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

Endry Nugroho Prasetyo, Tukayi Kudanga, Lars Østergaard, Jorge Rencore, Ana Gutiérrez, José C. del Río, J. Ignacio Santos, Lidia Nieto, Jesús Jiménez-Barbero, Angel T. Martínez, Jiebing Li, Göran Gellerstedt, Stéphane Lepiré, Carla Silva, Su Yeon Kim, Artur Cavaco-Paulo, Bente Seljebakken Klausen, Bjart Frode Lutnaes, Gibson S. Nyanhongo, Georg M. Guebitz

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) (Gargulak and Lebo, 2000; 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 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; Hütttermann 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. To be able to utilize lignin in polymer blends, it is essential to improve its incorporation and dispersion in the blends. 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; Hütttermann et al., 2001; Kosbar et al., 2001), are increasing.

The ability of laccases from Trametes villosa (TvL), Myceliophthora thermophila (MtL), 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, 13C 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/MtL-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.
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% 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 (Asikvik et al., 2001). In the presence of mediators, laccases (benzenediol: oxygen oxidoreductases, EC.1.10.3.2) can also oxidize the non-pheno
eric moieties in lignin (Call and Mücke, 1997; Leonowicz et al., 2004; Balakshin et al., 2001; Elegir et al., 2005; Rochefort et al., 2001). In the presence of mediators, laccases (benzenediol: oxygen oxidoreductases, EC.1.10.3.2) can also oxidize the non-pheno
eric moieties in lignin (Call and Mücke, 1997; Leonowicz et al., 2004; Balakshin et al., 2001; Elegir et al., 2005; Rochefort et al., 2004). Among the known mediators, 2,2’-azinobis-(3-ethylbenz
thiazoline)-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 (Almanza et al., 1999; 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 (ε450 = 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 nmoI 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 lignino
sulfonate 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 1H NMR, 13C NMR and heteronuclear single quantum correlation (HSQC) 2D-NMR spectra were recorded on 40 mg of lignosulfonate dissolved in 0.75 mL of DMSO-d6 using a Bruker AVANCE 500 MHz as previously described (Ibarra et al., 2006). A semiquantitative analysis of the HSQC cross

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., Yoriiyama, 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 even shown to be incorporated in polymerization products (Rittsathig 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
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 –SO₂– 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>
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<th>Time (h)</th>
<th>Mw</th>
<th>Mn</th>
<th>Pdi</th>
<th>Ar–OH (%)</th>
<th>COOH (%)</th>
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<td>8250</td>
<td>16.98</td>
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Mn-number average molecular weight; Pdi-polydispersity.

Table 1

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

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.
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

<table>
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<th>Mw</th>
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<th>Organic S (%)</th>
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(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–13C 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 DMSO-d6) 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 H2–C2, H5–C5 and H6–C6 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 H2–C2 correlation (with δ_H/δ_C 4.93/80.1 ppm), 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–13C 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 13C 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 13C 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-
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.

Fig. 5. Comparison of aromatic signals in (a) HSQC 2D-NMR spectrum (1H–13C correlation), (b) 1H NMR spectrum, (c) 13C NMR spectrum, and (d) CPMAS 13C 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.
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 pyromgrams was the decrease in the intensity of the lignin peaks (4-methylguaiacol, 4-ethylguaiacol, guaiacylacetone, 4-vinylguaiacol, homovanillic alcohol, eugenol, cis- and trans-isoeugenol, dihydroconiferyl alcohol and trans-coniferylaldehyde), 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 dimethylsulfide. 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 13C 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 discredit earlier comments by Cekapana et al. (2004) who emphasized a need for caution when analyzing 2D-NMR spectra of lignin data that must be complemented with 1D ($^1$H 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, 13C 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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