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Research Article
Study of *Ganoderma lucidum* in Laccase Production using Corncob and Paddies Straw Substrates on Submerged Fermentation System

[1]T. Yuliana, [1]N.Z. Putri, [1]D.Z. Komara, [2]E. Mardawati, [1]I. Lanti and [1]S. Rahimah

[1]Department of Food Technology, Faculty of Agro-industrial Technology, Padjadjaran University, Bandung, Indonesia
[2]Department of Agroindustrial Technology, Faculty of Agro-industrial Technology, Padjadjaran University, Bandung, Indonesia

Abstract

Background and Objective: *Ganoderma lucidum* a white rot fungi, produce laccase which capable to degrade lignin due to its activity as ligninolytic enzymes. The production of laccase by *G. lucidum* using various agroindustrial wastes, including corncob and paddies straw, as substrates have been studied. The purpose of this study was to determine substrate that able to produce the highest activity of the laccase from the *G. lucidum*. **Materials and Methods:** The method used an experimental design followed by descriptive analysis using 4 treatments with duplication including treatment *G. lucidum* growth into A (control, Potato Dextrose Broth (PDB)), B (PDB+corncob), C (PDB+rice straw) and D (PDB+corncob+rice straw). This study includes; (1) Qualitative assay determination of laccase, (2) Extraction and laccase activity, (3) Cell concentration of *G. lucidum* measurement and (4) pH measurement. **Results:** Laccase qualitative assay showed brownish red ring on PDA media that indicated a positive of the laccase enzyme secreted by *G. lucidum*. Enzyme activity under submerged fermentation condition was achieved by the treatment of adding corncobs with the highest activity accounting 68.75 U mL^{-1}. The fermentation process causes a decrease in pH during the incubation time to pH 4.83. The results of pH measurements showed that the laccase enzyme from *G. lucidum* worked optimally at pH 4-5 achieved after 5 day of incubation. **Conclusion:** Our results suggest that *G. lucidum* has potential to produce laccase enzyme by using substrate comprising corncob and rice straw on submerged fermentation.

Key words: Laccase, *G. lucidum*, ligninolytic enzyme, agroindustrial waste, corncob, paddies straw

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Corresponding Author: T. Yuliana, Department of Food Technology, Faculty of Agro-industrial Technology, Padjadjaran University, Bandung, Indonesia

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

Laccases are multicopper oxidases with high potential for industrial applications. The main substrate of laccase is an aromatic compound and the oxidation reaction carried out does not release hydrogen peroxidase. Application of laccase in the food industry can be used in the beverage industry as a stabilizer, such as; the fruit juice and wine industry. Laccase enzymes are also used in the bread industry to optimize the properties of dough, bleach and increase sensory intensity[1].

Generally, laccase is widely used in the agroindustrial sector because of its nature which can degrade lignocellulose will facilitate the handling of agroindustrial waste. Several studies on laccase and its applications have been widely reported[1]. Nevertheless, research on laccase in Indonesia is still minimal. Indonesia has a large potential for laccase production using substrates from agroindustrial waste. Moreover, the use of laccase is quite effective for the food industry, especially in Indonesia because of its rare availability which result in the price of this enzyme is quite high.

This study focus on the production of laccase produced by *Ganoderma lucidum* which known contains a variety of active compounds. According to Fang *et al.*[2], many of these fungal species have the potential to produce laccase. Laccase is widespread in white rot fungi including *G. lucidum* which can break down lignin by secreting enzymes such as laccase[3]. This fungus will be found in lignocellulosic agroindustrial industrial waste such as; oil palm empty fruit bunches, corncobs, cocoa shells, rice straw and so on.

According to Artiningsih[4], among of all *Ganoderma* species, G. *lucidum* species produced the highest ligninolytic activity. However, the results of the quantitative laccase enzyme test showed that the activity of the laccase enzyme produced by *G. lucidum* was at an average level of various types of *Ganoderma*. Laccase production by submerged fermentation method can provide optimum conditions for the growth of *G. lucidum*[4] and simplify the stirring process therefore the substrate degradation process can be more evenly made the fermentation process runs more efficiently[5]. Therefore, research to maximize enzyme activity by using various substrate variations by utilizing the surrounding agroindustrial waste is needed to produce laccase with economic value.

The potential production of enzymes from agroindustrial waste is expected to help optimize production from several food industries, especially the beverage industry. The large potential of this laccase production can also reduce production costs from the industry by producing laccase with additional substrates in the form of corncobs and rice straw. Laccase enzyme production from agroindustrial waste will help optimize the utilization of agroindustrial waste. Fungus added to lignocellulosic agroindustrial waste substrate can optimize the quality of the enzymes produced. Utilization of this waste can be one of the solutions to reduce agroindustrial waste that will increase in line with the increasing needs of the community for agroindustrial food stuffs.

Laccase enzymes have excellent lignin degradation properties. Therefore, adding lignocellulosic waste to the substrate will optimize the laccase enzyme produced. The substrates used in this study were rice straw and corncobs which had high enough lignin levels to produce optimal enzyme activity. Laccase enzymes are widely used in the process of lignin degradation which catalyzes the oxidation reaction of phenolic compounds by using oxygen as an electron acceptor[5]. This enzyme is able to act as an efficient biocatalyst for lignocellulosic pretreatment. Adding substrate from agroindustrial waste will help in processing agroindustrial waste and reduce the amount of agroindustrial waste. The purpose of this study was to determine the potential for the production of the laccase enzyme in *G. lucidum* with additional substrates agroindustrial waste, corncob and rice straw for media growth.

MATERIALS AND METHODS

Study area: All the experiments were performed during September, 2019-January, 2020 in the Food Microbiology Laboratory, Department of Food Technology and Central Laboratory, Padjadjaran University, Bandung city, Indonesia.

Microorganism: Isolate *G. lucidum* was obtained from the Research Center for Bioscience and Biotechnology at Institut Teknologi Bandung (ITB), Indonesia. *Ganoderma lucidum* was maintained at Potato Dextrose Agar (PDA) and stored at 4°C.

Qualitative assay determination of laccase: Laccase test was carried out using a guaiacol color indicator solution. The test was carried out by subculturing the mycelial of *G. lucidum* to the PDA plates with an additional 0.02% of guaiacol[6]. The mycelia of *G. lucidum* are placed at 5 points on the Petri dish, 4 points on the side and 1 point on the center. The sample was then re-incubated at 30°C for 3 days. The appearance of a brownish red color in the guaiacol agar added indicates the presence of the laccase enzyme.

Fermentation condition: The media used for fermentation culture was the Potato Dextrose Broth (PDB) media. About 20 g of dried rice straw and corncobs was added to the 100 mL

PDB in 250 mL Erlenmeyer flask. After that, the media was added with 1 mM of $CuSO_4$ as an inducer followed by sterilized at 121°C for 15 min[7].

The fermentation culture of *G. lucidum* was carried out by 4 treatments substrates variation with duplication including treatment A (control, only using PDB as substrate), B (PDB+corncob (5:1), C (PDB+rice straw (5:1) and D (PDB+corncob+rice straw (5:0.5:0.5)).

Erlenmeyer flask contain various medium was inoculated with 1 cm^2 *G. lucidum* followed by incubation at 30°C in a 100 rpm incubator shaker under dark conditions for 14 days. Sampling was carried out on days 1, 2, 3, 4, 5, 6, 7 and 8 for laccase enzyme analysis[5].

Extraction of laccase: Laccase extraction was carried out on cultures of day 4-7 referring to the modified method[6]. Laccase extraction was carried out by protein precipitation with saturated ammonium sulfate (80%), cooling at 4°C for 2 h then centrifuged at 10,000×g for 15 min.

Laccase analysis: Laccase analysis was performed spectrophotometrically[8]. Laccase activity measured with a solution of 50 µL enzyme filtrate added 100 µL 50 mM buffer phosphate pH 6 and 50 µL ABTS 0.45 mM then the sample was inserted into a microplate and incubated 20 min at room temperature by measuring the OD value in A_{420}. One unit enzyme was defined as the activity of laccase that oxidized 1 µmol of ABTS per minutes.

Data analysis: In this study, it was used experimental design followed by descriptive analysis with duplication for each treatment.

RESULTS

Qualitative assay of laccase: The existence of laccase activity was shown by the brownish red color resulting from the oxidation of guaiacol substrate by laccase. The higher the concentration of laccase would form a wider reddish zone. The qualitative laccase enzyme test with the guaiacol substrate observed in Fig. 1.

Based on observation, it showed a brownish red color on *G. lucidum* culture on PDA contain guaiacol after 7 days incubation indicated that *G. lucidum* has a potency to produce laccase. The reddish zone area produced around *G. lucidum* culture was large so that it was estimated to have a high laccase enzyme potential. Therefore, further tests were conducted in order to calculate the amount of enzyme activity present in the *G. lucidum*.

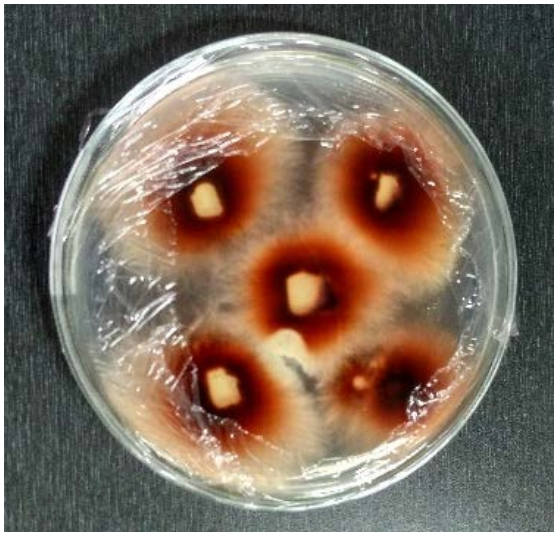

Fig. 1: Brownish red zone of *G. lucidum* indicating laccase activity

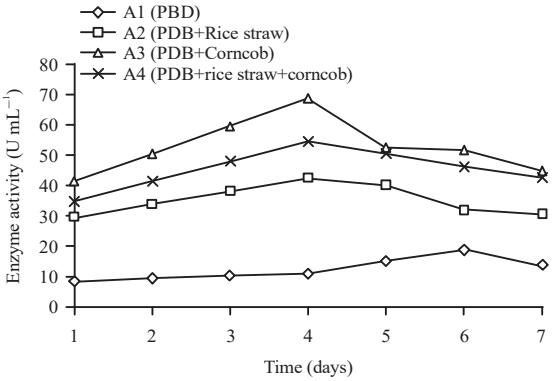

Fig. 2: Laccase activity of *G. lucidum* in various treatment; A1 (control), A2 (PDB+corncob), A3 (PDB+rice straw) and A4 (PDB+corncob+rice straw)

Laccase activity: The activity of the laccase was determined based on the rate of change of ABTS substrate into ABTS radical products. Laccase activity was expressed in international units (U mL^{-1}). Based on the observation results, laccase activity could be produced from *G. lucidum* by using an additional substrate of corncobs, rice straw and a mixture of both as presented in Fig. 2.

Based on the results above, it showed that *G. lucidum* on the first day of observation increased in enzyme activity, but the enzyme activity decreased on the second day of observation. Laccase production with the highest activity was *G. lucidum* which was treated with the addition of corncob substrate on the 4th day of observation which was

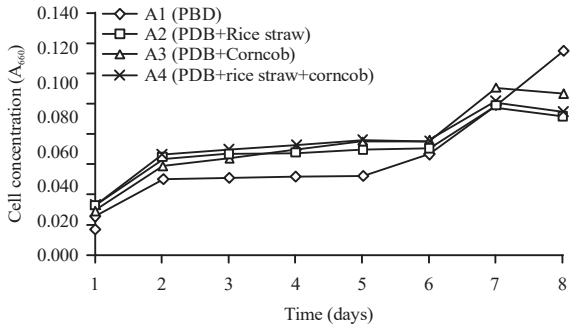

Fig. 3: Cell concentration of *G. lucidumin* various treatment; A1 (control), A2 (PDB+corncob), A3 (PDB+rice straw) and A4 (PDB+corncob+rice straw)

Table 1: pH during *G. lucidum* fermentation process

	pH			
Day of fermentation	A1	A2	A3	A4
6	5.22	5.13	4.98	5.38
7	5.05	4.95	4.92	5.27
8	4.94	4.81	4.83	5.21

68.75 U mL^{-1}. These results showed that substrate for *G. lucidum* by the addition of corncobs growing very well compared to other treatments. Treatment A2, A3 and A4 began to decrease in activity on the 6th day of observation. While in treatment A1, enzyme activity continued to increase slowly and began to decline on the 8th day of observation. The enzyme activity produced by the treated samples (A2, A3 and A4) indicates that the fungus *G. lucidum* used carbon sources derived from sucrose and dextrose from lignocellulosic waste.

Cell concentration of *G. lucidum*: Calculation of the number of cells were conducted using a spectrophotometer with the turbidimetry method. This method is a method of estimating the presence of microorganisms with the principle of turbidity. The results of this data were presented in the form of a standard curve where the goal was as a reference to count the number of cells on a growth curve as shown in Fig. 3.

pH : The optimal pH for laccase varies based on the substrate used. On the 6th, 7th and 8th day observations were made to check the pH which showed a decrease in pH caused by the fermentation process. This result was in line with observations where the enzyme activity of the *G. lucidum* which optimum in the pH range of 5. The results of pH measurements presented in Table 1.

The observations showed that pH at day 6, 7 and 8 continued to decrease along with the fermentation that occurred. In this study, the optimum pH in producing the laccase from the *G. lucidum* was in the range of pH 4.81-5.38.

DISCUSSION

Laccase could be produced by various microorganisms including bacteria and fungi. *Ganoderma lucidum* is one of fungi generally grows on wood or tree trunks which has been detected positive to produce laccase. The reddish zone area produced around *G. lucidum* culture was large so that it was estimated to have a high laccase enzyme potential. Calculation of enzyme activity present in the *G. lucidum* under submerged fermentation has also positive result by using variation of substrate from agroindustrial waste including corncobs and rice straws.

The qualitative laccase enzyme test was carried out to observe the presence or absence of laccase enzyme activity in the culture of *G. lucidum.* Guaiacol turn to brownish red when exposed to O_2 (oxidized) or exposed to light[9]. Laccase catalyzed the oxidation reaction on the guaiacol substrate which form a ketone group and produce H_2O. Before being oxidized, guaiacol has a slightly yellowish color, while after oxidizing, the color of guaiacol turned to be brown[9]. The presence of a brownish red zone on the PDA medium which indicated the activity of the laccase enzyme in the *G. lucidum*. Based on the Fig. 1, it was known that the five cultures added to show a brownish red color. This showed that every part of the *G. lucidum* culture able to produce laccase. The reddish zone area produced around the mushroom culture was large enough that could be estimated to have a high laccase potential. Therefore, further tests were conducted for calculating the amount of enzyme activity present in the *G. lucidum*.

Lacase is an extracellular enzyme which does not need to conduct cell lysis process during the isolation. Extracellular enzymes have a function to change the surrounding nutrients so that cells can be entered and absorbed directly by hyphae[10]. The laccase extraction process is one of the important things that must be considered to produce the highest enzyme activity and all laccase enzymes found in the fungus[11]. Based on these result, laccase activity from *G. lucidum* on the first day of observation has increased. The addition of $CuSO_4$ inducer on the first day can cause produce laccase activity up to two times. The addition of inducers plays a role in increasing the activity of enzymes called co-factors. Not all metal ions are co-factors, certain metal ions can act as inhibitors that inhibit enzyme activity[12].

The enzyme activity produced by the treated samples (A2, A3 and A4) indicated that the fungus *G. lucidum* can use carbon sources derived from sucrose and dextrose from lignocellulosic waste. Enzymes are produced as an effort of fungi to obtain nutrients from solid substrates that contain lignocellulose. Laccase which is a ligninolytic enzyme is secreted to degrade lignin, so that fungi can use cellulose and hemicellulose as a source of nutrition[13].

The highest activity of laccase production was the *G. lucidum* which was treated with the addition of corncob substrate on the 5th day of observation which was 68.75 U mL^{-1}. These results can be expected to see from the fungus *G. lucidum* given the addition of corncobs substrate treatment grows very well compared to other treatments. These results are comparable with research conducted by Asih[14], where the treatment of corncob substrate addition resulted in better enzyme activity compared to the treatment of rice straw substrate addition.

The observations showed the pH continued to decrease along with the fermentation that occurred. Based on research that has been done, the optimum pH in producing laccase enzymes from the fungus *G. lucidum* is in the range of pH 5. Acid conditions affect the amino acids that make up enzyme proteins. The pH variations cause ionic changes on the active side of the enzyme as well as the three-dimensional shape of the enzyme. Changes in three-dimensional shape can reduce the contact between enzymes and substrates so that it affects enzyme activity[15].

Our results suggest a possibility that laccase production from *G. lucidum* by using combination substrates from various agroindustrial wastes which is more economical for enzyme production and reduces the environmental burden. Further study is clearly needed to increase its enzyme activity and to generate pure laccase by purification.

CONCLUSION

Ganoderma lucidum has potential to produce laccase enzymes with the most preference enzyme activity was achieved after 5 days of incubation in the treatment of corncobs addition with enzyme activity of 68.75 U mL^{-1} with the optimum pH obtained ranged from pH 4-5. The results of cell concentration measurements showed the cell growth curve was in line with the enzyme activity curve.

SIGNIFICANCE STATEMENT

This study discovers the possible laccase production isolated from the white root fungi *G. lucidum* using synthetic medium (PDB) added with agroindustrial waste (corncob and rice straw) and $CuSO_4$ as inducer on submerged fermentation system. This study will contribute the researcher to find the potency of fungal laccase production by using agroindustrial waste which help to utilize and to reduce the agroindustrial waste. Thus, a new concept on this laccase production useful to meet the needs of laccase in industries such as; for food industry, pulp and paper industry and waste treatments etc., with lower costs and environmental friendly.

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