. Materials and methods

2.1. Reagents

The reagents 2,2-azino-bis-(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS), 2,5-xylidine, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDAC), 1,2-ethanodiamine (EDA), 2,3-Epoxy-1-propanol and $NaIO_4$ were acquired from Sigma–Aldrich, and 2,2-diphenyl-1-picrylhydrazyl (DPPH), Folin-Ciocalteu and $NaBH_4$ were acquired from Merck.

2.2. CF preparation and characterization

The fibers were recovered by hand-processing, dried at 60 °C, cut and passed through a 20-mesh screen. The CF was soaked with H_2SO_4 2% (v/v) or NaOH 2% (w/v) in a 1:10 ratio with thermal decompression. For thermal treatment, the mixture was heated to 121 °C for 15 minutes and the reactor was suddenly decompressed. Residual solids were washed with 10 volumes of water and air-dried.

The air-dried samples were milled to pass through a 0.84 mm screen. Approximately 3 g of the milled sample was extracted with 95% ethanol for 6 h in a Soxhlet apparatus. The percentage of extractives was determined on the basis of the dry weights of the extracted and non-extracted milled samples.

Ethanol-extracted samples were hydrolyzed with 72% sulfuric acid at 30 °C for 1 h (300 mg of sample and 3 mL of sulfuric acid). The acid was diluted with the addition of 79 mL of water, and the mixture was heated at 121 °C and 1 atm for 1 h. The residual material was cooled and filtered through a number 3 porous glass filter. The solids were dried to constant weight at 105 °C and considered insoluble lignin. The soluble lignin concentration in the filtrate was determined by the measurement of absorbance at 205 nm [27]. The concentrations of monomeric sugars in the soluble fraction were determined by high-performance liquid chromatography (HPX87H column; Bio-Rad, Hercules, CA, USA) at 45 °C and an elution rate of 0.6 mL/min with 5 mM H_2SO_4. Sugars were detected in a temperature-controlled refractive index detector at 35 °C. Under these conditions, xylose, mannose and galactose eluted at the same time and appeared as a single peak. Glucose, xylose, arabinose and acetic acid were used as external calibration standards. Minor concentrations of sugar degradation products formed during the acid hydrolysis were not considered in the mass balance of CF components. The factors used to convert sugar monomers to anhydromonomers were 0.90 for glucose and 0.88 for xylose and arabinose. Acetyl content was calculated as the acetic acid content multiplied by 0.72 [28]. This procedure was conducted in duplicate (data expressed as the mean ± SD). Glucose was reported as glucan after correction by the hydrolysis factor. The other sugars and acetic acid were used to calculate the non-cellulosic polysaccharide content [29].

The identification of functional groups on the CF surface was performed by Fourier transform infrared spectroscopy with attenuated total reflectance and readings were performed with 32 scan for each sample, in the range from 4000 to 500 cm^{-1} at 4 cm^{-1} resolution using a Thermo Science Nicolet device. Scanning electron microscopy was performed using a TOP COM-SM-300 device at a voltage of 10 or 20 kV. The samples were prepared in a BAL-TEC SCD050 sputter coater. Gold sputter coating was performed for 80 s at 25 °C and 41 mA in a vacuum.

The porosity expressed as the volume of pores/g of solids was determined employing the solute exclusion technique [30]. Wet samples of CF corresponding to 100 mg on dry weight basis were added to a dextran probe solution (1.5%, w/w). Six dextran probes were used, ranging from 20 to 553 Å. CF sample and dextran probe solutions were let to equilibrate for 24 h at 25 °C. Afterward, the concentration of the probe in the supernatant was determined using a HPLC's refractive index detector with water as mobile phase in a flow of 0.4 mL/min and injections of 20 μL. Pore volumes per gram of solids were calculated based on the solute exclusion technique as already reported [30,31].

2.3. Activation of coconut fiber

The activation methodology was modified from Guisán [32]. For activation with glyceryl groups, 1 g of CF was suspended in 11.25 mL of water, followed by the slow addition of 3.77 mL of NaOH 1.7 M containing 0.792 g of sodium borohydride. The mixture was placed in ice bath, and 3.6 mL of glycidol was dropped under mild agitation for 18 h at 25 °C. After filtration, the support was washed with 10 volumes of water.

Glyceryl-CF (1 g) was activated with glyoxyl groups by addition of 1.43 mL of $NaIO_4$ solution (100 mM) and 10 volumes of water. The mixture was shaken for 2 h, and the support was then washed with water. The glyoxyl groups were quantified through iodometry [32].

For activation with amino groups, 1 g of glyoxyl-CF reacted with 5.7 mL of ethylenediamine (1 M) at pH 10, for 2 h. Sodium borohydride (5.7 mg) was added for reduction of remaining aldehyde groups in to inert hydroxyl groups, and the support was washed with four volumes of sodium acetate buffer (pH 4.0) and distilled water.

Glutaraldehyde-CF was prepared through the bonding of glutaraldehyde to the amino groups in phosphate buffer 0.05 M (pH 7.0) with glutaraldehyde (3 mM) for 2 h and then washed with 20 volumes of phosphate buffer 25 mM (pH 7.0) and water. The long spacer observed in this support prevents any undesired restriction to the enzyme mobility or distortion of the active site [33].

2.4. Microorganism and growth conditions for enzyme production

The fungus *Trametes versicolor* was obtained from Instituto Nacional de Pesquisas da Amazônia (Brazil) and kept at 4 °C in petridishes containing oat (4%) and agar (2%). Laccase was produced in a liquid medium containing KH_2PO_4 (0.5%), NH_4NO_3 (0.2%), $MgSO_4{\cdot}7H_2O$ (0.02%), $CoCl_2{\cdot}2H_2O$ (0.01%), sodium citrate (1.5%), 2,5-xylidine (0.05 mM), glucose (0.5 mM), $CuSO_4$ (0.05 mM) and trace solution (0.05%). Each 100 mL of trace solution contained citric acid (5 g), $ZnSO_4{\cdot}7H_2O$ (5 g), $Fe(NH_4)_2(SO_4)_2{\cdot}6H_2O$ (1 g), $CuSO_4{\cdot}5H_2O$ (0.25 g), $MnSO_4{\cdot}H_2O$ (0.05 g), H_3BO_3 (0.05 g) and $NaMoO_4{\cdot}2H_2O$ (0.05 g). The culture was performed at 30 °C and 100 rpm for 160 h.

2.5. Determination of enzyme activity

Enzyme activity was measured continuously by the oxidation rate of ABTS at 420 nm ($\varepsilon = 3.6 \times 10^4\ M^{-1}\ cm^{-1}$) [34]. Free enzyme activity was quantified in acetate buffer (50 mM; pH 3.5) containing ABTS (5 mM) in a final volume of 2 mL at 25 °C. The activity of the derivative was assayed by the incubation of 10 mg of derivative in acetate buffer (50 mM; pH 3.5) containing ABTS (5 mM) at 25 °C. Activity was expressed in units (U), and each unit was defined as 1 μmol of ABTS oxidized per minute.

2.6. Chemical modification of laccase from T. versicolor

For the amination process, 5 mL crude extract (5 mg of protein/mL) was added to 5 mL of EDA 1 M containing EDAC 50 mM. The mixture was mildly shaken for 2 h and then dialyzed for 18 h and used for immobilization on the activated CF [35].

2.7. Preparation of laccase-CF derivatives

For immobilization in glyoxyl-CF, 5 g of fiber was added to 20 mL of sodium bicarbonate 100 mM (pH 10). For glutaraldehyde-CF, 5 g of fiber was added to 20.0 mL of sodium phosphate 100 mM (pH 7). In both cases, 6.55 U of chemically modified laccase (as described in Section 2.6) were added to each support suspension, and the mixtures were mildly shaken at 25 °C up to the enzyme activity dropped to less than 80% of the original value. Supernatant samples were periodically withdrawn for the analysis of enzyme activity. At the end of the reaction, sodium borohydride was added (1 mg/mL of solution), and the mixture was shaken mildly for 30 min. The derivatives were then washed in distilled water.

2.8. Thermal stability and reuse

For thermal stability studies, the chemically modified enzyme and derivatives were incubated in phosphate buffer (50 mM), pH 6.0 at 60 °C, and the activity was measured at each reported time interval. Residual activity was measured as described in Section 2.5. The reuse of the immobilized laccase was evaluated in a 10-times repeated batch experiment. On each batch (cycle) the apple juice phenols were oxidized in a mixture containing 0.2 g of enzyme derivative and 5 mL of apple juice shaken for 30 min at 38 °C.

2.9. Preparation of apple juice

Apples (*Malus domestica* cv. Fuji) were acquired from a local supermarket, rinsed in distilled water and triturated. The juice was pressed through three layers of gauze. Kaolin (0.1 mg/mL of juice) was immediately added. The mixture was shaken mildly for 30 min (25 °C) and then centrifuged at 4000 × *g* for 30 min at 4 °C and the pH was determined.

2.10. Enzyme treatment of apple juice in a packed bed reactor

For the treatment of apple juice a packed bed reactor was used [36], where 70 mL of centrifuged juice was eluted through a two-sleeve glass column (2.1 × 8 cm) containing 2 g (1.0–1.5 U) of enzyme derivative kept at a constant temperature of 38 °C. Juice flow though the column was maintained with the aid of a peristaltic pump (Amersham Pharmacia) at 2.5 mL/min for 60 min. Samples were withdrawn before and after treatment for the analysis of color, turbidity [37], total phenols [38] and antioxidant activity [39], the last one determined through the redox reaction between the DPPH radical and antioxidants compounds.

2.11. SDS-PAGE analysis

Derivative and free enzyme samples were analyzed using the method described by Laemmli [40]. The derivative was treated with SDS and β-mercaptoethanol for removal of any protein not covalently bonded. The supernatants were analyzed through SDS-PAGE (12%) and stained with Coomassie Brilliant Blue (R250). The Pharmacia LWM molecular mass marker was used (MW = 14,000–94,000 Da).

3. Results and discussion

3.1. Physicochemical characterization of CF

Chemical characterization of the CF indicated that 47.2% of the dry weight of untreated green coconut fiber was made up of ethanol-soluble components, which is characteristic of a waxy material (Table 1). CF also contained 13.6% of lignin or polyphenols and 27.9% of quantified polysaccharides. The remaining non-quantified components (11.3%) could include pectins, proteins and other minor components. After alkaline and acid treatments of CF the residual solids corresponded to 36.6% and 37.6% of the original CF, respectively. Low solids yield after alkaline and acid treatments indicate extensive dissolution of CF components in both cases. Chemical composition of treated materials and mass balance of the CF components indicate that the waxy materials were almost completely removed in both treatments (Table 1). The glucan fraction was mostly retained in both treatments. Lignin or polyphenols were removed in the alkaline treatment, whereas xylan was mainly removed by the acid treatment.

SEMs of the studied CFs indicated that untreated material presented a homogeneously covered external surface (Fig. 1a and d) that changed to fissured surface (Fig. 1b–e and c–f). These changes could be associated mostly with the waxy material removal during the acid and alkaline treatments [7,41,42]. After the acid treatment, which removed a significant part of the hemicellulosic fraction, the resulting material presented even more fissured surfaces (Fig. 1c and f). SEM data suggest that the treatments were efficient in the removal of the external surface layers that can hinder the derivatization of the fiber and, consequently, the immobilization of enzymes.

The porosity of the pretreated CF is given as accessible pore volumes in Fig. 2. The probes were used to give an overall view of pore size distribution and the volume of pores accessible to enzyme molecules with 40–50 Å, which corresponds to laccase approximate diameter [43]. The alkaline pretreated CF presented higher porosity showing more accessibility to the probes than the acid pretreated CF. In the range of laccase diameter, the accessible volume was 1.33 mL g^{-1} and 0.64 mL g^{-1} for the alkaline and the acid pretreated CF, respectively, indicating that the alkaline pretreated CF presented porosity higher than that reported for Sepabeads EC-EP3 (1.19 mL g^{-1}) [44].

Table 1
Quantification of chemical constituents of coconut fiber.

Sample	Treatment yield (%)	Chemical composition[a]						Mass balance[b]					
		Total Lignin	Glucan	Xylan	Arabinosyl Groups	Acetyl Groups	Extratives[c]	Total Lignin	Glucan	Xylan	Arabinosyl Groups	Acetyl Groups	Extratives[c]
Untreated	100	13.6 ± 0.2	16.0 ± 0.2	8.1 ± 0.1	2.4 ± 0.0	1.4 ± 0.1	47.2	13.6 ± 0.2	16.0 ± 0.2	8.1 ± 0.1	2.4 ± 0.0	1.4 ± 0.1	47.2
Steam/Alkali	36.6 ± 0.1	17.8 ± 0.1	48.0 ± 0.9	16.9 ± 0.4	2.9 ± 0.1	0.1 ± 0.0	1.0	6.6 ± 0.1	17.7 ± 0.5	6.3 ± 0.2	1.1 ± 0.0	0.0 ± 0.0	0.4
Steam/Acid	37.6 ± 0.1	49.0 ± 0.1	39.3 ± 0.4	2.5 ± 2.2	0.1 ± 0.0	0.7 ± 0.1	2.0	18.8 ± 0.0	15.1 ± 0.2	1.0 ± 1.2	0.0 ± 0.0	0.2 ± 0.1	0.7

The magnification was: 300× (a), 500× (b), 500 (c) and 2000× for (d)–(f).
[a] g/100 g of coconut fiber.
[b] g/100 g of initial coconut fiber.
[c] Ethanol soluble compounds.

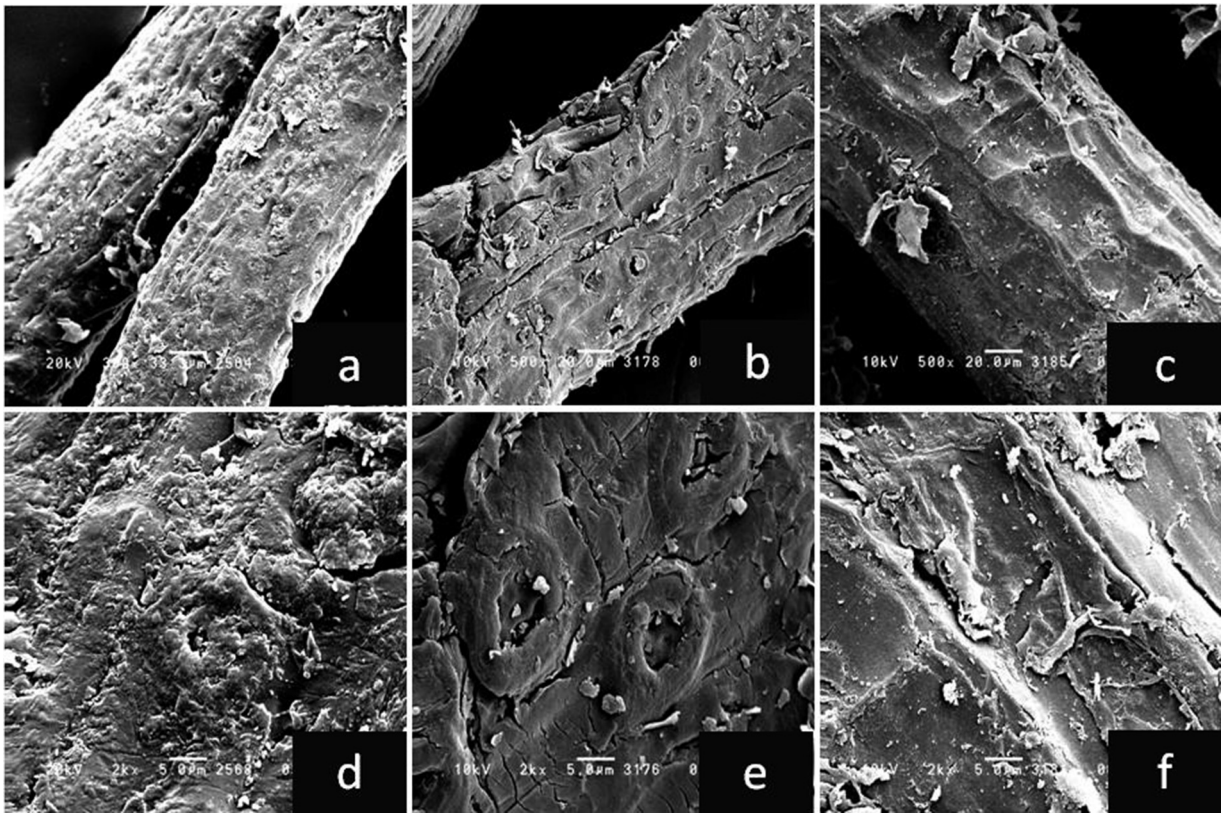

Fig. 1. Scanning electron microscopy of coconut fiber after chemical pretreatments: (a, d) Natural fiber; (b, e) Thermal decompression in NaOH 2%; (c, f) Thermal decompression in H_2SO_4 2%.

Acid or alkaline pretreatment associated with thermal decompression were performed to exposure the reactive groups in CF. Increases in the material porosity as well as in the hydroxyl contents in the material surface results in a better support for the activation methodology employed in the present study. For this reason, alkaline- or acid-treated CF showed to be useful for insertion of aldehyde groups (656 μmol aldehyde groups/g for alkaline-pretreated CF and 713 μmols aldehyde groups/g for acid pretreated CF. In all cases the FC-glyceryl was oxidized to give only 143 μmols/g of aldehyde groups, the maximum activation grade for agarose 6 BCL [31].

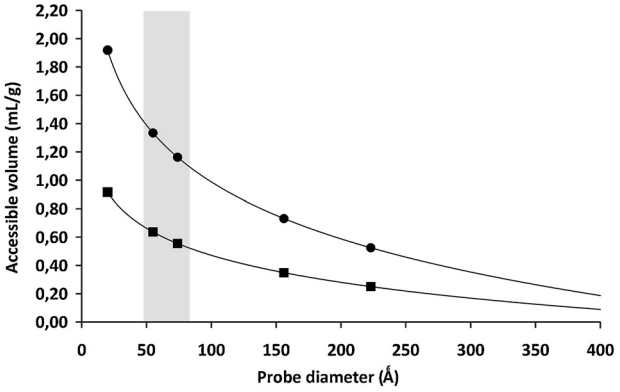

Fig. 2. Porosity of the pretreated coconut fiber: the accessible volume per gram of CF was performed for 6 probes ranging from 20 to 533 Å. Alkaline pretreated FC (●); acid pretreated FC (■).

The infrared analysis of activated fibers showed that the composition of the coconut fiber was strongly affected the activation process giving supports with distinct characteristics. The bands were show in Table 2 and agree with other studies [45]. After functionalization with aldehyde groups, CF exhibited an increase in all bands between 1027 and 1160 cm^{-1}, which may indicate the efficiency of the activation process after either treatments (Fig. 3). The increase in the 1596 and 1604 cm^{-1} bands that can be assigned to lignin carbonyl groups [45,46], indicating that lignin groups could be participating of the activation reactions, increasing the amount of aldehyde groups in CF pretreated with acid (Fig. 3B). The pretreated CF also showed an increase in bands between 1027 and 1038 cm^{-1}, which are assigned to C–O linkages in the lignin/polyphenols and polysaccharides [46,47]. The hydroxyls present on the surface of the CF were demonstrated by the band at 3313 cm^{-1} [48,49].

Table 2
Assignment of bands in the infrared spectra of coconut fiber samples.

Wavenumber (cm^{-1})	Assignment/functional group	Polymer
1038,1027	C—O, C=C and C—C—O stretching	Cellulose, hemicellulose, lignin
1095,1103	Weak aborbance	Lignin
1160, 1159	C—O—C asymmetrical stretching	Cellulose, hemicellulose
1265	Aromatic ring vibration	Guaicyl lignin
1313	CH_2 wagging	Cellulose, hemicellulose
1421	C—H in plane deformation	Lignin
1444	O—H plane bending	Cellulose, hemicellulose and lignin
1596, 1604	Aromatic ring vibration + C=O stretch	Lignin

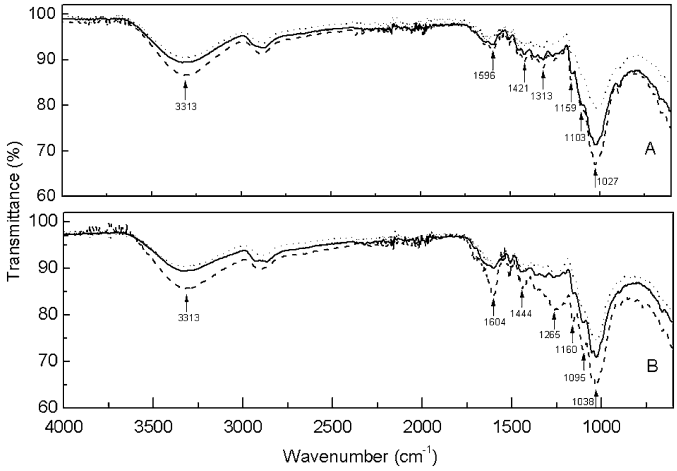

Fig. 3. Infrared analysis of coconut fiber activated with glyoxyl or glutaraldehyde after alkaline (A) or acid (B) pretreatment. Glyoxyl-CF (—); glutaradehyde-CF (—), Pretreated fiber used as control (. . ..).

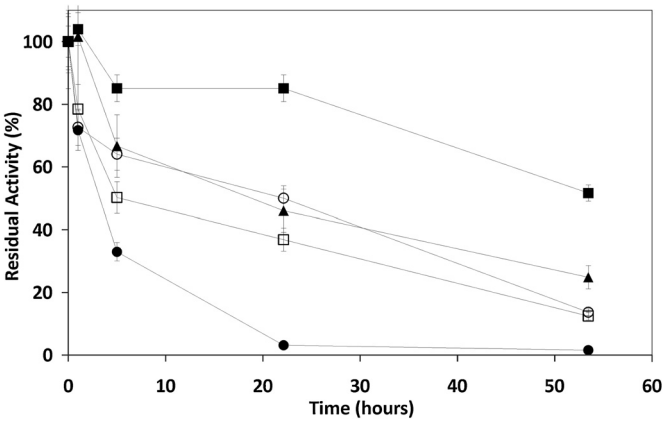

Fig. 4. Thermal stability of derivatives: free enzyme and derivatives incubated in phosphate buffer 100 mmol L^{-1} (pH 6.0) at 60 °C; laccase activity quantified in sodium acetate buffer 0.05 mmol L^{-1} (pH 3.0) at room temperature. Free laccase (●); CF1 (■); CF2 (▲); CF3 (○); CF4 (□).

3.2. Laccase immobilization

The aminated laccase was successfully immobilized on all tested supports, showing an excellent immobilization profile after two hours (Table 3). Chemical amination has been performed by activating the surface carboxylic groups (terminal carboxylic, Asp and Glu) with 1-ethyl-3-(dimethylamino-propyl)carbodiimide (EDAC) in the presence of ethylenediamine (EDA) as described by López-Gallego et al. [35]. We observed that the chemically modified enzyme showed better stability on alkaline pH, condition in which the reactions between the aminated laccase and the reactive groups of the support take place. The covalent nature of the bond between the enzyme and CF was confirmed by SDS-PAGE. The free enzyme has a molecular mass of approximately 63 kDa. However, no characteristic protein band of the enzyme was observed in the supernatant of the derivatives treated with β-mercaptoethanol and SDS. The laccase immobilization on coconut fiber activated with aldehyde groups using glycidyl 3-(trimethoxysilyl)propyl ether (GPTMS) showed an immobilization yield of 50–74% with recovery activity ranging from 0.87 to 0.47% [9].

The pretreatments affected directly the activity and stability of the derivatives. The alkaline pretreatment produced derivatives with greater enzyme activity, which may be related to the kind of structural changes that occurred in the fiber. Such changes affected the exposure of important groups and consequently influenced the activation of the fiber as well the activity of the derivatives.

Natural fibers have a highly hydrophilic surface [50], which may be partially attributed to the large number of hydroxyls distributed along the surface [51]. Pretreatments can modify some properties of the natural fibers as porosity, internal area and composition [52–54]. According to Mateo et al. [55], the internal morphology of the support exerts considerable influence on derivative activity and stability. In this sense the low porosity and high content of lignin could be related with the low activity of derivatives using acid pretreated fiber. In contrast, alkaline pretreatment leads to higher glucan and xylan content, increasing the porosity and internal area, and these changes could be related to the high enzyme activity (Table 3) and stability (Fig. 4) of derivatives using alkaline pretreated fiber.

3.3. Biochemical characterization of T. versicolor free laccase and derivatives

The optimum pH and temperature of free native laccase were 3.0 and 70 °C respectively. The half-life of free enzyme at 60°C was 3.24 h. These data are similar to that reported by the literature [56]. The kinetic properties of derivatives are shown in Table 3. The half-life of the glyoxyl derivative (CF1) increased 16.5-fold compared to the free enzyme. However the optimum pH of the derivatives was similar to the free enzyme.

3.4. Thermal stability of derivatives

All derivatives exhibited greater stability (60 °C; pH 6.0) in comparison to the free enzyme (Fig. 4). The glyoxyl-CF derivatives were more stable mainly because of the high reactivity of the groups involved in this type of immobilization and the formation of stable, multi-bridge bonds in the regions of the protein surface with a high density of lysine residues [57]. Laccase-glyoxyl-CF (NaOH) was 16.5-fold more stable than the free enzyme under the conditions tested, maintaining 85% of the initial laccase activity after 22 h at 60 °C (pH 6), whereas only 3% of the initial activity was maintained in the free modified enzyme. Laccase-glutaraldehyde-CF (NaOH) was 6.86-fold more stable than the free enzyme. The covalent immobilization of laccase on coconut fiber by Cristóvão et al. [9] using a different strategy lead to a stabilization factor of 2.5 (pH 7), whereas the pre-treatment and the activation methodology described in this study influenced positively the parameters of the derivatives. The laccase covalently immobilized on spent grain presented thermal stability of 1.72-fold [5]. The cross-linked of this enzyme with glutaraldehyde and ethylenediamine in the presence of BSA lead to an increased thermal stability of 4.7-fold (70 °C) [58]. The thermal stability of the laccase-glyoxyl-CF may be related to the type of interaction on these supports, where proteins are immobilized through the most exposed, most active group under such conditions (amino terminal group) [59,60]. Thus, glyoxyl derivatives offer greater stability as the multi-bridge bonds between the support and regions of the protein surface with a high density of lysine. This approach can make the protein structure more rigid and less susceptible to changes in conformation. In contrast, the bond between the amino terminal in glutaraldehyde derivatives may not provide sufficient rigidity to the tertiary structure to allow greater thermal stability.

3.5. Clarification of apple juice

As described above, laccase can be used to oxidize part of the phenolic compounds found in fruit juices by producing radicals, which interact to form insoluble polymers that can be separated by centrifugation. However, lighter oligomers may remain in the

Table 3
Kinetic properties of laccase from *T. versicolor* immobilized on coconut fiber.

Derivatives		IY[a] (%)	EA[b] (%)	OT[c] (°C)	Op[d] (–)	Half-lifetime (h)	SF (–)
Glyoxyl-CF*	(CF1)	60 ± 0.1	59 ± 0.9	40 ± 0.2	3.0 ± 0.02	53.48 ± 0.03	16.5 ± 0.01
Glyoxyl-CF**	(CF2)	90 ± 0.9	40 ± 0.2	60 ± 0.08	3.0 ± 0.04	18.63 ± 0.01	5.75 ± 0.05
Glutaraldehyde-CF*	(CF3)	97 ± 0.6	54 ± 0.1	50 ± 0.05	2.2 ± 0.01	22.24 ± 0.05	6.86 ± 0.1
Glutaraldehyde-CF**	(CF4)	98 ± 1.0	44 ± 0.0	40 ± 0.15	3.0 ± 0.01	5.10 ± 0.08	1.57 ± 0.08

*CF alkaline pretreated; **CF acid pretreated.
[a] Immobilization yield (IY) is defined as the percentage of laccase immobilized on different supports.
[b] Expressed activity (EA) is defined as the ratio between the activity in the derivatives obtained and the initial activity of the offered enzyme.
[c] Optimum temperature.
[d] Optimum pH.
[e] Stabilization factor (SF) is the ratio between the half-life of the immobilized enzyme and that of the corresponding soluble enzyme.

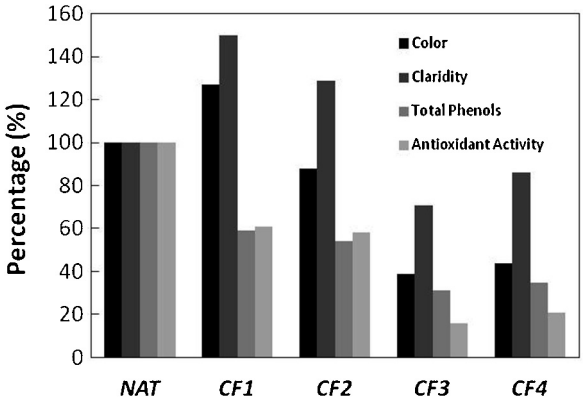

Fig. 5. Treatment of apple juice with laccase derivatives: Efficiency was measured through analysis of color, turbidity, total phenols and antioxidant activity before and after treatment with immobilized laccase. Natural juice (in natura); laccase-glyoxyl-CF (CF1 and CF2); Laccase-glutaraldehyde-CF (CF3 and CF4).

solution, darkening the juice and making it more turbid, as characterized by high absorbance at 420 nm and 650 nm, respectively. The treatment of natural apple juice (pH 4.2) with laccase-glyoxyl-CF led to an increase in color and turbidity (Fig. 5). This effect has previously been reported for pomegranate [20] and apple juice [61] and the authors attributed this effect to the accentuated formation of soluble oligomers. The laccase-glutaraldehyde-CF reduced the color and turbidity of the juice by 61% and 29%, respectively, proving to be effective in the removal of phenolic compounds from apple juice, making this derivative a potential biocatalyst for industrial application. Moreover, laccase-glyoxyl-CF removed approximately 40% of these compounds. As the gentle removal of phenols is the purpose of enzyme treatment, these derivatives have the greatest application potential, as the mild oxidation action reduced the antioxidant capacity of the juice by 40%. Laccase-glutaraldehyde-CF removed up to 65% of the phenolic compounds from the apple juice, leading to an 80% reduction in antioxidant capacity.

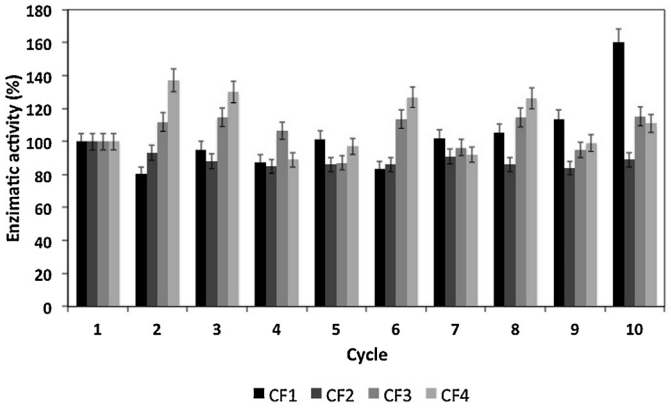

Fig. 6. Reuse assay of laccase-CF derivatives.

Laccase immobilized on CF proved efficient in the oxidation of phenolic compounds in apple juice, where the derivatives showed 80% or more of initial activity after 10 consecutive 30 min cycles of reuse (Fig. 6). Because of the fast, safe separation of the derivative and its reuse, these derivatives can be employed in the clarification of juices, as treatment time and temperature can be optimized to provide milder oxidation of these compounds, leading to a product that conserves the maximal amount of the natural antioxidant activity.

4. Conclusion

Based on the present findings, coconut fiber is an excellent alternative support for the immobilization of laccase. Laccase immobilized on CF with a combination of thermal decompression and alkaline treatment produced the most active and stable derivatives. Laccase-glutaraldehyde-CF derivatives exhibited better results regarding the clarification of apple juice and proved capable of efficiently oxidizing phenolic compounds. However, a better physicochemical characterization of the fiber after both pretreatments is necessary and also the optimization of the parameters involved in the apple juice clarification to obtain the mild oxidation of these compounds and preserve the maximal amount of antioxidant activity.

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