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Enzyme and Microbial Technology 33 (2003) 766–774
ENZYME and MICROBIAL TECHNOLOGY
www.elsevier.com/locate/enzmictec

An acid-stable laccase from *Sclerotium rolfsii* with potential for wool dye decolourization

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Received 17 July 2002; received in revised form 30 April 2003; accepted 3 May 2003

Abstract

The plant pathogen basidiomycete *S. rolfsii* secretes two laccases (SRL1 and SRL2) with molecular weights of 55 and 86 kDa, respectively. Laccase production was shown to be inducible by the addition of 2,5-xylidine to the cultural media. After treatment with a combination of chitinase and β-1,3-glucanase, two different laccases were isolated from the sclerotia depending on the stage of sclerotia development. The more prominent laccase, SRL1, was purified and found to decolourize the industrially important wool azo dye Diamond Black PV 200 without the addition of redox mediators. The enzyme (pI 5.2) was active in the acidic pH range, showing an optimal activity at pH 2.4, with ABTS as substrate. The optimum temperature for activity was determined to be 62 °C. Enzyme stability studies revealed that SRL1 was notably stable at 18 °C and pH 4.5, retaining almost full activity after a week. Oxidation of tyrosine was not detectable under the reaction conditions but the enzyme did oxidize a variety of the usual laccase substrates. SRL1 was strongly inhibited by sodium azide and fluoride. Dye solutions decolourized with the immobilized laccase were successfully used for redyeing.
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Keywords: Laccase; *Sclerotium rolfsii*; Purification; Characterization

1. Introduction

Laccases (benezenediol:oxygen oxidoreductase, EC 1.10.3.2) are multinuclear copper-containing enzymes that catalyse the oxidation of a variety of phenolic and inorganic compounds, with the concomitant reduction of oxygen to water [1]. Although primarily originating from fungi [2,3], laccases are widely distributed in nature and have also been detected in plants [4], insects [5] and bacteria [6]. These oxidative enzymes are particularly abundant in white-rot basidiomycete fungi, which are capable of degrading lignin in vivo. Accordingly, this class of fungi has been extensively researched as potential producers of laccase activity. To date, extracellular laccases have been purified from numerous fungal sources such as *Pleurotus eryngii* [7], *Coriolus hirsutus* [8] and *Pycnoporus cinnabarinus* [9].

Due to their wide substrate specificity laccases have gained much attention over the last number of years in many industrial and environment fields. Of particular commercial interest is the potential use of these enzymes to decolourize dyes. By means of enzymatic catalyzed oxidative reactions, laccase can detoxify phenolic contaminants, such as aromatic amines, to harmless/less harmful products [10]. The suitability of laccases for such processes has been known for some time [11] and successful results achieved have been well documented by our group [12] and others [13,14]. These being of primary importance to the textile industry, dyes and dyestuffs are commonly used in the pharmaceutical, food, cosmetic and leather industries. Stringent environmental legislation imposed to control the release of dyestuffs in wastewater poses particular problems for the textile industry. Up to 20% of dye is lost into the wastewater during the dyeing process although this figure depends on the class of dye application. It is known that 90% of reactive textile dyes entering activated sludge sewage treatment plants will pass through unchanged and be discharged to rivers [15]. Laccases, especially when used immobilized on carrier materials, are seen as attractive options in the development of an effective strategy for the biological treatment of wastewater. In particular, the reuse of enzymatically treated dyeing effluents could help to reduce water consumption [16].

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0141-0229/$ – see front matter © 2003 Elsevier Inc. All rights reserved.
doi:10.1016/S0141-0229(03)00162-5

The process of wool dying, carried out in the acidic pH range from pH 3.8 to 4.4 uses primarily acid dyes which promote the adhesion of the dye through ionic interactions between the dye and the wool. Acid dyes have hydroxyl, carboxyl or sulphonic groups with an overall negative charge. The enzymatic elimination of these dyes from the dyeing effluent requires an acid-stable laccase. The phytopathogenic fungus, *Sclerotium rolfsii*, which secretes an acid-stable laccase has been investigated in this study in terms of purification, biochemical characterization and for the ability of its laccase to decolourize acidic dyes. Although *S. rolfsii* has been extensively studied as a producer of cellulolytic and hemicelluloytic enzymes [17,18], its capacity to produce oxidative enzymes has been explored to a lesser extent [19]. Furthermore, until now there is no information available on whether the laccases produced during the formation of sclerotia are different to those secreted by the mycelium of *S. rolfsii* [20].

Sclerotia are small, compact, usually spherical reproductive bodies, comprising of a cell wall containing three layers; and ranging in size from 100 mm (plant pathogens) to 1 cm in omnivorous types. Sclerotia have a high resistance to chemical and biological degradation and play a vital role in the survival of the organism for extended periods of time [21]. Two types of germinating patterns exist: *Carpogenic sclerotia*, which produces a sexual fruiting body, as seen in the species *Claviceps* and the second form of sclerotia production, called *Myceliogenic sclerotia*, characteristic of *S. rolfsii*, develop from repeated localized hyphal branching followed by adhesion of the branches. Outer hyphae are crushed to form the rind of thick-walled melanized cells while the inner tissue stores nutrient reserves and become swollen, with extended vacuoles [21]. Laccases can be involved in pigment synthesis (polymerization of melanin) in differentiated fungal structures as shown for *Aspergillus nidulans* [22]. Laccases produced by *S. rolfsii* during formation of sclerotia and secreted by the mycelium could have different specificities and stabilities and thus, show a different behavior in dye degradation.

2. Materials and methods

2.1. Organism and culture conditions

S. rolfsii (CBS 350.80) was obtained from the University of Agriculture Science, Vienna, Austria. The culture was maintained on PDA-agar plates grown at 30 °C, stored at 4 °C and subcultured monthly. For laccase production *S. rolfsii* was grown in 1000 ml Erlenmeyer flasks at 30 ± 1 °C on an orbital shaker at 150 rpm using 300 ml culture medium containing the following ingredients: 42.6 g cellulose, 40 g meat peptone, 2.5 g NH_4NO_3, 1.5 g $MgSO_4$, 1.0 g KH_2PO_4, 0.5 g KCl, and 300 μl trace element solution [23] in 1 l distilled water. The initial pH was adjusted to 5.5 and the growth medium was sterilized at 121 °C for 15 min prior to inoculation with 1 cm^2 discs of fungus growing on agar plates. In order to induce laccase activity various phenolic agents (1 g l^{-1}) were added through sterile filters to the fermentation broth at the point of inoculation. These included caffeic acid, ferulic acid, gallic acid and pectin. 2,5-Xylidine in concentrations ranging from 10 to 100 μM was added to the culture medium after three days of cultivation. For the purpose of enzyme purification 10 μM of 2,5-xylidine plus 1% pectin was used to stimulate laccase activity. The fermentation continued for a total of 9 days after which the culture supernatant was harvested by centrifugation at 10,000 × *g* for 10 min.

2.2. Production of sclerotia

On agar plates, sclerotia development was visible after 9 days of growth. These structures were white (W) in appearance and matured to a light brown (LB) colour within another 6 days. The final stage of maturity was attained on approximately day 20 of growth when the sclerotia developed into a dark brown (DB) colour, characteristic of the mature sclerotia of the species. This process of sclerotia formation was continuous and at various stages of growth all forms were simultaneous present on the plate.

Sclerotia were harvested at the white (W), light (LB) and dark brown (DB) stages of development. To achieve this they were gently picked from their stems to detach them from the mycelial bed. Sclerotia were subsequently placed in a sieve and washed repeatedly with distilled water to remove any mycelia that remained attached. The wet weight (g) of the sclerotia was determined. Samples were ground using a mortar and pestle after which they were transferred to centrifuge tubes. Centrifugation at 8000 rpm and for 10 min was used to separate the insoluble material. The supernatant containing the enzyme of interest was decanted. The pellet was subsequently washed with 50 mM sodium-acetate buffer (pH 4.0) and re-centrifuged. The supernatant was added to that collected after the first centrifugation step. This sample was kept at 4 °C for future analysis and the pellet discarded. After some weeks of growing on agar plates a brown/yellow liquid was secreted onto the surface. This liquid (L) was also harvested, assayed for laccase activity and developed on SDS non-denaturing gels.

Trichoderma harzianum was cultivated and the major chitinase and β-1,3-glucanase purified as previously described [24]. Mature sclerotia were harvested and incubated overnight with chitinase and/or endoglucanase enzymes at 30 °C. After 24 h the samples were centrifuged and the pellet removed while the supernatant was assayed to determine if the added hydrolase activities successfully degraded the sclerotia cell wall and released the laccase activity. Control samples were run in parallel with buffer instead of enzyme.

2.3. Enzyme and protein assay

Laccase activity was measured at 25 °C by following the change in optical density at 436 nm using ABTS as substrate

($\varepsilon = 29.2\,mM^{-1}\,cm^{-1}$). The reaction mixture contained 1.15 ml of suitably diluted enzyme using 25 mM succinate buffer pH 4.5 and 0.35 ml ABTS according to published methods [25]. Activity on other substrates was measured similarly at their wavelength maxima. Protein concentration was routinely determined using the Bradford Reagent (Sigma) according to the manufacturer's instructions with bovine serum albumin as standard. All chromatographic runs were monitored for protein by absorbance at 280 nm. The inhibitory effects of a number of reagents as indicated below were studied at various concentrations using the standard ABTS assay to monitor the effects on laccase activity. The inhibitor was added 10 min prior to substrate addition.

2.4. Enzyme purification

Proteins were precipitated from the culture supernatent by the gradual addition of ammonium sulphate up to 85% saturation. The resultant pellet was resuspended in 10 mM sodium acetate buffer, pH 4.75 and $(NH_4)_2SO_4$ was added to a final concentration of 1.1 M. It was then loaded onto a Phenyl Sepharose CL-4B (Pharmacia) column (70 ml, pre-equilibrated with 1.1 M $(NH_4)_2SO_4$. Elution of proteins was achieved using an isocratic gradient of 1.1–0.0 mM $(NH_4)_2SO_4$. Laccase active fractions were pooled, concentrated and dialysed against 10 mM sodium acetate buffer, pH 4.75 and applied to a Q-Sepharose FF (Pharmacia) column (10 ml) pre-equilibrated with 10 mM sodium acetate, pH 4.75. The active fractions that remained bound to the column after the wash were eluted by a linear gradient of 0–0.5 M NaCl. The fractions were subsequently pooled, concentrated and dialysed against 1.0 mM sodium phosphate buffer pH 6.8. The sample was loaded onto a hydroxyapatite column (5 ml) pre-equilibrated with the same buffer. The active laccase fractions were eluted using a linear gradient of 1–25 mM sodium phosphate buffer (pH 6.8). Concentration steps were performed throughout the purification using an ultrafiltration stirred cell apparatus (Amicon Corp., Lexington, MA), using a 10-kDa PS membrane.

2.5. Electrophoresis and staining

SDS gel electrophoresis was performed according to the method of Laemmli (1970) using 10% gels and Coomassie Blue for protein staining. To detect laccase activity after SDS–PAGE, prior to gel application samples were incubated at 50 °C for 20 min. Following Coomassie Blue staining the gels were destained, rinsed with water and subsequently submerged in 3 mM ABTS where the laccase active band was highlighted in green. Isoelectric focusing (IEF) was carried out with the Mini-PROTEAN 3 cell from Bio-Rad using IEF Ready Gels (pH 3–10, Bio-Rad). Protein was visualized using Coomassie Blue.

2.6. Determination of temperature and pH optima and stabilities

Optimum temperature for the activity of the laccase was determined by carrying out the standard laccase assay at selected constant temperatures ranging from 25 to 65 °C. In each case the substrate was preincubated at the required temperature. The pH optimum was determined at a fixed assay temperature of 30 °C at various pH values between pH 2.2 and 7.0 using McIlvaine-type constant ionic strength citrate–phosphate buffer. In order to study the thermal stability, diluted enzyme samples (100 mM succinate buffer, pH 4.5) were incubated for fixed time periods at 30, 60, 80 °C. At time intervals, the pH of the incubation mixture was measured and aliquots were withdrawn and were cooled on ice before assaying to determine the residual enzyme activity, using the normal assay procedure. The stability of the enzyme at various pH values was determined by preincubating the enzyme at the desired pH for a fixed period at 30 °C. Residual enzyme activity was determined using the standard assay procedure.

2.7. K_m and V_{max} determination

K_m and V_{max} constants were determined using various concentrations of ABTS (0.05–20 mM) and syringaldazine (4-hydroxy-3,5-dimethoxybenzaldehyde azine; 2–85 μM) as substrate using the normal assay procedures. The experimental data were analyzed according to the Michaelis–Menten model by Lineweaver–Burk plots and by non-linear regression analysis using the program Origin 5.0.

2.7.1. Enzyme immobilization

Alumina pellets were silanized at 45 °C for 24 h in a 2.5% (v/v) solution of γ-aminopropyltriethoxy silane in acetone. The silanized pellets were washed with distilled water and immersed in 2% (v/v) aqueous glutaraldehyde for 2 h at 20 °C. Thereafter, the pellets were incubated with 60 mg l^{-1} of the crude enzyme preparation (obtained after ammonium sulphate precipitation of the culture filtrate) for 5 h at 20 °C. The immobilized enzyme pellets were washed with potassium phosphate buffer (100 mM, pH 7.0) and kept refrigerated until further use.

2.8. Decolourization experiments

The reaction mixture consisted of a final volume of 1 ml and contained 50 μl enzyme (3.3 nkat ml^{-1} final activity), buffer (50 mM sodium acetate, pH 4.5) and Diamond Black (DB) (Dye Star, maximum absorption 536 nm) dye solution at a specified concentration. The reaction was carried out at 40 °C and the colour change monitored spectrophotometrically from 700 to 400 nm. Control samples, containing denatured enzyme, were run in parallel under identical conditions. Alternatively, the dye solution was continuously pumped (0.1 ml^{-1} min, dual-piston-pump) through a column

(15 mm × 300 mm) filled with immobilized laccase corresponding to about 30 nkat total laccase activity. Both column reactors and the flow cell were kept at 30 °C. Decolourization was monitored on a spectrophotometer equipped with a flow cell.

2.9. Dyeing in enzymatically decolourised textile effluents

100% wool fabrics (samples of 0.5 g) were dyed with C.I. Acid Blue 113 and C.I. Acid Yellow 49 (0.5, 1 and 1.5% o.w.f. depth of shade) using an Ahiba Spectradye dyeing apparatus (Datacolor International, Luzern, Switzerland). The dyebaths were prepared with enzymatically decolourized liquors. Since starting concentrations of dyes in decolourization experiments were higher than in industrial effluents (to facilitate spectrophotometric monitoring) the decolourized solutions were diluted appropriately (1:3) with 0.1 M sodium acetate buffer, pH 5. The dye was added at 50 °C, and the temperature was raised to 100 °C with 1 °C min^{-1}; at 100 °C the dyeing continued for 1 h. Colour differences of the dyed fabrics were determined using a reflectance measuring apparatus (Spectraflash 600 from Datacolor, Luzern, Switzerland) according to the CIELAB colour difference concept at standard illuminant D_{65} (LAV/Spec. Excl., d/8, $D_{65}/10^{\circ}$) with a colour tolerance interval of one CIELAB unit.

3. Results and discussion

3.1. Induction of laccase in liquid culture

Although laccase activity is apparently constitutive in most basidiomycetes, inducers such as aromatic substances have been reported to enhance activity [9,26–28]. To investigate the effect of some known laccase inducers, the growth medium of *S. rolfsii* was supplemented with different phenolic compounds. Growth was monitored using caffeic acid, ferulic acid, gallic acid, pectin, 2,5-xylidine and 2,5-xylidine plus pectin as inducers. The time course of laccase production revealed a steady increase in activity detectable in the extracellular fluid. A selection of the data is presented in Fig. 1. A moderate increase in specific activity was detected after the addition of xylidine alone. However, a combination of both xylidine and pectin (day 3) resulted in a dramatic increase in activity on day 9 and then decreased sharply towards the end of the experiment.

Interestingly, polygalacturonase (PG) activity was also increased by the inclusion of xylidine in the culture media (data not shown). It has been previously reported that pectin acts as a second inducer of extracellular laccase formation by *Botrytis cinerea*, in the presence of a phenolic substance as a first inducer, but pectin alone failed to induce enzyme formation [29]. Similar findings were noted when *S. rolfsii* was cultivated on a pectin induced medium without the phenolic inducer, xylidine. Experiments to determine if xylidine was toxic to the fungi at higher concentrations revealed that

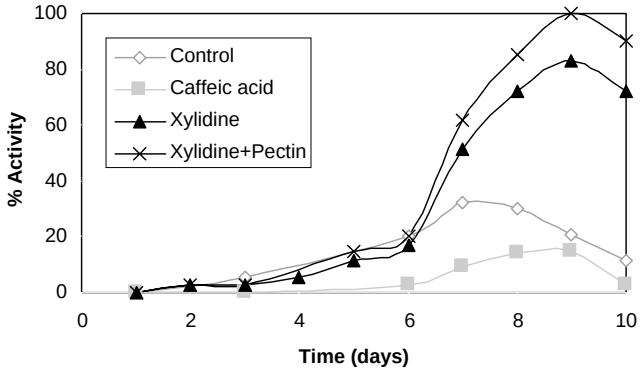

Fig. 1. Time course of laccase production by *S. rolfsii* in response to inducers added on day 3.

similar levels of activity were reached regardless of the concentration over a test range of 1.0–0.1 mM. The other inducers assessed resulted in a significant decrease in the level of laccase activity.

As previously noted with other fungal species which included *P. cinnabarinus* [30], *Rigidoporus lignosus* [31], *Trametes pubescens* [32] and *Phanerochaete chrysosporium* [33] more than one extracellular laccase isoform is often produced during cultivation. *S. rolfsii* secreted two electrophoretically distinct proteins displaying laccase activity. Their molecular masses were 55 and 86 kDa, respectively, as determined by SDS–PAGE with activity staining (Fig. 2). In general, after Coomassie Blue staining the same pattern of bands on SDS–PAGE gels was obtained regardless the temperature used for sample pretreatment (boiling or 50 °C for activity staining).

3.2. Laccases from sclerotia

Sclerotia grew increasingly dense as they reached maturity and a liquid, yellow in colour was secreted from the sclerotia,

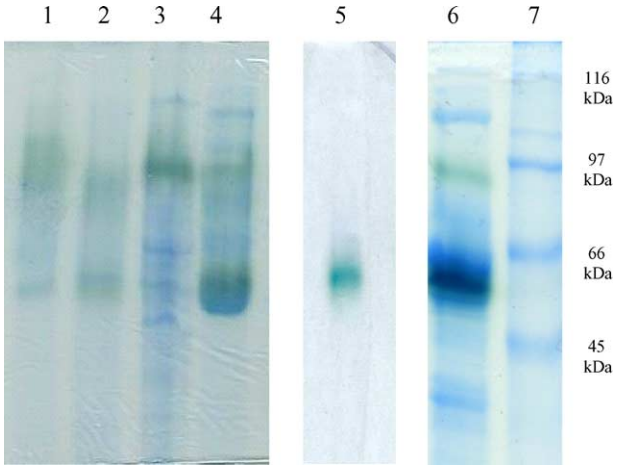

Fig. 2. Coomassie Blue and ABTS stained SDS–PAGE gel. Lane 1—LB (light brown), lane 2—DB (dark brown), lane 3—W (white), lane 4—L (liquid), lane 5—purified laccase, lane 6—crude extracellular laccases and lane 7—molecular weight standards (kDa).

Table 1
Laccase activity present at various stages of sclerotia growth

Stage of development	Weight of sclerotia harvested (mg)	Total laccase activity (nkat)	Protein ($\mu g\,ml^{-1}$)	Laccase activity per wet weight (nkat/g)
White (W)	50	0.156	16	3.1
Light brown (LB)	110	0.378	17	3.5
Dark brown (DB)	50	0.346	12	6.9
Liquid	n.a.	0.384	17	n.a

n.a.: not applicable.

apparently at the DB stage of development. This was assayed for laccase activity which was detected. Laccase activity, although present in all stages of sclerotia development, was slightly more predominant in the DB sclerotia (Table 1).

In order to determine if the mycelial and sclerotial laccases had different molecular masses samples harvested from the various stages of growth were separated on SDS-gels. From the activity stain (ABTS) it was clear that the sclerotial laccases had identical M_r to those of the extracellular laccases secreted by the fungi when grown on liquid media, i.e. 55 and 86 kDa, respectively (Fig. 2). It appears that the higher molecular weight laccase is present in the white sclerotia, as the development continues the second laccase appears. It is thought that the laccases are involved in the pigmentation of the sclerotia.

Since only low amounts of laccase could be isolated from sclerotia, an enzymatic method for the hydrolysis of sclerotia was developed. The fungal wall usually consists of layers of chitin, a linear polysaccharide polymer of *N*-acetylglucosamine, embedded in and often covered by glucans, branched polymers of glucose and other sugars. It was supposed that the action of chitinase together with β-1,3-glucanase could act to hydrolyse the chitin and β-1,3-glucan from the cell walls of the sclerotia structure and release the laccase activity. The 43 kDa chitinase and 74 kDa β-1,3-glucanase were purified from the *Trichoderma harzianum* T24 culture filtrate as described previously [24]. When the harvested sclerotia were incubated separately with either of the two purified hydrolases, the laccase activity detectable in the supernatant was higher than that of the control (Table 2). When β-1,3-glucanase was applied directly to the sclerotia, an increase of 36% in laccase activity was observed compared to the buffer alone. The combined treatment of chitinase and β-1,3-glucanase resulted in a substantial increase in the laccase activity detectable, 4.5 times greater than the control sample. This result suggests that when loaded together the enzymes acted in a synergistic manner leading to an increase in the laccase levels present in the supernatant. Synergistic interactions between chitinase and β-1,3-glucanase during biocontrol of *S. rolfsii* have previously been described [24].

3.3. Purification and molecular properties

The lower molecular weight laccase SRL1, which was secreted in much higher amounts than the higher molecular weight laccase SRL2, was purified to homogeneity using four chromatographic steps according to the procedure summarized in Table 3. The molecular mass of this polypeptide of 55 kDa falls within the M_r range reported for many fungal laccases, which can range from 50 to 90 kDa [2,3]. An approximate purification of 183 fold was achieved with a final yield of 12%. The isoelectric point of the pure enzyme was determined by IEF to be at pH 5.2.

Some properties of SRL1 are shown in Table 4. The 55 kDa enzyme was very active in the acidic pH range, showing a peak at pH 2.4 and a rapid decline in activity above pH 6.0, with ABTS as substrate. This property of SRL1 could potentially be exploited in the textile industry where acidic conditions predominate in wool dyeing. It should be noted however that the optimal pH for laccase activity is substrate dependent [34]. The optimum temperature for activity was determined to be 62 °C. Enzyme stability studies revealed that SRL1 was highly stable at 18 °C and pH 4.5, retaining almost full activity after a week.

3.4. Kinetic properties and substrate specificity

The apparent K_m and V_{max} values of SRL1 were determined to be 0.22 ± 0.03 mM and 1.70 ± 0.04 nkat ml^{-1}, respectively, with ABTS as substrate. When using

Table 2
Enzymatic treatment of sclerotia from *S. rolfsii*

Sample	Sclerotia weight (g)	Laccase activity (nkatal ml^{-1})	Volume of sample (ml)	Total activity (nkatal)	Activity/mg sclerotia
Chitinase + β-1,3-glucanase	0.76	0.56	1.20	0.67	0.87
β-1,3-Glucanase	0.72	0.31	0.60	0.19	0.26
Chitinase	0.68	0.26	0.60	0.16	0.23
Buffer	0.71	0.23	0.60	0.14	0.19

Table 3
Purification of the extracellular laccase SRL1 from *Sclerotium rolfsii*

Purification step	Total protein (mg)	Total activity (nkatal)	Specific activity (nkat mg^{-1})	Yield (%)	Purification factor
Culture extract	362	720	1.9	100	1
75% $(NH_4)_2SO_4$	308	616	2.0	85	1.02
Phenyl-Sepharose	33.8	431	12.8	60	7.9
QFF	3.04	209	68.8	29	42
Hydroxapatite	0.35	86	245	12	183

syringaldazine as substrate, the apparent K_m and V_{max} values were estimated to be 0.087 ± 0.005 mM and 3.18 ± 0.02 nkatal ml^{-1}, respectively. When compared with other fungal laccases, SRL1 has high K_m values with 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) and syringaldazine as substrates. Using syringaldazine as the substrate, laccases purified for both *Trametes villosa* I and *Rhus vernicifera* have K_m values of 0.058 and 0.043 mM, respectively [35].

The purified laccase was able to oxidize a diverse range of phenolic substrates as indicated by the increase in optical density measured at the appropriate wavelength (Table 5). While several of the compounds tested were oxidised by the laccase, syringaldazine appeared to be the best substrate.

Table 4
Properties of the purified laccase SRL1 from *S. rolfsii*

Molecular weight	59 kDa
Isoelectric point (pI)	5.2
Specific activity	245 nkatal mg^{-1}
pH optimum	2.4
T optimum	62 °C
Stabilities (half-life in min)	
pH 2.5, 30 °C	8 min
pH 3.0, 30 °C	12 min
pH 4.0, 18 °C	1 week
pH 4.5, 80 °C	<2 min
pH 4.5, 60 °C	60 min
pH 5.0, 30 °C	65 min

Table 5
Substrate specificity of laccase SRL1 from *S. rolfsii*

Substrate	Concentration (mM)	Wavelength (nm)	Percentage activity
Syringaldazine	0.5	525	100
ABTS	5	420	82
2,6-DMP	5	470	60
Guaiacol	5	436	21
Catechol	5	450	11
Lignin sulfonic acid	3	570	0.9
Pyrogallol	5	450	7
1,4-Fenilenediamine	5	500	0
Ferulic acid	5	287	0
Tyrosine	3	280	0

Values are given in percentage of activity in nkat ml^{-1} measured at standard conditions.

Typical of fungal laccases, this enzyme was unable to oxidize the simple phenolic compound, tyrosine [36,37].

3.5. Effect of inhibitors

The effects of a number of possible laccase inhibitors are shown in Table 6. As expected the most potent of the inhibitors assessed was sodium azide (NaN_3), requiring the presence of 9.0 μM to effect a 50% drop in laccase activity. The binding of NaN_3 to the types 2 and 3 copper sites effects internal electron transfer, thus inhibiting the activity of the laccase. The sensitivity of SRL1 to this inhibitor is demonstrated by the fact that enzyme activity was inhibited by approximately 30% by the presence of 3.3 μM NaN_3. These findings are in keeping with the general properties of laccase from a diverse range of fungal sources [38,39]. Addition of a water miscible solvent, such as ethanol or methanol, effected a decrease in enzyme activity, similar to those reported for the *Cerrena unicolor* laccase where ABTS was also used as the substrate [39]. However, organic solvents change the pH of aqueous solutions and hence, more factors could be contributing to the experimental data obtained [40]. Laccases are inhibited by metal chelators such as EDTA and more strongly by the copper chelators diethyldithiocarbamate (DDC) [2,41]. Type 1 copper can be easily removed by forming a complex since it is exposed to solvents [42]. EDTA up to a concentration of 300 mM showed no effect on SRL1. DDC (I_{50} = 1.2 mM) strongly inhibited the enzyme like many other fungal laccases such

Table 6
Laccase inhibitors of SRL1

Inhibitor	I_{50}
Methanol	37%
Ethanol	36%
Thiomine	0.95 mM
L-Cysteine	0.52 mM
NaN_3	9 μM
EDTA	>300 mM
NaBr	195 mM
NaCl	52 mM
NaF	0.08 mM
DDC[a]	1.2 mM
TGA[b]	0.5 mM

[a] DDC: diethyldithiocarbamate.
[b] TGA: thioglycolic acid.

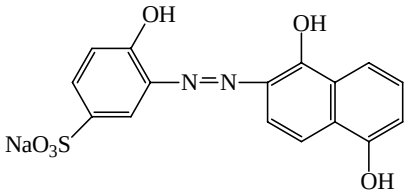

Fig. 3. Chemical structure of Diamond Black PV 200 (2-amino-1-phenol-4-sulfonic acid → 1,5-naphthalenediol).

as from *P. cinnabarinus* [43], from *B. cinerea* [44], from *Pleurotus ostreatus*, from *Trametes versicolor* [2] and *T. hirsuta* [45] where the I_{50} values were below 1 mM. Among the halogens tested, fluoride was the strongest inhibitor as shown previously in the literature [46].

3.6. Dye decolourization

A number of textile dyes was decolourized by SRL1 including, Remazol Brilliant Red, Indigo and Lancet Marine Blue (data not shown). Decolourization of Diamond Black PV 200 (MW 350.26), otherwise known as Mordant Black 9 or Eriochrome Black PV was investigated in more detail in this study since this dye is used in much higher quantities than any other dye in a collaborating German wool company. This acid-aniline dye contains a sulfonic group that imparts water solubility on the dye (Fig. 3). The dye was found to be a suitable substrate for the purified laccase and the addition of redox mediators was not necessary. Within a 55-min period the textile dye appeared to be completely degraded due to the laccase activity (Fig. 4). The kinetic parameters of laccase oxidation of the dye were described using the Michaelis–Menten model.

The apparent K_m was estimated to be $16.8 \pm 2.6\,\mu M$ DB or $5.9 \pm 0.9\,mg\,l^{-1}$ DB and the V_{max} was $1.34\,\mu M\,min^{-1}$.

Several other lignolytic fungi were shown to degrade azo dyes investigated including *P. chrysosporium* [47,48], *T. versicolor* [49], *T. modesta* [50] and *Aspergillus niger* [51]. Previously, a purified laccase from *A. niger* was shown to decolourize one of four diazo dyes tested, demonstrating a specificity for the type or position of substituents on the phenolic ring of the dye structure [14]. However, in most of these studies crude enzyme preparations or fungal cultures were used. One disadvantage of using fungal cultures to effect dye degradation is the accumulation of biomass.

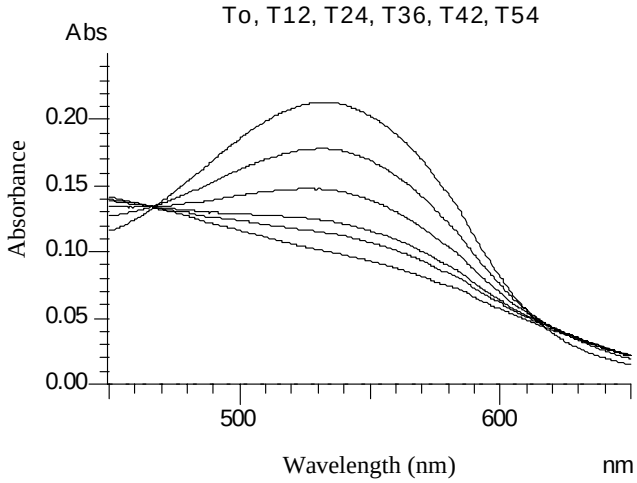

Fig. 4. Absorbance spectra of DB ($20\,mg\,l^{-1}$) after treatment with the *S. rolfsii* laccase. The scans were performed at 12 min intervals.

From an industrial point of view it would be simpler to add a laccase solution directly into the wastewater. An attractive feature of this enzyme is the fact that it does not require redox mediators to function efficiently in dye biotransformation. For the treatment of wool dyeing effluents with low pH values, an acid stable laccase would be desirable. Consequently, the laccase from *S. rolfsii*, which is very stable in the acidic pH range, may prove to be successful on an industrial scale. Immobilisation of the laccase could further reduce cost of the enzyme and avoid interaction of the enzyme protein with dyes when the treated effluents are reused in dyeing [45].

3.7. Redyeing

The crude laccase was immobilized on alumina support. Eighty-three percent of the protein bound to the carrier, corresponding to $0.13\,mg\,g^{-1}$ alumina and 72% of the laccase activity was retained on the carrier material. Previously, between 70 and 98% of the protein immobilised and 67–96% of laccase activity, recovery was reported with different carrier materials [52–54]. Out of a number of different carrier materials previously tested for laccase immobilization such as activated carbon [55], Eupergit® C [56], Sepharose [57], silica-based matrices [58] and porous glass [53,59] only alumina was used in this study as it seems to be more suitable

Table 7
Colour difference on fabrics dyed with C.I. Acid Blue 113 in enzymatically decolourised textile effluents coming from dyeing with C.I. Reactive Black 5

Depth of shade (% o.w.f.)	Colour difference, ΔE^*					
	Liquor 1	Liquor 2	Liquor 3	Liquor 4	Liquor 5	Liquor 6
0.5%	2.9	1.9	2.0	2.3	2.4	2.2
1.0%	1.0	1.3	1.0	1.5	1.9	1.9
1.5%	0.8	0.9	0.8	0.9	1.4	1.3

for application in dye decolourization, due to its mechanical stability.

Dyeing effluents decolourized with the immobilized laccase were suitable for redyeing (Table 7), providing considerable fresh water saving. The ΔE^* values for redyeing with 1.0 and 1.5 depth of shade (% o.w.f.) were in a range which is acceptable to the industry [60–62]. However, these values might certainly be improved by proper adjustments of the standard dyeing protocols. In summary, we have shown for the first time that an acidic laccase from *S. rolfsii* immobilized on alumina can be used for the continuous decolourization of wool dyeing effluents which allows usage of the water for redyeing.

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