Encapsulated Laccases for the Room-Temperature Oxidation of Aromatics: Towards Synthetic Low-Molecular-Weight Lignins


A new approach for the encapsulation of laccases with enhanced activity and stability by biomimetic silica mineralisation is reported. A range of lignin model compounds, which includes syringol, syringyl acid, 4-vinylphenol, gallic acid, vanillic acid and guaiacol, was oxidised to lignin-type polymers by the silica-immobilised laccase systems at room temperature. The oxidation rate of the immobilised systems was lower than that of the free enzyme counterparts, but interesting products were observed with the new bio-catalytic materials, which showed reusability and good stability.

Introduction

Bio-catalytic processes have attracted significant attention as alternatives to valorise biomass for the production of chemicals and bio-fuels, and many enzymes with enhanced catalytic capabilities have been identified for this purpose. In nature, many microorganisms produce “free enzyme” cocktails that work synergistically in a number of degradative catalytic processes. One-pot cascade processes (in continuous flow) that contain multiple enzymes do not require isolation steps and can offer significant benefits such as decreased unit operation, decreased reactor volumes, increased volumetric and space-time yields, and shortened cycle times as well as the inherent advantages of flow reactions such as simpler work-up and more controllable reaction conditions. Consequently, an enormous number of bio-catalytic systems are available naturally that can be used effectively in future processes on an industrial scale.

The application of oxidative enzymes in lignin de-polymerisation has attracted significant scientific interest as enzymes can work under mild and environmentally benign conditions. Many microbial systems (which include fungi and bacteria) produce metalloenzymes that oxidise aromatics using dependent radical pathways. As an example, the first isolated ligninolytic enzyme, the *Phanerochaete chrysosporium* lignin heme peroxidase (LiP), was able to oxidise sites with unusually high redox potentials such as aromatic rings. In addition to LiP, various fungi-secreted metalloenzymes, which include heme-containing manganese peroxidases (MnP) and versatile peroxidases (VP), as well as the multi-copper laccases are also efficient in aromatic oxidations. The ability of laccases to oxidise aromatic substrates in conjunction with reduct mediators is currently recognised as one of the potential “breakthrough” applications for the oxidation of lignin model compounds and ultimately lignin valorisation using oxygen as a green oxidant. The potential and mode of action of laccase-mediated systems in lignin biodegradation have been reviewed recently. However, there are still some essential limitations for the use laccases in terms of process applications, which include low catalytic efficiencies and operational stabilities, and high production costs, that limit its potential widespread and multi-purpose applications in various sectors.

To minimise these limitations in industrial applications, different heterogeneous immobilisation techniques have been explored recently that lead to the significantly improved catalytic activity, stability and reusability of laccases. In particular, mesoporous silica materials have attracted a great deal of attention towards enzyme immobilisation because of their large specific surface area, high mechanical strength and tuneable surface functionalities. Among different mesoporous silica materials, large-pore silica mesophases with 3D wormhole framework structures provide bio-catalytic nano-hybrids with excellent enzymatic activities because of the highly accessible reaction sites available on the pore surfaces. Wang et al. prepared large-pore magnetic mesoporous silica nanoparticles (MMSNPs) that exhibited a high adsorption capacity for laccases through chelation at Cu centres. Recently, our group developed a simple, innovative and highly efficient bio-silicification approach able to provide an advantageous enzyme en-
capsulation at room temperature in a one-pot process using a silicic acid precursor together with a buffer solution of the enzyme.\cite{20} With the use of this protocol, a new family of increasingly stable and active bio-nano-hybrid materials (i.e., lipases and metal nanoparticle-lipase hybrids) can be synthesised for various bio-catalytic applications, which could have a significant impact on future industrial bio-processes.\cite{21}

With the aim to expand the scope of the bio-silicification enzyme approach, in this contribution we disclose the design of bio-silicified laccases in the framework of a porous silica using a similarly simple and efficient one-pot method targeted at the oxidation of lignin model compounds. This work builds on the elegant previous studies by Galli et al. who conducted extensive research on hydrocarbon oxidations (which included lignin models) using laccase/mediator systems.\cite{22} Immobilised laccases were investigated in different solvent systems, as suggested in the studies of Gentili et al.,\cite{23} and evaluated during the encapsulation process to maximise the yields of bio-inorganic hybrid materials. The activities of the new materials were tested in the oxidation of different lignin model compounds such as syringol, 4-vinylphenol and syringic acid, which are all present as a result of lignin catalytic de-polymerisation reactions.

### Results and Discussion

#### Selection of optimum solvent and pH for the immobilisation yields and activity

The proposed one-pot encapsulation technique required the addition a specific amount of tetraethoxysilane (TEOS) to a buffer solution that contained the enzyme and organic solvents to obtain an optimum silica mineralisation, similar to that reported previously.\cite{20} In these experiments, 10 mg of enzyme with an activity of 0.92 U mg\(^{-1}\) was added typically to 2.5–5 mL of 50 mm acetic acid buffer at pH 4.5. The amount and type of solvent, DMSO, acetonitrile (ACN), and Britton–Robinson and acetic acid buffers (at pH 4–6 adjusted with concentrated NaOH and \(\text{CH}_3\text{COOH}\), respectively) were tested to determine the optimum conditions for the bio-silification step (Tables 1 and 2). Laccases from different sources with promising textural and structural properties were encapsulated successfully within the silica matrix (Table 3). In all cases, bio-silicified materials were obtained as white solids.

Significant differences can be seen clearly in the yields of the final bio-silicified products and, most importantly, in the measured laccase activities (Tables 1–3). These were found to be highly pH and solvent dependent. The use of DMSO led to low yields of material (0.88 g at 60% v/v) that showed an almost two-fold higher activity as compared with that obtained from ~40% solvent conditions (Table 1). ACN, employed previously as a solvent in lipase bio-silicification studies,\cite{20,24} provided similar yields and immobilised laccase activity as DMSO or MeOH under the same conditions (60% final concentration; Table 1). ACN was henceforth selected as the solvent used in the bio-silicification step in line with its more environmentally friendly nature than that of DMSO or MeOH. Other greener solvents (i.e., water, ethanol, ethyl acetate) investigated in the bio-silicification process provided almost negligible activities and low yields of final materials (data not shown).

The effect of the pH and buffer solution in the bio-silicification step was investigated (Table 2). At pH 4, both acetic acid and Britton–Robinson buffers provided good yields of bio-silicified material and a moderate immobilised laccase activity.

### Table 1. Bio-silification conditions and enzymatic activities in the presence of different solvents for the encapsulation of Tvl within a silica matrix.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Solvent</th>
<th>Solvent [%]</th>
<th>Buffer [mL]</th>
<th>Amine [mL]</th>
<th>TEOS [mL]</th>
<th>Solvent</th>
<th>Yield [g]</th>
<th>Specific activity [U mg(^{-1})]</th>
<th>Immobilised laccase activity [U g(^{-1})]</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>DMSO</td>
<td>0.62</td>
<td>2</td>
<td>38</td>
<td>1.46</td>
<td>0.92</td>
<td>6.30</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>DMSO</td>
<td>0.62</td>
<td>2</td>
<td>42</td>
<td>2.10</td>
<td>0.92</td>
<td>4.38</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>DMSO</td>
<td>0.32</td>
<td>1</td>
<td>60</td>
<td>0.88</td>
<td>0.92</td>
<td>10.45</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>ACN</td>
<td>0.32</td>
<td>1</td>
<td>60</td>
<td>1.03</td>
<td>0.92</td>
<td>8.89</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>MeOH</td>
<td>0.32</td>
<td>1</td>
<td>60</td>
<td>1.24</td>
<td>0.92</td>
<td>7.40</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

[a] Specific activity provided by Sigma–Aldrich using catechol as the substrate (at pH 6.0 and 25 °C).

### Table 2. Bio-silification conditions and enzymatic activities for various pH values and buffer solutions for bio-silicified Tvl.

<table>
<thead>
<tr>
<th>Sample</th>
<th>pH</th>
<th>Enzyme [mg]</th>
<th>Yield [g]</th>
<th>Specific activity [U]</th>
<th>Immobilised laccase activity [U g(^{-1})]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bio-a</td>
<td>4</td>
<td>100</td>
<td>2.86</td>
<td>92.0</td>
<td>32</td>
</tr>
<tr>
<td>Bio-b</td>
<td>4</td>
<td>100</td>
<td>3.04</td>
<td>92.0</td>
<td>30</td>
</tr>
<tr>
<td>Bio-c</td>
<td>6</td>
<td>20</td>
<td>0.43</td>
<td>18.4</td>
<td>43</td>
</tr>
<tr>
<td>Bio-d</td>
<td>4</td>
<td>20</td>
<td>0.25</td>
<td>18.4</td>
<td>72</td>
</tr>
<tr>
<td>Bio-e</td>
<td>6</td>
<td>30</td>
<td>0.61</td>
<td>27.6</td>
<td>45</td>
</tr>
<tr>
<td>Bio-f</td>
<td>4</td>
<td>30</td>
<td>0.38</td>
<td>27.6</td>
<td>73</td>
</tr>
</tbody>
</table>


### Table 3. BET surface area and pore volume measurements.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Surface area [m(^{2}) g(^{-1})]</th>
<th>Pore size [nm]</th>
<th>Pore volume [mL g(^{-1})]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bio-c</td>
<td>50</td>
<td>2</td>
<td>0.31</td>
</tr>
<tr>
<td>Bio-d</td>
<td>65</td>
<td>&lt; 2</td>
<td>0.38</td>
</tr>
<tr>
<td>Bio-e</td>
<td>32</td>
<td>&lt; 2</td>
<td>0.22</td>
</tr>
<tr>
<td>Bio-f</td>
<td>79</td>
<td>&lt; 2</td>
<td>0.45</td>
</tr>
<tr>
<td>Bio-CotA</td>
<td>45</td>
<td>&lt; 2</td>
<td>0.40</td>
</tr>
</tbody>
</table>

[a] All materials were essentially microporous.
Selection of the appropriate enzyme for immobilisation/encapsulation

In the next step, the activities of laccases were determined spectrophotometrically by monitoring the increase of absorbance from the oxidation of 2,2'-azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) according to the method proposed by Wolfenden and Wilson.[25] Firstly, enzyme tests using free *Trametes versicolor* (TvL) were conducted immediately after enzyme preparation and then repeated on the following days in aliquots taken from the preparation stored at 4 °C. The residual enzyme activity decreased remarkably (25% of the initial activity) after 72 h (Table 4). These results imply that laccase activity is reduced significantly because of the poor stability of the enzyme at pH 4 and at low temperatures. Subsequently, analogous experiments were conducted on bio-silicified enzymes but generally low or no activity after 24 h in buffer was observed for all of the bio-silicified samples tested (Table 2).

The relative activities of the bio-silicified enzymes were also compared with those of the free enzyme after storage at 4°C. As expected, the results indicate clearly that the bio-silicified enzymes exhibited reduced activities compared to those of the free enzyme (Table 5). However, the absorbance values of the reaction after 24 h for free and bio-silicified enzymes coincided, which indicates that the complete oxidation of the substrate could be achieved for bio-silicified preparations after 24 h of reaction in spite of their reduced initial activities.

The activities of the enzymes after immobilisation were measured (Table 6), and the recombinant *CotA*-laccase (*see Experimental Section*) from *B. subtilis*,[20] a non-commercial laccase, was used for comparison. This enzyme was bio-silicified using the conditions optimised for the commercial TvL enzyme in this work. Significant differences were observed in terms of laccase activity after immobilisation, which indicates that the non-commercial bio-silicified enzyme has remarkably improved activities after mineralisation (~20 times higher), almost comparable to those of the free enzymes (Table 6). However, the activity of native *CotA* was remarkably superior (approximately four times) compared to that of the bio-silicified *CotA*. Nevertheless, these results support the conclusion that the yields and activity of bio-silicified enzymes are complex and enzyme dependent and need careful analysis and investigation on a case-to-case basis. The differences observed suggest a more drastic effect of the silicification process on different laccases than that shown in previous work with lipases.[20,24]

### Table 4. Free enzyme stability in Britton–Robinson buffer at pH 4 and 4°C

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>Activity [U/mL⁻¹]</th>
<th>Residual activity [%]</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.89</td>
<td>100</td>
</tr>
<tr>
<td>24</td>
<td>0.53</td>
<td>59</td>
</tr>
<tr>
<td>72</td>
<td>0.22</td>
<td>25</td>
</tr>
</tbody>
</table>

### Table 5. Bio-silicified laccase activity for ABTS in Britton–Robinson buffer at pH 4.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Rate [abs min⁻¹]</th>
<th>Immobilised laccase activity [U]</th>
<th>Rate per amount of enzyme [abs min⁻¹ U⁻¹]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Free laccase</td>
<td>7.73×10⁻³</td>
<td>0.01</td>
<td>8.41</td>
</tr>
<tr>
<td>Bio-a</td>
<td>2.26×10⁻³</td>
<td>1.28</td>
<td>1.75×10⁻¹</td>
</tr>
<tr>
<td>Bio-b</td>
<td>5.12×10⁻³</td>
<td>3.03</td>
<td>1.69×10⁻¹</td>
</tr>
</tbody>
</table>

[a] After 48 h storage at 4 °C in Britton–Robinson buffer.

### Table 6. Enzymatic activities of bio-silicified laccases for ABTS oxidation.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Rate [abs min⁻¹]</th>
<th>Total volume [mL]</th>
<th>Enzyme [mg]</th>
<th>Specific activity [U mg⁻¹]</th>
<th>Total laccase activity after immobilisation [U]</th>
<th>Solid product [g]</th>
<th>Bio-silicified laccase activity [U g⁻¹ sol⁻¹]</th>
</tr>
</thead>
<tbody>
<tr>
<td>TvL</td>
<td>0.578</td>
<td>2</td>
<td>10</td>
<td>0.79</td>
<td>7.89</td>
<td>0.40</td>
<td>19.8</td>
</tr>
<tr>
<td>CvL</td>
<td>0.315</td>
<td>2</td>
<td>10</td>
<td>0.56</td>
<td>5.59</td>
<td>0.36</td>
<td>15.3</td>
</tr>
<tr>
<td>CotA</td>
<td>2.14</td>
<td>2</td>
<td>3.5</td>
<td>10.85</td>
<td>38.00</td>
<td>0.30</td>
<td>126.7</td>
</tr>
</tbody>
</table>
Bio-catalytic oxidation studies of lignin model compounds

Laccase enzymes show good oxidation properties towards many different substrates. In particular, we were interested to explore the possibility to oxidise molecules related to metabolic lignin pathways (i.e., aromatic compounds), namely, a number of phenols, which include syringol, syringic acid and 4-vinylphenol (Figure 1, Table 7) as well as gallic, syringol and vanillic acid (Figure 2, Table 8). Bio-catalytic tests were performed using samples Bio-c, Bio-f and Bio-a for comparison. After 24 h, the reactions were stopped by freezing, and the samples were lyophilised. The products were analysed by gel-permeation chromatography (GPC) as shown in Figures 1 and 2a, b and c, and the different properties of the final oxidation products are detailed in Tables 7 and 8.

The results pointed generally to the enzymatic oxidation of the different investigated substrates, although products were essentially different and depended on the monomeric substrates (M_w ≈ 160–200). Of particular relevance were the lignin-derived polymers obtained from the oxidation of syringic acid and, especially, 4-vinylphenol (M_w = 4370, high polydispersity of 10.68) characteristic of a typical low-molecular-weight poly-4-vinylphenol (PVP). A red to brown precipitate was obtained after the oxidation reactions, which is most likely a result of the presence of the formed oligo-polymeric polyphenolic compounds that lead to different colours depending on the degree of polymerisation.

Additionally, in the case of bio-silicified CotA, the first results could be obtained after only 1 h of reaction. Comparably, the molecular weight of the lignin-like products was not as significantly high as in the case of syringic acid and 4-vinylphenol oxidation in ACN, and low-molecular-weight lignin oligomers (dimers, timers and tetraters of M_w ≈ 300–800, low polydispersities) were obtained in all cases (Table 8).

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Two very important observations were extracted from these findings. Firstly and most importantly, the oxidation and polymerisation of certain 5-lignin units (syringic acid and, particularly, 4-vinylphenol; Figure 1) seems to be promoted preferentially by laccases in a similar way to that in nature in which 4-coumaryl alcohol essentially leads to the formation of G- and H-units in lignin polymers, in good agreement with previous reports. This has enormous potential for the understanding and overcoming of lignin de-polymerisation/re-polymerisation processes that occur if heterogeneous (bio)catalysis is used, particularly to target the quenching of such aromatic

monomers that are easily re-polymerised after lignin deconstruction to obtain gradually less recalcitrant lignin polymers with reduced molecular weights.\(^{29}\)

Secondly, the obtained oxidation products were similar for bio-silicified CotA (after 1h of reaction) and bio-silicified TvL (after 24h of reaction) at room temperature, which indicates the excellent oxidation capacity of bio-silicified isolated non-commercial CotA. These results can be visualised clearly for the particular example of syringol oxidation (Figure 1a vs. 2b).

To establish differences in activity between bio-silicified TvL laccases synthesised at different pH values and in different buffer solutions (Bio-c–Bio-f; see Table 1 for full details), a comparative experiment was conducted using syringol and guaiacol as model substrates (Figure 3, Table 9). After 24h reaction, all absorption values were very high, which shows further polymerisation, and a brown precipitate was formed in all samples. The products were analysed by GPC, which indicated significant differences in the molecular weight of the oxidation products compared to that of the initial monomers.

Of particular interest were syringol-derived polymeric compounds obtained after oxidation with bio-silicified TvL Bio-d (\(M_w = 2068\), polydispersity index = 2.7) and most guaiacol-derived products. These results seem to indicate once again a preferential oxidation of guaiacol as a substrate using bio-silicified laccases under the investigated conditions\(^{22,23,27,28}\) in good agreement with previous reports in which several complex mechanisms have been proposed (mostly for syringyl derivatives)\(^{30,31}\).

**Conclusions**

In view of the potential future applications of laccases in industrial oxidative processes (e.g., lignin deconstruction), in this work we aimed to design a new family of active and stable en-
capsulated laccases using a simple, reproducible and efficient bio-silicification approach. The immobilisation conditions were varied to achieve the maximum activity of the bio-silicified enzyme system. Gel-permeation chromatography results confirmed that the bio-silicified laccases could oxidise lignin model compounds successfully, which included S- and G-units. Lignin-like oligomer products could be obtained after 24 h at room temperature, and excellent activities were observed for a bio-silicified isolated laccase (CotA), which will be the subject of future studies in this area.

**Experimental Section**

**Chemicals and materials**

All reagents and solvents used were of analytical grade. Commercial laccases from Tvl and Coriolis versicolor (CvL); the same enzyme with different purification grades; Sigma Aldrich) were used in this study. Recombinant CotA-laccase from Bacillus subtilis was produced and purified as described previously, briefly. E. coli strain AH3517 was grown in Luria–Bertani medium with ampicillin (100 µg·mL−1) at 150 rpm. The cells were grown at 37 °C until an optical density (at 600 nm; OD600) of 0.6 was reached, after which 0.1 mM isopropyl-β-thiogalactopyranoside and 0.25 mM CuCl2 were added and the temperature was reduced to 25 °C. Incubation was continued for a further 4 h, after which a change to micro-aerobic conditions was attained by switching off the shaker. Cells were harvested by centrifugation after 20 h and disrupted by using a French-Press. Protein was purified using a cation-exchange S-Sepharose column followed by gel filtration by using a Superdex-200 column and kept at −20 °C until use.

One-pot encapsulation of laccase and activity measurements

Typically, TEOS (2 mL) was added to laccase acetic acid buffer (2.5 mL; pH 4–6), a stirred solution with the addition of n-dodecylamine (0.3–0.6 mL), and solvent (0.5–1 mL) at RT to obtain a final enzyme concentration of 1–10 mg·mL−1. The reaction mixture was stirred for 2 h, and the obtained solid was collected by filtration, washed with water, dried for 10 min under vacuum and dried at RT overnight. Enzymatic activities (see below) were designated as Specific Activity [Umgprotein−1] and Immobilised Laccase Activity [Umgsubstrate−1]. One unit of enzyme [U] corresponds to the amount of enzyme that converts 1 µmol of substrate per minute using either catechol (at pH 6 and 25 °C, as mentioned by the commercial manufacturer, Sigma) or ABTS as substrates. The Immobilised Laccase Activity corresponds to units of enzyme activity per gram of the final bio-silicified material synthesised.

The enzymatic assays with ABTS were performed in a 2 mL reaction mixture that contained 50 mM acetic acid buffer at pH 4.5 (0.9 mL), 11 mM ABTS (0.1 mL) and various amounts of free or immobilised laccase. ABTS oxidation was monitored spectrophotometrically at 436 nm ($F_{436} = 29300$ Lmol−1 cm−1).

The laccase activity was further assessed using guaiacol, 4-vinylphenol and syringic acid as substrates. The reaction mixtures contained 50 mM acetic acid buffer at pH 4.5 (1 mL), 20 mM substrate (0.05 mL) and various amounts of immobilised laccase unless otherwise stated. The reactions were monitored over 24 h by the molecular weight distribution of the products formed by using GPC.

**GPC**

GPC was performed at RT by using a Jasco HPLC equipped with a UV detector set at λ = 280 nm and a sequence of 50, 100 and 500 Å Phenogel columns (5 µm, Phenomenex). The eluent was THF at a flow rate of 0.3 mL·min−1. Calibration was achieved by using polystyrene standards. Samples were dissolved in THF (20–30 mg·mL−1) and filtered before injection.

**Reversed-phase liquid chromatography**

Reversed-phase liquid chromatography was performed at RT by using a JASCO HPLC equipped with a UV detector set at λ = 280 nm and a Zorbax Eclipse XDB-C18 (4.6×150 mm) column. Mobile phase A (water with 0.1% formic acid adjusted to pH 3 with ammonia) and B (acetonitrile) were employed. The gradient program at 1 mL·min−1 flow rate was: 10–20% B over 5 min, held for 3 min, to 70% B over 5 min, held for 5 min, to 100% B over 1 min and held for 2 min before an immediate return to the initial conditions.

**Materials characterisation**

N2 adsorption measurements were performed at 77 K by using an ASAP 2000 volumetric adsorption analyser from Micromeritics. Samples were degassed at 50 °C under vacuum (p < 10−2 Pa) before the adsorption measurements. Surface areas were calculated according to the BET equation. Pore volumes ($V_{BM}$) and pore size distributions ($D_{pore}$) were obtained from the N2 desorption branch.

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