3.4.1. Supplementary material

Table S1 Laccases and peroxidases used in this work.

Abbreviation	Source organism	Enzyme type	Supplier	Product number or reference
BALL	*Bacillus licheniformis*	Laccase	Our production	[10]
Ba-MnP	*Bjerkandera adusta*	Mn-peroxidase	Jena Bioscience	EN-203
Ba-VP	*Bjerkandera adusta*	Versatile peroxidase	Sigma-Aldrich	68528
HRP	/	horseradish peroxidase	ASA Spezialenzyme GmbH	
LAC 3	*Myceliophthora thermophila*	Laccase	ASA Spezialenzyme GmbH	[10]
LAC 4	*Trametes sp.*	Laccase	ASA Spezialenzyme GmbH	[10]
LAC 5	*Thielavia sp.*	Laccase	ASA Spezialenzyme GmbH	[10]
LAC A	*Agaricus bisporus*	Laccase	ASA Spezialenzyme GmbH	[10]
LAC C	*Trametes versicolor*	Laccase	ASA Spezialenzyme GmbH	[10]
LiP	Isoenzyme H8 from *Bjerkandera adusta*	Lignin peroxidase	Sigma-Aldrich	42603
MmPPOA-695-His	*Marinomonas mediterranea*	Polyphenol oxidase	Our production	[12]

Ms-MnP	*Mycetinis scorodonius*	Mn-peroxidase	ASA Spezialenzyme GmbH	
Nf-MnP	*Nematoloma frowardii* [a]	Mn-peroxidase	Jena Bioscience	EN-201
OB-1	variant from *basidiomycete* PM1	Laccase	Our production	[10]
Pc-MnP	*Phanerochaete chrysosporium*	Mn-peroxidase	Sigma-Aldrich	68528
Po-L	*Pleurotus ostreatus*	Laccase	Sigma-Aldrich	[10]
Ps-MnP	*Pleurotus sapidus*	Mn-peroxidase	ASA Spezialenzyme GmbH	
Tv-L	*Trametes versicolor*	Laccase	Sigma-Aldrich	[10]

[a] Newly reclassified as *Phlebia* sp. *Nf*b19 [11]

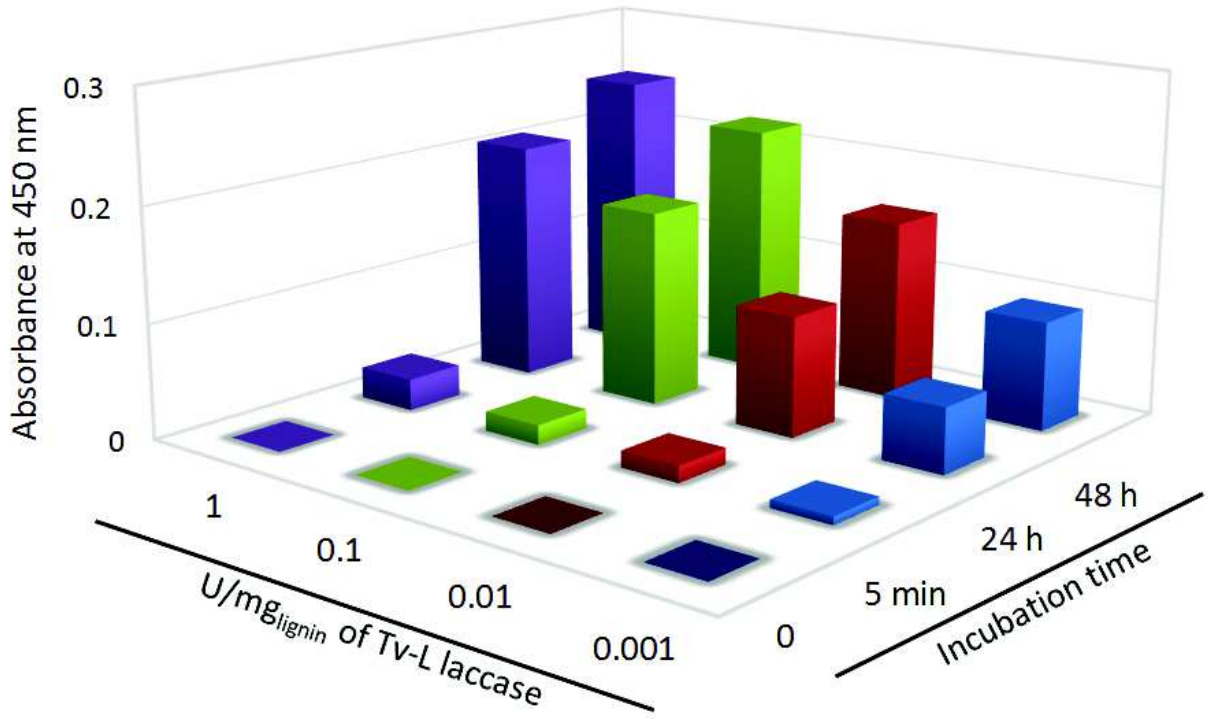

Figure S1. Screening for enzymatic lignin oxidation performed on a 96-well plate. Protobind lignin (0.05%, w/v) was treated with increasing amounts of Tv-L in presence of 1 mM ABTS as mediator resulting into a time-dependent increase in absorbance at 450 nm when the reaction mixtures were treated with 2,4-DNP at basic pH. Enzymatic reactions were performed at 25 °C in 50 mM NaAcO buffer, pH 5.0.

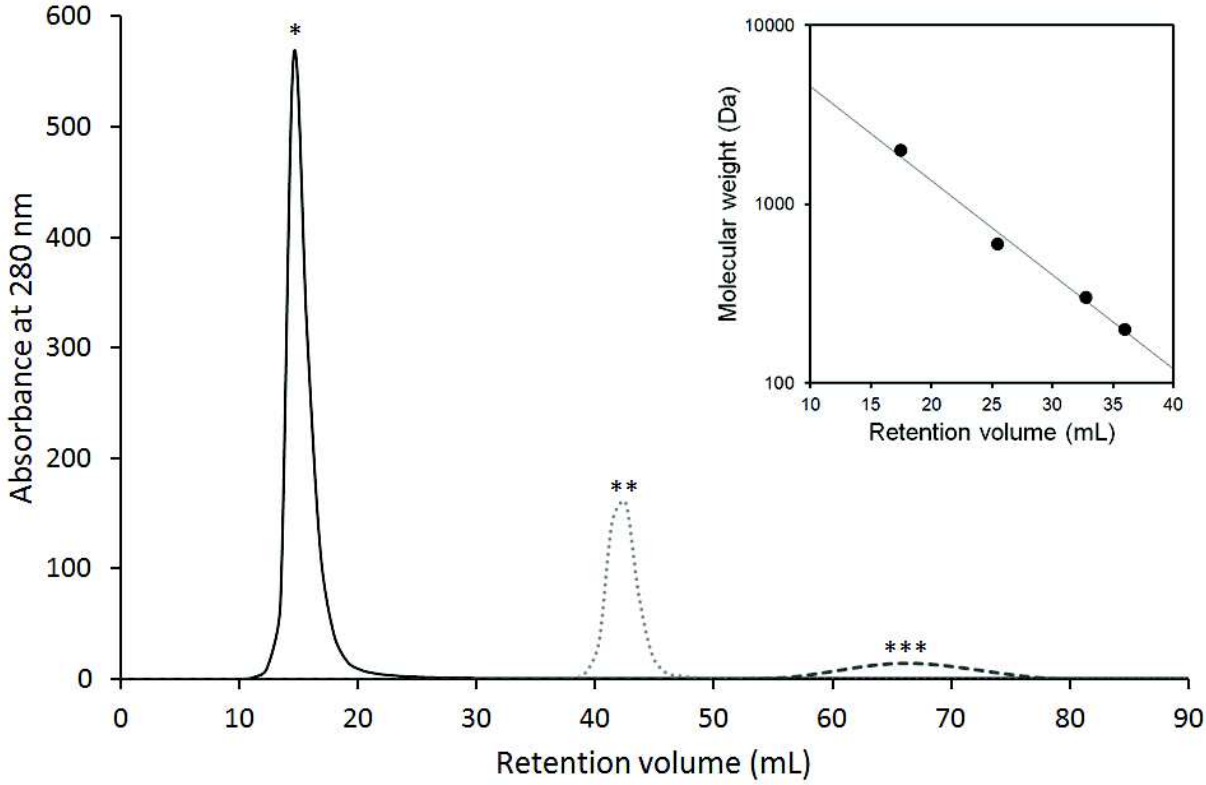

Figure S2 Size-exclusion chromatograms on Sephadex G25 column of 1 mg of Kraft lignin (continuous black line), 3 µmol of vanillic acid (dotty light grey line) and 3 µmol of sinapyl alcohol (dashed dark grey line). Elution buffer: 50 mM sodium acetate buffer pH 5.0, 0.1 M LiCl and 0.05% Tween-80; elution flow: 5 mL/min. The peak * at 16.0 mL contains lignin polymer; the peak ** at 42.6 mL contains vanillic acid; the peak *** at 66.1 mL contains sinapyl alcohol. Inset: calibration of the Sephadex G25 column using PEG standard samples in the 200-2000 Da range.

3.5. Enzymatic and chemo-enzymatic depolymerisation of lignin

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Keywords: lignin, laccases, TEMPO, formic acid, oxidation.

1. Introduction

Lignin is a three-dimensional branched aromatic heteropolymer normally found in plant-cell walls. It is derived by the co-radical polymerization of three monolignol monomers forming C-C and ether bonds: the relative abundance of linkages is variable depending on plant species and seasonality. In modern biorefinery, lignin is removed from the cellulose by aggressive media treatment and high technical efforts [1-4] giving a complex lignin mixture with a broad spectrum of size and chemistries. This complexity makes difficult and economically unsuitable the transformation of this feedstock into valuable product streams: an effective and cost efficient strategy is still to be identified.

The aromatic chemicals that could be obtained from lignin depolymerization represent a starting point for different valuable lignin-based products. Rahimi et al. (2015) [5] described a method for the depolymerization of oxidized lignin under mild conditions in aqueous formic acid that results in more than 60% yield of low-molecular-mass aromatics. However, the preliminary oxidation of lignin (or of the β-O-4 lignin linkage model compound) is necessary to obtain the reported yield [6]. The chemical oxidation is performed using high concentration of nitric and hydrochloric acid, high concentration of AcNH-TEMPO mediator and O_2 atmosphere. All these reaction conditions, taken together, are unsuitable for biorefinery use and not environmentally

friendly. On the other hand, the use of enzymes [7-9] and microorganisms [8, 10-12] to depolymerize lignin have been investigated by several groups: such processes show poor activity and thus are not commercially feasible.

In order to enable the controlled transformation of standardised lignin feedstocks to discrete families of high value product streams, here we report about the combined use of chemical and enzymatic treatments which have been optimized for selected fractions from different technical lignins and lignin linkage model compounds. The exclusive tool-box produced at the hosting laboratory [9], comprising 30 ligninolytic enzymes, allow to optimize the oxidation step by using a novel rapid screening procedure based on the reaction with 2,4-dinitrophenylhydrazine (2,4-DNP) [13]. According to proposed bio-catalytic models, the increase of carbonyl functionality in lignin samples was related to production of phenol monomers after the chemical treatment. The combination of high activity of chemical depolymerization with the specificity of enzymatic transformations lead to the formation of selected lignin monomers.

2. Materials and methods

2.1 Reagents and enzymes

LAC enzymes and chloroperoxidase from *Leptoxyphium fumago* (Lf-ClP) were supplied by ASA Spezialenzyme GmbH [14]. The laccases from *Trametes versicolor* (Tv-L) was purchased from Sigma-Aldrich (Milano, Italy). Recombinant laccase from *Bacillus licheniformis* (BALL) and basidiomycete PM1 (OB-1) were expressed and purified as reported in [14] (Table 1).

The mediators 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS), (2,2,6,6-tetramethylpiperidin-1-yl)oxyl (TEMPO), 1-hydroxybenzotriazole (HBT), N-hydroxyphthalimide (HPI), violuric acid (VA) and 3-hydroxyanthranilic acid (3-HAA) were purchased from Sigma-Aldrich. 1-(4-hydroxy-3-methoxyphenyl)-2-(2-methoxyphenoxy)propane-1,3-diol (GGE) was purchased from TCI (Tokyo, Japan). Kraft lignin was purchased from Sigma-Aldrich; Indulin AT lignin was purchased from MeadWestvaco (Richmond, Virginia, USA); Protobind lignin was provided by GreenValue SA (Orbe, Switzerland); Organosolv lignin was provided by ASA

Spezialenzyme GmbH. Protobind and Organosolv lignins were provided in the frame of the ValorPlus European project (see Acknowledgements).

2.2 Fractionation of Protobind lignin

Retentate and permeate fractions of Protobind lignin were obtained by dialyzing 0.5% (w/v) of lignin in 50 mM sodium acetate buffer, pH 5.0, containing 0.05% (v/v) Tween-80 using a 3-kDa dialysis tube, at 25 °C for 7 days.

2.3 Lignin sonication

Protobind lignin was dissolved in 0.1 M NaOH at the final concentration of 5% (w/v). Sonication process was performed using a Branson Sonifier 250 (Danbury, Connecticut, U.S.A.) in a ice-water bath for 2 h, with 50% output. The obtained lignin was diluited in a final volume of 1 mL in 50 mM NaAcO pH 5.0 (the pH was adjusted with 100 mM HCl). The samples were centrifuged for 2 min at 4000 xg and the supernatant removed. Once dried, the pellets representing the insoluble fraction were weighed. Control was carried out without sonication.

2.4 Methylation of GGE

1-(3,4-dimethoxyphenyl)-2-(2-methoxyphenoxy)propane-1,3-diol (Me-GGE) was obtained by methylation of GGE with MeI: to 51 mg of GGE dissolved in acetone were added 34 mg of MeI and 35 mg of K_2CO_3 and 2 drops of 1 N NaOH; the reaction was stirred at room temperature for 72 hours. Solvent was evaporated and the reaction mixture was re-dissolved in basic water (pH > 10). Me-GGE was extracted with CH_2Cl_2. Finally, 41 mg of Me-GGE were recovered (yield ≈ 80%).

2.5 Screening procedure and setup of incubations

Enzyme activities were assayed spectrophotometrically as reported in [14].

The dimeric model compounds GGE and Me-GGE were dissolved in 9 : 1 ratio of water : DMSO at 100 mM final concentration. The 0.5 mL assay mixtures containing 10 mM of GGE or Me-GGE, 0.2 U of laccases in 50 mM sodium acetate, pH 5.0, and 2 mM mediator (TEMPO, ABTS or HBT) were incubated on a rotatory wheel at 25 °C. At fixed times (0, 0.5, 1, 2, 4, 6 and 24 hours), 20 µL of the sample were withdrawn, added of 30 µL 100 mM HCl and used for colorimetric screening in a 96-well plate, as

reported in [13]. At the same times, 20 µL of reaction mixture were withdrawn for HPLC analyses.

Lignin samples were dissolved at the concentration of 1% (w/v) in 0.1 M NaOH and then diluted at 0.1% (w/v) in 50 mM sodium acetate, pH 5.0 (the pH was corrected with 0.1 M HCl, if not differently specified). Under these conditions, all technical lignin solutions appear homogeneous with no evidence of precipitate. Incubations were carried out in a final volume of 1 mL: to each sample, different combination of enzymes (0.2 U/mg lignin in 50 mM sodium acetate pH 5.0) and mediators (2 mM final) were added. When Lf-ClP was used, 0.2 mM of H_2O_2 and 200 mM NaCl were added to the reaction mixtures.

2.6 Chemical depolymerization of lignin

Reaction mixtures as described above were subjected to a chemical depolymerizing treatment: formic acid depolymerization was performed adding in a glass tube with screw cap 1 volume of reaction mixture, 1 volume of 98% formic acid and 3 equivalent of sodium formate. The tube was placed in autoclave at 120 °C for 20 minutes. Once cooled at room temperature the reaction mixture was concentrated to 4 mL by vacuum evaporation, centrifuged and analysed.

Hydrogen peroxide treatment was performed adding in a glass tube with screw cap 1 volume of reaction mixture, 2 M of NaOH, 0.88 mmol of H_2O_2, 0.5 volume of MeOH and 0.5. volume THF. The reactions were carried out al 50 °C for 12 hours.

2.7 HPLC analyses

The composition of reaction mixtures from GGE or Me-GGE oxidation before and after the chemical depolymerizing treatment, was determined by HPLC analysis. 20 µL of reaction mixture were withdrawn, added of 40 µL of mobile phase, centrifuged, and 20 µL of supernatant were analysed. HPLC analyses were performed on a Merck Hitachi apparatus with a Phenomenex Luna C18(2) column (5 µm, 4.6 x 250 mm) and 0.1% (v/v) trifluoroacetic acid, 5.1% (v/v) CH_3CN and 32.8% (v/v) CH_3OH in H_2O as mobile phase, at a flux of 0.8 mL/min; detection at 254 nm [13].

2.8 Size-exclusion chromatography (SEC)

The size-exclusion chromatography procedure, described in [13], was used to separate lignin components originating from the screening analyses. The samples were fractionated on a Sephadex G25 column (1.3 x 26.5 cm) at a flow rate of 5 mL/min using an Akta purifier system (GE Healthcare, Milano, Italy), which allows automation of the analysis and the recovery of the eluted fractions. The column was calibrated using PEG at different molecular mass (200-2000 Da). Lignin samples were diluted in 50 mM sodium acetate buffer pH 5.0, 0.1 M LiCl and 0.05% (v/v) Tween-80 (elution buffer) and 2 mL were injected in column.

3. Results

3.1 Lignin fractionation and pretreatments

In order to obtain a homogenous starting material, Protobind lignin was dialysed using a 3 kDa membrane: this simple method generated two fractions. The dialyzed sample is enriched of low molecular weight compounds while the retentate contains the high molecular weight components. The ratio between retentate and dialyzed is approximately 75% – 25% (w/v), respectively. As demonstrated by Gel Permeation Chromatography (GPC) (performed by Gianmarco Griffini, Politecnico of Milano) (Figure 1 A) and confirmed by a simple SEC analysis (Figure 1 B), the separation by dialysis was efficient and it can be scaled-up by employing tangential-flow filter. The obtained lignin fractions were used for the chemo-enzymatic treatments.

Interesting results, were also obtained by lignin sonication as pretreatment: with the final aim to improve the solubility and the dispersibility of lignin in aqueous environment [15], Protobind lignin was sonicated for 2 hours in a ice/water bath. Interestingly, an augmented solubility and dispersibility was apparent (Figure 2). The use of this pretreatment was targeted to decrease the use of cosolvents or surfactants during lignin degradation reactions.

3.2 Enzymatic and chemo-enzymatic treatment of β-O-4 lignin model compounds

The oxidative (Figure 3) and ligninolytic activities of different enzymes in the presence of different mediators was evaluated on two dimeric lignin model compounds GGE (previously reported in [13]) and Me-GGE. Different laccases were used in presence of TEMPO, ABTS and HBT as mediator. The reaction was followed by both colorimetric

screening and HPLC analysis. Concerning the reaction on GGE as substrate, the colorimetric screening showed a time-dependent increase in absorbance at 450 nm up to 4 hours of incubation when Tv-L was added to reaction mixture. HPLC analyses showed, together with the disappearance of GGE peak, the formation of both peaks corresponding to low molecular weight compounds and a number of peaks corresponding to dimeric and trimeric polymerization products (data not show). Indeed, the formation of low molecular weight compounds seems to be transient, because of the repolymerization reaction catalysed by the laccase that results in an uncontrollable reaction with the formation of precipitate. In order to prevent this drawback, BALL was used since it shows a low affinity for phenol compounds and this could prevent the repolymerization. When BALL was used in combination with TEMPO as mediator, the repolymerization process was slowed-down, but not fully eliminated: the formation of 2 peaks (retention time of 4.9 and 10.9 min), presumably corresponding to different keto-products, were observed (Figure 4 A).

A different scenario was apparent when Me-GGE was incubated with BALL and TEMPO: in this case, the phenolic group is unavailable to laccases attack and the only way to oxidize the model compound is through the mediator. Although much more slowly, also in this case an increase of absorbance at 450 nm was observed in the colorimetric screening. HPLC analyses (Figure 5 A) showed the formation of peaks that, based on retention time, we suppose to correspond to veratryl aldehyde (retention time = 6.7 min) and oxidised form of Me-GGE (retention time = 14.4 min - 10% yield in 24 hours) coupled with the partial disappearance of Me-GGE peak (retention time = 12.8 min). GC-MS analysis of the reaction products is under investigation.

The chemical degradation of enzymatic treated GGE and Me-GGE was performed in order to compare two different oxidation methods: a chemical oxidation reported in literature and an enzymatic oxidation proposed by us. When GGE was chosen as substrate, two chemical treatments were used: the formic acid [5] and the NaOH / H_2O_2 [6] treatment. In both cases the formation of low molecular weight compounds was observed: peaks with a retention time of 2.2 and 5.5 min –for NaOH/H_2O_2 treatment - and at 3.1 and 5.7 min – for formic acid treatment - were apparent in the HPLC chromatogram. Anyway, based on the area of HPLC peaks, a low yield of the chemo/enzymatic treatment is apparent. On the other hand, the chemical degradation

by formic acid of enzymatically treated Me-GGE result into 3 major peaks (Figure 5 B - retention time of 2.2, 3.5 and 7.6 min) in addition to other minor peaks: the overall yield seems higher than the one obtained with its non-methylated analogue. The peak corresponding to the Me-GGE (retention time= 12.8 min) is still observed also after chemical depolymerisation: this confirmed the hypothesis of [5] thus suggesting that the enzymatic oxidative step needs further improvement.

3.3 Enzymatic and chemo-enzymatic treatment of technical lignins

Based on the results obtained with lignin model compounds, enzymatic treatment of three different technical lignins by different enzymes and in the presence of different mediators was evaluated. In order to identify the best conditions for lignin oxidation a colorimetric screening was used: different combination of laccases/mediator were thus tested. The combinations used in the different trials are summarized in Table 2.

Different promising conditions, were taken into account and on these samples SEC analyses were performed using a Sephadex G-25 column. The sole enzymatic treatment of technical lignins with different combinations of enzyme/mediator resulted in an oxidation of lignin, as it is evident by the iperchromic effect at 280 nm, see [13] (data not show). The subsequent treatment of oxidised lignins with formic acid lead to the depolymerization of lignin: SEC analysis showed the generation of low molecular weight compounds (Figure 6, see peaks at an elution volume of 50-80 mL) only when lignins were pretreated with laccases. This result indicates that the synergistic effect of the enzymatic treatment and the chemical oxidation facilitates lignin depolymerization. GC-MS analysis are under investigation to demonstrate the products generated by the treatment. A preliminary GC-MS result (performed by Paola D'Arrigo, Politecnico of Milano) obtained by a sample of formic acid treated Kraft lignin (5 mg) after enzymatic oxidation in presence of 2 mM of TEMPO and 1 U of LAC C has shown the formation of 23.1 μg and 29.4 μg of dimethylbenzaldehyde and diterbutylphenol, respectively. Notably, a lower amount of the same molecules were obtained when Kraft lignin was treated with formic acid (only 18.2 μg and 13.3 μg, respectively): this suggests that laccase pretreatment could promote a more efficient degradation of lignin during the chemical degradation step.

Interestingly, the combination of lignin/mediator/enzyme seems to be a crucial choice for obtaining an efficient depolymerisation process. Of particular interest is the reaction that involves Protobind lignin: samples that were enzymatically pre-treated with BALL or Lf-ClP, after chemical hydrolysis did not show any degradation product. On the contrary, the incubation with the two enzymes simultaneously lead to the formation of low molecular weight products (Figure 6 C). This effect could be due to the peculiar structure of Protobind lignin that contains a low amount of β-O-4 linkages.

From estimation of the amount of degradation products, we can conclude that the yield obtained by chemical oxidation of lignin by [5, 6] is higher than the one we obtained with the chemo-enzymatic process. Further optimization of both enzymatic and chemical steps seems crucial to compete with the chemical process.

4. Discussion

Lignin degradation is one of the more challenging topics for chemists, biologists, engineers and, in particular, biotechnologists. Nowadays, a biocatalytic process for lignin depolymerization is not available yet for biorefineries and industrial uses. In order to develop an economically suitable method for lignin degradation several drawbacks must be solved. Degradation methods based on the use of fungal and microbial strains or enzymes alone have been reported but the low efficiency, in particular on polymeric lignin [16-19], make them inappropriate to large-scale use. In comparison, the chemicals methods for degradation present higher yield, but at the cost of high energy-consuming and environmental issues. Lignin degradation requires the use of different enzymatic activities [9, 20, 21] but also the support of chemistry: here we propose a chemo-enzymatic route for lignin degradation.

The process is made of two steps: the enzymatic oxidation of lignin catalysed by laccases in presence of different mediators and the chemical degradation of oxidised lignin by formic acid. This method shows different advantages: the oxidation step can be performed in mild conditions and without employ strong acidic oxidants. The only chemical reagent used for the subsequent degradation is formic acid, that could be partially recovered at the end of the process and is quite cheap in comparison to other solvents used in other processes.

The efficiency and applicability of this route on lignin linkage model compounds and on some technical lignins have been established: different lignins, differing in chemical composition and properties, were partially degraded into low molecular weight compounds, as show by SEC analyses. It is also interesting the effect on the yield of degradation exerted by different enzyme/mediator couples in the oxidation step,. On this side, the case of Protobind degradation by oxidation with BALL and Lf-ClP is emblematic. The enzymatic treatment is sometimes not necessary to obtain a degradation effect (e.g. in the case of organosolv lignin) but anyway it promotes a more efficient degradation. On this side, chloroperoxidases (interesting enzymes for biocatalysis [22]) favour the degradation [23] of the most recalcitrant of lignin sample in our possess.

For sake honesty, we need to clarify that further optimization is required on both steps (e.g., by identification of environmentally-friendly mediators, set-up of a one-pot reaction and optimization and use of enzyme mixtures). Anyway, this report demonstrates that the chemo-enzymatic route for degradation of lignin is feasible.

5. Acknowledgements

This work was done as part of the ValorPlus project that has received funding from the European Union's Seventh Framework Programme for research, technological development and demonstration under grant agreement no FP7-KBBE-2013-7-613802 to LP. We thank ASA Spezialenzyme GmbH for the generous gift of Lac and Pmsp1 enzymes. FT is a PhD student of the "Dottorato in Biotecnologie, Bioscienze e Tecnologie Chirurgiche" at Università degli studi dell'Insubria. We thank Gianmarco Griffini for GPC analyses, Paola D'Arrigo and Chiara Allegretti for GC-MS analyses and Elisa Vignali for technical support.

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Table 1 Laccases and peroxidases used in this work.

Abbreviation	Source organism	Enzyme type	Supplier	Product number or reference
BALL	*Bacillus licheniformis*	Laccase	Our production	[10]
Lf-ClP	*Leptoxyphium fumago*	Chloroperoxidase	ASA Spezialenzyme GmbH	2525
LAC C	*Trametes versicolor*	Laccase	ASA Spezialenzyme GmbH	[10]
OB-1	variant from *basidiomycete* PM1	Laccase	Our production	[10]
Tv-L	*Trametes versicolor*	Laccase	Sigma-Aldrich	[10]

Table 2 List of best conditions (lignin/enzyme/mediator) identified by the colorimetric screening assay and analyzed by SEC chromatography.

Technical lignin (amount)	Enzyme(s)	U_{tot}	Mediator	[Mediator] (mM)	Volume (mL)
Kraft – (50 mg)	LAC C	10	ABTS	2	50
Indulin AT – (50 mg)	OB-1	10	ABTS	2	50
Protobind – (50 mg)	BALL Lf-ClP	10 10	TEMPO	2	50
Organosolv – (5 mg)	Tv-L	1	TEMPO	2	5

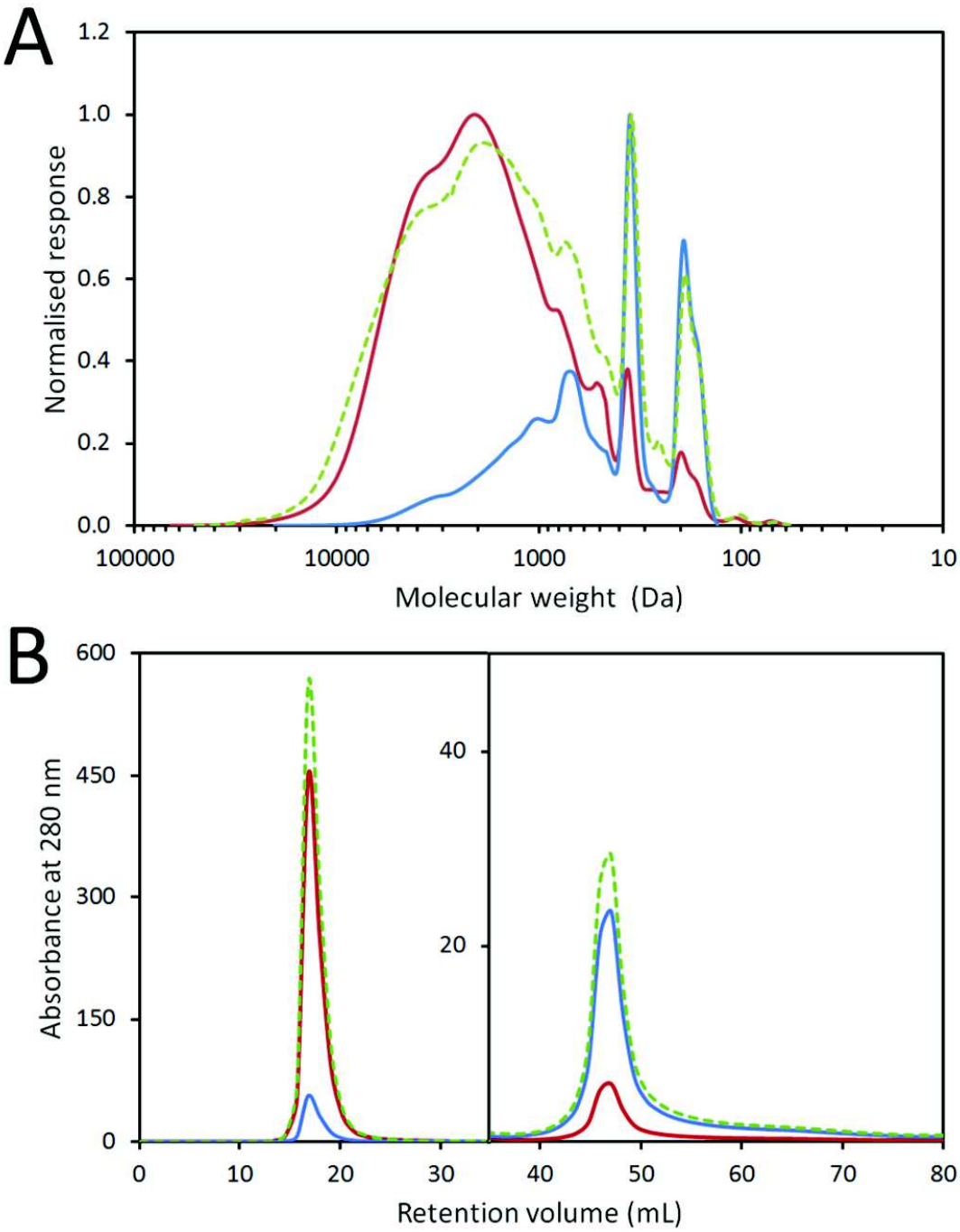

Figure 1 Comparison between **(A)** gel permeation chromatography (GPC) and **(B)** SEC chromatograms of fractionated Protobind lignin: Protobind lignin not fractionated (dotted green line), rententate fraction (red line), dialysate fraction (blue line).

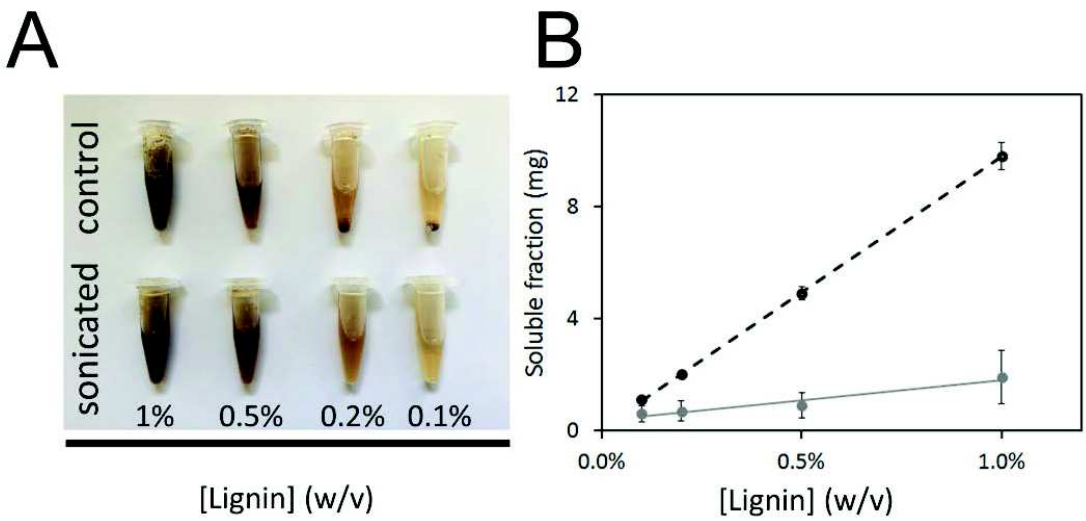

Figure 2 Analysis of sonicated lignin: (**A**) samples at different concentration of sonicated and untreated Protobind lignin; (**B**) solubility of sonicated (dotted black line) and untreated (continuous grey line) Protobind lignin.

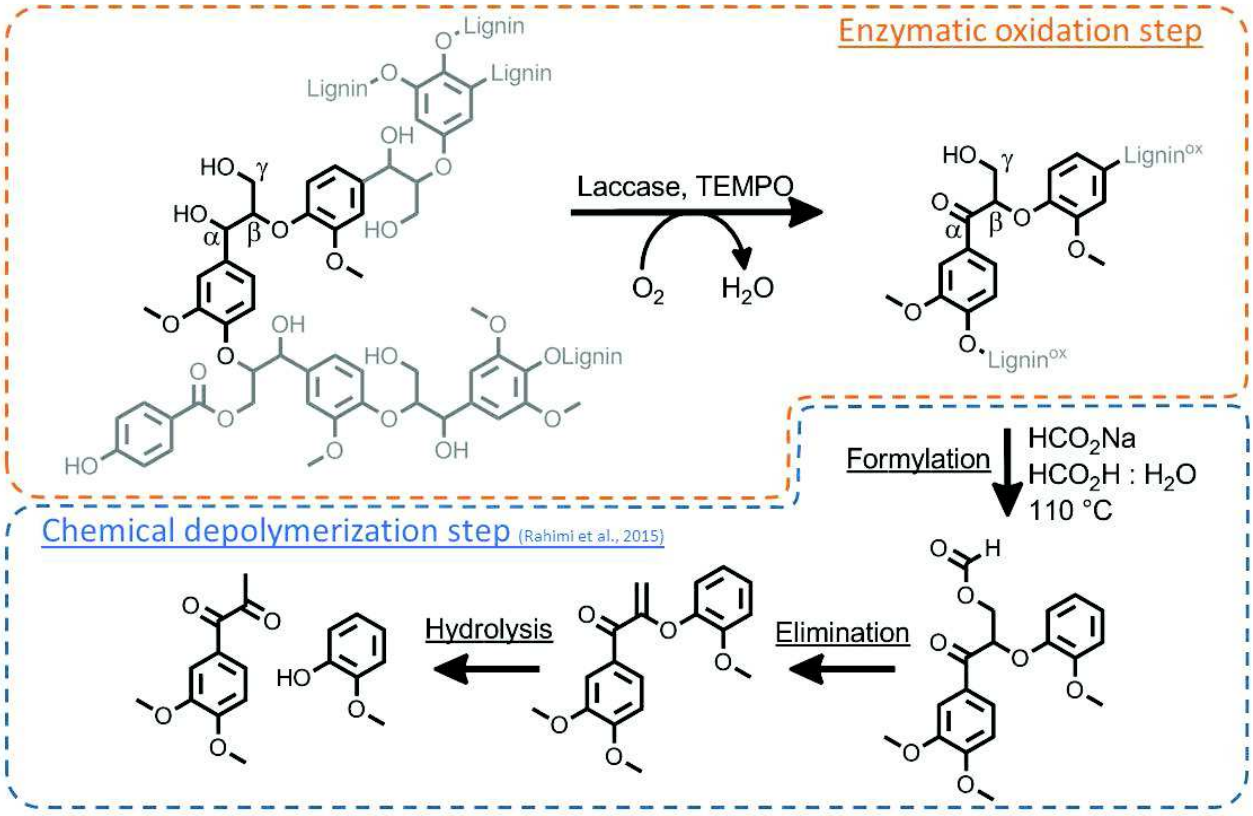

Figure 3 Proposed reaction of oxidation of β-O-4 lignin with laccases mediated by TEMPO and subsequential chemical depolymerisation reaction using formic acid.

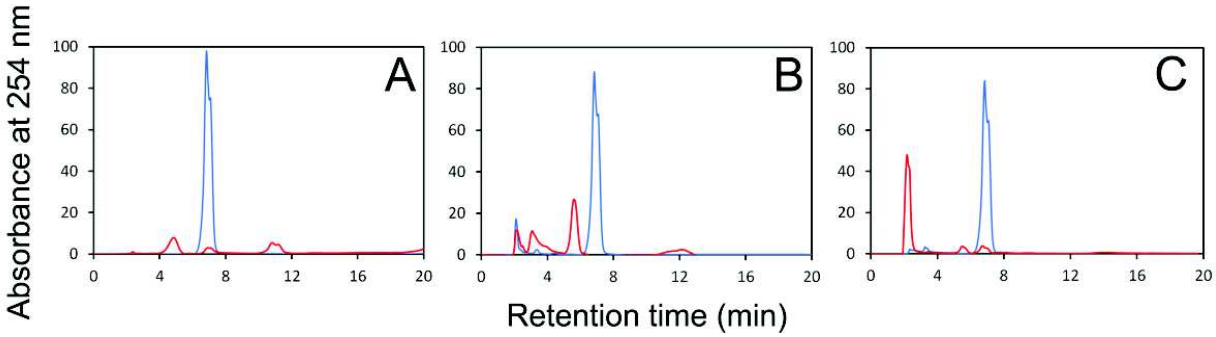

Figure 4 HPLC analyses of reaction mixtures of: (**A**) 10 mM GGE previous (blue line) and after 4 h of incubation with BALL (0.4 U/mL) and 2 mM TEMPO at 25 °C in 50 mM NaAcO buffer, pH 5.0 (red line); (**B**) GGE (blue line) and enzymatic treated GGE (red line) after chemical treatment with formic acid (1 volume) and 3 equivalent of sodium formate at 120 °C for 20 minutes; (**C**) GGE (blue line) and enzymatic treated GGE (red line) after chemical treatment with 2 M NaOH and 0.88 mmol H_2O_2 at 50 °C for 12 hours.

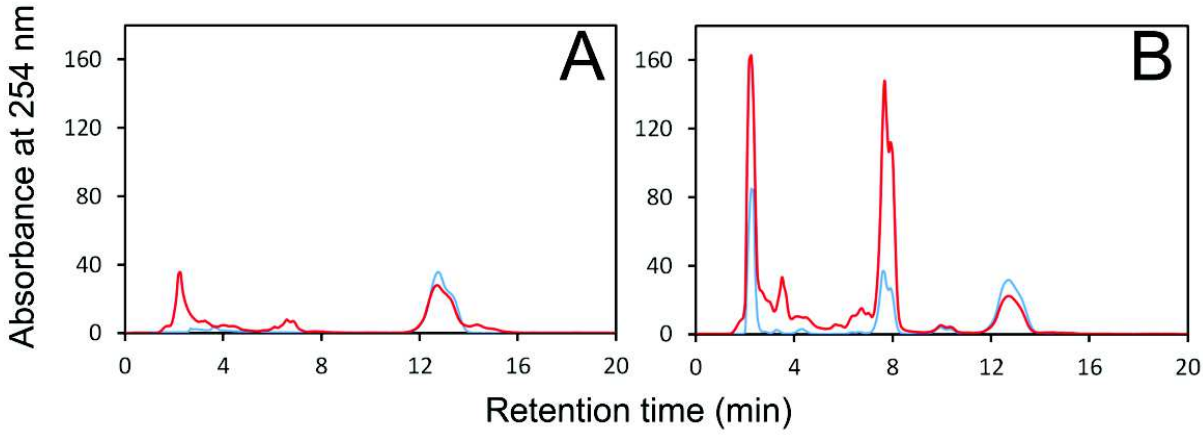

Figure 5 HPLC analyses of reaction mixtures of: (**A**) 10 mM Me-GGE previous (blue line) and after 4 h of incubation with BALL (0.4 U/mL) and 2 mM TEMPO at 25 °C in 50 mM NaAcO buffer, pH 5.0 (red line); (**B**) Me-GGE (blue line) and enzymatic treated Me-GGE (red line) after chemical treatment with formic acid (1 volume) and 3 equivalent of sodium formate at 120 °C for 20 minutes.

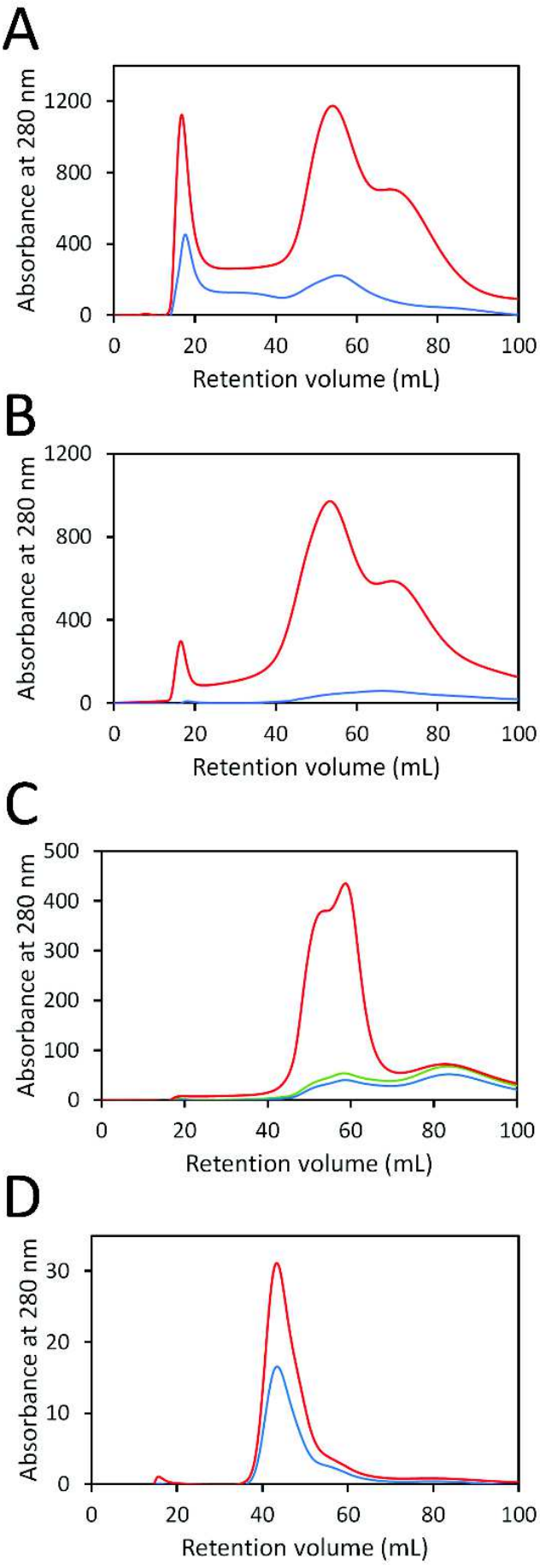

Figure 6 SEC analyses of reaction mixtures of: (**A**) Kraft lignin (blue line) and LAC C treated Kraft lignin (red line) after chemical treatment with formic acid; (**B**) Indulin AT lignin (blue line) and OB-1 treated Indulin AT lignin (red line) after chemical treatment with formic acid; (**C**) Protobind lignin (blue line), BALL treated (green line) and BALL / CPO treated Protobind lignin (red line) after chemical treatment with formic acid; (**D**) Organosolv lignin (blue line) and Tv-L treated Organosolv lignin (red line) after chemical treatment with formic acid. Chemical treatments were performed adding 1 volume of formic acid and 3 equivalent of sodium formate to 1 volume of enzymatic reaction mixture, at 120 °C for 20 minutes. In all cases, enzymatic reaction were setup as indicated in Table 2.

4. Discussion

“You can make anything you want out of lignin... except money”: this was cited in 2007 [1] and is a frequently heard joke in the scientific community.

Indeed, the growth of the cellulosic fuel industry has created a stream of lignin that the industry (as well as the society) needs to find valuable ways to use for commercial applications. At the same time, federal agencies and industries are funding research to simplify the process of taking biomass to fuels. Today, lignin is principally destined to energy production via combustion to supply part of power requirements in ethanol biorefineries. Ideally, lignin could become much more valuable to industry as it represents the only renewable source of phenol compounds and could be used for the production of low-molecular-weight chemicals.

In nature, lignin degradation is a multi-enzymatic process: four classes of oxidases, acting through a radical-mechanism, are involved. In addition, several accessory enzymes play an important ligninolytic role. Understanding the role of each enzymatic activity and of small molecule mediators in lignin degradation is crucial to make a step-forward in this technology.

The first part of this project focused on the preparation of a tool-box of enzymes which activity could be used for *in vitro* lignin degradation. In addition, because of the possibility to use different enzymes belonging to the same class, it is of utmost relevance to evaluate the specific working conditions of each enzyme. In this respect, different approaches were used: at first, different commercial enzymes were biochemically characterized, in order to assess their general features. In addition, interesting enzymes identified by literature evaluation were expressed in a recombinant form in *E. coli* or *S. cerevisiae*. A particular interest has been paid into laccases, as they are important biocatalysts in a number of applications: the properties of ten different laccases have been investigated under identical conditions. The influence of temperature, of pH and of the presence of sodium chloride, DMSO and surfactants on enzymatic activity were taken in account.

The outcome of this study was a comparative overview of the enzymes in our tool-box. In details, the bacterial laccase from *B. licheniformis* showed peculiar features in comparison to the other tested enzymes since its activity on phenolic and nonphenolic substrates is modulated by changing changes in the pH (the higher activity on 2,6-DMP and ABTS was observed at pH 7.0 and 4.5, respectively). Moreover, it possesses a good activity and stability at high temperature and is not significantly affected by sodium chloride and Tween-80. A further bacterial enzyme showing peculiar features is the membrane-bound polyphenol oxidase A from *Marinomonas mediterranea* (MmPPOA): it differs from prototypical laccases since it oxidizes substrates of both laccases and tyrosinases. Three MmPPOA variants were expressed in *E. coli* in a recombinant form*:* the full-length enzyme, the corresponding His-tagged form and a His-tagged variant lacking 58 residues at the N-terminal end which correspond to the putative sequence for membrane binding. Purification processes were set up for both His-tagged rMmPPOA variants. The kinetic and stability properties, the high thermostability and the peculiar substrate specificity render the recombinant rMmPPOA-695-His (full length variant) well suited for biotechnological applications. Notably, the removal of the signal peptide generated a soluble enzyme form and strongly affected the enzymatic activity (a lower enzymatic activity and substrate affinity was apparent): anyway this enzyme could represent a suitable starting point for the evolution of soluble polyphenol oxidase variants.

During this phase, novel enzymes were also taken into account: a novel peroxidase produced by a novel species belonging to the *Nonomuraea* genus was discovered and characterized. *N. gerenzanensis* peroxidase shows a dye-decolorizing activity that expands its substrate range; dye-decolorizing peroxidases were recently reported as bacterial lignin degrading enzymes [2, 3]. Furthermore, *Nonomuraea* peroxidase could be interesting for different industrial sectors, such as the textile (for bleaching) and dye industry. To the best of our knowledge, this is the first report on the lignin-modifying activity from a microorganism belonging to *Nonomuraea* taxon.

The main result of this first part of the project was the preparation of a panel of enzymes, differing each other in terms of activity and stability. Anyway the activity of each enzyme on lignin-like compounds was still an unexplored field. The analysis of the behaviour of the tool-box components on different substrates and in several

conditions is unfortunately too laborious and time-expensive, also for the modern analytical methods. For this reason, a colorimetric screening assay based on the reaction of carbonyl groups of the reaction substrates with DNP was developed. The rational of this assay arises from the observation that in different proposed mechanisms of lignin degradation the first step consists in the oxidation of alcoholic groups of lignin to carbonyl groups. In addition, many degradation products of lignin, reported in several studies concerning both chemical and biological depolymerisation, contains a carbonyl group (e.g. veratryl aldehyde, etc.).

The lignin modifying activity of all the enzymes of the tool-box was thus evaluated by a novel optimized colorimetric screening assay: at first, ligninolytic activity was assayed on 2 model substrates; then, the activity on 4 different technical lignins was evaluated. Interestingly, some optimal conditions were identified. As an example, the combination of enzyme, mediator and technical lignin used seems to be crucial for the assay outcome. Successful elements of this screening protocol are: i) the applicability on different compounds and conditions, since it does not interfere with the enzymatic reactions, ii) it does not require pre-modification of lignin as required by the few screening methods available to date, iii) it requires a low amount of substrates and, iv) it could provide both qualitative and quantitative results. This novel colorimetric screening assay represents a new starting point for this research: accordingly, all the tool-box components were subjected to screening evaluation on lignin-model compounds and technical lignins.

In order to validate the ligninolytic activities observed by the colorimetric screening assay and identify the molecular weight composition of treated lignins, a water-based size-exclusion chromatography (SEC) was set-up. By this method, the reaction mixtures can be quickly analysed without further extraction or chemical treatment (that could results into a loss of material). Although it is not as effective as a traditional gel permeation chromatography (GPC) method used in lignin analyses, the possibility to recover the low molecular weight fractions, the lack of solvents and the simplicity of analysis make it an additional tool for scientific studies concerning lignin modification. The SEC analyses of different reaction mixtures of technical lignins treated with mediators and laccases show the formation of oxidation compounds while only in few reactions the formation of low molecular weight compounds was observed. This result

was confirmed by HPLC analysis of reaction products of β-O-4 linkage model compounds treated with laccases: the main reaction consists in the oxidation of GGE and Me-GGE due to both laccase radical oxidation and TEMPO.

Different experimental evidences highlighted that the enzymatic treatment alone is not suitable for an industrial use: for this reason, we focused on a chemo-enzymatic depolymerisation. Formic acid depolymerisation is largely known and diffused [4, 5]: recently, Rahimi et al. [6] reported an efficient depolymerisation method of oxidised lignin using formic acid. This treatment represents an optimal candidate for the use in combination with laccase-mediated oxidation since it has a low cost, it is biocompatible and it gives high yield of depolymerisation. Moreover, the TEMPO mediated chemical oxidation method used by [7] can be replaced by the enzymatic oxidation since laccases are able to oxidise this widely used mediator. Using this method, we have demonstrated that the chemo-enzymatic treatment of four different technical lignins resulted into their partial depolymerisation into low molecular weight compounds.

To the best of our knowledge, this is the first report that uses this approach reaching a reasonable depolymerisation level.

In general, lignin valorisation methods aimed to the production of high-value compounds are attracting a great attention from the scientific community and industries [8]. The structural complexity and general recalcitrance of lignin could be addressed by a cascade approach for lignin valorisation, entailing a depolymerisation process that consists of several stages. One step of this process can be addressed by an enzymatic treatment and, accordingly, the selected examples reported in this project clearly exemplifies how the combination of chemistry and biology can make a step forward regarding this technology.

Keeping in mind that the lignin valorization may only effectively reach the market if cheap, efficient and “green” [8], the study and evolution of ligninolytic enzymes through different approaches (novel enzyme discovery and protein engineering) can lead to interesting results pushing these technologies. In particular, the knowledge of the enzyme/lignin interaction from a kinetic point of view, can allow the choice of a depolymerisation route based on a deterministic basis.

I would like to conclude this discussion citing Xu [8]: “Scientist around the world should also join forces in this fascinating crusade of lignin depolymerisation aiming to set multidisciplinary teams to come up with more innovative solutions for betterment of future generations”.

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