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Linoleic acid peroxidation initiated by Fe^{3+}-reducing compounds recovered from *Eucalyptus grandis* biotreated with *Ceriporiopsis subvermispora*

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ABSTRACT

This work evaluates linoleic acid peroxidation reactions initiated by Fe^{3+}-reducing compounds recovered from *Eucalyptus grandis*, biotreated with the biopulping fungus *Ceriporiopsis subvermispora*. The aqueous extracts from biotreated wood had the ability to reduce Fe^{3+} ions from freshly prepared solutions. The compounds responsible for the Fe^{3+}-reducing activity corresponded to UV-absorbing substances with apparent molar masses from 3 kDa to 5 kDa. Linoleic acid peroxidation reactions conducted in the presence of Fe^{3+} ions and the Fe^{3+}-reducing compounds showed that the rate of O_2 consumption during peroxidation was proportional to the Fe^{3+}-reducing activity present in each extract obtained from biotreated wood. This peroxidation reaction was coupled with in-vitro treatment of ball-milled *E. grandis* wood. Ultraviolet data showed that the reaction system released lignin fragments from the milled wood. Size exclusion chromatography data indicated that the solubilized material contained a minor fraction representing high-molar-mass molecules excluded by the column and a main low-molar-mass peak. Overall evaluation of the data suggested that the Fe^{3+}-reducing compounds formed during wood biodegradation by *C. subvermispora* can mediate lignin degradation through linoleic acid peroxidation.
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1. Introduction

Fe^{3+}-reducing compounds are frequently associated with wood biodegradation. These compounds seem to be the driving reagents that convert available Fe^{3+} to Fe^{2+}. In turn, the Fe^{2+} ions react with hydrogen peroxide generated in these biological systems, resulting in the formation of hydroxyl radicals (Fenton reaction). These radicals and other reactive oxygen species (ROS) are able to degrade and transform wood components. In brown-rot fungi, hydroquinone derivatives are the main Fe^{3+}-reducing compounds (Goodell et al. 1997; Hammel et al. 2002; Goodell 2003; Goodell et al. 2006).

Few studies have evaluated Fe^{3+}-reducing compounds in white-rot fungi. Some reports have indicated the occurrence of dihydroxy-benzoic acid and hydroxy-methoxy-aromatic derivatives in naturally decayed wood (Ferraz et al. 2001). Similar compounds were found in softwood biotreated by *Ceriporiopsis subvermispora* (Aguiar and Ferraz 2008). Low-molar-mass Fe^{3+}-reducing glycopeptides were detected in white-rot submerged cultures as well as in biotreated wood (Tanaka et al. 1999, 2007). Catecholate derivatives were produced by *Perenniporia medulla-panis* in submerged cultures (Arantes and Milagres 2006) while alkene-esters called Pc-reducers were detected in submerged cultures of *Phanerochaete chrysosporium* (Hu et al. 2009). Enzymatic reduction of Fe^{3+} has also been attributed to extracellular cellobiose-dehydrogenase (CDH), which is produced by several white-rot fungi (Henriksson et al. 2000; Baldrian and Valaskova 2008).

In cultures of the biopulping fungus *C. subvermispora*, Fe^{3+}-reducing activity has been associated with the occurrence of lignin degradation products (Aguiar et al. 2006; Aguiar and Ferraz 2008). Cellobiose-dehydrogenase has not been detected in enzymatic extracts recovered from wood biotreated by this species (Souza-Cruz et al. 2004; Aguiar et al. 2006; Aguiar and Ferraz 2008), although a recent work reported CDH secretion by *C. subvermispora* in submerged cultures grown under specific nutritional controls with stirred tank reactors (Harreither et al. 2009). This fungus also secretes alkylitaconic acids and copious amounts of oxalic acid, which are both able to inhibit in-vitro Fe^{3+} reduction (Enoki et al. 2000; Gutierrez et al. 2002; Aguiar and Ferraz 2008; Nishimura et al. 2009; Wei et al. 2010).

The occurrence of Fe^{3+}-reducing compounds in white-rot fungi cultures is relevant not only because they could be involved in polysaccharide degradation based on the Fenton reaction, but these compounds could also contribute to the occurrence of unsaturated fatty acid peroxidation reactions. These reactions seem to be

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involved in the degradation of some recalcitrant molecules and non-phenolic lignin structures (Moen and Hammel 1994; Kapich et al. 1999). It has been suggested that peroxyl (Kapich et al. 1999) and acyl radicals (Watanabe et al. 2001) formed during unsaturated fatty acid peroxidation can initiate degradation of non-phenolic lignin structures.

A number of oxidant species, including ROS and metallic ions, can initiate peroxidation of unsaturated fatty acids (Spiteller 2006). In the particular case of Fe^{2+}, its rapid oxidation by molecular oxygen generates a superoxyanion or hydroperoxyl radical, depending on the medium's pH (pKa 4.7) (Stumm and Lee 1961). In turn, these ROS can act as proximal oxidants of unsaturated fatty acids (Spiteller 2006).

In a recent report, we have shown that MnP produced by *C. subvermispora* can peroxidize linoleic acid in vitro. Carrying out this reaction in the presence of milled *Eucalyptus grandis* wood caused lignin depolymerization and the release of water-soluble lignin degradation products (Cunha et al. 2010). In this work, we continued to evaluate linoleic acid peroxidation reactions initiated by extracts recovered from *C. subvermispora*-biotreated wood. Enzyme-free extracts were assayed for Fe^{3+}-reducing activity. Fe^{2+} ions released in these reactions were able to initiate in-vitro linoleic acid peroxidation and to promote lignin degradation in insoluble milled wood.

2. Materials and methods

2.1. Wood biodegradation and recovery of the aqueous extracts

E. grandis wood chips measuring approximately 2.5 cm × 1.5 cm × 0.2 cm were obtained from 8-year-old trees supplied by a Brazilian pulp mill. Wet chips were air-dried and stored under dry conditions until used. *C. subvermispora* cultures were kindly provided by Dr. M Akhtar, from Biopulping International Inc., Madison, WI, corresponding to the L-14807, SS-3 strain. The fungus was maintained on 20 g l^{-1} malt-extract (OXOID Ltd., England) agar plates containing a slice of wood at 4 °C. Prior to the biodegradation experiments, 50 g (oven-dried basis) of wood chips were immersed in water inside 2-l Erlenmeyer flasks for 16 h. Then, the surplus water was drained and the flasks were sealed with cotton stoppers and aluminum foil and autoclaved at 121 °C for 15 min. The moisture of the autoclaved wood chips was approximately 55% (w/w). Each 2-l Erlenmeyer flask was then loaded with 10 ml of a blended mycelium suspension of *C. subvermispora* (corresponding to a fungal mycelium/wood ratio of 500 mg kg^{-1} of wood), shaken by hand, and stored stationary at 27 °C for 7, 14, and 28 days. Three flasks were incubated for each culture period. One set of wood chips was sterilized, but not inoculated, and it represented the control for all experiments.

Biotreated and control wood chips were extracted with 300 ml distilled water (pH 7.0) for 24 h at 25 ± 1 °C at 120 rpm. Extracts were treated with a nitrogen stream for 10 min and stored at −18 °C.

2.2. Characterization of the aqueous extracts

Fe^{3+}-reduction by the aqueous extracts was determined based on the ferrozine-Fe^{2+} complex formation (Ferraz et al. 2001), using two different procedures. Procedure 1 utilized a mixture containing 350 μl of a 50 mM sodium acetate buffer at pH 4.5, 375 μl of a 0.45 μm-filtered aqueous extract, and 25 μl of an 8 mM freshly prepared $FeCl_3$ solution. After reaction periods lasting between 10 and 60 min, 50 μl of 1% (w/v) NaF and 200 μl of 1% (w/v) ferrozine (3-(2-pyridyl)-5,6-bis(4-phenylsulfonic acid)-1,2,4-triazine) were added to each tube. The absorbance of the solutions was measured at 562 nm ($\varepsilon_{562\ nm}$ = 27,900 M^{-1} cm^{-1}; Stookey 1970). Procedure 2 utilized a mixture containing the ferrozine reagent from the initial reaction time. In this case, the mixture contained 400 μl of a 50 mM sodium acetate buffer at pH 4.5, 375 μl of a 0.45 μm-filtered aqueous extract, 200 μl of 1% (w/v) ferrozine, and 25 μl of an 8 mM freshly prepared $FeCl_3$ solution. Some Fe^{3+}-reduction reactions initiated by vanillin (3-methoxy-4-hydroxy-benzaldehyde) were evaluated as well. In those cases, 5 μM or 30 μM vanillin solutions replaced the aqueous extract.

The aqueous extracts were characterized by size exclusion chromatography (SEC) in a Superose 12 10/300GL column eluted at 0.7 ml min^{-1} with 50 mM Na_2HPO_4 and 150 mM NaCl at pH 12 using an AKTA 10 GE chromatograph set with a UV detector fixed at 280 nm. Fractions of 0.5 ml eluting from the column were collected and assayed for Fe^{3+} reduction and for total carbohydrate content. The fractions were neutralized by the addition of 20 μl of 1.6 M HCl. For Fe^{3+} reduction assays, the fractions were analyzed according to the previously described Procedure 1, with a reaction time of 90 min. Total carbohydrates were assayed by mixing 500 μl of sample, 25 μl of 40% (v/v) phenol, and 1200 μl of concentrated sulfuric acid. After a 30-min reaction, the mixture was monitored at 490 nm (Dubois et al. 1956). Molar mass markers were also run in the same chromatographic system: albumin (66 kDa), carbonic anhydrase (29 kDa), cytochrome c (12.4 kDa), aprotinin (6.5 kDa), and acetone (28 Da) (Forss et al. 1989).

Wood extracts were examined for low-molar-mass compounds using gas chromatography-mass spectrometry (GC/MS). Aqueous wood extracts were first ultrafiltrated at 10 °C through 5-kDa cut-off membranes (AmiconUltra-Ultracel 5k, Millipore) for 15 min at 3500 × g. The filtrates were then concentrated under reduced pressure and dried over P_2O_5 under vacuum for 3 days. Dried material was dissolved in pyridine to give 1 mg ml^{-1} solutions. These solutions were treated with a stream of nitrogen for 1 min. Next, 100 μl of each solution was treated with 100 μl of BSTFA at 60 °C for 1 h (Aguiar et al. 2006). After silylation, the solution was analyzed in a Finningan MAT-GCQ instrument equipped with a RTX-5MS (L 30 m × ID 0.25 mm) column (Restek). Column temperature was initially maintained at 60 °C for 1 min and then heated up to 100 °C at 20 °C/min. This temperature was maintained for 1 min and then raised to 290 °C at 5 °C/min. This final temperature was maintained for 8 min. Helium at 33.0 cm/s was used as the carrier gas. Injector and transfer line temperatures were 250 °C and 275 °C, respectively. One microliter of the sample was injected using a split of 1:30. Retention time in the GC and mass spectrum of each identified peak were identical to that of silylated authentic standards. The compounds, their retention time (RT), and main mass spectrum information (*m*/*z*, relative intensity) are as follows: oxalic acid (RT = 7.87 min), 219(M^+-15, 8), 190(11), 147 (68), 73(100); glycerol (RT = 11.19 min), 218(M^+-90, 20), 205(26), 150(100), 117 (8), 73(16); vanillin (3-methoxy-4-hydroxy benzaldehyde) (RT = 17.72 min), 224(M^+, 47), 209(76), 194(100), 193(25), 73(4); vanillic acid (3-methoxy-4-hydroxy benzoic acid) (RT = 22.87 min), 312(M^+,85), 297(100), 282(24), 267(30), 181(31), 73(6); syringic acid (3,4-dimethoxy-4-hydroxy benzoic acid) (RT = 25.71 min), 342(M^+,60), 327(100), 313(46), 297(60), 211(11), 73(10); gallic acid (3,4,5-trihydroxybenzoic acid) (RT = 26.85 min), 462(M^+, 2), 459(M^+-3, 100), 446(15), 280(50), 73(6); palmitic acid (RT = 28.18 min), 313(M^+-15, 9), 285(9), 201(13), 129(39), 117(100), 75(78), 73(35); oleic acid (RT = 31.30 min), 339(M^+-15, 11), 264 (20), 199(29), 129(64), 117(69), 75(100), 73(36); stearic acid (RT = 31.73 min), 341(M^+-15, 7), 257 (10), 201 (14), 129(47), 117 (100), 75(60), 73(28).

Aqueous extracts were also fractionated by ultrafiltration through cut-off membranes of 5 kDa, 10 kDa, and 30 kDa (AmiconUltra, Millipore, Ultracel 5 k, 10 k, and 30 k, respectively). Filtrates

were assayed for Fe^{3+} reduction following the previously described Procedure 1.

2.3. In-vitro peroxidation of linoleic acid

Reactions were carried out inside a 27 °C-thermo-controlled oxygraph cell (Hansatech oxygraph) according to Kapich et al. (2005). The oxygraph cell was filled with 1130 µl of 50 mM sodium acetate buffer at pH 4.5. This buffer solution was stirred at 100 rpm for 3 min to ensure oxygen saturation. In reactions carried out with Fe^{2+} ions, 500 µl of water was added to the cell followed by 40 µl of freshly prepared 8 mM $FeSO_4$ and 40 µl of 0.2% (w/v) linoleic acid previously emulsified in 1% (w/v) Tween 60. The oxygraph cell was sealed, and the dissolved oxygen concentration was monitored during all reaction periods and recorded by Hansatech software. In reactions carried out with Fe^{3+} ions, 500 µl of aqueous extracts replaced the water, while 40 µl of freshly prepared 8 mM $FeCl_3$ replaced the Fe^{2+} solution.

2.4. Preparation and chemical characterization of ball-milled E. grandis

Approximately 200 g of wood chips was milled in a knife mill to pass through a 0.5-mm screen. This material was further ground at room temperature for 160 h. Grinding was performed at 96 rpm in a rotary stainless steel jar (2.5 l) containing stainless-steel balls. Ball/wood weight ratio was 20:1. The ball-milled material was extracted by using a Soxhlet apparatus with a sequence of solvents: dichloromethane, acetone, 95% ethanol, and distilled water. Each extraction lasted 8 h. The remaining solids were further extracted for 72 h in a rotary shaker at 120 rpm and 27 °C with 1 l of 50 mM sodium acetate buffer at pH 4.5. The resulting solids were centrifuged at 2000 × g for 15 min. Insoluble material was finally washed with distilled water to remove residual salts and air-dried for 1 week. All milling, extraction, and washing procedures were designed to produce a milled wood substrate free of low-molar-mass fractions that would be solubilized by buffer systems used in the reaction medium. Recovery yield of the milled wood was 95% (w/w), and the chemical composition was 45.6% glucan, 16.2% polyoses, and 29% total lignin based on a chemical characterization using previously described methods (Ferraz et al. 2000).

2.5. Treatment of milled wood in a reaction medium containing Fe^{2+} or Fe^{3+}/aqueous extract and linoleic acid

Two milligrams of previously prepared milled wood were treated in 10-ml test tubes containing 1.3 ml of 50 mM sodium acetate buffer pH 4.5, 600 µl of water, 47 µl of 8 mM $FeSO_4$, and 47 µl of 42 mM linoleic acid previously emulsified in 1% (w/v) Tween 60. The test tubes were maintained under rotary shaking at 120 rpm for 72 h at 27 °C. Then, the reaction mixture was filtered through a 0.45-µm membrane and diluted five times for UV spectroscopic analysis. For SEC analysis, 800 µl of the soluble fraction were diluted with 200 µl of 1.0 M NaOH and eluted through a Sephadex G-50 column (3 cm in diameter × 50 cm long) with 0.5 M NaOH at 2.0 ml min^{-1} by using an Akta 10 GE chromatograph set with a UV detector fixed at 280 nm. Some molecular mass markers were also run in the same chromatographic system: aprotinin (6500 Da), previously characterized kraft eucalypt lignin (950 Da) (Ferraz et al. 1997), and vanillin (150 Da) (Forss et al. 1989).

Similar reactions were also carried out with Fe^{3+} ions and aqueous extracts. In those cases, 600 µl of aqueous extracts (from the 14-day biotreated sample and ultrafiltrated through 5 kDa cut-off membrane) replaced the water, while 47 µl of 8 mM $FeCl_3$ replaced the Fe^{2+} solution.

Treated milled wood was analyzed by thioacidolysis (Rolando et al. 1992). In this case, the reaction mentioned above was scaled up to 800 mg of milled wood, maintaining the same wood/Fe^{2+} ratio. For thioacidolysis, 20 mg of milled wood was treated with 15 ml of a reagent composed of dioxane/ethanodiol 9:1 containing 0.2 M of BF_3-etherate. To this mixture, 1 ml of 0.6 g l^{-1} tetracosane was added as an internal standard, and the vial was tightly capped with a Teflon-lined screw cap and put into a silicone oil bath at 100 °C for 4 h. The reaction tube was cooled in ice water, and its contents were transferred to a 125-ml Erlenmeyer flask using 15 ml of water. The pH of the aqueous phase was adjusted to 3–4 by addition of 0.4 M $NaHCO_3$ and extracted with CH_2Cl_2 (3 × 20 ml) and dried over Na_2SO_4. The solution was concentrated under reduced pressure at 40 °C and dissolved in 1 ml dichloromethane. Ten microliters of this solution were mixed with 50 µl of BSTFA plus 5 µl pyridine and allowed to react for 1 h at 60 °C. Silylated compounds were analyzed in a Clarus 600 Perkin Elmer gas chromatograph equipped with a DB-5 (L 30 m × ID 0.535 mm) column and a flame ionization detector. Nitrogen at 33.0 cm s^{-1} was used as the carrier gas. Injector temperature was maintained at 240 °C. One microliter of the sample was injected using a split of 1:30. The column temperature was initially maintained at 140 °C for 1 min and then heated to 240 °C at 3 °C min^{-1}. This temperature was maintained for 1 min and then raised to 290 °C at 30 °C min^{-1}. The final temperature was maintained for an additional 7 min. The amounts of each monomer were determined using a response factor (1.50) derived from pure monomer standards with tetracosane as the internal standard.

3. Results

3.1. Aqueous extracts recovered from biotreated wood

E. grandis wood chips were biotreated with *C. subvermispora* in 2-l Erlenmeyer flasks for 7, 14, and 28 days. Wood chips were extracted to recover water-soluble compounds produced by the fungus or released from the biotreated wood. Appropriate buffer solutions containing surfactants are able to extract enzymes from solid-state cultures (Machuca and Ferraz 2001; Souza-Cruz et al. 2004; Abbas et al. 2005). On the other hand, the present work evaluated aqueous extracts that had very low enzyme concentrations. The determination of MnP levels in the aqueous extract recovered after 14 days of biotreatment, a period in which a peak of MnP had been detected in a previous work (Cunha et al. 2010), confirmed the low enzyme concentration. In fact, the aqueous extract contained no detectable MnP levels. Ultrafiltration through a 30-kDa cut-off membrane yielded a ten-times concentrated extract presenting 0.97 IU MnP l^{-1} of the original extract, while an appropriate enzyme extraction from similar cultures yielded 22.5 IU MnP l^{-1} (Cunha et al. 2010).

The aqueous extracts recovered from the wood presented continuously decreasing pH values, from pH 5.2 in the undecayed control to pH 3.5 in the 28-day biotreated sample. The decreasing pH values could be attributed to oxalic acid, which is the main organic acid produced by *C. subvermispora* when growing on *P. taeda* softwood, *E. grandis* hardwood, and wheat straw grass lignocellulose (Galkin et al. 1998; Aguiar et al. 2006; Vicentim and Ferraz 2007).

The aqueous extracts also had the ability to reduce Fe^{3+} ions from freshly prepared solutions. Here, Fe^{3+} reduction reactions were monitored by complexing formed Fe^{2+} with ferrozine (Stookey 1970; Aguiar et al. 2006). Two different reaction procedures were employed initially. When the reaction was carried out in the presence of the ferrozine reagent, an intense red color rapidly developed. This is in accordance with a rapid equilibrium

displacement, owing to the high affinity of ferrozine to the newly formed Fe^{2+} ions (Stookey 1970). Under these reaction conditions, the aqueous extract recovered from untreated wood promoted an intense Fe^{3+} reduction in such a way that only extracts diluted 20 times provided reliable absorbance data and enabled determination of the Fe^{2+} concentration (data not shown). On the other hand, when the reaction was carried out without ferrozine in the test tube, Fe^{3+} reduction was slower and the final concentrations of Fe^{2+} were significantly lower: 120 μM and 20 μM Fe^{2+} after 60-min reactions with and without ferrozine in the test tube, respectively. Considering that ferrozine can displace the reaction equilibrium and favor Fe^{2+} accumulation, the remaining assays reported here were performed without ferrozine in the reaction tube. This could avoid incorrect data interpretation when considering the in-vivo role for such Fe^{3+}-reducing compounds. Based on these assays, the data reported in Fig. 1 showed that the Fe^{3+}-reducing activity remained low during the first 7 days of biotreatment and increased significantly after 14 days.

The nature of the compounds responsible for the Fe^{3+}-reducing activity was evaluated by molar mass distribution studies. SEC showed a wide distribution of compounds absorbing at 280 nm. The molar mass and quantity of these compounds varied during the biotreatment period (Fig. 2). On the other hand, Fe^{3+}-reducing activity was detected only in the fractions eluting from 16 ml to 19 ml, corresponding to molar masses ranging from 3 kDa to 5 kDa. Eluted fractions were also assayed for carbohydrates as shown in Fig. 2. The entire SEC data indicated the occurrence of a complex mixture of compounds, instead of a single polydisperse macromolecule.

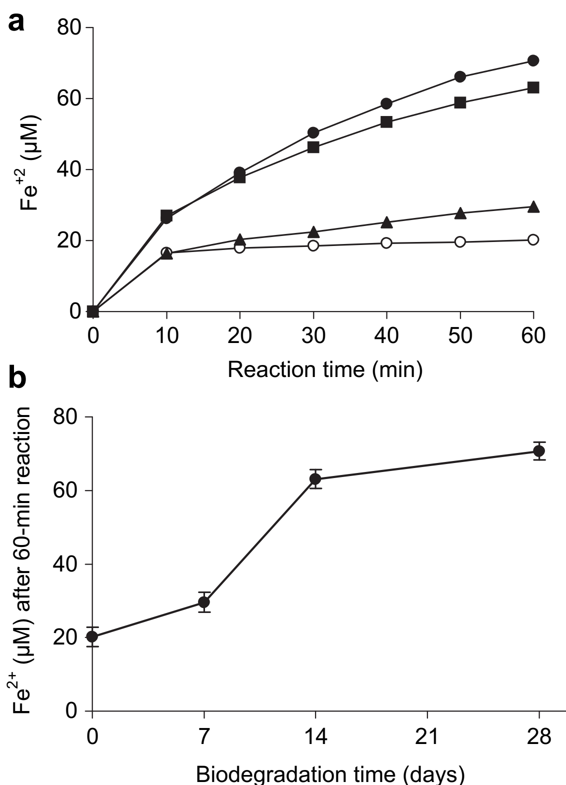

Fig. 1. Fe^{3+} reduction by aqueous extracts recovered from *E. grandis* wood biotreated by *C. subvermispora*. (a) Reaction kinetics: (open circles) untreated control; (triangles, squares, and filled circles) 7, 14, and 28-day biotreated samples, respectively. (b) Accumulated Fe^{2+} after 60-min reaction.

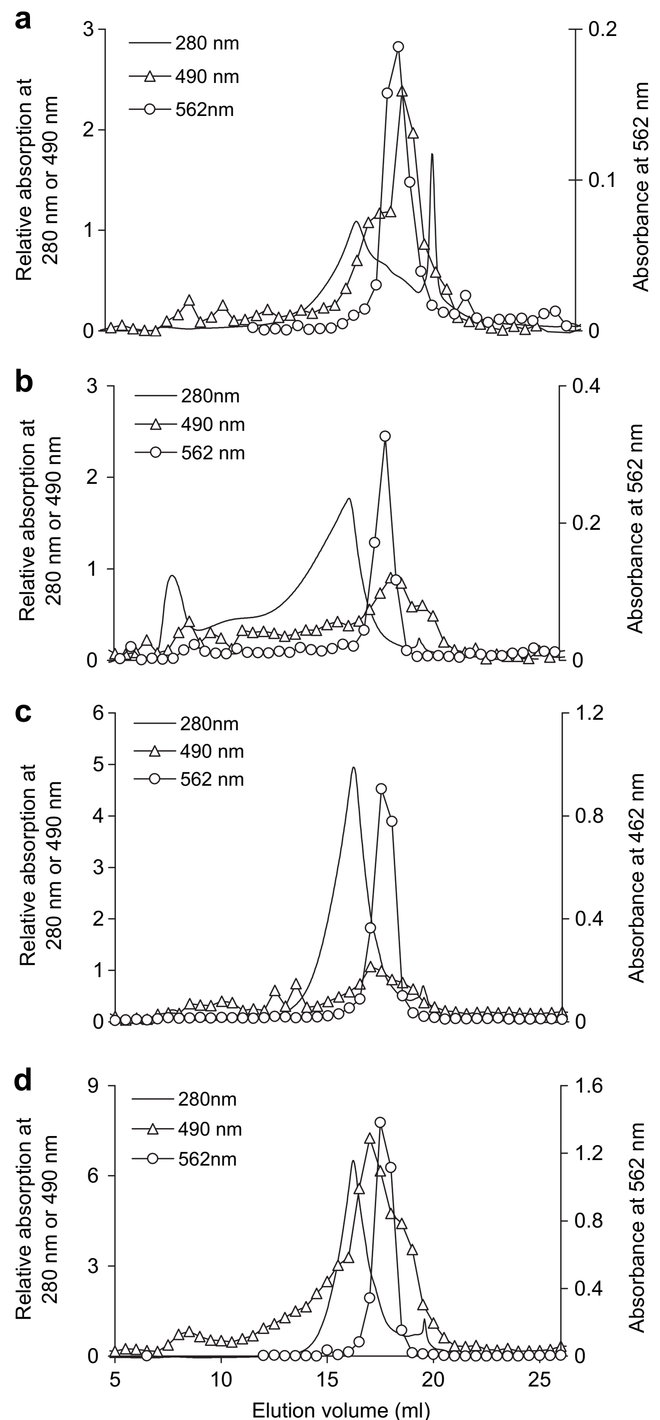

Fig. 2. Molar mass distribution of the aqueous extracts recovered from *E. grandis* wood biotreated by *C. subvermispora*. Relative absorption at 280 nm and 490 nm relates to aromatics and carbohydrates, respectively. Absorbance at 562 nm relates to Fe^{3+} reducing activity. (a) Undecayed control. Figures (b), (c) and (d) represent 7, 14, and 28 days of biotreatment, respectively. Molar mass markers and their elution volume correspond to albumin (66 kDa, 10,0 ml), carbonic anhydrase (29 kDa, 12,0 ml), cytochrome c (12.4 kDa, 14,4 ml), aprotinin (6.5 kDa, 16,0 ml), and acetone (28 Da, 19,2 ml).

The chromatogram of the aqueous extract from untreated wood (Fig. 2a) showed two main peaks detected at 280 nm, eluting from 13 to 19 ml and from 19 to 20 ml. Carbohydrates eluted as a broad peak from 13 to 20 ml, while Fe^{3+}-reducing compounds predominated in the fraction from 16 to 19 ml. The carbohydrates present in

the sample recovered from the undecayed control seemed to be consumed by the fungus during the initial stages of wood decay because these compounds were almost absent in chromatograms obtained from the aqueous extracts recovered after 7 days and 14 days of biotreatment (Fig. 2b and c, respectively). The UV-absorbing compounds detected in the 19–20-ml fraction from the control sample were also consumed, but higher-molar-mass compounds were released during these biodegradation periods. After long biodegradation periods (28 days), the aqueous extract re-accumulated significant contents of soluble carbohydrates and continued to accumulate UV-absorbing compounds (note the absorbance scale changes in Fig. 2d).

Ultrafiltration of the aqueous extracts though cut-off membranes of 5 kDa, 10 kDa, and 30 kDa was performed to confirm the molar mass range of the Fe^{3+}-reducing compounds. In all cases, the filtrates retained 80–100% of the initial Fe^{3+}-reducing activity and presented a single 16-ml to 19-ml peak in the SEC system, confirming that the compounds responsible for the Fe^{3+} reduction corresponded to a fraction with 3 kDa to 5 kDa (data not shown). The presence of low-molar-mass compounds was assayed in the 5-kDa ultrafiltrated fraction. Analysis using GC/MS indicated the presence of oxalic acid, long-chain fatty acids, glycerol, and some aromatic compounds (Table 1). Despite the fact that silylation was used to improve volatility, some compounds with higher molar mass could not be detected in GC/MS analysis owing to limited volatility under the evaluated conditions (injector and final column temperature of 250 °C and 290 °C, respectively). Among the detected compounds, only vanillin, galic, and vanillic and syringic acids have been reported to reduce Fe^{3+} in aqueous solutions (Jordan and Xu 1988; Aguiar and Ferraz 2007).

Vanillin was detected in all samples and was used as a model to evaluate the contribution of low-molar-mass compounds on the Fe^{3+}-reducing activity of the extracts. Vanillin concentration in the 5-kDa ultrafiltrates was estimated to vary from 5 μM to 22 μM, depending on the culturing time. Therefore, pure vanillin solutions (5 μM and 30 μM) were prepared and assayed for Fe^{3+} reduction. These solutions produced very low Fe^{2+} concentrations (0.5 μM and 0.7 μM after 10-min reactions carried out in the presence of ferrozine, respectively), when compared with the values produced by the 5-kDa ultrafiltrate recovered from the 14-day biotreated sample (43 μM under similar reaction conditions). These data corroborate that most of the Fe^{3+}-reducing activity can be attributed to the macromolecular compounds with molar mass of approximately 3 kDa to 5 kDa, instead of low-molar-mass compounds such as vanillin.

Table 1
Low-molar-mass compounds detected after GC/MS analysis of trimethylsilyl derivatives prepared from aqueous extracts recovered from *E. grandis* wood biotreated by *C. subvermispora*.

Compounds[a]	Biotreatment time (days)				Fe^{3+} reducing ability[b]
	0	7	14	28	
Oxalic acid	+	+	+	+	No
Glycerol	+	+	+	+	No
Vanillin	+	+	+	+	Yes
Vanillic acid	−	−	−	+	Yes
Syringic acid	−	−	−	+	Yes
Gallic acid	+	−	−	−	Yes
Palmitic acid	+	+	+	+	No
Oleic acid	−	+	+	+	No
Estearic acid	+	+	+	+	No

[a] The signals + and − mean, respectively, that the compounds were detected in the extract, or not.
[b] From Aguiar and Ferraz, 2007.

3.2. *Linoleic acid oxidation by Fe^{2+} ions and Fe^{3+} plus aqueous extracts recovered from biotreated wood*

Freshly prepared Fe^{2+} solutions were initially assayed for the capacity to degrade linoleic acid *in vitro*. The reactions were monitored in an oxygraph because fatty acid peroxidation occurs via O_2 consumption (Kapich et al. 2005; Cunha et al. 2010). When freshly prepared Fe^{2+} solutions were added to the reaction cell, a small amount of O_2 consumption was observed, probably resulting from Fe^{2+} oxidation. After linoleic acid addition, O_2 consumption was intense, as shown in Fig. 3. The rate of initial O_2 consumption in the Fe^{2+}-catalyzed reactions was 115 ± 13 nmol ml^{-1} min^{-1}. Control reactions, either lacking iron ions or replacing Fe^{2+} with Fe^{3+}, were unable to promote significant O_2 consumption. In these control reactions, the maximum rate of O_2 consumption was 0.9 nmol ml^{-1} min^{-1} (Fig. 3).

To test for the involvement of different reactive oxygen species, the reaction was performed in the presence of 10% methanol, which is known as an efficient hydroxyl radical-trapping agent (Rodriguez et al. 2003). In the presence of 10% methanol, the O_2 consumption rate (124 ± 30 nmol ml^{-1} min^{-1}) was similar to the one observed in the absence of methanol (115 ± 13 nmol ml^{-1} min^{-1}), which suggests a minor participation of hydroxyl radicals. All data considered together indicated that linoleic acid was rapidly oxidized under the evaluated reaction conditions only when enough Fe^{2+} ions were present in the solution.

Based on the previous data, the aqueous extracts recovered from biotreated wood were assayed for the ability to initiate linoleic acid peroxidation in vitro when freshly prepared Fe^{3+} solutions were provided in the reaction medium. Table 2 presents the initial rate of O_2 consumption observed in each experimental run. The rate of O_2 consumption was proportional to the Fe^{3+}-reducing activity present in each extract, as illustrated in Fig. 4.

It was relevant to determine if the radicals produced during linoleic acid peroxidation would be able to start in-vitro lignin degradation, as already demonstrated for similar reactions initiated by the enzyme MnP (Hofrichter et al. 2001; Cunha et al. 2010). Therefore, both systems (Fe^{2+} ions and Fe^{3+} ions/biotreated wood aqueous extract) were used to treat ball-milled *E. grandis* wood in the presence of linoleic acid. The aqueous extract recovered from biotreated wood chosen for these experiments corresponded to the 5-kDa cut-off ultrafiltrate extract obtained from a wood sample biotreated for 14 days.

Fig. 5 shows UV and SEC data for the soluble fraction released after treatment of the milled wood with the Fe^{2+}/linoleic acid system. Ultraviolet data (Fig. 5a) show that the reaction system released UV-absorbing compounds from milled wood. The chromatogram of the released material showed a minor fraction

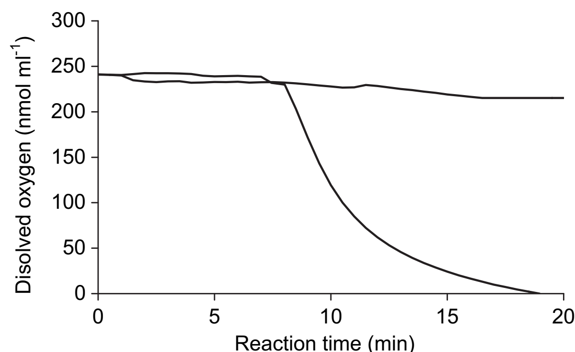

Fig. 3. Oxygen consumption during linoleic acid peroxidation initiated by Fe^{3+} and Fe^{2+} ions in solution.

Table 2
Rate of O_2 consumption during linoleic acid peroxidation initiated by Fe^{3+} ions and aqueous extracts recovered from *E. grandis* wood biotreated by *C. subvermispora*.

Aqueous extracts	Rate of O_2 consumption during linoleic acid peroxidation (nmol ml^{-1} min^{-1})
Without wood extract	0.9 ± 0.2
Wood extract from non biotreated wood	8 ± 1
Wood extract from 7-day biotreated wood	24 ± 2
Wood extract from 14-day biotreated wood	37 ± 1
Wood extract from 28-day biotreated wood	39 ± 2

representing high-molar-mass molecules excluded by the column and a main low-molar-mass peak, compatible with monomeric aromatic moieties such as vanillin and vanillic acid, which were used as molar mass markers in the column. Fig. 6 presents data for the treatment of the milled wood with the Fe^{3+} ions/biotreated wood aqueous extract/linoleic acid system. Also, in this case, UV-absorbing compounds were released from milled wood (Fig. 6a), while SEC data confirmed the low molar mass characteristic of the released material. Both reaction systems produced similar reactions; however, the Fe^{2+}/linoleic acid system released a slightly higher amount of UV-absorbing compounds, which was evidenced by the total area calculated from SEC data obtained for each evaluated reaction (Table 3). For comparison, Table 3 includes data previously obtained from the treatment of the same *E. grandis* milled wood using the MnP/linoleic acid system (Cunha et al. 2010). The residual milled wood was also analyzed by thioacidolysis to determine the yield of monomers recovered from aryl ether linkages in lignin (Table 3). These data taken together indicate that the radicals formed during linoleic acid peroxidation in reactions initiated by Fe^{2+} ions, or even Fe^{3+} reduced by aqueous extracts recovered from wood biotreated by *C. subvermispora*, can degrade lignin in the lignocellulose complex, releasing low-molar-mass lignin fragments. However, the iron-based systems released lower amounts of UV-absorbing compounds and residual lignin in the milled wood was not depolymerized as compared with the MnP/linoleic acid initiated reactions (Table 3).

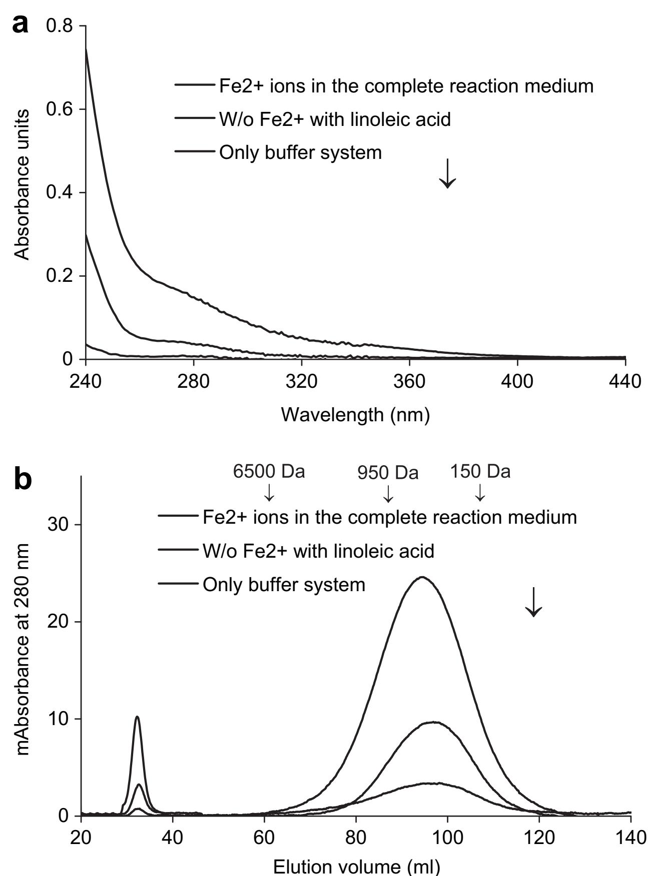

Fig. 5. UV spectra (a) and size exclusion chromatography (b) of the soluble fraction released from *E. grandis* lignocellulose treated with Fe^{2+} ions and linoleic acid. The arrows indicate that the legend and the graphs are in the same sequence. For the sake of clarity, only the reaction controls without Fe^{2+} ions and the buffer system are presented in the figure. Other related reaction controls lacking linoleic acid produced data similar to those of the buffer system. Molar mass markers correspond to aprotinin (6500 Da), kraft eucalypt lignin (950 Da), and vanillin (150 Da).

4. Discussion

In the present work, wood chips biotreated by *C. subvermispora* were extracted to recover water-soluble compounds produced by the fungus or released from the biotreated wood. A parallel work evaluating enzyme secretion under identical culture conditions (Cunha et al. 2010) showed that MnP prevailed in the oxidative complex, while xylanases and endocellulases were the main hydrolytic enzymes. Weight and component losses were typical for the *C. subvermispora*'s action on wood, which degraded mainly lignin and hemicelluloses, while preserving cellulose. The cultures also accumulated thiobarbituric acid-reactive substances (Cunha et al. 2010) that suggest the occurrence of lipid peroxidation reactions (Enoki et al. 1999).

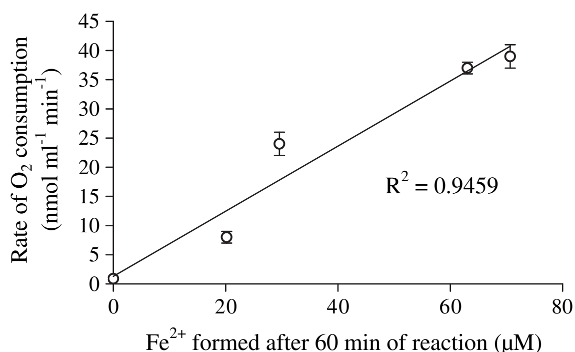

Fig. 4. Correlation between Fe^{3+} reduction activity in aqueous extracts from biotreated *E. grandis* and the rate of O_2 consumption during in-vitro linoleic acid peroxidation.

The aqueous extracts had the ability to reduce Fe^{3+} ions from freshly prepared solutions. The nature of the compounds responsible for the Fe^{3+}-reducing activity was evaluated by molar mass distribution studies and GC/MS analysis. The entire SEC data indicated the occurrence of a complex mixture of compounds, instead of a single polydisperse macromolecule, whereas GC/MS studies showed minor contribution of low molar mass compounds. The origin of such compounds in a culture medium containing wood is not clear. They could be secreted by the fungus or released from the wood undergoing biodegradation. In any case, it is noteworthy that these compounds take part in the wood degradation process because they were able to reduce Fe^{3+} ions.

Either freshly prepared Fe^{2+} solutions or Fe^{3+} solutions reduced by the extracts recovered from biotreated wood were able to degrade linoleic acid in vitro. The rate of O_2 consumption was proportional to the Fe^{3+}-reducing activity present in each extract, as illustrated in Fig. 4. Several reactions would occur simultaneously in the system containing Fe^{2+} ions. Fe^{2+} oxidation rapidly produces hydroperoxyl radicals under acidic pH. These radicals can

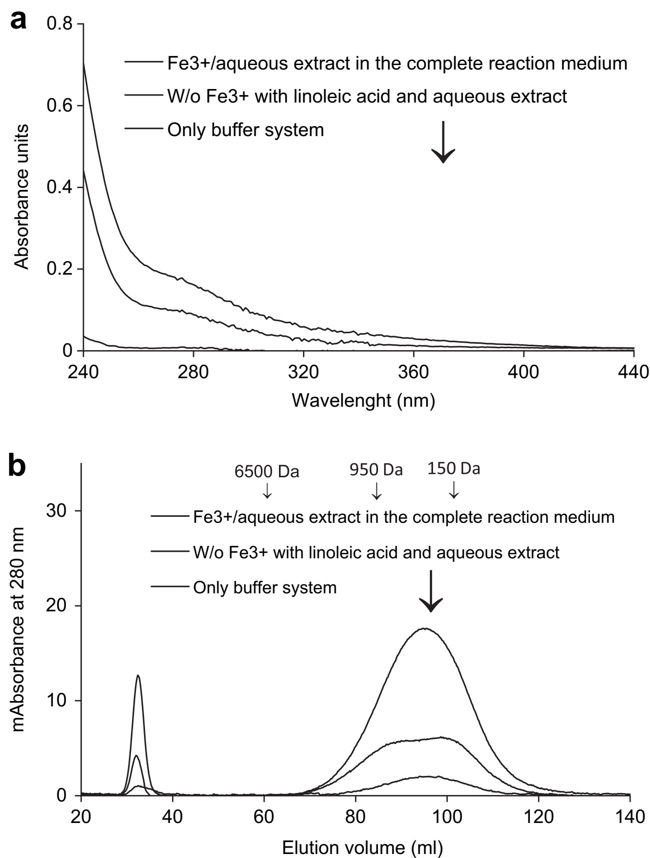

Fig. 6. UV spectra (a) and size exclusion chromatography (b) of the soluble fraction released from *E. grandis* lignocellulose treated with Fe^{3+} ions/aqueous extract from biotreated *E. grandis* and linoleic acid. The arrows indicate that the legend and the graphs are in the same sequence. For the sake of clarity, only the reaction controls without Fe^{3+} ions and the buffer system are presented in the figure. Other related reaction controls lacking linoleic acid produced data similar to those of the buffer system. Molar mass markers correspond to aprotinin (6500 Da), kraft eucalypt lignin (950 Da), and vanillin (152 Da).

continue to react with other Fe^{2+} ions to produce hydrogen peroxide, which ultimately can produce hydroxyl radicals via the Fenton reaction (Stumm and Lee 1961). Hydroperoxyl and/or hydroxyl radicals can rapidly oxidize linoleic acid (Rodriguez et al. 2003; Spiteller 2006). However, the aqueous extracts recovered from biotreated wood also contain phenolic compounds with antioxidant capacity, which would inhibit linoleic acid peroxidation to some extent (Kapich et al. 2005; Carvalho et al. 2008; Cunha et al. 2010). For example, 2,3-dihydroxybenzoic acid was shown to

Table 3
Size exclusion chromatography areas for UV-absorbing compounds released from *E. grandis*-milled wood and the yield of the main thioacidolysis monomers recovered from treated material.

Sample treatment	Total area for UV-absorbing compounds (mAbs ml)	Yield of thioacidolysis monomers (μmol g^{-1} of milled wood)		
		Guaiacyl	Syringyl	Total
Untreated	20	121 ± 2	344 ± 3	465 ± 5
Fe^{2+}/linoleic acid	649	123 ± 1	343 ± 6	466 ± 7
Fe^{3+}/aqueous extract/linoleic acid	471	Na	na	na
MnP/linoleic acid[a]	4308	81 ± 3	275 ± 13	356 ± 17

na, not analyzed.
[a] From Cunha et al. (2010).

inhibit linoleic acid peroxidation in MnP-initiated reactions (Kapich et al. 2007).

Fe^{3+} reduction during wood decay has been widely reported (Goodell et al. 1997; Hammel et al. 2002). Most cases indicate that Fe^{3+} is able to support the Fenton reaction, which generates an OH radical able to oxidize several wood components, including cellulose. As shown in the previous results, Fe^{3+} reduction also seems capable of initiating linoleic acid peroxidation. In this context, a key result from the present work was to demonstrate that the radicals produced during linoleic acid peroxidation were able to start in-vitro lignin degradation, as was already demonstrated for similar reactions initiated by the enzyme MnP (Hofrichter et al. 2001; Cunha et al. 2010). Ultraviolet and SEC data supported the idea that the treatment of milled wood with the iron/linoleic acid systems released low-molar-mass UV-absorbing compounds from wood. The soluble fraction released from wood differed from that released by the MnP/linoleic acid system previously described (Cunha et al. 2010). In the former work, intense oxidation of the released material was suggested because some adsorption of the material to the size exclusion column occurred. In contrast, data from Figs. 5b and 6b suggest that the released materials were simple low-molar-mass compounds similar to the vanillin and vanillic acid markers. Freshly prepared Fe^{2+} solutions or Fe^{3+} solutions reduced by the extracts recovered from biotreated wood produced similar reactions; however, the Fe^{2+}/linoleic acid system released a slightly higher amount of UV-absorbing compounds (Table 3). These data taken together indicate that the radicals formed during linoleic acid peroxidation in reactions initiated by Fe^{2+} ions, or even Fe^{3+} reduced by aqueous extracts recovered from wood biotreated by *C. subvermispora*, can degrade lignin in the lignocellulose complex, releasing low-molar-mass lignin fragments. However, it is relevant to compare the data reported herein with the data obtained from the treatment performed with the MnP/linoleic acid system (Cunha et al. 2010). The results show that the extent of lignin degradation in the MnP-initiated system was more pronounced because the iron-based systems released lower amounts of UV-absorbing compounds and residual lignin in the milled wood was not depolymerized, as was observed in the case of the MnP/linoleic acid initiated reactions (Table 3). This suggests that the Fe^{3+}-reducing compounds formed during wood biodegradation by *C. subvermispora* can effectively degrade lignin based on linoleic acid peroxidation, but MnP-based systems are more efficient in these reactions.

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