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Full Length Research Paper

Lignocellulosic enzymes from *Flavodon flavus*, a fungus isolated from Western Indian Ocean off the coast of Dar es Salaam, Tanzania

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Marine basidiomycetes fungus *Flavodon flavus* (Klotzsch) Ryvarden was isolated from sea grass at Mjimwema in the Western Indian Ocean off the Coast of Dar es Salaam, Tanzania, and cultured in the laboratory. Protein content and lignocellulosic enzyme activities were measured by photometric methods. Desalted and size-separated enzyme filtrates were resolved by sodium docecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and isoelectric focusing (IEF). The fungal filtrate had maximum lignin peroxidase (LiP), manganese peroxidase (MnP) and Laccase (Lac) activities of 42, 25 and 15 U/mL, respectively. At low carbon medium, *F. flavus* showed effective (92 - 100%) decolorization of raw textile wastewater and synthetic dyes such as rhemazol brilliant blue-R (RBB-R), Brilliant green, Congo red, Reactive black and Reactive yellow. SDS-PAGE analysis showed major bands of size-separated enzymes from *F. flavus* at relative molecular weights between 45 and 70 kDa. The LiP of *F. flavus*, purified by ion exchange chromatography, revealed that it has a molecular weight of 46 kDa and isoelectric point (pI) of 3.8. The study confirmed extracellular enzymes from *F. flavus* to be potential degraders of organic pollutants and showed that facultative marine fungi that live under harsh seawater conditions are suitable for bioremediation of recalcitrant environmental pollutants.

Key words: Marine basidiomycetes fungi, extracellular enzymes, biodegradation, electrophoresis, isoelectric focusing.

INTRODUCTION

Biodegradation of sea grasses and mangroves contribute to the primary detrital biomass which is important as source of food in the estuarine and near shore food web. Sea grasses and mangrove trees contain about 50% lignocellulosic polymers and the rest are soluble organics which include tannins and fenolics (Raghukumar et al., 1999). These compounds are mainly decomposed by bacteria and fungi. Bacteria colonize the maximum surface area and form the initial biofilm on submerged plant parts, while fungi penetrate into dead plants' surface, causing active decomposition (Newel, 1993).

Depending on the type of wood decay they cause, fungi are classified as white-rots, brown-rots or soft-rots. Among the three groups, white-rot fungus is the one that attack all the wood components, making them superior to other types. The ability of fungi to degrade lignocellulose is due to their possession of extracellular enzymes, mainly lignin peroxidase (LiP), manganese peroxidase (MnP) and laccase (Lac) (Pointing, 1998). These enzymes have been shown to degrade not only lignocellulose, but also recalcitrant environmental pollutants such as crude oil wastes, textile effluents, organochloride agrochemicals and pulp effluents which are a cause of serious environmental pollution (Mtui and Nakamura, 2002, 2004; Kiiskinen et al., 2004). Obligate and facultative marine fungi that live under harsh seawater conditions are better suited for bioremediation pollutants

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that have high load of inorganic salts of chlorides and sulphides (Raghukumar, 2005).

Degradative enzymes from marine fungi have been least studied compared to their terrestrial counterparts. Only limited reports covering narrow geographical area have so far been covered (Raghukumar et al., 1994, 1996, 1999; Raghukumar, 2000; Raghukumar and Rivonkar, 2001; Raghukumar et al., 2004a, 2004b; Raghkumar, 2005). Studies on *Flavodon flavus*, a basidiomycetes white-rot fungus belonging to order Polyporales (Ryvarden and Johansen, 1980; Hattori, 2000), have revealed that the strain produce all important lignocellulosic enzymes that could decolorize several individual synthetic dyes (Raghukumar et al., 1999), but no attempt has been made to test for their decolorization of raw textile effluents.

Purification and characterization of lignocellulosic enzymes is important in order to identify potential specific isoforms for bioremediation purposes. Chromatographical techniques are best suited to separate individual proteins into isoenzymes whose properties can be evaluated. The separated fractions can be resolved in gel electrophoresis which is a powerful tool in the analytical separation of proteins. In this technique, proteins are separated according to their molecular sizes. On the other hand, in isoelectric focusing (IEF), protein molecules are separated as they migrate through a pH gradient under a strong electric field (Scopes, 1982; Deutscher, 1990). Although it has been shown by Raghukumar et al. (1999) that concentrated filtrates of *F. flavus* (strain 312) from Arabian Sea cost has a wide range of isoelectric points of pI 3 - 6 and relative molecular masses of 43 - 99 kDa, very limited studies have been done on the characteristics of enzymes from fungi of the West Indian Ocean (Mtui and Nakamura, 2007).

This study investigated the production and application of lignocellolosic enzymes from marine *F. flavus* isolated from decayed sea grass off the coast of Dar es Salaam, Tanzania. Furthermore, the active fraction of LiP was purified to determine its characteristics.

MATERIALS AND METHODS

Sampling site, fungi isolation and identification

The strain was isolated from decaying sea grass leaves of *Thalla-sodendon ciliatum* collected about 50 m off the Coast of Mjimwema, 20 km South of Dar es Salaam, Tanzania. It was identified to be basidiomycete fungus, *F. flavus* (Klotzsch) Ryvarden, based on morphological features (Ryvarden and Johansen, 1980; Hattori, 2000; Zmitrovich et al., 2006). The identity of the fungus was later confirmed by phylogenetic analysis of internal transcribed spacers containing rRNA gene sequence (Kamei et al., 2005).

Culture media and cultivation of mycelia

Diluted (1:1) seawater was filter-sterilized and used instead of distilled water. Solid media consisted of 5% (w/v) malt extract agar (MEA), 10 g/L glucose, 0.02 g/L yeast extract and 20 mL of Kirk medium (Nakamura et al., 1997, 1999). The liquid medium contained 12.7 g/L malt extract, 10 g/L glucose and 25 mL Kirk medium. Solid medium cultivation was done in 10 cm – diameter Petri dishes. Leave discs (1 cm diameter) containing *F. flavus* were cut and surface-sterilized with 0.5% sodium hypochlorite and then cultured in the Petri dished at 30ºC for 5-7 days for production of mycelial mats. Cultivation in liquid medium was done in 250 mL conical flasks (25 mL working volume) plugged with cotton wool and then covered with aluminium foil. The culture medium was sterilized by autoclaving at 121ºC for 20 min. 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.

Decolorization of textile wastewater

Dark-blue raw effluent from *Karibu* Textile Mill Ltd, Dar es Salaam, Tanzania, was sampled at various outlets and mixed thoroughly. The effluent was diluted (1:1) by using formulated seawater followed by addition of 0.02% yeast extract. Fungal mycelia were then aseptically inoculated into the 250 mL flask (working volume 50 ml) and the reaction mixture was incubated at 30ºC for 2 weeks in stationary condition or gently shaken in a rotary shaker (*Fujitsu*, Co., Japan) at 30 and 60 rpm Supernatant of the culture was drawn at 2-day intervals and centrifuged at 5,000 rpm for 15 min. Control experiments were conducted using the same medium without inoculum. The color intensity (absorbance) was determined by using UV-Visible *Thermo Stonic* spectrophotometer, (UK) at 535 nm which was the λ_{max} of the dye (Yang et al., 2003).

Decolorization of synthetic dyes

Reagent grade Rhemazol Brilliant Blue R (RBBR), Congo red, Brilliant green, Reactive black and Reactive yellow (Sigma) were used. Stock solutions (1% w/v) were filter-sterilized with 0.2 µm pore diameter membrane and the fungal mycelia were cultivated in stationary 250 mL Erlenmeyer flasks (working volume 50 ml). Decolorization of the supernatant was detected by UV spectrophotometer after centrifugation at 5000 rpm for 10 min. Absorbance was measured at λ_{max} of each dye (Raghukumar et al., 1999). Color removal was reported as % decolorization = $(A_i-A_t)/A_i \times 100$. Where A_i is the initial absorbance before incubation and A_t is the absorbance after incubation time, t.

Sample concentration and dialysis

Crude enzyme filtrates were successively filtered in 0.45 µm and 0.2 µm *Acrodisc* syringe filters (*Pall* Gelman Lab, USA). The 30-mL samples were concentrated 10-fold by ultrafiltration using 10 kDa cut-off *Microsep* centrifugal devise containing omega membrane (*Pall* Life Sciences, USA) at 5000 rpm 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 supernatant was collected and the total protein, total activity and specific activity were measured. The concentrated proteins were stored at 4ºC for further analysis.

Desalting of proteins by gel chromatography

Enzyme desalting was done by using PD-10 column (void volume 3.5 ml) parked with Sephadex G-25 (dextran) gel (Pharmacia Co., Sweden). The column was equilibrated with 10 mM Bistris [bis(2-hydroxyethyl)imino-tris (hydroxymethyl) methane] – HCl buffer (pH 6.5) and 3.5 mL of the buffer was used for elution. The eluate was collected and its absorbance at 280 nm and 260 nm was determin-

ed by UV-Visible spectrophotometer (Shimadzu Co. Ltd., Japan).

Enzyme purification by ion exchange chromatography (IEC)

An ion exchange column HighLoad Q Sepharose 26/10 (Pharmacia, Sweden) packed with polystyrene matrix with quaternary ammonium functional groups carrying net positive charge was equilibrated with 50 mM Bistris (pH 6.5) buffer and connected to a peristaltic pump (Miniplus 2 Gilson, Sweden) set at a flow rate of 1.4 ml/min. 5 mL of the desalted sample was introduced into the column followed by rinsing with 10 mL buffer. Continuous linear gradient elution of 0.2 M NaCl in Bistris buffer (pH 6.5) was achieved by using two flasks connected by a siphon; the first flask with buffer was constantly stirred while the second flask had the NaCl counterion solution. The enzyme eluate was separated by a fraction collector (Microcol TDC 80, Gilson, Sweden) set at 3.5 min intervals. The protein content of the eluted fractions was monitored by a spectrophotometer (Shimadzu, Japan) at 280 nm for Lac, while the heme proteins (LiP and MnP) were monitored at 405 nm. Total proteins (mg/L) were determined by a Bradford method (Bradford, 1976) using Bio-Rad protein dye and bovine serum albumin standard (Bio-Rad, USA).

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 N,N,N',N'-tetramethylethylenediamine (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 250 followed by de-staining at 10% methanol-acetic acid solution (Scopes, 1982).

Isoelectric points (pIs) were determined by using IEF Unit, incorporating commercial ampholytes and protein markers (*Pharmacia,* Sweden) at a pH range of 2.5 - 9.5. Electrofocusing 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 (Deutscher, 1990).

Determination of enzymes activities

Activity of LiP 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 two 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 interval after addition of H_2O_2. One unit (U) of LiP activity was defined as activity of an enzyme that catalyzes the conversion of 1 µmole of veratryl alcohol per minute (Nakamura et al., 1997; 1999).

MnP activity was measured following the method described by Wunch et al. (1997). In this method, guaicol was used as a substrate, then 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), 300 µL 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°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 interval after addition of hydrogen peroxide. One unit of MnP activity was defined as activity of an enzyme that catalyzes the conversion of 1 µmole of guiacol per minute.

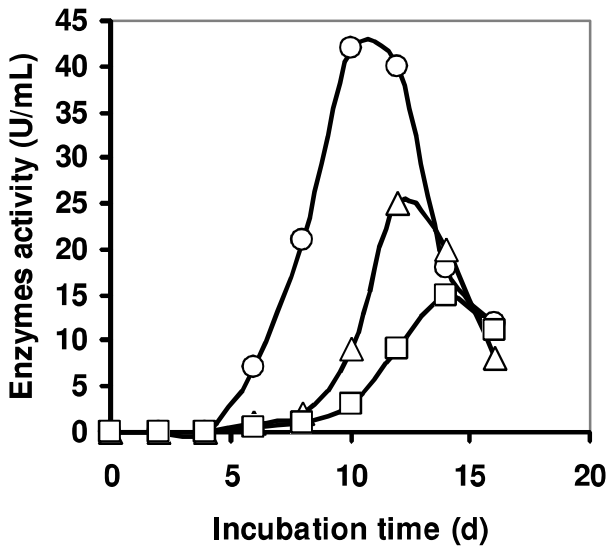


Figure 1. Enzyme activities from *Flavodon flavus* cultured in stationary Kirk's basal medium. The results are an average of quadruplicate samples. Symbols: Lignin Peroxidase (○), Manganese peroxidase (□), Laccase (Δ).

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 °C), 300 µl ABTS (5 mM), 300 µL mycelial liquid fraction and 1400 µL distilled water. The mixture was incubated for 2 min at 30°C and the reaction was initiated by addition of 300 µL hydrogen peroxide. The absorbance was measured immediately in one-minute interval after addition of hydrogen peroxide. One unit of Lac activity was defined as activity of an enzyme that catalyzes the conversion of 1 µmole of ABTS per minute.

RESULTS AND DISCUSSION

Production of extracellular enzymes

Figure 1 shows the time courses of lignocellulosic enzymes activities by *F. flavus.* The enzymes were produced during the idiophase when the fungus was carrying out secondary metabolism in nitrogen-deficient condition, starting from 4th day of incubation. LiP increased sharply from day 5 and peaked at 42 U/mL in day 11. MnP increased steadily from day 10 and peaked at 25 U/mL in day 12. Lac was the least produced; the maximum of 15 U/mL being recorded at day 14. The results were consistent with the reports by Raghukumar et al. (1999) and Kondo et al. (2004) who demonstrated the ability of some coastal marine fungi to produce major lignocellulolytic enzymes. The amounts of LiP, MnP and Lac are comparable to the amounts produced by the Tanzania's terrestrial mushrooms (Mtui et al., 2003).

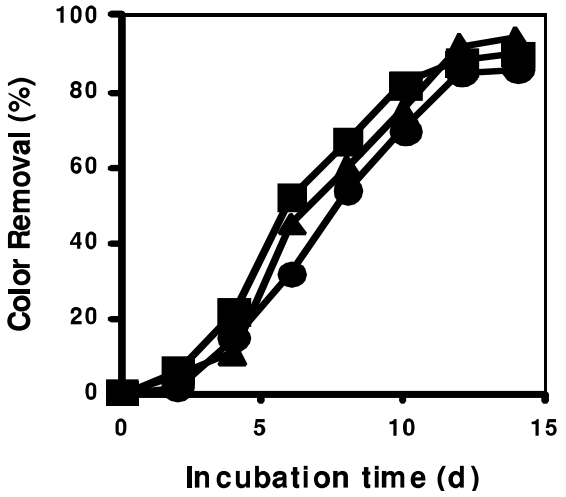


Figure 2. decolorization of 1:1 diluted raw textile effluent by *Flavodon flavus* at stationary culture (▲) and shake-flask cultures at 30 rpm (■) rpm and 60 rpm (●).The results are an average of quadruplicate samples.

The decrease in enzyme activities after their peaking is attributable to the production of protease in the medium (Nakamura et al., 1999; Mtui and Nakamura, 2002). The filtrate was therefore harvested for purification after 12 days of cultivation. The fact that all major lignocellulolytic enzymes were present in the culture of *F. flavus* is an indication that the strain could be a strong degrader of organic substrates.

Decolorization of raw textile effluent

Extensive decolorization of raw textile effluent by extracellular enzymes secreted by *F. flavus* at different culture conditions is shown in Figure 2. It was observed that slow shaking (30 rpm and 60 rpm) had little effect on docolorization rates and efficiency. At stationary culture condition, the highest color removal of 94% was established after 14 days of cultivation. Therefore, the subsequent incubation experiments were carried out at stationary culture condition. The decolorization of azo dyes contained in the raw textile wastewater in the absence of a redox mediator shows that LiP, MnP and Lac produced by *F. flavus* have broad substrate range. The findings are comparable to the report by Nilsson et al. (2006) who found that terrestrial white-rot fungus *Pleurotus flabellatus* could degrade real textile waste water by 67%. Color removal by filamentous fungi has been attributed to be mainly due to biosorption to the mycelium (Yang et al., 2003), but Gonzalo et al. (2005) and Raghukumar (2005) have shown that LiP, MnP and Lac are capable of carrying out catalytic and free-radical mediated breakdown of aromatic compounds including ring cleavage reactions.

Figure 3a-c demonstrates the direct involvement of LiP, MnP and Lac in decolorization of raw textile effluent. A strong positive correlation between enzymes production and decolorization efficiency was observed, indicating that when the enzymes were produced maximally, the decolorization was highest. The findings were in agreement with assertions by Martinez et al. (2005) and Nilsson et al. (2006) that lignolytic enzymes act synergistically to facilitate complete degradation of aromatic and aliphatic compounds.

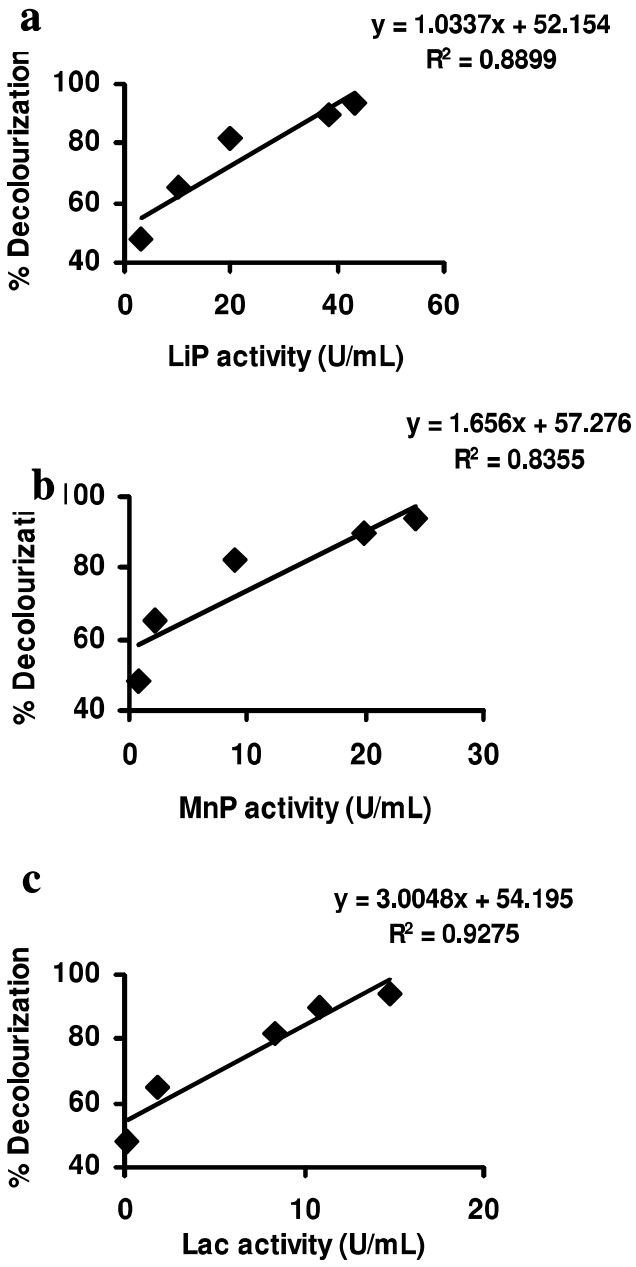


Figure 3a-c. Positive correlation between enzymes activities and decolorization of raw textile effluent.

Table 1. Decolorization of 0.2% w/v synthetic dyes by stationary culture of *Flavodon flavus*.

Dye	% Decolorization after time [t]		
	[4 days]	[6 days]	[12 days]
Rhemazol brilliant blue R	80.2	91.9	100
Congo red	73.6	85.5	95.6
Brilliant green	46.2	76.1	92.7
Reactive black	49.9	71.4	96.3
Reactive yellow	52.3	73.7	98.1

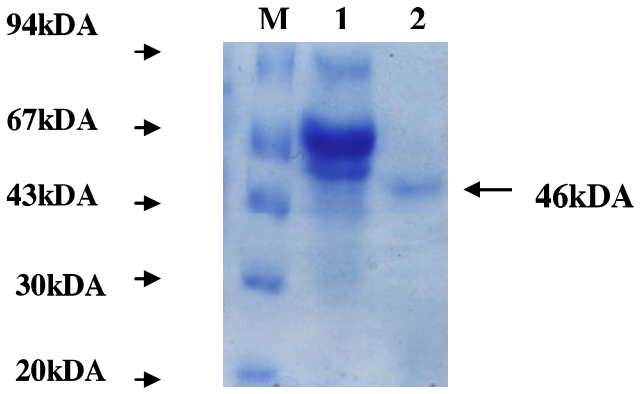


Figure 4. SDS PAGE of extracellular enzymes from *Flavodon flavus*. Lane 1: concentrated enzyme filtrates containing 2.4 mg protein; Lane 2: LiP (0.3 mg/mL) purified by ion exchange chromatography. M: Marker proteins: Phosphorylase b (94 kDa), Bovine Serum Albumin (67 kDa), Ovalbumin (43 kDa), Carbonic Anhydrase (30 kDa), Soybean Trypsin inhibitor (20.1 kDa).

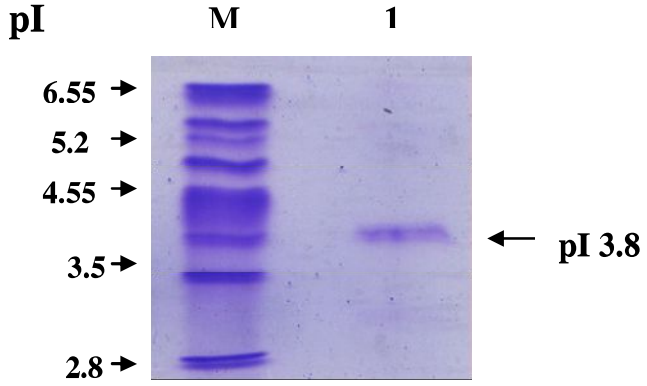


Figure 5. Isoelectric focusing of LiP (0.3 mg protein) purified by ion chromatography (Lane 1). Marker proteins: Pepsinogen (pI 2.8), amyloglucosidase (pI 3.5), methly red (dye (pI 3.75), soybean trypsin inhibitor (pI 4.55), B-lactoglobulin A (pI 5.2), bovine carbonic anhydrase B (pI 5.85), human carbonic anhydrase B (pI 6.55), horse myglobin-acidic band (pI 6.85).

Decolorization of synthetic dyes

Table 1 shows the decolorization of 0.2% synthetic dyes by *F. flavus*. Except for RBBR, all other tested compounds were azo dyes used in textile industries. All the dyes were decolorized almost completely after 12 days of incubation. The results are consistent with the reports by Shin et al. (1997) and Yang et al. (2003) that demonstrate significant decolorization of aromatic dyes by terrestrial fungi while Raghukumar et al. (1999, 2004) and Raghukumar (2005) reported the same trends for facultative marine fungi.

Decolorization of effluents and synthetic dyes may not always solve the pollution problem as they may still be toxic (Raghukumar, 2005; De Souza et al., 2006). Therefore, detoxification studies and mechanisms involved should be studied simultaneously. Future research should be focused towards searching for or creating fungal strains which overproduce lignin-degrading enzymes. Such organisms could be used in scaled-up bioreactors for large-scale treatment of recalcitrant pollutants.

Purification of lignin peroxidase from *F. flavus*

The concentrated *F. flavus* filtrate, pretreated by desalting gel chromatography, was subjected to size separation chromatography and gave dominant peaks containing peroxidases and laccases. As shown by the SDS-PAGE analysis, broad bands between 45 and 70 kDa were observed (Figure 4, Lane 1). Purification by ion exchange chromatography resulted to one active fraction at 405 nm (heme protein) which was pooled and its SDS-PAGE analysis gave a sharp band at 46 kDa (Figure 4, Lane 2). The separated fraction was found to be LiP based on substrate specificity experiments (data not shown). The results are comparable to studies by Raghukumar (1999) which showed relative molecular weight of LiP from *F. flavus* (strain 312) to be 41.5 kDa. Spectral analysis at 280 for other eluted fractions (MnP and Lac) had too little (<0.001 µg/ml) protein content that could not be resolved under SDS-PAGE analysis.

Figure 5 shows a single band of LiP purified by IEF analysis. A distinctive band was resolved at pI 3.8. Some other smaller non-distinctive bands appeared at pI 3.0

Table 2. Purification of lignin peroxidase from the culture filtrate of *Flavodon flavus.*

Procedure	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Yield (%)	Purification (fold)
Culture filtrate (0.2 μm diameter membrane)	1050	85.5	12.3	100	1
Microsep 10kDA membrane	998.8	51.3	18.5	95.12	1.5
Sephadex G-25 size exclusion chromatography	991.3	33.3	29.8	94.4	2.4
Q Sepharose 26/10 ion exchange chromatography	142.2	1.4	101.6	13.5	8.3

and 4.5 for fractions that showed little resolution at 280 and 405 nm signifying that the separated MnP and Lac were also observed (data not shown). The low pI values indicate that the number of acidic groups in the structures of lignocellulosic enzymes from *F. flavus* exceeds the number of basic groups. The LiP's pI value obtained from this study is comparable to the value of pI 4.0 reported by Raghukumar (1999) for *F. flavus* strain 312.

It is worth noting that the MnP and Lac fractions could not be clearly resolved in SDS-PAGE or IEF because the fractions collected had very low protein content. Optimized enzymes production will be the focus of the future research.

Table 2 summarizes the purification procedures for LiP from *F. flavus*. The purification of LiP by ion exchange chromatography gave a final specific activity of 101.6 U/mg protein with 8.3 purification-fold. The results are comparable to reports by Shin et al. (1997), Nakamura et al. (1999) and Mtui and Nakamura (2007) who demonstrated successful purification of lignocelluosic enzymes from terrestrial and marine fungal filtrates.

Conclusion

Lignocellulosic enzymes from white-rot fungus *F. flavus* isolated from decayed sea grasses from Mjimwema creek in the Indian Ocean coast of Dar es Salaam, Tanzania, were investigated. The fungal culture filtrate had maximum LiP, MnP and Lac activities of 42, 25 and 15 U/mL, respectively. The enzymes from *F. flavus* under seawater conditions could decolorize 94% of raw textile wastewater and almost completely decolorized RBB-R dye, Congo red, Brilliant green, Reactive black and Reactive yellow at low carbon culture medium. SDS-PAGE analysis showed major bands of concentrated enzymes from *F. flavus* at relative molecular weights between 45 and 70 kDa. The purified LiP resolved by SDS-PAGE and IEF analyses revealed the fungus to have a relative molecular weight of 46 kDa and Isoelectric point of 3.8. So far, the study provided basic information on lignocellulosic enzyme profiles and characteristics of marine *F. flavus* and elucidated its potential for environmental bioremediation. Optimized enzymes production and toxicity studies are the focus of future research.

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