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Polymerization of lignosulfonates by the laccase-HBT (1-hydroxybenzotriazole) system improves dispersibility

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ABSTRACT

The ability of laccases from *Trametes villosa* (TvL), *Myceliophthora thermophila* (MtL), *Trametes hirsuta* (ThL) and *Bacillus subtilis* (BsL) to improve the dispersion properties of calcium lignosulfonates 398 in the presence of HBT as a mediator was investigated. Size exclusion chromatography showed an extensive increase in molecular weight of the samples incubated with TvL and ThL by 107% and 572% from 28400 Da after 17 h of incubation, respectively. Interestingly, FTIR spectroscopy, ^{13}C NMR and Py-GC/MS analysis of the treated samples suggested no substantial changes in the aromatic signal of the lignosulfonates, a good indication of the ability of TvL/ThL-HBT systems to limit their effect on functional groups without degrading the lignin backbone. Further, the enzymatic treatments led to a general increase in the dispersion properties, indeed a welcome development for its application in polymer blends.

1. Introduction

Lignin, the second most abundant polymer on Earth constituting 30% of non-fossil organic carbon, is currently under-utilized (Boerjan et al., 2003). Industrial lignins constitute the main by-products of the pulp and paper industry regarded as waste material, imposing disposal problems and their major applications have been limited to providing fuel for firing the pulping boilers (Mohan and Karthikeyan, 1997). Only approximately 2% of the lignins are used commercially (1 million tons/year of lignosulfonates and less than 100,000 tons/year of kraft lignins) (Gargulak and Lebo, 2000; Gosselink et al., 2004; Lora and Glasser, 2002). However, over the recent years there has been a renewed interest in using lignin as a renewable raw material. This is partly due to new stringent environmental waste management regulations together with the demand for replacement of oil based products with renewable materials and the new possibilities offered by emerging technologies. Consequently, investigations into increasing the application of lignin in existing and novel polymer blends for mortar, construction materials, adhesives, biodegradable plastics, polyurethane copolymers, paints, dye dispersants, in pesticides and printed circuit boards (Sena-Martins et al., 2008; Stewart, 2008; Lora and Glasser, 2002; Hüttermann et al., 2001; Kosbar et al., 2001), are increasing.

Nevertheless, massive exploitation of lignin is hampered by its huge physico-chemical heterogeneity owing to its inherent variety of aromatic units, inter-unit linkages, functional groups, and molecular size (Vázquez et al., 1999; Glasser and Sarkanen, 1989; Li et al., 1997). This is a result of both the heterogeneity of this plant polymer and its degradation during the pulping process resulting in a polydisperse material (Dence and Lin, 1992) which has high interfacial tension and lacks interfacial adhesion properties, making it difficult to achieve desired degree of dispersion in polymer blends (Cazacu et al., 2004). Most lignin polymer blends

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have been reported to be immiscible due to low entropy (Flory, 1953) indicating that the ability of lignin to mix strongly depends on its active properties (Ekeberg et al., 2006). For example, increasing lignin concentration in thermoplastics and rubber blends negatively affects the tensile force and melt flow index of the product (Alexy et al., 2000), while its polydispersity limits its addition between 5% and 10% the level of the resin weight in adhesive synthesis (Turunen et al., 2003). In short, the deterioration of the mechanical properties of all polymer blends where lignin has been used, is attributed to the poor adhesion and dispersion of the lignin particles which create defects that act as stress concentrators (El Mansouri and Salvado, 2006). Therefore, industrial applications of lignins require modifications to improve its dispersion properties among other physico-chemical characteristics.

In line with this demand, in this study, the effect of laccase-mediator treatment on dispersion properties of lignosulfonates was investigated and correlated to chemical changes of the polymer. Lignosulfonates are highly cross-linked anionic polymers in which the essentially hydrophobic backbone is rendered hydrophilic by substitution with sulfonate groups (Askvik et al., 2001). In the presence of mediators, laccases (benzenediol: oxygen oxidoreductases, EC.1.10.3.2) can also oxidize the non-phenolic moieties in lignin (Call and Mücke, 1997; Leonowicz et al., 2001) and this has been widely studied in the pulp and paper industry in relation to delignification (Chandra and Ragauskas, 2005; Balakshin et al., 2001; Elegir et al., 2005; Rochefort et al., 2004). Among the known mediators, 2,2′-azinobis-(3-ethylbenzthiazoline)-6-sulfonate (ABTS) and 1-hydroxybenzotriazole (HBT) are the most commonly used (Ibarra et al., 2006; Leonowicz et al., 2001; Baiocco et al., 2003; Call and Mücke, 1997) and effective. Although, a number of studies have demonstrated the ability of laccase to modify technical lignins (Sena-Martins et al., 2008; Milstein et al., 1990, 1994; Hernández Fernaud et al., 2006; Lund and Ragauskas 2001; Popp et al., 1991), detailed characterization of the resulting chemical changes is still lacking. Here, we relate for the first time the enzymatic improvement of the dispersion properties of lignosulfonates to the chemical changes of the polymer. Detailed analysis was carried out by employing a number of different complementary techniques among them fluorescence monitoring, different nuclear magnetic resonance (NMR) techniques, Fourier transform infrared (FTIR) spectroscopy, size exclusion chromatography and chemical analysis.

2. Methods

2.1. Materials

Calcium lignosulfonate samples were provided by Borregaard (Sarpsborg, Norway). Among the enzymes used, NS51002 – *Trametes villosa* laccase (TvL) and NS 51003 – *Myceliophthora thermophila* laccase (MtL) were supplied by Novozymes (Bagsvaerd, Denmark). *Trametes hirsuta* laccase (ThL) and *Bacillus* spore laccase (BsL) were produced as previously described (Almansa et al., 2004; Held et al., 2005). All the other reagents used were of analytical grade purchased either from Sigma–Aldrich or Merck.

2.2. Laccase activity assay

The activity of laccase was determined spectrophotometrically by monitoring the oxidation of 2,2′-azinobis-(3-ethylbenzthiazoline)-6-sulfonate (ABTS) to its cation radical (ε_{436} = 29,300 M^{-1} cm^{-1}) as substrate at 436 nm in 50 mM sodium succinate buffer at pH 4.5 and 30 °C using quartz cuvette of path length 10 mm (Nugroho Prasetyo et al., 2009) and activity expressed in nano katals (nkat) corresponding to 1 nmol of substrate converted per second.

2.3. Polymerization of calcium lignosulfonates

Calcium lignosulfonate samples were incubated with each of the above laccases separately. Briefly, 1 g of the calcium lignosulfonate sample was dissolved in 50 ml double distilled water in 250 ml Erlenmeyer flasks and 1-hydroxybenzotriazole (HBT) or ABTS (1 mM final concentration) added to the reaction mixture. The reaction was started by adding a 30 nkat ml^{-1} laccase activity as determined at the optimum pH value of the individual experiments. Samples were then incubated at 30 °C while shaking at 150 rpm. Samples were withdrawn at regular intervals and fluorescence intensity measured instantly while the other part of the sample was immediately frozen by immersing in liquid nitrogen. The frozen samples were lyophilized using the Labconco Freeze Dry System / FreeZone© 4.5 Liter Benchtop Model 77500 (Vienna, Austria). The freeze drier was operated at a temperature of −48 °C and at a vacuum pressure of 3×10^{-4} mbar. These freeze dried samples were stored in the dark in sealed tubes at 4 °C until further analysis.

2.4. Fluorescence intensity measurements

During enzymatic polymerization fluorescence intensity was monitored (Ex 355 nm/Em 400 nm) at defined time intervals using TECAN Infinite M200 plate reader (Tecan Austria GmbH, Grödig, Austria). A lignin sample of 100 µl was added to a solution of 2-methoxyethanol (Thomson et al., 2005) and water (2:1 v/v) and then thoroughly mixed before measuring.

2.5. FTIR analysis

FTIR spectra were obtained on a Perkin–Elmer Spectrum 2000 instrument by the attenuated total reflectance (ATR) technique. Spectra were recorded in the 4000–600 cm^{-1} range with 16 scans at a resolution of 4.0 cm^{-1} and an interval of 1.0 cm^{-1}. Sulfonate groups were also detected by FTIR at 1145 and 647 cm^{-1}.

2.6. Size exclusion chromatography

All lignosulfonates, being highly soluble in water, were analyzed using three TSK-gel columns (3000 PW, 4000 PW, 3000 PW) coupled in series with 0.1 M sodium hydroxide as the eluant. Flow rate was 1 mL/min and detection was done by UV at 280 nm.

2.7. NMR analysis

Solution NMR spectra, including ^{1}H NMR, ^{13}C NMR and heteronuclear single quantum correlation (HSQC) 2D-NMR spectra were recorded on 40 mg of lignosulfonate dissolved in 0.75 mL of DMSO-d_6 using a Bruker AVANCE 500 MHz as previously described (Ibarra et al., 2006). A semiquantitative analysis of the HSQC cross-signal intensities was performed (Heikkinen et al., 2003; Zhang and Gellerstedt, 2007) including separate volume integrations and comparison in each of the regions of the spectrum, which contain cross-signals of chemically analogous carbon–proton pairs. Cross-polarization magic-angle spinning (CPMAS) ^{13}C NMR spectra of solid lignosulfonate samples were recorded for 9 h on a Bruker AVANCE DSX 300 using the standard pulse sequence, a time domain of 4 K, a spectral width of 41,666 Hz, a contact time of 2 ms, and an interpulse delay of 4 s. Signals were assigned by comparison with the literature (Bardet et al., 2006; Capanema et al., 2004; Lebo et al., 2008; Liitiä et al., 2003; Lundquist, 1981; Lutnaes et al., 2008; Martínez et al., 1999; Ralph et al., 1999; Ralph et al., 2004; Robert, 1992).

2.8. Photon-correlation spectroscopy (PCS) and Zeta-potential measurements

The surface charge of the oxidized solutions was measured in terms of Zeta-potential in a Zetasizer Nano Series (Malvern Instruments Inc., Worcester, UK). This method measures how fast a particle moves in a liquid when an electrical field is applied i.e. its velocity. The aggregation behaviour of treated lignosulfonate particles in solution was therefore performed by determining its electrophoretic mobility. The size distribution of the oxidized samples was also measured by photon-correlation spectroscopy.

2.9. Dispersion properties

The Turbiscan MA 2000 from Sci-Tec Inc (Sandy Hook, USA) was used to assess the stability of suspensions (Mengual et al., 1999). Different enzyme-modified lignosulfonates are rated after their ability to stabilise a standard suspension. A similar procedure has been used to follow the sedimentation of suspensions (Balastre et al., 2002) and creaming of emulsions (Roland et al., 2003). The Turbiscan technology consists in measuring backscattering and transmission intensities versus the sample height in order to detect particle size change (coalescence, flocculation) and phase separation (sedimentation, creaming).

2.10. Py-GC/MS

The pyrolysis of the lignosulfonates (approximately 100 μg) was performed in duplicate with a model 2020 micro-furnace pyrolyzer (Frontier Laboratories Ltd., Yoriyama, Japan) directly connected to an Agilent 6890 GC/MS system equipped with a 30 m × 0.25 mm i.d., 0.25 μm HP 5MS fused silica capillary column. The detector consisted of an Agilent 5973 mass selective detector (EI at 70 eV). The pyrolysis was performed at 500 °C. The GC/MS conditions were as follows: the oven temperature was held at 50 °C for 1 min and then increased up to 100 °C at 30 °C/min, from 100 to 300 °C at 10 °C/min and isothermal at 300 °C for 10 min. The carrier gas used was helium with a controlled flow of 1 ml/min. The compounds were identified by comparing the mass spectra obtained with those of the Wiley and NIST computer libraries and that reported in the literature (Faix et al., 1990; Ralph and Hatfield, 1991). Sulfonate groups were also detected by Py-GC/MS.

3. Results and discussion

3.1. Fluorescence intensity

Fluorescence spectroscopy was used as a sensitive and simple analytical tool to optimize modification of calcium lignosulfonates with different laccases at different pHs. A similar trend was observed in fluorescence changes (decrease in fluorescence) when lignosulfonates were treated with laccases in the presence of either HBT or ABTS (Fig. 1a and b). TvL and ThL were effective in reducing fluorescence under acidic conditions (pH 4.0 and 4.5) while the BsL and the MTL were more effective at pH above 6 (Fig. 1a and b). The MTL performed slightly better than the BsL in the presence of both ABTS or HBT as mediators (Fig. 1a and b). Although the decrease in fluorescence measured for TvL and ThL treated lignin at pH 4.0 and 4.5 was almost similar in the presence of ABTS or HBT, samples incubated with the latter performed slightly better. Similarly, at the same pH (4.5), the ThL reduced fluorescence was 2058 AU in ABTS supplemented samples and 2828 AU in HBT incubated samples. Fluorescence is an intrinsic property of lignin attributed to conjugated carbonyl, biphenyl, phenylcoumarins and stilbene groups (Albinsson et al., 1999; Lundquist et al., 1978). Therefore, the observed decrease in fluorescence intensity in this study upon incubation with laccases indicated modification of these functional groups present in lignin. The destruction or modification of biphenyl groups, for example, has been shown to affect fluorescence intensity (Castellan et al., 1992). Here, the decrease in fluorescence was used as an indication of the extent of modification of the calcium lignosulfonates. The observed different modifications by the different enzymes maybe attributed to the different redox potential of the laccases. For example TvL and ThL are high redox potential laccases with redox potentials of approximately +790 mV (Rebrikov et al., 2006; Tadesse et al., 2008) while MTL and BsL are low redox potential laccases (460 mV and 455 mV, respectively) (Tadesse et al., 2008; Melo et al., 2007).

3.2. Gel permeation chromatography

The calcium lignosulfonate samples incubated with TvL and ThL underwent extensive polymerization. The Mw increased by 74% after 17 h of incubation with ThL and by 370% in TvL-treated samples (Table 1) supplemented with 0.5 mM HBT. Polymerization as a central feature during laccase oxidation of lignin moieties has also been reported by previous authors (Ishihara and Miyazaki, 1972; Hüttermann et al., 1980; Elegir et al., 2007). As indicated earlier by Karhunen et al. 1990 a and b, the radicals generated by laccases underwent resonance stabilisation forming different mesomeric forms that coupled in many possibilities forming inter-unit linkages which include β-O-4, β-5, 5-5, β-β, 5-O-4 resulting in polymers of different sizes. In this study, the increase in Mw was accompanied by a decrease in phenolic groups and carboxylic groups. Several authors have observed a similar decrease in phenolic groups (Shleev et al., 2006, Grönqvist et al., 2005, Rittstieg et al., 2002; Buchert et al., 2002). This indicates that the laccase-HBT oxidized phenolic substituents and generated phenoxy radicals which underwent coupling reactions leading to the observed polymerization. The content of carboxylic groups in lignin decreased by 2% for ThL and by 2.4% for TvL after 17 h of incubation, respectively (Table 1).

The effect of increasing incubation time and doubling HBT concentration was investigated in subsequent experiments (Table 2). Increasing incubation time to 83 h and HBT concentration to 1 mM (final concentration) resulted in 107% and 572% increase in Mw of ThL and TvL incubated calcium lignosulfonate samples, respectively. The changes in Mw are clearly visible in size exclusion chromatography (Fig. 2 ThL, TvL). The Mw of TvL incubated samples clearly changed resulting in a narrower Mw band, comparing chromatograms of samples incubated for 0 h and 83 h. Although there was a clear modification of lignosulfonates in ThL samples, the modifications are different from those obtained in TvL samples. Previous researchers have also reported polymerization of lignosulfonates by laccases (Leonowicz et al., 1985; Hatakka et al., 1996; Bae and Kim, 1996), although the use of ABTS as a laccase mediator was repeatedly resulted in depolymerizaion of lignin (Hernández Fernaud et al., 2006; Bourbonnais et al., 1995) and was even shown to be incorporated in polymerization products (Rittstieg et al., 2002).

An increase in incubation time and HBT concentration lead only to a marginal further decrease in phenolic and carboxylic groups. The phenolic content decreased from 1.4 mmol g^{-1} in the untreated lignosulfonate sample to 0.85 mmol g^{-1} after 83 h incubation with ThL and to 0.45 mmol g^{-1} after incubation with TvL (Table 2). There was a small decrease in the organic sulfur content and a slight increase in inorganic sulfur content in both samples upon enzyme treatment (Table 2). This loss of sulfonic acid groups might be responsible for the in the Zeta-potential. This parameter increased from 0.65 to 2.4 mV in ThL-treated samples and from 0.6

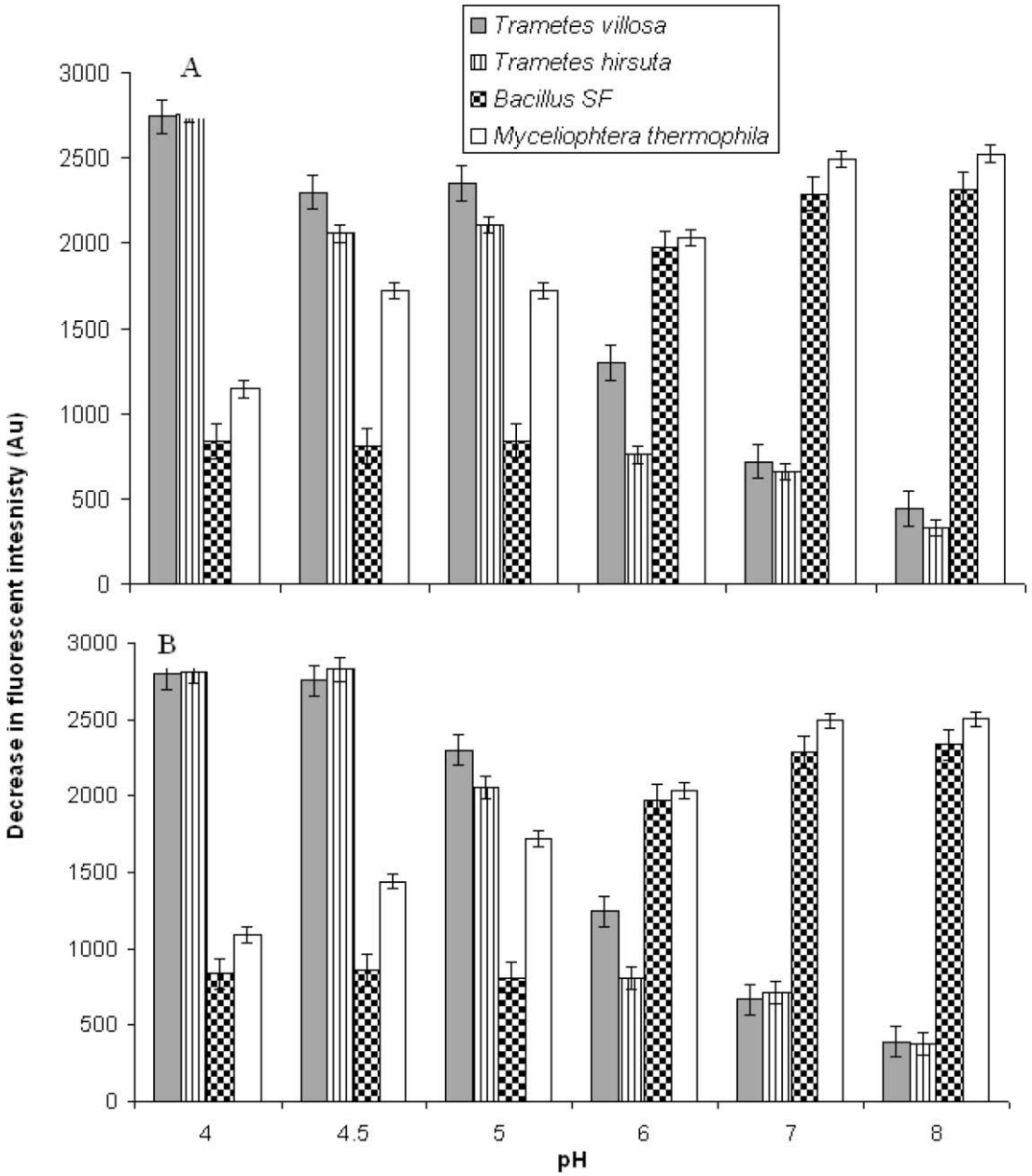

Fig. 1. Fluorescence decrease of lignosulfonate after incubation with different laccases in the presence of HBT (A) and ABTS (B) at different pHs after 83 h of incubation. Data is an average of 3 independent replicates ± standard deviation.

Table 1
Changes in molecular weight (Mw) and functional group content after treatment of lignosulfonates with different laccases in the presence of HBT.

Laccase	Time (h)	Mw	Mn	Pdi	Ar–OH (%)	COOH (%)
Trametes hirsuta	0	30800	1900	16.21	1.9	7.5
	0.5	29700	1900	15.63	–	–
	17	53800	4300	12.51	1.4	5.6
Trametes villosa	0	29400	1800	16.33	1.9	7.5
	0.5	40400	2950	13.69	1.5	5.6
	17	140100	8250	16.98	1.0	5.1

Mn-number average molecular weight; Pdi-polydispersity.

to 2.2 mV in TvL-treated samples. The particle size [Z-average (d.nm)] increased from 369.4 to 942.8 in ThL and 311.0 to 421.2 in TvL samples indicating aggregation of particles. Unlike TvL-treated samples for 83 h, ThL samples became partly insoluble.

3.3. FTIR analysis

FTIR spectroscopy at mid-infrared region (4000–600 cm^{-1}) was applied to monitor structural changes occurring during incubation of the calcium lignosulfonate with the ThL and TvL (Fig. 2 ThL and TvL). In the FTIR spectra bands at 1595 cm^{-1} and 1520 cm^{-1} suggest aromatic ring vibrations and at 1033 cm^{-1}, aromatic C–H in-plane deformation (Fig. 2 ThL and TvL). Sulfonate groups are shown by bands at around 1145 cm^{-1} (asymmetric and symmetric $–SO_2–$ vibrations) and one band at around 647 cm^{-1} (from S–O structure). Further the stretching vibrations of alcoholic and phenolic OH groups involved in hydrogen bonds were detected between 3500–3200 cm^{-1}. Further analysis of the region between 1645

Table 2
Changes during incubation of *Trametes hirsuta* and *Trametes villosa* laccase-HBT systems with lignosulfonates.

Laccase	Time (h)	Mw	Mn	Inorganic S (%)	Organic S (%)	Ar–OH (%)	COOH (%)
T. hirsuta							
	0	28400	2650	0.8	5.4	1.8	7.4
	17	43100	3800	0.8	5.0	1.4	6.1
	83	58800	5250	1.0	5.1	1.1	5.4
T. villosa							
	0	28400	2650	0.9	5.2	1.9	7.4
	17	142400	9200	1.0	4.9	1.1	5.2
	83	191100	10500	1.0	5.1	1.0	5.0

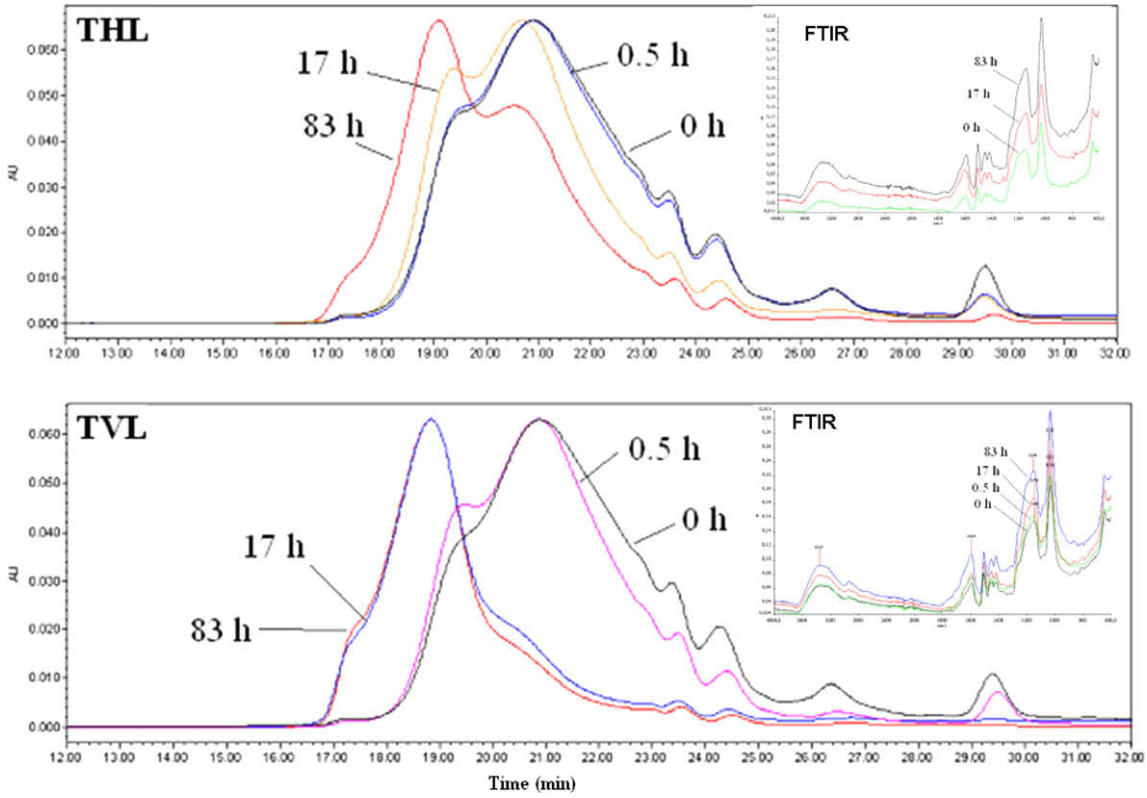

Fig. 2. Polymerization of lignosulfonates by *Trametes hirsuta* laccase (THL) and *Trametes villosa* laccase (TVL) in the presence of HBT after 0, 0.5, 17 and 83 h of incubation as monitored by size exclusion chromatography. Inserts show results of FTIR analysis of the lignosulfonates.

and 1760 cm^{-1}, reveals no noticeable changes in carbonyl or carboxylic acid group present or generated by the treatments in the structures. As a general conclusion, according to the FTIR data, no substantial changes were introduced in the calcium lignosulfonates samples during incubation with both TvL and ThL.

3.4. Dispersion properties

Indeed, the dispersant properties of the enzyme treated lignosulfonate were significantly improved as shown in Fig. 3. A low value of delta backscattering indicates a more stable suspension. The increased Mw and reactivity of the enzyme treated lignosulfonates could have enhanced its miscibility. Previously, prepared lignosulfonates by phenolation were shown to increase dispersibility by over 30% for gypsum paste than the commercial lignosulfonate (Matsushita and Yasuda, 2005). They attributed the improvement in dispersion properties to increased Mw and sulfur contents of the preparations. This is inline with the observation in this study where an increase in Mw and reactivity were noted and a very marginal loss of sulfur. The fact that laccase did not remove sulfur is very encouraging because sulfonate groups are important for imparting solubility properties to lignins.

For example, sulfonation leads to water-soluble anionic polymers and high-dispersibility gypsum paste (Matsushita et al., 2008; Li et al., 2009). The dispersing efficiency increased as the surface tension decreased, suggesting that the fluidity of the gypsum paste increased with the polymer adsorption on the gypsum particle surface (Matsushita et al., 2008). This phenomenon may also be attributed to the observed increase in dispersion properties in this study.

3.5. NMR analysis of enzymatically-modified lignosulfonates

In contrast to FTIR data, the HSQC NMR analysis (Fig. 4) showed decreases in the intensities of cross-signals in the three main regions of the lignosulfonate spectrum, corresponding to aromatic

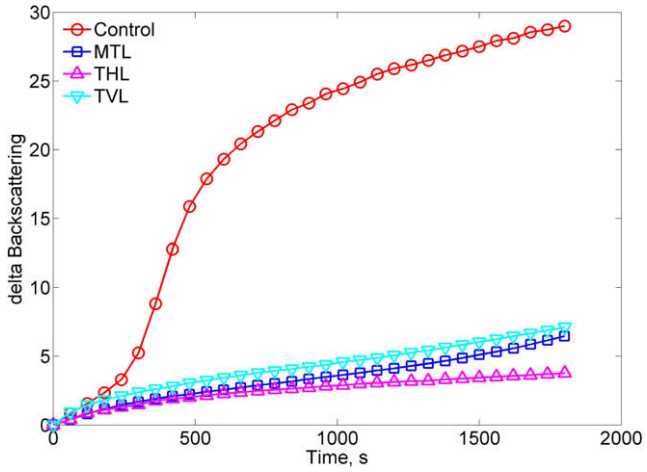

Fig. 3. Dispersibility of lignosulfonates polymerized with laccases from *Myceliophthera thermophilia*, *Trametes hirsuta* and *Trametes villosa* (MTL, THL and TVL) in the presence of HBT, based on multiple light scattering (BS - Backscattered and T - transmitted light compared to a control and to chemically modified lignosulfonate after 17 h incubation.

(δ_H/δ_C 5.5–8/105–140 ppm), aliphatic oxygenated (δ_H/δ_C 2.5–5.5/50–105 ppm) and aliphatic non-oxygenated (δ_H/δ_C 0–3/0–50 ppm) ^1H–^{13}C correlations, albeit with very different decrease intensities in each of them. The HSQC spectra were obtained from the same amount of sample (40 mg in 0.75 mL of DMSOd_6) and normalized to the residual DMSO cross-signal (δ_H/δ_C 2.5/40 ppm). The above decreases were observed in both the samples treated with TvL (Fig. 4a and b) and ThL (Fig. 4c and d).

After 83 h incubation the aromatic cross-signals found in the HSQC spectrum of the control (0-h) lignosulfonate, which corresponded to H_2–C_2, H_5–C_5 and H_6–C_6 correlations (with δ_H/δ_C 6.91/114.7, 6.65/114.9 and 6.75/122.9 ppm, respectively) completely disappeared in the TvL-treated samples. On the other hand, they were still partially visible in the ThL-treated samples although with very strongly reduced intensities. In addition, the methoxyl cross-signal (with δ_H/δ_C 3.72/56.2 ppm) significantly decreased in both the TvL and ThL-treated samples, together with those of the most abundant β-O-4′ linked α-sulfonated side-chains including H_β–C_β correlation (with δ_H/δ_C 4.93/80.1 ppm), while polysaccharide and other oxygenated aliphatic cross-signals remained practically unaffected by the laccase-mediator treatment. Finally, only a few and small cross-signals of non-oxygenated aliphatic correlations were observed in the lignosulfonate spectra including that from the methyl of the acetate buffer used for the enzymatic treatment (with δ_H/δ_C 1.1/19 ppm).

The disappearance of the aromatic ^1H–^{13}C correlation signals in the TvL treated lignosulfonate after 83 h of incubation, as shown by HSQC 2D-NMR, was initially unexpected. Therefore this sample and its (0-time) control were further analyzed by ^1H NMR, and by both liquid and solid state ^{13}C NMR (Fig. 5). The latter was used to solve eventual solubility problems due to enzymatic polymerization, although no DMSO insoluble material was observed in the NMR tubes.

The loss of aromatic cross-signals in the HSQC spectra obtained after the enzymatic treatment (Fig. 5a) was due to deprotonation of the lignin benzenic rings, as revealed by the 1D-NMR spectra. In this way, no aromatic proton signals were found in the 1H NMR spectrum of the lignosulfonate treated with TvL for 83 h (Fig. 5b) while strong signals of aromatic carbons appeared in the ^{13}C NMR spectra obtained either in solution (Fig. 5c) or in the solid state using the CPMAS technique (Fig. 5d). This suggests formation of new ether and C–C aryl–aryl or aryl–alkyl linkages as a result of the enzymatic attack on the lignosulfonate aromatic nuclei causing the strong polymerization observed by SEC. After initial condensation reactions between the phenoxy radicals formed by the action of the enzyme on the phenolic units present in the initial lignosul-

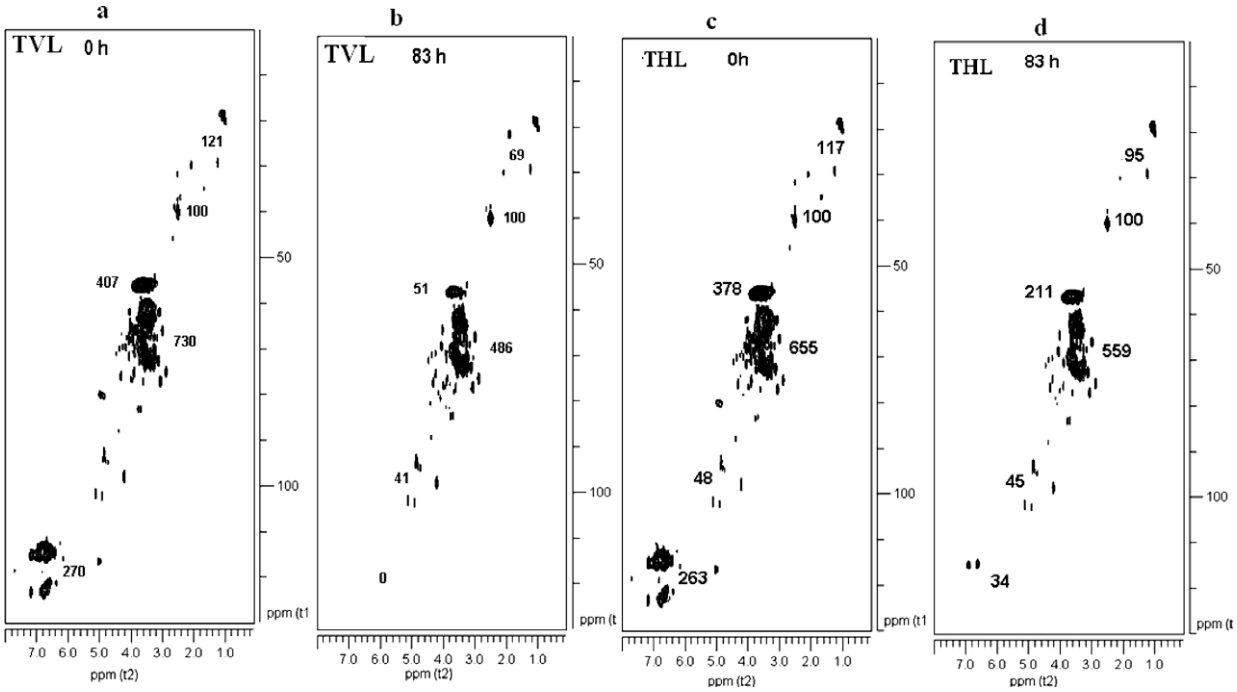

Fig. 4. HSQC 2D-NMR analysis of lignosulfonate modification by *Trametes hirsuta* laccase (THL) (right) and *Trametes villosa* laccase (TVL) (left) in the presence of HBT after 0 h (a and c, respectively) and 83 h (b and d, respectively) of incubation. The integrals of the main groups of ^1H–^{13}C correlation signals (from bottom to top: aromatic signals, anomeric polysaccharide signals, different oxygenated aliphatic signals, methoxyl signal, and non-oxygenated aliphatic signals) are indicated, referred to the residual DMSO signal (as 100%).

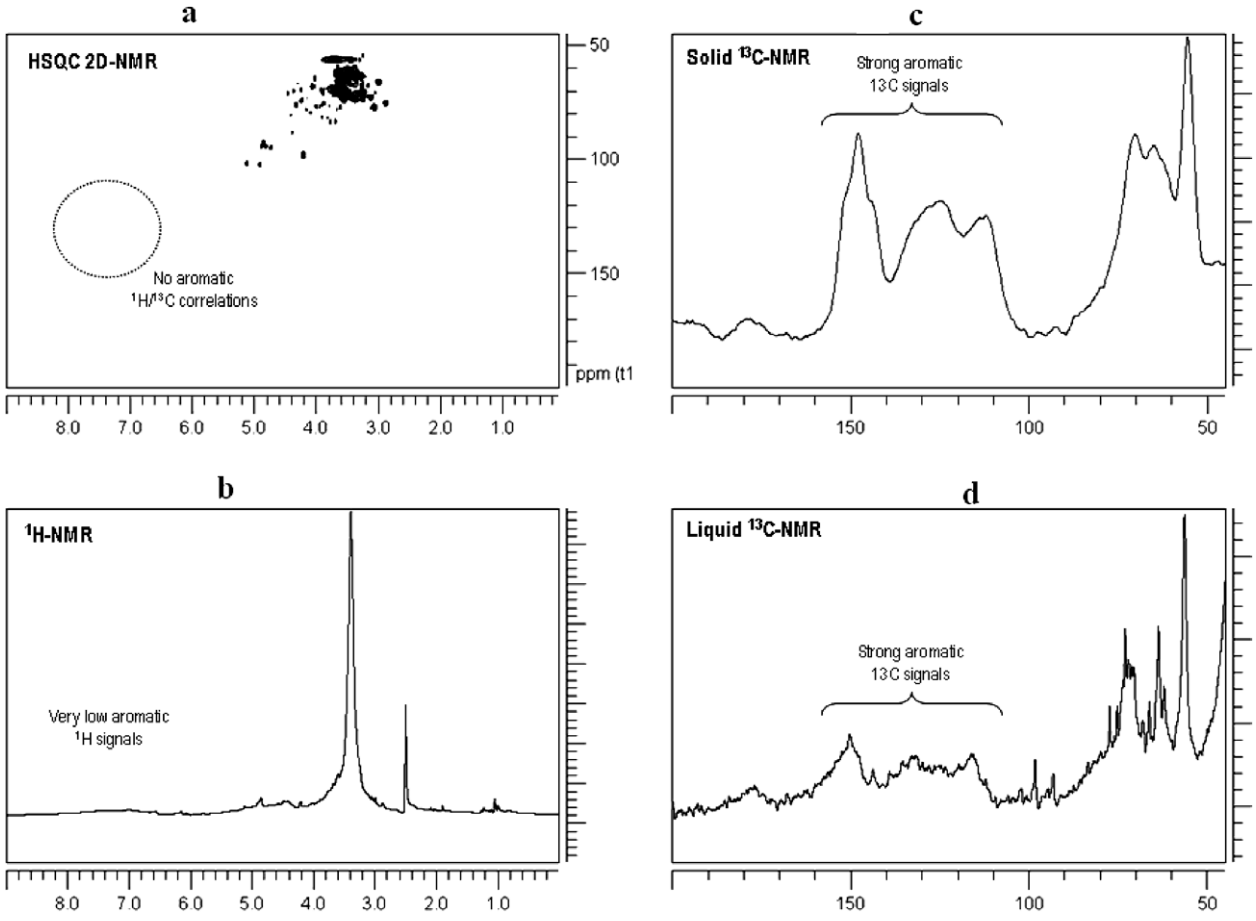

Fig. 5. Comparison of aromatic signals in (a) HSQC 2D-NMR spectrum (^{1}H–^{13}C correlation), (b) ^{1}H NMR spectrum, (c) ^{13}C NMR spectrum, and (d) CPMAS ^{13}C NMR spectrum of spruce lignosulfonate after 83 h incubation with *Trametes villosa* laccase (TVL)-HBT system.

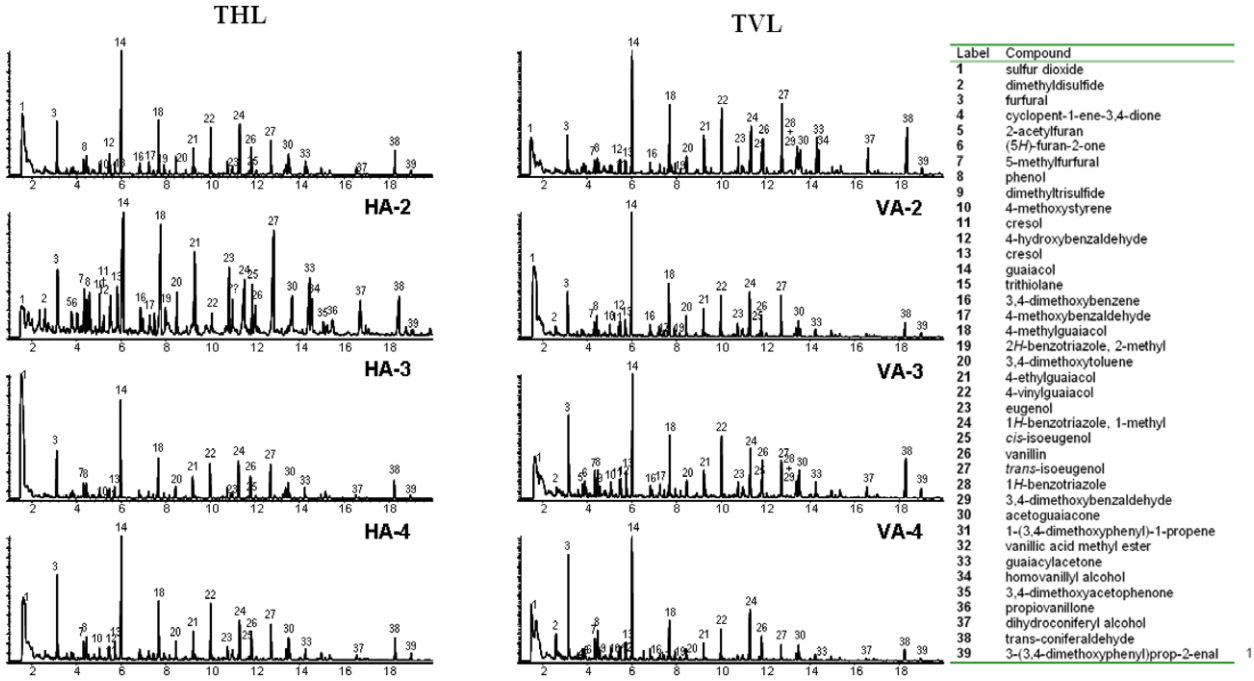

Fig. 6. Py-GC/MS of calcium lignosulfonate during incubation with *Trametes hirsuta* laccase (THL) and *Trametes villosa* laccase (TVL) in the presence of HBT.

fonate sample, the high redox-potential laccase-HBT system most probably cause additional oxidative attack on the non-phenolic lignin nuclei resulting in additional deprotonation and condensation reactions.

3.6. Py-GC/MS of enzymatically-modified lignosulfonates

The chemical composition of the lignosulfonates was analyzed by Py-GC/MS (Fig. 6). The compounds released arise mainly from lignin moieties with minor amounts of carbohydrates and sulfur compounds being present. Among the lignin derived compounds, only guaiacyl derivatives were detected, as corresponds to a lignosulfonate from softwood. The most interesting observation obtained from Py-GC/MS pyrograms was the decrease in the intensity of the lignin peaks (4-methylguaiacol, 4-ethylguaiacol, guaiacylacetone, 4-vinylguaiacol, homovanillylalcohol, eugenol, *cis*- and *trans*-isoeugenol, dihyroconiferyl alcohol and *trans*-coniferaldehyde), in both TvL and ThL-treated samples (Fig. 6). The decrease in the different lignin moieties was accompanied by a concomitant increase in sulfur dioxide and dimethyldisulfide. Lignin markers were very much present as detected by Py-GC/MS in all the samples during the whole incubation period despite a slight decrease at longer incubation periods (Fig. 6).

The FTIR and ^{13}C NMR spectra together with Py-GC/MS chromatograms suggesting no substantial structural changes in the calcium lignosulfonate aromatic structure are a good indication of the ability of TvL and ThL to limit their effect to effective cross-linking, without degrading the lignin backbone. These data are in line with reports by Martinnen et al. (2008) who also did not observe substantial differences in the aromatic signals after laccase treatment of lignin. The seemingly contradictory aromatic data by HSQC 2D-NMR, showing the disappearance of the aromatic cross-signals, may be due to the strong polymerization produced by the laccase-HBT treatment. This resulted in new carbon–carbon and carbon–oxygen linkages leading to condensation and/or modification reactions in such a way that most lignin aromatic carbons were unprotonated, the remaining ones being below the HSQC detection level. Some problems associated with 2D-NMR spectroscopy are related to the short T_1 and T_2 relaxation times (Garver et al., 1996 and Zhang and Gellerstedt, 2007 suggested the degree of polymerization as one of the factors affecting short T_2 values in HSQC NMR. Our observation seems to also vindicate earlier comments by Capanema et al. (2004) who emphasized a need for caution when analyzing 2D-NMR spectra of lignin data that must be complemented with 1D (^1H and ^{13}C NMR) spectra.

4. Conclusions

Size exclusion chromatography analysis of ThL-HBT and TvL-HBT treated lignosulfonate resulted in extensive polymerization leading to 107% and 572% increase in Mw from 28 400 Da, respectively. New ether and C–C aryl–aryl or aryl–alkyl linkages were detected as causing the strong polymerization as confirmed by FTIR, ^{13}C NMR spectra and Py-GC/MS chromatograms. Nevertheless, the treatment did not affect the lignin backbone, a good indication of the ability of TvL and ThL-HBT systems to limit their effect to the functional groups only. As a result, the dispersant properties of the enzyme treated lignosulfonate increased significantly.

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