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Research Article
Optimization of Laccase Production from *Marasmius* sp. in a Submerged Fermentation System

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Abstract

Background and Objective: *Marasmius* sp., is a lignin-degrading basidiomycetes that naturally grows in woods or plants containing lignocellulose. Laccase is one of the ligninolytic enzymes produced by this fungus which is potentially utilized as natural food additives in food and beverage processing industries. The purpose of this study was to obtain the activity of laccase produced by *Marasmius* sp., which is grown in media with high concentrations of lignocellulosic waste, such as rice straws and corn cobs. **Materials and Methods:** The laccase obtained from the *Marasmius* sp., isolate was then measured for its enzyme activity. The laccase activity produced was qualitatively measured from potato dextrose broth (PDB) media supplemented with rice straws and/or corn cobs. In addition, this experiment used experimental method followed by descriptive analysis. Each treatment was carried out in duplicate. Parameters include analysis of substrate water content, qualitative analysis of laccase, laccase enzyme activity, cell concentration and pH analysis during fermentation. **Results:** The results of laccase activity by qualitative test on potato dextrose agar (PDA) media produced reddish-brownish zone showing the potential activity of *Marasmius* sp., to produce laccase enzyme. Supplementation with rice straws and corn cobs into PDA agar demonstrated both the highest value of enzymatic activity (68.38 U/mL) and optical density (OD) 0.058. Besides, the laccase enzyme from *Marasmius* sp., was optimally produced at pH 5 on day 5 incubation. **Conclusion:** The *Marasmius* sp., has potentials to produce high levels of laccase in media supplemented with rice straws and corn cobs through submerged fermentation system.

Key words: Laccase, lignocellulose, rice straws, corn cobs, enzyme activity, *Marasmius* sp.

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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 classified as an oxidoreductase enzyme that can catalyze the oxidation process of phenolic compounds (polyphenols), methoxy polyphenol substitution, aromatic diamines and other compounds by utilizing Oxygen (O_2) as an electron acceptor[1,2]. As a biocatalyst, laccase reduces O_2 to Water (H_2O), mediated by phenolic substrates through a one-electron reaction resulting in a free radical which similarly occurs in the manganese peroxidase (MnP) reaction[3]. In nature, laccase is produced by white rot fungi which naturally grow in humid wastes, such as wood and plants containing lignocellulose. There are several types of white rot fungi, for example *Trametes versicolor*, *Marasmius* sp. and *Ganoderma lucidum*.

The *Marasmius* sp., one of the members of basidiomycetes, is the most abundant fungi in secreting laccase to decompose lignin, by the addition of nitrogen[4]. This fungus grows well at 30°C with 60-70% humidity level in aerobic conditions. Meanwhile, according to the study conducted by Vantamuri and Kaliwal[5] *Marasmius* sp., can optimally grow and produce laccase on potato dextrose broth (PDB) media at 37°C and pH[5,6].

The production of laccase can be increased by adding lignin as a natural inducer[6]. In nature, lignin can be found in waste with high lignocellulose concentrations, such as rice straws and corn cobs which have been shown as good substrates to produce laccase[7]. This study aims to qualitatively measure the level of laccase activity produced by *Marasmius* sp. which is grown in media supplemented with rice straws and corn cobs.

MATERIALS AND METHODS

Study area: All the experiments in the studies were performed during January to June, 2023 in the Food Microbiology Laboratory, Food Chemistry Laboratory, Food Processing Technology Laboratory, Engineering Laboratory Food Processing Department of Food Industry Technology, Faculty of Technology Agricultural Industry, Padjadjaran University and Central Laboratory Padjadjaran University.

Microorganism preparation: Isolate *Marasmius* sp., obtained from the Center for Biological Sciences, Bandung Institute of Technology. It was maintained at potato dextrose agar (PDA) stored at 30°C.

Qualitative assay of laccase: The laccase test is carried out using a guaiacol color indicator solution. The test was carried out by subculturing the fungal mycelia onto PDA agar plates with the addition of 0.02% guaiacol[8]. Mycelia were placed at 5 points in the petri dish, namely 4 points on the sides and 1 point in the middle using a loop. The samples were then incubated again at 30°C for 3 days. A positive indication of the presence of the laccase enzyme is marked by the appearance of a brownish-red zone on the agar to which guaiacol was added.

Fermentation process: The media used for fermentation culture is potato dextrose broth (PDB) media. Preparation begins by sterilizing the PDB media, followed by dissolving the PDB media in 100 mL of distilled water in a 250 mL Erlenmeyer flask. The substrate raw materials in the form of dried rice straw and corn cobs were added to the media. Next, the media was added with $CuSO_4$ inducer with varying concentrations of 1 mM. The media was then sterilized at a temperature of 121°C for 15 min[9].

The investigation of laccase extract produced by *Marasmius* sp., grown in PDB media was conducted by applying different conditions of fermentation media, as follows: 100 mL PDB+20 g rice straws (5:1), 100 mL PDB+20 g corn cobs (5:1) and 100 mL PDB+10 g rice straws+10 g corn cobs (5:0.5 and 5:0.5, respectively) and 100 mL PDB only, as control. The media was then added with $CuSO_4$ inducer with a concentration of 1 mM. Erlenmeyer flasks were inoculated with 1 cm^2 pieces of mushroom mycelium from PDA Petri plates, 3 pieces each. Incubate at 30°C in a water bath shaker incubator (Maskot Sic 50, China) at 100 rpm in dark conditions for 8 days. The extraction of the crude enzyme was carried out by precipitation of the protein with saturated ammonium sulfate (80%) at 4°C for 2 hrs then centrifugation at a speed of 10,000 g for 15 min. Extraction was carried out on culture day 1 to day 8 referring to the modified method.

Laccase activity assay: Laccase activity was measured by spectrophotometric enzymatic assay following the study by Buswell *et al.*[10]. The enzymatic activity test was conducted by using a spectrophotometer (Thermo Fisher Scientific, Madison, USA) at 420 nm. In the microplate, 50 µL of enzyme filtrate was supplemented with 100 µL of 50 mM phosphate buffer (pH 6) and 50 µL of 0.45 mM ABTS and the microplate was incubated for 20 min at room temperature. Laccase activity level expressed as unit (U) is the level of enzymes that can catalyse the oxidation process of phenolic compound in the presence of ABTS as substrate resulting 1 mmol product per minute under certain conditions.

Laccase pH characterization: The pH analysis during the fermentation process on days 6, 7 and 8 at a temperature of 30°C[8].

Statistical analysis: All experiments in this study were duplicates and the results were expressed as mean standard deviation (SD). Data collected was then descriptively analyzed.

RESULTS

Raw material characteristics: Apart from the lignin content, the water content in the substrate also influences the growth of the fungus *Marasmius* sp., in the production of the laccase enzyme. The desired moisture content from drying rice straw and corn cobs does not exceed 14%. The water content of corn cobs and rice straws was tested by gravimetric method. The results of measuring the water content in corn cobs and rice straw were 10 and 10.2%, respectively.

Laccase qualitative assay of laccase: A qualitative test of laccase activity was conducted by adding guaiacol substrate to *Marasmius* sp., culture. Figure 1 demonstrated the formation of a reddish-brown zone representing laccase activity resulting from the oxidation process of guaiacol substrates by laccase produced by *Marasmius* sp. The laccase zone was detected around fungi colonies after 3 days of incubation.

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 lignocellulose wastes-enriched fermentation media. The highest activity of laccase was produced from A4 samples (68.38 U/mL) which was treated with rice straws+corn cobs on day 5 incubation.

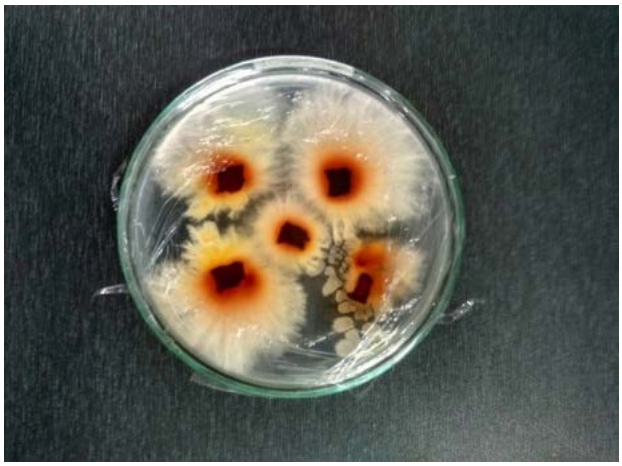

Fig. 1: Reddish-brown zone of *Marasmius* sp., colony on PDA media

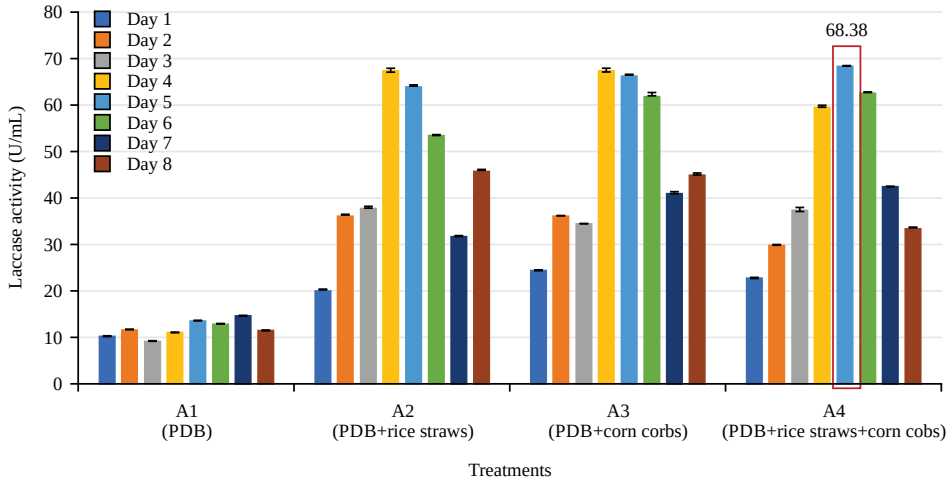

Fig. 2: Levels of laccase activity produced by *Marasmius* sp., in various fermentation conditions

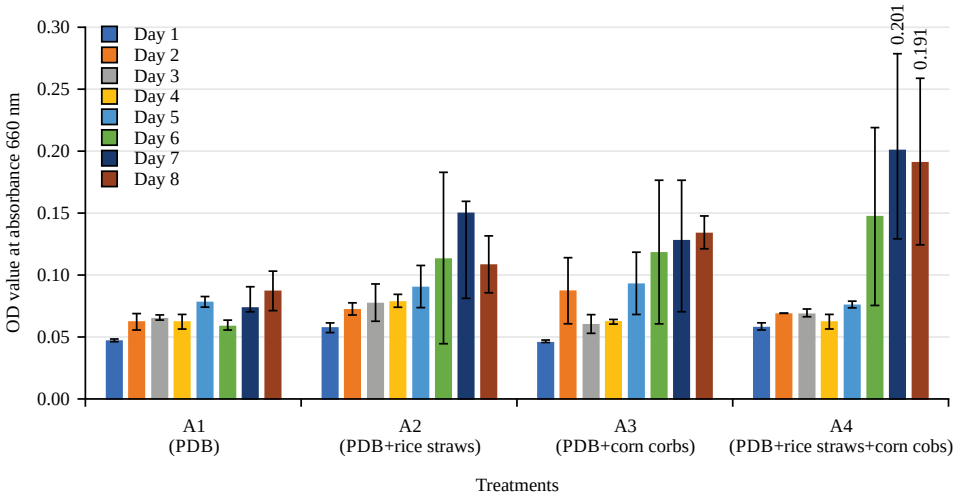

Fig. 3: Cell concentrations of *Marasmius* sp. measured at 660 nm

Table 1: pH value of fermentation media of *Marasmius* sp.

	pH			
Days	A1	A2	A3	A4
6	5.02	5.00	5.64	5.37
7	5.12	5.07	5.69	5.46
8	5.05	5.03	5.53	5.34

A1: PDB, A2: PDB+rice straw, A3: PDB+corn cobs and A4: PDB+rice straw+corn cobs

Cell concentration of *Marasmius* sp.: The results of cell concentration measurements were shown in Fig. 3. Based on this figure, the highest optical density (OD) value for A4 samples was 0.201, which was achieved on day 7 incubation.

pH: According to Table 1, the pH value of the fermentation media of *Marasmius* sp., remained stable at pH 5 showing that the catalytic activity of laccase did not occur during the fermentation process.

DISCUSSION

Rice straw and corncob fiber contain lignocellulose consisting of cellulose, hemicellulose and lignin which influences the growth of the *Marasmius* sp.[11,12]. *Marasmius* sp., can decompose lignin by secreting enzymes such as laccase. Lignin degradation occurs through the oxidation process of phenolic compounds via a single electron transfer mechanism, producing reactive free radicals and simultaneously reducing O_2 molecules to H_2O[13]. The ability of fungi to produce the laccase enzyme is greatly influenced by the production medium. The addition of a substrate containing lignocellulose can be used to produce laccase. Lignocellulosic waste which contains quite high levels of lignin, cellulose and hemicellulose can be used as a natural inducer and nutrient in enzyme production[6]. The high lignin content in the media will trigger the induction of laccase activity which will cause an increase in the activity of the laccase enzyme produced[14].

Lignin can be found in lignocellulosic waste such as rice straw and corn cobs. Apart from natural inducers, laccase activity can also be increased by adding metal ions. Copper (Cu) is the best inducer to increase the activity of the laccase enzyme. Based on research by Bertrand *et al.*[15], the addition of the $CuSO_4$ inducer can increase enzyme activity twofold compared to without the $CuSO_4$ inducer in the production of the laccase enzyme.

The laccase enzyme can be produced through submerged culture fermentation or solid culture fermentation[6]. Submerged culture fermentation involves microorganisms in a liquid medium that is rich in nutrients and has a high concentration of oxygen. The advantage of using a submerged fermentation system is that it makes stirring easier so that the substrate degradation process is more even and the induced enzymes can more easily spread and reach the substrate[16].

Qualitative testing of the laccase enzyme was carried out by subculturing the fungus on PDA media with the addition of 0.02% guaiacol. Qualitative tests were carried out to see whether or not the laccase enzyme was produced by the fungus *Marasmius* sp. From the qualitative test results, it was found that there was a reddish-brown color in the culture of *Marasmius* sp. This indicates the presence of the laccase enzyme. This color is caused by the oxidation reaction of the laccase enzyme against the guaiacol indicator, resulting in a reddish-brown color. Before oxidization, guaiacol has a slightly yellowish color, whereas, after oxidization, guaiacol's color will change to brownish[17].

Laccase extraction is carried out to obtain the laccase enzyme found in the media and fungal cell walls with the addition of saturated ammonium sulfate (80%) for protein precipitation, which is followed by a precipitation process at 4°C to prevent protein damage caused by heat during centrifugation[18].

Measurement of laccase enzyme activity with ABTS substrate is the principle of phenolic oxidation. The oxidation reaction that occurs in ABTS will convert ABTS into the cation radical compound $ABTS^{+}$, which causes the color change when measuring enzyme activity to bluish-green. The activity value of the laccase enzyme can be related to the number of ABTS cations produced[19].

Based on the results of measurements of laccase enzyme activity carried out on PDB fermentation media with the fungus *Marasmius* sp., showed that laccase activity was visible from the first day in all types of treatment, both controls and samples added with lignocellulosic waste material. Laccase production with the highest activity was sample A4, namely the fungus *Marasmius* sp., those treated with the addition of rice straw+corn cob substrate on the 5th day of observation were 68.38 U/mL. Research conducted by Jeon *et al.*[1], also shows that the optimum incubation time for producing the laccase enzyme is on day 5 or 6. In the control treatments, A2, A3 and A4, the activity of the laccase enzyme decreased starting from day 6 due to the lack of nutrition found in the media has begun to decline.

Cell numbers were counted using a spectrophotometer with the turbidimetric method. Turbidity in the media indicates the presence of microorganisms that are growing and developing. The more turbid the media, the more microorganisms there are in the growth media[20]. The cell concentration value is called the optical density (OD) value. Optical density is the amount of light scattered and absorbed by cells in a solution. The interaction between light and microorganism cells can be used to estimate cell quantity. According to Mira *et al.*[19] the best OD (optical density) value is the lowest OD value because the higher the OD value, the more turbid the solution. So, if you see at the graph, the best OD value on sample A4 with a substrate of rice straw and corn cobs with the best OD value 0.058 after the first day of observation.

The difference in the curve between enzyme activity and cell number growth is influenced by many factors, one of which is the substrate used. The diagram shows that each treatment results in the growth of *Marasmius* sp., cells different ones. This could be due to the influence of enzyme substrates and inducers added during the fermentation process of the *Marasmius* sp., fungus where on the 6th day of observation the nutrients from the substrate for the enzyme had decreased so that the enzyme activity decreased.

The catalytic activity of enzymes is also influenced by pH and temperature. When the temperature is low, the chemical reaction will take place slowly, while at high temperature the chemical reaction will take place quickly. Based on research that has been carried out, the optimum pH conditions in producing the laccase enzyme from the fungus *Marasmius* sp. is in the pH range of 5. Based on research conducted by Vantamuri and Kaliwal[5], the optimal pH obtained from *Marasmius* sp., is relatively stable at pH 5-6 according to Halaburgi *et al.*[21], calculation of laccase enzyme activity using ABTS substrate, many laccase enzymes show optimal catalytic pH in the acidic range.

The results of current research showed that there is potential for the production of laccase enzymes from the *Marasmius* sp., fungus using lignocellulosic waste in the form of rice straw and corn cobs with the addition of $CuSO_4$ inducer. Further research is needed to try to use longer incubation times and certain temperatures and pH so that more optimal enzyme quality can be produced. Apart from that, this study also recommend carrying out a lignocellulose content test so that you know the specific lignin content in the substrate used in the research.

CONCLUSION

This study discovered the optimal condition of laccase production from *Marasmius* sp., by supplementing PDB media with lignocellulose-rich waste (rice straws and corn cobs).The utilization of rice straws and corn cobs as a media substrate for laccase production is one of the potential efforts to escalate value of lignocellulosic waste which is produced in abundant amount, particularly in developing countries.

SIGNIFICANCE STATEMENT

This study highlighted the utilization of lignocellulose-rich waste (rice straws and corn cobs) as media supplementation to increase the production of laccase from the *Marasmius* sp. In addition, this study also provides various factors to obtain optimal condition of laccase production, such as the use of natural substrates and inducers, pH, incubation time and temperature.

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