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Production of Laccase Enzyme by *Marasmius* sp. from the Bark of Cocoa Beans

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ABSTRACT
Laccase is an enzyme that can be used in various processes in the field of pharmaceutical, biotechnology and food. This is due to the laccase that has broad substrate specificity, is environmentally friendly, and uses oxygen as the last electron acceptor. The laccase enzyme can be produced by white-weathered fungi, one of them is *Marasmius* sp. by utilizing the lignin components of lignocellulose of agricultural waste and plantations such as the bark of cocoa beans. The utiliziation of the bark of cocoa beans can reduce the cost of enzyme production and answer the need for high-laccase enzymes. The production of laccase enzyme by *Marasmius* sp. from the bark of cocoa beans can be done using a simple bioreactor with an incubation time of 0-14 days that can affect the growth and metabolic activities of *marasmius* sp. The research is aimed to determine the optimum incubation time of 0-14 days which is able to produce laccase enzyme with highest activity using a simple bioreactor. The production of enzyme is done using the bark of cocoa beans with a size of 60 mesh added with the solution of yeast extract and glucose with the temperature condition of 32°C. The sample collection is done every 12 hours to measure the activity of laccase enzyme using the spectrophotometry method. The result shows that the highest and lowest laccase activity was successfully produced by day-10 (15,1 U/l); day-5 (2,88U/l). Based on the results, it can be concluded that the optimal time growth of *marasmius* sp. is on the day-10 for the production of laccase enzyme.

Keywords: Laccase Activity, Enzyme, Bark of Cocoa Beans, Laccase, Fermentation Time

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INTRODUCTION

Marasmius sp. is a white-weathered fungus included in *Basidiomycota*. This fungus is commonly found in nature, exactly at the wood, trees, or dead plant containing lignocellulose components. *Marasmius* sp. has an ability to degrade lignin in the lignocellulose by producting a lignolytic enzyme, one of which is the laccase enzyme [١]. After degrading the lignin component, *Marasmius* sp. can reach the cellulose and hemicellulose which are then broken down into glucose molecules that are easily metabolized by microorganism and used as a substart for its growth.

Laccase enzyme is an extracellular enzyme that is environmentally friendly, so it is widely used in several industries such as pharmaceutical and food industries. That is due to the laccase enzyme that is able to oxidize the phenolic compounds and only produce water as a side product [١]. In the food and beverage industry, laccase is used to eliminate the phenolic compounds in the baking process, juice making and wine stabilization [٢]. In the textile industry, the laccase enzyme is utilized as a liquid waste management because it has the ability to degrade the synthetic colors [٣]. In the pharmaceutical, laccase enzyme can degrade lignin to take the cellulose fibers, the cellulose that has been successfully released from the constituent structure of lignin, potentially in the formation of composite of biomaterials including the development of nanon for paper, medicines, cosmetics, and edible packaging [٤].

One of the plantation wastes that is abundang in Indonesia and can be used as a substart for the production of the laccase enzyme is the waste of cocoa beans. Cocoa is one of the field commodities that has a significant role in the economy of Indonesia and is one of the state's revenues. Indonesia is the third largest cocoa producer in the world after Ivory Coast and Ghana with a total production of 486,000 tonnes. The amount of cocoa export is 365,000 tonnes and 121,000 tonnes are processed domestically [٥]. The cocoa waste consists of the skin of the fruit, cocoa bark, and sludge (pulp) cacao.

[٦] Indonesia has produced 70,919 tonnes of dry cocoa. [٧] The amount of cocoa skin ranges from 2.59 to 2.42% of total seed. Based on the condition, with the production capacity of 70,919 tonnes of dried beans per year, the number of raw materials of cocoa bark that can be used ranged from 1836.80-1717.23 tonnes per year. The waste of cocoa bark can be a problem for the environment if it continues to accumulate and not be further processed [٨].

The production of laccase enzyme by *marasmius* sp. using the bark of cocoa beans can reduce the problem of waste and suppress the cost of enzyme production because it uses lignocellulose waste as a substart.

METHODOLOGY

Time and Place

The research is conducted at Bandung Institute of Technology, Pasundan University of Bandung, and Research and Development Center for the Marine Geology of Bandung, Indonesia, from April 2019 – December 2019. These locations are selected intentionally due to the consideration of adjacent locations so that the samples do not need to be transported too far.

Tools and Materials

The tools required are a simple plastic bioreactor, ose wire, spatula, large basin, micro-pippete for 100 and 1000 µl, scale, refrigerator, incubator, centrifuge, ph meter, Bunsen, Erlenmeyer 500 ml, Erlenmeyer 250 ml, measuring cup, oven, spectrophotometer, scissors, petri cups, chemical cups, reaction tubes, electric cooker, autoclaves, acid cabinets, tube racks, herb bottles, falcon tubes 50 ml.

The materials needed are Potato Dextrose (PDA), cocoa bark, rubber, heat-resistant plastic, aluminium foil, adhesive plastic, microtube, alcohol, rubbing alcohol, aquades,

gloves, tissue, NaCl, label paper, yeast extract, NaOH, HCl, glucose and alcohol

Research Method

The research is conducted in three main phases, namely:

1. Material preparation including the substart such as the bark of cocoa, *Marasmius* sp. inoculum and yeast extract.
2. Laccase production by *Marasmius* sp. from the substrate of bark of cocoa using simple bioreactor for fourteen (14) days.
3. Sample analysis for the result of fermentation that is the activity analysis of laccase enzyme.

1. Material Preparation

Treatment for the Bark of Cocoa Beans

The samples of cocoa bark are obtained from the cocoa plantation in Yogyakarta, Indonesia. The bark of cocoa used has been dried at a temperature of 70 °C for 24 hours. After it is dried, the cocoa barks are grinded and sifted up to 60 mesh.

Marasmius sp. Fungi Cultivation

The *Marasmius* sp culture is obtrained from the chemical engineering laboratorium of Bandung Institute of Technology, West Java, Indonesia. The cultures obtained are subcultured into petri bowls containing Potato Dextrose (PDA) and incubated for fourteen (14) days until the reaction tube is covered by fungus mycelium.

Making an Inoculum of *Marasmius* sp.

The making of *Marasmius* sp. is performed by making a bag-log consisting of the bark of cocoa beans then added with 200 ml of aquades. The bark of cocoa beans is inserted into the heat-resistant plastic and sterilized using autoclaves at 112°C. After being sterilized, it is inoculated by moving 1.5x1.5 cm of *marasmius* sp. block which has been grown in PDA medium. The bag-log is incubated at the temperature of 32°C for fourteen (14) days until the mycelium covers the surface of bag-log.

2. Laccase Production

The fermentation process is done on a simple bioreactor of a plastic crates that has been given a faucet with a volume of 10 L. Before use, the chemical glass is sterilized using the solution of ethanol 70%. The solid substart of cocoa beans bark as much as 275 grams that have been given the initial treatment is put into the chemical glass and arranged in an intermittent way with *marasimus* sp. inoculum as much as 25%. The operating parameters that can be defined suring fermentation are temperature and pH. The operating condition during the fermentation process can be seen in Table 1.

Table 1: Operating Condition for Bioreactor during Fermentation

Operating Condition	Experiment 1	Experiment 2	Experiment 3
Temperature	32°C	32°C	32°C
pH	5	5	5
Fermentation Time	1-14 days	1-14 days	1-14 days

Analysis of Fermented Samples

The effluent produced from the bioreactor were taken as much as 15 ml using the falcon tube every 12 hours and were centrifuged with 4500 rpm at a temperature of 4°C for 20 minutes. The supernatant obtained from the process of centrifuge was separated for analysis of the activity of the laccase enzyme. In addition, the solid substart of the bark of cocoa beans from 14-day fermentation was separated for the analysis of lignin level.

RESULT AND DISCUSSION

Initial Treatment for Bark of Cocoa Beans

Before being used for the research, the bark of cocoa beans that have been dried up was reduced in size using a grinded and then sifted using the 60 mesh-sieve machine. The bark of cocoa beans that have been grinded with a size of 60 mesh is aimed to increase the surface area of the substrate thereby increasing the efficiency of contact between the particles and mycelia of fungi during the fermentation process. The particle size used is one of the parameters that should be considered where it can affect the fermentation process, i.e if it is too small, it can cause a substrate coagulation that can interfere with microbial respiration process, leading to a poor microbial growth. In contrast, a too large particle size provides a good influence for respiration where it increases the inter-particle space but reduces the efficiency of inter-surface contact with fungus mycelia [9]. After grinded, the bark of cocoa beans is sterilized using an autoclaved heat at 121°C for 15 minutes aiming to remove the contaminants because the temperature used is capable of denaturing the protein of contaminant cells and killing the endospore of most microbial species [10].

Proximate Test for Bark of Cocoa Beans

The barks of cocoa beans are analyzed using the proximate test on the sample of the bark in the laboratory of Food Technology Departement of Pasundan University, Bandung, Indonesia. The purpose of proximate us to test identify the content of lignin in the sample of cocoa bean barks prior to the fermentation which further serves as the main carbon source used for the production of laccase enzyme. In addition, through the proximate test, it can also identify other nutrients that are contained in the sample used, such as cellulose, hemicellulose and coarse fat. The

results of proximate test analysis from the bark of cocoa beans that have been given the initial treatment can be seen in Table 2. He results obtained are almost identical to those mentioned in the literature that the bark of cocoa beans consists of

Table 2: The Results of Proximate Test of Bark of Cocoa Beans Prior to Treatment

Nutrients	Percentage
Water	5,53 %
Ash	2,46 %
Protein	10,33 %
Coarse Fat	3,99 %
Carbohydrate (Starch)	54,83 %
Lignin	14,40 %
Cellulose	40,20 %
Hemicellulose	21,18 %

Result of Laccase Production Optimization

The optimization of laccase production is done for 14 days of different fermentation time, that is 1 day to 14 days. The result of the production of these laccases are depicted in Figure 1. Based on the research, it is known that the highest value of laccase enzyme activity is produced when the fermentation time ranges between 0-15,1 U/l. The enzyme activity is expressed in unit (U), i.e. the number of enzymes needed to oxidize 1µmol substrate per minute.

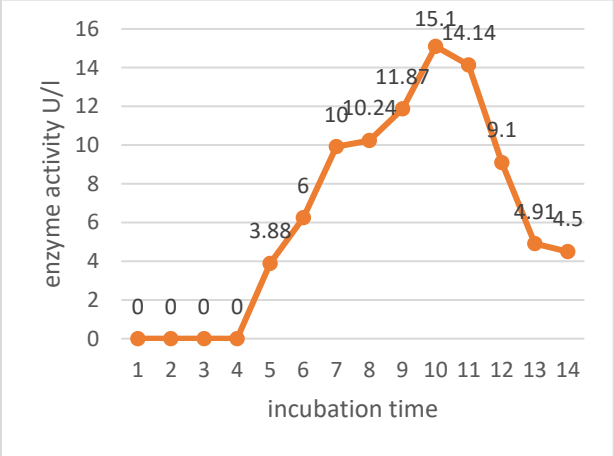

Figure 1: The activity of laccase enzyme with incubation time variation

The above curve shows the highest level of laccase enzyme on the day-10 where the *marasmius* sp. is included in the logarithmic phase where the cell reproduces with maximum numbers. The highest laccase enzyme level is on the day-10 of incubation with a value of 15,1 U/l, while the lowest level of laccase enzyme is on the day-5 incubation time with the value of 2,88 U/l. It occurs due to the fact that the *marasmius* sp. fungus has not grown a lot and has not produced a maximum laccase enzyme so that the value on day-5 is still minimal. On the day-11, it is seen that the number of laccase enzyme suffers caused by the mildew phase of the fungus. Supposedly, the longer the time incubation of *marasmius* sp. fungus, there will be more laccase enzyme, but at a certain level, the incubation time cannot increase the activity of enzyme-produced laccase, because according to [11] the microorganisms have a logarithmic/exponential phase starting after 2 hours of incubation up to 72 hours depending on the adaptation of each strain at 37°C with pH of 8.

Since the beginning of the fermentation to the day-10, it is seen that the activity of laccase has increased. The activity of laccase enzyme decreased on the day-11. The activity of enzyme-readable laccase is an enzyme that has been produced in the bark of cocoa beans. The increase on the enzyme activity of laccase is suspected due to the addition of yeast and glucose in cocoa bean barks. The increased activity of the laccase enzyme is a result of the shortage of carbon and nitrogen sources, so that the laccase activity increases to decompose the lignin and expose the cellulose to be degraded [12].

The decrease in enzyme activity is caused by the decrease of growth in the medium of *marasmius* sp. fungus. This results in the lower production of laccase enzyme and it triggers the growth of *marasmius* sp. due to the growth medium of *marasmius* sp. has suffered a decrease in the lignocellulose levels. For the growth of *marasmius* sp. an energy is required from the tearing down of simple sugar from the degradation of lignin and cellulose in the substrate. Lignin is composed of monolignol p-koumaril alcohol, konifenil alcohol, as well

as the synaptic alcohol, and does not contain the simple sugar [13]. In lignocellulose, cellulose is wrapped in lignin. Therefore, to obtain the glucose from the cellulose degradation, lignin needs to be degraded first [13]. The highest activity of laccase occurs on the day-10 with 15,1 U/l. After the day-10 of fermentation the laccase activity decreases. The decreased enzyme activity can be caused by the repression of catabolite.

The curve indicates that the laccase enzyme is produced continuously during the growth, even before entering the secondary metabolism phase occurring at the end of the first week of fermentation [14]. [15] the laccase enzyme involved in the degradation of lignin is not only produced when entering the phase of secondary metabolite, but rather manufactured a constituent phase of the primary metabolite, but with low concentration. Then, when the levels of carbon and nitrogen are at a low concentration, the production of the laccase enzyme will increase [14]. On the first day of fermentation process, the system has been arranged so the metabolism is at the low levels of carbon and nitrogen to support the production of laccase enzyme. The carbon source used by *marasmius* sp. to produce the laccase enzyme is lignin, which is located on the bark of cocoa beans and to trigger the activity of laccase during the fermentation process, co-metabolism and co-substrate are needed in form of carbon source which can be obtained from glucose, cellulose, in minimal concentration [15]. [16] explain that the glucose is known to be the best co-substrate for producing the laccase enzyme from white-weathered fungi. Co-metabolism is a change from the non-growth substrate in the condition that other substrates are used for growth. Non-growth substrates used in the co-metabolism are referred to as co-substrates and are not used to support the cell replication or biomass growth [17].

The *marasmius* sp. fungus is the one that produces laccase enzyme. The fermentation of *marasmius* fungus is arranged in such a way to produce the laccase enzyme. *Marasmius* sp. is an obligate aerob organism. When the oxygen levels are low in the incubation container, then the metabolism of the fungus becomes less optimum. In addition, oxygen is required in redox reactions catalyzd by the laccase enzyme. On the day-11, there is a decline in enzyme activity. That is suspected due to the optimum age of *marasmius* sp., as a producer of laccase enzyme, is in the lag phase. The logarithmich phase is observed on the day-11 to the end of the observation, the day-14.

The size of bark of cocoa bean particles has an influence in the production process of laccase enzyme. The smaller particles provide a wider area of contact surfaces between the particle and fungal mycelia so that the fermentation process can take place efficiently. Although it provides a good effect, a too small particle can cause an accumulation of substrates (substrate coagulation) that can interfere with the distribution process of the air to the buffer medium. This can cause the respiration process of the fungus to be disturbed so that the fungus growth will be impaired. The opposite effect is indicated by the particles of greater size. The greater the particle size will result in higher fraction of empty space between particles thereby facilitating the diffusion of oxygen, but can reduce the contact area between the particle surface and fungal mycelia [18]. Other research shows the larger the mesh of bark of cocoa beans, the higher the enzyme activity.

In the fermentation process for the delignification process, glucose and yeast extract are added. Yeast extract is used as a supplement in microbiological medium because it contains amino acids, peptides and vitamins that are very beneficial for the growth.

The yeast extract, as a source of nitrogen, plays a role in physiological process because nitrogen is a component of proteins, nucleic acids and other important substances. [19] Report that the yeast extract is a primary source of nitrogen used for the production of lactic acid because it has high peptides and B-complex vitamin. Therefore, one of the important sources for nutrients in culture media for the growth is nitrogen source. The concentration of yeast extract that are added to the medium which can affect the microbial growth of yeast is a single-cell-type microorganism with a size of 5 to 20 microns, with no flagella and some genera that form a filament. The yeast extract contains components of water-soluble cells, i.e. amino acids, peptides, carbohydrates and salts. The specification of yeast extract in *Indian Standards Institution* mentions that generally, yeast extract is used for microbiological work or as a supplement in microbiological medium that serves as a source of nitrogen. In this research, the enzyme crude in the bark of cocoa beans that have been fermented by *marasmius* sp. then shaked with a speed of 175 rpm for 1 hour. Compared to the other researches regarding the production of laccase enzyme, in this research, the enzyme activity tends to be good. This can be reached due to the fact that in the fermentation system of solid substrate, there is a possibility that the growth of *marasmius* sp. is homogenous, while the growth of fungal mycelia is uneven, because there is no stirring or agitation given on the substrate, leading to less homogenous substrate. That can make an uneven medium on the fermentation container.

REFERNCES

1. Risdianto, H., 2008. LACASE PRODUCTION AND ITS POTENTIAL IN PULP BLEACHING PROCESS. jurnal selulosa 43 No 1. http://dx.doi.org/10.25269/jsel.v43i01.162
2. Shradda, Shekher, Sehgal, 2011. Laccase microbial sources, production, purification, and potential biotechnological applications. Enzyme research.
3. Felde AZ, Pocasangre LE, Monteros CAC, Sikora RA, Rosales FE, Riveros AS., Rosales FE, Riveros AS., 2016. Effect of combined inoculations of endophytic fungi on biocontrol of Radhopholus similis 12–18.
4. Ibrahim, R., Adnan, S., Saleh, M., 2014. CHARACTERIZATION OF BLACK LIQUOR FROM OIL PALM EMPTY FRUIT BUNCH SODA-ANTHRAQUINONE PULPINGRushdan.
5. Departemen Perindustrian Republik Indonesia, 2007. Kakao.
6. Badan Pusat Statistik, 2017. Statistik Kakao Indonesia 2017, in: Statistik Kakao Indonesia 2017. BPS Indonesia, pp. 1–88.

7. Hugg, B., Golay PA, Giuffrida, F., Dionisi, F., Destaillats, F., 2006. Development of a gas liquid chomatographic method for the analys of fatty acid tryptamides in cocoa products. J. Agric 3199–3203.
8. Risza, S., 1994. Kelapa Sawit (Upaya Peningkatan Produktivitas). Yogyakarta.
9. Pandey, A., 2010. The realm of microbial lipases in biotechnology. https://doi.org/10.1111/j.1470-8744.1999.tb00541.x
10. Madigan, M.T, Martiko, J. M. Parker, J., 2012. Book Biology of Microorganism, 10th ed. New Jersey, Lentis Hall.
11. Bansal, K.S., N.,. Raman, S.,. Chetna, J.,. dan Sanjeev, 2012. Production of Xylanase-Cellulase Complex by Bacillus subtilis NS7 for Biodegradation of Agro-Waste Residues. Peer-Reviwed Article Lignocellulose 1, 196–209.
12. Brijwani K, A, R., Vadlani PV, 2010. Fungal Laccases : production, function, and application in food Processing. Enzime Research
13. Liu, W., Jiang, H., Yu, H., 2015. Thermochemical conversion of lignin for functional materials: a review and future direction Green Chemical. https://doi.org/10.1039/C5GC01054C
14. Fitriyanti, M., n.d. Produksi enzim lakase dara jamur pelapuk putih menggunakan continous packed bed bioreactor. ITB.
15. B. R. M. Vyas, J. Volc & V. Šašek, 1994. Ligninolytic enzymes of selected white rot fungi cultivated on wheat straw.
16. Patel, H, 2016. Optimization of different culture condition for enhanced laccase production and its purification from ,Tricholoma giganteum, Bioresources and Bioprocessing
17. Kunamneni A, 2007. Funggal laccase a versatile enzime for biotechnological applications. Microbiology 233–245.
18. Board, 2005. Handbook on plant and cell tissue culture. Asia Pacific Bisiness Press Inc, New Delhi, p. 451.
19. Pandey, A., 2010. The realm of microbial lipases in biotechnology. https://doi.org/10.1111/j.1470-8744.1999.tb00541.x
20. Altaf, M., Naveena, B. J., Reddy, G, 2005. Screening of Inexpensive Ntrogen Sources for Production of L(+) Lactat Acid from Starch by Amylolitic Lactobacillus amylophilus GV6 in Single Step Fermentation. Food Technol. Biotechnol., 3 Vol 43, 235–239.
21. July, J., As'Ad, S., Budhianto Suhadi, F., Islam, A. Cortisol dynamics are associated with electrocardiographic abnormalities following the aneurysmal subarachnoid hemorrhage (2012) Journal of Cardiovascular Disease Research, 3 (4), pp. 315-318. DOI: 10.4103/0975-3583.102717