BIOCHEMISTRICAL AND ENVIRONMENTAL APPLICATIONS: UTILIZATION OF LACCASE ENZYME AS IOREMIDIATION IN PETROLEUM INDUSTRY WASTE BY REDUCING PAH (POLYAROMATIC HYDROCARBON)

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Abstract- Laccase (benzendiol: oxygen oxidoreductase, EC 1.10.3.2) is an extracellular enzyme using oxygen compounds to run the oxidation reaction of various aromatic and nonaromatic compounds. Laccase enzyme are found in fungi, such as the Basidiomycetes, Ascomycetes, and Deutromycetes. Other sources of this enzyme are plants, insects, and bacteria, especially prokaryotes. Acer pseudoplatanus, Pinus taeda, Aesculus parviflora, and Populus eruamericana are the kinds of plants that are capable of producing the laccase enzyme. There are a lot of application of laccase enzyme, such as laccase in the paper industry, laccase in the textile industry, Medical and Personal Care of laccase, laccase in the food industry, Biosensors and Diagnostic Applications of laccase and for bioremediationThis research focus on aplication of laccase enzim as bioremidiation in waste of petroleum industry with reducted the PAHs (Poliaromatic Hydrocarbon) on it. The purpose of this study was to determine the utilization of laccase enzyme as bioremidiation in petroleum industry waste by reducing PAHs (Poliaromatic Hydrocarbon). The study was conducted with a sample media preparation, analysis of oil content (gravimetric), pH analysis, and analysis of the number of microbes TPC (Total Plate Count). Parameters were observed to determine the optimal conditions include: temperature, pH, water content, TPC (Total Plate Count) and degradation of pencentage of TPH (Total Petroleum Hydrocarbon). This review help to understand about propertise, aplication and optimalization laccase enzyme as bioremidiation in petroleum industry waste for its biochemistrical and environmental applications.

Keywords- laccase enzyme, PAH (Poliaromatic Hydrocarbon), bioremediation.

I. INTRODUCTION

Laccase is a extra celluler enzym that is a belongs to the group of the large blue copper proteins or blue copper oxidases. Laccase (EC 1.10.3.2) is a blue copper protein, but it also falls within the broader description of polyphenol oxidases. Polyphenol oxidases are copper proteins with the common feature that they are able to oxidize aromatic compounds with molecular oxygen as the terminal electron acceptor (Mayer, 1987). At the first time, the laccase enzyme exudate extracted from the Japanese lacquer tree, Rhus vernicifera by Yoshida in 1883 and demonstrated by Bertrand and Laborde in 1896 as a fungal enzyme. In generally, laccase enzyme was founded in plants, fungi, bacteria and insects. Laccase enzyme is also an enzyme that has the potential to catalyze the breakdown of lignin. So, in the industrial process by laccase enzyme utilized. Laccase enzyme major function in the degradation of lignin and phenolic compounds have been evaluated in a large number of biotechnological applications such as the production of bioethanol, the degradation of textile dyes, paper industry, crosslinking of polysaccharides, as well as bioremediation. Utilization of laccase enzyme is very interesting because has high efficiency, high selectivity, and friendly environment. Especially in prevent of industrial pollution and the environment. At this time, industry in the State of Indonesia is growing rapidly, especially in the petroleum industry. Waste is one of the problems in the industry, especially waste from these petroleum industry. It can be seen that the petroleum industry dispose of these wastes into the water area. Industrial development is not matched with the handling and control of industrial waste generated is what causes the risk of environmental damage. Waste generated from the petroleum industry one of them is PAHs (Poly Aromatic Hydrocarbon). PAHs contaminated sediment dense covering of soil, water, and air. PAHs received more attention for humans because PAHs are aromatic ring compound which is a pollutant which the character identified as carcinogenic, mutagenic and teratogenic until now there is still no treatment at wastewater petroleum (Larsson, Sahlberg, Eriksson & Busk, 1993).

In this study focuses on the application of laccase enzyme as bioremediation. Laccase enzyme can be produced by utilizing a mold that grows on trunk of oil palm. Mold growth on the trunk of oil palm, it was indicated that contains laccase enzyme has the potential to degrade PAHs compound of liquid waste petroleum industry (Daljit Singh Arora and Paramjit Kaur Gill, 2000). Laccase enzyme utilization as industrial wastewater bioremediation of petroleum is the proper technique to overcome. This is motivated because the processing of industrial processes that are environmentally friendly and cheaper than using chemical physics methods that require high energy

consumption and very expensive as well as the use of chemicals that can cause secondary pollution.

Therefore, this study aims to optimize the enzyme laccase of insulating oil palm empty fruit bunches mold as the solution handling petroleum industrial wastewater containing PAHs (Poly Aromatic Hydrocarbon).

II. MATERIALS AND METHODS

Isolation Process Laccase Enzyme by Trunk of Oil Palm: Preparation of enzyme made from isolates trunk of oil palm at chemistry laboratory, Universitas of Airlangga. Mold that has grown in PDA media, inoculum prepared by dissolving conidia or spores with sterile distilled water. This inoculum is inserted as 200µL into GMY liquid media by the addition of 0.05 mM CuSO4 solution. GMY media containing inoculum is incubated for best time on mold at room temperature and shaken at a speed of 150 rpm. At best time on mold, GMY media that has been incubated and shaken, centrifuged at 3300 rpm, 4 ° C for 2 minutes. Formed on the medium supernatant after centrifugation is called laccase enzyme. (Gytha, 2010)

The Activity Test of Laccase Enzyme: The activity test of laccase carried out by Bourbonnais and Paice methods (1990). The principle of this test is as follows: non-phenol dye Acid azinobis-2,2'-di- (3-ethylbenzthiazolinesulphonate) (ABTS), oxidized by laccase into a radical cation (ABTS+) that is more stable. Radical cation concentration turquoise (wavelength of 420 nm) correlated with laccase activity. Laccase activity measurement performed using optimum conditions on the enzyme. The measurement is done in the following way: a solution of 0.4 mM ABTS in sodium acetate buffer (pH 4.5) of 1160 mL inserted into the cuvette 1.5 mL, 40 mL enzyme is then added and shaken to mix homogeneous. Then the cation radical absorbance was observed at a wavelength of 420 nm (εmM = 36mm-1 cm-1) for five minutes using a UV-Vis spectrophotometer. Cation radical absorbance changes observed every minute. Laccase activity has shown as International Units (IU) per liter, with 1 IU is defined as the amount of enzyme which can oxidize 1 mol ABTS per minute. (Ghyta, 2010) Laccase activity was determined using the following equation (1) is:

$$aktivitas\left(IU/l\right)=2\left(\frac{V}{v\,x\,\varepsilon\,x\,d}\right)x\,\Delta A.\min^{-1}\,x10$$

that:

V = total reaction volume (ml)

v = enzyme volume (ml)

ε = extinction coefficient ABTS at 420 nm = 36 $mM^{-1}cm^{-1}$

d = Light path of cuvette (cm)

$\Delta A.min^{-1}$ = change in absorbance per minute at 420 nm

Extraction of PAHs : Optimum extraction conditions were determined after preliminary assays. The following conditions were selected. After 1 month incubation, petroleum

waste were oven-dried (30◦C, overnight) before extraction. Samples (5 g) were extracted in a Soxhlet apparatus for 16 hours with dichloromethane. With this method, 96% of PAH initially present in petroleum waste of soils was recovered. The extracts were concentrated and analysed by gas chromatography (GC) using a model Auto-System GC (Perkin-Elmer Co, Inc.) equipped with a Kame ionisation detector. The capillary column used was a RTX-5 (30 m × 0:53 mm i:d:×1 m %lm thickness, Restek, Evry, France). The oven temperature was programmed at 90◦C (1 min), followed by linear increase of 4◦C min−1 to 310◦C (2 min). The detector temperature was maintained at 320◦C. The carrier gas was hydrogen at a constant Kow rate of 15 ml min−1. Calibration standards of 16 PAHs according to EPA at various concentrations were used to calibrate this instrument. The results were calculated in an internal standard mode using the hexachlorobenzene as internal standard. All analyses of inoculated treatments and parallel controls were conducted in triplicate. The percentage of PAHs depletion (%D) of eachisolate was given by the formula: %D = 100[(MHgCl2 − MT)=MI], in which MHgCl2 was the quantity of PAHs obtained in HgCl2-killed mycelial controls, MT was the quantity of PAHs obtained in each treatment and MI was the initial PAHs quantity present in soil. The mean (m) and the standard deviation (SD) were calculated and are shown in Fig. 1 according to the formula m ± SD. (Yucheng, et al, 2007)

Bioremediation of petroleum contaminated waste by laccase enzyme : PAHs contaminants of petroleum (1%) as sole carbon source into 250 ml Erlenmeyer flasks containing CDA broth and autoclaved at 120° C for 20 min. The test fungi were inoculated and incubated at 30° C for 28 days. Each experiment was carried out in triplicate and laccase activity was measured. The fungal trunk of oil palm was removed by filtration and clear culture fluid was centrifuged at 10,000 rpm for 10 min. The pure of supernatant was used to measure laccase activity of the test fungal strain. The experiment was also performed in autoclaved soil to test the efficiency of fungal consortia.

Gas Chromatography analyses: The soil samples were collected from two wheeler service station in Chevron Indonesia . The laccase enzyme was inoculated in the medium with contaminated soil and incubated at 25° C for 28 days. After incubation, extracted the whole flask and analyzed in GC. To the contaminated soil from garages (1.0 g), 50 ml of acetone was added. The hydrocarbons in the contaminated soil were extracted using acetone twice. The whole medium in the flasks were extracted with acetone (v/v) for hydrocarbon analysis. The extract

was filtered through sodium sulphate and condensed to 1 ml with rotary evaporator unit. A 10 µl of extracted sample was injected into the injection port. The chromatograph consist of CHEMITO GC 8610 Flame Ionization Detector, carrier gas as nitrogen and hydrogen, oxygen for ignition purpose and a BPX-70 (50% cyanopropyl 50% methylsiloxane) column. Temperature regimen maintained during the operation was as follows: Injection port
250°C, detector port 260°C, oven starting temperature 160°C and increase by 7.5°C per minute to a final oven temperature of 240°C. A winchrom software package in the GC apparatus was used to analyze the data. (V.Balaji, 2013)
TPC Test of Fungal Isolates: Soil samples that have been stored as 1 gram. And then decimal diluted (10-1 to 10-10 using test tubes each filled with 9 ml of sterile Nutrient Broth). Taken 1 ml of dilution, pipetted into sterile petri dish (Duplo). Plate Count Agar medium (PCA) which has been cooled to approximately 44oC as 10 ml is poured into a petri dish and homogenized. Incubated at room temperature for 24 hours. Cup used is a cup containing 30-300 colonies. Then measured the amount of enzyme population (delish enzymes or bacteria) in total. In the ideal amount of microbial composting system is needed as much as 104-107 or a minimum of 103 CFU / g soil (Sulistyowati, 2001).
Analysis of TPH (Total Petroleum Hydrocarbon): before analysis of TPH, that calculated moisture content and dry weight of the sample that is the way the soil sample is weighed ± 5 grams, and oven for 4 hours at a temperature of 100 ° C. Dried soil samples were weighed again and calculated water content. For the calculation of TPH levels in the soil sample preparation and measured using soxhlet. Soil samples were weighed ± 5 grams and sodium sulfate was added, then inserted into the cellulose thimble and covered with glass wool to taste. Hexane 75 ml and 75 ml acetone inserted into the still pot / flask and assembled into soxhlet apparatus. Soxhlet apparatus switched on for 4 hours. Crude oil that has been extracted channeled entirely into the still pot. Empty weight still pot weighed. Solvent present in the still pot was evaporated with a rotary evaporator for 5 min at 80 ° C until the solvent evaporated and left behind only crude oil. Still pot is weighed again after the temperature stable. Then the calculated levels of TPH. The rate of degradation showed impairment TPH each day of the results of bioremediation. The formula rate of degradation (decrease in oil content), equation (2) is:

$$PM = \frac{A-B}{A} X\ 100\%$$

So that:
PM = decreased levels of oil (%)
A = initial oil content (mg/kg)
B = the oil content after bioremediation (mg/kg)
(Bagariang, 2008)

III. RESULTS AND DISCUSSION

Isolation of the laccase enzyme by Trunk of Oil Palm : Figure below shows that on PDA, there is about one mold growing. And then, it is purified on PDA slant. Purification is done by making oblique PDA media, then inoculating spores on PDA slant. Mold that has been marked by a pure color uniformity that grows on PDA slant.

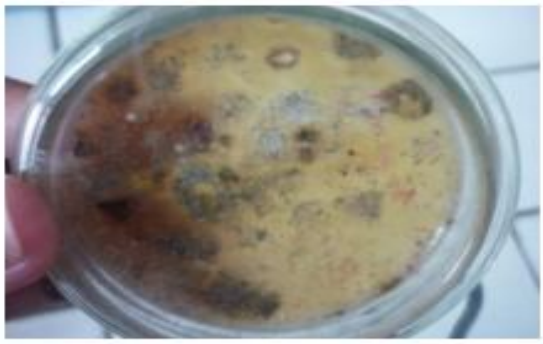


Fig. 1. Mold growing on PDA (Universitas Airlangga)



Fig. 2. Mold that has been pure (Universitas Airlangga)

Laccase Enzyme Activity Test
Laccase activity carried out by Bourbonnais and Paice methods (1990). Non-phenol dye Acid azinobis-2,2'-di- (3-ethylbenzthiazolinesulphonate) (ABTS) is oxidized by laccase into a radical cation (ABTS +) more stable. Radical cation concentration turquoise (wavelength of 420 nm) correlated with the activity of laccase (Bar, 2001). This is a picture change ABTS radical cation ABTS

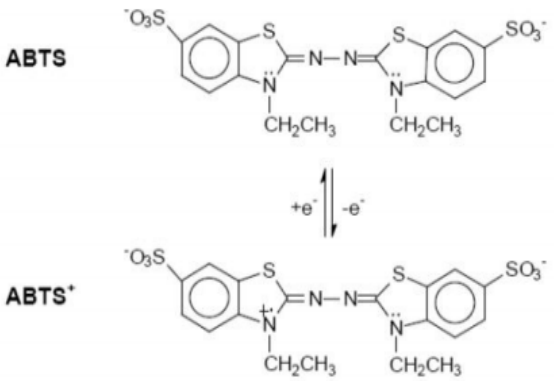


Fig. 3. Asam 2,2'-azinobis-di-(3-ethylbenzthiazolinesulphonate) (ABTS)

Cation radical absorbance changes observed every minute. Laccase activity was expressed as International Units (IU) per milliliter, with 1 IU is defined as the amount of enzyme which can oxidize 1 mol ABTS per minute. Based on the growth curve, it can be seen the log phase of mold. In the log phase, mold producing primary metabolites, including the production of some enzymes. So in this study tested the laccase enzyme activity at all points contained in log phase line at 5 points, day 2, day 4, day 5, day 6 and day 7. The next, laccase activity was tested on each of these points.

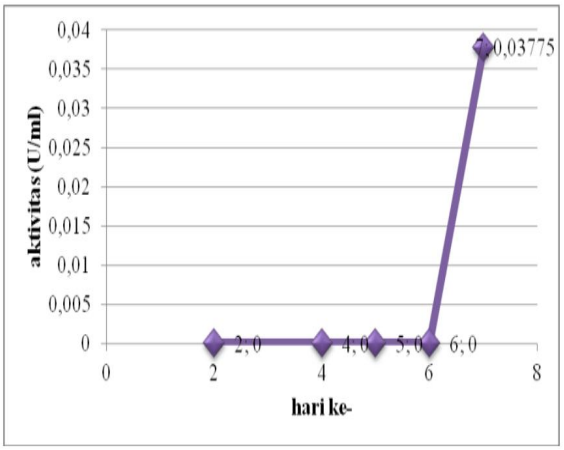


Fig. 4. Laccase enzyme production curve

The curve can be seen that mold secretes an enzyme laccase best on the 7th day. On the 7th day, there was an increase absorbance. While on the other days absorbance unstable and some have decreased, so that the activity was considered zero. After calculation, the best laccase enzyme activity obtained on the 7th day in the amount of 37.75 x10-3 U / mL.

Temperature stability shown that the stability of laccase enzyme activity when incubated at optimum temperature. Observations carried out for 10 hours and sample researched every 2 hours. Measurements carried out on the residual enzyme activity of the enzyme. If the residual activity of enzyme laccase more than 50%, then it was stable condition. Laccase enzyme from mold is stable at a temperature of 50ºC for 6 hours. At the 6 hours, the residual activity of enzyme laccase is still above 50% at 62.25%. But after that the activity has decreased to below 50%.

The stability of laccase enzyme shows the range of pH 4-8. The quality of enzyme can be determined through pH stability. Enzymes quality comparable to a wide range of pH. The wider range of pH, the more superior enzyme, because enzymes can work on a wide pH conditions. Laccase enzyme from trunk of oil palm is stable at pH 5 to 7. At pH 5% residue activity is 65.38% and at pH 7 at 52.85%. Determination of pH stability is based on the residual activity of the enzyme, if over 50%, then the enzyme is said to be stable.

Oxidatin of PAHs by laccase enzyme:

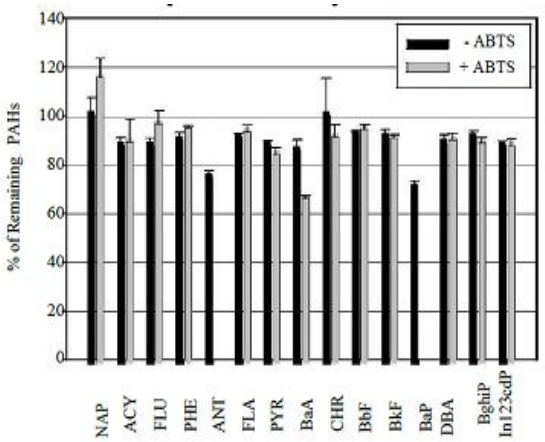


Fig. 5. Oxidation (% of control) of 15 PAHs by laccase in the presence or in the absence of 1mM ABTS after 24 h of reaction in reaction mixtures. Naphthalene (NAP); acenaphthylene (ACY); fluorene (FLU); phenanthrene (PHE); anthracene (ANT); fluoranthene (FLA); pyrene (PYR); benzo[a]anthracene (BaA); chrysene (CHR); benzo[b]fluoranthene (BbF); benzo[k]fluoranthene (BkF); benzo[a]pyrene (BaP); dibenz[a,h]anthracene(DBA); benzo[g,h,i]perylene(BghiP); indeno[1,2,3-cd]pyrene (In123cdP).

Before testing soil remediation, capacity formation of PAHs by laccase in the reaction mixture used to determine the effects of the presence or absence of a mediator ABTS (Figure 1). On the acquired data, the absence of ABTS, 24.972.3% produce anthracene and 29.572.9% benzo (a) pyrene were transformed by laccase. Which of the Anthracene and benzo (a) pyrene is more significant than the other PAHs. The addition of a mediator ABTS enhanced the oxidation of several PAHs. The presence of 1 mM ABTS, anthracene and benzo (a) anthracene can be changed completely and the third transformable was benzo (a) anthracene, 4-ring PAHs, which 33.671.0% is loss was observed. However ABTS was not significantly increase (p40.05) residual PAHs. The process of PAHs degradation was occur through the enzymatic oxidation-reduction events where the enzyme acts as a mediator of PAHs oxidation so that the bonding in benzene rings separated constituent PAHs and PAHs can be degraded.

Gas Chromatography Analyse : The contaminated soil with the mixture of petroleum waste contained different hydrocarbons from C12- C24 on 28th day, most of the compounds were completely degraded by enzyme strains in relation to laccase production (Fig 6) The extracellular enzyme filtrate were extracted and tested for their laccase activity by native gels using tributyrin as substrate. The highest laccase activity and clear zone was detected in fungal consortia growing in presence of petrol, grease and PAHs contaminated soil. The saturate profile of the contaminated soil showed successful and complete degradation of n- alkanes. The GC profile also show that the fungal strains were capable of degrading branched alkanes isoprenoids such as pristine (C17) and pyrene (C18) (April et al., 2000 and Husaini et

al., 2008). The present study, revealed that PAHs contaminated soil associated fungi and laccase from Trunk of Oil Palm can be used in bioremediation of PAHs contaminated habitat is petroleum waste. The present study also details about laccase production in relation with hydrocarbon biodegradation by the fungal strains.

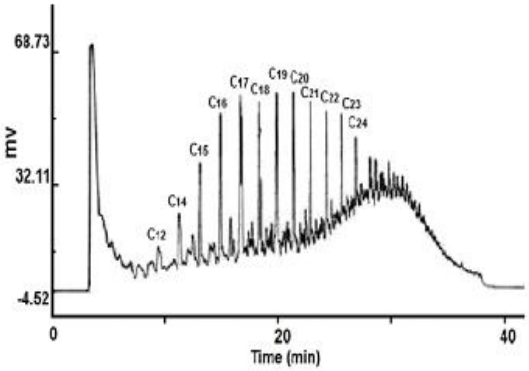


Figure .6. Gas chromatography profiles hydrocarbon degradation (C12 – C24) present in the contaminated soil by fungal strains after 28 days of incubation

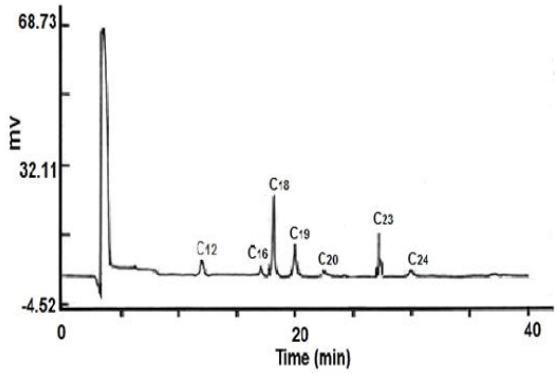


Fig. 7. Gas chromatography profiles hydrocarbon degradation (C12 – C24) present in the contaminated soil by fungal strains after 28 days of incubation

Enzymatic remediation of PAHs-contaminated soil : Regardless of the initial amount of enzyme added, the activity of laccase declined smoothly over the whole incubation period shown in Fig. 3. By the 14th day there was no apparent enzyme activity in any of the soil microcosms. After application to the soil, laccase transformed several PAHs efficiently and the more the enzyme that was amended, the greater the removal of the PAHs. After incubation for 24 h, anthracene and benzo(a)pyrene were the most degradable of the 15 PAHs determined, which was similar to the aqueous reaction mixtures. Moreover, the degradation rate of dibenzo(a,h)anthracene in soil was very high compared with the aqueous reaction mixtures. Relatively low total PAHs removal was observed for microcosms receiving enzymatic treatment at the end of the incubation period: 67.6–82.4% of PAHs (compared with control microcosms) were still recovered from soil. The dissipation of benzo(a)pyrene and the toxic equivalence (presented as toxic equivalent concentration based on benzo(a)pyrene) were notably significant, suggesting the potential of laccase to detoxify PAHs-contaminated soil

(Fig. 7).

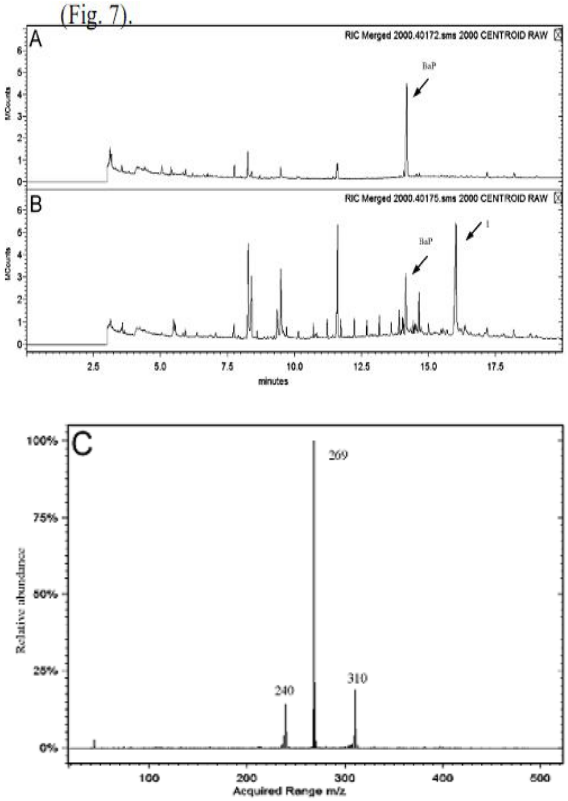


Fig. 8. Identification of the main products obtained from benzo(a)pyrene oxidation with laccase and ABTS. (A) GC of the control reaction, (B) GC of the products after 24 h of incubation and (C) mass spectrum of peak 1 in (B).

Analysis of TPH (Total Petroleum of Hydrocarbon): Bioremediation of crude oil with laccase enzyme for 3 months showed a difference between the reduction in TPH on media without the enzyme laccase and media using laccase enzyme. In the media using laccase enzyme-treated crude oil with the addition of 1% and 3%, the average value of TPH (%) with 3 repetitions on the 3rd month fell to 0.81% and 0.99%. In the treatment of crude oil 10% increase in repeat 1 and 3, a decrease of TPH fully occur without any interaction with the enzyme, because enzymes laccase die in the first week. According to Frick et al., the general degradation of hydrocarbons may not be done directly by enzyme. But the organism will work with enzymes or depending on type of pollutants, type of enzyme and the environment. The test results further Duncan 5% the effect of the interaction of various concentrations of crude oil and the use of enzyme laccase on the value of TPH after 3 months showed that the greater concentration of crude oil then decreased activity of enzyme and the smaller concentration of crude oil, the activity of the enzyme to degrade crude oil more effectively , However, the media without using enzyme laccase showed that no degradation process. Hydrocarbon was degraded by

the enzyme will produce CO2 and H2O and energy. Otherwise the plant will utilize the CO2, H2O and energy to make the process of metabolism.

Applications Laccase of Enzyme: There are so many applications of laccase. Like as laccase in the paper industry, textile industry, medical and personal care of laccase, laccase in the Food Industry, biosensors and diagnostic applications of laccase and for bioremediation. In the paper industry, Laccase is used for the separation and degradation of lignin in replacing chemical oxidant, based chlorine and oxygen are expensive, toxic, and difficult to recycle. In the Textile Industry, laccase may prevent staining on textile materials, therefore by reducing the time, processes, and water needed. Utilization of laccase enzyme in substitute chemical oxidants also can produce a variety of color display with good quality. In the Medical and Personal Care of laccase, laccase can be used as catalysts in the manufacture of anti-cancer drug, to treat inflammation due to poisonous plants (poison ivy), oxidizes iodide to iodine (agent disinfection and sterilization), perfect cosmetic effects, and material for deodorant. In the Food Industry, Phenolic compounds in food ingredients can undergo polymerization and oxidation that changes the scent and color (enzymatic darkening). Laccase used to remove unwanted phenolic compounds, such as in the combustion process, the manufacture of juice, wine stabilization, etc.

In the biosensor, laccase can be used to detect the presence of oxygen, phenols, aniline, as well as reduced other substrates. However, in this study, Utilization of Laccase enzyme focus on bioremediation using laccase enzyme produced from Trunk of oil palm to reduce PAHs in the petroleum waste.

CONCLUSION

Utilization of laccase enzymes is very large in the industrial fields, among others in the paper industry, textile industry, medical and personal care of laccase, laccase in the food industry, biosensors and diagnostic applications of laccase. However, in this study about the utilization of laccase enzyme from the trunk of oil palm on bioremediation method to reduce PAHs in the petroleum waste produced. The results of discussion can be concluded that the utilization of laccase enzyme can increase degradation of Polycyclic Aromatic Hydrocarbons (PAHs) compounds. Optimization of laccase enzyme in soil contaminated with PAHs compounds made with activity test of laccase enzyme and TPH (Total Petroleum of Hydrocarbon) test.

The higher concentration of crude oil in the soil caused by the degradation rate of laccase enzyme decreases. However at certain concentrations of crude oil that is 3% causes degradation by the enzyme laccase levels more efficiently. The total Petroleum Hydrocarbon (TPH) can be accumulated as Polycyclic Aromatic Hydrocarbons (PAHs) compounds.

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