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Research Article

Purification and Characterization Laccase from *Trametes versicolor* (L.) Lloyd in Submerged Fermentation

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Abstract

Background and Objective: Laccase is classified as an oxidoreductase enzyme that catalyzes oxidation reactions of phenolic groups by using oxygen as its electron acceptor. Laccase isolated from *Trametes versicolor* (L.) Lloyd has a wide range of applications in the industrial sector. The use of enzymes in the industrial sector requires pure enzyme conditions from impurities so that the enzyme can maximize its ability in converting the substrate. This study aims to obtain enzyme activity and the characteristic of purified laccase enzymes isolated from *Trametes versicolor* (L.) Lloyd. **Materials and Methods:** This study was conducted with an experimental method followed by descriptive analysis. The steps of this research consist of a qualitative assay of laccase enzyme, crude laccase extract desalting by Sephadex G-25, laccase purification by Sephadex G-100 and laccase optimum pH characterization. **Results:** The result of this study showed that purification of laccase from *Trametes versicolor* (L.) Lloyd with Sephadex G-25 increases laccase enzyme-specific activity which is 10.966 U mg^{-1} and reaches 2.93-fold purity. The highest laccase enzyme activity was achieved at pH 4 with a value of laccase activity 62.39 U L^{-1}. **Conclusion:** Based on current results, purifying laccase from *Trametes versicolor* (L.) Lloyd with Sephadex G-25 was recommended which resulting higher enzyme specific activity.

Key words: Laccase, purification, specific activity, *Trametes versicolor* (L.) Lloyd

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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

The industrial demand for enzymes in Indonesia tends to increase every year, based on the data recorded in 2017, enzyme demand reached 2500 tons and an average volume growth rate of 5-7% per year[1]. In addition to driving the country's economic growth, enzyme production and use can also replace chemicals used in industry. Research on enzyme production, purification and characterization enriches industry knowledge and must be implemented to meet the industrial need for enzymes and to make them commercially viable.

Laccase is an extracellular enzyme that contributes to the process of lignin degradation by using oxygen and producing water as the by-product. This characteristic of laccase has the potential to be applied in various fields such as industrial and environmental fields[2]. Some of the laccase applications in various fields are bioethanol[3], biopulping[4], biofuel[5], biobleaching[6], decolorization of synthetic dyes[7], detoxification and biodegradation[8]. Laccase can also have applications in the food industry, such as juice clarification, wine stabilization and bread processing[9].

Laccase can be extracted from plants, insects, fungi, or bacteria. One of the known laccase producers with high laccase enzyme activity was isolated from *Trametes versicolor*. The advantages of laccase extraction from microorganisms are low production costs, high yield in a short time and productivity is also easier to increase[10]. The production of laccase can be conducted with a submerged fermentation system. The advantage of submerged fermentation is that it is more manageable and sustainable and industrial applications have been favoured over submerged fermentation. Different species or different strains belonging to the same species significantly affect the activity of laccase[11].

Enzyme purification is an essential step to determine the exact kinetics of the enzyme due to the possibility that a compound from a fungal source could act as a natural intermediate or the presence of a similar enzyme may exhibit significantly different reaction kinetics[12]. The reason for the need for enzyme purification is the problem that arises when unpurified enzymes allow interference from other enzymes in the extract using the same substrate or cofactor. There may also be enzymes that cause changes in UV absorbance that may interfere with UV-based testing[13]. Purification steps include desalination/dialysis and fractionation by column chromatography.

This study focused on the purification of laccase produced by the fungus *Trametes versicolor* (L.) Lloyd with the addition of kepok banana peel and sawdust and a previously undetected $CuSO_4$-inducible substrate. Purification of laccase by the addition of substrate and inducer will increase the enzyme activity of the laccase and remove impurities, thus the purity of the laccase will also be increased. The increased activity of the enzyme laccase due to the purification process will also increase the possibility of using laccase for industrial purposes.

MATERIALS AND METHODS

Study area: All the experiments in the studies were performed during December, 2021-March, 2022 in the Food Microbiology Laboratory, the Food Chemistry Laboratory, Department of Food Technology and Central Laboratory, Universitas Padjadjaran, Bandung City, Indonesia.

Microorganism preparation: Isolate *T. versicolor* (L.) Lloyd was obtained from the Indonesian Culture Collection (InaCC) LIPI, Indonesia. *Trametes versicolor* was maintained at Potato Dextrose Agar (PDA) and stored at 4°C. The culture was grown in a modified submerged fermentation system to produce crude laccase.

Qualitative assay of laccase: Laccase assay was determined using guaiacol. The test was performed by culturing the fungus *Trametes versicolor* (L.) Lloyd's in a mixture of PDA agar plates with 0.02% guaiacol. The mycelium was placed at 5 points on the Petri dish. Samples were incubated at 30°C for 3 days. A reddish brown or orange colour around the colony is a positive sign of the laccase enzyme in guaiacol agar[14].

Laccase desalting using column chromatography: Crude laccase enzyme with an amount of 3 mL was poured into the column chromatography contained with Sephadex G-25 matrix[15]. The crude laccase enzyme was then eluted using 0.5 M acetate buffer pH 5. The pooled fraction was then tested for protein content and enzyme activity.

Laccase purification using column chromatography: The 3 mL enzyme fraction from the desalting process was poured into the column chromatography contained with Sephadex G-100 matrix[16]. The enzyme was eluted using 0.5 M acetate buffer pH 5. The pooled fraction was then tested for protein content, enzyme activity and pH characterization.

Laccase activity assay: Laccase activity was determined spectrophotometrically[17]. The mixture of 60 µL acetate buffer pH 5 (0.5 M), 20 µL 2.2'-azino-bis (3-ethylbenzothiazoline-6-sulfonate) (ABTS) (1 mM) and 50 µL crude laccase enzyme in

microplate was incubated at 30°C for 30 min. The absorbance was measured at 420 nm. Enzyme unit was defined as the laccase activity that oxidized 1 μmol of ABTS per minute.

Protein content determination: Protein content was determined by the Bradford method[18]. The bovine serum albumin (BSA) standard curve was performed by performing different concentrations with a mixture of 5 μL sterile aqua dest and 100 μL Bradford reagent as blank. The BSA standard solution concentration variations were 1, 3, 5, 7 and 9 ppm, 5 μL each. About 100 μL of Bradford's reagent was added to each microplate containing the BSA standard solution, then homogenized for 10 sec and incubated for 10 min. Absorbance was measured by a spectrophotometer at 595 nm. The protein standard curve was generated by plotting the BSA concentration (x) against the absorbance of the standard solution (y) to obtain the linear regression equation:

$$y = ax + b$$

The 5 μL enzyme fraction was mixed with 100 μL Bradford's reagent in the microplate. The mixture was then homogenized for 10 sec and incubated for 10 min. The absorbance was measured with a spectrophotometer at 595 nm. The sample protein content was calculated by entering the absorbance value of the sample (y) into the linear regression equation:

$$y = ax+b$$

so, that the concentration or protein content of the sample (x) can be determined.

Laccase pH characterization: About 40 μL of enzyme purified by column chromatography Sephadex G-100 was mixed with 60 μL of acetate buffer pH 3, 4, 5, 6 and 7, respectively, which had been added with 20 μL ABTS 1 mM. The mixture with a total volume of 120 μL was then homogenized. The absorbance of the mixture was measured at 420 nm for 0 and 30 min at 30°C[19].

RESULTS

Qualitative assay of laccase: A positive indicator of laccase activity in PDA substrate-guaiacol supplemented was demonstrated by the appearance of a reddish brown or orange colour around the colony. The reddish brown colour appearing on PDA was the result of the oxidation of guaiacol by the enzyme laccase. This result indicated that *Trametes versicolor* (L.) Lloyd can secrete laccase.

Laccase desalting using column chromatography: The crude laccase enzyme sample was then desalted by column chromatography. The gel substrate used is Sephadex G-25 which separates the enzymes from the smaller molecules. The total number of enzyme fractions pooled was 24. The results were shown in Fig. 1, there were 5 parts with the highest protein content, which were parts 8, 9, 14, 15 and 16. The highest absorbance peaks were represented by parts 8 and 14, indicating the existence of laccase in the mentioned sections, with absorbance values of 0.448 and 0.515, respectively. The 5 fractions were then pooled and purified by Sephadex G-100.

Laccase purification using column chromatography: The desalting enzymes obtained from the desalting step were

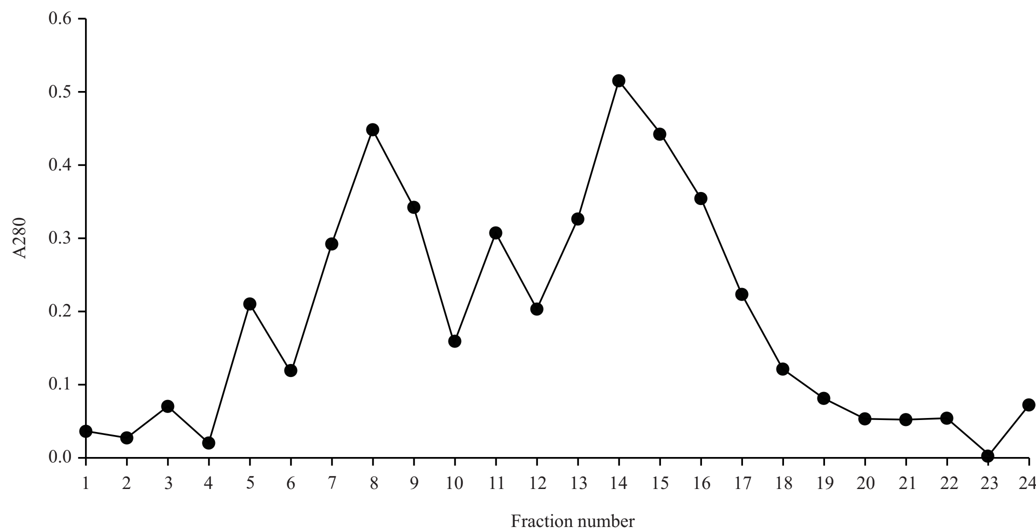

Fig. 1: Chromatogram of protein from desalting using Sephadex G-25 matrix

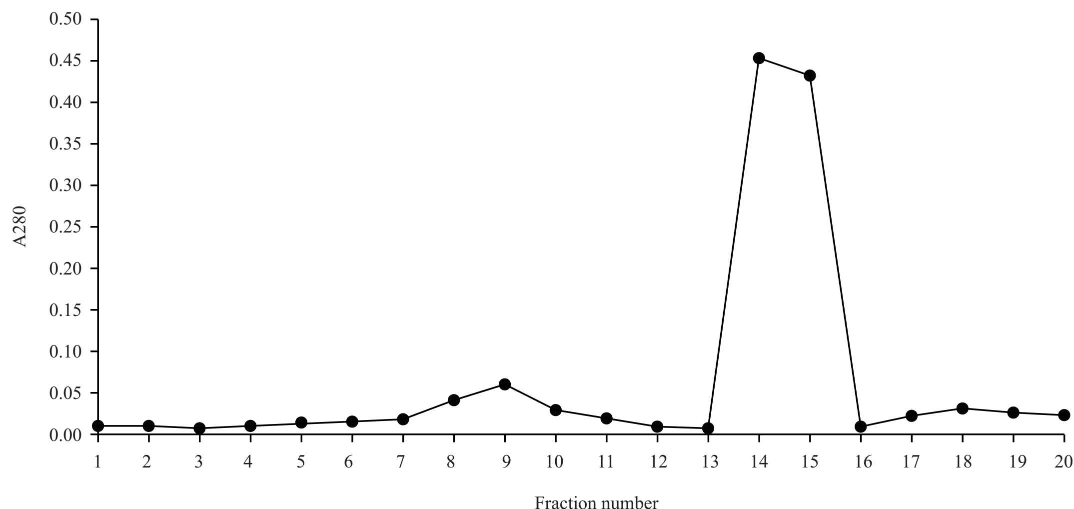

Fig. 2: Chromatogram of protein from purification using Sephadex G-100 matrix

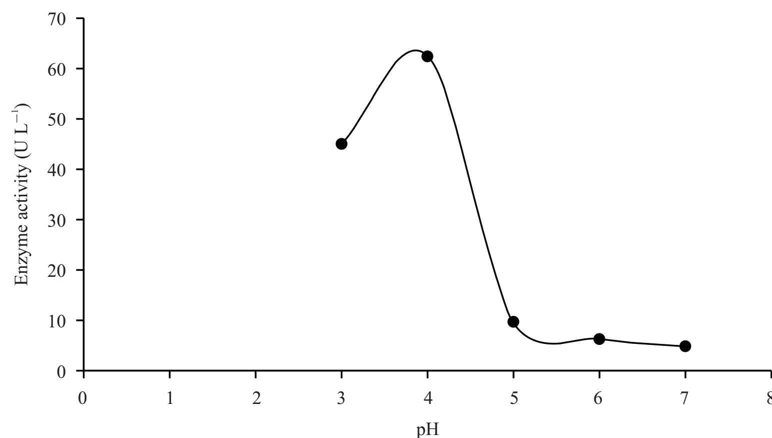

Fig. 3: Effect of pH on laccase activity

Table 1: Purification summary of laccase from *Trametes versicolor* (L.) Lloyd

Purification step	Enzyme activity (U L^{-1})	Protein content (mg L^{-1})	Total activity (U)	Total protein (mg)	Specific activity (U mg^{-1})	Purification fold
Crude enzyme	1865.9	499.2	190.32	50.92	3.738	1.00
Sephadex G-25	5398.3	492.2	53.98	4.92	10.966	2.93
Sephadex G-100	18.8	458.7	0.11	2.75	0.041	0.01

then purified by column chromatography using the Sephadex G-100 substrate. Sephadex G-100 was used to separate a specific laccase enzyme from another molecule based on the size of the laccase molecule and the pores of the substrate. The total amount of synthetic enzyme was 20 fractions. The results of the laccase purification were shown in Fig. 2, the highest peaks of absorbance were in fractions 14 and 15 with the absorbance value of 0.453 and 0.432, respectively. The high absorbance value in the collected fraction was assumed to be an indicator of the presence of the purified laccase enzyme.

Quantitative laccase assay: Laccase enzyme samples from each purification step were assayed quantitatively by measuring the enzyme's protein content, enzyme activity and specific activity. The result of the tests can be seen in Table 1, the desalting process using Sephadex G-25 can increase the enzyme activity of the laccase enzyme. The same result can also be observed at the laccase enzyme's specific activity. The increasing value of specific activity affects the purity of laccase which go up to 2.93 fold.

Laccase pH characterization: Characterization of enzymes against pH is important to obtain the optimum enzyme condition so, that the enzyme can maximize its ability in converting the substrate. The tests were carried out by measuring the enzyme activity under various pH conditions. The results of this test were presented in Fig. 3, laccase enzyme activity at various pH above, the laccase enzyme produced by *Trametes versicolor* (L.) Lloyd has the highest activity at pH 4. This result showed that the laccase enzyme works optimally at pH 4.

DISCUSSION

Trametes versicolor (L.) Lloyd has shown the reddish-brown colour around the mycelia based on the qualitative assay. This condition indicate that *Trametes versicolor* (L.) Lloyd can produce laccase enzyme, which the reddish brown colour that emerged on the PDA added with guaiacol was the result of guaiacol oxidation by laccase enzyme. Before the oxidation process occurred, guaiacol has a slightly yellowish colour and then turns brownish after the oxidation has taken place. This result was in line with[20], red-brown oxidized zones developed around colonies picked for laccase production. The wider the reddish brown zone indicates the greater concentration of laccase enzyme[14]. This laccase enzyme can catalyze the oxidation process of guaiacol which produces a ketone group and H_2O[21]. The enzymatic reaction carried out by laccase is one-electron oxidation. Oxygen is needed as the electron acceptor and then produces water. When oxidation takes place, the substrate will lose one electron and form free phenoxy radicals. These free radicals can do further enzymatic oxidative reactions or non-enzymatic reactions such as hydration and polymerization[22]. In this case, laccase enzymes convert guaiacol into radical quinine and with the help of oxygen will be converted again into a quinone. The result of that oxidation process initiated the polymerization reaction forming the brown-coloured melanoidin.

Purification of crude laccase enzyme was conducted in 2 steps using the column chromatography method. The basic principle of protein separation with gel filtration column chromatography is the difference in protein molecular weight. The Sephadex gel was used as a stationary phase, while the acetate buffer acted as a mobile phase[23]. The first step is the desalting process with Sephadex G-25 gel matrix and then continued to the purification process using Sephadex G-100 gel. The desalting process using Sephadex G-25 involves the removal of salts and smaller molecule size than a protein, in which those molecules will be retained in the gel matrix pores. The bigger molecule size such as proteins will be separated and fractionated first[24]. Sephadex G-100 was then used to separate protein with a molecular weight of 4-150 kDa[25]. In this study, laccase was indicated by the highest peak of absorbance in each step of purification. Those obtained samples were then assayed to determine their protein content, enzyme activity and specific activity.

The result of enzyme activity shows that laccase enzymes desalted with Sephadex G-25 have a greater value than the purified ones. The used of Sephadex G-25 increased laccase activity on laccase production from *Botrytis cinerea* (DSMZ 877)[26]. The decreased laccase enzyme activity after purification may occur due to the laccase enzyme molecular weight being too small for the molecular weight that was able to pass through the pores of the Sephadex G-100 matrix, which only allow molecules with 4-150 kDa molecular weight. Based on this study, the molecular weight of the laccase enzyme studied does not match the pore size allowed to pass through the Sephadex G-100 matrix. However, the Sephadex G-100 was used for partial laccase purification of *Bacillus* sp. The AKRC01 with a molecular weight of 61 kDa[27]. The same method was also carried out for laccase purification from white-rot fungus, *Marasmius* sp., BBKAV79 using Sephadex G-100[16]. An alternative that can be used to solve this problem is to reduce the pore capacity that can be passed by a molecule in the column chromatography system. This can be applied by performing the column chromatography system using Sephadex G-75 which has a molecular fractionation range of 3-80 kDa[28]. Sephadex G-75 has been used for the purification of laccase from the rice blast fungus, *Magnaporthe grisea* which resulted in 282-fold purification with a specific enzyme activity of 225.91 U mg^{-1} and a yield of 11.92%[29]. Further investigation of the laccase molecular weight can be determined by the electrophoresis SDS-PAGE method. Thus, it can be said that the laccase enzyme produced by *Trametes versicolor* (L.) Lloyd used in this study tended to be more effectively purified using the Sephadex G-25 gel matrix.

In this study, laccase purification using Sephadex G-25 increased the specific activity, which was 3.738 U mg^{-1} and then increased to 10.966 U mg^{-1} with a purity of 2.93 fold after purification. The protein content in the sample decreases along with the purification process. Purification in this experiment was proven by the decreased protein content and increased enzyme purity. The crude laccase applied to the desalting treatment has increased in purification level up to 1.6-2.5 times after precipitation and dialysis[30]. The same result was also found in research conducted by the use of a gel filtration chromatography purification step increased the enzyme purity to 3.7-fold (28-fold purification) over the cold acetone precipitation obtained above and showed 57%

recovery of enzyme activity[31]. The difference in specific activity values obtained compared to the literature can be influenced by the source of the enzyme and the growth media used. The higher the total protein, the lower the specific activity of the sample. This is because there are several protein contaminants besides laccase that inhibit substrate changes in the product. The increased enzyme activity in purified enzymes is due to a decrease in the number of contaminants that prevent the active side of the enzyme from binding to the substrate[32].

Laccase in this study has the optimum pH of 4. A low optimum pH can be associated with fungi that adapt to acidic growing conditions. Laccase enzyme produced by fungi is generally at a pH range of 4-6[33]. The laccase activity of the ascomycete fungus *Nectriella pironii* had an optimum pH of 2.0 or 6.0[34]. Jin *et al.*[35] reported the purified laccases from *Trametes versicolor* were optimized at a pH range from 5.0-7.0. Enzyme activity is influenced by the degree of acidity (pH) during the enzymatic reaction[36]. The active side and three-dimensional shape of enzymes can undergo ionic changes caused by variations in pH. The change of structure can reduce the contact between enzyme and substrate so that it will affect the enzyme activity[37]. When the enzyme is at the optimum pH condition, the proton donor and acceptor groups in the catalytic site of the enzyme are at the desired ionization level so that the conformation of the active site matches the substrate. This condition will stimulate the formation of a maximum complex between the enzyme and the substrate so that the enzyme will be effective in producing products[38]. Overall, the results of this study showed the potential for partially purified white rot fungus termites versicolor to increase the specific activity with 2.9 times higher purity. So, it can be considered for the production of laccase on a larger scale.

CONCLUSION

This research has obtained the specific activity and the increased purity value of laccase enzyme produced from *Trametes versicolor* (L.) Lloyd can be effectively purified using Sephadex G-25, with the specific activity of purified laccase was 10.966 U mg^{-1} with the purity of 2.93 fold. Laccase enzyme has the optimum pH of 4. This study will inform researchers or industries about a pH condition that is suitable with the laccase enzyme activity which can maximize its application for analytical purposes or everyday life needs.

SIGNIFICANCE STATEMENT

This study was carried out to determine the activity of purified laccase enzyme and the properties of laccase enzyme isolated from *T. versicolor* by submerged fermentation system. Purification of laccase enzyme in this study by chromatography gel filter column. The principle of protein separation by filter column chromatography gel is the difference in molecular weight of a protein. After undergoing purification, the specific laccase activity of *T. versicolor* and the degree of purity increased and the optimum pH was obtained.

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