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Enzymatic grafting of natural phenols to flax fibres: Development of antimicrobial properties

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ABSTRACT

Unbleached flax fibres for paper production were treated with laccase from *Pycnoporus cinnabarinus* and low molecular weight phenols (syringaldehyde – SA, acetosyringone – AS and p-coumaric acid – PCA) to evaluate the potential of this treatment to biomodify high cellulose content fibres. After the enzymatic treatment with the phenols, an increase in kappa number was found, probably due to a covalent binding of the phenoxy radicals on fibres. Grafting was more evident in pulps treated with PCA (an increase of 4 kappa number points with respect to the laccase control was achieved). Paper handsheets from treated pulps showed antimicrobial activity against the bacteria tested: *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Klebsiella pneumoniae*. An important reduction on microbial count was obtained after incubation of liquid cultures of the bacteria with grafted handsheets. AS and PCA grafted fibres showed a high antibacterial activity on *K. pneumoniae*, getting a nearly total growth inhibition. AS fibres also caused a high reduction in bacterial population of *P. aeruginosa* (97% reduction). Optical properties of handsheets from treated pulps were also determined, showing a brightness decrease and increase in coloration, evaluated by CIE $L^*a^*b^*$ system, caused by the laccase induced grafting of the phenols. The results suggest that these low molecular weight phenols, covalently bound to the flax fibres by the laccase treatment, can act as antimicrobial agents and produce handsheets with antimicrobial activity.

1. Introduction

Laccases (oxidoreductases) are one of the most important enzymes in terms of application versatility in the forest products industry. Laccases are multicopper oxidases secreted by white-rot fungi and other organisms that play a crucial role in the terrestrial carbon cycle by helping the synthesis and the degradation of lignocellulosic materials. In co-operation with other ligninolytic (lignin-degrading) fungal enzymes such as lignin peroxidase (LiP), manganese peroxidase (MnP), and versatile peroxidase (VP) (Bajpai, 2004; Call & Mücke, 1997; Martínez et al., 2005), laccase oxidizes lignin, an aromatic polymer that together with the polysaccharides cellulose and hemicelluloses are the principal components of wood.

As a result, laccase biotechnology is applicable in those sectors of the forest products industry where the goal is to either remove (e.g., in pulp bleaching, Aracri & Vidal, 2011; Bourbonnais, Paice, Freiermuth, Bodie, & Bornemann, 1997; Fillat & Roncero, 2010; Sigoillot et al., 2004; Valls, Vidal, & Roncero, 2010) or co-polymerize lignin (e.g., in grafting, Chandra & Ragauskas, 2002; Widsten & Kandelbauer, 2008).

Recently, laccase research has shifted towards fibre modification, a new research field of increasing interest (Aracri, Colom, & Vidal, 2009; Liu, Shi, Gao, & Qin, 2009). In the last years several authors have shown that laccase treatments can improve physical and chemical properties of different fibres for a better performance, or to create new value-added products. Laccase-catalyzed biografting is a versatile method of functionalization due to the enzyme's nonspecific substrate requirements, which allow bonding a wide range of phenolic compounds to fibres (Chandra, Lehtonen, & Ragauskas, 2004; Kenealy & Jeffries, 2003). Wood surfaces have been enzymatically modified either for aesthetic purposes or for preservation (Kudanga et al., 2008). Lignocellulosic fibres have been grafted with laccases and phenolic compounds (Aracri et al., 2009; Chandra & Ragauskas, 2002; Gronqvist et al., 2006) in order to confer them hydrophobicity, antimicrobial properties or to improve mechanical properties as wet tensile strength (Chandra et al., 2004; Elegir, Kindl, Sadocci, & Orlandi, 2008; Garcia-Ubasart et al., 2011; Lund & Felby, 2001; Schroeder, Aichernig, Güebitz, & Kokol, 2007).

In recent years, interest has grown in the preparation of materials with antibacterial properties, for use in a wide range of fields such as food packaging, sanitary materials, and household, medi-

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cal, and military items (Hou, Zhou, & Wang, 2009). The best way to obtain antimicrobial surfaces is by incorporating antimicrobial agents through covalent bonding (Roy, Knapp, Guthrie, & Perrier, 2008). Fibre modification by an eco-friendly approach, such as the enzymatic grafting of natural antimicrobial organic molecules to lignocellulosic fibres, can represent a valid solution to meet the growing consumers' expectation of higher hygiene standards and safer products together with environment protection concerns (Elegir et al., 2008).

Natural phenols are potential antibacterial agents of low molecular weight. In a previous work, treatment of flax pulp with laccase and natural single phenols such as syringaldehyde (SA), acetosyringone (AS) and p-coumaric acid (PCA) as mediators, improved pulp properties (decrease in kappa number and rise in brightness) after a bleaching sequence (Fillat, Colom, & Vidal, 2010). However, the cross-linking of some of these natural compounds to fibres was evidenced after an enzymatic stage with *Pycnoporus cinnabarinus* laccase (Aracri et al., 2010; Cadena et al., 2011).

In this work, we have evaluated the capacity of laccases to graft natural phenols (syringaldehyde, acetosyringone and p-coumaric acid) on unbleached flax fibres and analyzed the antimicrobial properties conferred to the paper products obtained.

2. Materials and methods

2.1. Pulp sample, enzyme and natural phenols

Unbleached soda-anthraquinone pulp from *Linum usitatissimum* was provided by CELESA mill in Tortosa (Spain). Pulp was washed with sulphuric acid at pH 4 for 30 min to remove impurities, reduce the content in metal ions and adjust the pH to the requirements for the enzymatic stage. Initial high cellulose content pulp had kappa number of 7.00 and 38.8% ISO brightness. Laccase from *P. cinnabarinus* (PcL) was produced by the INRA (Marseille, France) from the monokaryotic hyperproducing strain ss3 (Herpoël, Moukha, Lesage-Meessen, Sigoillot, & Asther, 2000). Activity was monitored by measuring the ABTS oxidation at 436 nm (ε_{436} = 29,300 M^{-1} cm^{-1}). One laccase activity unit was defined as the amount of enzyme that transforms 1 μmol/min of ABTS at 25 °C. All measurements were carried out using a Thermo Scientific Evolution 600 spectrophotometer. The natural phenols acetosyringone (AS), syringaldehyde (SA) and p-coumaric acid (PCA) were purchased from Sigma–Aldrich.

2.2. Laccase assisted grafting treatments

Washed flax pulp was firstly extracted with acetone in a Soxhlet extractor for 2 h and 15 min. Grafting treatments with laccase and simple phenols were carried out with an Ahiba Spectradye apparatus (Datacolor) in closed vessels containing 10 g (dry weight) of pulp at 5% consistency in 50 mM sodium tartrate buffer (pH 4), 40 U/g of *P. cinnabarinus* laccase and 3.5% (w/w) natural phenols (relative to dry pulp weight). Tween 80 from Sigma (0.05%, w/v) was added as surfactant. Samples were incubated with shaking (30 rpm) at 50 °C for 4 h. Control pulps were treated under identical conditions in the absence of either the phenolic compound (lacasse control) or both the phenols and laccase (initial control). Once treated, pulps were extensively washed with water and filtered, and the residual liquor was collected for subsequent analysis. Handsheets from treated pulps were made according standard ISO 3688.

2.3. Kappa number and optical properties

Kappa number and brightness were determined following ISO 302 and ISO 3688, respectively. Other optical properties were analyzed by a paper reflectance measuring Technidyne Colour Touch apparatus at standard illuminant D65 (LAV/Spec. Excl., d/8, $D_{65}/10°$). The reflectance spectra of paper sheets were obtained from scattering (s) and absorption (k) coefficients using the Kulbelka–Munk theory (Dence & Reeve, 1996). The colour of the samples was described according to the CIE $L^*a^*b^*$ colour system, where L^*, a^* and b^* are the coordinates of the colour in the cylindrical colour space, based on the theory that colour is perceived by black–white (L^* = lightness), red–green (a^*) and yellow–blue (b^*) sensations (Hunt, 1998). Other optical parameter used was the Chroma (C^*) = $(a^{*2} + b^{*2})^{1/2}$, that represents the perpendicular distance from lightness axis.

2.4. Antimicrobial properties of the natural phenols

The antimicrobial properties of the natural phenols studied were tested on three microorganisms: *Staphylococcus aureus* (Gram+), *Pseudomonas aeruginosa* (Gram−) and *Klebsiella pneumoniae* (Gram−). The bacterial strains were inoculated in 5 ml of LB medium supplemented with increased concentrations, 0–25 mM, of the natural phenols and the cultures were incubated overnight at 37 °C. After overnight incubation, $OD_{600\,nm}$ was measured as an estimation of the growth of the bacterial strain. The percentage of growth inhibition was calculated using the following formula:

$$\text{Growth inhibition}\,(\%) = 100 - \left(\frac{I}{B} \times 100\right)$$

where $I = OD_{600\,nm}$ of the culture of a bacterial strain with a natural phenol at a given concentration and $B = OD_{600\,nm}$ of the culture of the same strain without added phenol.

2.5. Antimicrobial properties of laccase–phenols treated paper

Antimicrobial activity test of the papers treated with laccase and phenol was based on the ASTM Standard Test Method E 2149-01. Overnight shaken cultures of *S. aureus* (Gram+), *P. aeruginosa* (Gram−) or *K. pneumoniae* (Gram−) were diluted in KH_2PO_4 buffer (working bacteria dilution). 1 g (dry weight) of the treated paper handsheets or control papers cut into small portions were added to flasks containing 50 ml of working bacterial solution and shaked 200 rpm for 1 h at 37 °C. Samples were taken before or after 1 h incubation with papers, and bacterial concentration as colony forming units (CFU/ml) was determined by standard viable plate count in T.G.E. Agar (Scharlau). The percentage of reduction in CFU was calculated using the following equation:

$$\text{CFU/ml}\quad \text{Reduction}\,(\%) = \frac{B - A}{B} \times 100$$

where A = CFU/ml after 1 h contact time and B = CFU/ml before the contact.

The presence of antimicrobial leaching from treated pulps was determined by evaluating the production of growth inhibition haloes on agar plates inoculated with the microorganisms studied. Treated paper samples (1 g odp) were added to 50 ml of sterile buffer solution and shaked in flasks at 200 rpm for 1 h at 37 °C. 100 μl of these samples were poured into 8 mm diameter holes made in the centre of T.G.E. agar plates previously inoculated with a confluent lawn of the bacteria studied (1×10^5 CFU/ml) and incubated at 37 °C overnight. Finally, the presence of a zone of inhibition surrounding the agar holes was recorded.

2.6. Effluent properties: spectrophotometric curves and residual activity

The effluents from enzymatic treatments were diluted to 1:20 and their absorbance measured between 200 and 400 nm in a

UV–vis Thermo Scientific Evolution 600 spectrophotometer. The residual activity of the laccase on the effluents was also measured.

3. Results and discussion

In preliminary assays, some intrinsic pulp interference that made complicated the measurement of the antimicrobial properties of enzyme treated fibres was observed. We assumed that pulp extractives could cause unreal outcomes. Extractives are relatively small molecules that can be removed using solvents. These molecules are non-cell wall components and some of them protect the plant against bacterial or fungal attack. Gutiérrez and del Río (2003) identified the main extractives present on bast fibres of flax (long fibres from the stem used for manufacturing specialty papers). Their results revealed that waxes, series of long chain n-fatty alcohols, n-aldehydes, n-fatty acids, and n-alkanes were present in the pulps. To eliminate extractives and avoiding the interference of these substances in the determination of antimicrobial properties of grafted papers, unbleached pulps used in the studies were washed with acetone in a Soxhlet extractor before the lacasse–phenol grafting stage.

3.1. Kappa number

Treatment conditions were previously optimized in order to get the maximum possible amount of phenolic substrates coupled onto the fibres. So, some variables were modified respect to the conditions used in the laccase mediator bleaching treatments reported in previous works (Fillat et al., 2010). In this way, in these new grafting treatments, the pulp consistency, phenolic compounds dose and laccase units were increased (laccase units were doubled respect to the bleaching assays), while the reaction time was reduced by 1 h. Chandra and Ragauskas (2002) observed that oxidative polymerization and grafting copolymerization of phenoxy radicals in lignin caused an increase of kappa number. For this reason, kappa number and optical properties were measured after the enzymatic treatment in order to assess the tendency of the natural phenols to couple to fibres.

Treatments with laccase and simple phenols produced an increased kappa number of pulps when compared to control samples (Fig. 1a). These results suggest that laccase leads to the crosslinking or grafting of these phenolic compounds on fibres. The highest degree of grafting was obtained with PCA (4 points respect to the laccase control), while treatments with SA and AS also produced a higher kappa number than that of the laccase control (1.5 and 1.3 points, respectively). These results match up with previous studies where biobleaching capacity of this enzymatic system was evaluated (Fillat et al., 2010). Laccase-induced grafting was also reported for *P. cinnabarinus* and these phenols by Aracri et al. (2010) that evidenced the incorporation of these compounds into the fibres by pyrolysis analysis coupled to gas chromatography/mass spectrometry in the absence and/or in the presence of TMAH as methylating agent. Therefore, natural phenols may be involved simultaneously in oxidative degradation and grafting reactions.

The higher grafting degree caused by PCA is probably due to both the higher pK_a of its phenolate group and the lower stability of its phenolic radical, that facilities condensation reactions at the C_3 and C_5 atoms with residual lignin (Camarero et al., 2007). Furthermore, PCA phenoxyl radicals could form several kinds of dimers and oligomers with a lower phenolic content (Camarero et al., 2008). On the other side SA and AS have similar structure: two methoxy radicals with steric hindrance that protect the phenolic groups (Chandra & Ragauskas, 2002). These phenols present lower pK_a values and form more stable radicals. Their substituents increase the lifetime of their phenoxy free radicals by preventing the coupling reactions with lignin, resulting in less grafting (Astolfi et al., 2005) but higher bleaching efficiency (Fillat et al., 2010).

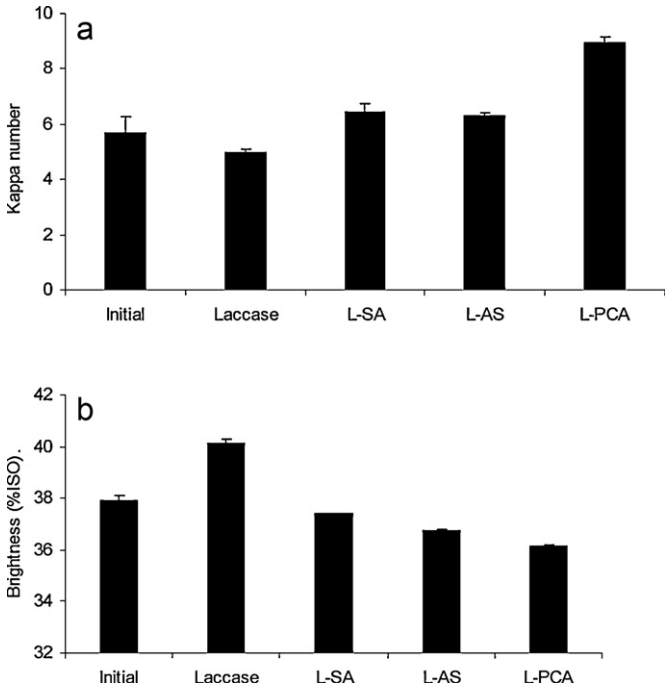

Fig. 1. Kappa number (a) and brightness (b) of flax pulp after laccase-assisted grafting of phenols. Initial sample corresponds to flax pulp after the acetone extraction; laccase control sample corresponds to flax pulp treated with laccase in the absence of phenols.

3.2. Brightness and optical properties

After the enzymatic treatment with phenols, brightness decreased when compared to the laccase control pulp (7–10% decrease) suggesting again a possible grafting of the phenolic compounds (Fig. 1b). However, pulps treated with laccase alone resulted in an increase of brightness and a decrease of kappa number respect to the initial pulp, showing the potential of the laccase used in flax pulp delignification. So, it could be assumed the coexistence of two simultaneous reactions during the treatments: lignin oxidation and phenol grafting. In our previous work, we demonstrated the capacity of *P. cinnabarinus* laccase and these natural phenols in flax delignification by getting higher final delignification rates, after a chemical bleaching stage, than the laccase alone (Fillat et al., 2010). Because of this ability, the differences of the laccase–phenol treated pulps respect to the laccase control pulps (without phenol) do not reflect the total grafting of phenols on pulps.

The optical properties of treated pulps were further analyzed. The CIE $L^*a^*b^*$ is defined as a three-dimensional space based on opposite colours. The L^* coordinate (lightness) indicates the amount of light present in a given colour, in our case; whether the pulp was lighter or darker. A positive a^* coordinate is indicative of red colour and a negative one of green colour; a positive b^* coordinate is indicative of yellow colour and a negative one of blue colour. The Chroma (C^*) is a parameter based on the CIE $L^*a^*b^*$ system and it is indicative of strong or weak colour (intensity).

Treated pulps with phenols were darker than laccase control pulps, L^* coordinate was smaller in all the pulps grafted with simple phenols (Fig. 2a). Simultaneously, these pulps presented a higher colour saturation (C^*) than the laccase control.

In all the treatments, the chromatic coordinates a^* and b^* were positive (Fig. 2b). Furthermore, pulps treated with laccase and phe-

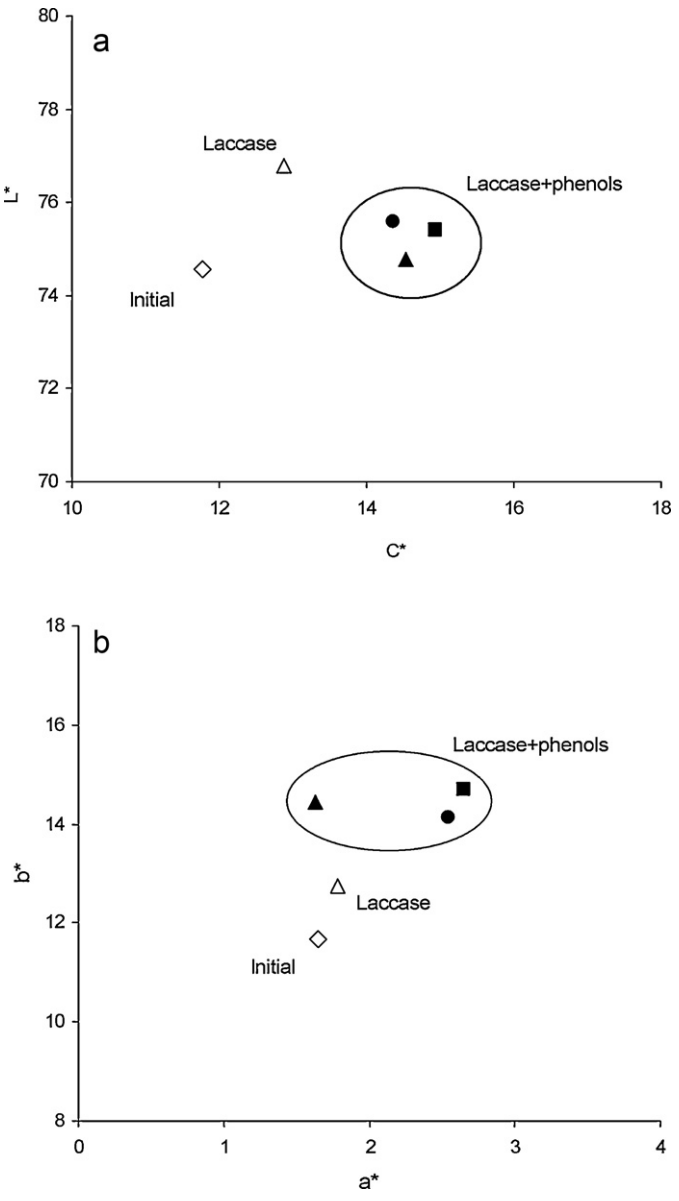

Fig. 2. CIE $L^*a^*b^*$ coordinates. Variation of the chromatic values L^* and C^* (a), and variation of coordinates a^* and b^* (b) of the flax papers. L–SA paper (●), L–AS paper (■) and L–PCA paper (▲).

nols showed an increase of a^* and b^* coordinates respect to laccase control (except in the case of PCA treated pulp, where only a b^* coordinate increase was observed), which supposed an increase in red and yellow colour, respectively.

The k/s index allows evaluating the amount of chromophoric groups presents in pulp. Papers obtained from pulps treated with laccase and natural phenols presented higher k/s values than initial and laccase control papers (Fig. 3), mainly at 400 nm, confirming the formation or addition of chromophores in these pulps. Oppositely, laccase control papers resulted in a k/s values decreased respect to the initial control papers, showing a loss of chromophoric groups (lignin removal) caused by the action of the laccase.

3.3. Antimicrobial properties of the grafted fibres

The antimicrobial properties of the grafted flax fibres against three bacteria: *S. aureus*, *K. pneumoniae* and *P. aeruginosa* were tested.

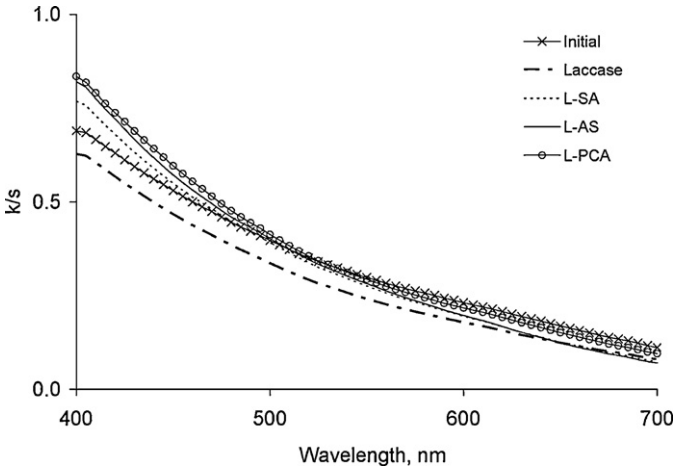

Fig. 3. k/s curves of paper obtained from initial pulp, laccase control pulp and laccase–phenol treated pulps.

Phenolic compounds are known to exhibit antimicrobial activity against a variety of microorganism. They have been reported some studies about antifungal and antibacterial activity of SA (de Souza et al., 2005; Zaldivar, Martínez, & Ingram, 1999) and PCA (Salomão et al., 2008; Wen, Delaquis, Stanich, & Toivonen, 2003).

Some of the requirements for an "ideal" antimicrobial agent would include effectiveness against a wide range of microorganism, low cost, easiness to apply and resistance to leaching from the material. According to these needs, natural phenols are potentially good antimicrobial substrates to test in grafting.

The method used for evaluating the antimicrobial activity effectiveness of an antimicrobial fibre was based on the ASTM E2149 Standard Test Method. The type of test challenge applied in this method is extreme, and very effective to test antimicrobials that are covalently bonded to the fibres. Furthermore, this method ensures good contact of inoculums to treated fibres by constant agitation during the test period.

The results showed that incubation of the tested microorganisms with the enzyme–phenol treated fibres caused a decrease in the microbial viable count, indicating an antibacterial activity of the grafted papers. The Gram negative bacteria tested, *K. pneumoniae* and *P. aeruginosa* showed an important growth inhibition by the phenol grafted papers, as a notable reduction in the number of bacterial cells (colony forming units, CFU) was caused by the contact with these fibres. AS and PCA grafted fibres showed an important antibacterial activity on *K. pneumoniae*, producing a nearly total growth inhibition, while SA fibres caused a less pronounced effect. AS fibres also caused a high reduction in bacterial population of *P. aeruginosa* (97% reduction), while SA and PCA coupled fibres reduced the bacterial population around 70%. Contact with the phenol grafted fibres produced a lower reduction in the bacterial cell count of the Gram positive bacterium tested, *S. aureus* (Table 1). The major reduction was caused by the PCA grafted fibres (73% reduction), while AS and SA fibres caused a reduction between 40 and 55% in the microbial population.

Table 1
Antibacterial activity against different bacteria of flax fibres grafted with natural phenols.

	Bacterial population reduction (%)		
	S. aureus	*K. pneumoniae*	*P. aeruginosa*
Control	17	5	0
Laccase–Syringaldehyde (L–SA)	55	61	71
Laccase–Acetosyringone (L–AS)	40	99	97
Laccase–p-coumaric acid (L–PCA)	73	97	70

Immobilized antimicrobial agents, as surface bonded materials, are not free to diffuse or be released into the environment under normal conditions of use. The method used to determine the antimicrobial activity of these materials ensures good contact between the microorganisms and the treated sample. To verify the suitability of this methodology for the specimens tested, the presence of antimicrobial leaching was determined evaluating the effect on bacterial growth of the supernatants obtained from incubation of the grafted fibres in sterile buffer, as detailed under Section 2. The absence of inhibition in every of the bacterial lawns cultured on agar plates indicated the absence of leaching, i.e. all the phenolic compounds tested remained bound to the fibres during the test.

Previous results reported by Elegir et al. (2008) showed that laccase mediated grafting of softwood kraft pulps can give antimicrobial properties to fibres. The phenols used in this report were different to those tested in our work. We have shown the antibacterial activity of pulp flax fibres grafted with acetosyringone, syringaldehide or p-coumaric acid. To our knowledge this is the first report that shows the antimicrobial activity of grafted pulp flax fibres, and also the first reported study on the antimicrobial effect of these natural phenols when grafted to lignocellulosic materials. The results obtained show the potential of functionalized fibres to produce safer paper products for sanitary and food uses, even with high quality fibres, as those from flax, of important alimentary use.

3.4. Antimicrobial properties of the natural phenols

To analyze the correlation between the antimicrobial properties of the grafted flax fibres and the antimicrobial properties of the corresponding natural phenols used, the antibacterial effect of these compounds against *K. pneumoniae*, *P. aeruginosa* and *S. aureus* was tested.

PCA was the most effective growth inhibitor, leading to inhibitions greater than 90% at 10 mM concentration for *K. pneumoniae* and *S. aureus*, and at 15 mM for *P. aeruginosa* (Fig. 4a). SA caused more than 50% growth inhibition on *K. pneumoniae* at 10 mM, and on all the tested strains at 25 mM, the highest concentration assayed (Fig. 4b). On the other hand, AS had a lower inhibition effect, only showing high antimicrobial effect on *K. pneumoniae* (more than 50% inhibition above 15 mM). At 25 mM concentration AS reduced the growth of *S. aureus* around 3%, and that of *P. aeruginosa* by 35% (Fig. 4c).

It is interesting to indicate that the lower concentration tested with the free phenols (5 mM) is higher than that used in the grafting experiments (3.5 mM). Growth inhibition values caused by 5 mM free phenols, with the exception of PCA and SA on *K. pneumoniae*, are below 30%, while inhibition caused by grafted fibres was always above 40%. The differences observed in the antibacterial behaviour between the free natural phenols, and those coupled onto laccase treated flax fibres, may be explained due to the modifications produced in these compounds by coupling to fibres by the laccase treatments. At this regard while PCA was the most effective antimicrobial agent both free or grafted onto fibres, AS showed a pronounced effect in grafted pulps, while it showed the lower antimicrobial activity when tested as a free compound.

3.5. UV spectra and residual activity in effluents from grafting

It was not possible to estimate the amount of simple phenols not grafted onto the fibres or the quantity of dissolved lignin on the effluents, as information given by spectra is only qualitative, and the signals that appeared overlapped and corresponded to different products. Fig. 5 shows the effluents spectra from the enzymatic stage. In the graph for each simple phenol three spectra are shown: laccase control, phenol control (effluents from pulp incubated with

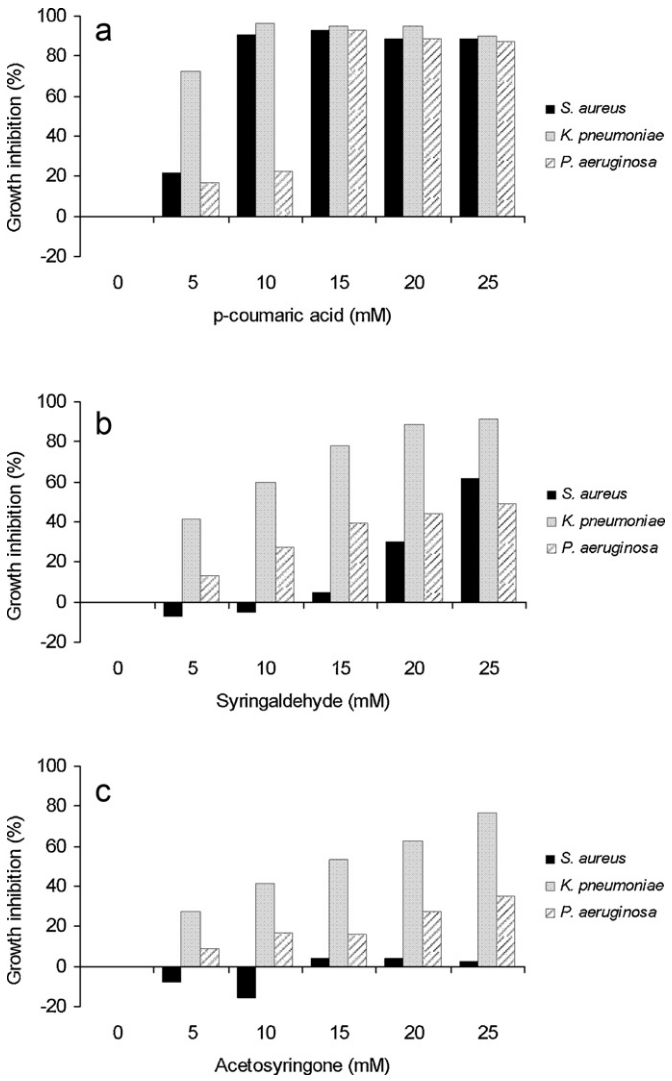

Fig. 4. Antimicrobial properties of the natural phenols PCA (a), SA (b), and AS (c) against *S. aureus* (black bars), *K. pneumoniae* (grey bars) and *P. aeruginosa* (striped bars).

phenol but without laccase) and laccase assisted grafting of phenols. As an additional measurement laccase was incubated with the phenols studied without pulp to monitor their oxidation (data not showed). The reduced state of the phenols produced a spectrum with the same peaks that those exhibited by the effluents of the phenol controls. Laccase control spectrum (pulp treated with laccase, 40 U/g, but without phenol) showed a very low signal; though a signal increase was observed under 250 nm (caused by the buffer used on the treatments). SA control exhibited a peak at 308 nm corresponding to SA solution spectra (Fig. 5a). This signal diminished strongly in the laccase SA treatments, though a lower peak was detected at 287 nm. This peak could be due to a SA not oxidized that remained in the effluents or/and SA degradation and/or oxidation products as well as the presence of dissolved lignin. On its side, AS control showed a peak at 300 nm also detected in AS solution (Fig. 5b). Furthermore, AS oxidation produced an increasing peak at 362 nm which was not detected in Laccase–AS samples, indicating AS grafting onto the pulp or AS degradation, although a signal below 330 nm was detected. Fig. 5c shows the PCA control spectrum exhibiting a high peak at 286 nm that corresponds to PCA reduced form. Laccase–PCA treatments exhibited a very low signal below 330 nm suggesting high grafting to pulps. The signal changes between the laccase–phenols treatments effluents

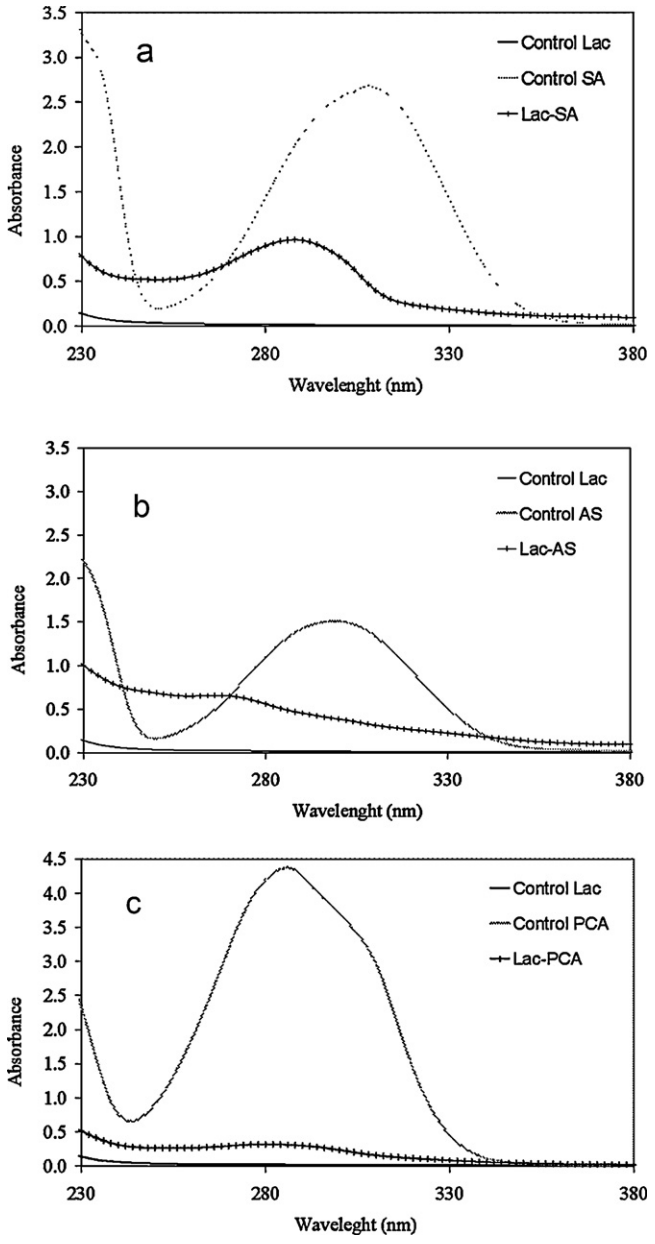

Fig. 5. Spectra of effluents from enzymatic treatments with SA (a), AS (b) or PCA (c). Control SA, AS or PCA: effluents from control phenols. Control Lac: control laccase. All spectra were carried out with 1:20 dilution of the effluents.

and the control–phenols would indicate a phenol grafting onto the pulp, as well as degradation and/or oxidation products, although an increased concentration of dissolved lignin cannot be excluded.

The inactivation of the laccase after the treatments was also investigated (Table 2). The control experiment without phenol resulted in 50% remaining activity. Treatments with AS and SA caused a laccase inactivation of 10% and 32%, respectively. In contrast, only 22% of the initial laccase activity was found after PCA treatments. This high laccase inactivation rate matched up with a high grafting rate caused by PCA. The loss of laccase activity caused by SA and AS would allow longer treatment time and maybe higher amount of grafted phenols onto the pulps, as well as the reutilization of the laccase.

Table 2
Laccase residual activity after the enzymatic treaments.

	Laccase	L–SA	L–AS	L–PCA
Residual activity (%)	50	68	90	22

4. Conclusions

In this study, a laccase was used to initiate the grafting of three different antimicrobial phenol structures onto unbleached flax fibres with a high cellulose content. Pulp and paper properties of the treated samples were analyzed to assess the tendency of the natural phenols to couple to fibres, suggesting that laccase lead to the crosslinking of these compounds. Furthermore, grafted pulps presented high antimicrobial activity against three bacteria analyzed and non-leaching of these phenolic compounds to the media; demonstrating the immobilization of the antimicrobial agents. This system can be seen as a valid method to create covalently bound bio-active papers with a new high added value: antimicrobial activity.

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