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Original Article

Isolation of White Rot Fungi from Rotten Wood from Bogor Botanical Garden in Indonesia and its Ligninolytic Enzymes Activity

T Sumiati, H Suryadi*, Harmita, Sutriyo

ABSTRACT

Background: White rot fungus is one of the microorganisms that can naturally decompose lignocellulosic biomass. Indonesia's nature with its tropical forests has enormous potential for the development of white rot fungi that can be used as biological resources, one of which is in the bio delignification process. This paper aims to study the isolation and qualitative and quantitative screening of ligninolytic enzymes from white rot fungi found on rotten wood. **Methods:** In this study, white rot fungus was isolated from rotten wood from Bogor Botanical Gardens area, Indonesia. The isolated fungus were screened qualitatively by the Bavendamm test and decolorization of Remazol Brilliant Blue R test. Furthermore, the activity of laccase enzymes, manganese peroxidase and lignin peroxidase were measured by UV-Vis Spectroscopy. Laccase enzyme as an enzyme that has the highest activity is produced in liquid media containing rice husks and purified and its activity is measured. **Result:** The results showed that of the 5 isolates that were positive for Bavendamm test and decolorization of RBBR test, they were KRB1, KRB8, KRB9, KRB10 and KRB12. The highest laccase activity was produced by isolates KRB 12 at 8244.72 U/ml. Laccase was purified by precipitation of ammonium sulfate at a saturation level of 0-80%. Laccase was precipitated optimally in ammonium sulfate saturation 0-20%. The overall yield of the purification was 44.92%, with a purification fold of 1.72 and a specific activity of 5579.95 U/mg protein. **Conclusion:** The result for isolate KRB12 laccase as compared of the reported laccases suggests isolate KRB12 is a potential isolate for the production of laccase enzymes.

Key words: White rot fungi, Isolation, Bavendamm test, Decolorization of RBRR, Laccase, Manganese peroxidase, Lignin peroxidase.

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INTRODUCTION

The potential of microorganisms in Indonesia is very large. Microorganisms naturally have a very important function in the decomposition process. An example of a microorganism is a white rot fungus. White rot fungi secrete extracellular enzymes that can degrade lignocellulosic organic matter. The extracellular enzymes produced by these fungi are not selective but effective in attacking lignin. Lignin-degrading enzymes such as lignin peroxidase (LiP), manganese peroxidase (MnP), and laccase (Lac) are also known as lignin converting enzymes (LMEs).[1,2] Laccase has advantages over other ligninolytic peroxidases because it uses oxygen as an electron acceptor in its catalytic activity, making it more suitable for industrial and environmental purposes.[3] Another advantage is that laccase is an effective lignin-degrading enzyme with high selectivity and low cellulose decomposition rate.[4]

Laccase enzymes have been widely used in various fields such as pulp bleaching processes,[5] bioethanol production[6] and glucose production.[6,7] Research on the isolation of the laccase enzyme has been carried out on *Trametes harzianum,*[8] *Schizophyllum commune,*[9] *Pycnoporus cinnabarinus,*[5] *Trametes versicolor* using the method solid fermentation,[10] *Pleurotus djamor.*[11] From the results of this study, it is known that the laccase enzyme produced from the purified product has a greater lignolytic activity than the crude extract of the enzyme.[8,10]

Rice husk is one of the lignocellulosic wastes whose availability in Indonesia is abundant. Rice husk is produced from 20-22% by weight of grain during the rice milling process. Rice production in Indonesia at 2020 reached around 31,33 million tons, so that around 6.266 million tons of rice husk will be produced.[12] Rice husk is usually used as fuel, animal feed, and a source of silicon manufacture.[13] The chemical content of rice husk is lignin (26 to 31%), cellulose (25 to 35%) and hemicellulose (18 to 21%).[14] In a previous study, it was found that the addition of lignocellulosic waste to the liquid production medium was shown to significantly increase the activity of *Trametes versicolor* laccase.[15] The potential of this ligninolytic enzyme can be used as a bioresource to support economic development towards the development of a green environment. This study aims to isolate and select white rot fungi obtained from natural areas in the Bogor Botanical Gardens in Indonesia. Furthermore, the selected isolates were purified and the levels of their laccase-specific enzymes were analyzed and grown in liquid media containing rice husks. This research is expected to provide information about the potential of laccase produced by local isolates so that it becomes a reference for its development and utilizes rice husks as a substrate for the production of laccase produced.

MATERIALS AND METHODS

Materials

The material used in this study is rotten wood taken from the Bogor botanical garden area. Other materials consist of PDA (Potato Dextrose Agar) media, aquadest, gallic acid (Merck), tannic acid (Merck), Remazol Brilliant Blue R (RBRR) (Merck),

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guaiacol (Merck), acetic buffer pH 5, peroxide, veratryl alcohol, 80 mesh Cibinong rice husks, distilled water, Potato Dextrose Agar (PDA), 2,2'-azino-bis (3-ethylbenzothiazolineacid)-6-sulfonate) (ABTS) (Merck), ammonium sulfate ($(NH_4)2SO_4$), CH_3COOH, CH_3COONa, malt extract (Difco), Tween80 (Merck), KH_2PO_4, $MgSO_4.7H_2O$, $CaCl_2$, $FeSO_4.7H_2O$, $MnSO_4.H_2O$, $NaMoO_4.2H_2O$, H_3BO_3, $CuSO_4.5H_2O$, $ZnSO_4.7H_2O$, NH_4NO_3, $Co(NO_3)2.6H_2O$, Bovine Serum Albumine (BSA) (Merck), Bradford's reagent, ethanol (Merck), and ethyl diamine tetra acetate (EDTA). All of chemicals used in this research were of analytical grade. The apparatus used in this study were analytical balance (aculab), petri dish, test tube (pyrex), ose, hot plate stirrer (Corning), refrigerator, autoclave (Tomy), oven (WTB Binder), incubator (Memmert), filter paper, water bath shaker, pH meter (Hanna), centrifugator (Kubota 6800), volume pipette and other glassware commonly used in laboratories. Analysis of the instrument used is a UV-Vis spectrophotometer (Shimadzu UV-1601).

Sampling

In this study, the visual method was used for the sampling process based on direct observation of wood infected with fungi. Furthermore, the sample is cleaned and put into a clean plastic bag and stored in a room at room temperature until the isolation process.

Isolation of enzyme-producing fungi

Isolation of the fungi is done by taking a sample of 1g of rotten wood, then washing it with distilled water. Then crushed in a mortar that has been cleaned with 70% alcohol. The crush is aseptically inserted into an Erlenmeyer flask which has been filled with sterile distilled water as much as 10 ml to obtain a cell suspension or hyphae cut suspension. The suspension was shaken for 1 minute, then diluted the sample to 10^{-8} using a sterile distilled water solution. From the last three series of dilutions, 0.1 ml each was taken with a micropipette and spread by means of a spread plate on PDA medium containing 0.05% chloramphenicol in a petri dish. Petri dishes were incubated at room temperature for 2-7 days. The growth of the formed fungi was observed and the growing fungi colonies were transferred to a new sterile PDA medium.

Ligninolytic enzyme activity screening

The fungal isolates that had been obtained were then tested for their lignolytic ability to degrade lignin using the Bavendamm test and decolorization of Remazol Brilliant Blue R (RBBR).

Bavendamm test

In the Bavendamm test method, each isolate is tested and isolated on a selective medium for lignin degradation by the point method, then incubated for 2-7 days at room temperature 28 ° C. The selective medium used was PDA medium with the addition 0.5% gallic acid or 0.5% tannic acid as a carbon source.

Decolorization of RBBR test

In the decolorization of RBRR method, PDA selective medium was used with the addition of 0.05% RBRR. Remazol Briliant Blue R-PDA media consists of two layers. The first layer is made of PDA media. The autoclaved PDA solution was poured into 10 mL petri dishes and allowed to solidify. The second layer was made of agar which was added with RBBR. After the media cooled and solidified, one agar plugs (Ø = ± 0.5 cm) of PDA culture was inoculated on the medium and then incubated at room temperature. Mycelium growth and change in media colour were analyzed every day.

Enzyme production medium

The media used for enzyme production is the original culture medium according to Kirk's base medium.[16,17]

Enzyme activity analysis

The enzymes to be measured are laccase (Lac), manganese peroxidase (MnP) and lignin peroxidase (LiP). Enzyme sources for quantitative testing were prepared by growing fungal isolates on enzyme production media. At the end of the incubation period, the liquid cultures were separated by filtration to separate the fungal mycelia. The obtained liquid media contains crude enzymes that are ready to be tested.[18] Laccase enzyme activity was measured by mixing 0.4 mL of enzyme filtrate plus 0.5 mL of 0.05 M acetate buffer pH 5 and 0.1 mL 1 mM ABTS. The solution was read at a wavelength of 420 nm minute 0 and minute 30.[19] MnP enzyme activity was measured by mixing 0.1 mL 50 mM Na-lactate buffer pH 5 with 0.1 mL 4 mM guaiacol; 0.2 mL 1mM MnSO4; 0.1 ml of 1 mM H_2O_2, 0.3 ml of distilled water and 0.2 ml of enzyme filtrate. The solution was read at 0 and 30 min at a wavelength of 465 nm.[19] LiP enzyme activity was measured by mixing 0.1 mL of 8 mM Veratrile alcohol added with 0.2 mL of 0.05 M acetate buffer pH 5, 0.4 mL of distilled water, 0.1 mL of 1 mM H_2O_2 and 0.2 mL of enzyme filtrate. The solution was read at 0 and 30 min at a wavelength of 310 nm.[20] The enzyme activity measured was expressed in units per mL (U/mL) which was defined as the amount of enzyme required to catalyze 1µmol. substrate in one minute.

Enzyme production time

Rice husk substrate as much as 1%, 2%, and 3% were added to the enzyme production media according to the kirk medium.[16,21] The media was then autoclaved at 121 °C for 20 minutes. Then three of agar plugs (Ø = ±0.5 cm) 7-day-old fungus were inoculated into the enzyme production medium and incubated at 28°C for 13 days. Sampling was carried out every day until 13 day. The enzyme was extracted with 0.05 M acetate buffer pH 5 and the enzyme activity was measured. The incubation time with the highest enzyme activity was used as the optimum production time of crude extract.

Crude extract production

Three of agar plugs (Ø = ±0.5 cm) of 7-day-old fungus were inoculated into the enzyme production medium with the addition of rice husk as substrate. Then the production medium was incubated at room temperature with 125 rpm shaking. Cells were harvested during fermentation which indicated the optimum enzyme production time. The mycelium growing on the production media was ground with a buffer using a sterile mortar at a temperature of 4°C. The enzyme was extracted with 0.05 M acetate buffer pH 5. The mashed mycelium with the production medium was then centrifuged at 7000 rpm for 10 minutes at 4°C. The resulting supernatant is a crude extract of the enzyme.[22]

Partial purification by precipitation of ammonium sulfate

The method used is based on Asgher, Nasir Iqbal and Asad (2012) which has been modified.[10] The supernatant which is a crude extract of lignolytic enzymes was precipitated using ammonium sulfate at saturation levels of 20-40%, 40-60%, and 60-80%.

Determination of protein content

Determination of laccase protein levels using Bovine Serum Albumin (BSA) as a protein standard. The concentration of BSA used was 0.1-1.00 mg/ml. Then the protein standard was measured by adding 0.1 ml of the standard solution in series with 5 ml of Bradford's reagent. This solution gives a blue color and is read at a wavelength of 595 nm. By using linear regression, a mathematical equation for the protein standard solution obtained from the standard absorbance value will be obtained which will be used to measure the dissolved protein content.[23]

RESULTS AND DISCUSSION

White rot fungi of isolation

The rotten wood was taken from the Bogor Botanical Gardens area, then the fungi was isolated using Potato Dextrosa Agar (PDA) media. The mold isolates were incubated for 3-5 days at room temperature and purified of the mold isolates growing in PDA media. The purification results obtained 13 fungi isolates. From the observations, it was found that the colony color of all isolates was white like cotton and there were hyphae in all colonies.

Qualitative screening of ligninolytic enzyme activity using Bavendamm test and decolorization of remazol brilliant blue R (RBRR)

The fungal isolates that had been obtained were then tested for their lignolytic ability to degrade lignin using the Bavendamm test and decolorization of RBRR test. In the Bavendamm test method, each isolate is tested and isolated on a selective medium for lignin degradation by the point method, then incubated for 2-7 days at room temperature 28 ° C. The selective medium used is PDA medium with the addition of 0.5% gallic acid or 0.5% tannic acid as a carbon source.[24] The Bavendamm test showed a positive reaction which was indicated by the formation of a light to dark brown zone in the medium containing gallic acid and tannic acid around the colony. It is suspected that the cultured isolates secrete extracellular oxidase enzymes by oxidation reactions with gallic acid or tannic acid. In the RBBR decolorization test, the ligninolytic ability of isolates was evaluated by looking at the color change of the medium from blue to colorless, this was due to the oxidation process and the breaking of anthraquinone bonds.[25] The results of the Bavendamm test and decolorization of RBRR test can be seen in Table 1.

In the Bavendamm test using gallic acid, the color change to brown on the media was seen in eight isolates, namely KRB1, KRB2, KRB5, KRB7, KRB8, KRB9, KRB10 and KRB12. The change in media color was the fastest and the greatest intensity occurred at KRB12. The colour change in the media containing KRB12 isolate for 1-7 days can be seen in Figure 1.

In the Bavendamm test using tannic acid, the change in colour to brown on the media was seen in nine isolates, namely KRB1, KRB2, KRB5, KRB7, KRB8, KRB9, KRB10, KRB12 and KRB13. The change in media colour was the fastest and the greatest intensity occurred at KRB 12. The color change in the media containing KRB12 isolate for 1-7 days can be seen in Figure 2.

Screening for decolorization of RBBR on media showed positive results in eight isolates, namely KRB1, KRB3, KRB4, KRB6, KRB8, KRB9, KRB10 and KRB12. The blue colour of the RBBR comes from the anthraquinone chromophore moiety. The cleavage of anthraquinone bonds by ligninolytic enzymes causes the medium to change colour from blue to colorless, dark brown or light brown. Isolates that were able to change the colour of RBBR media indicated that these isolates produced ligninolytic enzymes. The fastest change in media colour and the greatest intensity occurred at KRB12. The colour change in the media containing KRB12 isolate for 1-7 days can be seen in Figure 3.

Based on the results of the Bavendamm test on gallic acid and tannic acid medium as well as the decolorization of RBRR test, the isolates suspected to have ligninolytic extracellular enzymes were isolates KRB1, KRB8, KRB9, KRB10 and KRB12. Screening of ligninolytic activity was based on the formation of a brown colour zone on media containing gallic acid and tannic acid. The formation of a brown zone proves that the fungus is able to decompose lignin in the presence of ligninolytic enzymes. The result of screening Ligninolytic Activity based on the colour Zone in Gallic acid and Tannic Acid Media can be seen in Table 2.

Quantitative screening of ligninolytic enzyme activity

A total of 5 isolates isolated from fungi were selected from weathered wood and re-cultured in PDB liquid medium for 7 days at room temperature and the production of laccase enzymes (Lac), Manganese Peroxidase (MnP) and Lignin Peroxidase (LiP) were measured. Determination of the highest production of laccase enzyme was produced by KRB12 isolate at 8244.72U/mL, followed by KRB8 isolate at 3243.54 U/mL and KRB1 isolate at 3239.68 U/mL as shown in Table 3.

Table 1: Bavendamm test results and decolorization of RBBR on white rot isolates.

Fungi code	Bavendamm Test		Decolorization of RBRR
	Gallic acid oxidation	Tannic acid oxidation	
KRB1	√	√	√
KRB2	√	√	-
KRB3	-	-	√
KRB4	-	-	√
KRB5	√	√	-
KRB6	-	-	√
KRB7	√	√	-
KRB8	√	√	√
KRB9	√	√	√
KRB10	√	√	√
KRB11	-	-	-
KRB12	√	√	√
KRB13	-	√	-

Table 2: The result of screening ligninolytic activity based on the colour zone in gallic acid and tannic acid media.

No	Isolate	Diameter of colour zona (cm)	
		Gallic acid	Tannic acid
1	KRB 1	0.66	0.9
2	KRB 8	0.45	2.5
3	KRB 9	1.25	1.8
4	KRB 10	0.95	0.95
5	KRB 12	5	5

Table 3: Measurement of laccase (Lac), manganese peroxidase (MnP) and lignin peroxidase (LiP) activity.

Isolate	Enzyme activity (U/mL)		
	Lac	MnP	LiP
KRB 1	3239.44	1106.04	3881.91
KRB 8	3243.33	82.31	6626.31
KRB 9	2344.64	5486.49	5302.89
KRB 10	412.39	282.94	362.61
KRB 12	8244.72	66.88	3261.62

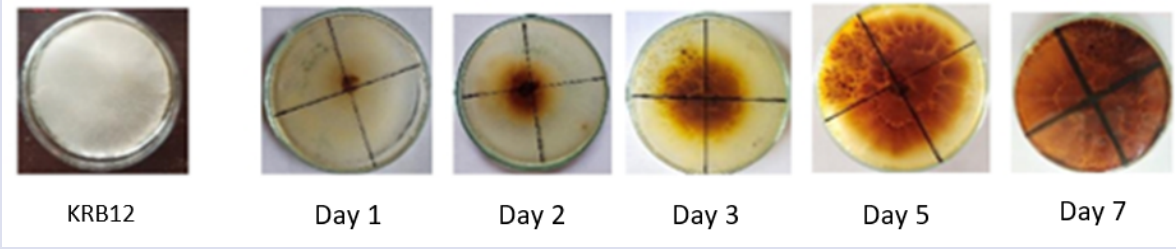

Figure 1: Colour changes in the Bavendamm test using gallic acid on KRB12 isolates.

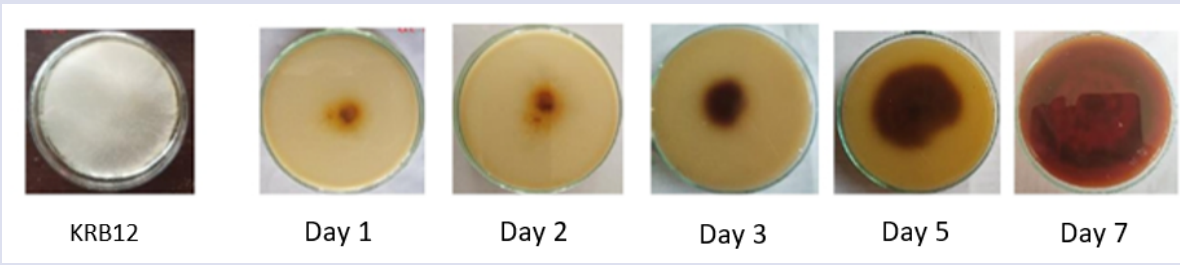

Figure 2: Colour changes in the Bavendamm test using tannic acid on KRB 12 isolates.

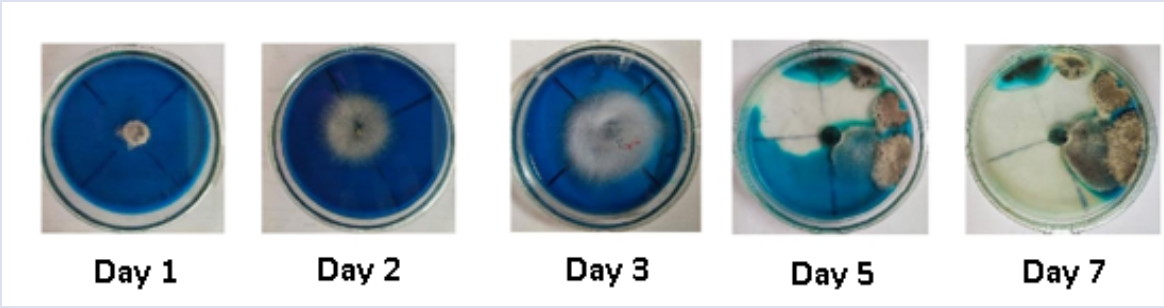

Figure 3: Colour changes in the Bavendamm test using tannic acid on KRB12 isolates.

The highest production of the MnP enzyme was produced by isolate KRB9 at 5486.49 U/mL, KRB1 at 1106.04U/mL, followed by KRB10 at 282.94 U/mL. The highest production of the LiP enzyme was produced by isolate KRB8 at 6626.31U/mL, followed by KRB9 at 5302.89 U/mL and KRB1 at 3881.91U/mL as shown in Table 3.

Fungus can grow on PDB media that are not rich in nitrogen sources because their composition only contains potato juice and dextrose. Fungi are able to secrete the enzymes laccase, manganese peroxidase and lignin peroxidase in the medium. From the results of the study, in general the laccase enzyme had higher activity than the activity of manganese peroxidase and lignin peroxidase at the stage of screening ligninolytic enzyme activity with PDB growth media. This is also in accordance with the research of Vaitanomsat *et al.* (2013) who reported higher laccase activity than manganese peroxidase with the same medium during the screening stage.

From table 3, it can be seen that there are differences in the activity of each isolate. This was suggested to occur because it is influenced by differences in the ability of fungi to change the existing substrate. The enzymes produced by each isolate had different speeds and abilities in converting substrates into simpler compound products.

Time and substrate concentration for optimum lac enzyme production

In this study, a liquid fermentation technique was used to produce laccase. The advantage of liquid fermentation is that it is easier to stir so

Table 4: The results of laccase activity.

Fraction	Total volume (mL)	Total enzyme activity (U)	Total protein content (mg)	Specific enzyme activity (U/mg)	Purification fold	Yield(%)
Crude enzyme	100	91641.59	28.28	3240.34	1	100
Fraction (0-20%)	15	41160.91	7.38	5579.95	1.72	44.92
Fraction (20-40%)	15	2775.18	1.13	2452.63	0.76	3.03
Fraction (40-60%)	15	233.43	0.46	509.16	0.16	0.25
Fraction (60-80%)	15	33.07	0.41	79.85	0.02	0.04

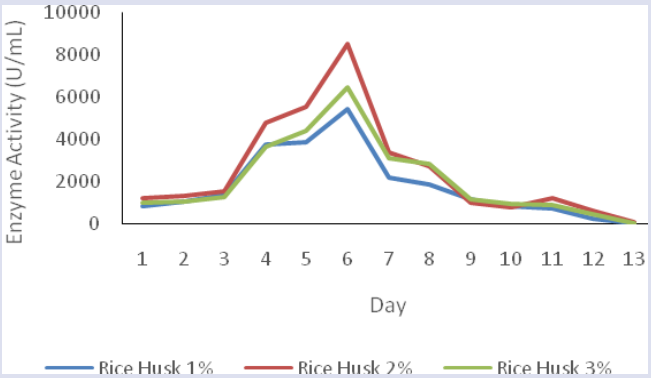

Figure 4: Optimization of laccase production time of white rot fungi KRB12 isolate.

that the substrate degradation process is more evenly distributed and the induced enzymes are easier to spread and reach the substrate. The use of suitable production media for fungal growth also affects laccase activity.[10] Rice husk was added to the production medium which was used as a carbon source, yeast extract served as a nitrogen source and carbon source at the beginning of fungal growth.[10]

In this study, an optimization of enzyme production time was carried out to determine the time required for KRB12 to produce laccase with the addition of husk as a substrate of rice lignocellulosic material at concentrations of 1% (w/v), 2% (%w/v) and 3% (w/v). In another study it was reported that the laccase activity of *T. versicolor* increased significantly with the addition of lignocellulosic material.[15] The optimization results showed that laccase activity was seen on the first day. This is in accordance with the results of previous studies which stated that laccase was produced at the beginning of mycelium growth.[26] Nutritional needs increased the next day so that laccase production also increased and reached its peak on day 6. After on 6 day, laccase activity decreased because the nutritional needs of the fungus had been met so that the role of laccase was no longer needed. Laccase activity was detected on the first day after fungal mycelium was planted on the media. Enzyme activity continued to increase significantly until day 6. The activity on 6 day was the highest activity and on 7 day the enzyme activity decreased sharply, after that the enzyme activity decreased until 13 day.

On the addition of rice husk substrate, it was found that for the addition of 1%, 2% and 3% husk from the first day to the sixth day there was an increase and decrease after on 7 until 13 day. In other studies it has also been reported that the laccase activity of *Trametes versicolor* reaches its maximum on 5 day of incubation,[27] and *T. versicolor IBL-04* also reaches its maximum on 5 day of incubation.[10] In this study, the highest laccase enzyme activity occurred on 6 day of adding 2% rice husk substrate with an activity at 8551.16 U/mL. The addition of 1% rice husk gave an activity at 5471.82 U/mL. The addition of 1% rice husk gave an activity at 6456.24 U/mL. The results can be seen in Figure 4.

Laccase partial isolation and purification results

The crude laccase extract obtained was purified by the ammonium sulfate precipitation technique in stages. The advantage of using ammonium sulfate salt to precipitate protein is because of its high solubility even at low temperatures, low price, and does not affect the structure of the protein. The activity specific of crude laccase extract produced in this study was 3240.34 U/mg. The results of the calculation of laccase activity can be seen in table 4.

Determination of laccase protein content in this study used the Bradford method. The purity of an enzyme can be measured by the specific activity value. The value of specificactivity indicates the amount of enzyme concentration contained in the sample. Specific activity was defined as the unit of enzyme activity per milligram of protein. Based on the purification results (Table 1), laccase had the highest specificactivity in the 0-20% fraction among the four tested fractions. This indicates that the laccase has been maximally precipitate data saturation level of 0-20% ammonium sulfate. In previous studies, it was found that the laccase of *T. versicolor* IBL-04 precipitated maximally at 90% saturation[10], *T.versicolor* sdu-4 at 58% saturation[28] and *T. versicolor* MTCC 138 at 70% saturation.[9] The difference in thesaturation level of ammonium sulfate is related to the amount of hydrophilic and hydrophobic aminoacids on the tertiary surface of the enzyme. Enzyme proteins containing more hydrophilic amino acids on the surface will interact strongly with water, therefore large amounts of salt ions are needed to interfere with these interactions so that precipitation can occur. On the other hand, enzyme proteins containing more hydrophobic amino acids require low salt concentrations to precipitate them. The specific activity of laccase at a saturation level of 0-20% (5579.95 U/mg) was greater than the specific activity of laccase crude extract (3240.34 U/mg). This indicates that the laccase was successfully purified. Purification of laccase with ammonium sulfate precipitation technique increased the purity to 1.72 times of the crude extract with a yield of 44.92%. Theyield value in this study was greater than *T. versicolor* IBL-04[10] was 13.80 with a Purification fold at 1.16. The difference in the specific activity obtained wasinfluencedby the source of the enzyme producing and the growth medium used.

CONCLUSION

In this study, the 5 isolates observed showed a positive reaction to Bavendamm test and RBRR decolorization in isolates, namely KRB1, KRB8, KRB9, KRB10 and KRB12. The highest laccase activity was produced by isolate KRB12 at 8244.72 U/mL. The highest MnP enzyme activity was produced by isolate KRB9 at 5486.49 U/mL. The highest LiP enzyme activity was produced by isolate KRB8 at 6626.31 U/mL. The optimization time of laccase enzyme production in 2% rice husk media was 6 days with an enzyme activity at 8551.16 mU/mL. Laccase isolated from KRB12 isolate was successfully purified by ammonium sulfate precipitation technique. The results of purification of ammonium sulfate 0-20% were able to increase the purity of laccase 1.72 times compared to crude extract of the enzyme with a yield of 44.92% and a specific activity of 5579.95 U/mg

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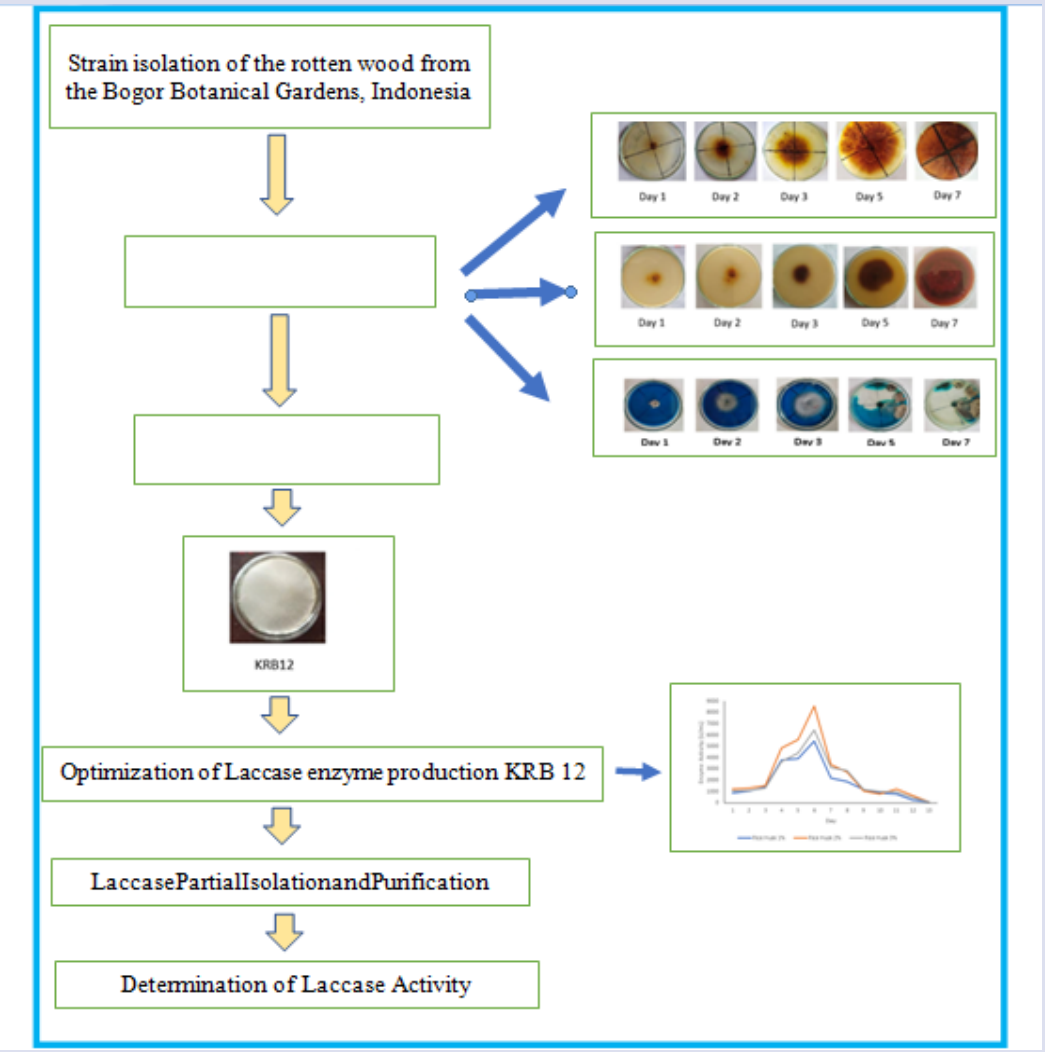
GRAPHICAL ABSTRACT



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