Screening for Potential Laccase Producers from *Trichoderma* Strains Isolated From Riau Citrus Rhizosphere and Palm Tree Plant Parts

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Abstract. The enzyme laccase has wide biotechnology applications in the textile, pulp, food and pharmaceutical industry. Fungal laccases have higher redox potential than bacterial laccases, therefore are more preferred for industry. This study aimed to screen for potential laccase producers from several *Trichoderma* strains isolated from citrus rhizosphere and as endophytes of palm trees. Four strains were initially screened in a qualitative colour assay on PDA supplemented with substrate indicators of laccase activity. The qualitative assay showed that *Trichoderma asperelloides* LBKURCC2 demonstrated strong laccase activity on PDA supplemented with guaiacol or 2,2'-azino-di-3-ethylbenzotiazol-6-sulfonate (ABTS). *Trichoderma virens* LBKURCC70, 71 and 85 showed very weak laccase activity on media supplemented with guaiacol, and no detectable laccase on media supplemented with ABTS. Quantitative assays for laccase activity were performed on crude enzyme extracts from solid state fermentation systems with rice straw as the laccase inducer, and the test fungal strain as the prospective producer. From the quantitative assays it was found that *T. asperelloides* LBKURCC2 produced the highest laccase activity (208 ± 76 U/L). Laccase activity produced by *T. virens* LBKURCC70 was 19 ± 5 U/L, while there were no detectable laccase activities produced by *T. virens* LBKURCC71 and 85.

1. Introduction
The oxidoreductase multicopper enzyme, laccase (EC 1.10.3.2) has wide biotechnology applications in the textile [1-2], pulp [3-4], food [5], biofuel [6] and pharmaceutical industry [7]. A wide variety of organisms from bacteria to plants produce laccase. However, since fungal laccases have higher redox potential than the bacterial laccases, and fungi are easy to handle and grow at room temperature, fungal laccases are more preferred in industry than bacterial laccases [8]. Laccases from different fungal species and strains have differences in substrate preference, catalytic activity, response to activators and inhibitors, and stability characteristics, which reflects difference in protein structures [8-9]. Therefore isolation of new fungal strains producing laccase continue to be important, to add to the understanding of structure function relationship of the enzyme, and inventory of fungal laccases to choose from for a certain application.
One fungal genus with potential species and strains that can produce laccase is the ascomycete *Trichoderma*. Members of the *Trichoderma* genus produce a wide variety of hydrolytic enzymes that are used in many industrial processes. Many *Trichoderma* species also can act as biocontrol agents, biofertilizers for promoting plant growth, and are isolated from various environments such as soil and as endophytes [10-11]. A *Trichoderma* strain recently reported to produce laccase is *Trichoderma asperellum* LBKURCC1 [12]. *T. asperellum* LBKURCC1 was previously identified as *Trichoderma harzianum* TNC52, and isolated as a biocontrol strain from cacao rhizosphere soil in Riau [13]. The identification has been corrected to *T. asperellum* LBKURCC1, based on molecular and phylogenetic analysis using ITS rRNA sequences [12]. The finding that a biocontrol strain of *Trichoderma sp.* produces laccase, may be an indication that other biocontrol strains are potential producers of laccase. Another source of laccase may be endophytic *Trichoderma sp.* An example of an endophytic fungi that produces laccase is the endophyte *Myrothecium verrucaria* MD-R-16 isolated from Pigeon Pea [14].

Based on the hypothesis that biocontrol fungi and endophytes may be laccase producers, we aimed to screen a collection of *Trichoderma* sp. strains that were isolated as biocontrol strains from Riau for their ability to produce laccase. One strain was isolated as a biocontrol strain from citrus rhizosphere from a plantation located in Riau. It was formerly identified as *T. viride* TNJ63 [13], but has since been identified based on molecular and phylogenetic analysis using ITS rRNA sequences as *Trichoderma asperelloides* LBKURCC2 [12]. Three other biocontrol strains were isolated as endophytes from palm oil plant parts and identified as *Trichoderma virens* LBKURCC70, LBKURCC71 and LBKURCC85 based on ITS rRNA sequences [15-16]. In this paper we report our results of the qualitative and quantitative screening for laccase production by these four Riau isolate *Trichoderma* biocontrol strains.

2. Methodology

2.1. Qualitative assay for laccase production

Qualitative assay for laccase production was done following the method described by Daasi *et al.* [17] with slight modification. Briefly the procedure is described as follows: Fungi cultures to be tested for their ability to produce laccase where first grown as lawns on Potato Dextrose Agar (PDA) plates. For the assay PDA plates containing 4 mM guaiacol (SIGMA-Aldrich Cat. No. A1888) or 2 mM 2,2'-azino-di-3-ethylbenzotiazol-6-sulfonate (ABTS) (SIGMA-Aldrich Cat. No. G5502) were prepared, without or with addition of Cu$^{2+}$ ions. For experiments where Cu$^{2+}$ ions were added, the plates were supplemented with 0.15 mM CuSO$_4$. Plugs (1 cm in diameter) from fungal culture lawns on PDA plates were transferred aseptically to the center of PDA plates containing the laccase test substrates (guaiacol or ABTS, without or with CuSO$_4$ supplements). The plates were incubated for 3 to 6 days at room temperature (±30°C). The appearance surrounding the fungal colony of a brownish red halo for guaiacol or blue green halo for ABTS, signaled a positive test for laccase production. Results were recorded by digital camera photography.

2.2. Production of Laccase by Solid State Fermentation

For the quantitative assay of laccase production by the fungal strains, the test fungi were grown in a Solid State Fermentation (SSF) system using rice straw as the inducer for laccase production. SSF was done in 500 mL flasks. Prior to fermentation, an inoculum of the test strain was prepared by growing 2 lawn plugs of the test strain in 30 mL of Potato Dextrose Broth (PDB), until the fungi reached its mid-exponential growth phase. The 30 mL inoculum of mid-exponential phase fungi was then added to a prepared SSF system. The prepared SSF system was 40 g of rice straw sterilized together with 30 mL of nutrition supplement media in a 500 mL flask. The nutrition supplement media had the following composition for 1 L media: 23 g Soybean meal, 10 mL glycerol, 1 g Tween-20, 1 g MgSO$_4$, 0.6 g KH$_2$PO$_4$, 1 g (NH$_4$)$_2$SO$_4$, 0.0487 g CuSO$_4$.7H$_2$O. Inoculated SSF systems were incubated at room
temperature (±30°C) for 8 days, with daily shaking of flasks for 1 minute for even distribution of growing mycelia in the media.

2.3. Extraction of Laccase from SSF Systems and Laccase Activity Assay

Enzymes were extracted from the SSF system media on the 8th day of incubation, the crude enzymes washed and concentrated, following the procedure as described by Rahayu et al. [18]. Laccase activity was determined following the procedure described by Agrawal et al. [19], using 5 mM ABTS as laccase substrate, and measuring the absorbance of the enzymatic reaction at 420 nm at times 0, 5 and 10 minutes after reaction started. For every enzymatic reaction, controls were used, comprising ABTS in pH 5.5 0.5 M sodium acetate buffer, and replacing the same volume of enzyme with buffer. The enzyme activity was calculated using equation (1). Where EA is enzyme activity, $A_t$ is absorbance of enzyme reaction at times t, $A_0$ is absorbance of enzyme reaction at times zero, $A_c$ is absorbance of control solution at time t, $A_0c$ is absorbance of control at times zero, $V_{tot}$ is total reaction volume, Df is dilution factor, $\varepsilon_{420nm}$ is the molar extinction coefficient for ABTS at 420 nm which is 36,000 M⁻¹cm⁻¹, $V_{enzyme}$ is the enzyme volume added to the reaction, d is the optical path length, which was 1 cm, and t is reaction time. One unit of laccase activity (U) is the amount of enzyme needed to oxidize 1 µmol of substrate per minute.

$$EA=\frac{(A_t-A_0)-A_0c \times V_{tot} \times Df}{\varepsilon_{420nm} \times V_{enzyme} \times d \times t}$$

(1)

3. Results and Discussion

Four Trichoderma sp. biocontrol strains were qualitative and quantitatively screen for their ability to produce laccase. Figure 1 gives the results on the qualitative assay on PDA indicator plates using guaiacol as a laccase substrate, where a laccase positive result is indicated by a brown reddish halo or area surrounding the fungal colony. Figure 2 gives results on the qualitative assay on PDA indicator plates using ABTS as a laccase substrate, where a laccase positive result is indicated by a dark green to bluish (turquoise) halo or area surrounding the fungal colony.

From Figure 1, it could be clearly seen that T. asperelloides LBKURCC2 gave strong laccase positive signals since day 3 of colony growth on the PDA guaiacol indicator plates without CuSO₄, with halo diameters of 3.8 cm (day 3), and 7 cm (day 6). These strong laccase positive signals became more enhanced on PDA guaiacol indicator plates containing 0.15 mM CuSO₄ supplements, with halo diameters reaching 4.5 cm (day 3) and 9 cm (day 6). Strong laccase positive signals were also seen since day 3, when T. asperelloides LBKURCC2 were grown on PDA ABTS indicator plates (Figure 2). Without CuSO₄ supplements, turquoise halo diameters surrounding T. asperelloides LBKURCC2 was 5.4 cm (day 3), and 9 cm (day 6). With CuSO₄ supplements, the turquoise halo’s diameters were not much different, compared to without CuSO₄, which was 5.6 cm (day 3), and 9 cm (day 6). This indicates that CuSO₄ activation of laccase produced by T. asperelloides LBKURCC2 is dependent on the substrates used, which may reflect slight differences in binding sites of the strains laccase towards guaiacol and ABTS.
Figure 1. Qualitative assay photograph results using guaiacol as the substrate indicator of laccase. Shown are fungal growth on: A. Control PDA plates without indicator; B. PDA plates with 4 mM guaiacol indicator; and C. PDA plates with 4 mM guaiacol indicator and 0.15 mM CuSO₄.

Figure 2. Qualitative assay photograph results using ABTS as the substrate indicator of laccase. Shown are fungal growth on: A. Control PDA plates without indicator; B. PDA plates with 2 mM ABTS indicator; and C. PDA plates with 2 mM ABTS indicator and 0.15 mM CuSO₄.
From Figure 1 and 2, it could also be seen that *T. virens* LBKURCC70, 71 and 85 showed very weak laccase activity on media supplemented with guaiacol, and no detectable laccase on media supplemented with ABTS. Weak laccase activity towards guaiacol was shown by the faint appearance of brownish orange halos only after 6 days growth of *T. virens* LBKURCC70, 71 and 85 on the indicator plates. Due to the weak signals, it was difficult to measure the halo diameters that developed. Dependence of laccase activation by CuSO$_4$ was variable in these strains. On PDA plates supplemented with guaiacol, both *T. virens* LBKURCC70 and 85 gave stronger halo signals in plates without CuSO$_4$, indicating that these strains may be sensitive to high concentrations of CuSO$_4$ which may hamper its growth. On the other hand, *T. virens* LBKURCC71 gave stronger positive laccase signals on the addition of 0.15 mM CuSO$_4$. Although production of laccase requires the presence of Cu$^{2+}$ in its growth media, as laccase is a copper containing enzyme, the amounts may be very minimal, and sufficiently provided by the potato extracts in PDA.

To confirm the qualitative assay, a quantitative assay was performed on enzyme extracts from Solid State Fermentation (SSF) systems of the fungal test strain grown on a synthetic media containing rice straw as laccase inducer. Previous studies using *Trichoderma asperellum* LBKURCC1 showed that rice straw could induce production of laccase [20], so that this was an ideal media to induce production of laccase. The quantitative assay showed confirmed that *T. asperelloides* LBKURCC2 produced laccase extracts with a high average activity of 208 ± 76 U/L. In the same quantitative assay, only *T. virens* LBKURCC70 produce laccase extracts with activity that could be detected, but with an average activity that was tenfold lower that the activity of *T. asperelloides* LBKURCC2 laccase extracts (Table 1).

| Fungal identity | Laccase activity (U/L) |
|-----------------|------------------------|
| *T. asperelloides* LBKURCC2 | 208 ± 76 |
| *T. virens* LBKURCC70 | 19 ± 5 |
| *T. virens* LBKURCC71 | 0 ± 0 |
| *T. virens* LBKURCC85 | 0 ± 0 |

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*T. asperelloides* LBKURCC2 is a strain isolated from rhizosphere soil around citrus trees [12-13], while *T. virens* LBKURCC70 was isolated as an endophyte from the roots of an oil palm tree [21]. These strains may have laccase ability to utilize surrounding lignocellulosic material in the soil. Being an endophyte, regulating sequences for the laccase gene expression in *T. virens* LBKURCC70 may have evolved to produce minimal laccase as not to be harmful to the host root, but enough to utilize surrounding biomass lignocellulose as nutrition for both the endophyte and the host. *T. virens* LBKURCC71 and LBKURCC85, being endophytes that were isolated from the stem of palm trees, may have higher gene suppression or lost a large part of their ability to produce laccase, so as not to harm any part of the host plant stem. The weak laccase production demonstrated qualitatively by *T. virens* LBKURCC71 and LBKURCC85 on PDA media supplemented with guaiacol, is not sufficient for production in an SSF system.

*T. asperelloides* LBKURCC2 maintains high laccase ability, as it is not an endophyte. *T. asperelloides* LBKURCC2 may also have high laccase ability, as part of its defence mechanism against antagonistic organisms in the soil. A biocontrol strain of *T. viride* was also reported to produce laccase as part of its defence mechanism against the antagonistic *Aspergillus ochraceus* [22]. Laccase production by SSF in this study has not been optimized yet. By an unoptimized system *T. asperelloides* LBKURCC2 already produced laccase 4 times higher, than that optimized for *T.
asperellumLBKURCC1. Optimization for laccase production by T. asperellum LBKURCC1 in a SSF system using rice straw only produced laccase with activity of 58 U/L [18].

4. Conclusion
From this study it can be concluded that the qualitative assay using PDA supplemented with laccase substrate indicators such as guaiacol and ABTS to screen for fungal producers of laccase is a good preliminary screen. The method can aid in predicting high to low producers of laccase. T. asperelloides LBKURCC2 is a high laccase producer, while T. virens LBKURCC70 is a low laccase producer. T. virens LBKURCC71 and LBKURCC85 cannot produce laccase in sufficient amounts to be detected in an SSF system with rice straw as the laccase inducer.

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