1. Introduction

Laccase (EC 1.10.3.2) is a multicopper oxidase which reduces oxygen to water and simultaneously performs one-electron oxidation of many aromatic substrates [7]. Laccases catalyze the removal of a hydrogen atom from the hydroxyl group of methoxy-substituted monophenols, ortho- and para-diphenols, but it be able also to oxidize other substrates such aromatic amines, syringaldazine, and non-phenolic compounds to form free radicals [8].

2. Materials and methods

2.1 Instruments

2.2 Covalent Immobilization

Alumina (Al₂O₃) tablets pellets (7 mm) and sferical pellets (3 mm) were silinized, immerse in γ-aminopropyltriethoxy silane 2.5% (V/V) [1] in solution of acetone at 45°C for 24 h. The pellets were washed whit distilled water and immersed in 2% (V/V) of aqueous glutaraldehyde solution [2] for 2 h at room temperature. Re-washed whit distilled water, therafter the support was dry at 60°C for 1h. At 5 g of pellets was add in 10 ml of the crude enzyme preparation, in 0.1 M sodium acetate (pH 5), for 5 h at room temperature. Immobilized protein was determined by protein analysis according to the method of Bradford [3] by using bovine serum albumin for the calibration.

The immobilized enzyme pellets were washed whit phosfate buffer solution of pH 7.0 and kept a refrigerated until further use.

2.3 Enzyme activity

A Laccase (benzenediol: oxygen oxidoreductase, EC 1.10.3.2) from Trametes hirsutus were supplied by VTT Biotechnology (Espoo, Finland). Initially for the immobilezed laccase it was used the Jong method [4] but its three steps were laborius. It was not possible used the best alternative that is a direct measurement of the oxygen consumption. Finally it was used the ABTS test [5] based on a direct one electron oxidation of substrate that is still one of the best indirect tests for determining the activity of oxidative enzymes.

2.3.1 Method Jong

Laccase activity was determined using 2.6-dimethoxyphenol (DMP) as a substrate [4]. The reaction mixture contained 50 mmol sodium malonate (pH 4.5), 1mmol DMP, 1mmol MnSO₄, and 700 μl sample in a total volume of 1ml. The formation of an orange/brownish dimer was followed spectrophotometrically at 468 nm. Activities for both these enzymes were calculated using the molecular extinction coefficient of 49.6 mM⁻¹ cm⁻¹ and expressed as IU which is defined as one μmol.min⁻¹ of substrate converted.

2.3.2 Method ABTS

Laccase activity was determined by using ABTS [2,2´-azino-bis-(3-ethylbenothiazoline-6-sulfonic acid)] as a substrate [5]. The reaction mixture
contained 0.5 mmol ABTS, and 1 ml sample, diluted in 0.1 M sodium acetate (pH 5), in a total volume of 2 ml. Oxidation of ABTS was followed spectrophotometrically at 420 nm. Activities for both these enzymes were calculated using the molecular extinction coefficient of 3.6*10^4 mM^-1 cm^-1 and expressed as IU which is defined as one μmol min^-1 of substrate converted.

2.4 Dyes

Two model dyes were used to study their influence in the pH profile activity of Laccase. A cationic dye, crystal violet (basic violet 3, C.I. 42555) and an anionic dye, indigo carmine (acid blue 74, C.I. 73015). The dyes were purchased from Sigma with 90% of purity. The chemical structure of these two dyes is represented in Figure 1.

Dye solutions in buffer (95 mL) were incubated with 100 μL of enzyme solution in a shaker bath operating at 90 rpm. Samples of the reaction mixture were taken at different periods of time to measure the dye absorbance and the percentage of decolourisation was calculated. In the temperature activity profile experiments, the enzymatic reaction was performed at pH 5.0. The activity-pH profile of laccase enzyme was studied in the range of pH 3-9 and in these experiments the temperature was set at 50 °C. The buffer systems used in these experiments are listed in Table 1 and were prepared at 0.1 M concentration. Control tests were also performed in order to observe the effect of buffer and temperature alone on dye decolourisation and this effect was subtracted to the activity observed in the enzymatic reactions.

<table>
<thead>
<tr>
<th>pH</th>
<th>Buffer system</th>
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<tbody>
<tr>
<td>3</td>
<td>Tartaric acid-NaOH</td>
</tr>
<tr>
<td>4</td>
<td>Acetic acid-NaOH</td>
</tr>
<tr>
<td>5</td>
<td>Acetic acid-NaOH</td>
</tr>
<tr>
<td>6</td>
<td>Phosphoric acid-NaOH</td>
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<tr>
<td>7</td>
<td>Phosphoric acid-NaOH</td>
</tr>
<tr>
<td>8</td>
<td>Tris-HCl</td>
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<tr>
<td>9</td>
<td>Tris-HCl</td>
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</tbody>
</table>

Table 1: Buffer systems used in activity-pH profile experiments.

3 Results and Discussion

3.1 Differences of allumina supports

2.5 Decolourisation
3.2 Effect of temperature on enzyme activity

3.3 Effect of pH on enzyme activity
Figure 5: Activity-pH profile of *Trametes hirsutus* laccase for the two dyes (measured as percentage of decolourisation after 24 hours incubation, 50 °C).

5 Conclusion

References


