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Novel multienzyme oxidative biocatalyst for lignin bioprocessing

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ABSTRACT

A novel multienzyme biocatalyst, based on coimmobilization of the laccase and horseradish peroxidase by cross linking and layer-by-layer coating with polyelectrolyte, was designed, synthesized and applied at the development of an oxidative cascade process on lignin. The efficiency and specificity of the new LbL-multienzyme system, the occurrence of a synergy of the co-immobilized enzymes, the lignin oxidation pathway and the nature of the structural modifications occurred in treated lignins have been investigated in the present effort by means of GPC analysis and quantitative ^{31}P NMR techniques.

1. Introduction

Today the rising energy consumption and the depletion of fossil fuel feedstocks have focused the attention on the use of alternative renewable materials and on the development of environmentally friendly processes that operate in mild reaction conditions.

Lignin is the second most abundant organic polymer in plant kingdom and constitutes up to 30% of wood.[1] It constitutes to date the bottleneck to the development of integrated biorefinery since it is the residue of modern saccharification processes. Current bioethanol production from wood originate about 500 g of lignin each liter of bioethanol. From this viewpoint the development of processes of lignin upgrade through oxidative depolymerization or functionalization is mandatory.[2–4]

In Nature the selective oxidation of lignin is carried out by white-rot basidiomycetes fungi that produce a pool of extracellular ligninolytic enzymes such as laccases and peroxidases.[5,6] In particular laccases can easily oxidise phenolic groups[7] and in presence of radical mediator, such as 1-hydroxybenzotriazole, their reactivity can be extended towards other functional groups as phenyl–aryl ethers.[8] Mn-peroxidase and lignin peroxidases are able to oxidize lignin at the phenolic and non-phenolic aryl–ether positions respectively.[9–16]

Laccases and peroxidases constitute an interesting tool for the development of alternative oxidative processes due to their low substrate specificity and relatively wide pH of action. In the last years both laccases and peroxidases have been used in several biotechnological applications such as oxidation of organic pollutants, pulp delignification, bleaching and development of biosensors or biofuel cells. Unfortunately, the exploitation of their potentiality is prevented, especially in the case of peroxidases, by their low stability.[17]

There are a number of constraints in the use of the enzymes; the common perception is that enzymes are sensitive, unstable and have to be used in water, features that are not ideal for a catalyst and undesirable in most syntheses.[12]

Several approaches have been proposed to overcome these limitations; among them immobilization is generally considered favorable for industrial scale applications since it allows for continuous processes.[13] The basic requirement for the development of economically sustainable enzymatic processes are the possible recycle of the catalyst and a high stability of the enzyme. As such a number of different immobilization techniques have been developed.[17–19]

The layer-by-layer (LbL) adsorption technique, introduced by Decher et al.[20] is a general and versatile tool for the controlled fabrication of multimaterial surface coatings on a large variety of surfaces.[21] By means of this technique the construction of multilayer films is possible by the consecutive deposition of alternatively charged polyelectrolytes on a solid surface.[21] The LbL technique has been demonstrated to be an effective means for the immobilization of enzymes.[22] In fact, polyelectrolyte coatings have the ability to protect immobilized proteins from high-molecular-weight denaturing agents or bacteria and allow regulation of the permeability

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towards small substrates, which can enter and leave the protecting layers to react with the biomolecules in the interior.[23]

Another key aspect of enzyme immobilization is the possibility to perform multi-enzyme immobilization with the development of multi-enzyme or chemoenzymatic cascade processes.[24]

Biocatalysis is becoming a transformational technology for chemical synthesis as a result of recent advances in large-scale DNA sequencing, structural biology, protein expression, high throughput screening, and enzyme evolution technologies. To truly impact chemical synthesis at the industrial scale, enzyme discovery, biocatalyst optimization, process design and development must be integrated in order to deliver cost-effective and green chemistry solutions for processes. Domino or cascade reactions involve the transformation of materials through several non-separable steps in a concurrent fashion, which often proceed via highly reactive intermediates. These processes show a remarkable synthetic advantage despite the fact that the cascade is proceeding through one (or more) highly unstable intermediate(s), which are prone to decomposition reactions, the final product can often be isolated in good yields, because undesired side reactions of the reactive intermediate are largely avoided since the intermediate is transformed in the same instant as it appears.[25]

Recently, significant efforts have been made to develop organocatalytic cascade reactions with the objective to mimic the biosynthetic strategy, but to date the innovative potential of biocatalysis by promoting the multistep catalytic concept by multienzyme systems has not been fully explored.

Most of the multienzyme systems synthesized to date were targeted towards sensors development[26–33] however, examples dealing with preparative cascade processes have been described.[34–36] Many immobilised HRP and laccase have been reported in literature.[37–46]

Recently both laccase[47,48] and horseradish peroxidase[49] were immobilised onto alumina supports and coated by the LbL technique with polyelectrolytes. The stability of such systems was found significantly enhanced upon coating. As a consequence the reactivity toward lignin oxidation was found increased, probably due to the enhanced stability.

In this work, novel multienzyme biocatalysts were developed for bioprocessing applications.

More specifically, the present study describes a new process for the co-immobilization of oxidative enzymes by cross linking and layer by layer coating and their application to the development of an oxidative cascade process on lignin. The efficiency and specificity of the new multienzyme system, the occurrence of a synergy of the co-immobilizsed enzymes, the lignin oxidation pathway and the nature of the structural modifications occurred in treated lignins have been investigated in detail.

2. Results and discussion

2.1. Enzymes immobilization and coating

Laccase and HRP were immobilized onto 2 mm alumina particles suitably silanized and activated by glutaraldehyde treatment. The two enzymes were both singularly and co-immobilized in order to subsequently point out the possibility of different behavior of the LbL multienzyme biocatalyst from the separately immobilized enzymes (Fig. 1). At pH 6 the enzymes are both negatively charged; it was thus possible to coat them by use of a first layer of polyallyl amine hydrochloride (PAA). Overall three layers of PAA and polystyrene sulfonate were coated on both singularly supported laccase and HRP and on the multienzyme biocatalyst.

Figure 1 shows the SEM of the multienzyme biocatalyst before (A) and after (B) LbL coating. It is evident that the polyelectrolyte coating leaves pores on the surface of the average dimensions of 100 μm. Such pores allow substrate diffusion to the enzyme active site.

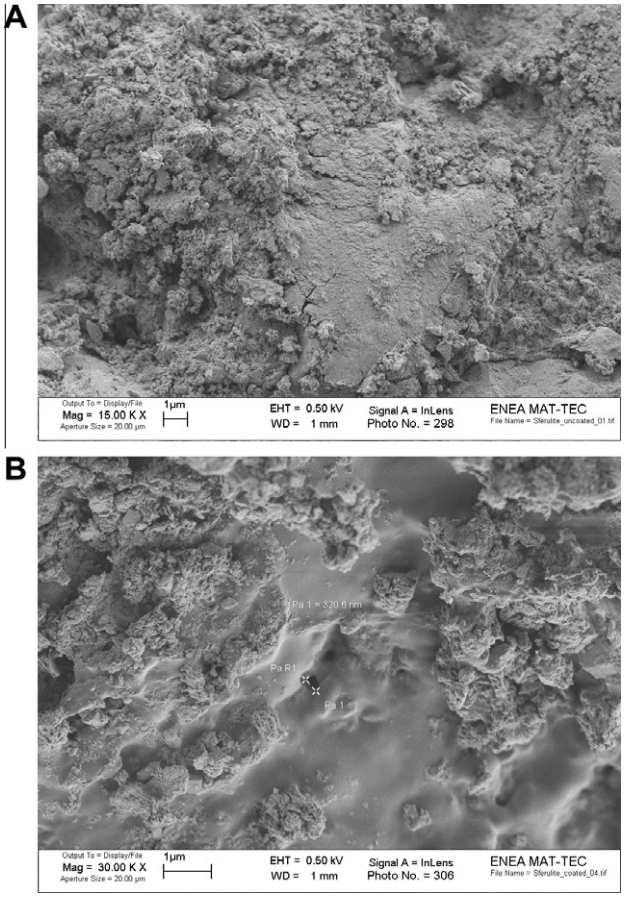


Figure 1. SEM images of multienzyme biocatalyst before (A) and after (B) LbL coating.

The amount of immobilized enzyme was evaluated spectrophotometrically by the Bradford assay, analyzing the residual enzymatic content in the waste waters after the reaction of immobilization and successive washings. About 90% of both enzymes was successfully immobilized respect to starting material, in accord with previously reported results for the chemical immobilization of enzymes on alumina pellets under similar experimental conditions.[50–52]

Figure 2 shows the immobilization efficiency as evaluated by the residual enzymatic activity after immobilization and coating. Under these conditions it was found always higher for HRP than for laccase. After the deposition of a triple layer of polyelectrolytes,

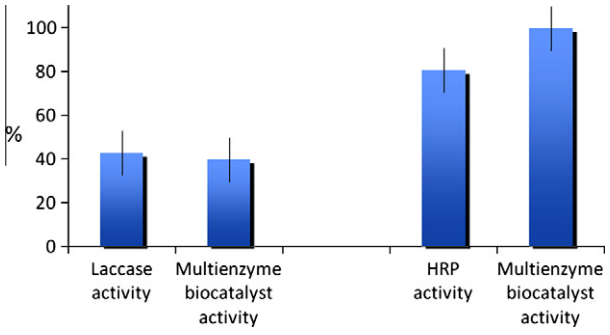


Figure 2. Enzymatic activity after immobilisation and coating.

the immobilized enzymes retained about 40% and 90% of activity with respect to the parent enzymes, laccase and HRP, respectively. The presence of another co-immobilized enzyme, in the case of the multienzyme biocatalyst, did not significantly modify the immobilization efficiency.

2.2. Enzyme stability

The enzyme stability was evaluated by 12 h reaction batches as previously reported 46. It is noteworthy that the residual activity after 10 cycles was found much higher in supported systems rather than in soluble enzymes even in the multienzyme biocatalyst (Fig. 3). This is in accord with the previously hypothesized role of the polyelectrolyte coating as protection from enzyme deactivation.

2.3. Wheat straw milled lignin enzymatic oxidation

Very In order to systematically investigate the efficiency and reaction pathways of laccase and HRP treatments on wheat lignin (WL), a panel of different experiments was designed. More specifically, with respect to soluble enzymes, wheat milled lignin (WL) was treated with laccase, laccase + HBT as oxidation mediator[7], HRP, HRP + laccase and HRP + laccase + HBT respectively (Table 1 entries 1–5). The percentage conversions of lignin are reported in Table 1. Such conversions were evaluated on the basis of unsoluble residues recovered after treatments. No residual lignin was found adsorbed on the catalysts surface after treatments.There was not lignin on the immobilized enzymes. Enzyme beads (2 mm diameter) were filtered off and washed with water. No lignin was adsorbed on the catalysts surface.

The amount enzymes used were chosen in such a way to have the same lignin conversion yield with laccase and HRP. This approach was designed in order to compare the different enzymes reaction pathways at the same conversion efficiency. Thus the conversions ranged from 14.6% to 17.1% when the lignin was treated with 23 U of laccase or 165 U of HRP (Table 1, entries 1–3) and increased to 23–36% when laccase and HRP were used in association (Table 1, entries 4, 5). This accounts for the occurrence, in the latter cases, of two distinct enzymatic oxidations carried out contemporarily. The conversion yields roughly correspond to the sum of the lignin conversion of each single enzymatic treatment.

A second panel of experiments was designed in the same fashion as described above and carried out with LbL-laccase or LbL-HRP (Table 1, entries 6–10). The conversion of WL treated with LbL-laccase or LbL-HRP (Table 1, entries 6–8) was found higher than that obtained under otherwise identical experimental conditions with free enzymes (Table 1, entries 1–3). This finding was expected on the basis of previous efforts carried out on the oxidation of softwood MWL.[49]

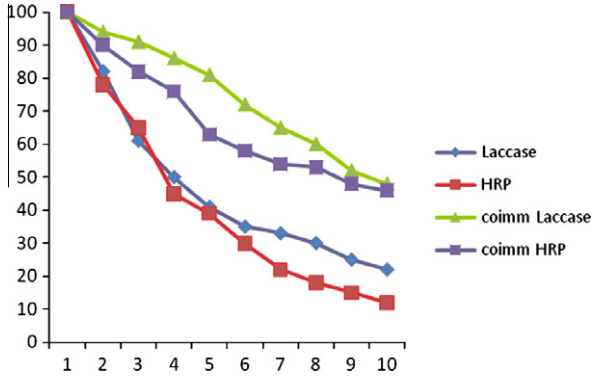


Figure 3. Enzymatic residual activity after 10 successive 12 h batch reaction.

Table 1
Conversion of WL after treatment with free, immobilised and coimmobilised enzymes, in presence or absence of HBT as oxidation mediator

Entry	Biocatalyst (U)	Conversion[e] (%)
1	Laccase [a]	17.1
2	HRP [a]	15.1
3	Laccase + HBT [a,d]	14.6
4	Laccase + HRP [a]	23.1
5	Laccase + HRP + HBT [a,d]	36.4
6	LbL-Laccase [b]	34.7
7	LbL-HRP [b]	41.6
8	LbL-Laccase + HBT [b,d]	58.2
9	LbL-Laccase + LbL-HRP [b]	27.8
10	LbL-Laccase + LbL-HRP + HBT [b,d]	35.3
11	LbL multienzyme system [c]	59.8
12	LbL multienzyme system + HBT [c,d]	61.3

[a] Native enzyme.
[b] Immobilised layer-by-layer enzyme.
[c] Coimmobilised layer-by-layer enzyme.
[d] In some case the reaction was performed in presence of HBT as oxidation mediator (1 mM), under identical experimental conditions.
[e] Conversion is defined as percentage of converted lignin.

Differently from what happened under homogeneous conditions, when WL was treated with the LbL-immobilized enzymes in mixture (Table 1, entries 9, 10) the conversion did not significantly change.

The last experiments were carried out treating WL with the LbL-multi-enzyme system (Table 1, entries 11 and 12). In this latter case the oxidation was slightly more efficient than in presence of the two LbL-immobilised enzymes in mixture.

2.4. WL structural modifications upon enzymatic treatments

Structural modifications in WL were evaluated by GPC analysis of both water insoluble and soluble lignin fractions after enzymatic treatments, respectively.

In Table 2 the data of Mw, Mn and Mw/Mn for WL after each experiment (insoluble fraction) compared with the starting WL are reported. Confirming previously reported results,[48, 49] laccase and HRP treatments induced an increase in the Mn and Mw values, probably due to oxidative coupling processes (Table 2, entries 2 and 3), while the treatment carried out with the laccase-mediator system yielded a residual lignin with a distribution of molecular weight similar to substrate (Table 2, entry 4). When the two free enzymes were used in association, an increase in the molecular weight distribution was found both in the absence and in the presence of HBT as oxidation mediator (Table 2, entries 5 and 6) the increase being less pronounced in the presence of mediator. This confirms that in the presence of HBT two different oxidation

Table 2
Mw, Mn and Mw/Mn of WL recovered after each experiment (insoluble material) compared with the starting material (WL)

Entry	Biocatalyst	Mn	Mw	Mw/Mn
1	—[a]	2.62E + 04	1.69E + 05	6.5
2	Laccase	3.51E + 04	1.87E + 05	5.3
3	HRP	3.58E + 04	2.08E + 05	6.0
4	Laccase/HBT	2.77E + 04	1.84E + 05	6.6
5	Laccase + HRP	3.84E + 04	2.18E + 05	6.0
6	Laccase + HRP/HBT	3.33E + 04	1.96E + 05	5.9
7	LbL-Laccase	1.96E + 04	1.05E + 05	5.4
8	LbL-Laccase/HBT	1.29E + 04	4.21E + 04	3.3
9	LbL-HRP	1.81E + 04	8.28E + 04	4.6
10	LbL-Laccase + LbL-HRP	2.64E + 04	1.72E + 05	3.1
11	LbL-Laccase + LbL-HRP/HBT	2.08E + 04	9.84E + 04	4.7
12	LbL-multienzyme biocatalyst	1.37E + 04	1.14E + 05	6.5
13	LbL-multienzyme biocatalyst/HBT	8.55E + 03	3.52E + 04	4.1

[a] Starting material (WL).

processes can occur in competition, as previously suggested in literature, the former with prevalence of oxidative coupling (that yields ultimately to polymerization) the latter with prevailing side-chain cleavage processes yielding depolymerization,[48] (Scheme 1 routes A and B, respectively). On the contrary, when LbL-laccase was used, the Mn and Mw were decreased with respect to the starting value irrespective to HBT (Table 2, entry 7, 8). This finding confirms that LbL-Laccase oxidizes lignin with a reaction pathway different from the free enzyme, depolymerization prevailing over oxidative coupling processes. (Table 2, entries 7, 8 vs 2, 3 and Scheme 1 routes A and B). Analogous behavior was evident when LbL-HRP was used as oxidative system (Table 2, entry 9). This result is in contrast with the reactivity of an analogously immobilized HRP toward spruce lignin.[49] In that case an increase of Mn and Mw was evident. Such difference could be rationalized considering that WL is less prone to polymerization due to the high content in siringyl units typical for grass lignins that prevents, to some extent, oxidative coupling reactions. Another relevant issue is the enzyme load. In fact HRP is accepted to act mainly by generation of phenoxy radicals that in turn can couple or react by atom abstraction.[16] In the present effort the HRP load was higher and the reaction time longer than used before.[49] Thus the different reaction conditions might have driven the reaction in a different direction.[53] When LbL-laccase and LbL-HRP were used in mixture (Table 2, entries 10, 11) no significant modification of the Mn and Mw of WL were found even in the presence of HBT. On the contrary when the LbL-multienzyme biocatalyst was used, the WL molecular weight distribution was found decreased and the lignin conversion increased. Addition of HBT improved lignin depolymerization and conversion (Table 2, entries 12 and 13). these data suggest that the LbL-multienzyme system is able to depolymerize WL with a efficiency higher than the other biocatalytic systems tested. This implies a possible synergy in the reaction of the co-immobilized enzymes.

The structural analysis of oxidative modification in WL was determined by phosphorus nuclear magnetic resonance technique (^{31}P NMR), able at characterizing and quantifying the labile OH groups present in lignin after the treatment with enzymes.[54–56]

Table 3
Aliphatic OH, phenolic OH groups and carboxylic acids as evaluated by ^{31}P NMR after phosphytilation

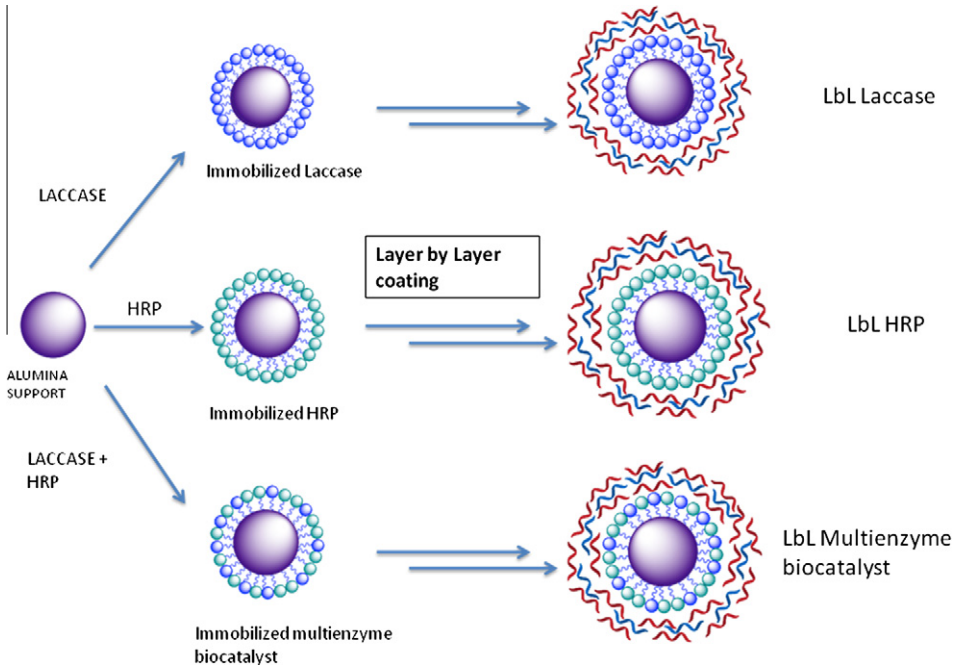
Entry	Biocatalytic treatment/ insoluble lignin [a]	Aliphatic OH (mmol/g)	Phenolic OH (mmol/g)	COOH (mmol/g)
1	Blank[b]	1.46	0.80	0.27
2	Laccase	1.46	0.71	0.25
3	HRP	1.50	0.78	0.30
4	Laccase/HBT	1.49	0.46	0.30
5	Laccase + HRP	1.48	0.51	0.31
6	Laccase + HRP/HBT	1.48	0.53	0.29
7	LbL-laccase	2.49	0.95	0.12
8	LbL-laccase/HBT	2.62	1.09	0.17
9	LbL-HRP	2.84	1.20	0.18
10	LbL-laccase + LbL-HRP	1.99	0.78	0.12
11	LbL-laccase + LbL-HRP/ HBT	2.55	1.10	0.15
12	LbL-multienzyme biocatalyst	1.48	0.54	0.28
13	LbL-multienzyme biocatalyst/HBT	1.15	0.50	0.50

[a] Residual insoluble lignin collected after enzymatic treatment.
[b] Starting material (WL).

After lignin functionalization with 2-chloro-4,4′,5,5′-tetramethyl-1,3,2-dioxaphospholane in pyridine/deuterated chloroform mixture (1.6:1.0, v/v ratio), in presence of cholesterol as internal standard, it is possible to identify and quantify the aliphatic phenolic, condensed and acidic OH groups. The assignment of signals was carried out on the basis of previous works..[54–56]

Table 3 summarizes the NMR data for the oxidation of wheat lignin, carried out with free, LbLimmobilized and LbL-multienzyme biocatalysts.

The content in aliphatic OH groups after treatment with soluble laccase or HRP was nearly unchanged with respect to the starting lignin. Also the content in carboxylic acid moieties did not change significantly. On the contrary, the content of phenolic OH groups decreased upon treatment with free laccase/HBT, free laccase + HRP and free laccase + HRP/HBT. Overall these treatments yielded a lignin that, besides having a higher Mn value, showed



Scheme 1. Scheme of preparation of immobilised catalysts coated by means of layer-by-layer technique.

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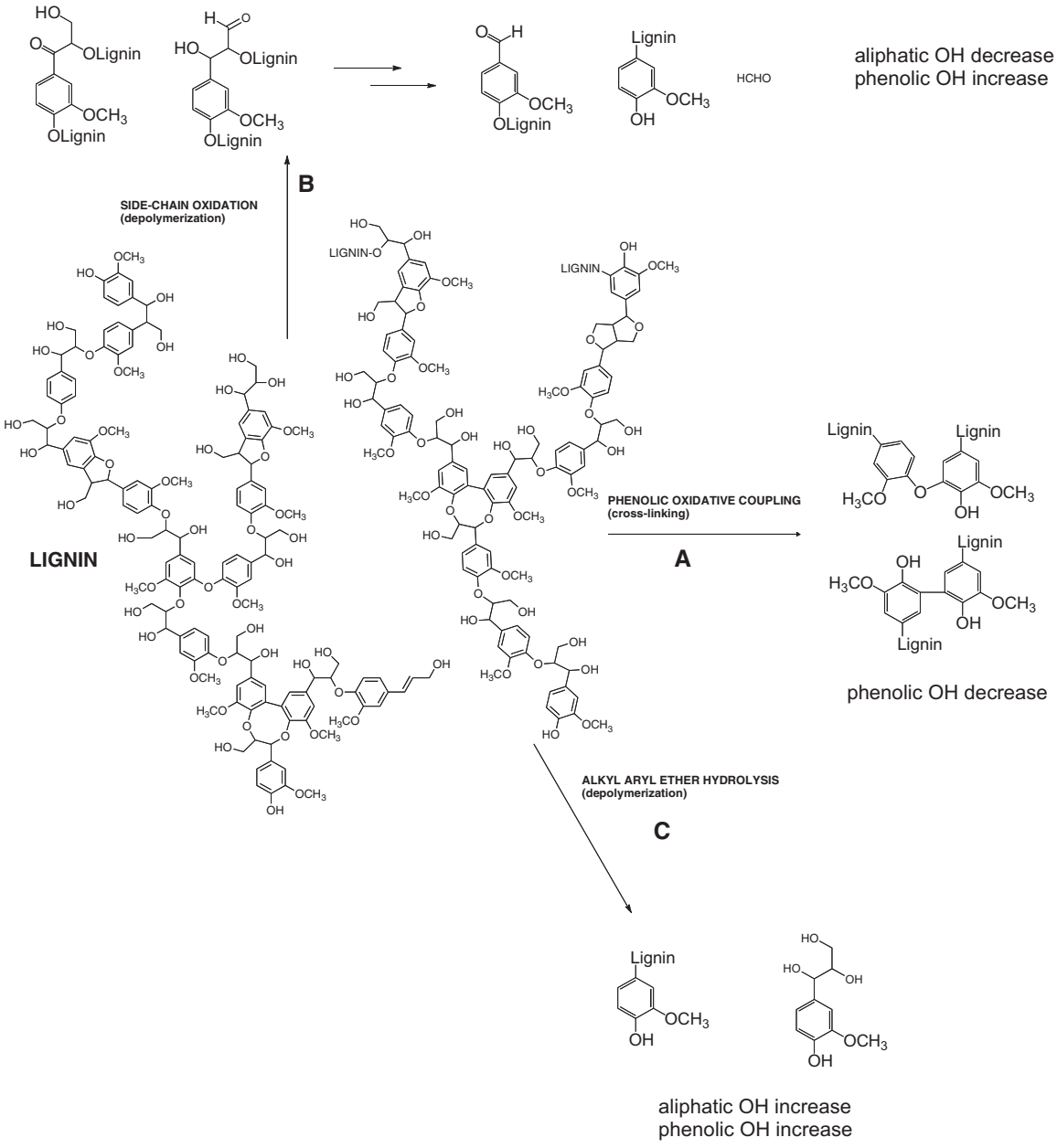

Scheme 2. Lignin oxidation pathways.

an overall lower hydrophilic groups content. This is consistent with an oxidation pathway in which oxidative coupling is the prevalent process (as shown in Scheme 1A). When lignin was treated with LbL Laccase, LbL-laccase/HBT or LbL-HRP both the aliphatic and the phenolic OH groups increased after treatments, while the COOH groups decreased. Under these experimental conditions, lignin was recovered in amount lower than after soluble enzymes treatments (Table 2), and resulted more hydrophilic with a high value of OH content and low value of Mn. Thus, while soluble enzymes catalyze the oxidative coupling of lignin to yield a residual polymer with decreased solubility and increased Mn and Mw, the LbL-enzyme treatments result in a overall depolymerization (higher conversion yields) yielding a more soluble polymer. The contemporary increase of aliphatic and phenolic OH groups and the decrease of Mn and Mw further suggests the presence of a hydrolytic reaction pathway associated to oxidative processes (as shown in Scheme 2C). A similar behavior was observed with LbL-laccase and LbL-HRP used in mixture. (Table 3 entries 10–11). When the LbL-multienzyme biocatalyst was used, the amount of aliphatic OH groups did not vary significantly, while the phenolic OH groups were found decreased. In the presence of HBT, a significant reduction of both aliphatic and phenolic OH groups and an increase in the carboxylic acid content was evident. The latter finding indicates that when the LbL-multienzyme biocatalyst is used, especially in the presence of HBT, the reactivity pattern toward lignin oxidation changes and yields ultimately to a residual lignin characterized by a lower content of hydrophilic groups and Mn and Mw values. This is consistent with a reaction pathway in which the oxidative coupling and side-chain oxidation processes, that result in phenols and aliphatic OH decrease and COOH increase, occur in addition to hydrolytic reactions (Scheme 2 routes A and B vs C).

In order to further investigate the nature of lignin modifications induced by the enzymes, a GPC analysis was carried out for the water soluble fractions. Figure 4A shows the GPC analysis of WL before and after treatments with laccase or HRP. The starting

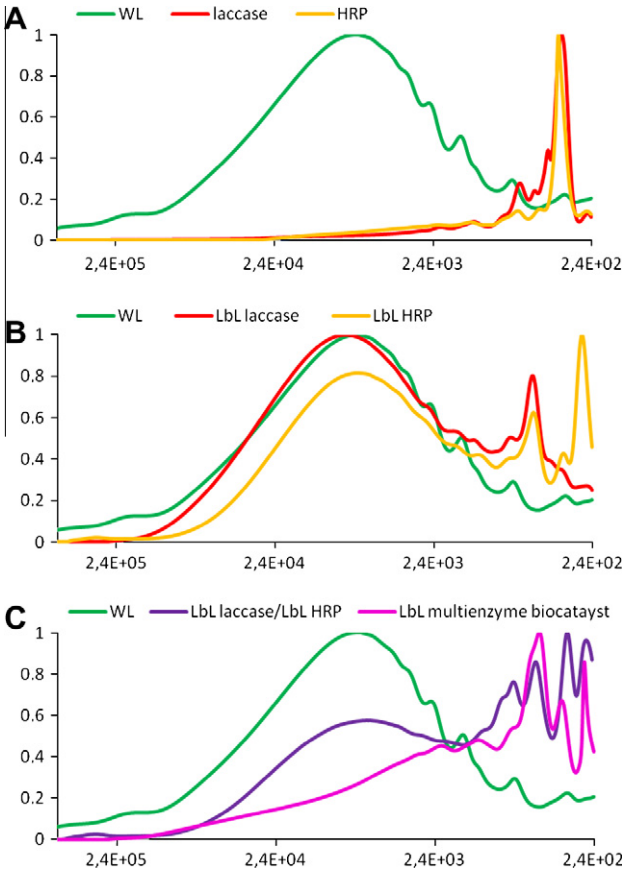

Figure 4. GPC analysis of soluble fraction after enzymatic treatment.

MWL did not show any soluble fraction. For this reason the treated soluble fraction has been compared with the starting lignin. Blank experiments showed that lignin was not significantly affected by the treatment.

It is evident an overall decrease of the Mn of the soluble fraction with the presence of discrete peaks due to dimers, trimers and tetramers. This suggests a high reactivity at phenolic end groups in lignin. On the contrary, after treatment with LbL-laccase or LbL-HRP, the solubilised fraction (Fig. 4B) showed, besides low molecular weight compounds, also the occurrence of a broad peak due to polymeric material. Figure 4C shows the GPC of the soluble fraction after treatment with the mixture of LbL-Laccase and LbL-HRP and with the LbL-multienzyme biocatalyst. While the treatment with the mixture of LbL-Laccase and LbL-HRP yielded a soluble fraction containing oligomers and a consistent polymeric fraction, the LbL-multienzyme biocatalyst gave rise to the formation of a predominant oligomeric soluble fraction. Finally, when the enzymes were reacted with lignin in the presence of HBT, they showed only the presence of low molecular weight compounds (available supplementary material). However, since low molecular weight peaks overlap with HBT in the GPC chromatogram, this result can be overestimated.

Thus, LbL-multienzyme biocatalyst was able to depolymerise WL not only with a higher efficiency than the other biocatalytic systems tested, but also that in lignin degradation depolymerization prevails over lignin solubilisation (increase of phenolic OH).

3. Conclusions

Chemical co-immobilization of laccase and HRP onto alumina particles followed by layer-by-layer coating with alternatively charged polyelectrolytes was found to be a valuable approach for the design and synthesis of a novel LbL-multienzyme oxidative biocatalyst. The co-immobilized enzymes maintained their activity and the LbL coating improved enzyme stability. The oxidative cascade processes were studied on wheat straw lignin. It was thus possible to draw a detailed picture of soluble and immobilized laccase and HRP reactivity. The soluble enzymes undergo a partial depolymerization at the lignin terminal units with a contemporary increase of the lignin Mn and Mw due to oxidative coupling processes, leaving into solution only low molecular weight fractions and behaving as exo-depolymerizingenzymes. LbL-laccase, LbL-laccase/HBT and LbLHRP undergo a higher lignin conversion possibly due to the higher stability of coated enzymes with respect to the soluble ones. The depolymerization was also evident from the lower Mn and Mw of the residual lignin. In this case the higher content in aliphatic and phenolic OH groups indicates the prevalence of hydrolytic processes that cause lignin random depolymerization. This is also supported by the distribution of the Mn and Mw of the soluble fractions that were characterised by the presence of a significant amount of lower molecular weight polymeric material and not only by oligomers. LbL-laccase and LbL-HRP behave as endo-depolymerizing enzymes in the hydrolytic process.

When LbL-multienzyme biocatalyst was used, a higher lignin conversion was associated with a lower Mn and Mw of the lignin residual after treatment. However in this case also the soluble fraction was found being essentially oligomeric. This implies that in the case of the LbL-multienzyme biocatalyst both endo- and eso-depolymerizing activities are occurring with hydrolytic and oxidative pathways respectively. The difference in the behaviour of the singly immobilised laccase and HRP and the multienzyme biocatalyst, points out the occurrence of a cascade reaction pattern originated by the co-immobilised enzymes.

4. Experimental section

4.1. Reagents

All solvents and chemicals were of analytical grade and high purity. Laccase from Trametes versicolor, 1-hydroxybenzotriazole (HBT), Horserhadish Peroxidase from Armoracia rusticana G. (type VI), 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS), H202 solution (35% w/v), poly(allylamine hydrochloride) (PAH, Mw = 70,000), poly(sodium 4-styrenesulphonate) (PSS, Mw = 70,000), alumina (Al2O3) spherical pellets (3 mm diameter), γ-aminopropyltriethoxysilane (γ-APTS), glutaraldehyde and 2-chloro-4,4′,5,5′-tetramethyl-1,3,2-dioxaphospholane were purchased from Sigma–Aldrich.

The polyelectrolyte solutions as well as the buffer solutions were prepared using Millipore Milli-Q deionised water (r = 18 MV cm).

4.2. Isolation of milled wheat straw lignin

WL was isolated from wheat straw. WL was obtained by using a slightly modified acidolysis procedure as previously reported.[57,58] The yield was 38%, and purity was confirmed by UV and Klason lignin content standard measurements as reported in the literature.[59] WL was prepared from ultraground extractive-free powder according to Bjorkman's procedure with some modification[60]

4.3. Enzymes immobilization

Support activation: alumina pellets were silanised with 2% (v/v) γ-aminopropyltriethoxysilane in acetone at 45 °C for 20 h. The silanised supports were washed once with acetone and silanised again for 24 h. They were then washed several times with deionised water and dried through air. In the second stage, the alumina pellets were treated with 2% (v/v) aqueous glutaraldehyde (50%,

v/v) during 2 h at room temperature, washed again with deionised water and dried through air.

4.3.1. Laccase immobilization

One fifty gram of activated support were put in contact with the enzyme, by immersion, during 48 h at 25 °C, in 250 mL of laccase (5,000 U/L) solution obtained by dissolving the enzyme in 100 mM citrate buffer pH 5 with 100 mM NaCl. The particles were then washed several times with 0.05 M phosphate buffer (pH 7) until no enzymatic activity was found in the washing solution.

4.3.2. HRP immobilization

One fifty gram of support was put in contact with the enzyme, by immersion, for 48 h at 25 °C, in 250 mL of HRP (5000 U/L) solution obtained by dissolving the enzyme in 100 mM citrate buffer pH 7 with 100 mM NaCl. The particles were then washed several times with 0.05 M phosphate buffer (pH 7) until no enzymatic activity was found in the washing solution.

4.3.3. Multienzyme biocatalyst immobilization

One fifty gram of activated support were put in contact with the enzymes, by immersion, during 48 h at 25 °C, in 250 mL of a laccase/HRP (2,500 + 2,500 U/L) solution obtained by dissolving the enzymes in 100 mM citrate buffer pH 6 with 100 mM NaCl. The particles were then washed several times with 0.05 M phosphate buffer (pH 7) until no enzymatic activity was found in the washing solution.

4.4. LbL coating of immobilized enzymes

During the LbL coating, the pellets were first washed three times with 0.1 M NaCl and then the sequential deposition of polyelectrolyte (PAH+, PSS−) layers onto the alumina particles was performed. Polyelectrolyte solutions (0.01 M) with 0.5 M NaCl were prepared and the supports were immersed inside each solution for 20 min. Since the alumina pellets with immobilised enzymes were negatively charged at pH 6, coatings consisting of three layers were created, starting with the positively charged polyelectrolyte polyallyl amine hydrochloride ((PAH+ + PSS− + PAH+). After each layer, the excess of polyelectrolyte was removed by washing with 0.1 M NaCl. The red particles of chemically-immobilised LbL laccase or HRP or of the multienzyme biocatalyst were obtained by simple filtration from the reaction mixture.

4.5. Enzyme activity assay

Free HRP activity was determined spectrophotometrically using ABTS as the substrate. The assay mixture contained 0.02 M ABTS, 0.01 M H2O2, 0.1 M phosphate buffer pH 7 and an amount of enzyme; the substrate oxidation was followed by an absorbance increase at 405 nm for 2 min for the free enzyme and for 6 min for the immobilized enzyme, as previously reported. 28 One activity unit was defined as the amount of enzyme that oxidised 1 mmol ABTS/min. The immobilisation yield was calculated as the difference between the activity present in the immobilisation solution and that remaining in the supernatant at the end of the adsorption procedure.

Free laccase activity was determined spectrophotometrically using ABTS as the substrate. The mixture contained 0.5 mM ABTS, 0.1 M sodium acetate pH 5 and an amount of enzyme; the substrate oxidation was followed by an absorbance increase at 415 nm for one minute for the free enzyme and for 3 min for the immobilized enzyme.[60] One activity unit was defined as the amount of enzyme that oxidised 1 μmol ABTS/min. The immobilisation yield was calculated as the difference between the activity present in the immobilisation solution and that remaining in the supernatant at the end of the adsorption procedure.

To determine the concentration of free enzymes in a solution Bradford assay was applied. Bradford reagent consists of Coomassie Brilliant Blue G-250 and phosphoric acid dissolved in ethanol 95% according to the method developed by Bradford.[62] 50, 100, 150, 200 μl of the test solution in 2,600 ml of deionised water were added to 400 μl of Bradford reagent and mixed well. The absorbance at 595 nm was then measured against a blank made of 2,600 ml of deionised water and 400 μl of Bradford reagent.

4.6. Scanning electron microscopy (SEM) analysis

High resolution scanning electron microscopy was performer by a FE–SEM LEO1530 apparatus. The samples were fixed on an adhesive graphitic support.

4.7. Enzymatic treatments of lignin

4.7.1. Laccase oxidation

Wheat lignin (80 mg) was suspended in acetate buffer pH 6 (40 mL) and treated at 30 °C with the enzyme (23 U, either in solution or immobilized and coated) under vigorous stirring to optimize the contact of the solution with air. After 24 h, the mixture was cooled, acidified at pH 3, the immobilized enzyme was filtered off and washed with water, and reaction mixture was centrifuged. The residue was washed with water three times to eliminate solubilized lignin oligomers and then freeze-dried. The residual lignin structure after oxidation were analyzed by GPC and ^{31}P NMR. The combined waters were freeze-dried and the water soluble fraction was analyzed by GPC. Parallel reactions were carried out in the presence of 1-hydroxybenzotriazole (HBT) as oxidation mediator 1 mM.

4.7.2. HRP oxidations

Lignin (80 mg) was suspended in acetate buffer pH 6 (40 mL) with 0.5 mM H_2O_2 and treated at 30 °C with the enzyme (165 U, either in solution or immobilized and coated). After 24 h, the mixture was cooled, acidified at pH 3 the immobilized enzyme was filtered off and washed with water,and centrifuged. The residue was washed with water three times to solubilised lignin oligomers and then freeze-dried. The residual lignin structure after oxidation was analysed by GPC and ^{31}P NMR. The combined waters were freeze-dried and the water soluble fraction was analysed by GPC.

4.7.3. Multienzyme oxidations

Lignin (80 mg) was suspended in acetate buffer pH 6 (40 mL) with 0.5 mM H_2O_2 and treated at 30 °C with the enzymes (23 U laccase + 165 U HRP either in solution or immobilized and coated) under vigorous stirring to optimise the contact of the solution with air. After 24 h, the mixture was cooled, acidified at pH 3 the immobilized enzyme was filtered off and washed with water, and centrifuged. The residue was washed with water three times to eliminate solubilised lignin oligomers and then freeze-dried. The residual lignin structure after oxidation was analyzed by GPC and ^{31}P NMR. The combined waters were freeze-dried and the water soluble fraction was analyzed by GPC. Parallel reactions were carried out in the presence of 1-hydroxy-benzotriazole (HBT) as oxidation mediator 1 mM.

4.8. GPC analysis of lignins

Acetobromination of lignin samples for GPC analysis was carried out following the procedure described previously.[61] Briefly, 10 mg of lignin is suspended in acetic acid glacial/acetyl bromide mixture (2.5 ml of 92:8 v/v) and stirred at room temperature. After 2 h the solvent is evaporated under reduced pressure and then the residue is dissolved in 5 ml THF. The GPC analyses were performed using a

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Shimadzu LC 20AT liquid chromatography with a SPD M20A ultraviolet diode array (UV) detector set at 280 nm. The sample (20 μl) is injected into a system of columns connected in series (Varian PL gel MIXED-D 5 μm, 1–40 K and PL gel MIXED-D 5 μm, MW 500–20 K) and the analysis is carried out using THF as eluant at a flow rate of 0.50 ml min^{-1}. The GPC system has been calibrated against polystyrene standards (molecular weight range of 890–1.86×10^6 g mol^{-1}) and lignin monomers and model dimers. In particular apocynol and (3-methoxy-4-ethoxy-2-phenyl)-2-oxo-acetaldehyde were synthesized according to literature procedure[63,64] and used as monomer and dimer lignin standard respectively.

4.9. Quantitative ^{31}P NMR

Derivatization of the sample with 2-chloro-4,4′,5,5′-tetramethyl-1,3,2-dioxaphospholane was performed as previously described.[1,25,38,39] Samples of lignin (30 mg), accurately weighed, were dissolved in a solvent mixture composed of pyridine and deuterated chloroform, 1.6:1.0 (v/v) ratio (0.4 mL). 2-Chloro-4,4′,5,5′-tetramethyl-1,3,2-dioxaphospholane (0.1 mL) was then added, followed by the internal standard and the relaxation reagent solution (0.1 mL). The NMR spectra were recorded on a Bruker 300 NMR spectrometer using previously published methods.[65,54] To improve resolution, a total of 256 scans was acquired. The ^{31}P NMR data reported in this regard are averages of three phosphitylation experiments followed by quantitative ^{31}P NMR acquisition. The maximum standard deviation of the reported data was 2×10^{-2} mmol/g, while the maximum standard error was 1×10^{-2} mmol/g.

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A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2011.05.058.

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