Isolation of a New Efficient Dye Decolorizing White Rot Fungus *Cerrena* Sp. WICC F39

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Lignin degrading system of white rot fungi and its exploitation in the decolorization of synthetic dyes has been used in a wide array for human benefits. In this study, decolorization of two model synthetic dyes, Reactive Black 5 (RB5) and Methylene Blue (MB) with the ability of Kraft lignin degradation were investigated using new fungal isolates from Malaysian soils. The most potent isolates WICC F38 and WICC F39 were selected for further investigation along with reference strain *P. chrysosporium* DSMZ 6909. To prove their dye decolorization capabilities, the qualitative and quantitative methods were conducted, while their potential for biodegradation of kraft lignin was carried out using gravimetric method. The degree of lignin degradation, dyes decolorization (dyes intensity reduction) and productivity of lignin peroxidase, manganese peroxidase, and laccase were studied. It was noticed that 86-88% of decolorization rate for both synthetic dyes was obtained by WICC F38 and WICC F39; respectively. However, MB showed a more decolorization rate than RB5 by the selected isolates. The efficiency of the selected fungal isolates comparisons with reference strain for decolorization rate of synthetic dyes could be ordered as follows; WICC F39 > *P. chrysosporium* DSMZ 6909 > WICC F38. The fungal decolorization capabilities in the present study were concomitant with the production of lignin peroxidase, manganese peroxidase and laccase. The isolates under the study were identified as *Phanerochaete* sp. WICC F38 (ac., KU141330) and *Cerrena* sp. WICC F39 (ac., KU141331) using molecular identification strategy.

**Keywords:** White Rot Fungi, Dyes Decolorization, Lignin Degradation, Methylene Blue, Reactive Black 5, *Cerrena* sp

**Introduction**

Textile’s effluents contributed to about 17-20% fresh water pollution and mostly are recalcitrant to biodegradation¹. These untreated dyes effluent created hazardous environmental effect by releasing the toxic compounds into the receiving water bodies. Recently, exploitation of green technology using fungi have been successfully applied for many industrial processes as high potential biofactory for the production of complex enzymes, and wide range of primary and secondary metabolites²–⁶. White rot fungi which characterized by their ability to produce versatile isoforms of extracellular oxidases including laccase (EC 1.10.3.2), manganese peroxidase (EC 1.11.1.13), lignin peroxidase (EC 1.11.10.14) act as an alternative strategy to conventional chemical and physical treatments, which present serious limitations. Therefore, the main objective of the current study was to isolate a new potent white rot fungi which produce lignin degrading enzymes with high capacity for decolorization and degradation of recalcitrant dyes.

**Experimental section**

**Dyes, chemicals and microorganisms**

All the chemicals used in the present study were high purity and analytical grade. Guaiacol, veratryl alcohol, 2,2’-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), 2,6-dimethoxy-phenol (DMP), methylene blue were purchased from Sigma-Aldrich (USA) except Kraft lignin (Merck). Reactive black 5 was obtained from textile industries (Tradrite Marketing Sdn. Bhd., Malaysia). *Phanerochaete chrysosporium* DSMZ 6909 as reference strain was purchased from Leibnitz Institute DSMZ-German collection of microorganisms and cell cultures, Braunschweig, Germany.

**Collection of samples and isolation of interest fungal isolates**

The different soil sources, including Permanent Reserve Forests (PFR), National/State Park (NP), Total Forest Cover (TFC) and Agriculture and Build
Up Areas (ABUA) with 11 different substrata localities around Johor, Malaysia were used in the sampling of 77 soil samples under aseptic conditions. The isolation of fungal isolates was performed according to the method described by Shrestha et al. The isolates were purified by repeated transfer to malt extract agar (MEA) plates and grown at 30°C for 7 days. Pure isolates were sub-cultured maintained on MEA slants and stored at 4.0°C and sub-cultured regularly.

Qualitative screening for ligninolytic activities of the fungal isolates

Qualitative estimation was carried out by inoculated 90 mm diameter Petri dishes containing PMM medium composed of: glucose, 20.0 g L⁻¹; ammonium tartrate, 2.0 g L⁻¹; malt extract, 2.0 g L⁻¹; KH₂PO₄, 0.26 g L⁻¹; Na₃HPO₄, 0.26 g L⁻¹; MgSO₄, 0.50 g L⁻¹; CuSO₄, 10 mg L⁻¹; CaCl₂, 6.6 mg L⁻¹; FeSO₄, 5 mg L⁻¹; ZnSO₄, 0.5 mg L⁻¹; Na₂MoO₄, 0.02 mg L⁻¹; MnCl₂, 0.09 mg L⁻¹; H₃BO₃, 0.07 mg L⁻¹. This medium was supplemented with 0.01% of guaiacol, 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid (ABTS) and polymeric dye (Poly R-478) with agar disc (5 mm) of 7-day old mycelium, and incubated at 30°C for 10 days. The intensity of color in the vicinity of the mycelium on guaiacol medium was measured. Isolates which showed notable decolorization activities were further exploited in dye decolorization assay.

Dyes decolorization assays

Qualitative decolorization of the fungal strains was carried out by inoculated middle of malt extract agar plates supplemented 0.2% Reactive black 5 (RB5) and Methylene blue (MB) with a disc of fungal isolates growth, and incubated at 30°C for 28 days. The decolorization rate was calculated by measuring the halo zone diameter (mm). While, quantitative screening of decolorization capability was performed by inoculating 50 ml malt extract broth medium supplemented with 0.5% tween 80 dispensed in 250 ml Erlenmeyer flask with 5% of spore suspension for each tested isolate. The inoculated flasks were incubated at 30°C for initial 2 days using reciprocal shaker (150 rpm). A control experiment was run under similar conditions without inoculum, after that, 1 ml of 0.2% dyestuff was added to each flask and incubation time was continued for 8 days. All experiments were carried out in triplicates and data represented are their mean values. Aliquots were withdrawn aseptically, cell free supernatant was obtained by centrifugation at 8,000 xg for 15 min, the decolorization rate was estimated at pre-determined λmax of the respective dyes solution, and also, ligninolytic enzymes activity was determined. The rate of decolorization for the tested dyes was calculated from the following equation:

\[ \text{decolorization rate} = \frac{[(\text{Abs}_1 - \text{Abs}_2)/\text{Abs}_1] \times 100}{\text{time}} \]

Where Abs₁ is the initial absorbance and Abs₂ is the absorbance after incubation for time t.

Screening of ligninolytic activities

Ligninolytic enzymes productivity for the selected isolates was investigated qualitatively by plate assay method using Kraft lignin (KL), 0.025% as a substrate. The clear zone was visualized directly after 10 days incubation at 30°C, by flooding the plates with 1% (w/v) FeCl₃, 6H₂O and K₃[Fe(CN)₆] reagent. The precipitate was obtained by centrifugation at 8,000 xg for 30 min, dried at 60 ± 5°C for 48 h, and weighed, the results were elucidated by comparison with control samples.

Ligninolytic enzymes assays

Lignin peroxidase (LiP) activity was assayed according to Kheiralla et al. Mn-peroxidase (MnP) was measured by oxidation of 2,6-dimethoxyphenol, \( \epsilon_{310} = 9300 \text{ M}^{-1} \text{ cm}^{-1} \) and laccase (Lac) activity has been estimated using (ABTS) as substrate with an extinction coefficient \( \epsilon_{420} = 36,000 \text{ M}^{-1} \text{ cm}^{-1} \) at 420 nm. All calculations of enzymes were derived from a study carried out by Baltierra-Trejo et al. All of the enzymatic units were defined as the quantity of enzyme which oxides 1 μMol of substrate per minute per litre.

Molecular identification of ligninolytic enzymes producing fungal isolates

The isolates showing the highest ligninolytic activities were subjected to genotypic identification. Amplification of the 18s rRNA gene from the genome of fungal isolates under investigation was carried out via the polymerase chain reaction technique. After completion the PCR reaction, the amplification products were examined using 1.0% agarose gel electrophoresis and purified by standard method and sequenced using BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems).

Alignment and phylogenetic analysis

After a BLAST search (www.ncbi.nlm.nih.gov /blast) to assess the DNA homologous, the sequence of
isoles were deposited in the GenBank to get an accession number. The boot-strap test of phylogeny of the isolates and closely related species were analyzed using the multi-sequence alignment program (MEGA 6.0). Neighbor-joining with p-distance methods were used for computing the evolutionary distance matrix, thousand bootstraps replicates were used to build the corresponding phylogenetic trees\textsuperscript{12}.

Results and Discussion

Isolation, screening and characterization of the fungal isolates

From isolation program, an abundant pure white cottony mycelium with the growth margin reaching the edge of the plate was obtained within 5-6 days of incubation, and the color of the subsurface mycelium was very similar to that on the surface. After 7 days of incubation, it was noticed that the fungal mat became denser and more felt-like, with slight yellowish color. Most of the morphological images (data not shown) presented the germination of basidiospores to form septate hyphae (filaments), which is important in developing phylogenetic hypotheses in the Basidiomycota. Moreover, it was observed that most of the cells produced clamp connections to ensure each cell, or segment receives a set of different nuclei.

In a preliminary screening program for ligninolytic activities of selected fungal isolates, it was found that 119 isolates showed brown-colored zones with varying intensity around mycelia growing on PMM-guaiacol medium. 75 out 119 fungal isolates were selected as apture culture. Table 1, illustrated the number of the collected samples and number of positive isolates growing on PMM-guaiacol medium.

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<td>TPNP</td>
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Classification involved four zones of characterization type: 4+ represented a strong or complete red color reaction, 3+ was medium red color reaction, 2+ was weak red color reaction, 1+ was very weak (light) red color reaction and 0 for no reaction.

Dye decolorization by the selected fungal isolates

The dye decolorization studies showed great variation in the ability of selected fungal isolates to degrade dyes belonging to diverse categories. The decolorization rate depends on the type of stains, type and concentration of enzymes, chemical structure and concentration of used dyes\textsuperscript{13}. The comparison between qualitative and quantitative decolorization methods were exploited in the present study. The obtained results confirmed that there was a linear correlation between two methods, whereas the isolates which exhibited large and obvious decolorization zone on solid medium, also showed the highest percentage of decolorization rate in liquid medium. Traditionally, the synthetic dyes were classified into acidic, reactive, direct, basic, disperse, metal complex, mordant and sulphur dyes. Reactive black 5 which chosen in current study is the example of one of the greatest group of synthetic dyes belong to azo dyes, which characterized by low solubility expense, high stability, color variety and structure complexity as a result of the presence of double azo bonds (-N=N-). Therefore, removal of this azo dye from a medium by fungal isolates under study can be exploited as a basic screening for their future application in bioremediation study. The recorded result revealed that the WICC F39 isolate exhibited high potential for decolorization of RB5 and MB more than other selected isolates with decolorization rate 86-88%; respectively, and as well showed the greatest decolorization zone on plate assay. Results obtained in the present study are in a good agreement with the results obtained by others investigators, Adnan et al.\textsuperscript{14} reported that the RB5 was decolorized by T. atrovinide up to 91.1%, which laccase was involved in the RB5 biodegradation process. In another study, Daâssi et al.\textsuperscript{15} reported that C. gallica...
could decolorize RB5 but the decolorization efficiency was relatively low. While up to 92% of RB5 decolorization was obtained by using *Polyporus sp.* S133. Conversely, MB was decolorized more efficiency than RB5 by WICC F38 isolate with decolorization rate 69% and 43% respectively. Likewise, the decolorization rate of MB 73.07±0.08% was obtained by reference strain *P. chrysosporium*, additionally, the resistance of RB5 to decolorization was noticed through decrease of decolorization efficiency by 29.83±2.68%. Previous study obtained by other investigator showed that the methylene blue was only decolorized about 10% by *Trametes* sp. LAC-17. Consequently, dye decolorization performance of local fungal isolates was more efficient compared to the reference strain. It can be clearly seen from the obtained results that the intensity of colored mycelial mat during decolorization process was directly proportional with dye adsorption/biosorption activities. The efficient decolorization may be attributed by the productivity of ligninolytic enzymes, which responsible for degradation of chromophore in dye molecule or/and adsorption by the fungal biomass. Ligninolytic productivity has been investigated by using the used synthetic dyes in the present study. Referring to Figure 1, laccase (Lac) was the highest titer among other ligninolytic enzymes with 340 U ml\(^{-1}\) produced from WICC F38, 229 U ml\(^{-1}\) from WICC F39 and 34 U ml\(^{-1}\) from *P. chrysosporium* DSMZ 6909; respectively. These results were in compliance with the earlier reports that the laccase from the white-rot fungus *C. unicolor* showed high efficiency for the decolorization of the different classes of dyes either by using purified or crude enzyme preparations.

On the other hand, manganese peroxidase (MnP) and lignin peroxidase (LiP) activities were detected with 14 – 57 U ml\(^{-1}\); respectively. Optimization of culture conditions may needed for enhancement the enzymes productivity and decolorization rate as a consequence. Additionally, another study carried out by Cheng *et al.* showed that *Coriolopsis* sp. strain arf5 isolated from Malaysia has completely degraded different azo dyes under varying times such as Ponceau 2R and Basal Scarlet in 9 days while Orange G and Direct Blue 71 in 8 days. It is noteworthy to mention that identification of responsible enzymes, determination of degradation products as well as their toxicity is utmost important while choosing the best method to introducing them into highly contaminated effluent. One study has been conducted in Malaysia to demonstrates degrading ability and enzymatic applications for dyes treatment using different standard strains. However, the decolorization or degradation system of local isolates white rot fungi have not yet been studied in significant extent.

**Biodelignification of kraft lignin by ligninolytic activities of the selected isolates**

Ligninolytic productivity by the selected fungal isolates was further investigated in the current study. Results in Figure 2 shows that Kraft lignin was degraded efficiently WICC F38 followed by WICC F39 and PC DSMZ 6909.
F39 in comparison with reference strain. It was found that the presence consortium of ligninolytic enzymes contributed to lignin loss about 35% in case of WICC F38 whereas, manganese peroxidase was found in the highest titers, 266 U ml\(^{-1}\), laccase 180 U ml\(^{-1}\) and lignin peroxidase 29 U ml\(^{-1}\); respectively. In addition to this, around 30-32% of lignin loss was detected from lignin biodegradation by WICC F39 and \textit{P. chrysosporium} DSMZ 6909 with the same trend of enzymes titre as follows; MnP>Lac>LiP (Figure 3). The present results are consistent with that obtained by another investigator who indicated that the degradation of lignin accompanying with the presence of a consortium of ligninolytic enzymes worked together\(^{20}\). Munir \textit{et al.}\(^{21}\) reported that a lignocellulosic substrate like corn stover, corn cobs, sugarcane bagasse, wheat straw, rice straw and banana stalk core accelerated the degradation of lignin with concomitant production of peroxidases and laccase. It has been reported that the Kraft lignin was degraded by using laccase with ABTS as mediator at low pH range (3-6)\(^{22}\) and the more efficiency of degradation was obtained by using purified laccase compared to the crude\(^{23}\).

**Phylogenetic analysis for selected fungal isolates**

The cladogram in Figure 3 indicated that the most potent strains WICC F38 (Accession no KU141330.1) and WICC F39 (Accession no KU141331.1) fell within a clade comprised of \textit{Phanerochaete} and \textit{Cerrena} species, with the closest were to \textit{Phanerochaete} sp. (Accession no GU190187.1) and \textit{Cerrena unicolor} (Accession no AY85007.1); respectively. Both strains were later named as \textit{Phanerochaete} sp. WICC F38 and \textit{Cerrena} sp. WICC F39.

**Conclusions**

In the current study, the exploitation of the most potent and promising local fungal isolates with ligninolytic activities in degradation of recalcitrant dyes was investigated. The fungal isolates have undergone for molecular identification and were identified as \textit{Phanerochaete} sp. WICC F38 and \textit{Cerrena} sp. WICC F39 and Subsequently, the sequences have been deposited in the GenBank to get an accession number as KU141330 for WICC F38 and KU141331 for WICC F39. The potential of these fungal strains to utilize lignin containing compound and to decolorize various synthetic dyes belonging to different categories was also evaluated. The results obtained from study of dye removal efficiency and lignin loss capacity for the selected strains reflected the importance of the local fungal stains under study to solve wide world pollution problems.

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**References**

15 Daássi D, Rodríguez-Couto S, Nasri N & Mechichi T, Biodegradation of textile dyes by immobilized laccase from Cordylospis gallica into Ca-alginate beads, Int Biodeter Biodegr, 90 (2014) 71-78.
19 Mohamed R, Lim M T & Halis R, Biodegrading ability and enzymatic activities of some white rot fungi on kenaf (hibiscus cannabinus), Sains Malays, 42 (2013) 1365-1370.