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Full Length Research Paper

Purification and characterization of a laccase from the basidiomycete *Funalia trogii* (Berk.) isolated in Tanzania

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A lignolytic basidiomycete fungus, *Funalia trogii* (Berk.), was isolated from decayed wood in coastal Tanzania and cultivated in submerged culture. Initially screened crude enzyme filtrate showed complete rhemazol brilliant blue - R (RBBR) decolorization 2,2'-azino-bis (3-ethylbenzthiazoline)-6-sulfonate and guaiacol oxidation after 7 days of incubation. The fungal filtrate had maximum laccase activity of 593 U/ml after 15 days of incubation. A laccase was purified by anion exchange and size exclusion chromatography to good purity as judged by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and isoelectric focusing (IEF). The isolated main component had a molecular weight of ca 58 kDa as determined by MS and an isoelectric point (pI) of 3.8. The optimal pH and temperature range for the purified laccase were 4.0 - 5.0 and 50 - 70 ºC, respectively, using 2, 6-dimethoxyphenol as a substrate.

Key words: *Funalia trogii*, laccase, anion exchange chromatography, size exclusion chromatography, electrophoresis, isoelectric focusing.

INTRODUCTION

Laccase (benzenediol: oxygen oxidoreductase; EC. 1.10.3.2), is a polyphenol oxidase that contains four copper atoms and is able to oxidize its substrates with molecular oxygen serving as electron acceptor (Thurston, 1994). The ability of laccases to oxidize phenolic compounds and reduce molecular oxygen to water has led to intensive studies of these enzymes (Nagai et al., 2002; Murugesan et al., 2006; Lu et al., 2007; Liersa et al., 2007). Laccases have various functions such as participation in pigmentation in fungi (Clutterbuck, 1990), plant pathogenicity (Iyer and Chattoo, 2003), and biodegradation of many aromatic compounds (Xiao et al., 2004). Due to their low substrate specificity and strong oxidative abilities, laccases have a number of industrial applications including biopulping, prevention of wine decolouration, detoxification of environmental pollutants, textile dye bleaching, enzymatic conversion of chemical intermediates and the production of valuable compounds from lignin (Thurston, 1994; Xu, 1996; Xu et al., 1999; Gianfreda et al., 1999; Mayer and Staples, 2002; Nyanhongo et al., 2002). The ideal laccases for industrial use would exhibit stability at high temperature and pH conditions (Quaratino et al., 2007; Niladevi et al., 2008). Thus, fungal strains with high laccase activity and substrate affinity that exhibit such stabilities have a potential for biotechnological applications.

Comparative studies of fungal laccases have shown that these enzymes are similar in their specificity for different phenolic compounds, regardless of their origin, but differ markedly in their inducibility, number of enzyme forms, molecular mass and optimum pH and temperature (Fortina et al., 1996; Eggert et al., 1996; Giatti et al., 2003; Palonen et al., 2003; Baldrian, 2004; Minussi et al., 2006, 2007; Park and Park, 2008).

In Tanzania, previous studies by Masalu (2004) and Mtui et al. (2003) screened lignin-degrading fungi and their lignolytic enzymes with emphasis on their ability to degrade lignocellulosic substrates. Mtui and Nakamura (2007) further reported biochemical characteristics of lig-

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nolytic enzymes from a marine white-rot fungus, *Phlebia chrysocreas* isolated from the sea coast of Tanzania. Recent studies have elucidated characteristics of purified enzymes from facultative and obligate marine fungi isolated from mangrove forests and in the Indian Ocean waters, respectively (Mtui and Masalu, 2008; Mtui and Nakamura 2008). However, the characteristics of purified enzymes from Tanzania's terrestrial fungi have not been investigated. Therefore, this study reports on the biochemical characteristics of purified laccase from *Funalia trogii*, a Tanzania's terrestrial lignolytic mushroom isolated from decayed wood.

MATERIALS AND METHODS

Collection, screening and cultivation of the fungus

F. trogii (Berk.) was collected from decayed wood in coastal Tanzania. The fungus was preliminary identified on the basis of morphological and microscopic features (Buczacki, 1992; Härkönen et al. 2003) and confirmed by phylogenetic analysis of internal transcribed spacers containing rRNA gene sequence (Kamei et al., 2005). Pure fungal mycelia were obtained by tissue culture on 5 % (w/v) malt extract agar. Rhemazol Brilliant blue R (RBBR) dye, 2, 2'-azino-bis (3-ethylbenzthiazoline)-6-sulfonate (ABTS) and guaiacol were added in the solid medium to screen for laccase expression. The modified Kirk's medium (Dhouib et al., 2005) was used in the submerged culture fermentation.

PURIFICATION OF LACCASE

Precipitation of proteins by ammonium sulfate

The crude fungal culture was filtered through Whatman No 1 filter paper using a vacuum pump and the resulting filtrate was concentrated by freeze-drying and kept at 4°C. Proteins were precipitated by ammonium sulfate from the mycelia filtrate obtained after submerged culture fermentation. Solid ammonium sulfate was added slowly to the crude extract of each isolate to give 80% saturation and the solution was stirred gently for at least 1 h at 4°C and then left to stand overnight. The precipitate was collected by centrifugation at 8,000 g for 1 h at 4°C. The supernatants were discarded and the pellets were dissolved in a minimum amount of 5 mM Bis-Tris [bis-2-hydroxyethyl) imino-tris (hydroxymethyl) methane]-HCl buffer, (pH 6.5).

Desalting/Buffer exchange of proteins

The re-dissolved protein precipitate was desalted through PD-10 columns (*GE Healthcare, Uppsala,* Sweden) equilibrated with 5 mM Bis-Tris-HCl buffer (pH 6.5) according to the manufacturers instruction.

Anion exchange chromatography (AEC)

A HiLoad^{TM} 26/10 quaternary ammonium (QA)-Sepharose column (GE Healthcare, Uppsala Sweden) was equilibrated with 5 mM Bis-Tris-HCl buffer, pH 6.5, and the desalted protein solution was loaded into the column at a flow rate of 0.67 ml/min. Bound proteins were eluted with a 400 ml linear gradient of NaCl (0 – 0.5 M) in 5 mM Bis-Tris HCl-buffer, pH 6.5 at the same flow rate. 3 ml fractions were collected. Protein concentration was estimated from the absorbance at 280 nm and laccase activity was determined for each fraction using ABTS as the substrate (Beeckmans, 1999). One unit of laccase activity was defined as activity of an enzyme that catalyzes the conversion of 1 µmole of ABTS per minute. Fractions showing laccase activity were pooled for further purification.

Sample concentration

Pooled protein fractions were concentrated by ultrafiltration using a *Filtron* membrane concentrator (*Filtron* Technology Corp., USA) with 10 kDa nominal cut-off at a gauge pressure of 2.5 bars with N_2. Three pools (pool 16 - 35, 48 ml; pool 56 - 60, 30 ml; and pool 61 - 68, 120 ml) were obtained after anion exchange chromatograph and concentrated to 8, 5 and 5 ml respectively. Laccase activity was measured before and after concentration.

Gel filtration chromatography (GFC)

The pooled fractions were further fractionated on a Superose^{TM} 12 prep grade gel column with VT = 104 ml coupled to a LKB 2150 pump (GE, Healthcare, UK). The column was equilibrated using 0.1 M NaCl in 5 mM Bis-Tris-HCl buffer, pH 6.5, as eluent. 2 ml sample was injected in each run and chromatographed at a flow rate of 1 ml/min and collected in fractions of 2 ml. The fractions were assayed for laccase activity and the active fractions were pooled for SDS-PAGE and IEF analyses and characterization. Specific activities, percent yields and purification folds were calculated according to Wilson and Walker (2005).

ELECTROPHORETIC ANALYSIS AND CHARACTERIZATION OF LACCASES

SDS-PAGE and IEF analyses

The pools obtained after chromatography were analyzed by 12% SDS-PAGE (Laemmli, 1970). Electrophoresis was carried out at 150 Volts for 1 h and the gel was stained with 0.25% w/v Coommasie Brilliant Blue R-250 followed by de-staining overnight in 5:1 methanol-glacial acetic acid solution.

Isoelectric points were determined using Ampholine PAG plates in an LKB Multiphor Unit and broad pI (pH 3.0 - 10.0) standard markers (GE Healthcare, Uppsala, Sweden). The IEF gel was fixed in 5% sulphosalicylic acid and 10% trichloroacetic acid solution for 60 min followed by staining in 0.2% Coomassie Brilliant Blue R-250 in methanol-acetic acid solution (3: 1) for 30 min, and de-staining in 3:1 methanol-acetic acid solution (Scopes, 1982).

Mass spectroscopic analysis

The protein pools 16 - 35, 56 - 60 and 61 - 68 were analyzed by MALDI-TOF MS. The pure laccase in pool 56-60 was also analyzed by peptide mass fingerprinting after trypsin cleavage.

Optimum pH, temperature and thermostability

The pH-dependence of the laccase activity was examined at room temperature (20°C) in the pH range of 2.5 - 8.0 at a pH interval of 0.5 using 2, 6-dimethoxyphenol (DMP) as substrate. A 0.1 M Britton- Robinson buffer (0.1 M boric acid, 0.1 M acetic acid and 0.1 M phosphoric acid) was used and the required pH was adjusted by 1 M NaOH (Xu, 1996).

The effect of temperature on laccase activity was determined following the laccase-catalyzed oxidation of 1 mM DMP for 2 min at temperatures ranging from 20 - 90°C at 10°C intervals in 0.1 M Britton-Robinson buffer at the optimum pH obtained for each lac-

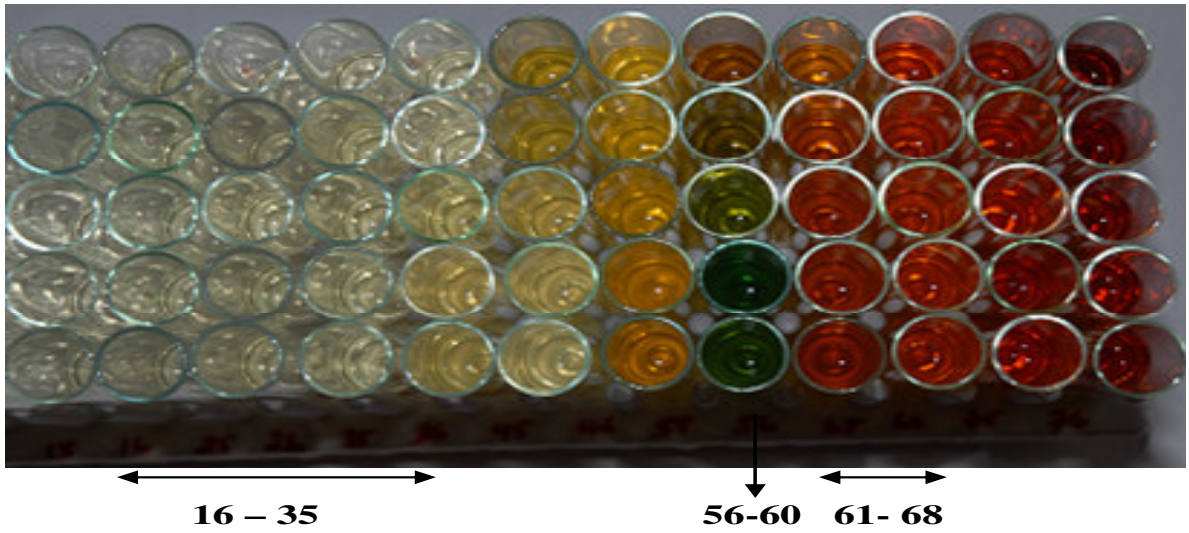

Figure 1. Fractions collected after anion exchange chromatography of *Funalia trogii* culture filtrate. Note: The greenish fractions correspond to pool 56 - 60, orange fractions to pool 16-35 and dark red fractions to pool 61 - 68.

case.

The thermal stability of the purified laccase was determined by following the oxidation of 1 mM DMP for 2 minutes at 20 °C after pre-incubation of laccase for 20, 40, 60, 90 and 120 min at 50, 55, 60 and 65°C. The reaction was started by the addition of pre-incubated laccase at the above temperatures in 1 mM DMP dissolved in 0.1 M Britton-Robinson buffer at optimum pH.

Laccase assay

DMP was oxidized to its quinone, 2, 6-dimethoxycyclohexa-2, 5-diene-1-one, by laccase-catalysed reaction resulting in the formation of the yellow-orange colour. The reaction mixture (1.5 ml) contained 1490 µl of 1 mM DMP in 0.1 M Britton-Robinson buffer and 10 µl of the enzyme. The oxidation of DMP was followed spectrophotometrically at 477 nm, with extinction coefficient (ε_{477}) of 14800 $M^{-1}cm^{-1}$ (Palmieri et al., 1997).

RESULTS AND DISCUSSION

Initial screening for laccase activities

In the initial screening, complete RBBR dye decolorization, ABTS and guaiacol oxidation was observed after 7 days of incubation. Dye decolorization and halo formation as a result of oxidation of coloured compounds is due to lignolytic enzymes production (Rodrı´guez et al., 2000; Kiiskinen et al., 2004). It is an evidence of multi-enzymatic actions that could be applied in xenobiotic biodegradation studies as well as an indication of the physiological conditions of basidiomycetes during bioremediation process (Machado et al., 2005). The use of dyes offers a series of advantages in relation to conventional substrates because they are stable and soluble substrates with high rates of molar extinction and low toxicity (Machado et al., 2005). Also, the use of dyes and coloured indicators that enable visual detection of lignolytic activities is a simple method of screening as no measurement is required (Okino et al., 2000). RBBR dye used in the initial screening is an antracene derivative and it is thus related to an important group of organopollutants (Machado et al., 2005). The results obtained in this study support previous studies that plate-test is an efficient and simple method for bioprospecting fungi with novel lignolytic enzymes for industrial application purposes (Masalu, 2004).

Purification of laccase from *F. trogii* culture filtrate

The fungal filtrate had maximum laccase activity of 593 U/ml after 15 days of incubation. Anion exchange chromatography gave fractions with detectable laccase activity in a breakthrough region, where fractions 16 - 35 (orange) were pooled; a main peak (56 - 60, green) and a small side peak (61 - 68, reddish brown) displayed a noticeable absorbance at 280 nm (Figure 1 and 2).

The final gel chromatography carried out for all of the concentrated pools revealed distinctive activity peaks, suggesting single (and probably very similar) active components (Figure 3a - c). However, only the 56 - 60 fractions gave a pure component, with the activity peak coincident with the blue colour typical for copper laccases (Figure 3d). It is likely that the activity in the breakthrough fraction was due to traces of the major laccase, whereas the small activity peak in pool 61 - 68 corresponds to a small heterogeneity in physical properties but most likely also corresponds to the same gene. The orange-red coloration found in many fractions is most likely due to non-protein components.

Pools16 - 35 and 61 - 68 fractions did not show the typical blue colour, most likely due to the low concentration of active laccase, in accordance with the low specific activity. Laccase-like enzymes that lack the typical

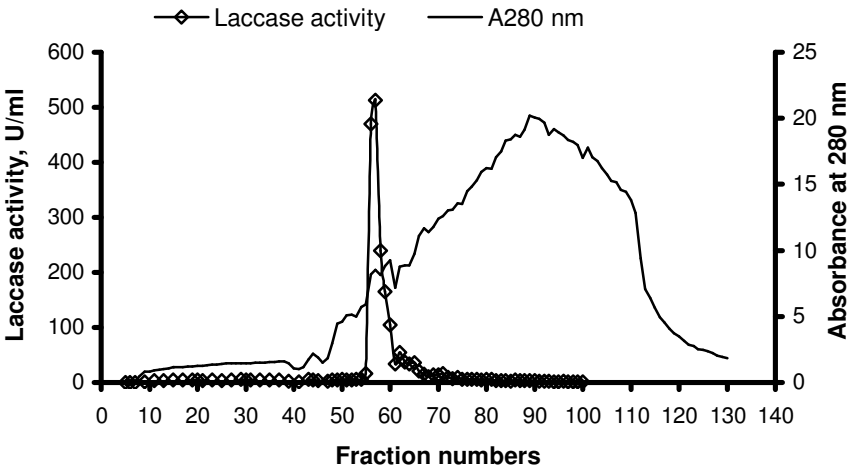

Figure 2. Elution profile of laccase from *F. trogii* separated on QA-sepharose anion exchange chromatography.

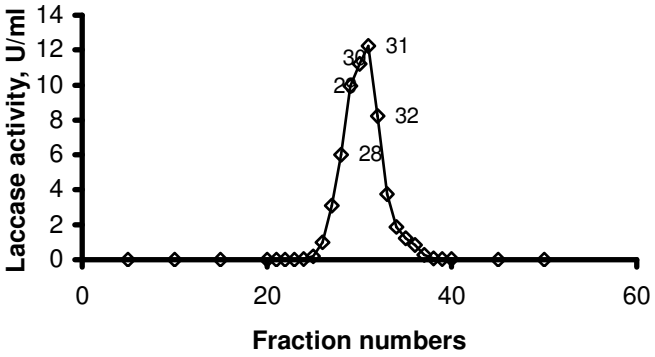

Figure 3a. Laccase activity for pool 16-35 after gel chromatography.

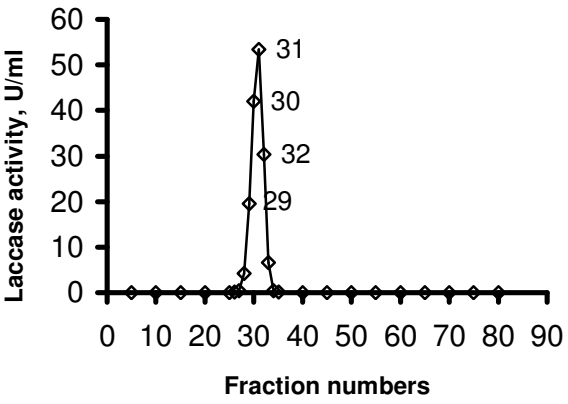

Figure 3c. Laccase activity pool 61-68 after gel chromatography

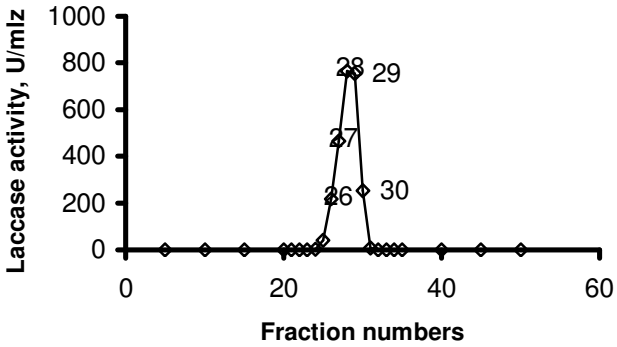

Figure 3b. Laccase activity of pool 56 - 60 after gel chromatography

Figure 3d. Fractions collected after gel filtration chromatography of "green fractions" (pool 56-60), the blue fractions are pure laccase.

absorption around 600 nm have, however, been reported (Meyer and Staples, 2002; Leontievsky et al., 1997; Palmieri et al., 1997; Edens at al., 1999) where copper appears in the reduced form. It has been found that laccases which do not have the blue color can have copper, zinc and iron atoms instead of the classical four copper atoms and these are referred to as 'white' laccases (Palmieri et al., 1997). The other possibility is that the lac-

Table 1. Summary of purification procedures for laccase from *F. trogii.*

Purification steps	Fractions pool	Total activity, (Units)	Total protein (mg)	Specific activity, U/mg	Yield (%)	Purification fold
Culture filtrate		19900	600	33	100	1.00
Freeze drying		19100	530	36	96	1.09
$(NH_4)_2SO_4$		17800	486	37	89	1.12
Desalting		15100	400	38	76	1.15
	16 - 35	600	150	4	3	(0.12)
AE chromatography	56 - 60	10000	210	48	50	1.45
	61 - 68	1800	n.d.*	n.d.*	9	n.d.*
	16 - 35	550	121	5.0	2.5	(0.15)
Ultrafiltration	56 - 60	9200	164	56	46	1. 7
	61 - 68	1300	n.d*.	n.d.*	7	n.d*.
	16 - 35	520	98	5.3	1	(0.16)
GF Chromatography	56 - 60	8800	96	92	44	2.8
	61 - 68	1130	n.d*.	n.d.*	6	n.d.*

*Measurement was impossible due to non-protein contaminants.

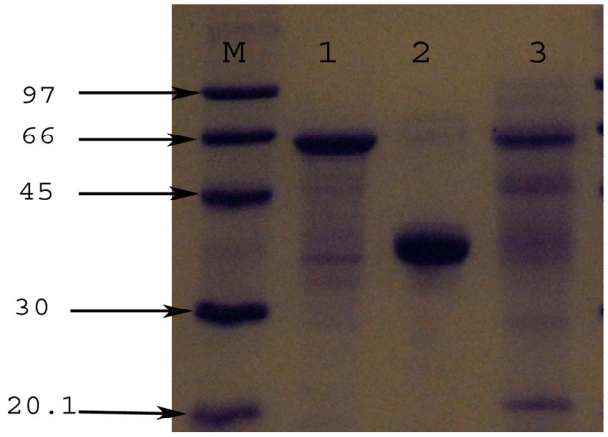

Figure 4. SDS-PAGE analysis of laccase from *F. trogii* after gel filtration chromatography, M = marker proteins, lane 1 = pool 56 - 60, lane 2 = pool 16 - 35 and lane 3 = pool 61 - 68.

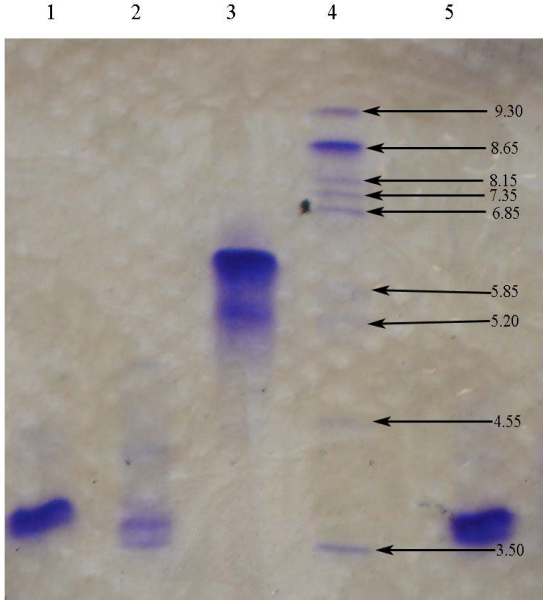

Figure 5. Isoelectric focusing of laccase, lane 1 and 5 = pool 56-60, lane 2 = pool 61-68, lane 3 = pool 16-35 and lane 4 = pI marker.

cases could be yellow instead of blue, as a result of the modification of blue laccases by products of lignin degradation (Leontievsky et al., 1997; Pozdnyakova et al., 2006).

Table 1 summarizes the purification procedures for the laccase from *Funalia trogii.* In the gel filtration chromatography, fractions 56 - 60 gave the highest specific activity, yield and purification fold of 92 U/mg, 44% and 2.8 respectively. The results are comparable to reports by Nakamura et al. (1999), Mtui and Nakamura (2008) and Mtui and Masalu (2008) who demonstrated successful purification of lignocelluosic enzymes from terrestrial and marine fun-gal filtrates.

SDS-PAGE and IEF analyses

The SDS-PAGE analysis in Figure 4 displayed a single band with apparent molecular weight of ~65 kDa for pool 56 - 60, whereas several components still were found in the other pools also after the additional gel chromatography. Comparative 65 kDa laccase has been isolated in the culture medium of *F. trogii* ATCC 200800 (Deveci et al., 2004), 72 and 74 kDa from *Trametes* sp AH28-2 (Xiao et al., 2003, 2004), 70 kDa from *Trametes. Trogii* strain 201 (Garzillo et al., 1998), 62 kDa from *T. trogii* B6J (Mechichi et al., 2006).

IEF analysis of the three pools after gel filtration chromatography gave a similar result with a distinct band at pI 3.8 for pool 56 - 60 whereas the other pools were heterogenous (Figure 5). These results are comparable to re-

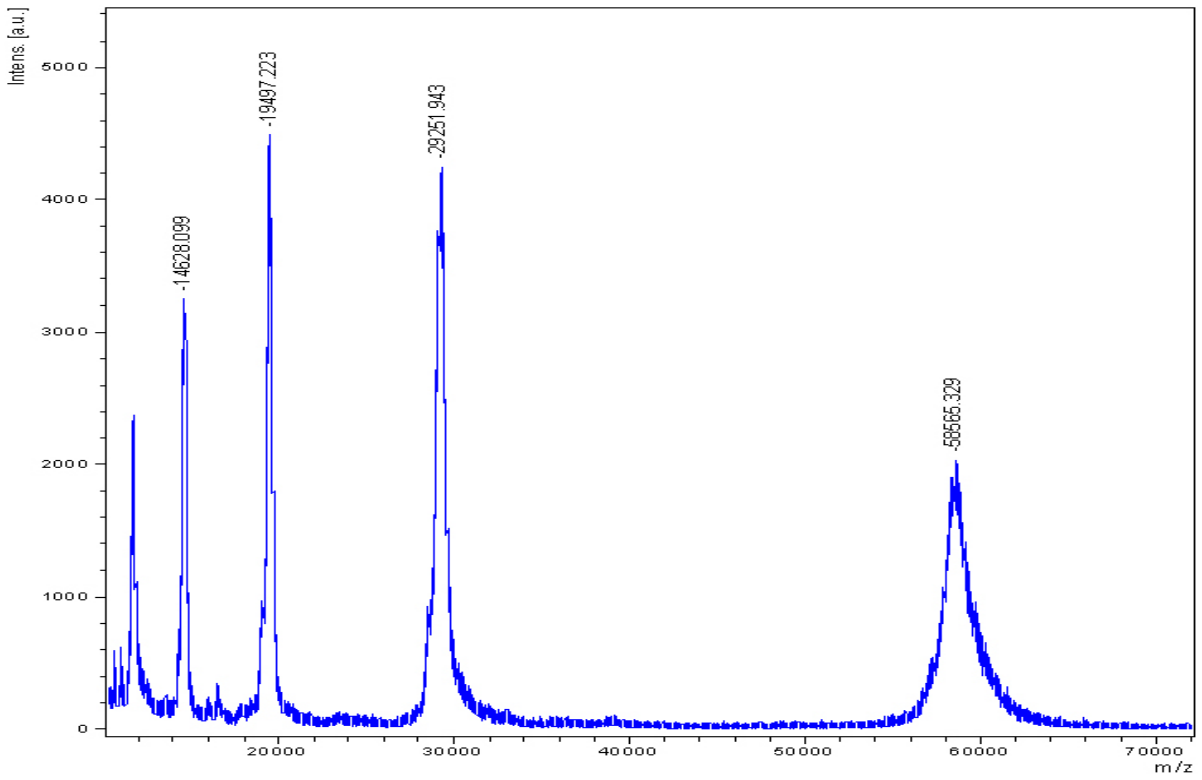

Figure 6. Full m/z spectrum of the pure laccase.

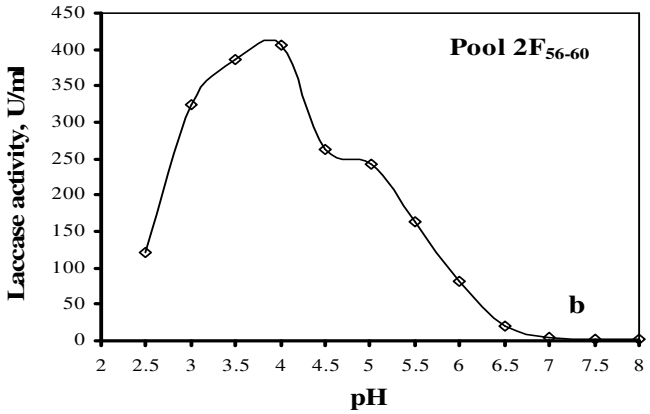

Figure 7. pH dependence on activity of the purified laccase pool 56 - 60.

ports by Garzillo et al. (1998) and Mechichi et al., (2006) who obtained *pI* values of 3.6 - 4.5 from *T. trogii* strains.

Mass spectroscopy (MS) analysis

MALDI-TOF-MS of the pure laccase in pool 56 - 60 revealed a mass envelope centered at 58 kDa (Figure 6). The width of the peak is undoubtedly due to heterogeneous glycosylation, and a more complete m/z spectrum confirmed the purity of the enzyme. Peptide mass fingerprinting after tryptic cleavage revealed a close homology with other fungal laccases such as *Trametes* sp I-62, *Volavariella volvacea* and basidiomycetes CECT 20197 (http://www.matrixscience.com/cgi/master). MS of the other pools confirmed the heterogeneity observed in SDS-PAGE and IEF. A 58 kDa component similar to that in pool 56 - 60 was, however, observed in pool 61 - 68.

pH optimum

The curve in Figure 7 shows the optimum pH for the purified laccase in pool 56-60 to be 4.0. It is noteworthy that the pH range is very broad, suggesting the possibility of using laccase from *F. trogii* for many biotechnological processes, including those that require alkaline conditions. The bell-shaped pH profile is the result of two opposing effects: The first effect is due to the redox potential difference between a reducing substrate (phenolic compound) and the Type 1 copper center of laccase, where the substrates dock and electron transfer rate is favored for phenolic substrates at a high pH. The second effect is generated by the binding of a hydroxide anion to the type 2/Type 3 copper centers of laccase, which inhibits the binding of O_2, the terminal electron acceptor, and therefore inhibits the activity at a higher pH because of the increased amount of OH^- ions (Munoz et al., 1997). Both the enzymatic rate of oxidation and pro-

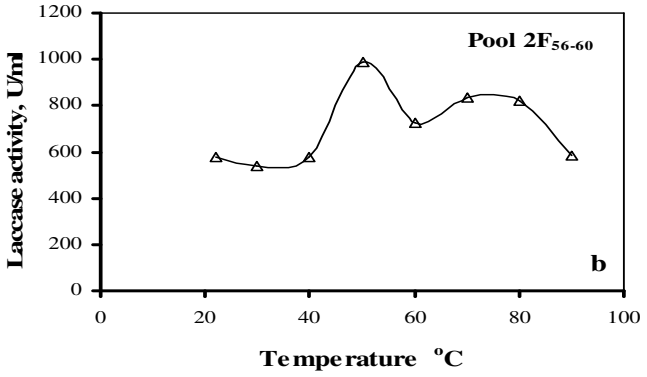

Figure 8. Temperature dependence on the activity of the purified laccase in pool 56 - 60.

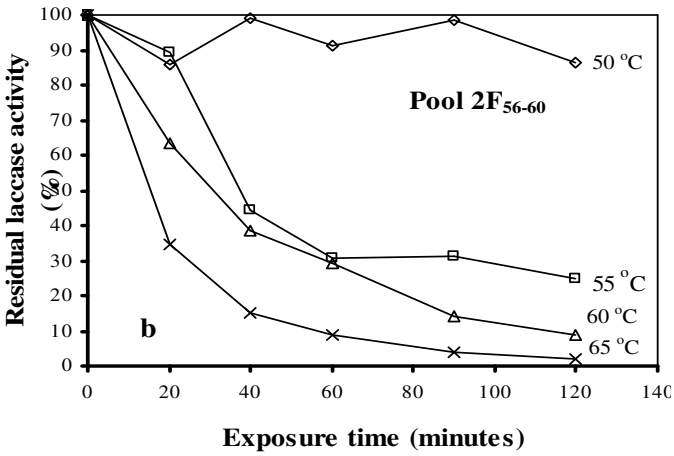

Figure 9. Thermal stability of the purified laccase.

products formed are pH dependent as it affects abiotic follow-up reactions of primary radicals formed (Baldrian, 2005). The results on pH optima are consistent with previous reports that suggest, depending on the substrates used, pH optima for fungal laccases on substrates like DMP, guaiacol and syringaldazine, range between 4.0 and 7.0 (Heinzkill et al., 1998; Min et al., 2001; De Souza and Peralta, 2003; Baldrian, 2005; Wang, 2006).

Temperature optima and thermal stability

The effect of temperature on the activity of the purified laccase is shown in Figure 8. The reaction rate had an optimum at 50°C and stayed high at a temperature range of 40 - 70°C below and above which a sharp or steadily decline in activity was observed. The optimum temperature for laccase activities can differ from one strain to another, with a range for most fungal laccases being 50 - 70°C (Luisa et al., 1996, Baldrian, 2005).

The thermal stability of the laccase is shown in Figure 9. At 50°C, very little activity was lost after the allocated 120 min. The results are in line with other works that have reported thermal stabilities of fungal laccases (Farnet et al., 2000; Sadhasivam et al., 2008). The tem-perature stability of fungal laccases varies considerably: Half life at 50°C ranges from few minutes in *Botrytis cinnerea* (Slomczynski et al., 1995), to over 2 - 3 h in *Lentinula edodes* and *Agaricus bisporus* (D'Annibale et al., 1996), to up to 50 - 70 h in *Trametes* sp. (Smirnov et al., 2001). While the mesophylic laccase from *Gano-derma lucidum* is immediately inactivated at 60°C, the thermostable laccase from *Melanocarpus albomyces* still exhibit a half life of over 5 h and thus a very high potential for selected biotechnological applications (Kiiskinen et al., 2002). The findings of this study suggest that the extracellular laccases isolated from *F. trogii* are adapted to tropical climates and therefore valuable for bioremediation of environmental pollutants.

Conclusion

This study has successfully isolated *Funalia trogii* (Berk.), a basidiomycetes fungus from decayed wood in coastal Tanzania. The crude filtrate was found to contain considerably high activity of laccase (593 U/ml) after 15 days of incubation and was capable of degrading rhemazol brilliant blue - R (RBBR) and guaiacol, showing that it has high potential for biotechnological applications. The main laccase fractions, purified chromatographically and resolved by SDS-PAGE and IEF, showed that the enzyme has a molecular weight of 58 kDA and *PI* of 3.8. Its identity was confirmed by mass spectrometry and its optimal pH and temperature range were found to be 4.0 - 5.0 and 50 - 70°C respectively. Future research is focused on molecular characterization and application of the laccase in biodegradation of recalcitrant environmental pollutants.

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