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Action of fungal laccases on lignin model compounds in organic solvents

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ABSTRACT

The stability and reactivity of five different thermostable fungal laccases from the species *Trametes hirsuta*, *Melanocarpus albomyces*, *Thielavia arenaria* (two laccases) and *Chaetomium thermophilum* were investigated in the presence of organic solvents. Oxidations of small organic phenolic compounds, matairesinol and 7-hydroxymatairesinol lignans, as well as synthetic lignin dehydrogenation polymer DHP in aqueous solutions of ethanol and propylene glycol solvents were investigated using analysis of oxidation rates, high performance liquid chromatography and size-exclusion chromatography. The laccases showed variability in their solvent tolerance. The redox potential of the laccases appeared not to be the main factor determining the efficiency of the polymerization reactions of complex phenolic model compounds in aqueous organic solutions. Nuclear magnetic resonance spectroscopic analysis of laccase treated DHP in 50% propylene glycol indicated that the formation of new biphenylic 5–5′ structures was favored in laccase-catalyzed radical coupling reactions over the other possible reactions through the phenolic groups forming new 5—O—4 ether bonds. The polymerization reactions took place even at high concentrations of solvents, which already inhibited the enzyme activity, encouraging enzymatic upgrading of lignin in organic solvents to be studied further. Thus, it was confirmed that thermostable laccases are potential enzymes for various industrial applications where organic solvents are required for the reaction systems.

1. Introduction

Laccases are an integral component of fungal metabolism due to the broad variety of oxidative reactions they catalyze. Laccase is an important component of the lignin-degrading enzyme system in wood-rotting fungi, such as *Trametes hirsuta*. However, it is an equally important enzyme in several other parts of fungal metabolism. Laccases function in multiple roles, including polymerization of phenolics and synthesis of pigments, as well as detoxification of various compounds [1]. In all cases, cross-linking of monomeric phenolics is initiated by laccase oxidation yielding polymers *via* radical intermediates. Phenolic substrates are oxidized to phenoxy radicals, which can spontaneously polymerize *via* radical coupling. Alternatively, depending on reaction conditions, the radicals can be rearranged leading to formation of quinones through disproportionation including *e.g.* Cα oxidation or cleavage of aromatic ring, alkyl–aryl or Cα—Cβ bond. In addition, the low substrate specificity of laccases, acting on diphenols, substituted phenols, diamines and even on inorganic substrates [1,2], has made it feasible to exploit laccases also in different industrial applications as reviewed by [3].

Laccases are copper-containing enzymes, in which the T1 copper site determines the redox potential of the enzyme. The redox potential of fungal laccases varies between 450 and 790 mV [1]. The catalytic efficiency depends on the redox potential of the T1 copper, but the steric hindrance and conformation of the substrate may further affect the oxidation capacity [4–6]. Oxidation of bulky substrates, such as the lignin macromolecule, is generally assumed to be restricted by the steric hindrance of phenolic moieties within lignin to become oxidized in the active site of the laccase, and consequently the concept of different mediators has been suggested [7]. These easily oxidizable low molecular weight compounds may mediate the oxidation of substrates, such as lignin, not accessible to the active site, *via* radical transfer reactions [8,9], and extend the oxidation even to non-phenolic lignin moieties, which are resistant to oxidation by laccase alone [10]. Laccases also utilize lignin degradation products as mediators in further oxidation reactions of lignin [9,11,12].

Lignin and other hydrophobic polymers are not soluble in aqueous solutions, a characteristic that restricts the reactivity of solid substrates with laccase. Recent studies with synthetic DHP lignin indicated a clear relationship between lignin solubility and

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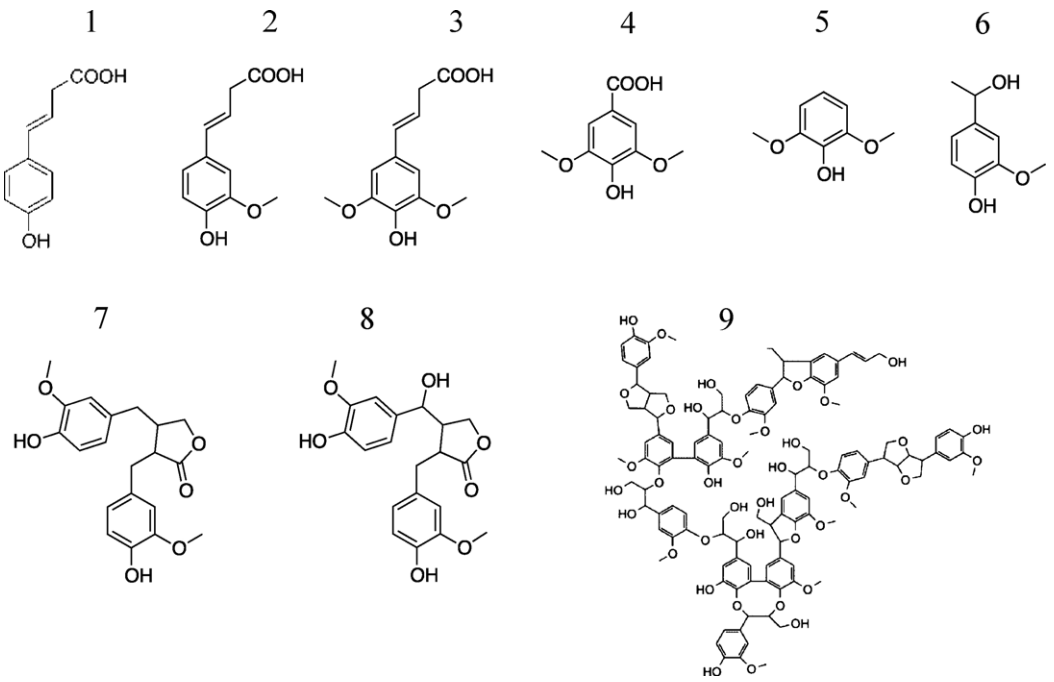

Fig. 1. Structures of phenolic laccase substrates. *p*-Coumaric (1), ferulic acid (2), sinapic acid (3), syringic acid (4), 2,6-dimethoxyphenol (5), apocynol (6), hydroxymatairesinol (7), matairesinol (8) and DHP (9). The redox potentials of the compounds are shown in Table 2.

reactivity [13]. The solubility of technical lignins at the pH optimum of the laccase seemed also to restrict the extent of enzymatic modifications [13,14]. In some cases, precipitation of the polymerized product may limit the further oxidative polymerization [15]. To overcome these problems, organic water-miscible solvents to increase the solubility of the substrate can be exploited. An increasing content of organic solvent in the reaction mixture, however, inactivates laccase gradually [13], although the activity loss may be partially compensated by the increased reactivity of the substrate.

Thus, in this study phenolic acids, 2,6-dimethoxyphenol, 4-(1-hydroxyethyl)-2-methoxyphenol (apocynol), synthetic lignin dehydrogenation polymer (DHP) as well as two softwood-derived lignans, which possess interesting properties as medical and nutritional substances [16–18], were used as substrates for five laccases from various fungal origins. Laccases differed in their redox potential and pH optimum. The rate of oxidation of substrates by laccases and enzyme stability was compared in the presence of organic solvents. The oxidation of substrates was examined by oxygen consumption method, high performance liquid chromatography (HPLC) and size-exclusion chromatography (SEC). The polymerization products of DHP in propylene glycol and ethanol were further investigated by nuclear magnetic resonance (NMR) spectroscopy.

2. Materials and methods

2.1. Chemicals

Low molecular weight organic molecules syringic acid (Fig. 1, (4)) (Fluka, no: 86230), *p*-coumaric acid (1) (Sigma, no: C-9008), ferulic acid (2) (Fluka, no: 46280), sinapic acid (3) (Bruker), and 2,6-dimethoxyphenol (2,6-DMP) (5) (Aldrich) were used as substrates with different redox potentials to compare the oxidation reaction rates of various laccases. The compounds were selected to represent the most important structural features in wood lignins. *p*-Coumaric acid was used as model compound for the *p*-hydroxyphenyl structure, sinapic acid was selected to represent syringylic structure and ferulic acid to represent the guaiacylic structure. In addition two other molecules, one lacking carboxylic acid group, *i.e.* 2,6-dimethoxy phenol and one with acidic group linked to aromatic ring *via* single covalent bond *i.e.* syringic acid were used to study the effect of size of the substrates to the reactivity of laccases. A simple phenolic monomer 4-(1-hydroxyethyl)-2-methoxyphenol (apocynol) was chosen to present the most abundant reactive structural unit in coniferous lignin as a substrate of oxidation by laccase. Apocynol (6) was prepared from acetovanillone (Aldrich) by sodium borohydride reduction according to [19]. The lignans 7-hydroxymatairesinol (7) (HMR, Mw 374,39 g mol^{-1}) and matairesinol (8) (MR, Mw 358,39 g mol^{-1}) were obtained as a gift from the Laboratory of Wood Chemistry, Åbo Akademi University, Finland. The DHP (9) (Mw *ca.* 3000 g mol^{-1}) of coniferyl alcohol was prepared according to methods described previously by [20] and [21]. The chemical structures of the model compounds are shown in Fig. 1. Organic solvents (acetone, ethanol, propylene glycol, diethylene glycol monomethyl ether) and all other chemicals used in this study were of analytical quality.

2.2. Laccases

The recombinant laccase from the ascomycete *Melanocarpus albomyces* (MaL), expressed and purified as described [22], and the high redox potential laccase from the basidiomycete *T. hirsuta* (ThL), produced and purified as described previously [23], were kindly donated by Dr. K. Kruus (VTT, Finland). The recombinant laccases TaLcc1 and TaLcc2, originating from the ascomycete *Thielavia arenaria* ALKO4197, and CtLcc1, originating from the ascomycete *Chaetomium thermophilum*, all produced in *Trichoderma reesei* [24,25], were obtained from Roal Oy (Rajamäki, Finland). The enzyme products were used without further purification. Optimal pH range and thermostability properties of the studied laccases are shown in Table 1.

2.3. Enzyme activity

Laccase activity was determined using 1 mM 2,6-DMP as the substrate in 25 mM Na-citrate buffer [26]. Instead, for

Table 1
Optimal pH range and thermostability properties of the studied laccases.

Enzyme	Optimal pH-range	$T_{1/2}$ at 50 °C (h)	$T_{1/2}$ at 60 °C (h)	References
ThL	3.0–5.0	65	4	[14,34,51]
MaL	4.0–6.0	50	5	[32,34]
TaLcc1	3.0–5.5	26	5.5	[24,34]
TaLcc2	4.5–6.0	25	6	[35]
CtLcc1	4.5–6.0	32	7	[35]

the oxygen consumption measurements, laccase dosage was based on the activity measurement with 1 mM 2,2′-azino-bis-3-ethylbenzthiazoline-6-sulphonic acid (ABTS) as a substrate [27]. TaLcc1 laccase was a granulate product, which was dissolved into the citrate buffer, centrifuged, and the clear liquid was collected and used for the enzymatic assays. To calculate the activity in the presence of solvents, the extinction coefficient of the 2,6-DMP oxidation product (ε = 27,500 M^{-1} cm^{-1}) was assumed to remain unaffected [28].

2.4. Stability experiments

The stability of various laccases in ethanol and in propylene glycol was investigated by incubating the enzymes in 25 mM Na-citrate buffer with varying amounts of solvent at 25 °C for 1 h, 2 h or 20 h. After incubation the residual activity against 2,6-DMP was determined in the solvent. Enzyme incubated in 25 mM Na-citrate buffer was used as the reference.

2.5. Oxygen consumption measurements

Oxygen consumption was measured with FIBOX 3 fiber-optic oxygen meter (PreSens, Regensburg, Germany) at 20 °C. The small phenolic molecules were dissolved in 25 mM Na-succinate buffer at pH 5 and 6 to a final concentration of 1 mM in a total volume of 1.86 ml. The reaction vessel was sealed and the solution was stirred for 10 min for stabilization. The enzyme (dosage: 70 nkat μmol^{-1} based on activity determination with ABTS as a substrate in 55 μl) was injected through a septum in the cap, and the consumption of oxygen was monitored for 10 min. Measurements were performed in duplicates and the consumption of oxygen (μg l^{-1} s^{-1}) was calculated from the initial slope of the oxygen consumption curve.

2.6. Oxidation of lignans and DHP

The oxidations of MR and HMR lignans were performed in the presence of 25, 50 and 70% (v/v/v) ethanol or propylene glycol in 200 μl total volume buffered at pH 5. In the buffer solution, pH 5 lignans were precipitated, and the reactions were not further followed. Enzyme dosage was 70 nkat μmol^{-1} reactive groups in lignan based on the ABTS activity at pH 5. The enzymatic reactions were terminated by adding 0.05% (w:v) NaN_3 prior to freeze drying. Dried samples were solubilized in 50 mM NaOH at a concentration of 1 mg ml^{-1} for chromatographic analyses.

Oxidation studies of DHP were conducted in 20 ml test tubes covered with parafilm. The total reaction volume was 1 ml and contained 10 mg of DHP and 25, 50 and 70% (v/v/v) of organic solvent. Laccase dose was adjusted according to the initial activity in different solvents to give the same initial activity in all reactions. The enzyme activity in reactions corresponded to 1 nkat mg^{-1} substrate in the aqueous solution. The reactions with laccases TaLcc1 and MaL were carried out using 25 mM Na-citrate buffer pH 5.5; in the case of ThL pH 3.5 was used. The reactions were stirred for 24 h and then stopped by adding 0.05% NaN_3. Milli-Q-water (3 ml) was then added to the reaction mixtures, centrifuged, and the solids were washed twice with 3 ml of water. The samples for ^{31}P NMR analysis were prepared by incubating the DHP sample (25 mg) in 50% (v:v) propylene glycol–25 mM citrate buffer pH 5.5 with 10 nkat ml^{-1} TaLcc1 in a total volume of 1.5 ml for 24 h. The samples for ^{1}H and ^{13}C NMR were prepared by incubating the DHP sample in 50% ethanol–25 mM citrate buffer pH 5.5 with TaLcc1 laccase. The reference DHP samples were prepared similarly without laccase.

2.7. High performance liquid chromatography

The conversion of the model compound 4-(1-hydroxyethyl)-2-methoxyphenol (apocynol) was followed by using high performance liquid chromatography (HPLC) Agilent 1200 with detection wavelength of 270 nm. Only the disappearance of the starting material was followed. In the HPLC studies, a sample of 5 μl was injected with autosampler and eluted with methanol:H_2O gradient Step 1: time: 0–10 min, gradient: 50:50 (v:v) isocratic, flow rate: 0.15 ml min^{-1}; Step 2: time 10–20 min, gradient: linear, 0.15 ml min^{-1} from 50:50 to 100:0; Step 3: time: 21 min, 0.2 ml min^{-1}. Before analysis the column was equilibrated by applying 22–28 min linear gradient from 100:0 to 50:50 0.2 ml min^{-1}; 28–31 min 50:50 0.15 ml min^{-1} and 31–40 min 50:50 0.15 ml min^{-1}.

2.8. Size exclusion chromatography

Laccase treated DHP samples as well as the control samples were dried under vacuum with an oil pump and 10 mg portions were subjected to acetylation. Samples were dissolved in 3 ml of 1:1 (v:v) acetic anhydride:pyridine. The reaction was allowed to proceed overnight and was terminated by addition of 5 ml ethanol. The solvent was removed by azeotropic distillation with toluene under reduced pressure.

Prior to analysis the samples (2–3 mg) were dissolved in 1 ml of THF and filtered through 0.45 μm Acrodisc GHP Membrane HPLC filter (Waters, Milford MA, USA). Agilent 1100 HPLC system (Agilent Technologies, Santa Clara California, USA) including degasser, pump, autosampler and column oven was used to run the chromatograms. Columns were Waters Styragel HR5E and HR1 (Waters Corporation, Milford, USA) connected in series. Injection volume was 30 μl and flow rate was 0.5 ml min^{-1}. The column temperature was not controlled and the ambient temperature was 23 °C. Agilent 1050 diode array detector at wavelength 280 nm was used for detection. The molar mass was calibrated using polystyrene standards (Mw: 434,000, 177,000, 42,300, 9000 and 1240 g mol^{-1} and Mn: 420,000, 174,000, 41,400, 9590 and 1210 g mol^{-1}) obtained from Polymer Standards Service – USA Inc. (Warwick, USA).

Polymerization of 2 mM MR and 3 mM HMR by MaL in ethanol (20–70%) and propylene glycol (25–70%) was studied by Waters (Milford, MA, USA) SEC apparatus equipped with Waters μHydrogel 2000, 250 and 120 Å columns using the alkaline elution method described by [29]. Pullulans were used as standards for molecular mass calibration.

2.9. Nuclear magnetic resonance spectroscopy

The DHPs were isolated as described in Section 2.6 and dried under vacuum for 24 h at 40 °C. The samples for ^{31}P NMR were prepared according to [30,31]. The accurately weighed DHP samples (*ca.* 15 mg) were dissolved in pyridine (0.100 ml) in a 10 ml screw-top vial and $CDCl_3$ (0.475 ml) was added. The mixture was stirred overnight. The phosphitylation reagent 2-chloro-4,4,5,5-tetramethyl-1,3,2-dioxaphospholane (0.100 ml), the internal standard endo-N-hydroxy-5-norbornene-2,3-dicarboximide (e-HNDI) (0.063 ml, 121.5 mM in Pyr: $CDCl_3$/3:2) was added and mixture was stirred for 1 min. Finally, the relaxation agent chromium(III)

Table 2
Oxidation of phenolics in buffer by laccases. Rate of oxygen consumption was measured at pH 5 and 6. Amount of laccase used (70 nkat mg^{-1} substrate) was based on the activity assay with ABTS as the substrate at pH 5.

Reactivity ($\mu g\, l^{-1}\, s^{-1}$)	E° (mV)	ThL		MaL		TaLcc1		TaLcc2	
		pH 5	pH 6	pH 5	pH 6	pH 5	pH 6	pH 5	pH 6
2,6-Dimethoxyphenol	530[b]	171	79	144	93	140	83	54	87
Sinapic acid	532[a]	126	126	133	114	119	128	140	96
Syringic acid	591[a]	157	84	200	111	198	87	107	122
Ferulic acid	661[a]	185	115	64	150	159	88	26	76
p-Coumaric acid	700[a]	122	74	3	5	10	12	25	35
Vanillic acid	708[a]	147	nd	14	nd	39	nd	15	nd
Phenol	1150[c]	20	nd	0	nd	0	nd	2	nd

nd, not determined
[a] Data at pH 4.0 from [52].
[b] Data at pH 4.5 from [34].
[c] Data at pH 5 from [53].

acetylacetonate ($Cr(acac)_3$) solution 0.5 ml (0.08 M in $CDCl_3$) was added.

^{31}P NMR spectra (^{31}P: 243 MHz) were recorded in a total volume of 0.750 ml. Chemical shifts were referenced to the phosphitylated water at 132.2 ppm in $CDCl_3$. The ^{31}P NMR spectra were recorded using inverse gated proton decoupling sequences on a Varian Unity Inova 600 spectrometer equipped with 5 mm direct detection broadband probe-head. ^{31}P spectra were collected at 27 °C with 256 transients using 90° pulse flip angle, 85,000 Hz spectral width, 1 s acquisition time, and 6 s relaxation delay.

The samples for ^1H and ^{13}C NMR were isolated as described earlier, acetylated and dried in vacuum before NMR analysis. ^1H and ^{13}C NMR spectra were recorded with Varian Inova 500 spectrometer (^1H: 500 MHz and ^{13}C: 125 MHz) in $CDCl_3$. The 2D HSQC and HSQC-TOCSY were used for the identification of the reaction products and measured using standard pulse sequences provided by the manufacturer in acquisition and data processing.

3. Results and discussion

3.1. Oxidation of substrates in buffer by laccases

Oxidation of structurally different phenolic substrates was monitored by oxygen consumption in buffer at pH 5 and 6. The five laccases that were selected for the study were known to have good thermal stabilities (Table 1). The laccase from *T. hirsuta* (ThL) has a high redox potential, and all the tested phenolics were efficiently oxidized by ThL (Table 2). ThL has an acidic pH-optimum, and clearly lower rate of oxidation was detected at pH 6 compared to pH 5. This is in contrast to other laccases, by which many phenolics were equally or even more efficiently oxidized at pH 6. Many ascomycete laccases preferentially oxidize phenolic compounds at a more neutral range than basidiomycete laccases [32–34]. The redox potential for phenolic substrates (E°) is pH-dependent, and decreases when the pH increases, while the E° for laccases appears to be unaffected by pH [4,35]. Considerable variation in the rate of oxidation of the substrates was found between the laccases; and although E° of the substrate affected the rate of oxidation by laccases, it was not the only factor contributing to the oxidation of phenolics. MaL and TaLcc1 possessed very different activity profiles depending on the pH, which may result from the differences in both the size and the shape of the substrate binding pocket between TaLcc1 and MaL [34]. Only TaLcc2 appeared to possess higher activities with all phenolics at pH 6. Laccases have in general high K_m-values for *para*-substituted phenols as substrates [1], and the difference in the oxidation of *p*-coumaric acid (1) clearly distinguished the high and low redox laccases: ThL possessed good activity, whereas with MaL, TaLcc1 and TaLcc2 the oxidation of *p*-coumaric acid was very slow. Interestingly, TaLcc2 had a relatively higher rate of *p*-coumaric acid oxidation compared to other ascomycete laccases, and was even able to slowly oxidize phenol, a substrate with a high redox potential.

The oxidation of the larger phenolic substrates, HMR (7) and MR (8) lignans, revealed a similar trend as detected with the small molecule phenolics. The high redox potential ThL showed the highest rate for oxidation of lignans, when the solvent content (ethanol, propylene glycol) in the reaction mixtures was kept low enough to ensure a complete solubility of the substrate, but on the other hand, to retain the laccase activity close to the same level as in aqueous buffer solution. The oxidation rates of ThL between the two lignans were similar, however HMR was slightly favored (data not shown). The hydroxyl group in the HMR significantly weakened its reactivity, and about four times lower oxidation rates of HMR with MaL and TaLcc1 were recorded.

3.2. Initial laccase activity in water organic solvent mixtures

All organic solvents tested; acetone, ethanol, propylene glycol and diethylene glycol monomethyl ether (DEGME), gradually inhibited the initial activity, as previously shown by [36,37]. Our study revealed that all selected low and high redox potential laccases steadily lost the activity as the organic solvent concentration exceeded 25% (Table 3). Acetone inactivated all laccases already at low solvent concentrations below 10% (data not shown). It has been reported recently [13] that ethanol and propylene glycol were the organic solvents best tolerated by MaL. Ethanol was significantly inhibiting than propylene glycol, although some variability was observed between the studied laccases: CtLcc1 exhibited even higher activity in 25% ethanol than in the buffer. A similar phenomenon has been reported for MaL when 5–30% DEGME was used as a co-solvent [13] as well as in some other enzyme-solvent systems [38,39]. However, reactions in DEGME varied among laccases, and poor oxidation of 2,6-DMP by ThL was observed (data not shown). Therefore, reactions of laccases in this solvent were not further studied.

Table 3
Initial activity of laccases in aqueous ethanol and propylene glycol. Data is based on the rate of oxidation of 2,6-DMP in the presence of the solvent.

Enzyme	Solvent						
	Buffer	Ethanol (%)			Propylene glycol (%)		
		25	50	70	25	50	70
ThL	100	36	11	nd	80	49	30
MaL	100	32	21	nd	90	57	45
TaLcc1	100	54	36	nd	80	37	9
TaLcc2	100	38	1	1	54	15	10
CtLcc1	100	125	42	32	102	70	61

nd, not determined.

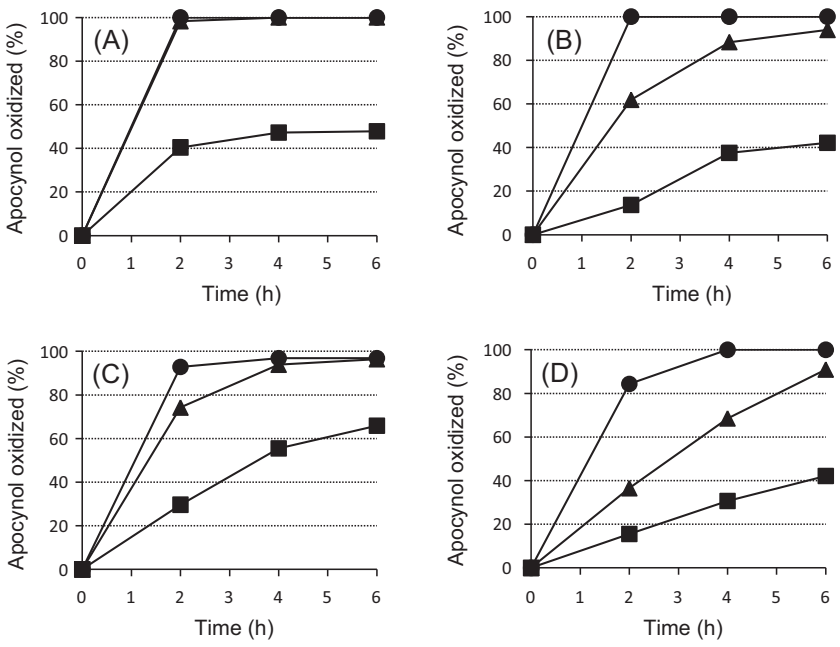

Fig. 2. Oxidation of apocynol (6) in ethanol (A and B) and in propylene glycol (C and D) by ThL and TaLcc1 laccases. Symbols: (●) 25%, (▲) 50%, (■) 70% of organic solvent.

Excellent solvent tolerance of CtLcc1 for both ethanol and propylene glycol was observed. Over 30% of the initial activity was still present at 70% ethanol concentration. Even higher activities were recorded in propylene glycol: over 60% of the initial laccase activity was retained at a solvent concentration of 70%. The TaLcc1 laccase also showed high activity in ethanol and in propylene glycol up to a solvent concentration of 50%, as shown in Table 3. The ThL laccase lost its activity in ethanol, but could tolerate propylene glycol, similarly to other studied laccases. The poor initial activity of *T. hirsuta* laccase, and several other high and low redox potential laccases, including the *Myceliopthora thermophila* laccase, in ethanol has been reported earlier [40].

To further compare the activities of laccases (ThL, MaL and TaLcc1), the oxidation of MR and HMR lignans in ethanol and propylene glycol were studied by oxygen consumption measurements. The activities of ThL and TaLcc1 laccases resembled that of MaL, reported by [13]. MR lignan was more reactive than HMR with all tested laccases. The activities decreased significantly, when the solvent concentration increased from 25% to 50%, and no oxidation was observed even after 20 min incubation when 70% solvent was present (data not shown).

3.3. Stability of laccases in solvents

Most of the tested laccases had excellent stability in aqueous propylene glycol solutions and only little loss of catalytic activity was evident even in the 70% solvent concentration, with the exception of TaLcc2 that showed only moderate stability in propylene glycol in concentrations over 50% (Table 4). TaLcc2 appeared to be the most sensitive laccase for both propylene glycol and ethanol. As the laccase activities decreased even when incubating the enzymes in buffer without solvent, stability values exceeding 100% were recorded especially in propylene glycol. In low ethanol concentrations this response was also observed, with the exception of TaLcc2 that lost most of its activity already in 25% ethanol concentration. High ethanol concentration above 50% clearly decreased the enzyme stability. Of the tested enzymes, TaLcc1 and MaL showed the highest stability in the presence of ethanol.

3.4. Oxidation of the model compound apocynol

Oxidation of apocynol (6) was studied with laccases ThL, TaLcc1 and TaLcc2. In accordance with experimental data on the oxidation of 2,6-DMP (Table 3), an increase of ethanol or propylene glycol content decreased the rate of apocynol oxidation by all laccases. Even in 75% solvent concentration, however, a considerable amount of apocynol was oxidized within 6 h by ThL and TaLcc1 (Fig. 2). The rate of oxidation of apocynol by laccases was higher in ethanol than in propylene glycol, although the stability of laccases was better in propylene glycol. The oxidation of apocynol was similar by both TaLcc1 and TaLcc2 in solvents and therefore data with only ThL and TaLcc1 laccases is presented. The HPLC-analysis of the oxidized products of apocynol indicated no alteration in the product profile, although the reactions were conducted in two different solvents (data not shown). The result is in accordance with our previous studies with MaL in the same solvent systems [13]. Structural elucidation of the polymerized products of lignans indicated that similar products were formed during the polymerization reactions in 20% ethanol and 50% propylene glycol [13].

3.5. Structural features of the selected laccases

It is thought that thermal and other stability characteristics of laccases usually coincide [34,40–42]. Therefore, thermally stable laccases are among those enzymes that potentially tolerate organic

Table 4
Stability of laccases in ethanol and propylene glycol. Stability of laccases was measured after 20 h incubation. The numbers indicate the rate of 2,6-DMP oxidation (%) compared to the activity in buffer.

Enzyme	Relative activity in buffer and solvents						
	Buffer	Ethanol			Propylene glycol		
		25%	50%	70%	25%	50%	70%
ThL	100	124	17	12	115	141	79
MaL	100	101	50	31	97	87	76
TaLcc1	100	120	50	44	116	109	148
TaLcc2	100	21	bdl	bdl	101	10	29
CtLcc1	100	90	33	17	102	94	104

bdl, below detection limit.

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                 ●                   ●
MaL     : TEVDNWMGPDGVVKEKVM :  58
MtL     : TEVDNWTGPDGVVKEKVM :  58
CtLcc1  : TEEDNWTGPDGVVKEKIM :  58
TaLcc1  : TEKENWIGPDGVLKNVVM :  58
TaLcc2  : SVVEGPCAPDGYN—RTCM :  55

                    ●     ●
MaL     : ITDYYYRAADDLVHFTQN :  189
MtL     : ISDYYYSSADELVELTKN :  189
CtLcc1  : LTDYYYKSADELVRHTQN :  189
TaLcc1  : LMDYYYRSADELVHFTQS :  189
TaLcc2  : LQDWSHVEAFTRWHEAKA :  185
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Fig. 3. Amino acid sequence alignment of two separate fragments of laccases. Residues forming salt bridges in MaL are indicated with symbol (●). Protein sequences obtained: *M. albomyces* (MaL): [41], *Myceliopthora thermophila* (MtL): [50], *Chaetomium thermophilum* (CtLcc1): [25], *Thielavia arenaria* (TaLcc1): [24], *T. arenaria* (TaLcc2): [25]. Predicted amino acid sequence alignment was built using ClustalW program of European Bioinformatics Institute.

solvents as well. Indeed, this assumption is in agreement with the data presented here. All studied laccases possess good thermal tolerance (Table 1), which is in accordance with their good solvent tolerance, as shown in Tables 3 and 4. MaL has high thermal stability and a broad pH range optima for different phenolics [32], similar to *Thielavia* laccases. The structure of MaL has been solved as one of the first complete laccases [41]. Its good stability characteristics may be related to the conformation of several amino acids and glycans within the protein structure making it challenging to identify single key affecting factor. Both MaL and TaLcc1 are glycosylated in a similar manner [34] and the typical extended carboxy terminus forming "a plug" in the solvent channel, may promote the stability for many ascomycete laccases [42,43]. Other possible stabilizing factors in MaL may be created through hydrogen bond formation with glycans [41,44], as well as through salt bridge formation between Lys56 and Glu42 and between Asp182 and Arg178, depicted in Fig. 3. Salt bridge formation on the surface of the laccase between the mutated residue Glu182Lys and Glu55 has recently been found to improve the tolerance against organic solvents in *M. thermophila* [40].

The TaLcc2 revealed lower tolerance against organic solvents compared to other laccases (Tables 3 and 4). It is likely that the 3D structure of TaLcc2 is only distantly related to the structures of TaLcc1 or MaL, as only 51% of the full-length sequence of TaLcc2 is similar to TaLcc1. The distinct amino acid composition of TaLcc2 compared to other laccases is clearly seen in the peptide alignment (Fig. 3). However, without actual structure determination or molecular modeling, structural arrangements in the TaLcc2 enzyme remain only hypothetical. The putative structure of TaLcc2 involves an insertion of nine amino acids in the loop fragment between residues 203 and 204 in MaL structure, [41] as well as a deletion of a fragment residues 453–464 in MaL structure [41], not present in either MaL or TaLcc1, which may affect its stability characteristics, presumably by decreasing the protein compactness.

Laccases from the basidiomycete genus *Trametes* are considered as potentially thermostable enzymes [45]. The ThL with good thermal stability values (Table 1) showed also good solvent stability characteristics. Structurally important amino acid residues for alleviating good stability were found at the surface of the protein also for basidiomycete laccases [46]. The stability and initial activity of laccases in organic solvents are features not directly linked to each other, and it may be possible to improve separately the stability and catalytic efficiency by mutations [42,46]. In this work, however, the catalytic effiency *per se* appears to be strongly compromised by the tolerance of the enzyme in organic solvents, *i.e.* in conditions, where the substrate availability for oxidation is facilitated [13].

Table 5
Effect of organic solvent on the polymerization of MR and HMR by ThL, MaL and TaLcc1 laccases. Values in the table (ΔMw) represent the change of the molecular weight of lignan compared to that of untreated lignan.

	ThL		MaL[a]		TaLcc1	
	MR	HMR	MR	HMR	MR	HMR
Ethanol (%)						
25	7700	5900	12,600	7400	17,300	9100
50	8500	8900	12,300	9200	11,500	9400
70	3800	3200	11,200	6900	10,000	6100
Propylene glycol (%)						
25	4400	6500	28,500	7700	22,900	10,600
50	5000	8800	39,200	9100	13,800	9400
70	5900	11,700	19,900	10,100	11,600	8200

[a] Data as presented in [13].

3.6. Polymerization of lignans and synthetic DHP lignin by laccases

3.6.1. Lignans

The water insoluble MR and HMR lignans were efficiently polymerized by all laccases even in the highest organic solvent concentrations (Table 5). In buffer solutions pH 5 all selected lignin model compounds were precipitated by laccases less than in 20% solvent mixtures (data not shown). Oxidation by the high redox potential ThL resulted in polymerized products with a lower molecular weight than oxidation by the low redox laccases MaL and TaLcc1. Although ThL was equally active in the initial oxidation of MR and HMR, reactions in propylene glycol resulted in the polymerization of HMR to products with a higher molecular weight than in the polymerization of MR. In ethanol no difference between the lignans was observed in the polymerization reactions by ThL. The highest molecular weight was obtained by ThL in 70% propylene glycol, whereas by MaL the polymerization in 50% propylene glycol was most efficient, and by TaLcc1 the reactions in 25% propylene glycol resulted in the highest molecular weight of polymerized lignans (Table 5). The structural difference of the MR and HMR lignans significantly affected the rate of polymerization by MaL and TaLcc1, which is in accordance with the estimates of the initial oxidation rate of these model compounds, shown by [13]. MR is more efficiently oxidized than HMR by low redox potential laccases. A high concentration of ethanol in the reaction mixture resulted in decreased molecular weight of the final product by all laccases, indicating that the initial laccase activity was significantly decreased to proliferate necessary radical formation for efficient polymerization of lignans.

3.6.2. DHP

Oxidative reactions by different laccases varied when DHP (9) was used as the substrate. Oxidations by the laccases ThL and TaLcc1 in 50% ethanol and by MaL in 70% ethanol resulted in the highest molecular weight reaction product (Table 6). The polymerization reactions by ThL in ethanol solutions were clearly more extensive than the reactions in propylene glycol, although the stability of ThL in ethanol was poor, as shown in Table 4. In propylene glycol, the highest molecular weight was obtained in 70% solvent concentration by all laccases. However, only by TaLcc1 extensive polymerization occurred (Table 6 and Fig. 4). Polymerization by ThL and MaL remained relatively inefficient regardless of the amount of solvent. The slight difference in the redox potential does not explain this behavior, as the use of high redox laccase ThL did not result in extensive polymerization. Although the physico-chemical characteristics of the solvents may partially explain the variation in the of polymerization reactions [13], differences in the catalytic efficiency and/or other structural features of laccases may facilitate the variability in the oxidation of complex substrates in presence of organic

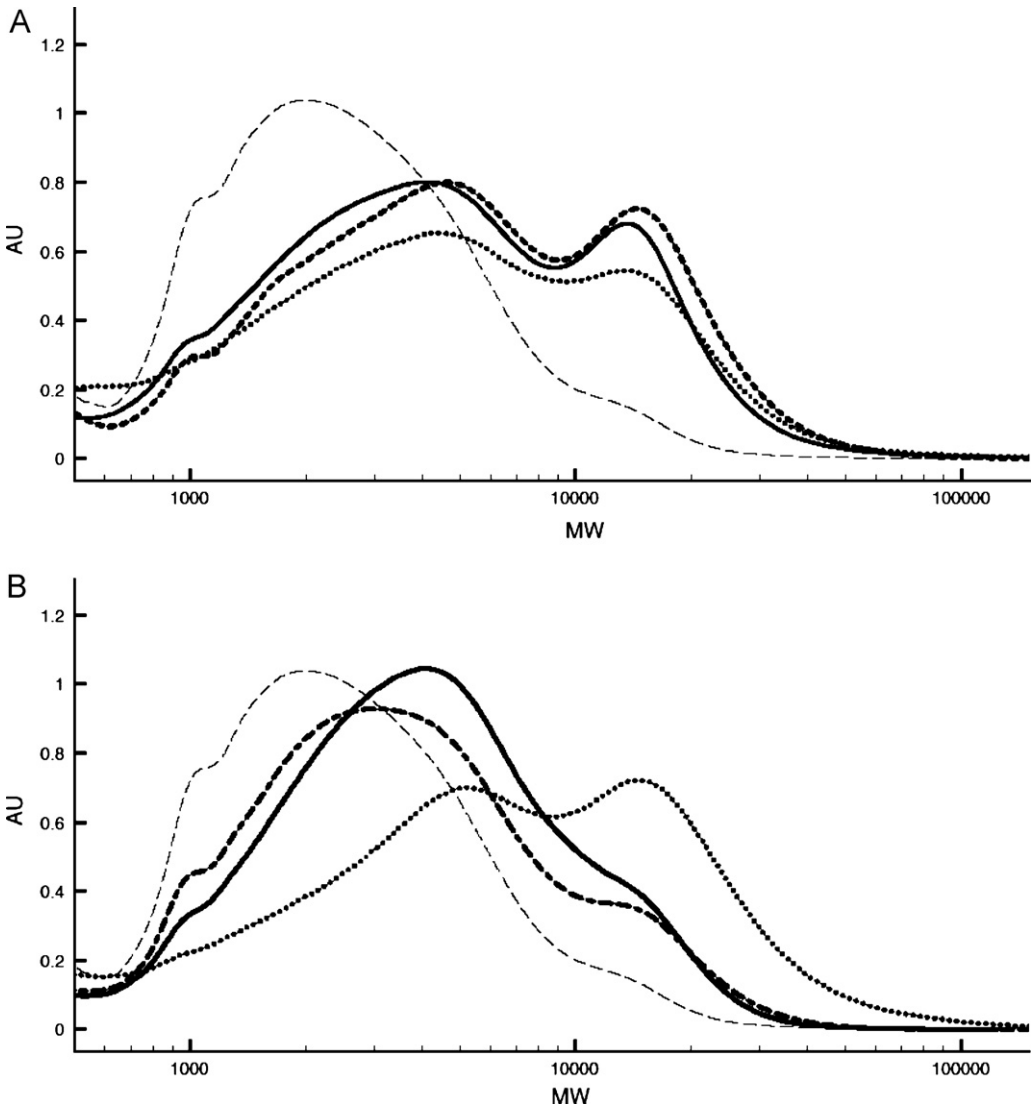

Fig. 4. Size exclusion chromatograms of DHP (9) incubated with ThL, MaL, and TaLcc1 laccases in reaction mixtures containing 70% ethanol (A) and 70% propylene glycol (B) for 24 h. The reference DHP incubated without enzyme is shown. (—) ThL; (●●●●●) TaLcc1; (- - - - - -) MaL; (- - - - - -) no enzyme.

solvents. Although MaL and TaLcc1 share structural and kinetic characteristics, the observed structural variability, as reported by [34], may confer also their different reactivity in propylene glycol.

Interestingly, depolymerization of DHP was observed with MaL in 25% ethanol, in contrast to the other studied laccases. This observation was unexpected, but was obtained in repeated experiments. MaL retained its activity in 25% ethanol, but a more detailed study would be required to find out, if the shift from depolymerization to repolymerization is linked to the solvent derived change of the molecular architecture of MaL or of other interactions between the enzyme and the substrate. During the oxidation of DHP in organic solvent both depolymerization and repolymerization have been observed with manganese peroxidase, depending on the type of DHP [28]. The results are encouraging, and open up possibilities for modification and valorization of technical lignins. The laccases ThL, MaL, TaLcc1 and TaLcc2 have shown potential for modification of different types of lignins even without organic solvents [14].

Table 6
Effect of organic solvent on polymerization of DHP by ThL, MaL and TaLcc1. Values in the table (ΔMw) represent change of the DHP molecular weight compared to that of untreated DHP. The molecular weight of the DHP without laccase treatments was 3000 g mol^{-1}.

	ThL ΔMw (g mol^{-1})	MaL ΔMw (g mol^{-1})	TaLcc1 ΔMw (g mol^{-1})
Ethanol (%)			
25	1400	−1600	2200
50	7500	3400	5000
70	4100	4900	3900
Propylene glycol (%)			
25	1400	1100	4300
50	1700	1900	6400
70	2300	1900	7200

3.6.3. Structural elucidation of repolymerized DHP

Structural analysis of the polymerized DHP by NMR was undertaken in order to obtain detailed information on the regiochemistry of the oxidative reactions by laccases. It has been previously shown with guaiacylic lignin model compounds that 5–5′ coupling products represented the only oxidation products of laccases [11]. DHP was repolymerized in the presence of TaLcc1 laccase in 50% propylene glycol and in 50% ethanol; the reactions which resulted in high molecular weight (Table 6). The ^{31}P NMR spectra of the polymer are shown in Fig. 5. The samples cannot be directly compared since all propylene glycol could not be removed from the NMR

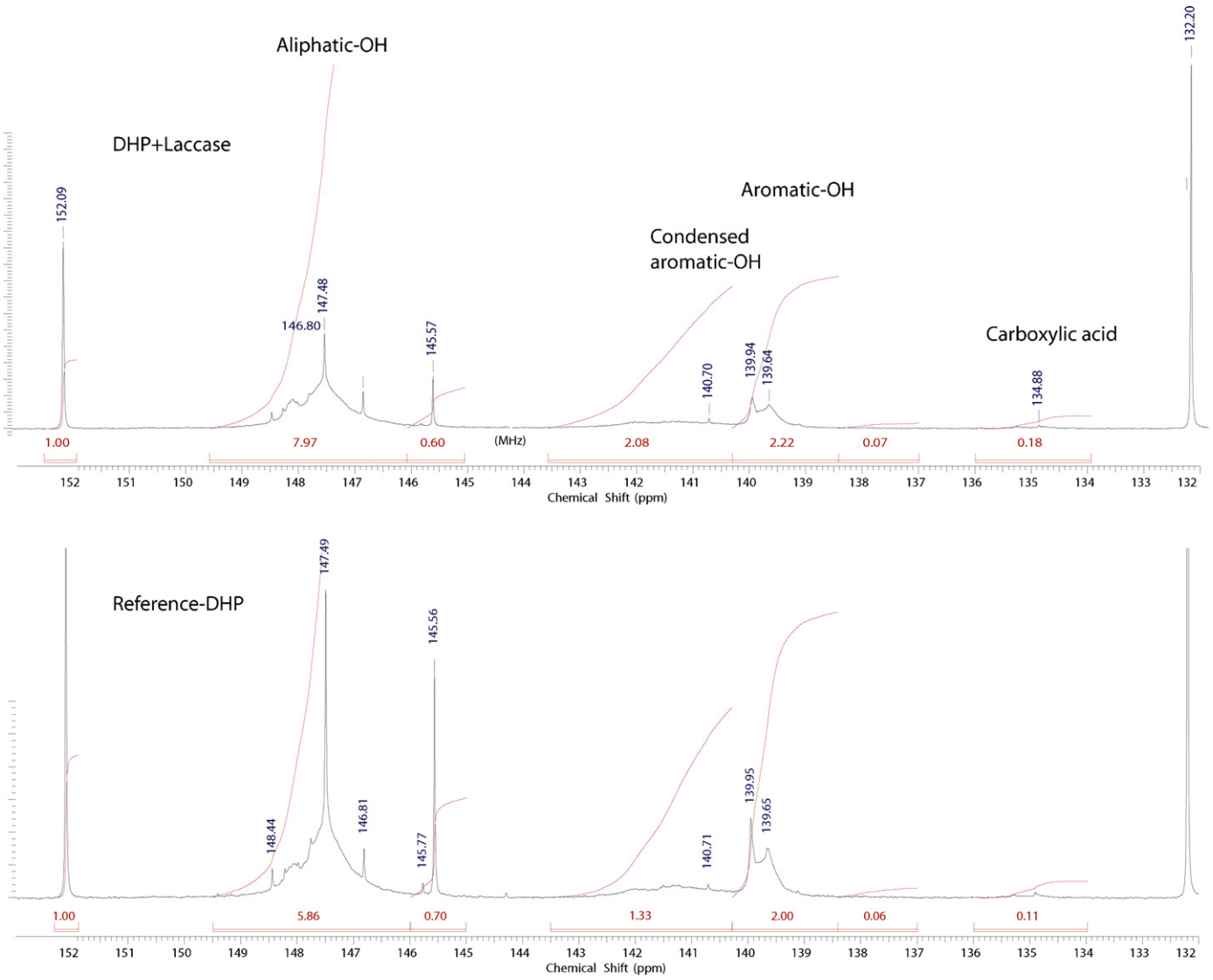

Fig. 5. ^{31}P NMR of DHP treated by laccase TaLcc1 in buffered solution pH 5.5 containing 50% propylene glycol (upper spectrum). The reference DHP sample was treated in a similar fashion but without laccase (lower spectrum).

sample (Fig. 5, peaks derived from propylene glycol at 145.56 and 147.49 ppm) and thus, it contributes by different amounts to the total DHP in each sample. However, the residual propylene glycol did not affect the amount of phenolic hydroxyl groups in the sample, and the relative amounts of these hydroxyl groups can be compared between the samples. Comparison of the condensed phenolic hydroxyl groups to non-condensed shows that the ratio was higher in laccase oxidized DHP (0.94) than in the reference (0.67). This suggests that the formation of new biphenylic 5–5′ structures was a favorable reaction in the other possible radical coupling reactions that would form new 5—O—4 ether bonds through the phenolic groups. Since it was practically impossible to completely remove propylene glycol from samples, TaLcc1 laccase treatment was repeated in 50% ethanol to be analyzed by 1D ^{1}H and ^{13}C NMR as well as 2D ^{13}C HSQC NMR spectroscopy. The analysis of ^{1}H NMR of the laccase treated DHP and the reference DHP showed widening of the peaks indicating that the molar mass of the polymer had increased. As expected, the different intermonomeric bonding units in DHP (β—O—4 arylglycerol-β-aryl ether, β-5 phenylcoumaran and β-β pinoresinol structures) seemed to remain intact during the reaction, as well as coniferyl alcohol end groups, which are not directly susceptible to radical reactions in the DHP polymer. The most relevant changes in ^{1}H NMR could be detected in the

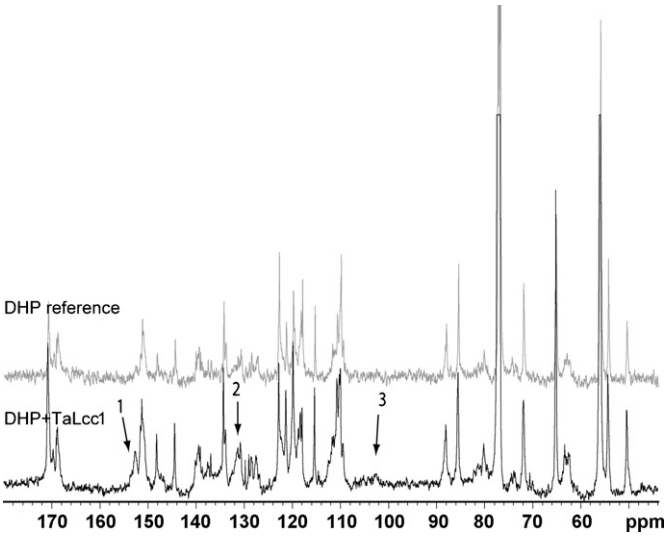

Fig. 6. ^{13}C NMR of DHP treated by laccase TaLcc1 in buffered solution pH 5.5 containing 50% ethanol. The reference DHP was treated in a similar fashion but without laccase. Arrows 1 at 152 ppm and 2 at 131 ppm indicate the signals for the increase of condensed biphenylic 5,5′-bonds during laccase treatment. Arrow 3 indicates the depleted signals of new 4′—O—5-structures.

spectral region of acetylated phenolic hydroxyls at 2.3 ppm, suggesting that some of the phenolic hydroxyl groups had shifted to a higher field (*i.e.* lower ppm value), typical for acetylated 5–5′ phenolic hydroxyls [47]. A similar peak pattern existed at the aromatic region around 7.0 ppm, suggesting that the amount of aromatic hydrogens had decreased (by about 15%).

The more detailed analysis of ^{13}C NMR (Fig. 6) showed that the amount of signals was increased at around 130 ppm, where the C5 signals of 5–5′ structures appear [48,49]. The carbon ^{13}C NMRs also revealed that the signal intensity increased significantly at 152 ppm in DHP, oxidized by TaLcc1, suggesting the formation of a new type of aromatic structure. Interestingly, both of the carbon ^{13}C spectra showed practically no signals at around 103–109 ppm where C2 and C6 are expected to resonate in 4—O—5′ bonds [54,55].

4. Conclusions

The thermostable laccases investigated in this study showed good stability even in high concentrations of ethanol and propylene glycol. The solvent tolerance of laccases varied to some extent, which is presumably related to their different three-dimensional structures. Thus, the redox potential value of laccase is not supposed to be the main factor determining the efficiency of polymerization reactions of phenolic lignin model compounds. Polymerization reactions varied between different laccases, encouraging searching, characterization and improvement of novel laccases for modification of lignin-type materials. Solvent-stable laccases are potential candidates for industrial applications. Even in conditions, which strongly inhibited the enzyme activity, polymerization reactions took place, which opens up further possibilities for the utilization of laccases in areas where the solubility of the reactants or products is limited. NMR data revealed that the polymerization of complex phenolics such as DHP proceeded in water-solvent mixtures mainly through biphenylic 5–5′ radical coupling. In addition, the results presented in this paper revealed interesting properties of two novel laccases from *T. arenaria*.

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