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Research Article
Laccase Activity of *Trametes versicolor* Using Various Pineapple and Arabica Coffee Wastes Under Solid-State Fermentation Process

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Abstract

Background and Objective: Laccase, a ligninolytic enzyme, can degrade lignin by utilizing lignocellulose. With this ability, laccase is useful as a pre-treatment enzyme of lignocellulosic materials in various industries. This study evaluated the laccase activity produced by *T. versicolor* using pineapple and coffee-derived waste as lignocellulosic-rich substrates using solid fermentation. **Materials and Methods:** This study tested laccase production activities from various pineapple wastes and coffee peels through a solid fermentation process by growing *T. versicolor* on the substrate mixture of Szabo media and pineapple skin or coffee peel. The laccase produced was analyzed for its activity and growth, qualitatively and quantitatively during a 12 days incubation time. The method used was experimental followed by descriptive analysis. **Results:** Various amounts of lignocellulose were obtained among pineapple wastes and coffee peel. Laccase from these substrates was optimally produced on day 6 incubation on Szabo+5 g coffee peel group showed the highest laccase activity levels, following its biomass weight (1949.13 U/L and 3.498 g). The optimum pH for laccase production was reached in the range 4-6. The produced laccase was indicated by the appearance of the blackish-brown zone on PDA agar supplemented with guaiacol. **Conclusion:** Lignocellulosic wastes from pineapple and coffee had the potential as substrates to produce laccase from *T. versicolor* with various activity levels which day 6 incubation showed the most optimum fermentation period.

Key words: Laccase, *Trametes versicolor*, pineapple waste, coffee peel, solid fermentation

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Agriculture commodity productions have given rise, leading to agriculture-by products and waste generations, including rice husks, rice bran, straw, cocoa husk, pineapple-derived waste (crown, leaves, peel and stump) and bagasse, which highly contain lignocellulose. Pineapple production in Indonesia reached 2.89 million tons in 2021, which previously increased by 17.95% to 2.45 million tons[1]. Moreover, pineapple production has been reported as the second largest commodity worldwide, increasing pineapple-derived waste[2]. Besides, Arabica coffee peel production in Indonesia is also abundant which is about 794.8 thousand tons produced every year[1].

The utilization of the waste derived from pineapple and coffee is required to escalate the value in the marketplace, which is applied as substrates for enzyme production in food industries. Based on the Indonesia Ministry of Research and Technology (2017), about 99% of the enzyme supply in Indonesia is still imported from other countries, so autonomous enzyme production is highly required to fulfill the needs in the future[3].

Laccase is an extracellular enzyme that degrades lignin and acts as a biocatalyst for the oxidation of organic and inorganic compounds, including mono-di-and polyphenols, aminophenols, methoxyphenols, substituted phenols, diamines, aromatic amines and ascorbic acid[4,6]. Laccase-based biocatalysts need only oxygen as the final electron receptor and are appropriate for efficient and environment-friendly industries, resulting in widespread applications, ranging from the food industry to the paper and pulp, textile, pharmaceutical and cosmetic industries[7,8]. Laccase is one of the enzymes commonly found in plants and fungi and some bacteria and insects. The development of laccase production using fungi with several divisions from ascomycetes, basidiomycetes and deuteromycetes, such as *Melanocarpus albomyces*, *Cerrena unicolor*, *Magnaporthe grisea* and *Trametes versicolor* has been widely applied with varying degree of purifications[9,11]. However, white-rot basidiomycetes are the most efficient lignin degraders and can produce high-yield laccase with high catalytic activity[12,13].

Trametes versicolor, also known as *Coriolus versicolor*, is a white-rot basidiomycetes that is commonly found in deciduous trees and some conifers, such as firs and pines. The fungi can produce extracellular enzymes, including laccase, manganese peroxidase (MNPs) and lignin peroxidase (LIPs)[14]. In particular, these fungi can produce large amounts of laccase by using affordable substrates, such as plants-derived wastes to increase laccase production of *T. versicolor*[15].

The solid fermentation process uses inert substrates and is suitable for fungi that require less moisture for growing conditions[16]. Compared to submerged fermentation, this technique requires less energy for aeration, as well as media preparation and its process is considered to be simpler.

This study aimed to evaluate the laccase activity produced from *T. versicolor* by utilizing various pineapple and Arabica coffee-derived waste using a solid fermentation process.

MATERIALS AND METHODS

Study area: The experiments were performed from November, 2023 to April, 2024 in the Food Microbiology Laboratory, the Food Chemistry Laboratory, the Department of Food Technology and the Central Laboratory, Padjadjaran University, Sumedang City, Indonesia.

Pineapple and coffee waste preparation: The pineapple waste (*Ananas comosus*) of as much as 5 kg was used with the smooth cayenne variety collected from Subang District, West Java, Indonesia while the Arabica coffee peel (*Coffea arabica*) was collected from Loa Village, Bandung District, Indonesia. *Trametes versicolor* isolate was obtained from the Laboratory of Microbiology and Bioprocess Technology, Chemical Engineering, Bandung Institute of Technology, Indonesia.

The procedure begins with pre-treating the raw material by pulverizing it. The next step was to dry the raw material in an oven (Esco Lifesciences Ltd., Singapore) at a temperature of 50°C for 72 hrs. After drying, it was pulverized using a grinder (B-One, Tangerang, Indonesia) and sieved through a 60-80 mesh sieve.

Lignocellulose content: The lignocellulose testing is performed by the van Soest *et al.*[18] using ADS (Acid Detergent Solution) and NDS (Neutral Detergent Solution) detergents. The calculation of the lignocellulose content is done as follows:

$$\text{ADF content (\%)} = \frac{(c + b) - b}{a} \times 100$$

$$\text{NDF content (\%)} = \frac{(c + b) - b}{a} \times 100$$

$$\text{Lignin content (\%)} = \frac{d - e}{a} \times 100$$

Cellulose content (%) = ADF-insoluble ash-lignin

Hemicellulose content (%) = NDF-ADF

a = Waste sample weight
b = Sintered glass weight
c = Dry residue weight
d = Constant weight residue after oven
e = Ash content weight

Qualitative laccase enzyme: The qualitative laccase enzyme produced by *T. versicolor* was conducted by using the color indicator, guaiacol, to detect the presence of laccase in the fungi. Qualitative tests were performed by subculturing mycelia on PDA media treated with 0.02% guaiacol. Fungal mycelia were placed at 5 points in a Petri dish, 4 points on the periphery and 1 point in the center. Duplicated samples were incubated at 30°C for 3 days.

Laccase enzyme production: This study used a solid fermentation process and applied a modified media in the previous study reported by Szabo *et al.*[17] consisting of (sulfuric acid pH 5 g/L). A NH_4NO_3 (3 g), $(NH_4)^2$ HPO_4 (3 g), KH_2PO_4 (5 g), $MgSO_4{\cdot}7H_2O$ (0.5 g), NaCl (0.5 g), $CaCO_3$ (0.5 g), $FeSO_4{\cdot}7H_2O$ (0.35 g) and dan $CuSO_4{\cdot}5H_2O$ (0.6 g). After the fermentation medium was filled into an Erlenmeyer flask, sterilized and allowed to reach room temperature, *T. versicolor* was added to the substrate. The inoculum was evenly distributed. Erlenmeyers containing substrate and fungus were then sealed and incubated at room temperature for 12 day.

After the fermentation process was completed, the enzyme was separated from the fermentation medium. Extraction of the laccase enzyme was performed by mixing the fermentation medium with 50 mL of acetate buffer (50 mM, pH 5). The mixture was then placed in a rotary shaker (Esco Lifesciences Ltd., Singapore) at 120 rpm for 60 min. The mixture was then filtered using Whatman No. 1 paper and centrifuged at 9000 rpm for 10 min at 4°C. The supernatant obtained is the crude laccase extract, which is tested for enzyme activity. This procedure refers to the method conducted by Perdani *et al.*[16].

Laccase activity analysis was used to measure the activity of laccase formed in the media. The laccase activity assay was performed by quantification using a spectrophotometer (Infitek, Shandong, China). Laccase activity was determined by mixing 50 µL of enzyme filtrate with 60 µL of acetate buffer 0.5 mM pH 5.0 and 20 µL of ABTS 1 Mm. All solutions were then added to a microtiter plate and the absorbance was calculated at min 0 and min 30 at 30°C. The absorbance was measured at a wavelength of 420 nm. The activity of the laccase enzyme was calculated using the modified van Soest *et al.*[18] method:

$$\text{Enzyme activity (U/mL)} = \frac{(A_t - A_0) \times (V_{tot} \times 10^6)}{V_{enzyme} \times d \times t \times \varepsilon_{max}}$$

Where:

A_t = Absorbance at 30 min
A_0 = Absorbance at 0 min
ε_{max} = ABTS molar absorptivity (36000 M^{-1} cm^{-1})
d = Thick solution (0.357 cm)
t = Time (30 min)
V_{tot} = Volume of total solution (0.13 mL)
V_{enzyme} = Volume of enzyme filtrate (0.05 mL)

This study was conducted with an experimental method followed by descriptive analysis. Each treatment was carried out in duplicate.

RESULTS

Lignocellulose content: The lignocellulose contents in the substrates were the indicator of laccase enzyme production levels. Figure 1 shows the lignocellulose contents varied among three different substrates. The pineapple peel contained hemicellulose 25.178%, cellulose 20.798% and lignin 7.237%. The pineapple leaves contained hemicellulose 20.278%, cellulose 21.690% and lignin 20.278%. The coffee peel contained hemicellulose 7.562%, cellulose 8.100% and lignin 16.184%.

Qualitative of laccase enzyme: A qualitative test was conducted to determine the production of laccase enzyme by *T. versicolor*. The fungal mycelia of *T. versicolor* were sub-cultured on PDA agar media with guaiacol which is an indicator for laccase production. The results were shown as the presence of blackish brown zone indicated the laccase activity on the plate (red circle, Fig. 2).

pH of laccase: The pH of laccase was measured every other day to calculate its activity and determine the optimum pH for laccase production. The optimum pH was distinctly shown among substrate groups day by day which was in range 4-6 which was the optimum pH for growth of *T. versicolor.* Optimum pH conditions will produce optimum laccase activity (Fig. 3).

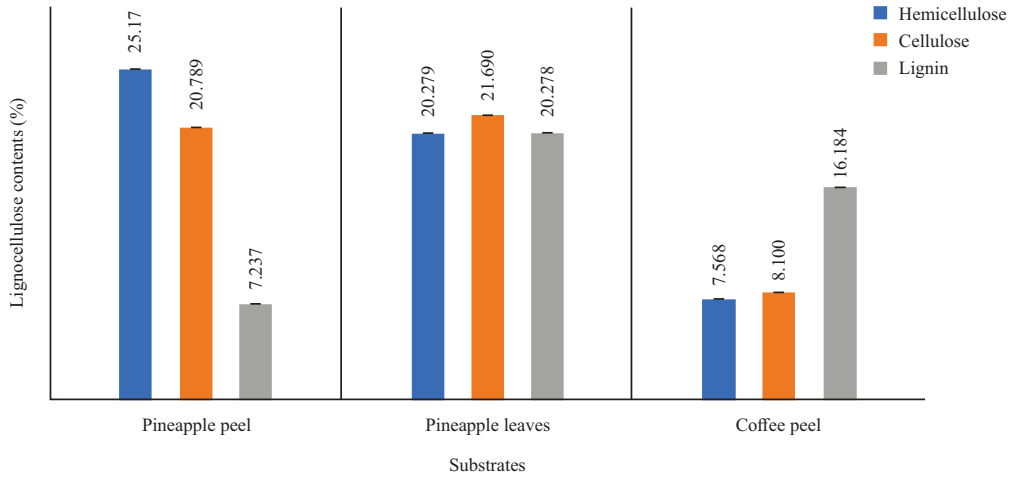

Fig. 1: Lignocellulose contents in various pineapple and coffee-derived wastes

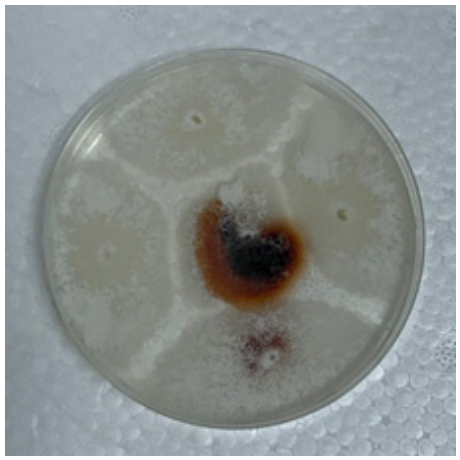

Fig. 2: Blackish brown zone of *T. versicolor* colony on PDA agar media supplemented with guaiacol

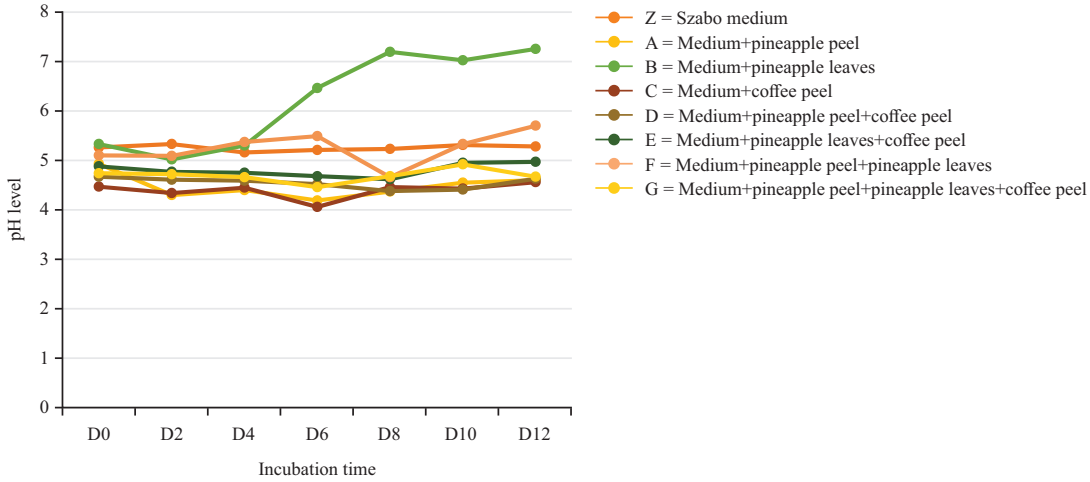

Fig. 3: pH of laccase enzyme using various substrates in solid-state fermentation process

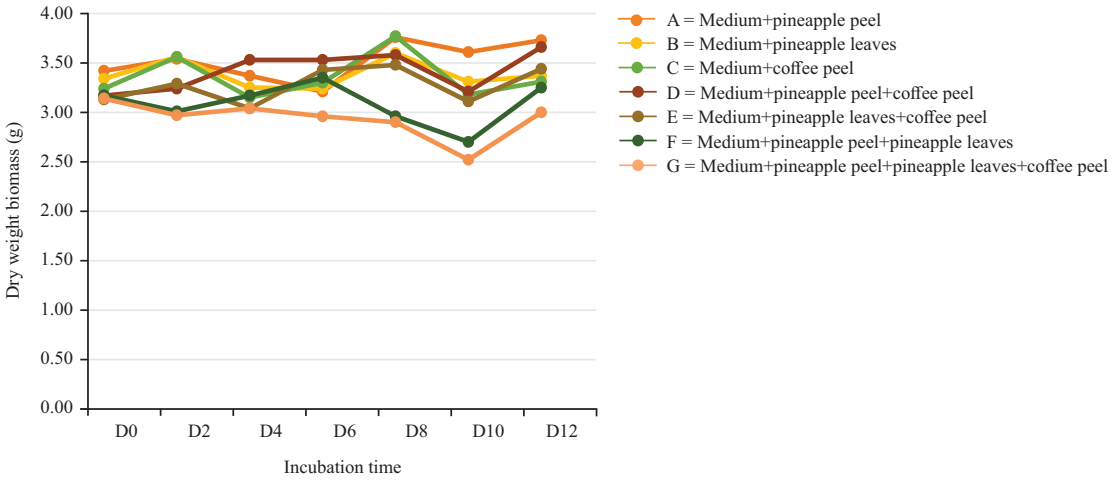

Fig. 4: Dry weight of mold biomass by various substrates during the solid-state fermentation process

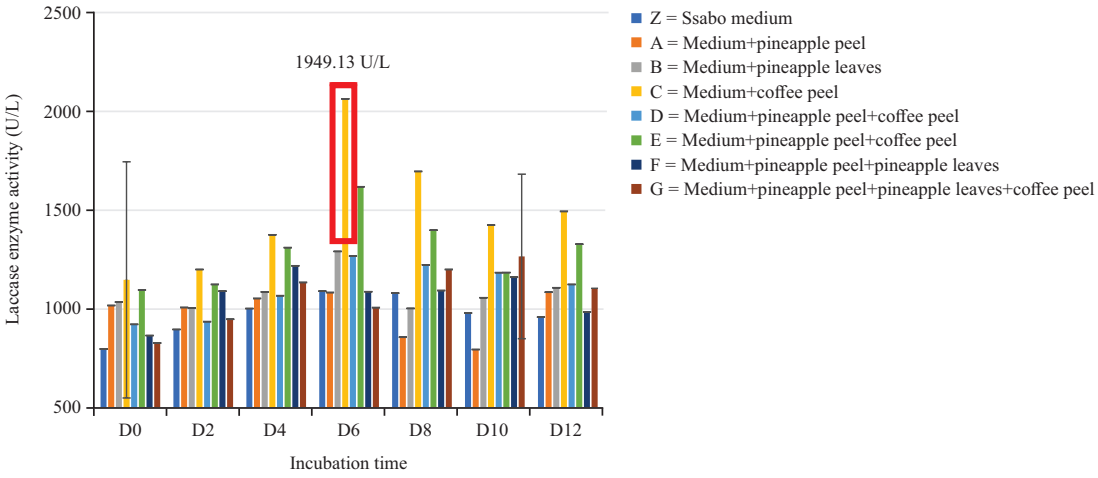

Fig. 5: Levels of laccase activity produced by *T. versicolor* by various substrates using the solid-state fermentation process

Dry weight of biomass: The dry weight of biomass was measured to determine the mold growth levels during the fermentation process. The samples were dried in an oven at 50°C and placed in a desiccator to stabilize the temperature before weighing. Figure 4 showed that substrate group C increased the weight from day 0 and day 6 (3.336 g and 3.498 g, respectively). Meanwhile, substrate group G had the highest weight on the 6th day, 3.625 g. Other substrate groups decreased their weight from day 6 to 12. These results indicated that optimum growth of *T. versicolor* was reached on day 6.

Laccase activity test: The laccase activity test was performed to measure the laccase activity produced by *T. versicolor* in the solid fermentation. The laccase was produced using the solid fermentation method with Szabo media and various combinations of lignocellulosic waste (pineapple leaves, pineapple and coffee peel) as substrates, with a control group which was media only. Figure 5 shows substrate group C contained coffee peel resulting in the highest laccase activity (1949.13 U/L) on day 6 incubation time. In contrast, substrate group A obtained the low est laccase activity, which contained pineapple peel (363.45 U/L) on day 12 incubation time.

DISCUSSION

This study provides an alternative application for pineapple and coffee-derived waste as substrates for optimizing laccase production. These lignocellulosic wastes contain lignin, cellulose and hemicellulose which serve as nutrients in the fermentation process, supporting an effective laccase production[19,20]. Laccase produced by *T. versicolor* in

solid fermentation was measured for its growth and activity. In this study, we obtained substrate group C with the addition of coffee peel provided the highest laccase activity on day 6 (Fig. 5). This result may be caused by the lignin contents in the coffee peel being higher than those in the pineapple peel and pineapple leaves (16.18, 7.56 and 8.10%, respectively) (Fig. 1). These results were also supported by the previous study conducted by Yuliana *et al.*[21] which found that the optimum incubation time for laccase enzyme production was obtained on day 6 incubation. Furthermore, the dry weight of biomass using coffee peel substrate (group C) also increased on day 6 (3.498 g, Fig. 4), suggesting that the optimum laccase production was achieved on day 6.

The qualitative test of laccase was indicated by the appearance of a blackish-brown zone on the sample (Fig. 2) indicating the oxidation process of various substrates catalyzing by laccase by converting oxygen (O_2) to form ketone groups and producing H_2O[22].

The optimum pH for laccase production was obtained in the range of 4-6 (Fig. 3), supported by Asgher *et al.*[23] and Yuliana *et al.*[24] which found that the optimum pH for fungal growth by *T. versicolor*. In addition, results found that pineapple-derived waste had a high pH (substrate group B with pineapple leaves = 6.21) because the presence of weak organic acids and potassium hydroxide in the pineapple can further increase the pH level. In contrast, we obtained a low pH (4.39) in the substrate using coffee peel (group C), which was consistent with previous work conducted by Mussatto *et al.*[25].

The waste utilization as substrates for enzyme production is necessary to fulfill the enzyme's demand as one of the essential components in the food industry. The discoveries in this study provide an initial judgment that wastes derived from pineapple and coffee which are highly produced in Indonesia are potentially utilized as substrates to produce enzymes by using a solid-state fermentation process. However, this study still needs further steps on laccase purification, which is then followed by the analysis of laccase characterization and its kinetic activity.

CONCLUSION

The lignocellulosic substrates composed of coffee peel, pineapple peel and pineapple leaves, can support the optimization of laccase activity produced by *T. versicolor*. We obtained that coffee peel provided the highest laccase activity (1949.13 U/L) with a biomass of 3.498 g and a pH of 4.39. For further confirmation, it is necessary to conduct some analysis related to enzyme characterization and its activity as catalysis in specific reactions that occur frequently in the food industry, so that we can broaden the functions of the produced enzyme, specifically for the improvement of food products.

SIGNIFICANCE STATEMENT

This study focuses on optimizing laccase production by utilizing the lignocellulosic substrates which are highly found in agricultural waste, such as pineapple and coffee-derived waste. A solid fermentation process was conducted using *T. versicolor*. This study was focused on the characterizations of the produced laccase by evaluating its growth and activity. Hopefully, the findings of this study can be used as an alternative solution to improve laccase production and its application in various industries, particularly in the food sector.

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