CHARACTERISTICS AND DYES BIODEGRADATION POTENTIAL OF CRUDE LIGNOLYTIC ENZYMES FROM WHITE-ROT FUNGUS *CREPIDOTUS VARIABILIS* ISOLATED IN COASTAL TANZANIA

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ABSTRACT

Lignocellulosic enzymes from Crepidotus variabilis *collected from mangrove forests of coastal Tanzania were investigated by using standard methods, and their ability to degrade aromatic compounds were elucidated. The fungal crude enzyme filtrates had maximum laccase (Lac), lignin peroxidase (LiP) and manganese peroxidase (MnP) activities of 70 U/mL, 16 U/mL and 8, U/mL respectively. The crude enzyme extracts were able to oxidize rhemazol brilliant blue-R (RBB-R) dye, phenol, α-naphthol and pyrogallol. Also, they could remove up to 58% and 92% color from raw textile effluent and aromatic dyes, respectively, after 14 days of incubation at 30ºC and pH 4.5. Desalted and size-separated enzyme filtrates, resolved by sodium docecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and isoelectric focusing (IEF), showed laccases and peroxidases from* C. variabilis *to have molecular weights of 67 kDa and 47 kDa, respectively, while the isoelectric points (pI) of laccases and peroxidases were found to lie in a range of 3.0 to 4.1. The study provided basic information on the characteristics of crude lignolytic enzymes from* C. variabilis *and confirmed it to be one of the potential biodegraders of aromatic compounds that could be applied in bioremediation of polluted ecosystems.*

INTRODUCTION

The raw industrial and domestic effluents that are discharged into the Indian Ocean pose a serious environmental pollution problem. Such wastewater contains recalcitrant organic compounds including crude oil wastes and textile effluents; organochloride agrochemicals such as DDT, PCBs and dioxins (Nakamura *et al*. 1997, 1999, Saparrat 2000, Mtui and Nakamura 2002, Gonzallo *et al.* 2005). However, Tanzania has rich diversity of basidiomycetes fungi with potential for application in both *ex situ* and *in situ* biodegradation of such pollutants (Härkönen *et al.* 2003, Mtui *et al.* 2003, Coulibaly *et al*. 2003, Martinez *et al.* 2005). Worldwide, there is little published information on lignin-degrading abilities of the obligate and facultative marine fungi, Mtui and Masalu 2008). Moreover, the presence of manganese-dependant peroxidase (MnPs), lignin peroxidases (LiPs) and laccases (Lacs) in such fungi have not been investigated except for few studies (Datta *et. al*. 1991; Raghukumar *et al*. 1999; Kondo *et. al*. 2004; Mtui and Masalu, 2008). In Tanzania, more attention on fungal research has been focused on edible mushrooms, with more than 50 edible species already identified (Härkönen *et al.* 2003, Mtui *et al*. 2003), but research on the non-edible types has so far received little attention.

Crepidotus variabilis (Pers) Gray is a wood-inhabiting white-rot fungus, a member of family Crepidotaceae and order Agaricales that is characterized by its saprotrophic habit, filamentous cuticle, and brown-pigmented basidiospores that lack either a germ pore or plage (Aime, 2001). Although terrestrial *C. variabilis* has been shown to be able to degrade lipophilic extractives in *Eucalyptus globulus* wood in solid fermentation (Gutiérrez *et al*. 1999, Martinez e*t al*. 1999) and lignocellulosic compounds in liquid cultures (Martinez *et al*. 2005), the *C. variabilis* strains inhabiting marine ecosystems have not been reported in the literature and their degradative enzymes have

not been elucidated. Therefore, this knowledge gap in marine and coastal fungal lignocellulosic enzyme profiles, properties and degradative abilities makes it an important and interesting area of research.

Characterization of lignocellulosic enzymes is important in order to identify potential specific isoforms that are suitable for bioremediation purposes. Chromatographical techniques have been shown to be conducive in separating individual proteins into isoenzymes in order to evaluate their properties. The separated fractions are resolved in sodium docecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) which is a powerful tool in the analytical separation of macromolecules whereby proteins are separated according to their molecular sizes under the influence of an electric field. On the other hand, isoelectric focusing (IEF) is another biochemical technique in which protein molecules are separated as they migrate through a pH gradient under a strong electric field (Deutscher 1990). SDS-PAGE analysis has shown that relative molecular weights of extracellular proteins from lignocellulosic fungi range from 40 kDa to 130 kDa (Matsubara *et al*. (1996), Heinfling *et al*. (1998), while IEF analysis shows considerable variation in isoelectric point (pI) values between 3 to 10 (Scopes, 1982; Deutscher 1990, Martinez *et. al*. 2005). There is very limited information available in the literature on the specific characteristics of extracellular ezymes from facultative and marine fungi.

This work reports the original research results on extracellular enzymes from Crepidotus *variabilis* isolated from Tanzania's marine ecosystem and elucidates their characteristics and dye degradative abilities.

MATERIALS AND METHODS

Study site, sample collection and identification

The fungus was collected at Oyster Bay, Mtoni and Mbweni mangrove forests along the Western Indian Ocean cost of Dar es Salaam, Tanzania. It was found growing on decomposing logs of marine vegetation at the periphery of the seashore. The fungus was identified as *Crepidotus variabilis* (Pers) Gray based on morphological characteristics (Ainsworth 1973, Arora 1986, Buckzaki 1992, Phillips 1994, Bougher and Katrina 1998, Harkönen *et. al*. 2003).

Culture media and cultivation of mycelia

Solid media consisted of 5% (w/v) malt extract agar (MEA), 1% glucose, 0.002% malt extract and 20 mL of Kirk medium and maintained at pH 4.5 using potassium phthalate buffer (Tien and Kirk, 1984). Cultivation was done in 10 mm – diameter petri dishes. Fresh 2 mm^2 tissues of fungal fruiting bodies were aseptically cultured in the petri dished and incubated at 30ºC for 5-7 days for production of mycelial mats. The liquid medium contained 1.0-3.0 ammonium tartrate, 1% glucose and 25 mL Kirk medium at pH 4.5. Cultivation in liquid medium was done in 250 mL conical flasks plugged with cotton wool and then covered with aluminium foil. The culture media was sterilized by autoclaving at 121ºC for 20 minutes. The flasks were then inoculated with 5 mm mycelial mats (from solid cultures) and incubated in stationary condition at 30ºC for up to 2 weeks.

Screening for RBBR decoloration, oxidation of guaiacol, α-naphthol and pyrogallol

Solidified 2% (w/v) MEA plates containing 0.005 g/L rhemazol brilliant blue-R RBBR (Sigma, UK) were inoculated with 5-mm agar plugs from a 7 day-old mycelium of *C. variabilis* previously grown on 2% (w/v) MEA. Plates were incubated at 30º C in the dark until they were completely colonized. Peroxidases and oxidases production were revealed by the halo in the medium resulting

from RBBR decolorization. For guaiacol, α-naphthol and pyrogallol tests, 7-mm agar plug from 7–day old mycelium grown in 2 % (w/v) MEA was used to inoculate triplicate plates with w/v 0.2% sugarcane bagasse powder, 0.01% guaiacol and 1.6% agar. The plates were incubated for 21 days before being evaluated on the basis of the formation of a reddish coloured zone in the culture medium resulting from guaiacol oxidation. The drop-test was then carried out on the same plates using 0.1 M α-naphthol and 1% (v/v) pyrogallol to detect laccases and peroxides, respectively (Okino *et al*. 2000).

Decolorization of textile wastewater and aromatic dyes

Half-strength raw effluent wastewater from *Karibu* Textile Mill Ltd in Dar es Salaam and 1% (w/v) of analytical grade synthetic dyes (Azure-B, Poly-B and Poly-R), were used. The wastewater and synthetic dyes were cultured in a low nitrogen medium. Fungal mycelia were then aseptically inoculated into 250 mL flask and the reaction mixture was incubated at 30ºC for 2 weeks. Supernatant of the culture was drawn at 2-day intervals and centrifuged (18,000 g for 15 minutes). Control experiments were conducted using the same medium without fungal inocula. The color intensity (absorbance) was determined by using UV-Visible *Thermo Stonic* spectrophotometer, (UK) at the maximum wavelengths (λ_{max}) of the dyes (Yang *et al.* 2003).

Sample concentration and dialysis

Crude enzyme filtrates were successively filtered in 0.45 µm and 0.2µm *Acrodisc* Syringe Filters (*Pall* Gelman Lab, USA). The 15 mL samples were concentrated 10-fold by ultrafiltration using *Microsep* devise containing omega membrane (*Pall* Life Sciences, USA). The samples were centrifuged (10,000 g for 3 h) at 4ºC. The *Micfrosep* devise retained in the reservoir the concentrated proteins of molecular weight larger than 10 kDa, while lower molecular weight proteins and solvent passed through the membrane into the filtrate reservoir. The concentrated proteins were stored at 4ºC for further analysis.

Protein spectrophotometric assay

The spectrometric assay at 280 nm wavelength was used to estimate the presence of proteins in the filtrates by measuring UV-visible spectrophotometer (Shimadzu Co. Ltd., Japan). The absorbance of the samples were also determined at A_{260} to detect the presence of nucleic acids. The protein concentrations were calculated according to the following empirical equation which takes into account the correction of the interference by nucleic acids (Deutscher 1990): Protein concentration (mg/ml) = 1.55 A_{280}- 0.76 A_{260}), where A = absorbance.

Determination of enzymes activities

LiP activity was determined spectrophotometrically at 310 nm through the oxidation of veratryl alcohol to veratryl aldehyde (molar absorptivity, ε_{310}, = 9300 $M^{-1}cm^{-1}$). The reaction mixture contained 300 µL veratryl alcohol (8 mM), 600 µl sodium tartrate buffer (0.5 M, pH 4.5) at 27 ^{0}C), 60 µL mycelia liquid fraction and 1890 µL distilled water. The mixture was incubated for 2 minutes at 30°C and the reaction was initiated by addition of 150 µL H_2O_2 (5 mM). The absorbance was immediately measured in one-minute intervals after addition of H_2O_2. One unit (U) of LiP activity was defined as activity of an enzyme that catalyzes the conversion of 1µ mole of veratryl alcohol per minute (Nakamura *et al*. 1997, 1999).

Activity of MnP was measured following the method described by Wunch *et al.* (1997). In this method, guaicol was used as a substrate, and the increase in absorbance at 465 nm due to oxidation of guaicol was measured (ε_{465} = 12,100 $M^{-1}cm^{-1}$). The reaction mixture contained 300 µL sodium succinate buffer (0.5 M, pH 4.5 at 27 °C), 300µL guaicol (4 mM), 600 µL manganese sulphate (1 mM), 300 µL mycelial liquid

fraction and 1200 µL distilled water. The mixture was incubated for two minutes at 30 ^{o}C and the reaction was initiated by addition of 300-µL hydrogen peroxide (1mM). The absorbance was measured immediately at 465 nm in one-minute intervals after addition of hydrogen peroxide. One unit of MnP activity was defined as activity of an enzyme that catalyzes the conversion of 1µ mole of guiacol per minute.

Lac activity was measured by using the method described by Bourbounnais *et al*. (1995) based on the oxidation of 2,2'-azino–bis (3-ethylbenzothiazoline)-6-sulphonic acid (ABTS). The rate of ABTS oxidation was determined spectrophotometrically at 420 nm (ε_{420}= 36,000 M^{-1} cm^{-1}). The reaction mixture contained 600 µl sodium acetate buffer (0.1M, pH 5.0 at 27 ^{o}C), 300 µL ABTS (5 mM), 300 µL mycelial liquid fraction and 1400 µL distilled water. The mixture was incubated for 2 minutes at 30^{o}C and the reaction was initiated by addition of 300 µL hydrogen peroxide. The absorbance was measured immediately in one-minute intervals after addition of hydrogen peroxide. One unit of laccase activity was defined as activity of an enzyme that catalyzes the conversion of 1µ mole of ABTS per minute.

Desalting and size separation of extracellular proteins by gel chromatography

Enzyme desalting and size separation was done by using PD-10 column (void volume 3.5 mL) parked with Sephadex *G-25* dextran gel (*Pharmacia*, Sweden). The column was equilibrated with 10 mM Bistris [bis(2-hydroxyethyl)imino-tris (hydroxymethyl) methane] – HCl buffer (Bistris-HCl) (pH 6.5). Culture filtrate sample (2.5 mL) was passed through the column by 3.5 mL Bistris-HCl buffer exchange. The eluent was collected and its absorbance at 280 nm and 260 nm was determined by UV-Visible spectrophotometer (*Shimadzu*, Japan).

SDS-PAGE and IEF analyses

Modified Laemmli (1970) method of SDS-PAGE analysis using 30% acrylamide:0.8% bis-acrylamide, 2 M Tris-HCl buffer (pH 8.8), 20% SDS, 0.02% $(NH_4)_2S_2O_8$ and 0.01 TEMED was used to resolve the purified proteins. Electrophoresis was carried out at 150 V and the gel staining was done using 0.05% (w/v) coomassie brilliant blue (CBB) R 150 followed by de-staining at 10% methanol-acetic acid solution.

Isoelectric points (pIs) were determined by using IEF Unit, commercial ampoulines and protein markers (*Pharmacia,* Sweden) at pH range of 2.5-9.5. Elecrofocusing was carried out at 1,500 V and 50 mA for 1.5 h. The gels were stained by 0.01% (w/v) of CBB R 250 followed by destaining in 20% (v/v) ethanol-acetic acid, solution (Scopes 1982, Deutscher 1990).

RESULTS AND DISCUSSION

Strain description

The *Crepidotus variabilis* strain inhabiting dry twigs and decomposing stems of mangroves is kidney-shaped. The white caps, turning creamy with age, are tiny (0.2 – 3 cm in diameter), sessile (lacking stalk) and the attachment to the substrate is via its cap rather than stipe (Fig. 1). The identity of the strain was confirmed by using descriptive keys (Bougher and Katrina, 1998, Aime 2001, Harkönen *et. al*. 2003). The salt tolerance of *C. variabilis* makes it a potential candidate for bioremediation of high-salt content effluents at alkaline condition and its ability to attach firmly to substrates makes it adaptive to the turbulent seashore environment especially during high tides.

Oxidation of RBB-R, guaiacol, α-naphthol and pyrogallol by *C. variabilis*

The ability of *C. variabilis* to degrade aromatic model compounds such as RBB-R and guaiacol in solid medium is shown in Figure 2. Pyrogallol and α-naphthol were also oxidized by the fungus. The degradation of the recalcitrant organic

compounds were evidenced by the appearance of a halo around the *C. variabilis* mycelia. The results imply that the same enzymes could also be used in detoxification of aromatic pollutants such as agro-industrial industrial effluents. The degrading ability of basidiomycetes fungi is thought to be due to the action of their extracellular oxidative enzymes (Wunch *et al*. 1997, George *et al.* 2000). The involvement of LiP and MnP in degradation of aromatic compounds suggests that hydrogen peroxide is responsible for the breakdown of aromatic molecules and oxygen may play a role in the initial degradation, while Lac and other enzymes interact with mediators during transformation of lignocellulose (Leonowicz *et al.* 2001). The *ex situ* degradation of aromatic molecules serves as a model system for *in situ* bioremediation of marine ecosystems polluted with recalcitrant hazardous chemicals. The results are consistent with the findings by Shin *et al*. (1997), Okino *et al*. (2000) and Mtui and Nakamura (2004) who demonstrated that both wood-inhabiting and non-wood inhabiting basidiomycetes fungi are capable of degrading RBB-R dye and other xenobiotic compounds. The decolorization ability is a result of synergetic action of multiple enzymes including peroxidases and oxidases (George *et al.* 2000).

Figure 1: Marine *Crepidotus variabilis* growing on a base of decomposing log in the mangrove-inhabited sediments. Note the peculiar lack of stalk and attachment to the substrate via its cap.

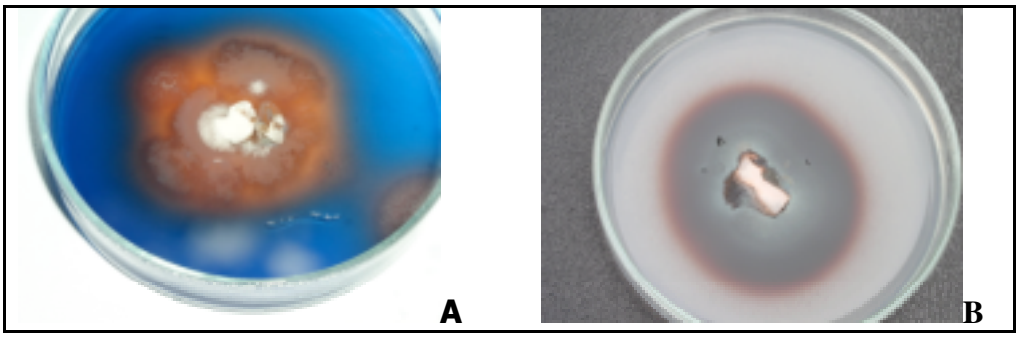

Figure 2. MEA agar plates showing RBB-R decolorization (A) and guaiacol oxidation (B) by *Crepidotus variabilis* in solid cultures after 5 days of incubation at 30ºC. The substrates were completely oxidized after 14 days of incubation.

Production of lignocellulosic enzymes

Figure 3 shows time courses of lignocellulosic enzymes activities by *Crepidotus variabilis*. The enzymes were produced during the idiophase when the fungi were carrying out secondary metabolism in nitrogen-deficient condition. Generally, the enzyme production started after 10 days of incubation, increasing sharply from day 11 and peaked at day 12 for Lac and day 13 for Lip and MnP. The maximum Lac, LiP and MnP activities were 50 U/mL, 10 U/mL and 8 U/ml, respectively. The decrease in enzyme activities after 12-13 days of incubation could be attributed to the presence of protease which digests proteins in solutions and addition of protease inhibitor in the reaction mixture would solve the problem (Nakamura *et al.* 1999, Mtui and Nakamura 2002).

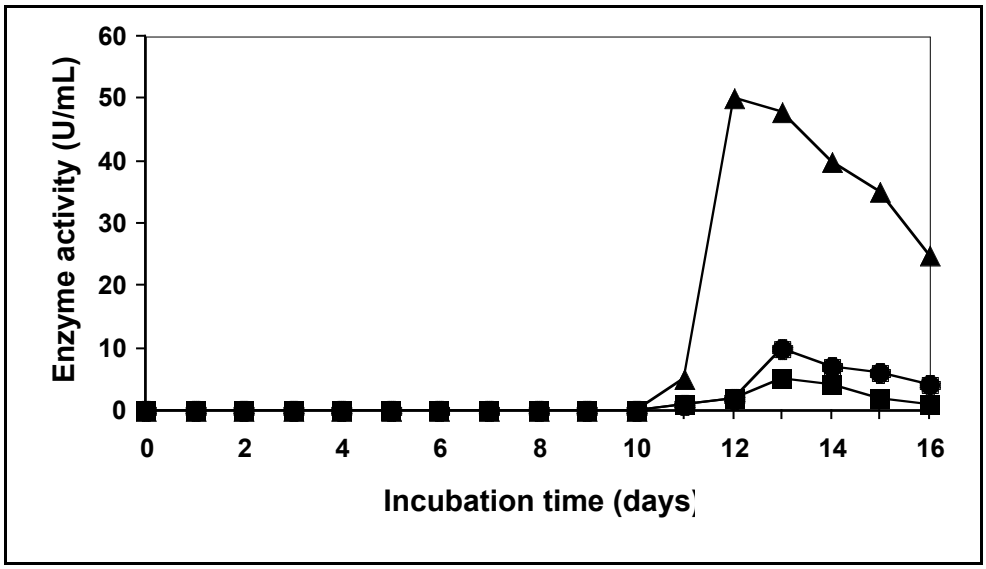

Figure 3: Time courses of lignocellulolytic enzymes production by *Crepidotus variabilis* grown in Kirk medium at pH 4.5 and temperature of 30ºC. Symbols: Laccase (▲), Lignin peroxidase (●); Manganese peroxidase (■).

The effect of nitrogen content in the culture media was investigated in order to address the problem of prolonged lag phase in enzyme production observed in Figure 3. Media containing 1.0, 2.0 and 3.0 mM ammonium tartrate were used for low nitrogen (LN), medium nitrogen (MN) and high nitrogen (HN) cultures, respectively. As shown in Table 1, LN medium was the most effective in producing larger quantities of extracellular enzymes after shorter incubation periods. Up to 70 U/mL Lac, 16 u/mL LiP and 8 U/ml MnP was produced maximally after 8 days of incubation. At HN medium, comparatively lower enzyme production peaking after 15 days of inhibition was observed. This trend could be attributed to the fact that at high nitrogen concentration, fungal mycelial growth is favored as the culture exhibits mostly primary metabolic activities (Nakamura *et. al*. 1999). The results were consistent with the work by Raghukumar *et al*. (1999) and Kondo *et al*. (2004) who demonstrated the

ability of some coastal and marine fungi to produce major lignocellulolytic enzymes at various culture growth conditions. The amounts of Lac, LiP and MnP are comparable to the amounts produced by the Tanzania's terrestrial fungi (Mtui *et al.* 2003, Mtui and Nakamura 2004). The presence of all major lignocellulosic ezymes, namely Lac, Lip and MnP in *C. variabilis* underlines its degradation potential for cellulose, lignin and other organic compounds. Since the amounts of LiP and MnP produced are comparatively low, it is suggested that for improved enzyme production, further research should focus on improvement of culture growth conditions including addition of potential LiP and MnP inducers.

Table 1: Effect of nitrogen content on the production of extracellular enzymes by *Crepidotus variabilis* in 1% glucose medium containing Kirk's Salts.

Incubation time (days)	Enzyme activity (U/ml)								
	Laccase			Lignin peroxidase			Manganese peroxidase		
	LN	MN	HN	LN	MN	HN	LN	MN	HN
1	0	0	0	0	0	0	0	0	0
2	0	0	0	0	0	0	0	0	0
3	0.2±0.1	0	0	0	0	0	0	0	0
4	4.7±0.3	0	0	0	0	0	0	0	0
5	15.6±2.4	0	0	0.3±0.2	0	0	0.4±0.2	0	0
6	35.9±6.7	0	0	0.6±0.3	0	0	1.7±0.5	0	0
7	67.4±11.0	0	0	1.8±0.9	0	0	3.7±1.2	0	0
8	70.0±13.1	0	0	15.9±3.6	0	0	7.7±2.1	0	0
9	52.1±9.3	0	0	11.3±1.9	0	0	6.2±1.6	0	0
10	43.6±9.1	0	0	9.8±1.1	0	0	4.3±1.0	0	0
11	31.7±4.4	5.2±2.2	0	6.2±0.8	1.2±0.6	0	4.0±0.8	0.5±0.1	0
12	21.8±3.3	50.3±9.1	0	5.3±1.0	2.3±0.5	0	2.3±0.4	1.5±0.2	0
13	22.1±3.0	48.4±7.7	0.9±0.2	3.9±0.8	9.9±1.7	0.2±0.1	2.1±0.5	5.2±1.7	0
14	19.2±2.6	40.7±5.3	6.2±1.3	3.0±0.8	7.6±1.4	1.1±0.3	0.8±0.3	4.1±1.9	1.7±0.2
15	14.7±2.1	35.1±4.9	15.8±3.3	2.8±0.4	6.0±1.1	6.2±1.2	0.5±0.2	3.4±1.1	2.6±1.1
16	10.8±1.9	15.2±2.3	8.7±2.1	2.2±0.5	3.9±0.6	5.4±0.9	0.2±0.1	1.5±0.6	1.9±1.0

Low nitrogen (LN), medium nitrogen (MN) and high nitrogen (HN) medium contained 1.0, 2.0 and 3.0 mM ammonium tartrate, respectively. Each value is an average of triplicate cultures ± standard deviation

Decolorization of textile effluent and synthetic dyes

Figure 4 shows the effect of the crude enzymes from *Crepidotus variabilis* in decolorization of 1:1 diluted raw wastewater and synthetic aromatic dyes cultured in low nitrogen medium It was found that the fungus could decolorize the half-strength raw wastewater by 58% after 14 days of incubation. In the case of 1% synthetic azo dyes, color removal from Azure-B, Poly-B, and Poly-R were 84%, 86% and 92%, respectively after 2 weeks of incubation. The decolorization of azo dyes in the absence of a redox mediator shows that the Lac, LiP and MnP from *C. variabilis* have broad substrate range (Yesilada 1995, Yang *et al.* 2003). The results are consistent with the findings by Gutiérrez *et al*. (1999) and Martinez *et al*.(1999, 2005) who reported the degradative abilities of white-rot fungi on recalcitrant organic substrates. Colour removal by filamentous fungi has been attributed to be mainly due to biosorption to the mycelium (Yang *et al.* 2003), but Leonowicz *et al*. (2001) and Gonzalo *et al*. (2005) have shown that Lac, LiP and MnP are capable of carrying out catalytic and free-radical mediated breakdown of aromatic compounds including ring cleavage. The fact that degradative enzymes from *C. variabilis* could not degrade all the organic compounds

in the wastewater shows the complexity of systems associated with the degradation of aromatic molecules. As lignocellulosic enzymes work in concert with other fungal enzymes (Leonowickz *et al*. 2001), a synegistic action of enzymes from different fungal strains is therefore imperative for complete degradation of recalcitrant organic compounds.

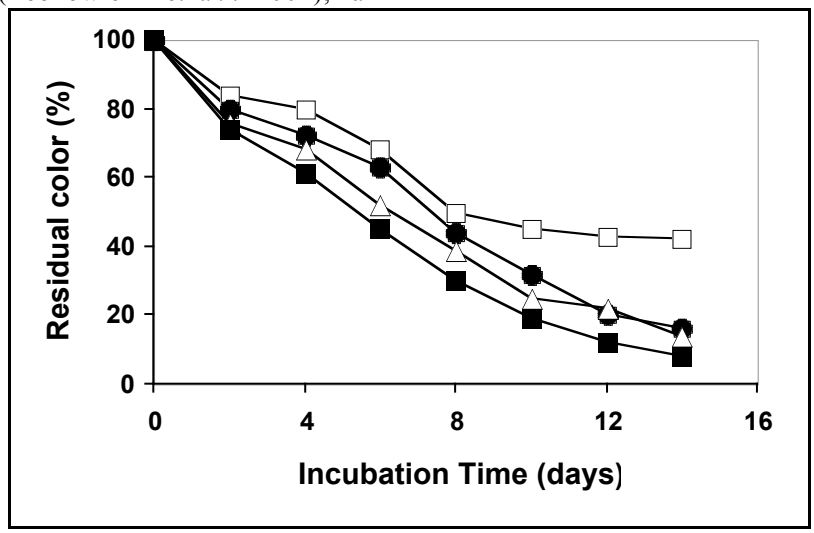

Figure 4: Decolorization of half-strength textile wastewater and 1% synthetic dyes by *Crepidotus variabilis* grown on low nitrogen medium at 30ºC and pH 4.5. Symbols: Textile wastewater (□), Azure-B (●), Poly-B (Δ), Poly-R (■).

Characterization of extracellular enzymes from *Crepidotus variabilis*

The filtrates of *C. variabilis* concentrated by ultrafiltration and subjected to spectrophotometric analysis at 280 and 260 nm had overall protein content of 4.3 mg/ml. In Figure 5, SDS-PAGE analysis showed distinct bands at relative molecular weights of 67 kDa and 47 kDa, which corresponds to laccases and peroxidases, respectively (Wang and Ng, 2006). A minor band resolved at 55 kDa could be an isoform of laccase. The results are comparable to studies by Karhunen *et al*. (1990), Bonarme *et al*. (1990), Matsubara *et al*. (1996) and Heinfling *et al*. (1998) who observed the molecular weights of peroxidases from *Phlebia radiata*, *IZU* 154, *Phanerochaete chrysosporium* and *Bjerkandera adusta* to be 49 kDa, 43 kDa, 42 kDa and 45 kDa, respectively. Fungal laccases have been shown to have various relative molecular weights as follows: *PM1* (CECT2971 Strain - 64 kDa (Coll *et al.* 1993); *Phenerochaete flavido alba* - 96 kDa (Perez *et al*. 1996), *Panaeolus sphincrinus* and *Trametes gallica* - 60 kDa (Heinzkill *et al*. 1998, Dong and Zang 2004, *Trametes trogii* - 70 kDa (Garzillo *et al*. 2004) and *Ganoderma lucidum* - 75 kDa (Wang and Ng 2006).

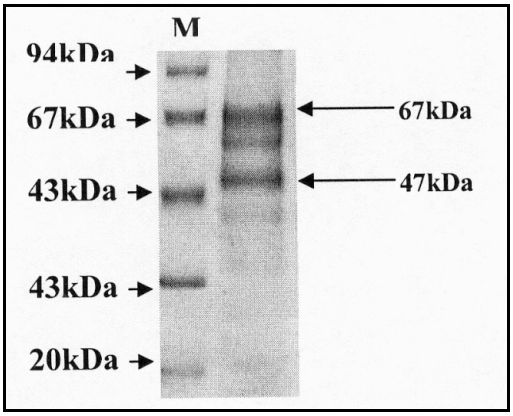

Figure 5: SDS-PAGE analysis of enzymes filtrate from *Crepidotus variabilis* containing 0.2 mg/mL proteins separated by gel filtration chromatography. Main bands appears at 67 kDa (laccases), and 47 kDa (peroxidases). A minor band of laccase isoenzyme was focused at pI 50. (Lane M: Standard Molecular Marker proteins: Phosphorylase b (94 kDa), Bovine Serum Albumin (67kDa), Ovalbumin (43 kDa) and Carbonic Anhydrase (30 kDa).

IEF analysis demonstrated distinctive bands focused at a range of 3.0 to 4.1, typical of fungal peroxidases and laccases (Fig. 6). Some smaller non-distinctive bands showed up at pI 3.0-3.5 and more minute bands fixed around pI5.3. When broad markers (pI3.5-9.5) were attempted, there was no any band fixed in the basic region. The low pI values indicate that the number of acidic groups in the structures of lignocellulosic enzymes from *C. variabilis* exceeds the number of basic groups. The IEF values for fungal laccases are comparable to the value of pI 3.5 reported by Perez *et al*. (1996) for *P. flavido-alba* and pI 3.1 reported by Dong and Zang (2004) for *T. gallica*. Peroxidases from *P. radiata* have been shown to have pI values ranging from 3.8 to 4.7 (Vares *et. al*. 1995, Karhunen *et al.* 1990), while Matsubara *et al*. (1996) recorded pI values of 4.9 to 5.1). The next stage of this research will involve purification of the enzymes using ion exchange chromatography including high performance liquid chromatography (HPCL) in order to completely separate individual enzymes and their isoforms.

In the present study, crude lignocellulosic enzymes from white-rot fungus *Crepidotus variabilis*, collected from Oyster Bay, Mtoni and Kunduchi mangrove forests along the coast of Dar es Salaam, Tanzania have been shown to exhibit maximum Lac, LiP and MnP activities of 70 U/mL, 16 U/mL and 8 U/mL, respectively, in low nitrogen medium. The enzymes were able to oxidize RBB-R dye, phenol, α-naphthol and pyrogallol. Also, the enzymes could decolorize 59% of raw textile wastewater and up to 92 % synthetic azo dyes. SDS-PAGE analysis showed major bands of concentrated enzymes from *C. variabilis* at relative molecular weights of 67 kDa and 47 kDa for laccases and peroxidases, respectively. IEF analysis revealed that *C. variabilis* produce acidic laccases and peroxidases, the main bands focusing at pI 3.8, 4.0 and 4.1. The study provided basic information on lignocellulosic enzyme profiles from facultative marine *C. variabilis* and elucidated its potential for environmental bioremediation. Optimized enzymes production and purification of fractionalized isoforms to determine their specific characteristics is the focus of future studies.

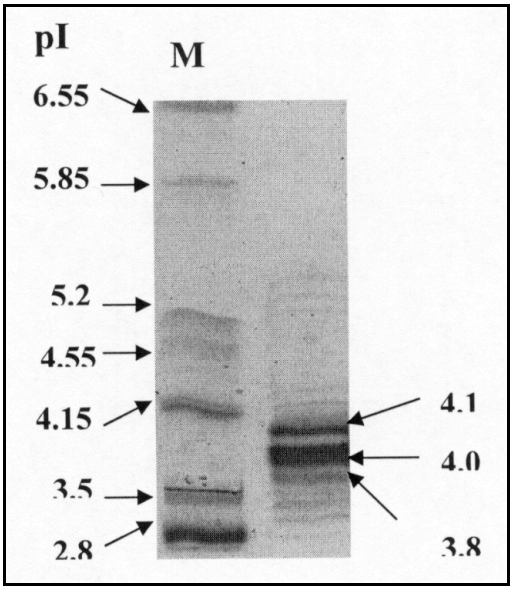

Figure 6: IEF of size-separated *C. variabilis* enzymes filtrate containing 0.2 mg/mL proteins separated by gel-filtration chromatography. Main peroxidase and laccase bands appeared at pI 3.8, 4.0 and 4.1 and other smaller bands were focused between pI3.0 and pI3.5. Markers (M): Pepsinogen (pI 2.8), amyloglucosidase (pI 3.5), Glucose oxidase 4.15, soybean trypsin inhibitor (pI 4.55), β-lactoglobulin A (pI 5.2), bovine carbonic anhydrase B (pI 5.85) and human carbonic anhydrase B (pI 6.55).

ACKNOWLEDGEMENTS

Major part of this research was carried out with the financial support from Western Indian Ocean Marine Science Association (WIOMSA), through the Marine Research Grant (MARG-1) program. I am grateful to the management of the University of Dar es Salaam through the Department of Molecular Biology and Biotechnology for logistical support. Ms. Rose Masalu is acknowledged for her expert execution of fungal cultivation and crude enzyme assays. Prof. Gunnar Johansson of the Department of Biochemistry and Organic Chemistry at Uppsala University, Sweden, is thanked for his kind facilitation and guidance on protein characterization. My family is appreciated for their warm moral support.

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