Characterization of alkaliphilic laccase activity in the culture supernatant of *Myrothecium verrucaria* 24G-4 in comparison with bilirubin oxidase

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Abstract

An enzyme showing alkaliphilic laccase activity was purified from the culture supernatant of *Myrothecium verrucaria* 24G-4. The enzyme was highly stable under alkaline conditions, showed an optimum reaction pH of 9.0 for 4-aminoantipyrine/phenol coupling, and decolorized synthetic dyes under alkaline conditions. It showed structural and catalytic similarities with bilirubin oxidase, but preferably oxidized phenolic compounds. The enzyme catalyzed veratryl alcohol oxidation at pH 9.0 with 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) as a mediator, suggesting that the laccase mediator system functioned well under alkaline conditions.

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Keywords: Laccase; Polyphenol oxidase; Alkaliphilic; Mediator; Bilirubin oxidase; Myrothecium verrucaria

1. Introduction

Laccase (benzenediol:oxygen oxidoreductase; EC 1.10.3.2) is a multi-copper-containing enzyme catalyzing the oxidation of a wide range of phenolic and aniline compounds. Because of its functional variability, laccase has been purified from various sources, especially plants and fungi, and is widely used for practical purposes. In its reaction, substrates are oxidized by one electron to generate the corresponding phenoxy radicals, which either polymerize to yield a phenolic polymer or are further oxidized to a quinone. Therefore, laccases can be applied for polymerization and decomposition. Laccase is used for the synthesis of phenol resins and also for the polymerization of lignin as a binder in particle boards [1]. Delignification of lignocellulosic materials can be performed to improve the digestibility of wood or straw for animal feeds or reduce the cost of pulp preparation in the paper industry [1].

The ability to decolorize and decompose dyes and phenolic compounds can be applied in the paper or textile industries, or to the removal of toxic aromatic compounds in wastewater from various industries [2–4]. Recently, the concerted action of fungal laccases and oxidizable low-molecular-mass compounds, so-called mediators, was found to extend or permit oxidation of non-substrate compounds by this enzyme class. These laccase–mediator systems enhanced their status in comparison to the generally more powerful peroxidases, and they have been applied to a wide range of applications, such as textile dye bleaching, polycyclic aromatic hydrocarbon degradation, and synthesis of aldehydes [5–9].

In these application fields, many processes involve high pH conditions. But there have been few reports on laccases with high activity and stability under alkaline conditions [10–12]. In this study, we screened for practically useful alkaliphilic laccases in microorganisms. An alkalitolerant fungus, *Myrothecium verrucaria* 24G-4, showed extracellular laccase activity. The enzyme showing alkaliphilic laccase activity (tentatively called ‘alkaliphilic laccase’ in this report) was purified and characterized.
2. Materials and methods

2.1. Enzymes and chemicals

Laccase from *Coriolus versicolor* and bilirubin oxidase (BOD) from *Myrothecium* sp. were obtained from Amano Enzyme (Nagoya, Japan). Laccase from *Trametes* sp. Ha-1 was a kind gift from Daiwa Kasei (Osaka, Japan). All other chemicals used in this study were of analytical grade and commercially available.

2.2. Screening for alkaliphilic laccases in alkali-tolerant fungi

Various fungi (about 300 strains isolated from soil) were cultivated at 28°C for several days on 2% agar plates (pH 9.0) containing 1% glucose, 0.5% yeast extract, 0.5% peptone, 0.1% KH$_2$PO$_4$, 0.1% K$_2$HPO$_4$, 0.02% MgSO$_4$·7H$_2$O, 0.5% Na$_2$CO$_3$, and 0.003% chloramphenicol. To pick up laccase producers, a piece of filter paper that had been soaked in the laccase assay solution (2 mM 4-aminoantipyrine, 2 mM phenol, and 100 mM Tris·HCl buffer, pH 9.0) was put on the colonies that grew on the plates. Extracellular laccase producers showing a red color due to quinoimine dye production were selected. The selected culture supernatants was assayed as described below.

2.3. Laccase assay

The standard reaction mixture comprising 2 mM 4-aminooantipyrine, 2 mM phenol, and 100 mM Tris–HCl buffer (pH 9.0) in 180 µl was incubated at 37°C. A 20-µl enzyme sample was added to the reaction mixture in a 96-well microplate, and then the increase in absorbance at 500 nm was measured with a microplate photometer (Spectra Max 190; Molecular Devices, Sunnyvale, CA, USA). A molar extinction coefficient of 12880 M$^{-1}$ cm$^{-1}$ for the quinoimine dye was used for calculation of the activity. One unit of laccase activity was defined as the amount that produced 1 µmol quinoimine dye per minute.

2.4. Purification

*M. verrucaria* 24G-4 (AKU 3560, Faculty of Agriculture, Kyoto University = FERM P-17525, International Patent Organisms Depository, National Institute of Advanced Industrial Science and Technology, Japan) was cultivated at 28°C for 4 days with shaking in 500 ml of liquid medium, in a 2-l flask, comprising 1% glucose, 0.5% yeast extract, 0.5% peptone, 0.1% K$_2$HPO$_4$, 0.02% MgSO$_4$·7H$_2$O, 0.5% Na$_2$CO$_3$, and 0.2% casamino acid, pH 9.0. The culture supernatant (500 ml) was concentrated by ultrafiltration, and then subjected to purification (total protein, 17 mg; total activity, 5.20 U; specific activity, 0.31 U mg$^{-1}$ protein). All the following purification steps were carried out at 4°C and the buffer used was 20 mM Tris–HCl, pH 8.5, unless otherwise stated. The concentrated culture supernatant was dialyzed against buffer and then applied to a MonoQ HR 10/10 column (Amerham Pharmacia Biotech, Uppsala, Sweden) equilibrated with buffer and eluted with an increasing salt gradient of 0–1 M NaCl. The active fractions were combined (total protein, 2.5 mg; total activity, 1.35 U; specific activity, 0.54 U mg$^{-1}$ protein) and then applied to a Sephacryl S-200 HR column (1.5×90 cm; Amerham Pharmacia Biotech) previously equilibrated with buffer containing 0.2 M NaCl. The protein was eluted with the same buffer and the active fractions were combined (total protein, 0.80 mg; total activity, 0.64 U; specific activity, 0.80 U mg$^{-1}$ protein). The sample was dialyzed against buffer, and then applied to a MonoQ HR 5/5 column (Amerham Pharmacia Biotech) equilibrated with buffer and eluted with an increasing salt gradient of 0–1 M NaCl. The active fractions were used for characterization (total protein, 0.30 mg; total activity, 0.37 U; specific activity, 1.23 U mg$^{-1}$ protein).

2.5. Comparison of BOD and laccase activities

BOD and laccase activities were assayed by monitoring oxygen consumption or by spectrophotometry. Reactions were performed at 37°C. Oxygen consumption: 2.49 ml of a reaction mixture comprising 0.2 mM bilirubin or 3.2 mM *N,N*-dimethylphenylenediamine (DMPD), and 150 mM phosphate buffer (pH 8.0) was put into an oxygen electrode chamber (DW1; Hansatech, Norfolk, UK). 10 µl of the enzyme sample was added to start the reaction. The slope for the oxygen consumption rate was used to calculate the relative activity. Spectrophotometry: 622.5 µl of a reaction mixture comprising 150 mM phosphate buffer (pH 8.0), and 0.2 mM bilirubin, 3.2 mM DMPD, or 10 mM 4-aminooantipyrine, and 10 mM phenol was used. 2.5 µl of the enzyme sample was added to start the reaction. The oxidation of bilirubin and DMPD was monitored at 450 nm and 550 nm, respectively. 4-Aminooantipyrine/phenol coupling was monitored at 500 nm.

2.6. Laccase–mediator system assay

The reaction mixture, in 150 µl, comprised 28 mM veratryl alcohol, 1.7 mM 2,2’-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) or 1-hydroxy-1H-benzotriazole (HBT), 100 mM buffer, and suitable amount of enzyme. The buffers used were sodium acetate-acetate buffer (pH 5.0), potassium phosphate buffer (pH 7.0), and Tris–HCl buffer (pH 9.0). The reaction was carried out at 28°C for 24 h. Production of vanillic acid in the reaction mixtures was monitored by high performance liq-
uid chromatography at 254 nm fitted with a Cosmosil 5C18-AR-II column (4.6x250 mm, Nacalai Tesque, Kyoto, Japan), and 50% methanol (pH 2.5) as the eluent at the flow rate of 1.0 ml min$^{-1}$ at 40°C. The specific activity of laccase-mediator systems was expressed as μmol veratraldehyde production per hour per mg enzyme protein.

3. Results

3.1. Screening of alkaliphilic laccase in alkali-tolerant fungi

Several fungi that grew at high pH exhibited extracellular laccase activity of below 0.5 U l$^{-1}$ culture supernatant at pH 10.0. Only one fungus, M. verrucaria 24G-4, showed high laccase activity at pH 10.0 (approximately 10 U l$^{-1}$ culture supernatant), and thus was subjected to further characterization.

3.2. Purification of alkaliphilic laccase

As described in Section 2, the alkaliphilic laccase was purified from the culture filtrate of M. verrucaria 24G-4 approximately four-fold to homogeneity with a specific activity of 1.23 U mg$^{-1}$ protein. It gave a single band corresponding to a molecular mass of 62000 on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Fig. 1). The purified enzyme showed maximum absorption at 274 and 595 nm, and a small shoulder at 325 nm. These results indicated that the enzyme is a copper-containing enzyme like common laccases and BOD.

3.3. Effects of pH and temperature on the laccase activity and stability

The optimum reaction pH of the laccase for 4-aminoantipyrine/phenol coupling was around pH 9.0 (Fig. 2A). The laccase retained more than 80% of the initial activity after 17 h incubation at 30°C at pH 8–11.5 (Fig. 2B). The laccase showed higher stability under alkaline conditions than the laccases from C. versicolor and Trametes sp. Ha-1, which exhibited optimum reaction pHs for 4-aminoantipyrine/phenol coupling of around pH 5.0. The laccase showed the highest initial reaction rate at 70°C for 4-aminoantipyrine/phenol coupling. Thermal stability was investigated by incubation in 100 mM Tris–HCl buffer (pH 8.5) at various temperatures for 60 min. The laccase retained more than 80% of the initial activity up to 50°C, but only 25% at 60°C (Fig. 2C).

3.4. Substrate specificity

Many phenolic and aniline compounds were good substrates for the laccase in the 4-aminoantipyrine coupling reaction (Table 1). 2,6-Dimethoxyphenol was a particularly good substrate. The laccase showed different substrate specificities for dye decolorization and the 4-aminoantipyrine coupling reaction from other common laccases under alkaline conditions. High decolorizing activity was found toward anthraquinone and azo dyes, particularly Direct Blue 53 (Table 2). An increase in absorbance was observed for the reaction with lignin, indicating the laccase catalyzed lignin polymerization under alkaline conditions.
The NH$_2$-terminal amino acid sequence of the laccase was determined by automated Edman degradation with an Applied Biosystem 476A pulsed-liquid-phase protein sequencer (Foster City, CA, USA). The obtained 17-amino acid sequence, APQISPQYPMFTVPLPI, exhibited high homology with that of BOD from *M. verrucaria* MT-1, VAQISPQYPMFTVPLPI [13], but not common laccases.

### 3.6. Comparison of the catalytic properties of the laccase with BOD

Since the laccase showed high sequence homology with BOD, its BOD activity was examined. The laccase showed BOD activity with an optimum reaction pH of 8.0–8.5, which was the same as that of BOD from *Myrothecium* sp. However, determination of the laccase/BOD activity ratio with an oxygen electrode and by spectrophotometry revealed that the laccase showed higher specificity toward phenolic and aniline compounds (Table 3). The alkaliphilic laccase from *M. verrucaria* 24G-4 catalyzed the oxidation of typical laccase substrates more efficiently than BOD from *Myrothecium* sp.

### 3.7. Evaluation of laccase–mediator system

Laccase–mediator system was examined using ABTS or Table 1

#### Table 1

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Wavelength (nm)</th>
<th>Relative activity (%)</th>
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</thead>
<tbody>
<tr>
<td>Phenols</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phenol</td>
<td>500</td>
<td>100 (1.23)</td>
</tr>
<tr>
<td>Catechol</td>
<td>500</td>
<td>1340 (1.13)</td>
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<td>Resorcinol</td>
<td>500</td>
<td>731 (0.97)</td>
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<td>Hydroquinone</td>
<td>500</td>
<td>404 (0.63)</td>
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<tr>
<td>Guaiacol</td>
<td>500</td>
<td>1500 (1.63)</td>
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<tr>
<td>Pyrogallol</td>
<td>500</td>
<td>473 (0.81)</td>
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<tr>
<td>$p$-Hydroxybenzoic acid</td>
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<td>52 (0.35)</td>
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<tr>
<td>Caffeic acid</td>
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<td>713 (1.26)</td>
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<tr>
<td>Hydrocaffeic acid</td>
<td>500</td>
<td>1200 (1.90)</td>
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<tr>
<td>$o$-Cresol</td>
<td>500</td>
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<tr>
<td>$p$-Toluidine</td>
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<td>348 (0.70)</td>
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<tr>
<td>$o$-Chlorophenol</td>
<td>500</td>
<td>190 (1.02)</td>
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<tr>
<td>$m$-Chlorophenol</td>
<td>500</td>
<td>80 (0.45)</td>
</tr>
<tr>
<td>2,4-Dichlorophenol</td>
<td>500</td>
<td>80 (0.89)</td>
</tr>
<tr>
<td>2,6-Dichlorophenol</td>
<td>500</td>
<td>49 (1.17)</td>
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<td>2,6-Dimethoxyphenol</td>
<td>500</td>
<td>3840 (1.04)</td>
</tr>
<tr>
<td>$n$-Propylgallate</td>
<td>500</td>
<td>94 (–)</td>
</tr>
<tr>
<td>Anilines</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TOOS$^a$</td>
<td>555</td>
<td>15 (0.15)</td>
</tr>
<tr>
<td>Dimethylaniline</td>
<td>555</td>
<td>40 (0.16)</td>
</tr>
<tr>
<td>Diethylamine</td>
<td>555</td>
<td>10 (0.60)</td>
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<tr>
<td>$N,N$-Dimethyl-$p$-phenylenediamine</td>
<td>555</td>
<td>900 (2.78)</td>
</tr>
<tr>
<td>$p$-Phenylenediamine</td>
<td>555</td>
<td>563 (–)</td>
</tr>
<tr>
<td>Others</td>
<td>ABTS$^b$</td>
<td>500 (53)</td>
</tr>
</tbody>
</table>

The reaction mixture consisted of 100 mM Tris–HCl buffer (pH 9.0), 2 mM 4-aminoantipyrine, and 2 mM substrate. 10 µl of the enzyme sample was added to the 190 µl reaction mixture in a 96-well microplate, and then the increase in absorbance at the indicated wavelength was measured at 30°C. Relative activity was defined as the percent increase in absorbance, that obtained with phenol being taken as 100%. The values in parentheses are specific activities (U mg$^{-1}$ protein). –, not determined.

$^a$TOOS, N-ethyl-N-(2-hydroxy-3-sulfopropyl)-3-methylaniline.

$^b$ABTS, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid).

3.5. NH$_2$-terminal amino acid sequence of the laccase

The NH$_2$-terminal amino acid sequence of the laccase was determined by automated Edman degradation with an Applied Biosystem 476A pulsed-liquid-phase protein sequencer (Foster City, CA, USA). The obtained 17-amino acid sequence, APQISPQYPMFTVPLPI, exhibited high homology with that of BOD from *M. verrucaria* MT-1, VAQISPQYPMFTVPLPI [13], but not common laccases.

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3.7. Evaluation of laccase–mediator system

Laccase–mediator system was examined using ABTS or

Fig. 3. Effect of pH on the laccase–mediator systems. The reactions were carried out as described in Section 2 with ABTS as a mediator. ■, alkaliphilic laccase from *M. verrucaria* 24G-4; ○, laccase from *Trametes* sp. Ha-1; Δ, BOD from *Myrothecium* sp.
given in Section 2. BOD activity was examined with bilirubin as a sub-
electrode and (B) a spectrophotometer under the reaction conditions
and BOD from
Myrothecium
The laccase and BOD activities of laccase from
M. verrucaria
Phenol 7.3 0.54 14
Bilirubin 100100
(D) Spectrophotometry
in absorbance, that obtained with bilirubin beingtaken as 100%.
(B) Relative activity was de¢ned as the percent change
¢ned as the percent speci¢c activity, that obtained with bilirubin being
4-aminoantipyrine and DMPD or phenol. (A) Relative activity was de-
strate, and laccase activity was examined in couplingreactions for
DMPD 360 38 9.6
Bilirubin 100 100
HBT as the mediator in oxidation of veratryl alcohol that
is a non-laccase substrate. The alkaliphilic laccase oxidized
veratryl alcohol to veratraldehyde with ABTS as a medi-
ator but not with HBT. The pH dependence of the alka-
philic laccase^ABTS mediator system showed considerable activ-
ity under pH 7.0 and 9.0. The speci¢c activities of laccase^-
laccase activity was examined in coupling
reaction mixture in a 96-well microplate, and then the decrease in absorbance at the indicated wavelength was mea-
sured at 30°C. Relative activity was de¢ned as the percent decrease in absorbance, that obtained with RBBR being taken as 100%. A minus sign indi-
cates that an increase in absorbance was observed.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Conc. (mg ml^-1)</th>
<th>Wavelength (nm)</th>
<th>Relative activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RBBR</td>
<td>0.23</td>
<td>600</td>
<td>100</td>
</tr>
<tr>
<td>Reactive Blue 2</td>
<td>0.010</td>
<td>600</td>
<td>829</td>
</tr>
<tr>
<td>Reactive Blue 5</td>
<td>0.30</td>
<td>600</td>
<td>−63.0</td>
</tr>
<tr>
<td>Acid Blue 80</td>
<td>0.030</td>
<td>600</td>
<td>385</td>
</tr>
<tr>
<td>Direct Blue 53</td>
<td>0.0070</td>
<td>600</td>
<td>6340</td>
</tr>
<tr>
<td>Acid Orange</td>
<td>0.015</td>
<td>430</td>
<td>629</td>
</tr>
<tr>
<td>Poly R 478</td>
<td>0.070</td>
<td>500</td>
<td>214</td>
</tr>
<tr>
<td>Poly S 119</td>
<td>0.0070</td>
<td>430</td>
<td>177</td>
</tr>
<tr>
<td>Acid Violet 17</td>
<td>0.010</td>
<td>500</td>
<td>−304</td>
</tr>
<tr>
<td>Phenol Red</td>
<td>0.030</td>
<td>500</td>
<td>−181</td>
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<td>600</td>
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<td>Acid Red 52</td>
<td>0.030</td>
<td>500</td>
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<tr>
<td>Lignin</td>
<td>1.0</td>
<td></td>
<td>−1390</td>
</tr>
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</table>

The reaction mixture consisted of 100 mM Tris^HCl bu¡er (pH 9.0) and the respective concentration (Conc.) of each substrate. 20 µl of the enzyme sample was added to the 180 µl reaction mixture in a 96-well microplate, and then the decrease in absorbance at the indicated wavelength was mea-
sured at 30°C. Relative activity was de¢ned as the percent decrease in absorbance, that obtained with RBBR being taken as 100%. A minus sign indi-
cates that an increase in absorbance was observed.

HBT as the mediator in oxidation of veratryl alcohol that
is a non-laccase substrate. The alkaliphilic laccase oxidized
veratryl alcohol to veratraldehyde with ABTS as a medi-
ator but not with HBT. The pH dependence of the alka-
philic laccase–ABTS mediator system was examined in
comparison with those of the laccase from
Trametes sp., Ha-1 and BOD. As shown in Fig. 3, only the alkaliphilic
laccase–ABTS mediator system showed considerable activity
under pH 7.0 and 9.0. The specific activities of laccase–-
ABTS mediator systems with alkaliphilic laccase, laccase

from Trametes sp. Ha-1 and BOD at pH 7.0 were 27.6, 2.2, and 3.8 µmol h^-1 mg^-1, respectively, and those at pH 9.0 were 10.3, 0 and 1.1 µmol h^-1 mg^-1, respectively.

4. Discussion

To our knowledge, the optimum pHs of known laccases are around 4–5, or at the highest 7.0 as in the case of
Rhizoctonia practicola laccase [10], Rigidoporus lignosus [11], and edible burdock laccase [12]. On the other hand,
the laccase from
M. verrucaria
24G-4 showed a higher optimum reaction pH of 9.0 for the 4-aminoantipyrine/
phenol coupling reaction. It also showed dye decomposi-
tion and lignin polymerization activities under high pH conditions. Remarkably, this laccase was very stable under alkaline conditions, indicating that it is useful for process-
es involving alkaline conditions. The laccase showed sequence homology with BOD from
Myrothecium sp., and also showed BOD activity. It is known that BOD oxidizes some laccase substrates under alkaline conditions [14,15]. However, the alkaliphilic laccase from
M. verrucaria
24G-4 showed higher speci¢city toward laccase substrates than common BOD. Moreover, the alkaliphilic laccase well catalyzed veratryl alcohol oxidation at pH 9.0 with ABTS as a mediator, suggesting that the laccase–mediator system functioned even under alkaline conditions. These results indicated that
M. verrucaria
strains are good sources of alkaliphilic laccase, and that BOD could be a potential starting material for the protein engineering of alkaliphilic laccase.

Table 2
Dye-decolorizing activity of laccases from
M. verrucaria
24G-4 (24G-4),
Trametes sp. Ha-1 (Tr), and
C. versicolor
(Cv)

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Conc. (mg ml^-1)</th>
<th>Wavelength (nm)</th>
<th>Relative activity (%)</th>
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<tr>
<td>Anthraquinone dyes</td>
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<td>RBBR</td>
<td>0.23</td>
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<td>Lignin</td>
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<td>−1390</td>
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</table>

The laccase and BOD activities of laccase from
M. verrucaria
24G-4 and BOD from
Myrothecium sp. were measured with (A) an oxygen
electrode and (B) a spectrophotometer under the reaction conditions
given in Section 2. BOD activity was examined with bilirubin as a sub-
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efined as the percent specific activity, that obtained with bilirubin being
taken as 100%. (B) Relative activity was defined as the percent change
¢ned as the percent speci¢c activity, that obtained with bilirubin being
1 mg
3
1, respectively, and those at pH
3
9.0 were 10.3, 0 and 1.1 µmol h^-1 mg^-1, respectively.

Table 3
Comparison of the substrate specificities of laccase from
M. verrucaria
24G-4 and BOD from
Myrothecium sp.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Relative activity</th>
<th>Ratio of laccase (24G-4)/BOD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Laccase (24G-4)</td>
<td></td>
<td>BOD</td>
</tr>
<tr>
<td>(A) Oxygen consumption</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bilirubin</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>DMPD</td>
<td>110</td>
<td>74</td>
</tr>
<tr>
<td>(B) Spectrophotometry</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bilirubin</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>DMPD</td>
<td>360</td>
<td>38</td>
</tr>
</tbody>
</table>

The laccase and BOD activities of laccase from
M. verrucaria
24G-4 and BOD from
Myrothecium sp. were measured with (A) an oxygen
electrode and (B) a spectrophotometer under the reaction conditions
given in Section 2. BOD activity was examined with bilirubin as a sub-
strate, and laccase activity was examined in coupling reactions for
4-aminoantipyrine and DMPD or phenol. (A) Relative activity was de-
efined as the percent specific activity, that obtained with bilirubin being
taken as 100%. (B) Relative activity was defined as the percent change
¢ned as the percent speci¢c activity, that obtained with bilirubin being
100%.
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References