Degradation potential of basidiomycetes *Trametes ljubarskyi* on Reactive Violet 5 (RV 5) using urea as optimum nitrogen source

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**ABSTRACT**

Dye wastewater management has been a challenging task for the textile industry. In this study, basidiomycetes fungi was explored for its ability to degrade dye. Isolate EL2, collected from the decaying woods around Kampar, Perak, Malaysia was selected for this purpose. The collected isolate was identified as *Trametes ljubarskyi* by using a molecular characterization method and morphological observation. Then, it was screened with solid agar supplemented with 2,2-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid, guaiacol and Remazol Brilliant Blue R dye for laccase production. Four different nitrogen sources were involved in further liquid screening for optimization of laccase production in EL2. Based on the results obtained, urea is the best candidate, as it was able to produce maximal laccase activity of 50.82 U/(L·d), as compared to three other nitrogen sources (1.11–5.08 U/(L·d)). Besides, EL2 was able to significantly decolourize Reactive Violet 5 (97.92%) on day 7.

**KEYWORDS**

Basidiomycetes; laccase; dye; nitrogen; fermentation

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**Introduction**

In the textile industry, azo dyes are the predominant colourants in textile wastewater. They represent 70% of the total amount of the dyes discarded in wastewater [1]. About 10% of dyes are reported to end up in industrial effluents during the dyeing processes. These textile dyes, especially azo dyes, are recalcitrant, since they are designed to be resistant to light, water and oxidizing agents. They are difficult to degrade naturally once they enter the aquatic system [2].

Azo dyes may undergo reduction that leads to the formation of aromatic amines which is hazardous and can cause cell mutation as well as cancer. Besides, discharge of dye wastewater into the environment will cause colourization of water sources, alteration of water pH, increase of chemical oxygen demand (COD) and biochemical oxygen demand (BOD). High colour intensity of textile wastewater will reduce the passage of sunlight through the water and deteriorate water quality by lowering the gas solubility. This will lead to the reduction of aquatic diversity. Therefore, textile dye wastewater management has become a major concern for textile industries [3].

Currently, textile effluents in Malaysia are treated with chemical methods, such as coagulation, flocculation, as well as a physical method such as adsorption by activated carbon. Besides, physicochemical and biological methods are other alternative techniques being used to treat textile effluents. However, most of these available applications are costly. Coagulation and flocculation will produce sludge whose disposal poses additional problems. The adsorption method is the only one that complies with the discharge standard, but it is relatively expensive [3]. Hence, it is necessary to search for an approach that is more effective, affordable and environmentally friendly.

Biodegradation techniques using fungi can be applied to degrade azo dyes completely by reducing the dyes with secreted enzymes [4]. Basidiomycetes, the white rot fungi, serve as potential candidates to be used in bioremediation to treat textile wastewater. Many studies have proved that basidiomycetes are capable of degrading recalcitrant organic pollutants with their ligninolytic enzymes, predominantly laccase [5]. These extracellular enzymes are produced with low substrate specificity that enables them to mineralize organic pollutants with structural similarity to wood lignin. Thus, there are growing interests in screening fungal strains with high laccase activity for dye degradation.
The objective of this study was to investigate the laccase activity of isolate EL2, collected from the decaying woods around Kampar, Perak, Malaysia, using both solid and liquid screening. Subsequently, different nitrogen sources were screened to determine the best nitrogen source for cultivating EL2. Lastly, EL2 was tested in the decolourization assay for its ability to degrade azo dyes, namely Reactive Violet 5 (RV5).

Materials and methods

**Basidiocarps morphological identification**

Basidiocarps of EL2 were rinsed with distilled water and further sterilized with 70% alcohol to remove environmental contaminants. The morphology of the basidiocarps was observed and recorded. A small portion of the basidiocarps was cut and cultured on malt extract agar (MEA) at 30 °C. The pure culture of EL2 was preserved on MEA slants and stored at 4 °C [5].

**Molecular identification**

Genomic DNA of EL2 was extracted by using the ethanol precipitation method as described by Liu et al. [6]. The internal transcribed spacer (ITS) region in fungal DNA was amplified with forward primer ITS-1 (5'–TCCGTAAGTGAACCTGCG-3') and reverse primer ITS-4 (5’–TCTTCCGCTTAATTGATATGC-3'). The polymerase chain reaction (PCR) conditions used were: initial denaturation at 94 °C for 5 min, followed by denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s and extension at 72 °C for 30 s for 35 cycles. The PCR amplifications were completed with a final extension at 72 °C for 5 min (SuperCycler Trinity, Kyratec, Australia). After purification, the PCR products were sent to Genomics Bioscience and Technology Co. (New Taipei, Taiwan) for sequencing. Ten most homologous sequences were determined by comparing to GenBank database using BLAST (basic local alignment search tool) software. The genetic distance was analyzed using MEGA 7 software [5,6].

**Solid agar screening for laccase production**

EL2 was screened for laccase production via solid agar method. MEA plates supplemented with 2,2-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS), guaiacol and Remazol Brilliant Blue R (RBBR) dyes as substrates were used [5,7]. The plates were incubated at 30 °C for one week to allow mycelium growth and halo development. The diameters of the fungal colony and halo zone were measured and recorded after one week. The halo zone ratio of the isolate was determined by using the following formula [5]:

\[ \text{Ratio of halo zone} = \frac{\text{diameter of halo}}{\text{diameter of fungal colony}} \]

**Liquid screening for laccase activity**

**Culture conditions.** Liquid screening was done to quantify the activity of laccase produced by EL2. It was cultured in submerged fermentation by using Mineral Salt Broth (MSB). The fermentation medium consists of: 2 g/L KH₂PO₄; 0.5 g/L MgSO₄·7H₂O; 0.1 g/L CaCl₂·2H₂O; 2 g/L glucose; 0.2 g/L ammonium tartrate; 10 mg/L thiamine hydrochloride and 10 mL/L of trace elements solution. The chemical components in the trace element solution: 1.5 g/L nitrilotriacetic acid; 0.48 g/L MnSO₄·H₂O; 1 g/L NaCl; 10 mg/L CoCl₂·6H₂O; 10 mg/L FeSO₄·7H₂O; 10 mg/L ZnSO₄·7H₂O; 8 mg/L CuSO₄·5H₂O; 8 mg/L H₃BO₃ and 8 mg/L Na₂MoO₄·2H₂O [8]. Four mycelial discs (5 mm in diameter) were inoculated into 150 mL of culture medium in a conical flask (250 mL). The experiment was conducted in triplicates. The culture flasks were then placed in a shaking incubator at 140 r/min at 30 °C for 20 days.

**Optimization of nitrogen sources for laccase production**

Three different nitrogen sources (ammonium nitrate, urea and yeast extract) were used to substitute the original nitrogen source. The assessed concentration was the same as that of the original fermentation broth (0.2 g/L). All cultured flasks were incubated for 20 days at 30 °C in a shaking incubator at 140 r/min.

**Laccase assay**

One milliliter of sample was collected from each of the culture flasks daily and centrifuged at 14,416 g (Sorvall Legend Micro 17R, Thermo Scientific, Waltham, USA) for 5 min to obtain the extracellular cell-free enzyme extracts (CFEE). Laccase activity of CFEE harvested from submerged fermentation was determined by the oxidation of ABTS. One hundred microliters of 20 mmol/L ABTS solution was mixed with 870 μL of 0.1 mol/L sodium acetate buffer (pH 4). After incubated in a water bath at 30 °C for 10 min, 20 μL of CFEE was added. The absorbance was read at 420 nm for 2 min (Genesys 10S UV-Vis, Thermo Scientific, Waltham, USA). One unit of enzyme activity is defined as the amount of enzymes that oxidize 1 μmol of ABTS per minute [9].
Dye decolourization analysis

As described in the liquid screening for laccase activity, 80 mg/L of RV 5 dye was added into the fermentation broth, with urea as the nitrogen source. In order to monitor the decolourization of the dyes, CFEE collected daily were measured spectrophotometrically at 558 nm. The absorbance was recorded and the percentage of decolourization was calculated as follows [5]:

\[
\text{% of decolorization} = \left( \frac{I - F}{I} \right) \times 100,
\]

where \( I \) is the initial absorbance and \( F \) is the final absorbance.

Results and discussion

Fungal identification

Basidiocarps morphological identification

Figure 1 shows a picture of EL2 at its sampling site and its basidiocarps measurement. The basidiocarps of EL2 were creamy white in colour with spots of pale greyish brown. It is 4 cm in length and 6 cm in width, with a glabrous and dull surface. All these characteristics match with the description of white rot basidiomycetes fungi *Trametes ljubarskyi* [10].

Molecular identification

Genomic DNA of EL2 was successfully extracted and amplified by PCR using fungal-specific primers. The PCR amplification of the ITS region gave approximately 650-bp PCR products. Comparison of the ITS sequence was made by alignment with highly similar sequences in the National Centre for Biotechnology Information (NCBI) nucleotide sequence database with the aid of the BLAST algorithm. The result showed that EL2 was 95% homologous, with a 0% gap, to *Trametes ljubarskii* (Accession no. AY684174.2). A phylogenetic relationship tree for EL2 was constructed using the neighbour-joining method as shown in Figure 2. EL2 displayed maximum identity with *Trametes ljubarskii* (Figure 2). Thus, EL2 was confirmed as *T. ljubarskyi* based on its basidiocarps morphology and ITS rDNA gene sequence analysis.

Solid agar screening for laccase production

Laccase is a phenol oxidase with the capability to catalyze oxidation of several substrates, particularly phenols and aromatic amines [11]. In this study, primary screening was done on solid agar plates that were supplemented with different substrates to detect the presence of laccase. Three selected substrates, ABTS, guaiacol and RBBR dyes were used to compare their susceptibility to laccase produced by EL2. ABTS is a non-phenolic heterocyclic compound that could be oxidized to cation (ABTS⁺) and dication (ABTS⁻²⁺) radicals [11]. ABTS is colourless, while the oxidized radicals are blue-green in colour [12]. Therefore, a blue-green coloured halo would form under the fungal colonies that produce laccase [13]. Guaiacol is a methonymy-substituted monophenol, which is oxidized to aldehydes [11]. Oxidative polymerization of guaiacol by laccase causes the formation of a reddish-brown halo underneath the fungal colonies [14–16]. On RBBR dye plates, the presence of laccase is confirmed with the decolourization of blue colour agar [14,15] and the formation of a colourless halo on the plate. Many studies suggest that the ability to decolourize polymeric dyes, such as RBBR, is solely due to laccase activity [17–19]. In this study, EL2 was confirmed to be able to produce laccase, as it formed halo zones on all three different screening plates. The ratios of the halo zones to the colony diameter were 1.78 ± 0.03, 1.06 ± 0.01 and 0.73 ± 0.04 on ABTS, guaiacol and RBBR dye plates, respectively (Figure 3).

The results clearly showed that laccase activity assayed with ABTS was significantly higher than that observed with the other two substrates. This was in agreement with Eggert et al. [20] and Kalmış et al. [21].
where ABTS is a more sensitive substrate as compared to guaiacol in the detection of laccase. Apart from this, a comparative study carried out by Li et al. [11] also indicated ABTS as the most sensitive substrate to evaluate laccase from Trametes sp.

Liquid screening for laccase activity

Optimization of nitrogen sources for laccase production

The purpose of liquid screening by using submerged fermentation is to quantify laccase secreted by EL2. Laccase, being one of the secondary metabolites produced during secondary metabolism [22], will be directly secreted into the fermented broth [23]. This eases the process of collecting and purifying the secreted enzymes. The fermented broth was collected daily for laccase assay. Nitrogen is essential for amino-acid synthesis, the building blocks for proteins and other value-added substrates. Nitrogen-sufficient broth has been reported to enhance the production of ligninolytic enzymes in many fungi [7]. In this study, organic nitrogen sources, urea and yeast extract, and inorganic nitrogen sources, ammonium nitrate and ammonium tartrate, were used to identify the most suitable nitrogen source for cultivating EL2.

Based on their effect on laccase production, the studied nitrogen sources ranked as follows: urea > ammonium tartrate > yeast extract > ammonium nitrate (Figure 4). Among the tested nitrogen sources, urea gave the highest laccase activity (457.42 U/L) on day 9 with 50.82 U/(L·day) of laccase produced. Complex nitrogen sources are known with the capability of stimulating ligninolytic activity and laccase accumulation of the fungi [24]. However, the addition of ammonium nitrate into the fermentation broth had no significant effect on laccase production as compared to urea. The result seen here corroborates an earlier study by Revankar and Lele [25], which showed that there was lower yield of laccase with inorganic nitrogen sources. On the other hand, fermented broth supplemented with yeast extract resulted in lower laccase activity as compared to that with ammonium tartrate, but still much higher than the one achieved with ammonium nitrate. These results contradicted with our initial hypothesis that organic nitrogen sources would always be expected to be a better choice for laccase production. However, a proper comparison between the findings in the present study and previous ones is difficult due to differences in the concentration and brand of the nitrogen sources used.

Dye decolourization analysis

Preliminary results of this study revealed that urea was the best nitrogen source for laccase production in EL2. Hence, EL2 was cultured in MSB containing 80 mg/L of RV 5 with urea as the nitrogen source to evaluate its dye decolourization ability. EL2 showed profiles of rapid decolourization activity in liquid cultivation, as the violet colour was gradually decolourized during the incubation period (Figure 5). The graph indicated that the decolourization of RV 5 by EL2 began on day 2 (dye decolourization rate: 5.30% ± 1.56) and reached a maximal dye decolourization rate (97.92% ± 0.23) on day 7. Meanwhile, the production of laccase was assayed in order to

Figure 3. Halo zone formation as a result of ABTS oxidation (A), guaiacol oxidation (B), RBBR decolourization (C) by laccase produced by isolate EL2.

Figure 4. Effect of different nitrogen sources on laccase production by isolate EL2 along 20 days of submerged fermentation. Mean values from duplicate experiments. Error bars represent standard error of the means.
investigate the dye decolourization mechanism through biodegradation by ligninolytic enzymes. The laccase production profile shown in Figure 6 indicated that the increase in laccase activity is relative to the RV 5 dye decolourization activity. This result is in agreement with previous studies reporting laccase as being solely responsible for decolourization and degradation of various industrial and textile dyes [26]. However, the results obtained here for EL2 are only preliminary and there are limited studies on similar isolates; therefore, comparison with similar earlier studies would be relatively difficult.

Conclusions
In this study, EL2 was identified as T. ljubarskyi via a molecular approach and morphological identification. Based on the results obtained from solid agar screening, ABTS was found to be the most sensitive substrate for laccase detection, while urea was the best nitrogen source for laccase production. EL2 achieved a maximal dye decolourization rate of 97.92% for RV 5 on the seventh day. Further studies on EL2 laccase-production ability and decolourization of other industrial dyes are suggested to be carried out to enhance our understanding of the potential of isolate EL2 in dye decolourization.

Disclosure statement
No potential conflict of interest was reported by the authors.

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