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Screening and identification of *Trametes lactinea* and *Pycnoporous coccineus* isolated from Kampar for laccase production

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ABSTRACT

Aims: Laccase is a blue copper oxidase that catalyses four electron reduction of molecular oxygen to water. It is able to oxidise aromatic compounds with molecular oxygen as the terminal electron acceptor. The aim of this study was to screen for laccase producing basidiomycetes isolated from decaying woods and tree trunks around Kampar, Perak. **Methodology and results:** The isolated basidiomycetes were screened for their laccase activity on different agar plates supplemented with 2, 2-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS), guaiacol and Remazol Brillant Blue R (RBBR), respectively. In the presence of laccase, the colourless ABTS and guaiacol were oxidised to form blue-green and reddish-brown coloured zone around the fungal colony, respectively; whereas the blue RBBR was decolourised by the enzyme. Colour or colourless halo zones that are formed on the agar plates indicate the presence of ligninolytic enzyme activities. Isolates KA1 and TR9 indicated the highest enzymatic hydrolysis on ABTS plates with the halo zone ratio of 1.43 ± 0.04 and 0.98 ± 0.01, respectively. Based on the BLAST results from the amplicon of ITS1 and ITS4 primers, Isolates KA1 and TR9 were identified as *Trametes lactinea* and *Pycnoporous coccineus*, respectively. Under submerged fermentation, *P. coccineus* has higher laccase production (0.72 U/mL) compared with *T. lactinea* (0.16 U/mL).
Conclusion, significance and impact of study: Both *T. lactinea* and *P. coccineus* are potential strains for laccase production which can be used for dye decolourisation and degradation. Future studies will focus on the application of the laccase in textile dye degradation.

Keywords: ligninolytic enzymes, internal transcribed spacer, textile dye

INTRODUCTION

There are more than 3000 azo dyes widely used in different manufacturing industries, including textile, leather, cosmetics, food colouring and paper production industries. About 80% of these dyes are used for dyeing in the textile industries (Asad *et al.*, 2007). It was reported 10 to 15% of azo dyes were lost in effluent during dyeing process (Saharan and Ranga, 2011). Through mineralisation, these dyes can be broken into aromatic amines which are suspected to be carcinogenic (Sudha *et al.*, 2014). This increases the probability of serious environmental pollution and health-risk issues due to the application of azo dyes in textile dyeing process.

Textile industries have significantly contribute to the economy of Malaysia. However, it does bring some significant negative impacts to the environment. Textile dyes that are discharged from factories have caused serious pollutions to the environment and human health. Hence, proper management of textile wastewater has been a major concern.

Small scale industries in Malaysia, such as the batik industry are unable to apply expensive textile wastewater treatment (Pang and Abdullah, 2012). It is necessary for alternative dye removal strategy that is more effective, affordable and environmental friendly. Biodegradation of azo dyes by fungal laccase is one of the best alternatives to detoxify the recalcitrant compounds in the textile wastewater (Sudha *et al.*, 2014). The ability of fungal laccases in decolourising azo dyes has prompted the idea of employing the enzymes in textile wastewater treatments.

The white rot fungi, under basidiomycetes phyla have been reported as potential bioremediation agents in azo dye decolourisation. This is due to their ability to produce ligninolytic enzymes, mainly laccase, manganese peroxidase and lignin peroxidase to decay the lignocellulosic residue present in the woody plants (Patrick *et al.*, 2011; Manikandan and Shoba, 2013). Among these enzymes, laccase is found to be the most efficient enzyme that is able to degrade azo dyes (Patrick *et al.*, 2011).

Laccase producing fungi can be screened on solid agar media containing coloured indicator compounds that enable the detection of laccase activity. Common substances that are used to detect laccase activity are guaiacol, ABTS, syringaldazine and polymeric dyes such

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as Remazol Brilliant Blue-R (RBBR) (Kumar *et al.*, 2011). The aims of this study were to screen for laccase producing basidiomycetes isolated from Kampar, Perak by plate screening method, identified the isolated basidiomycetes and production of laccase using submerged fermentation.

MATERIALS AND METHODS

Sample collection and isolation of fungi

The fungal fruiting bodies were collected from the decaying woods and tree trunks around Kampar, Perak. Fresh fruiting bodies were subjected to surface sterilisation using 75% ethanol and thoroughly washed with sterilised water. A small portion of the fruiting body was cut and cultured on malt extract agar (MEA) at 30 °C. The pure cultures of the fungi were preserved on MEA slants and stored at 4 °C (Jebapriya and Gnanadoss, 2014).

Screening of laccase activity

MEA plates supplemented with ABTS, guaiacol and RBBR dyes as substrates were used to screen for the laccase of the isolates (Ang, *et al.*, 2010; Jebapriya and Gnanadoss, 2014). The plates were incubated at 30 °C. The diameters of the fungal colony and decolourisation zone were measured and recorded after one week. Each isolate was screened in triplicate. The ratio of the decolourisation zone of the isolate was determined by using the following formula (Ang, *et al.*, 2010):

$$\text{Ratio of decolorisation zone} = \frac{\text{Diameter of coloured halo}}{\text{Diameter of fungal colony}}$$

Fungi identification

Genomic DNA of the isolates was extracted by using conventional method as described by Liu *et al.* (2000). The gene encoding ITS region was amplified with forward primer ITS-1 (5' TCCGTAGGTGAACCTGCG 3') and reverse primer ITS-4 (5' TCCTCCGCTTATTGATATGC 3'). The PCR profile used for amplification was as following: initial denaturation stage at 94 °C for 5, 35 cycles of amplification stage involving denaturation for 30 sec at 94 °C, annealing for 30 sec at 55 °C, and extension for 30 sec at 72 °C; eventually was the final extension at 72 °C for 5 min (Liu *et al.*, 2000; Jebapriya and Gnanadoss, 2014). After purification, the PCR products were outsourced for sequencing. The most homologous sequences were determined by comparing with GeneBank database using BLAST software. Genetic distance and Neighbor-Joining algorithm was analysed by using MEGA 7 software.

Submerged fermentation

For the production of laccase, the isolates were cultured in Mineral Salt Broth (MSB) under submerged fermentation in a 250 mL conical flask which consists of 4 mycelial discs (5 mm in diameter) in 150 mL of culture medium. The medium consists of: KH_2PO_4, 2 g; $MgSO_4 \cdot 7H_2O$, 0.5 g; $CaCl_2 \cdot 2H_2O$, 0.1 g; glucose, 2 g; ammonium tartrate, 0.2 g; thiamine hydrochloride, 10 mg and trace elements solution, 10 mL. The chemical components in the trace element solution were: nitrilotriacetic acid, 1.5 g; $MnSO_4 \cdot H_2O$, 0.48 g; NaCl, 1 g; $CoCl_2 \cdot 6H_2O$, 10 mg; $FeSO_4 \cdot 7H_2O$, 10 mg; $ZnSO_4 \cdot 7H_2O$, 10 mg; $CuSO_4 \cdot 5H_2O$, 8 mg; H_3BO_3, 8 mg and $Na_2MoO_4 \cdot 2H_2O$, 8 mg (Chander and Arora, 2007). The culture flasks were then incubated at 30 °C for 20 days in a shaking incubator at 140 rpm. 1 mL of sample was collected from each culture flask daily and centrifuged at 13,200 rpm for 5 min to obtain cell free enzyme extracts (CFEE) (Jing, 2010). The experiment was carried out in triplicate.

Laccase assay

Laccase activity of CFEE harvested from the submerged fermentation was determined by the oxidation of ABTS. 100 µL of 20 mM ABTS solution was mixed with 870 µL of 0.1 M sodium acetate buffer (pH 4). After incubating in water bath at 30 °C for 10 min, 20 µL of CFEE was added. The absorbance was read at 420 nm for 2 min. One unit of enzyme activity is defined as the amount of enzymes that oxidises 1 µmol of ABTS per minute (Jing, 2010).

DNS assay

DNS assay was carried out to determine glucose concentration in the CFEE. 1 mL of the CFEE was mixed with 1 mL of DNS solution and a few drops of NaOH. The mixture was then incubated in boiling water bath for 15 min. The absorbance of the reaction mixture was read at 540 nm. A glucose standard curve was constructed with its concentration ranging from 0-2 g/L. The glucose concentration of the CFEE was determined based on the constructed glucose standard curve (Gusakov *et al.*, 2011).

RESULTS AND DISCUSSION

White rot fungi are well-known laccase producers (Madhavi and Lele, 2009). In the present study, in total 10 basidiomycetes fruiting bodies were collected from the environment and only 5 were successfully isolated on MEA agar plates, and were screened for its laccase activity by using different indicator plates. From Table 1, all the isolates gave positive results on ABTS plate with the presence of green halo surrounding the fungal colonies. Only 3 isolates, i.e. isolates TBB5, TR9 and KA1 showed the presence of decolourisation zone on the guaiacol plate. ABTS was the most sensitive indicator for laccase, followed by guaiacol and RBBR dye (Fonseca *et al.*, 2015; Patrick *et al.*, 2011). Isolates TR9, KA1 and KB2 gave positive results on the RBBR plate.

On the other hand, RBBR dye decolourisation was indicated by the appearance of colourless halo zones.

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Table 1: Ratio of decolourisation zones of the isolates.

Isolate	Ratio of decolourisation zones		
	ABTS plate	Guaiacol plate	RBBR plate
KA1	1.43 ± 0.04	0.73 ± 0.00	0.62 ± 0.01
TR9	0.98 ± 0.01	0.80 ± 0.01	0.52 ± 0.15
KB2	1.00 ± 0.00	0.00 ± 0.00	0.84 ± 0.05
TBB4	1.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
TBB5	0.83 ± 0.01	0.70 ± 0.02	0.00 ± 0.0

According to Patrick *et al.* (2011), the formation of coloured halo zone and dye decolourisation were due to the oxidation of the dyes by fungal ligninolytic enzymes produced. Kiiskinen *et al.* (2004) reported that the reaction with guaiacol was well correlated in the reaction with polymeric dyes, including RBBR dye.

Isolates TR9 and KA1 were chosen for further analysis due to they gave positive results in all the laccase screening tests. Basidiocarps of isolate KA1 was thick and spongy with cream white colour and some brown stains on its surface (Figure 1a). Thin and soft basidiocarps with noticeable bright reddish-orange colour can be observed in isolate TR9 (Figure 1b), it was suspect to be from the genus *Pycnoporous* (JGI, 2016). To further identify the species level of isolates TR9 and KA1, the ITS region of these isolates were amplified using primer set, ITS-1 and ITS-4, the PCR amplicon with band size of 650 bp was successfully obtained and sequenced. The amplified DNA sequences were aligned against sequences in GeneBank at National Centre for Biotechnology Information (NCBI). The phylogenetic relationship of these two isolates was inferred using the Neighbor-Joining method as shown in Figures 2 and 3. The Neighbour-Joining trees showed that isolate KA1 is closely related to *T. lactinea* strain BRFM 1119 with 99% homology and 0% gap; therefore it is identified as *T. lactinea*. As for isolate TR9, it is closely related to *P. coccineus* voucher UOC SIGWI-S26 with 99% homology and 0% gap; hence it is identified as *P. coccineus*. The production of laccase was carried out in submerged fermentation by *T. lactinea* and *P. coccineus*, respectively. In this study, the depletion of glucose in the culture medium was measured as a result of the fungus consumed the glucose to support its growth, which can be observed in Figures 4 and 5. Both *T. lactinea* and *P. coccineus* started to utilise the glucose as carbon source to support its growth starting from Day 1 till Day 8. In white rot fungi, the first metabolism generally involved mycelium growth along with easily accessible glucose consumption. When the mycelium growth stops, secondary metabolite will be expressed in response to glucose depletion (Rouches *et al.*, 2016).

This phenomenon can be observed in *P. coccineus* (Figure 4), as it started to produce laccase in Day 6 and the glucose concentration had reduced 86.13% as compare to the initial glucose concentration supplied in the culture medium. Rouches *et al.* (2016) also stated that supplementary of glucose caused a transient repression of ligninolytic enzymes production. Erden *et al.* (2009)

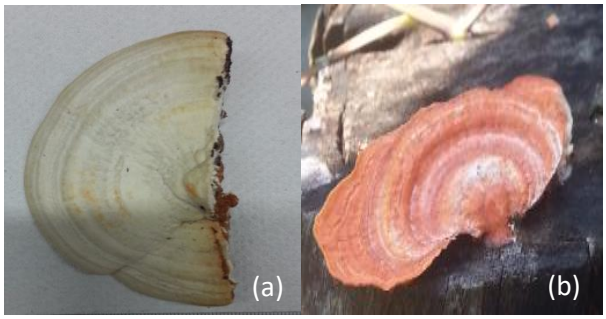

Figure 1: Basidiocarps of isolated fungi. (a) KA1, (b) TR9.

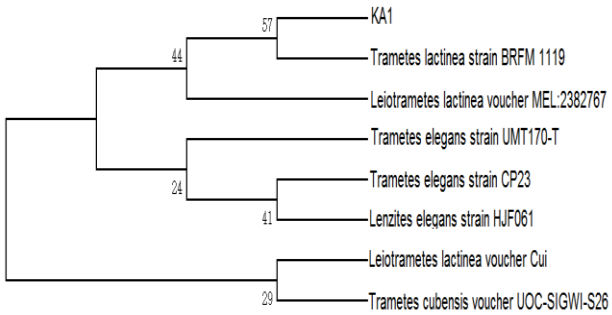

Figure 2: Neighbour-Joining tree of isolate KA1 and its higher similar sequences from the GeneBank selected from the results of BLAST search.

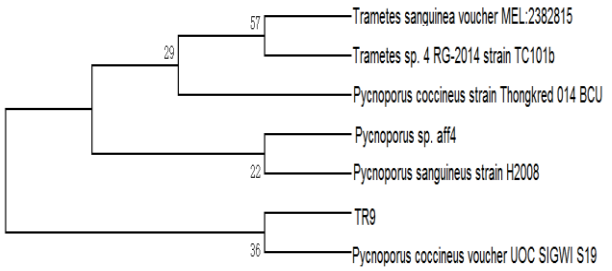

Figure 3: Neighbour-Joining tree of isolate TR9 and its higher similar sequences from the GeneBank selected from the results of BLAST search.

stated that ligninolytic enzymes will only be expressed when carbon source is limited. Besides, this was also in agreement with Rivera-Hoyos *et al.* (2013) that glucose is a typical repressor for laccase expression for many fungal species. With that, peak for laccase production can be observed on Day 7 (0.72 U/mL). Our result showed similarity with findings by Jaouani *et al.* (2005), where highest laccase activity was produced by *P. coccineus* in all their media with different substrates after 7 days of submerged fermentation. Besides, literature also showed that *P. coccineus* was able to produce maximal laccase activity as high as 3.61 U/mL on Day 8 (Thongkred *et al.*, 2011). In accordance with other reports, *P. coccineus* was capable to produce laccase with enzyme activity of 2.95

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U/mL in submerged fermentation with mandarin peels, 0.05 U/mL with tree leaves, 1.42 U/mL with apple peels and 2.62 U/mL with banana peels (Elisashvili *et al.*, 2008; Elisashvili *et al.*, 2011). Laccase production is highly dependent on the cultivation conditions of the fungi. There are several factors that can alter enzyme production, such as type of fungi, the concentration of carbon and nitrogen sources, pH of the medium, temperature, agitation rate, and cultivation period. High nitrogen concentrations with limited carbon or sulphur source are able to activate the ligninolytic enzyme system of white rot fungi. Low carbon to nitrogen ratio is preferred for better laccase production (Kunamneni *et al.*, 2007). However, various media composition, cultivation condition and different activity assay procedures have been used by different group of researchers (Elisashvili and Kachlishvili, 2009).

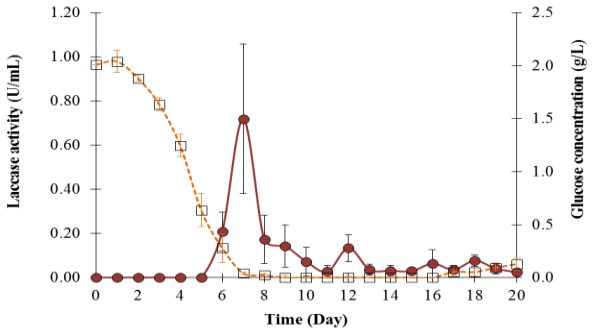

Figure 4: Laccase production and glucose consumption by *P. coccineus* via submerged fermentation for 20 days. •, laccase activity; □, glucose concentration.

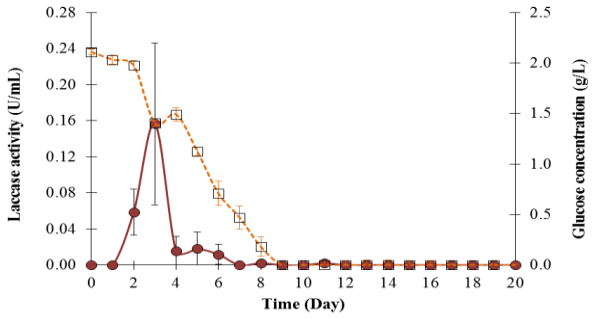

Figure 5: Laccase production and glucose consumption by *T. lactinea* via submerged fermentation for 20 days. •, laccase activity; □, glucose concentration.

CONCLUSION

Among 5 successful isolated basidiomycetes, only isolates KA1 and TR9 gave positive results on all the three indicator plates tested. Based on their morphology identification and molecular identification on the ITS region, isolate KA1 was *T. lactinea* and isolate TR9 was identified as *P. coccineus*. From the submerged fermentation, it shows that there is no direct correlation of the ratio of decolourisation zone with the enzyme activity. This is due to *P. coccineus* produced higher laccase (0.72 U/mL) compare to *T. lactinea* in the submerged fermentation, while, *T. lactinea* has higher values on the ratio of decolourisation for ABTS and RBBR plates, 1.43 ± 0.04 and 0.62±0.01, respectively.

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