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Research Article
Potential of Lignocellulosic Waste for Laccase Production by *Trametes versicolor* under Submerged Fermentation

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Abstract

Background and Objective: Laccase is one of the ligninolytic enzymes classified as a multicopper oxidoreductase group that has the ability in oxidizing phenolic compounds and has widespread use in both food and non-food industries. This enzyme is extracellularly secreted by white-rot fungi, *Trametes versicolor* (L.) Lloyd in the media containing lignocellulose, for example, kapok banana peels and sawdust. The objective of this study was to evaluate lignocellulosic substrate that able to produce the highest activity of the laccase from the *T. versicolor* (L.) Lloyd. **Materials and Methods:** Three substrate variations used in this work included the cultivation media with the addition of either kapok banana peels or sawdust and without using both materials. The inducer ($CuSO_4$) was added to each substrate variation and the laccase activity was subsequently measured. **Results:** The qualitative test result for laccase detection showed that *T. versicolor* (L.) Lloyd was able to produce this enzyme indicated with a reddish-brown surrounding fungal colony. The fungi cultivated in media with the content of sawdust and 1 mM $CuSO_4$ yielded the highest laccase activity, reaching 573.6 U L^{-1} with an OD value of 0.5567 and a pH of 5.3 after 7 days of incubation. Meanwhile, the addition of kepok banana peels and 1 mM $CuSO_4$, showed the maximum laccase activity (297.7 U L^{-1}) with the OD value of 0.6932 and a pH of 5 after incubation for 6 days. **Conclusion:** The white-rot fungi of *T. versicolor* (L.) Lloyd could produce optimal laccase activity by adding sawdust substrate and 1 mM $CuSO_4$ inducer on submerged fermentation.

Key words: Laccase, lignocellulosic waste, *T. versicolor* (L.) Lloyd., white-rot fungi, sawdust, submerged fermentation, oxidizing phenolic compounds

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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

Laccase is extracellular enzymes predominantly found in various types of fungi, insects, bacteria and higher plants[1]. The use of laccase has been extensively applied in both food and non-food industries. In the non-food industries, laccase is broadly used in the bleaching process of pulp, bioethanol production and decomposition of textile factory wastes[2-4]. On the other hand, the fruit juice, wine and bakery industries, widely employed laccase for decolourization, the undesirable colour removal process of food and beverage to enhance the appearance of products[5].

Fungi are naturally capable of synthesizing laccases inside the cell and secreting this enzyme outside the cell for plant lignin degradation. The free-lignin plant facilitates other enzymes of these fungi such as cellulase to degrade cellulose and acquire glucose as fungi nutrients. Hence, the preferred source of industrial laccase production originates from fungi. White-rot fungi are the best-known laccase producer. One of the potential strains from this fungi type is *Trametes versicolor*[6,7]. According to Tišma *et al*.[8], *T. versicolor* had a higher laccase activity of 2.378 U $dm^{3\ -1}$ after improved by the substrate using waste from the paper industry containing microparticles of $CaCO_3$. This substrate stimulates the formation of freely dispersed mycelium and laccase production during submerged cultivation of *T. versicolor*.

The ability of *T. versicolor* fungus to produce laccase is strongly influenced by the production media. Lignocellulosic waste obtained from agricultural and agro-industrial activity can be used as a substrate for laccase production. Lignocellulosic wastes contain lignin, cellulose and hemicellulose insufficient amounts, so these materials can be used as a natural inducer and nutrition in producing enzymes[3]. Laccase production can be significantly improved by the addition of xenobiotic compound in the production media including lignin to increase and induce laccase activity[9].

Lignocellulosic wastes such as kepok banana peels and sawdust are abundantly available in Indonesia and still not optimally utilized. Banana peels consist of 16.8% lignin, 18.7% cellulose and 20.3% hemicellulose[10] whereas the cellulose content of sawdust is reached 45.42% and the rest are 21% hemicellulose, 26.50% lignin and 7.08% ash[11]. Due to the sufficient lignocellulose availability in kepok banana peels wasted and sawdust, these materials are highly potential to be utilized for laccase production.

The performance of laccase production can also be improved by the addition of metal ions which act as an inducer leading to the increase of laccase activity. Copper is one of the important micronutrients needed by white-rot fungi to produce laccase[12]. According to Gomaa and Momtaz[13], the addition of metal sources such as copper sulfate ($CuSO_4$) could increase the laccase activity of the white-rot fungi and optimize the lignocellulose degradation process. Cu metal specifies the sufficiently potent inducer for laccase production. However, the concentration added to the media also must be controlled since it can influence the production of the laccase. Adding more $CuSO_4$ into the fermentation medium will give a negative impact on fungal growth[14].

Laccase enzymes can be produced through both submerged fermentation and solid-state fermentation[3]. Submerged fermentation involves the inoculation of fungi into a medium containing a high amount of liquid and supplies oxygen concentration. Téllez-Téllez *et al*.[15] reported the submerged fermentation system showed higher laccase production than solid-state fermentation because of the presence of oxygen within the fermentation system and laccases activity determined to indicate accelerator stability throughout the cultivation period.

The purpose of this study is to obtain the best substrate type from lignocellulose waste between kepok banana peels and sawdust and the best concentration of $CuSO_4$ inducers giving high laccase activity during the submerged fermentation by *T. versicolor*.

MATERIALS AND METHODS

Study area: All the experiments in the studies were performed during December, 2019-March, 2020 in the Food Microbiology Laboratory, the Food Chemistry Laboratory, Department of Food Technology and Central Laboratory, Universitas Padjadjaran, Bandung city, Indonesia.

Microorganism: Isolate *T. versicolor* (L.) Lloyd was obtained from the Indonesian Culture Collection (InaCC) LIPI, Indonesia. It was maintained at Potato Dextrose Agar (PDA) stored at 4°C.

Qualitative laccase assay: The first stage of this study was qualitative laccase assay using a guaiacol colour indicator solution to determine whether or not *T. versicolor* could produce laccase. The media for this test was prepared by adding 0.02% guaiacol to the PDA and then sterilized. The mycelial of *T. versicolor* were inoculated to this media and they placed at 5 points on the petri dish, 4 points on the side and 1 point on the centre. The sample was re-incubated at 30°C for 3 days[2]. A positive result was indicated by the presence of a reddish-brown zone surrounding the fungal colony in the media.

Fermentation process: The fermentation media used in this study consisted of 10 g L^{-1} malt extract; 0.2 g L^{-1} KH_2PO_4; 0.05 g L^{-1} $MgSO_4.7H_2O$; 0.01 g L^{-1} $CaCl_2$; 10 mL L^{-1} Tween 80; and 10 mL L^{-1} microelement solution (1.0 g L^{-1} $FeSO_4.7H_2O$; 1.0 g L^{-1} $MnSO_4.H_2O$; 0.25 g L^{-1} $NaMoO_4.2H_2O$; 0.10 g L^{-1} H_3BO_3; 0.25 g L^{-1} $CuSO_4.5H_2O$; 0.25 g L^{-1} $ZnSO_4.7H_2O$; 0.10 g L^{-1} NH_4NO_3 and 0.5 g L^{-1} $CO(NO_3)_2.6H_2O$). Either kepok banana peels or sawdust (3 g) was added to 150 mL of this media. The control composed of merely fermentation media without adding both substrates was also prepared as a comparison. Each prepared substrate and the control were added by $CuSO_4$ inducer with concentration from 0-2 mM. This media was then autoclaved at 121°C for 15 min[16].

The production of crude laccase extract was initiated with a pre-incubation process (inoculum preparation) in which 3×1 cm^2 of mycelium obtained from PDA was inoculated to 250 mL flasks containing substrates and 150 mL of fermentation media. Incubation was performed at 30°C and 125 rpm for 2-3 days using a rotary shaker incubator. After pre-incubation, 1% inoculum was transferred to the production media consisting of substrates of $CuSO_4$ inducers with concentration from 0-2 mM and subsequently incubated at 30°C and 125 rpm for 13 days using a rotary shaker incubator (Daihan Scientific, Wonju city, Gang-Wang Do, Korea). The fermentation broth was sampled from days 0-12 and each sample was analyzed for laccase activity, the cell concentration following the turbidimetric method and pH. However, before the analysis of laccase activity, the extraction of the crude enzyme was precipitated by using saturated ammonium sulfate (20%) cooled at 4°C for 2 hrs and followed by centrifugation at 10,000×g for 15 min. This process referred to the modification methods[17].

Laccase analysis: Laccase activity was analyzed by spectrophotometric enzyme assay following the method of Buswell *et al.*[18]. The analysis was conducted by adding 60 µL of 0.5 mM acetate buffer with pH 5 and 50 µL of 1 mM ABTS into 50 µL enzyme filtrate and this solution was then thoroughly mixed. The mixed solution was inserted into a microplate and incubated at 30°C for 30 min. The OD value was measured in $\lfloor_{420}$. Laccase enzyme activity expressed as Unit (U) was defined as the number of enzymes that was able to catalyze the release reaction of 1 mmol product per minute under certain conditions. The basic principle for estimating the laccase activity by using the ABTS substrate followed the reaction of phenolic oxidation. This reaction turned ABTS into $ABTS^+$ radical cation.

RESULTS

Raw material characteristics: The composition of lignin, cellulose and hemicellulose to kepok banana peels and sawdust used as substrates were quantitatively analyzed to determine how the potential of these materials for laccase production. The data in Table 1 showed the content of these components. According to this table, sawdust contained a higher lignin and cellulose content than kepok banana peels.

Laccase qualitative assay of laccase: The qualitative laccase enzyme test was carried out to see whether or not there was a laccase enzyme activity in *T. versicolor* culture. The existence of laccase activity produced by fungi was shown by the formation of a reddish-brown zone resulted from the oxidation of guaiacol substrate by laccase. The qualitative laccase enzyme test with the guaiacol substrate was shown in Fig. 1. After 7 days of incubation, the reddish-brown was detected around the fungal colony.

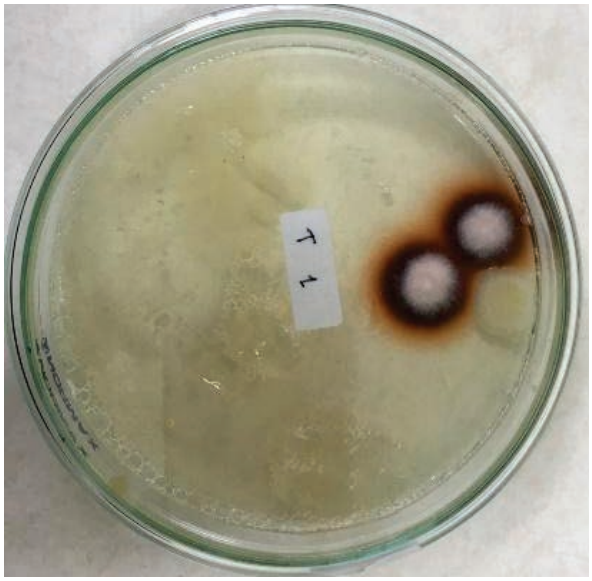

Fig. 1: Reddish-brown zone of *T. versicolor* colony on PDA medium

Table 1: Lignocellulose composition in kepok banana peels and sawdust

Lignocellulosic waste	Hemicellulose (%)	Cellulose (%)	Lignin (%)
Kepok banana peels	36.13±0.941	16.26±1.568	14.76±2.102
Sawdust	15.73±0.254	52.58±1.468	23.63±0.762

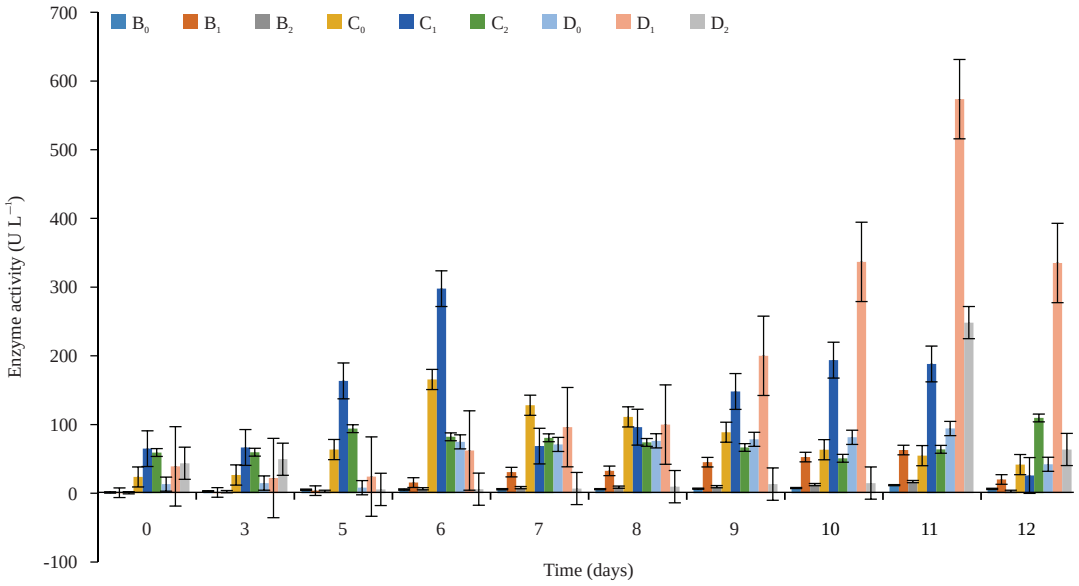

Fig. 2: Laccase activity of *T. versicolor* in various treatment

B_0 (control), B_1 (control+1 mM $CuSO_4$), B_2 (control+2 mM $CuSO_4$), C_0 (Production media+kepok banana peels+0 mM $CuSO_4$), C_1 (Production media+kepok banana peels+1 mM $CuSO_4$), C_2 (Production media+kepok banana peels+2 mM $CuSO_4$), D_0 (Production media+sawdust+0 mM $CuSO_4$), D_1 (Production media+sawdust+1 mM $CuSO_4$) and D_2 (Production media+sawdust+2 mM $CuSO_4$)

Table 2: Analysis of pH media after 12 days of fermentation

	Acidity degree (pH) of each sample								
Days	B_0	B_1	B_2	C_0	C_1	C_2	D_0	D_1	D_2
0	5±0.00	5±0.00	5±0.00	5±0.00	5±0.00	5±0.00	5±0.00	5±0.00	5±0.00
3	5±0.00	5±0.00	5±0.00	5±0.00	5±0.00	5±0.00	5±0.00	5±0.00	5±0.00
5	5±0.00	5±0.00	5±0.00	5±0.00	5±0.00	5±0.00	5±0.00	5±0.00	5±0.00
6	5±0.00	5±0.00	5±0.00	5±0.00	5±0.00	5±0.00	5±0.00	5±0.00	5±0.00
7	5±0.00	5±0.00	5±0.00	5±0.00	5±0.00	5±0.00	5±0.00	5±0.00	5±0.00
8	5±0.00	5±0.00	5±0.00	5±0.00	5±0.00	5±0.00	5±0.00	5±0.00	5±0.00
9	5±0.00	5±0.00	5±0.00	5±0.00	5±0.00	5±0.00	5±0.00	5±0.00	5±0.00
10	5±0.00	5±0.00	5±0.00	5±0.00	5±0.00	5±0.00	5±0.00	5±0.00	5±0.00
11	5±0.00	5±0.00	5±0.00	5±0.00	5.5±0.00	5.5±0.00	5.3±0.29	5.3±0.29	5.17±0.29
12	5±0.00	5±0.00	5±0.00	5.5±0.00	5.5±0.00	5.5±0.00	5.5±0.00	5.5±0.00	5.5±0.00

B_0 (control), B_1 (control+1 mM $CuSO_4$), B_2 (control+2 mM $CuSO_4$), C_0 (Production media+kepok banana peels+0 mM $CuSO_4$), C_1 (Production media+kepok banana peels+1 mM $CuSO_4$), C_2 (Production media+kepok banana peels+2 mM $CuSO_4$), D_0 (Production media+sawdust+0 mM $CuSO_4$), D_1 (Production media+sawdust+1 mM $CuSO_4$) and D_2 (Production media+sawdust+2 mM $CuSO_4$)

Laccase activity: The results of measurements of laccase enzyme activity were shown in Fig. 2. According to Fig. 2, the laccase activity had been detected since day 1 of fermentation for all media types, both controls and the fermentation media with the addition of lignocellulose wastes. The media with the addition of sawdust and 1 mM $CuSO_4$ gave the highest laccase activity, going to 573.6 U L^{-1}. This result showed the synergistic effect between the addition of sawdust and 1 mM $CuSO_4$.

Cell concentration of *T. versicolor*: The results of the cell concentration measurement were shown in Fig. 3. According to this Fig. 3, the average growth of the cell in the media with the addition of kepok banana peels (C_0, C_1 and C_2) showed higher results than both the other samples including the control, but this case did not affect the activity of the laccase enzymes produced. The highest cell concentration value was achieved on the seventh day after incubation with an OD value of 0.8909 in the addition of kepok banana peels substrate and 2 mM of $CuSO_4$.

pH: The occurrence of catalytic activity by the laccase could be observed from the pH change of the media during fermentation. According to Table 2, the pH of the production media always remained stable at pH 5. However, the pH in the media with the addition of kepok banana peels substrate

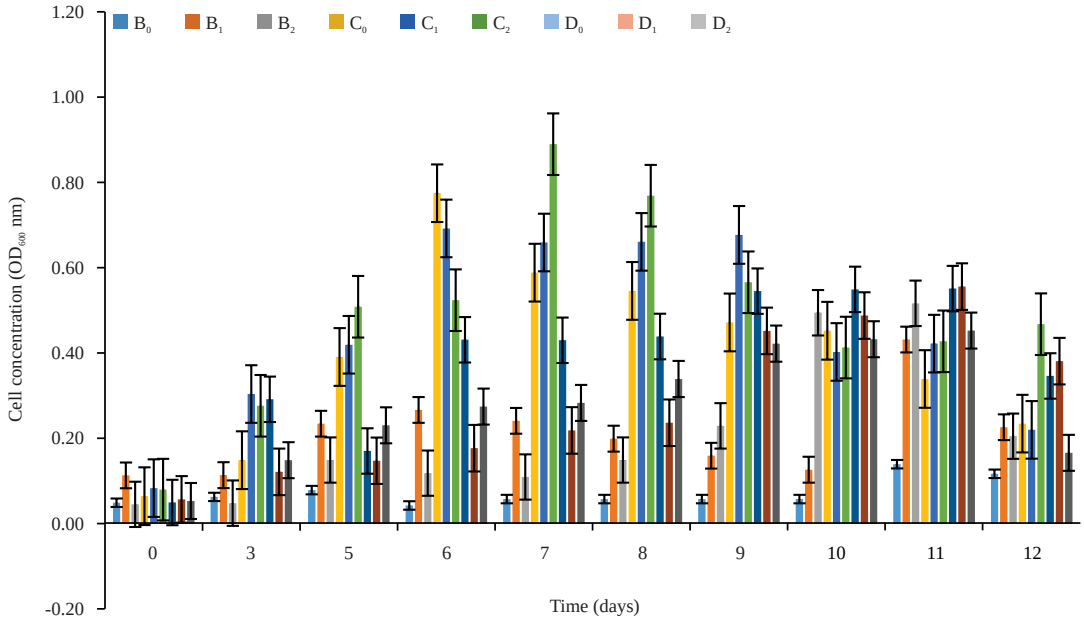

Fig. 3: Cell concentration of *T. versicolor* in various treatment

B_0 (control), B_1 (control+1 mM $CuSO_4$), B_2 (control+2 mM $CuSO_4$), C_0 (Production media+kepok banana peels+0 mM $CuSO_4$), C_1 (Production media+kepok banana peels+1 mM $CuSO_4$), C_2 (Production media+kepok banana peels+2 mM $CuSO_4$), D_0 (Production media+sawdust+0 mM $CuSO_4$), D_1 (Production media+sawdust+1 mM $CuSO_4$) and D_2 (Production media+sawdust+2 mM $CuSO_4$)

and sawdust increased on day 11. The results of the pH measurement for 12 days could be seen in Table 2. In this study, the optimum pH in producing the laccase from the *T. versicolor* was in the range of pH 5.00-5.5.

DISCUSSION

Trametes versicolor is one of the best types of white-rot fungi that produce laccase enzymes. The ability of this fungus to produce laccase enzymes is highly influenced by the production media. The addition of substrates such as lignocellulosic waste can induce fungi to produce laccase in a higher amount. This is due to the presence of lignin in lignocellulosic waste that stimulates fungi to secrete laccase. The Lignin-free material containing cellulose and hemicellulose can easily be hydrolyzed to the monomeric sugars[19]. The more lignin content is available in the production media substrate, the more laccase is produced to degrade the lignin. The highest lignin content was found in sawdust. Accordingly, the use of this material could provide a higher activity of laccase than the addition of sawdust substrate.

Mycelia of *T. versicolor* grown on PDA containing guaiacol can form a Reddish-brown zone as an indication that there is laccase enzyme activity in the fungal culture. The laccase enzyme produced by *T. versicolor* fungus will catalyze oxidation reactions in guaiacol which will form a ketone group and produce H_2O[20]. The laccase enzymes will convert guaiacol to radical quinine with the help of oxygen and then convert it to a quinone. This oxidation results in polymerization which results in brown colour, an insoluble polymer known as melanoidin. The activity of laccase produced varies, it depends on the species of fungi used. Therefore, the laccase activity was quantitatively measured.

In this study, laccase was produced through a submerged fermentation system utilizing media for fungal growth and enzyme production. The result showed that the addition of lignocellulosic waste into the fermentation media gave higher laccase activity than the media without the addition of lignocellulosic waste. The laccase enzyme that had been produced was able to degrade the lignin of the waste. Delignified waste still consisting of cellulose and hemicellulose was easily hydrolyzed for the nutritional addition of fungi. Lignin degradation occurs through the oxidation process of phenolic compounds following a single electron transfer mechanism so that free radicals which are reactive and simultaneously with O_2 molecules are also produced by the H_2O solution[21].

The laccase activity in media with the addition of kepok banana peels showed a higher value than the addition of sawdust. However, this result did not correspond to the lignin content of kepok banana peels which was lower than sawdust. This occurred because apart from the lignin amount in the

material, the value of laccase activity also was affected by the lignin structure of lignocellulosic waste. The lignin structure affected the ability of the white weathered fungus to carry out the de-lignification process by producing the laccase enzyme[22]. Kepok banana peels are a part of a plant originally from a monocot plant that contains lignin with a mixture of the three components of lignin, named p-kumaril alcohol, coniferyl alcohol and sinapyl alcohol. The laccase can be induced very well because the lignin in kepok banana peels is composed of three monolignol monomer structures at once[23]. It is different from the sawdust. This waste less significantly induces the laccase because the lignin content in sawdust only contains guaiacyl structures derived just from coniferyl alcohol[22].

The result showed that the addition of metal ion inducer of $CuSO_4$ influenced laccase activity. The high laccase activity was attained at the media with the addition of 1 mM $CuSO_4$. When the concentration of the inducer additions was increased up to 2 mM, the result of laccase enzyme activity tended to decrease. Cu^{2+} ion that was added into the production media fill up the type 2 Cu bond that worked to promote molecular oxygen. Accordingly, the work of molecular oxygen increased and it led to the occurrence of electron transfers to oxygen in the process of substrate oxidation[24].

The production of laccase with the addition of sawdust substrate and 1 mM $CuSO_4$ gave the high enzyme activity, reaching 573.6 U L^{-1}. This led to a synergetic effect between the addition of sawdust and the addition of 1 mM $CuSO_4$. The interaction mechanisms of both were initiated by the addition of a $CuSO_4$ inducer that acted as a laccase catalyst to produce radical compounds related to the lignin degradation process. The result of the radical compound could separate the aromatic ring from phenol and form a new quinonoid structure formation. The new substructure formed induced tridimensional structure changes of lignin, so the fungus could easily utilize cellulose and hemicellulose as a carbon source[25]. Therefore, the sample with the addition of $CuSO_4$ and lignocellulosic waste could increase the activity of the laccase enzyme produced.

The average growth of cell concentration in media with the addition of kepok banana peels showed a better result than the sample without the addition of substrate or with the addition of sawdust, but this did not affect the activity of the resulting laccase enzymes. This was because the laccase production process occurred in the final stage or after the nutrients in the media had started to run out which was affected by the process of lignin degradation and low availability of lignin in kepok banana peels. High cell concentration did not always indicate that the activity of the laccase enzyme was also high too. This case was related to fungus ability to degrade the lignin which was influenced by the availability of nutrients, lignin structure and the addition of appropriate inducer[22-26].

Optimum laccase enzymes activity was shown on day 1. There was a change in the initial pH that was 5 at the media with the addition of kepok banana peels substrate and sawdust. This case was related to the growth of the fungi in the media. The high cell concentrations were also shown on the eleventh day both in the media with the addition of kepok banana peels and in the media with the addition of sawdust. This showed that on the eleventh day the fungus was still undergoing the growth phase so that the process of breaking down the sugar into ethanol took place quickly. This caused an increase in OH groups derived from ethanol. Ethanol has an OH group (alkaline), so the increase of OH groups at the fermentation media will cause a pH increase[27].

Our study result indicated that there was a potential for the production of the laccase enzyme from the *T. versicolor* fungus using lignocellulose waste and the addition of $CuSO_4$ inducer. Further research was needed for the purification and characterization of the laccase enzyme that had passed the purification.

CONCLUSION

The highest laccase enzyme activity was obtained from the *T. versicolor* (L.) Lloyd fungus at the media with the addition of sawdust substrate and 1 mM $CuSO_4$ inducer as much as 573.6 U L^{-1} on the eleventh day of incubation with OD values of 0.5567 and pH of 5.3. Meanwhile, the addition of kepok banana peel substrate and 1 mM $CuSO_4$ inducer showed the highest laccase enzyme activity on the sixth day of 297.7 U L^{-1} with an OD value of 0.6932 and pH of 5.

SIGNIFICANCE STATEMENT

This study found the potential for laccase enzyme production from the white-rot fungi *T. versicolor* (L.) Lloyd using basic production media with the addition of lignocellulosic waste and $CuSO_4$ as the inducer in the submerged fermentation system. This study will help researchers to find the optimum conditions in the selection of lignocellulosic waste substrates and concentrations of $CuSO_4$ inducer to produce optimum laccase activity.

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