

Isolation and Screening of Laccase Producing Basidiomycetes via Submerged Fermentations

Mun Yee Chan, Sin Ming Goh, Lisa Gaik Ai Ong

Abstract—Approximately 10,000 different types of dyes and pigments are being used in various industrial applications yearly, which include the textile and printing industries. However, these dyes are difficult to degrade naturally once they enter the aquatic system. Their high persistency in natural environment poses a potential health hazard to all form of life. Hence, there is a need for alternative dye removal strategy in the environment via bioremediation. In this study, fungi laccase is investigated via commercial agar dyes plates and submerged fermentation to explore the application of fungi laccase in textile dye wastewater treatment. Two locally isolated basidiomycetes were screened for laccase activity using media added with commercial dyes such as 2, 2-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid (ABTS), guaiacol and Remazol Brilliant Blue R (RBBR). Isolate TBB3 (1.70±0.06) and EL2 (1.78±0.08) gave the highest results for ABTS plates with the appearance of greenish halo on around the isolates. Submerged fermentation performed on Isolate TBB3 with the productivity 3.9067 U/ml/day, whereas the laccase activity for Isolate EL2 was much lower (0.2097 U/ml/day). As isolate TBB3 showed higher laccase production, it was subjected to molecular characterization by DNA isolation, PCR amplification and sequencing of ITS region of nuclear ribosomal DNA. After being compared with other sequences in National Center for Biotechnology Information (NCBI database), isolate TBB3 is probably from species *Trametes hirsutei*. Further research work can be performed on this isolate by upscale the production of laccase in order to meet the demands of the requirement for higher enzyme titer for the bioremediation of textile dyes.

Keywords—Bioremediation, dyes, fermentation, laccase.

I. INTRODUCTION

IN textiles industry, the most used colorants are known as azo dyes. Azo dyes represent 70% of the total amount of dyes produced annually because of its ease and cost effectiveness in synthesis. Hence, it constitutes the predominant colorants in textiles wastewater [1]. Azo dyes are characterized by the presence of nitrogen-nitrogen (N=N) in their chemical structure. Azo dyes can be divided into different subclasses because many different structures are possible in azo dyes, such as mono-, di- and tri-azo dyes [2]. Most of the azo dyes have azo groups that linked to benzene or naphthalene rings, as well as to aromatic heterocyclic or enolizable aliphatic groups too [2].

Generally, the textiles wastewater consists of about 10 % of dyes that escape from dyeing processes. The textiles dyes, especially azo dyes, are recalcitrant since they are designed to be resistant to light, water and oxidizing agents. Pang and Abdullah [3] stated that once discharged, the azo dyes in the

textile wastewater have high possibility to undergo reduction. The cleavage of the azo linkage that will leads to formation of aromatic amines, known as mutagens and carcinogens. Discharge of dye wastewater into environment will also leads to colorization of water sources, alteration of water pH, increment of chemical oxygen demand (COD) and biochemical demand.

Basidiomycetes, also known as wood white rot fungi have been studied for their ability to decolorize several dyes. This indicates that their potential to be used in textile wastewater treatment [4]. According to [5], extracellular ligninase, especially laccase is found to be the most efficient enzyme that is able to degrade azo dyes [6]. Laccase decolorize azo dyes through a highly nonspecific free radical mechanism forming phenolic compounds, thereby avoiding the formation of aromatic amine. Fermentation is suggested to improve the enzyme production [7]. Basidiomycetes constitutively produce low concentration of laccase when they are cultivated in submerged culture or on wood. With regard to this, a preliminary study was conducted on two locally isolated basidiomycetes for their laccase activity via dye plate screening and submerged fermentation.

II. MATERIALS AND METHODS

A. Sample Isolation, Cultivation and Primary Screening

The fungal fruiting bodies were collected from decaying woods and tree trunks from local environment. A small portion of the cap fresh was cut out and inoculated on malt extract agar (MEA) and incubated at 30°C. The pure culture of the fungal was preserved on MEA slant and store at 4°C. Primary screenings of the fungal isolates were performed on the agar plates supplemented with ABTS [8] guaiacol, and Ramazol Brilliant Blue R (RBBR) [9] dyes as substrates. The plates were incubated at 28°C. The diameters of the colour halo zone were measured and recorded after one week [8]. Each sample was screened in triplicate. The ratio of the decolorization zone of the sample were determined by using the formula [10]:

$$\text{Ratio of decolorization zone} = \frac{\text{diameter of colour halo}}{\text{diameter of mycelia}}$$

B. Submerged Fermentation

Secondary screening of fungal isolates was done by submerged fermentation. Four fungal discs from each isolate were inoculated into modified Mineral Salts Broth (MSB)

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containing KH_2PO_4 2 g, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.5 g, $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ 0.1 g, glucose 2 g, ammonium tartrate 0.2 g, thiamine hydrochloride 10 mg and trace elements solution 10 ml. Trace elements solution: nitrilotriacetic acid 1.5 g, $\text{MnSO}_4 \cdot \text{H}_2\text{O}$ 0.48 g, NaCl 1 g, $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ 10 mg, $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ 10 mg, $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ 10 mg, $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ 8 mg, H_3BO_3 8 mg and $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$ 8 mg [11]. Four mycelia discs were inoculated into the culture medium. The culture flasks were incubated at 30°C for 20 days on a shaking incubator at 140 rpm. One mL of sample was harvested from the culture flask daily and centrifuge at 13,200 rpm for 5 minutes to obtain supernatant [12].

C. Laccase and DNS Assay

One hundred μL of 20 mM ABTS solution was mixed with 870 μL of 0.1 M sodium acetate buffer (pH4). After incubated in water bath at 30°C for 10 minutes, 20 μL of the enzyme extract was added. The absorbance was read by UV-spectrophotometer at 420 nm after 3 minutes. One unit of enzyme activity is defined as the amount of enzymes that oxidizes 1 μmol of ABTS per minutes [12].

DNS assay was used to measure the glucose concentration in the extract from fermented culture. One mL of the extract was mixed with 1 mL of DNS solution. Upon the addition of a few drops of 1 N NaOH, the mixture was kept in water bath for 5 minutes. After 20 minutes, the absorbance of the mixture was read at 540 nm. A standard curve was drawn by plotting the absorbance reading of each standard versus concentration. The glucose concentration of the extract was determined by using the standard curve [13].

D. Molecular Characterization

Comparison of the internal transcribed spacer (ITS) ribosomal DNA (rDNA) gene sequence was employed for molecular characterization. Genomic DNA of the fungal isolates was extracted by using fungi genomic DNA isolation kit. The gene encoding ITS region was amplified by the polymerase chain reaction (PCR). After purification of amplified products by agarose gel electrophoresis, the amplified products were sent for sequencing with ITS 1 and ITS 4 primers. The most homologous sequence was determined by comparison to GeneBank database using BLAST software. Genetic distance and neighbor-joining algorithm is analyzed by using MEGA 5 software [10], [14].

III. RESULTS AND DISCUSSION

Isolates TBB3 (Fig. 2) and EL2 (Fig. 1) showed positive reactions on all the indicator plates tested. In the initial screening, ABTS oxidation was observed clearly, with the ratio of decolorization zone for Isolate TBB3 at 1.70 ± 0.06 while Isolate EL2 at 1.78 ± 0.08 (Table I). Laccase can oxidizes model lignin compounds, given the appropriate primary substrates such as 2, 2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) is present [15]. Both isolates are qualified as a high extracellular ABTS oxidizing activity producer as the dark green coloration appeared in the first week of incubation and the ratio [diameter of the halo/diameter of the colony] was greater than 1 [8].

Positive guaiacol oxidation was confirmed by the formation of reddish-brown halo zone around the microbial colony, while colorless halo zone was observed for RBBR decolorization [16]. Based on Table I, guaiacol was more sensitive than RBBR [9]. ABTS is the best detector as compared to RBBR and guaiacol in ligninolytic enzymes activity. This was in agreement with [17], where ABTS is stated as a very sensitive substrate in detection of extracellular ABTS-oxidising enzymes compared to guaiacol.

TABLE I
RATIO OF DECOLOURIZATION ZONE

	ABTS	Guaiacol	RBBR
TBB3	1.70 ± 0.06	0.70 ± 0.15	0.62 ± 0.20
EL2	1.78 ± 0.08	1.06 ± 0.02	0.73 ± 0.11

Ligninolytic enzymes produced by fungi, which include laccase, lignin peroxidase and manganese peroxidase, is being referred as lignin-modifying enzymes (LMEs) [18]. Since 3 types of screening solid media are without the presence of hydrogen peroxidase (H_2O_2), thus the dyes decolorizing was attributed to the laccase activity [19].

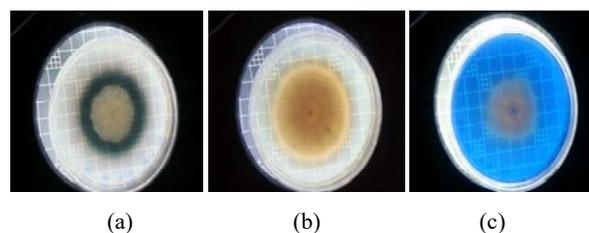


Fig. 1 The positive result of each dye decoloration of Isolate EL2; (a) ABTS plate (b) Guaiacol plate and (c) RBBR plate

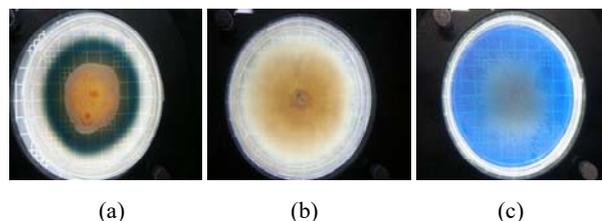


Fig. 2 The positive result of each dye decoloration of Isolate TBB3; (a) ABTS plate (b) Guaiacol plate and (c) RBBR plate

Comparatively, although Isolate EL2 displayed higher values of dye decolorization rate on all 3 types of plates, Isolate TBB3 secreted higher amounts of laccase enzyme. Laccase production for both isolates were highest on Day 17 (3.91 U/ml/day) and Day 13 (0.251 U/ml/day) for Isolate TBB3 (Fig. 3) and EL2 (Fig. 4), respectively.

Laccase is being produced as secondary metabolite during secondary metabolism [20]. It is only being produced when nutrient depletion in order to prevent self poisoning with the accumulation of primary compounds. Besides, secondary metabolites might have other functions, such as ecological function [21], [22]. In this study, the secretion of laccase from Isolate TBB3 started to increase significantly from Day 10 (19.20 U/mL) to the highest on Day 17 (66.41 U/ml).

Production of laccase from Isolate EL2 was much lower, with the maximum laccase secretion observed on Day 13 (3.27 U/ml).

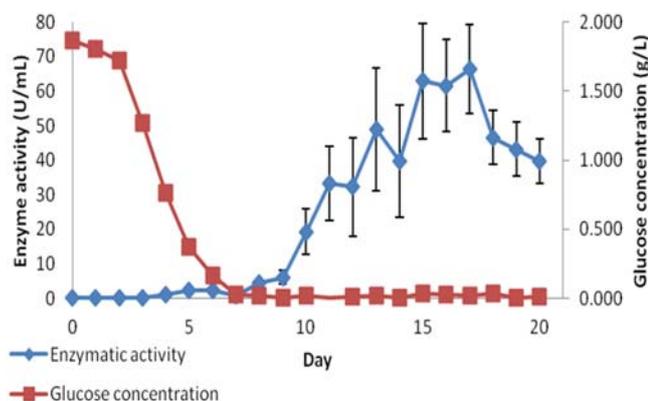


Fig. 3 Laccase production of Isolate TBB3

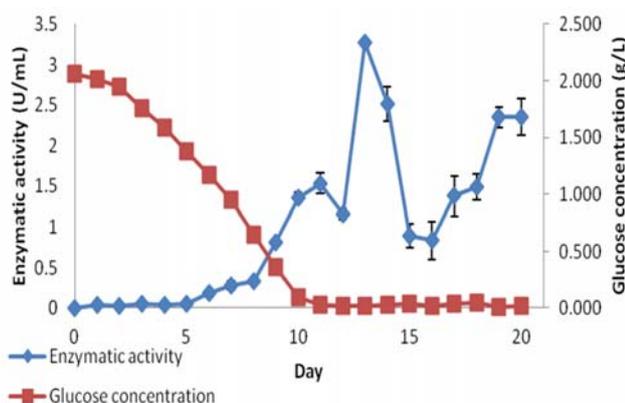


Fig. 4 Laccase production of Isolate EL2

Nitrogen sources and concentration was one of the culture condition factors in laccase production. In this study, 0.2 g of ammonium tartrate was used for submerged fermentation medium. Gold and Alic [23] showed that laccase was produced when nitrogen level is limited. Carbon source in the medium will also influence laccase production. High concentration may suppress laccase production [24], however [9] stated that 10 g/l was the highest laccase production concentration for their sample.

The glucose consumption test showed that laccase was only produced by Isolate TBB3 when the glucose concentration was depleting. The same situation was noticed for Isolate EL2. This was found in agreement with [20] which stated that ligninolytic enzyme will be synthesized when carbon source is limited.

Based on the higher results of laccase production by submerged fermentation, we further identify Isolate TBB3 by Polymerase Chain Reaction (PCR). The Internal Transcribed Spacer (ITS) regions nested among the 18S (small subunit) rRNA gene, 5.8S rDNA gene, and 28S (large subunit) rRNA gene were amplified using ITS-1 and ITS-4 primers. Polymerase Chain Reaction (PCR) targeting the Internal Transcribed Spacer (ITS) regions of fungal ribosomal DNA was done using universal primers ITS-1 and ITS-4 followed by

DNA sequence analysis. Amplicon size of Isolate TBB3 was estimated to be 650 bp (Fig. 5) and sequencing result confirmed this isolate are under the genus of *Trametes*, probably from species *T. hirsute*. Isolate TBB3 can be the target for further research on upscale production of laccase for bioremediation purposes.

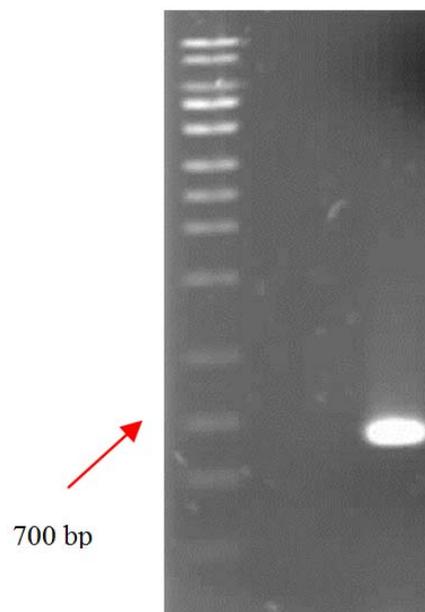


Fig. 5 PCR gel picture of Isolate TBB3

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REFERENCES

- [1] L., Mendoza, "Laccases from new fungal sources and some promising applications", PhD., Lunk University, 2011.
- [2] H. B. Bhimani, "Bacterial degradation of azo dyes and its derivatives", PhD. Saurashtra University, 2011.
- [3] Y. L. Pang, and A. Z. Abdullah, "Current status of textile industry wastewater management and research progress in Malaysia: A Review", *Clean-Soil, Air, Water*, vol. 41, no. 8, pp. 751-764, 2012.
- [4] D. Balan, and R. Monteiro, "Decolorization of textile indigo dye by ligninolytic fungi", *J. of Biotech.*, vol. 89, pp. 141-145, 2001.
- [5] G. Sumbali, "The Fungi", 2nd ed., USA: Alpha Science Intl Ltd., 2011.
- [6] F. Patrick, G. Mtui, A. M. Mshandete, and A. Kivaisi, "Optimization of laccase and manganese peroxidase production in submerged culture of *Pleurotus sajorajau*", *African J. of Biotech.*, vol. 10, no. 50, pp. 10166-10177, 2011.
- [7] M. Narkhede, R. Mahajan, and K. Narkhede, "Ligninolytic enzyme production and Remazol Brilliant Blue R (RBBR) decolorization by a newly isolated white rot fungus: Basidiomycota spp. L-168.", *Int. J. of Pharma and Bio Sciences*, vol. 4, no. 1, pp. 220-228, 2013.
- [8] A. Dhoubi, M. Hamza, H. Zouari, T. Mechichi, R. H'midi, M. Labat, M.J. Martinez, and S. Sayadi, Autochthonous fungal strains with high ligninolytic activities from Tunisian biotopes, *African J. of Biotech.*, vol. 4, no. 5, pp. 431-436, 2005.
- [9] L.L. Kiiskinen, M. Ratto, and K. Kruus, Screening for novel laccase-producing microbes, *J. of App.Microbiol.*, vol. 97, pp.640-646, 2004.
- [10] G. R. Jebapriya, and J. J. Gnanadoss, "Screening and molecular characterization of white rot fungi capable of laccase production and dye

- decolourization”, *Int. J. of Life Science & Pharma Research*, vol. 4, no. 2, pp. 12-20, 2014.
- [11] M. Chander, and D. S. Arora, “Evaluation of some white-rot fungi for their potential to decolourise industrial dyes”, *Dyes and Pigments*, vol. 72, no. 2, pp. 192–198, 2007.
- [12] D. Jing, “Improving the simultaneous production of laccase and lignin peroxidase from *Streptomyces lavendulae* by medium optimization”, *Bioresource Tech.*, vol. 101, pp. 7592-7597, 2010.
- [13] Y. Y Sim, “Molecular identification of *Schizophyllum commune* and its production of Schizophyllan”, BSc. University Tunku Abdul Rahman, 2014.
- [14] D. Lii, S. Coloe, R. Baird, and J. Pederson, “Rapid mini preparation of fungal DNA for PCR”, *J. of Clin. Microbiol.*, vol. 38, no. 1, pp. 471, 2000.
- [15] G. M. Soares, M. T. de Amorim, and M. Costa-Ferreira, “Use of laccase together with redox mediators to decolourize Remazol Brilliant Blue R”, *J. Biotechnol.*, vol. 89, pp. 123–129, 2001.
- [16] F. Patrick, G. Mtui, A. M. Mshandete, and A. Kivaisi, “Optimized production of lignin peroxidase, manganese peroxidase and laccase in submerged cultures of *Trametes trogii* using various growth media compositions”, *Tanz. J. Sci.*, vol 36, pp 1-18, 2010.
- [17] E. Kalmış, İ. Yaşa, F. Kalyoncu, B. Pazarbaşı, and A. Koçyiğit, “Ligninolytic enzyme activities in mycelium of some wild and commercial mushrooms”, *Afr. J. of Biochem. Research*, vol. 7, no. 23, pp. 4313-4320, 2008.
- [18] C. M. Maganhotto de Souza Silva, I. Soares de Melo, and P. Roberto de Oliveira, “Ligninolytic enzyme production by *Ganoderma* spp.”, *Enz. and Microbiol. Tech.*, vol. 37, pp. 324-329, 2004.
- [19] E. Gomes, A. P. Aguiar, C. C. Carvalho, M. R. B. Bonfá, R. D. Silva, and M. Boscolo, “Ligninases production by basidiomycetes strains on ligninocellulosic agricultural residues and their application in the decolorization of synthetic dyes”, *Brazilian J. of Microbiol.*, vol. 40, pp. 31-39, 2009.
- [20] E. Erden, M. C. Ucar, Tekin Gezer, and N. K. Pazarlioglu, “Screening for ligninolytic enzymes from autochthonous fungi and applications for decolorization of remazole marine blue”, *Brazilian J. of Microbiol.*, vol. 40, pp. 346-353, 2009.
- [21] H. W. Heldt, “Plant Biochemistry”, 3rd ed., Elsevier Academic Press, 2005.
- [22] E. M. Fox, and B. J. Howlett, “Secondary metabolism: regulation and role in fungal biology”, *Curr. Opinion in Microbiol.*, vol. 11, pp. 481-487, 2008.
- [23] M. H. Gold, and H. Alic, “Molecular biology of the lignin-degrading basidiomycete *Phanerochaete chrysosporium*”, *Microbiol. Rev.*, vol. 57, no. 3, pp. 605-622, 1993.
- [24] C. Eggert, U. Temp, and K. E. L. Eriksson, “The ligninolytic system of the white rot fungus *Pynoporus cinnabarinus*: purification and characterization of the laccase”, *App. and Env. Microbiol.*, vol. 62 no. 4, pp. 1151-1158, 1995.