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Submission date: 26-Apr-2021 11:18AM (UTC+0800)
Submission ID: 1569781973
File name: 5._Charactyerization_of_fungal_laccase.pdf (1.33M)
Word count: 6049
Character count: 30303

Biocatalysis and Agricultural Biotechnology 27 (2020) 101676

Contents lists available at ScienceDirect

Biocatalysis and Agricultural Biotechnology

journal homepage: http://www.elsevier.com/locate/bab

Characterization of Fungal Laccase Isolated from oil palm empty fruit bunches (OPEFB) and Its Degradation from The Agriculture Waste

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ARTICLE INFO

Keywords:
Laccase
Lignin
Fungi
Scanning electron microscope

ABSTRACT

In recent years, many studies have been developed to overcome the abundance of lignocellulosic waste. Laccase is a metalloenzyme that can degrade lignin. This enzyme has useful properties that make it applicable for the agricultural sector in Indonesia. The present study is designed to isolate and determine the activity of laccase found in the fungi of oil palm empty fruit bunch (OPEFB), and the fungal laccase was precipitated and partially purified by using ammonium sulfate. The optimum temperature of fungal laccase was 40 °C for fungus lacc-B and 50 °C for fungus lacc-A, C, and D. The optimum pH was obtained 5.0 for fungus lacc-A and D, pH 6.0 for fungus lacc-C and pH 7.0 for fungus lacc-B. Furthermore, the activity of crude extract were 1.4 U.mL^{-1}, 1.5 U.mL^{-1}, 1.3 U.mL^{-1}, and 1.3 U.mL^{-1} for fungus lacc-A, B, C, and D. Purification levels of fungus lacc-A, B, C, and D increased 1.7, 1.6, 1.9, and 2.5 times, respectively after ammonium sulfate precipitation and dialysis. Fungus lacc-D has the highest level of laccase activity than other fungi. Physical analysis of the agricultural waste after fungus lacc-D addition indicated that the surface profile is damaged, hollow, and broken. The delignification from corn cob and rice straw was observed with scanning electron microscope (SEM) which showed the presence of morphological changes in the lignocellulose waste sample.

1. Introduction

In recent years, there has been quite a lot of studies that have been developed to overcome the abundance of lignocellulosic waste. Lignocellulose is a renewable plant resource (Da Silva et al., 2013). Conversion of this lignocellulosic waste into useful materials, such as organic fertilizer, animal feed and bioethanol, will increase the value of lignocellulosic waste. However, the utilization of these wastes has not shown optimal results, such as the utilization of rice straw waste into bioethanol (Devendra and Pandey, 2016).

Palm oil is one of the export commodities from the agriculture sector that has an important contribution to the economy of Indonesia. Based on the data for 2019 by The Central Bureau of Statistics (Badan Pusat Statistik Indonesia, 2020), Indonesia's palm oil production reaches 34.40 million tons at 2017. About 25–26% of the total palm oil production is extracted from empty bunches, which are the by products (Dewanti, 2018); only 10% of the oil palm empty fruit bunches (OPEFB) has been used for boiler or composting, and the rest is still in the form of waste (Ngadi and Lani, 2014). OPEFB are composed of 25.83% lignin (Sudiyani et al., 2013). This component is so high that it can be ascertained that the fungus from OPEFB contains laccase, which can disintegrate the lignin content. It has been reported that the fungi found in woody plants are able to degrade lignin (Arora and Gill, 2000), so it can be ascertained that fungi are one of the laccase producing organisms (Ire and Ahuekwe, 2016). Interestingly, there are no reported the existence of fungal laccase from palm oil empty fruit bunches (OPEFB) yet, meanwhile Indonesia is one of the biggest palm oil plantation in the world with Malaysia and Thailand.

In general, the high content of lignin is pre-treated with acidic or alkaline solutions. However, the use of chemicals in pre-treatment of

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https://doi.org/10.1016/j.bcab.2020.101676
Received 29 March 2020; Received in revised form 23 May 2020; Accepted 4 June 2020
Available online 7 June 2020

lignin degradation can rising the new environment problems. Improper handling of these chemicals can pollute the environment. In order to overcome this problem, there is an alternative method of using enzymes capable of degrading lignins like laccase. Laccase can be isolated from fungi or mushrooms (Senthivelan, 2019; Pandey et al., 2018; Lin et al., 2018). Laccase is a metalloenzyme that can degrade lignin during the bio-catalysis process (Kameshwar and Qin, 2016). The laccase is used in the pulp, paper, and textile industries to efficiently obtain environment friendly products as this enzyme requires only oxygen and produces water as the only side product (Riva, 2006). In addition, laccase effectively and spontaneously catalyze the oxidation process of both phenolic and non-phenolic aromatic compounds by reducing molecular oxygen to water. Laccase is an oxidizing enzyme group, it can oxidize various substrates such as di-phenol, substituted methoxy-phenol, and aliphatic and aromatic amines to a radical form and reduce oxygen to water (Muthukumarasamy,and S.Murugan, 2014). Laccase can be used as an alternative solution to avoid the lignin interference on lignocellulosic waste. The laccase enzyme works specifically to degrade lignin. Lignin degradation causes the hemicellulose and cellulose in the lignin complex to disconnect, followed by the degradation of hemicellulose and cellulose by xylanase and cellulose respectively (Chandra, 2015). In addition to the specificity of enzyme performance, another reason for using laccase as an alternative solution is the rate of reaction, as a biocatalyst enzyme reactions are faster than non-enzymatic reactions (Pelczar and Chan, 1986).

This study aims to isolate the fungal laccase from OPEFB and optimize the temperature and pH during mycelium growth in order to study the activity of fungal laccase. Fungal laccase was added to the corn cob and rice straw waste to determine the effect of fungal laccase when degrading hemicellulose and cellulose covalent linker in the lignocellulosic waste.

2. Material and methods

2.1. Isolation of fungi from OPEFB

Fungi were collected from OPEFB waste, located at a palm oil farm in Riau, The Central Eastern Coast of Sumatra, Indonesia. The fungi from OPEFB was isolated using the spread plate method. Five grams of OPEFB were dissolved in sterilized water to minimize the possibility of contaminants (i.e., other organisms that grow on OPEFB). Furthermore, sterilized water containing spores were grown on the medium of Potato Dextrose Agar (PDA) for 5 days at room temperature. After incubation, molds of varying colors grew in the PDA. The color variations of the mold that grew in the PDA indicate that they are heterogeneous (Fig. 1a). Furthermore, purification was done using a slanted PDA medium by inoculating with each spore on a slanted PDA medium. The pure fungus was characterized by a uniform color that grew on a slanted PDA medium (Fig. 1b).

2.2. Selection of isolate

The culture was placed on a Petri dish selective black liquor medium consist of 0.01% yeast extract, 0.05% $MgSO_4$, 0.15% KH_2PO_4, 0.01% NH_4NO_3, 5% NaCl (v/v), 1.5% bacto agar and 95% black liquor from pulping waste. The final pH was 7.2 and incubated at 37 °C for 5 days. The halo (clear area) was observed around the growing colony, and then one isolate with the largest halo area was calculated to find the index value. The largest halo also indicated the highest activity of fungal laccase. The Halo index value was measured by calculating the diameter of halo area divide to diameter of the colony area with equation of Halo index = (d halo)/(d koloni).

2.3. Fungal mycelium growth from OPEFB

Assessment of the growth curve was carried out using dry cell weight measurement. A selected fungus was grown on the Glucose Malt Yeast (GMY) medium consist of 4% of glucose, 4% of Yeast Extract, and 10% of Malt Extract. This mixture were dissolve on distilled water until pH 7.2 using KOH. The inoculum was incubated at room temperature and shaken at 150 rpm for 14 days. The mycelium mass was measured every day, then the incubated GMY was centrifuged at 3300 rpm for 2 min at 4 °C.

2.4. Determination of laccase activity

The enzyme assay was carried out using the Bourbonnais and Paice method (Bourbonnais and Paice, 1990) The laccase activity was determined by adding 40 μl of the enzyme solution to 1160 μl of ABTS (2, 2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (0.4 mM) dissolved in sodium-acetic buffer with a pH of 4.5 (Kalyani et al., 2008). Mixture was then shaken until it became homogenous. Subsequently,

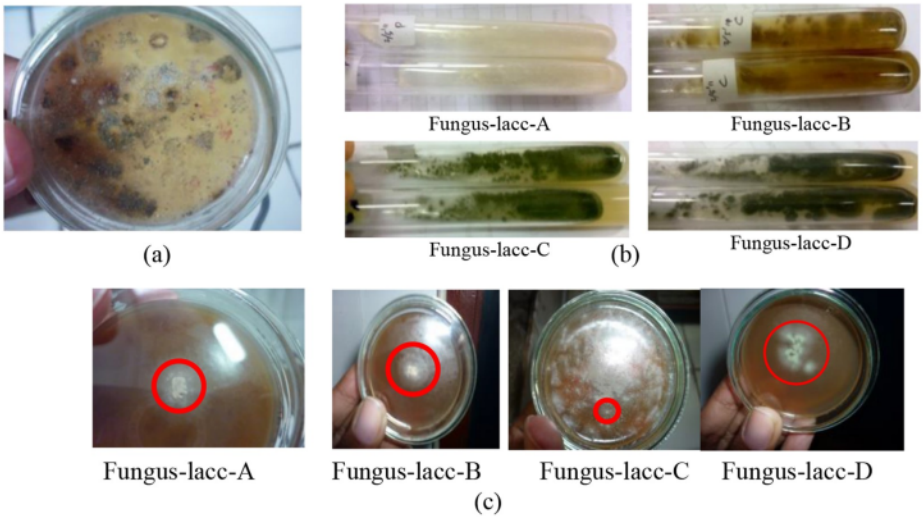

Fig. 1. (a) Fungal isolates from the isolation of OPEFB; (b) A pure colony of fungus isolation (Fungus lacc-A, B, C, and D), (c) Halo area of Fungus lacc-A,B,C, and D.

the absorption of the radical cation was measured at $\lambda = 420$ nm ($\varepsilon_{mM} = 36\ mM^{-1}cm^{-1}$) for 5 min using spectrophotometer UV–Vis (Bar, 2001). One unit (U) of laccase activity was defined as the amount of enzyme needed to release 1 μmol ABTS per minute per ml. The calculation of the protein concentration was performed using the Bradford method (Bradford, 1976).

2.5. Laccase characterization

Laccase is an extracellular enzyme secreted by fungi in its production medium. To obtain the laccase enzyme, growth media was separated from the fungus cells through filtering process. Supernatant was collected for further analyses. The optimum temperature measured was diverse, ranging from 20 to 80 °C, and the optimum pH was measured in the range of 4.0–8.0 (0.1 M phosphate citrate buffer) at optimum temperature. Thermostability was measured at an optimum temperature in the absence of substrate for 10 h of incubation time and sampling every 2 h. The pH stability was measured in the range of 4.0–8.0, sample was incubated for 1 h at the optimum temperature without substrate.

2.6. Ammonium sulfate precipitation

Proteins were sequentially precipitated from crude extract by the stepwise addition of solid ammonium sulfate, stirred until a certain degree of saturation (Bollag, 1996). This was followed by incubation on ice, slowly stirring for at least 30 min, then centrifugation at 6000 rpm for 20 min at 4 °C. The pellet obtained after each centrifugation was re-suspended in 5 mL of 0.1 M universal buffer pH 7.0. The above steps were followed by 40–100% saturation of ammonium sulfate.

2.7. Dialysis

The cellophane tube containing the laccase was stirred and immersed in a 0.05 M universal buffer solution with optimum pH (optimum pH for each fungus of lacc-A, B, C and D) for dialysis. This dialysis process was stopped when enzyme solution was separated from the ammonium sulfate salt. The dialysis process was declared complete when the enzyme solution was free of ammonium sulfate, characterized by the absence of a brown precipitate when Nessler's reagent was added into the buffer solution.

2.8. Pretreatment of cereal agriculture waste using fungal laccase

Five grams of rice straw and corn cob were added with 5 mL of laccase until the sample was immersed in the enzyme. The mixtures were incubated at an optimum temperature of fungal laccase i.e. 50 °C for 3 h. The surface profile of corn cob and rice straw as part of the cereal agriculture waste was analyzed by using a scanning electron microscope (SEM).

3. Result and discussion

3.1. Selection of fungus isolate

The fungi A, B, C, and D were selected because of their ability to degrade lignin (Fig. 1b). Fungi were grown in the selective medium of black liquor and the halo area was observed. Fungi A and B have a halo index value of 2.0, while Fungus C was 1.7 and Fungus D was 2.3, respectively. This result showed that all fungi produced laccase, as shown by the presence of the halo area around the fungi colonies. The laccase activity in these fungi is proven by the presence of the halo region in the selective media test. The fungi showed almost similar halo index in the halo test, which confirms the presence of lignin degradation in these selective media as in Fig. 1 (c).

The selective media is a source of carbon and minerals. The medium of black liquor is made from a mixture of black liquor and salts. Black liquor serves as the main carbon source (C), whereas the salts serve as a mineral source. Black liquor is the soluble solution of cooking chemicals in the pulping process, which contains many lignin. Black liquor still contains the chemical compounds of Sodium Hydroxide (NaOH) and Sodium Sulfide (Na_2S), which is alkaline (Wallberg et al., 2003). A neutral pH had to be obtained to prevent interference with the fungus growth, so 1 N Hydrochloric acid (HCl) was added to the black liquor.

Lignin was the only source of carbon in the selective medium. The halo area was appeared in the selective media contained of lignin indicated the degradation activity of fungus by secreting extracellular laccase. The halo area is a clear region of the medium around the fungus. The size of the halo area depends on the unit activity of laccase. The wider area of the halo indicates the higher the enzyme activity. These results indicated that all fungi have laccase activity, but the difference in the size of halo area was not significant.

3.2. Fungal growth from OPEFB

In this study, GMY was used to determine the growth curve and laccase activity of OPEFB isolated fungi. GMY is a selective growth medium with low pH useful for cultivating yeasts, fungi, molds, or other acid-tolerant or acidophilic organisms, importantly also deterring growth of most bacteria and other acid intolerant organisms. Other than that, the containing of malt in GMY can induce the laccase production.

The growth curve of fungal laccase production was made in 14 days. Based on Fig. 2, each fungi have different optimum incubation time for their growth; fungus lacc-A and fungus lacc-C reached the optimum growth of 7 days incubation time, while fungus lacc-B reached the optimum growth of 8 days incubation time, and fungus lacc-D reached the optimum growth of 10 days incubation time. Based on the growth curve, the rapid growth phase (log phase) can be determined at the point where cells divide rapidly and constantly. In the next phase, the stationary phase, the number of stem population is almost unchanged. The absence of changes in the stationary phase is due to the decreased nutrients, while the microorganisms continue to divide (Schlegel and Schmidt, 1994). After the stationary phase, there was a decrease in the dry cell weight due to the cell lysis that resulted in cell death. This phase is called the death phase. In this phase, the growth rate is lower than the death rate. The speed of fungus growth was decreased because the nutrients contained in the medium were increasingly exhausted.

The growth curve of fungus lacc-A showed the highest dry cell weight and the significant change in the log phase indicated the high of cell division. Fungus lacc-D showed the longest lag phase range, which was assumed to be the longer adaptation time of the fungus to the medium. Unfortunately, the growth curve for lacc-A and lacc-B were suddenly decreased after 7–8 days. Those were assumed caused by cell autolysis where in general the cell wall and macromolecular components are degraded and returned to the medium as the dead cells undergo necrosis (Moore, D. et al., 2019).

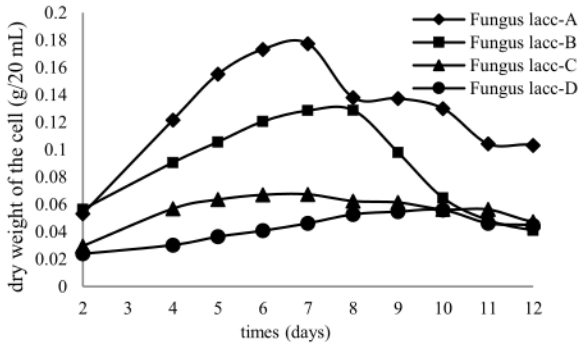

Fig. 2. Growth curve of Fungus lacc-A, B, C, and D.

3.3. Characterization of fungal laccase

3.3.1. Optimum temperature and pH

Fungal laccase activity was observed at temperatures of 20–80 °C. Fungus lacc-A, C and D showed the highest activity of laccase at 50 °C for 5.5×10^{-3} U.mL^{-1}, 37.7×10^{-3} U.mL^{-1} and 4.49×10^{-3} U.mL^{-1}, respectively. While, the fungus lacc-B showed the highest activity of fungal laccase at 40 °C, with 2.99×10^{-3} U.mL^{-1} (Fig. 3a). Temperature is related to the enzyme's energy required to perform a reaction. If the temperature is too low, the enzyme will not have enough energy to perform the reaction, so the reaction does not run optimally. At the optimum temperature, the energy obtained by enzyme is required to optimally run the reaction and produce the best activity (Champe et al., 1994). As shown in Fig. 3b, the result showed that the pH significantly affects the activity of fungal laccase. The activity of lacc-A and lacc-D increased from pH 4.0 to 5.0, achieving the highest activity of 1.7499 $\times 10^{-3}$ U.mL^{-1} and 2.666×10^{-3} U.mL^{-1} each at pH 5.0. The fungal laccase from fungus lacc-B increased from pH 6.0 to 7.0, and reached the highest activity of 8.2499×10^{-3} U.mL^{-1} at pH 7.0. The laccase activity of fungus lacc-C elevated the pH from 4.0 to 6.0, and achieved the highest activity of 4.333×10^{-3}U.mL^{-1} at pH 6.0. After passing each optimum pH, the laccase activity decreased (Fig. 3b).

The results of pH optimization showed that the fungal laccase had the ability to work at acidic pH levels, as shown by fungus lacc-A and lacc-D which could work optimally at pH 5.0, while optimal pH of fungus lacc-B was 7.0, and fungus lacc-C at pH 6.0.

3.3.2. Thermostability and pH stability

As shown in Fig. 4a, the fungus lacc-A was found to be stable at 50 °C until 8 h and retained 51.52% of laccase residual activity. Fungus lacc-B was stable at 40 °C until 6 h and retained 69.45%; fungus lacc-C was stable at 50 °C until 6 h and retained 62.25%; and fungus lacc-D was stable at 50 °C until 6 h and retained 54.59%. Each fungal laccase showed a fairly high temperature stability at each optimum temperature, around 6–8 h of incubation time. In other research, the laccase from *A. flavus* was found at a temperature range of 25–50 °C (Kumar et al., 2016). Fig. 4b showed that the fungus lacc-A was stable at the pH range 4.0–8.0 and the laccase residual activity retained 57.14% at pH 8.0; fungus lacc-B was stable at the pH range 6.0–8.0 and retained 52.52% at pH 8.0; fungus lacc-C was stable at the pH range 5.0–7.0 and retained 53.85% at pH 7.0; fungus lacc-D was stable at the pH range 4.0–7.0 and retained 53.12%. The pH stability of fungal laccase was used to determine the stability of laccase during incubation time at optimum temperature. This decrease in activity is likely to be caused by conformational changes between enzymes and substrates due to pH changes. Based on the curve above, it is known that all fungi have useful activities from pH 5.0 to 7.0, which is compatible to the paper industry. Variation of pH from 5.0 to 7.5 for writing and printing papers, Dutt reported that xylanases working in acidic pH levels are more effective for deinking of old newspaper pulp (Dutt et al., 2013). The results are similar to the laccase from *T. orientalis* (Zheng et al., 2017) and *A. flavus* (Kumar et al., 2016). Fungal laccases show the good activity to catalyze the substrate ABTS at its optimum pH around 5.0 and lose their activities at pH above 7.0 (Kumar et al., 2016; Zheng et al., 2017; Castano et al., 2015).

In this study, the fungal lacc-A has sufficient activity both at acidic and basic pH; the average pH stability obtained for all four fungi showed that fungi could work in acidic conditions (between pH 4.0 and 5.0) and alkaline conditions (pH 8.0). This good result considering that fungal laccase can be applied in the paper industry for environment friendly bio-deinking processes.

3.3.3. Precipitation of fungal laccase with ammonium sulfate

The crude extract produced from the four fungi was concentrated with ammonium sulfate. The addition of ammonium sulfate salt into the protein solution attracted the water molecules that initially protected the surface of protein molecules; consequently, each protein settles at the optimum saturation of ammonium sulfate. The presence of ammonium sulfate serves to attract water trapped in the hydrophobic area, thus allowing the aggregation and precipitation of enzyme molecules.

The optimum laccase activity after ammonium sulfate precipitation was dissolved in the optimum pH buffer of each fungi laccase, then the cellophane tube was immersed into the buffer solution having a lower concentration than the buffer solution in the tube. Ammonium sulfate precipitation is part of partial purification method. At the optimum percentage of saturation ammonium sulfate indicated the highest of laccase activity. As seen in Fig. 5 and Table 1, the deposition of fungal laccase from OPEFB using saturation ammonium sulfate showed that there was an increase in laccase activity. Fungus lacc-A, lacc-C, and lacc-D showed an activity of 0.7 U.mL^{-1}, 0.9 U.mL^{-1} and 0.8 U.mL^{-1} with an ammonium sulfate concentration of 80%, while fungus lacc-B showed an activity of 0.7 U.mL^{-1} with a concentration of 70%. Specific activity of fungal laccase after precipitation with ammonium sulfate were 0.7, 0.9, 1.3, and 1.3 U.mg^{-1} for fungus lacc-A, B, C, and D, respectively (Fig. 5, Table 1). The increasing of specific activity meant that the laccase protein was more precipitated and could be separated from contaminants protein in crude extracts. Due of that, the total protein after ammonium sulfate precipitation and dialysis were less than total protein

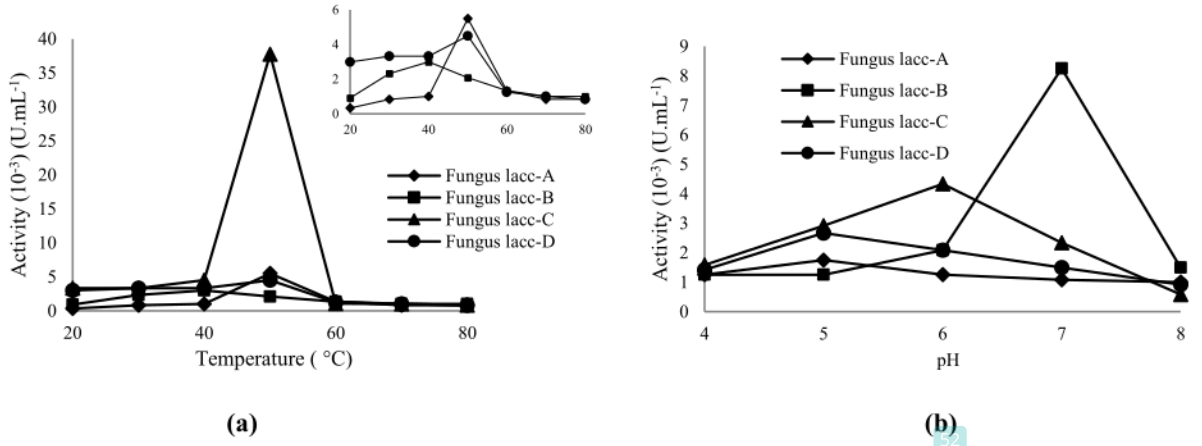

Fig. 3. (a) The optimum temperature of fungus lacc-A, B, C, and D; (b) The optimum pH of fungus lacc -A, B, C, and D.

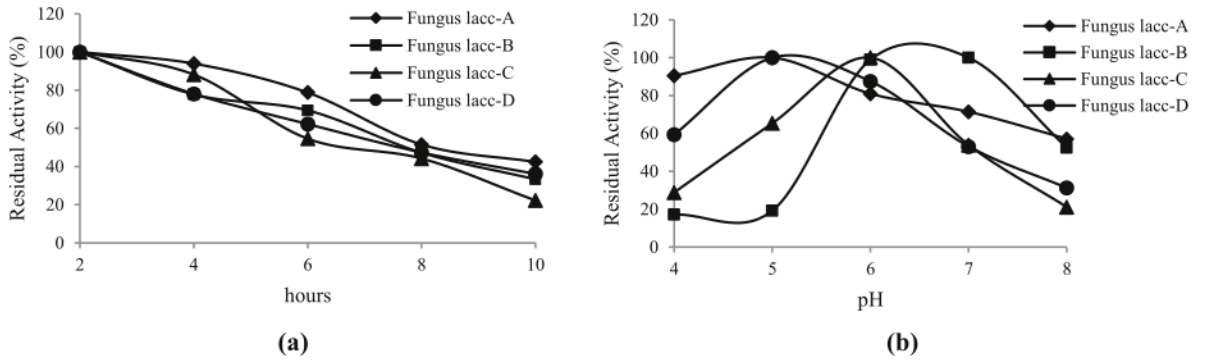

Fig. 4. (a) Thermostability of fungus lacc-A, C and D (T_{opt} = 50 °C), and fungus lacc-B (T_{opt} = 40 °C), based on incubation times using ABTS; (b) The pH stability of fungus lacc-A, B, C and D.

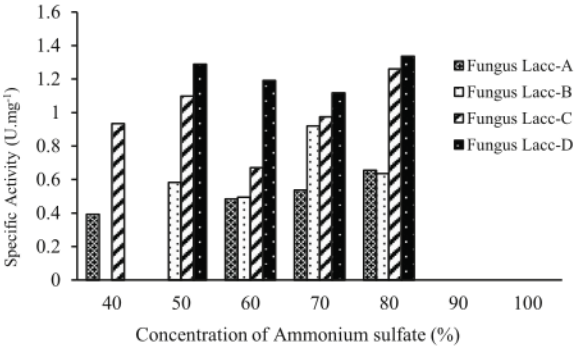

Fig. 5. The optimum saturation of Ammonium sulfate for fungus lacc-A, B, C and D.

in crude extract then resulting the higher of specific activity.

3.3.4. Dialysis

The specific activity of laccase after dialysis process increased because the enzyme solution was free from ammonium sulfate salts and other proteins were dissolved in the supernatant, so the specific activity of the laccase enzyme was increased. The specific activity of laccase were 1.2 U.mg^{-1}, 1.3 U.mg^{-1}, 1.5 U.mg^{-1}, and 2.0 U.mg^{-1} for lacc-A, lacc-B, lacc-C and lacc-D, respectively. The purification level of fungal laccase resulting from the precipitation of ammonium sulfate salt and dialysis of fungus lacc-A, B, C and D were 1.7, 1.9, 1.9, and 2.5 times, respectively from its crude extract. The dialysis process was done to remove the ammonium sulfate salt that had precipitated with the protein and the enzyme. A process within dialysis will separate proteins from ammonium sulfate molecules that have smaller sizes.

3.4. Studying cereal agricultural waste using scanning electron microscope (SEM)

The SEM was used for observing the corn cob and rice straw fibers in both treated and control as untreated samples. Irfan et al. (2011) used SEM method to analyze the increasing of delignification of sugarcane bagasse after using suitable concentration of KOH on specific heating time. Delignification of natural lignocellulose biomass waste is used to break down the lignin chain. After the lignin are removed, the hemicellulose will be easily to degrade. It will also change the crystalline structure of cellulose to improve the availability and the release of the cellulose (Chen et al., 2017). Corn cob and rice straw are the agriculture biomass waste. In this study, we choose Fungus lacc-D for delignification of agriculture waste of corn cob and rice straw because it showed the highest laccase specific activity and also showed the highest purification level (Table 1) after dialysis compared to the others three fungus of lacc-A, lacc-B, and lacc-C.

As illustrated in Fig. 6, the SEM data showed that delignification of corn cob and rice straw waste was corresponding to the superficial microstructural changes. The surfaces of corn cobs and rice straw showed a change in the surface profile between the control and the sample treated with fungal laccase. SEM images of rice straw and corn cob waste after being treated with laccase indicated the presence of degradation activity shown by its characteristics such as being hollow, damaged, and brittle. The holes fibers of corn cob and rice straw after lacc-D treatment were around 10 μm and 18 μm, respectively. This damage showed that the carbon linkage was broken by enzyme treatment. The distortion of the corn cobs and rice straw structures after

Table 1
Purification of laccase from OPEFB.

Fungus	Step of Concentration	Total Activity (U)	Total Protein conc. (mg)	Specific Activity (U.mg^{-1})	Yield (%)	Purification Level
Fungus Lacc-A	Crude extract	1.4	2.0	0.7	100	1.0
	80% of $(NH_4)_2SO_4$	0.7	1.0	0.7	50.0	1.0
	Dialysis	0.5	0.4	1.2	35.7	1.7
Fungus	Crude extract	1.5	1.9	0.8	100	1.0
Lacc-B	70% of $(NH_4)_2SO_4$	0.7	0.8	0.9	46.7	1.1
	Dialysis	0.4	0.3	1.3	26.7	1.6
Fungus	Crude extract	1.3	1.7	0.8	100	1.0
Lacc-C	80% of $(NH_4)_2SO_4$	0.9	0.7	1.3	69.2	1.6
	Dialysis	0.5	0.3	1.5	38.5	1.9
Fungus	Crude extract	1.3	1.7	0.8	100	1.0
Lacc-D	80% of $(NH_4)_2SO_4$	0.8	0.6	1.3	61.5	1.6
	Dialysis	0.4	0.2	2.0	30.8	2.5

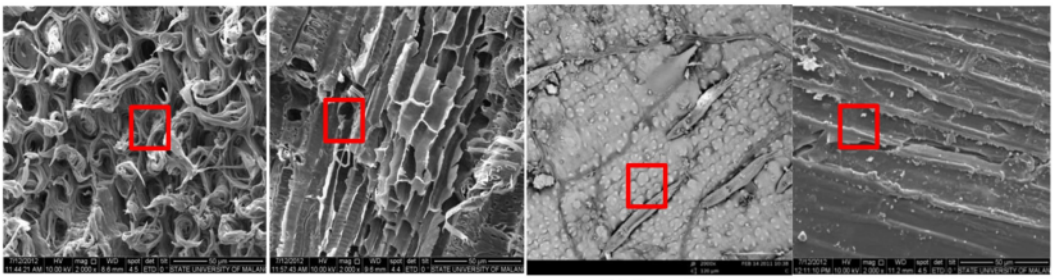

Fig. 6. Profile of Scanning Electron microscope on (a) corn cob control and (b) corn cob after lac-D treatment by magnification of ×1000 (c) rice straw control and (d) rice straw after lac-D treatment by magnification of ×2000. Red box indicated the holes in the fibers of corn cob and rice straw after lacc-D treatment were around 10 μm and 18 μm, respectively (b) and (c). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

laccase treatment is due to the lignin removal which causes the disruption of matrix structure from cellulose-hemicellulose-lignin. This result was consistent with previous studies from Sherpa (2018) used laccase from *P. djamor* to treat sugarcane tops for saccharification (Sherpa et al., 2018), and Mukherjee (2018) used the chemical treatment for rice straw delignification (Mukherjee et al., 2018).

4. Conclusion

Fungus lacc-A, B, C and D were successfully isolated from oil palm empty fruit bunch. The optimum growth period of fungus lacc-A, B, and C was 7 days and fungus lacc-D was 10 days. The optimum pH and temperatures of fungus-lacc A, B, C, and D were 5.0–7.0 and 40–50 °C. Additional analysis from SEM indicated that fungal laccase could degrade lignin with apparent damage to the lignocellulose substrate of corn cob and rice straw. The optimum pH and SEM results of fungal laccase assumed that the fungal laccase from OPEFB has potential to be used in bio-deinking process on pulp and paper industry. The results of this study highlight the important possibility of fungal laccase from OPEFB for the pulp and paper industry.

CRediT authorship contribution statement

Lailatul Fithri: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Resources, Software, Validation, Visualization, Writing - original draft. **Ni Nyoman Tri Puspaningsih:** Conceptualization, Formal analysis, Funding acquisition, Methodology, Resources, Supervision, Validation, Writing - review & editing. **One Asmarani:** Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Project administration, Resources, Software, Validation. **Ni'matuzahroh:** Conceptualization, Formal analysis, Methodology, Resources, Supervision, Validation. **Gytha Deasy Fitrah Dewi:** Data curation, Formal analysis, Investigation, Methodology, Resources. **Radita Yuniar Arizandy:** Data curation, Formal analysis, Investigation, Methodology, Resources.

Acknowledgements

We would like to thank the Ministry of Research, Technology, and Higher Education for the research funding (SK Rektor Unair No. 1149/UN3/2018). Ni Nyoman Tri Puspaningsih is a receiver of a Tahir Professorship of Universitas Airlangga.

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