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# Azo Dyes Decolourisation by ABTS-oxidases (Laccases) from a Fungus from Tropical Tree

**Syed Faraz Moin, Helmi Yousif Alfarra and Muhammad Nor Omar***

Department of Biotechnology, Kulliyyah of Science, International Islamic University Malaysia, Kuantan- 25200, Pahang, Malaysia.

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**Lignin is an important constitute of the wood and thus the organism like fungi and insects growing on wood produce some extracellular lignolytic enzymes. In fungi these enzymes include different oxidases and peroxidases. Lignin peroxidases, manganese peroxidases, versatile peroxidases and laccases have been characterised from fungi and their roles in wood decay has been established. Here we report ABTS-oxidases from an unidentified fungus isolated from tree bark sample from Kuantan, Malaysia. After the liquid media cultivation, the crude extracellular protein content was extracted and analysed by polyacrylamide gel electrophoresis. SDS-PAGE, native PAGE and zymography were carried out. Azo dyes, brilliant green, trypan blue, direct red 80 and eriochrome black T were used for decolourisation activity of the isolated fungus on solid medium. The results suggest that the extracellular proteins contain laccase enzyme activity. Out of the four azo dyes that were used for dye decolourisation assay, at least three were decolourised to different extents by the fungus.**

**Key words:** Laccases, White rot fungus, Extracellular, Azo dye, Plate assay,

Lignin is a complex polymer present in wood as a part of plant cell wall. It is degraded by several organisms like fungi, bacteria and insects. The extracellular lignolytic enzymes produced by for example white rot fungi includes mainly the lignin peroxidases, manganese peroxidases, versatile peroxidases and laccases[1-5]. Lignin peroxidase (EC. 1.11.1.14) and manganese peroxidase require $H_2O_2$ as a co-substrate for biodegradation[6-7]. The versatile peroxidise (EC. 1.11.1.16) has the combined catalytic characteristics of lignin peroxidase and manganese peroxidases[8-9].

Unlike the peroxidases which have the heme group as co-factor and require hydrogen peroxide as co-substrate, laccases are multicopper oxidoreductases, utilise oxygen molecule as terminal electron acceptor. Laccases (EC. 1.10.3.2) oxidises a variety of substrates such as monophenols, diphenols, non-phenolic compounds, and aromatic amines resulting in generation of free radicals which can undergo further reactions [10-12]. Laccase is a lignin degrading enzyme and frequently more than one isozyme may be produced by the organisms in a given time[13-15]. The vast affinity of laccases to oxidise different compounds have attracted interest towards becoming a subject of industrial and biotechnological applications. For example, laccases have been used for detoxification, bioremediation, as biosensors, in petrochemical and textile industries, as agent in some water purification system, as catalyst for manufacturing

* To whom all correspondence should be addressed.
Tel: +609 571 6400 ext: 2831 , Fax: +609 571 6781
E-mail: mnoromar@iium.edu.my

drugs, in cosmetics and other purposes[16-18]. Laccase has also shown the potential of a green catalyst that can be a promising alternative in organic synthesis[19-20]. Laccases in monomeric form have a molecular mass range of 43-100 kDa with dimeric to oligomeric states. The three peroxidases are found in monomeric from with lignin peroxidase 37-50 kDa, manganese peroxidase 32-62.5 kDa and versatile peroxidase with 42-45 kDa molecular mass range[21-22]. Fungi, due to their ability to produce cosmic amounts of extracellular lignin-modifying enzymes (LMEs) that facilitate the wood degradation process have attracted a great deal of interest as potential biomass degraders for large-scale applications. It is primarily basidiomycetes, the white-rot fungi that are responsible for the efficient lignin degradation in wood decay processes [20]. In the present studies a wood rot fungus has been isolated from tree samples. The extracellular protein content produced by the fungus in liquid medium have been analysed by polyacrylamide gel electrophoresis and zymography. The isolated fungus was also subjected to grow on media agar plates containing different azo dyes to test the ability of decolourisation.

## MATERIALS AND METHODS

**Sample collection**

The fungal samples were collected in sterile tube from the trees in the vicinity of Kulliyyah of Science, International Islamic University Malaysia, Kuantan campus, Malaysia. The collection was done in the month of January. The fungi can be seen growing on the tree bark throughout the area.

**Media conditions and screening**

Potato dextrose agar (PDA) and Sabouraud-4 %-dextrose agar (SDA) were used to isolate fungi. Chloramphenicol (0.01 %) was added to the media in order to inhibit the growth of bacteria. 0.1 % ABTS (2,2-Azinobis-3-ethylbenzthiazoline-6-sulphonate) was used as indicator. Serial dilutions were made and the plates were incubated at 30 °C until positive colour production was observed. The colour producing fungal colonies were purified by sub-culturing.

**Cultivation in liquid media**

The isolated fungus was maintained on SDA plates containing 0.1 % ABTS at 30 °C and placed at 4 °C for long storage. Sub-culturing was done every month. For crude enzyme production, 500 mL Erlenmeyer flasks containing 125 mL Sabouraud-2 %-dextrose broth (SDB) were inoculated by one 5-day-old culture fungal plug (6 mm diameter) and incubated at 30 °C and 150 rpm [23]. The enzyme inducers $CuSO_4$ (0.3 mM) and $MnSO_4$ (0.1 mM) [24] were added to the culture medium after 72 hr of inoculation and left for incubation of further 11 days.

**Preparation of crude enzymes**

After incubation, the culture broth was filtered using Whatman No. 1 and subjected to protein precipitation. The culture filtrate was brought to 80 % saturation with ammonium sulfate, and the precipitated proteins were collected by centrifugation at 8,500 rpm for 60 min. The precipitated proteins were re-suspended in 20 mM sodium acetate buffer pH 5.0 and dialysed (10 kDa cutoff) over night against 1L of the same buffer and then with 2L of the same buffer. The dialysed sample was then freeze dried.

**Gel electrophoresis**

The crude sample was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis with a 12.5 % polyacrylamide gel containing 0.1% SDS. Samples (10 mg of protein) were treated before they were loaded onto the gel with 0.5% SDS and 5% b-mercaptoethanol and were boiled at 95°C for 5 min. The gel was run for 45 min at constant voltage of 200. The protein was visualized by staining the gel with Coomassie blue G-250 (Bio-Rad) and was compared with molecular weight markers (Novagen).

**Native PAGE and zymography**

Polyacrylamide gel electrophoresis (PAGE) was performed as described by Ornstein and Davise[25-26]. The separating and stacking gels contained 10 % and 4 % acrylamide respectively. Visualization of the bands was achieved by Coomassie blue staining. For zymogram the gel was first submerged in 20 mM sodium acetate buffer (pH 4.0) for 30 min and then in 20 mM ABTS in the same buffer until the colour development.

**Dye decolourisation**

Decolourization of different dyes was performed on SDA plates containing 0.01 % of the respective dyes (brilliant green, trypan blue, direct

red 80 and eriochrome black T). The agar plates were inoculated at the center with mycelia discs (5mm) taken from the periphery of 5-day-old fungal cultures grown on SDA. The plates were analyzed for dye decolourization after 8 days of incubation at 30±1 °C.

## RESULTS

The screening of the tree samples resulted in isolation of at least three fungi which are producing oxidases. The production of deep reddish purple colour confirmed the presence of extracellular enzymes. The most active producer (Fig. 1) in terms of days of incubation was selected for culture in the liquid media.

The preparation of crude sample resulted in 85 mg of slightly yellowish dry concentrate from the processing of liquid media. The lyophilised sample was used for further investigations.

The SDS-PAGE analysis was done under reducing and denaturing (Fig. 2) as well as semi denaturing conditions (data not shown). In both the conditions the results obtained were compare able. Different concentrations, 10, 20, 30, 40 and 50 ìg of sample were loaded on to the gel. After destaining, the gel showed seven clear bands. With respect to the molecular weight marker, four bands were below 25 kDa and the rest more than 35 kDa. The native PAGE analysis revealed that there are few diffused bands (Fig. 3) with different mobility. The PAGE was compared with the zymogram. Both gels were loaded with the same concentrations of the sample as for SDS-PAGE. There is only one band, at the middle (Fig. 3) out of the three that is

**Fig. 1**. Isolated fungus producing reddish purple colour on SDA plate containing 0.1 % ABTS

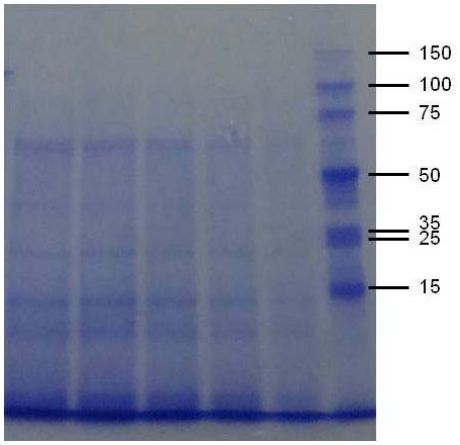


**Fig. 2**. SDS-PAGE of crude sample under reducing and denaturing conditions. From right to left, molecular weight marker, 10, 20, 30, 40 and 50 µg of sample

actively producing intense colour. The intensity of colour increases with the concentration of the loaded sample.

The oxidases produced by the fungi can decolourise brilliant green and trypan blue that can be observed after 7 days of incubation and then followed up to 14 days. There is no decolourisation of direct red 80 and some precipitate formation with Eriochrome black T which appears in the form of dark circle at the periphery of the growing fungus (Fig. 4).

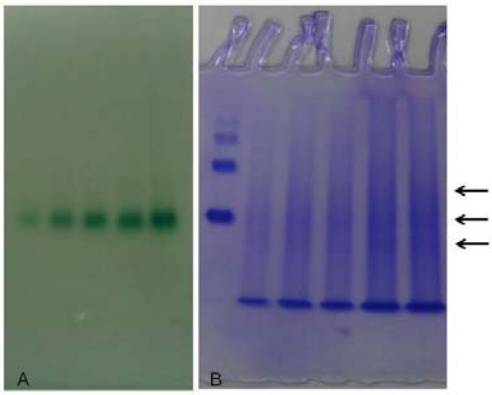


**Fig. 3**. Zymography (A) and native PAGE (B) of crude sample. From left to right, 10, 20, 30, 40 and 50 µg of sample was loaded. BSA was used as standard in native PAGE (B) 1st lane on the left

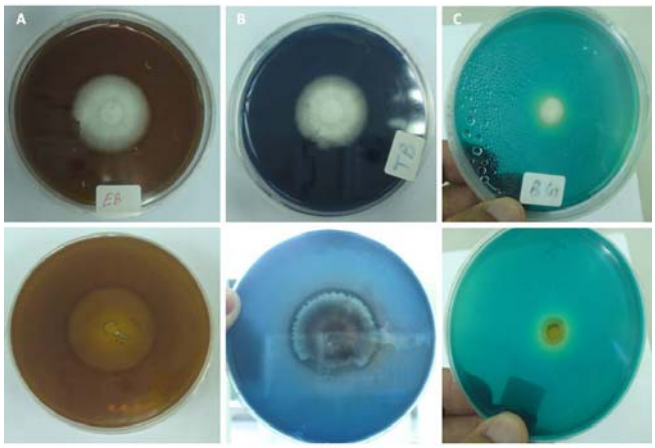


**Fig 4**. Azo dyes decolourization by the isolated fungus.
Eriochrome black T (A), trypan blue (B), and brilliant green (C)

## DISCUSSION

The screening of fungi from trees on the SDA plates containing 0.1 % ABTS produced a reddish purple colour for oxidases producing fungi. The colour is observed due to the oxidative polymerisation of enzyme such as laccases with ABTS produced during the screening of white rot fungus [26]. From the three isolates the most active producer of extracellular oxidases was cultured in broth and induction was carried out with $CuSO_4$ and $MnSO_4$ [22-23]. The crude protein sample that was obtained by precipitation and dialysis was subjected to PAGE analysis. The gel run under denaturing conditions showed multiple bands. The coomassie blue staining was performed repeatedly to observe bands as it was noted that the sample was not stained properly by just one time staining. Two sharp bands at about 60 kDa and at least one band of approximately 45 kDa with respect to the

molecular weight marker could be the potential oxidases produced by the fungus. The bands with less than 25 kDa may present some other proteins or degraded protein products [27]. The native PAGE analysis showed three bands while the zymography showed only one band (Figure 3). The appearance of greenish colour suggests the presence of enzymes such as laccase, as there is no hydrogen peroxide present for peroxidase activity in the staining solution (see material and methods). The colour appeared within 30 minutes and initially observed as two close bands and afterwards one intense band. The comparison of native PAGE and zymogram suggested that there may be only one protein component in the crude sample that is responsible for the oxidation of ABTS. The fungi showed decolourisation of brilliant green. This is in contrast with the marine fungi and *Armillaria* specie which were reported to have no effect on the dye [28-29]. The white rot fungus *Grammothele subargentea* strain that produces laccase as the sole source of lignolytic activity was able to decolourise brilliant green [30]. This suggests that the observe decolourised zone contain extracellular laccases. Trypan blue was also decolourised by the isolated fungi. Different fungi have been reported to decolourise trypan blue in different conditions due to the activity of laccase enzyme [31-32]. The decolourisation of direct red 80 was observed to a very low extent as compared with brilliant green and trypan blue. This is observed may be because the enhanced decolourisation needs optimisation of media conditions and maximum activity of lignin peroxidase [33]. Eriochrome black T was degraded by the fungus which appeared in the form of dark circle may be owing to the degradation of the dye. This might be for the reason that extracellular degradation or adsorption of dye and release of metabolic products [34]. In the current study a fungus producing extracellular ABTS-oxidases has been isolated from tree sample. Polyacrylamide gel analysis revealed the presence of three potential proteins bands responsible for the activity. Zymography experiment confirmed the presence of laccase enzyme in the crude sample that was analysed. The extracellular oxidases have shown azo dyes decolourisation activity in the solid medium. These results are promising to potential biotechnological application for industrial dye treatment. Identification of the fungus, purification and characterisation of the proteins are in progress and will be reported elsewhere.

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