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Research Article

Optimization of Enzymatic Bioremediation of Oil Contaminated Soil by Laccase from *Marasmiellus palmovorus* using Response Surface Methodology

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Abstract

Crude oil contamination is considered highly toxic and poses a significant environmental problem. As an alternative, the laccase enzyme showed high performance for removing various toxic contaminants, particularly oil-contaminated soil (i.e., total petroleum hydrocarbon). However, previous studies mainly tested the performance of laccase under irrelevant environmental conditions (i.e., artificially contaminated soil) with a limited number of soil samples, which can lead to bias optimization results for assessing laccase performance. Two types of natural oil-contaminated soils were tested under various reaction times and various concentrations of laccase extracted from *Marasmiellus palmovorus*. In addition, response surface methodology was used to find the maximum degradation of total petroleum hydrocarbon (TPH). The maximum degradation of TPH from soil A and soil B were 48.57% and 54.1%, respectively. Moreover, the performance of the laccase enzyme for oil recovery was also tested, with the percentage of oil recovery being 9.89% and 10.1 % for soil A and soil B, respectively. SARA fraction analysis indicated that laccase enzyme preferentially degraded highly polar SARA fraction (i.e., asphaltene and resin). In general, the application of laccase for the enzymatic remediation of oil-contaminated soils was practical. Hence, the use of laccase for environmental application is still promising.

Keywords: Laccase; enzyme; total Petroleum Hydrocarbon; bioremediation

1. Introduction

Oil contaminated soil is hazardous and toxic solid waste that can lead to environmental pollution and health problems for humans and other living beings (Coronel Vargas et al., 2020). Vast quantities of

crude oil produced and transported from upstream to downstream are associated with increased contamination by crude oil (Karthick et al., 2019). This activity cannot be separated from spills or leaks of crude oil that result in contamination of the environment. Oil spills on land affect whole ecosystems, wildlife, changing vegetation, microbial process, and overall soil health (Polyak et al., 2018). Therefore, restoring oil-contaminated soil's carrying capacity and initial function is necessary.

Among various remediation techniques, bioremediation of oil-contaminated soil has been applied widely due to cost-effective technology and potential application for oil recovery (i.e., microbial enhanced oil recovery) (Bhattacharya et al., 2019; Chaudhary and Kim, 2019; Lin et al., 2010). The capability of biological agents (e.g., bacteria and fungi) to degrade and recover the contaminants has opened the concept of green engineering. However, the use of whole living cells in the bioremediation process presents some limitations, including the need for the continuous supply of fresh inoculum, aeration, nutritional requirements, specific contaminants that could be toxic to the bacteria and fungi, and the need for acclimatized microbial populations (i.e., prolonged reaction time) (Concetta Tomei and Daugulis, 2013; Eibes et al., 2015; Megharaj et al., 2011; Rayu et al., 2012). Therefore, these limitations have boosted the utilization of enzymes rather than whole cells as bioremediation agents (i.e., enzymatic bioremediation).

Enzymatic remediation utilizes the catalytic ability of natural or engineered enzymes to degrade or transform crude oil into a harmless form. It could also avoid the possibility of releasing exotic or genetically modified organisms into the environment (Zhang et al., 2020). Among various types of enzymatic remediation techniques (e.g., oxygenase, laccase, peroxidase, and dehydrogenase), laccase is ubiquitous which is present in a wide range of organisms, including fungi, and can oxidize several organic compounds such as phenols, polyphenols, arylamines, aromatic thiols, and poly-aromatic hydrocarbons (Aranda et al., 2017; Arca-Ramos et al., 2012; Rahmani et al., 2020; Strong and Claus, 2011). *Marasmiellus palmivorus* is one of the white-rot fungi taxonomically included in the division of Basidiomycota, which can produce extracellular enzymes in the form of laccase (Arora and Gill, 2001). In general, laccase showed some advantages, including high activity, good activity in a broad pH range, high thermal stability, and not being significantly affected by NaCl (Zhang et al., 2020). Despite these advancements, the application of laccase for contaminated soils remediation is minimal compared to bacteria or fungi (Quintella et al., 2019).

To date, many studies are still focussing on the mycoremediation of petroleum hydrocarbon substances instead of enzymatic remediation. A mushroom species belonging to strain *Pleurotus osteatrus* was identified to degrade petroleum-contaminated soil up to 64.7% removal efficiency ((Mohammadi-Sichani et al., 2017). Mycoremediation of crude oil, diesel oil, and used engine oil using *Pupureocillium lilacinum* was found to have removal efficiency of 44.55%, 27.66%, and 14.39%, respectively (Benguenab & Chibani, 2020). Furthermore, Roshandel et al. (2021) investigated the ability of *Pleurotus Florida* to degrade gas oil and was found to remove Total Petroleum Hydrocarbon (TPH) by an average of 55% removal after 30 days. However, few comprehensive studies in the literature examine the enzymatic remediation of oil-contaminated soil (i.e., total petroleum hydrocarbon) by laccase. The limited observations in previous studies are due to the limitation in the number of soil samples tested under different experimental conditions. Most studies used synthetic oil-contaminated soil (i.e., not representative of the practical environmental condition), leading to bias optimization results.

In this study, our objective was to investigate the enzymatic performances of laccase from *Marasmiellus palmivorus* for the remediation of two oil-contaminated soils (e.g., heavily contaminated and lightly contaminated). We used natural oil-contaminated soil from Wonocolo Village, Bojonegoro Regency, Indonesia, to represent the experimental condition for enzymatic remediation. Further, process optimization was done using response surface methodology to attempt the higher enzymatic remediation performance. The results better understand the laccase activity for the enzymatic remediation of oil-contaminated soil.

2. Material and Methods

2.1 Fungi and Culture

Potato Dextrose Agar (PDA) medium inoculated *Marasmiellus palmivorus* fungi, incubated at 25°C for 7 days. Fungi were grown in the preconditioned medium before being cultured using SSF (Solid State Fermentation) technique. The precondition medium consists of 1000 grams of wood powder, 5% (w/w) rice bran, 2.5% (w/w) lime, and 1% (w/w) NPK fertilizer. The SSF technique was carried out with the same medium as the preconditioned medium, then incubated at 25°C for 14 days.

2.2. Soils

Oil contaminated soils were collected at lat. -7.04445 and long 111.66105 on 29th May 2018. Figure **1** shows the sampling point location for oil-contaminated soil. Oil contaminated soils were obtained from traditional oil wells in Wonocolo Village, Bojonegoro Regency. Oil contaminated soils were taken around the good lip (high concentration sample) at 2-3 meters from the well's edge (low concentration sample) and at a depth of 0 – 20 cm below the surface. After collection, the oil-contaminated soils were collected in a 30 L HDPE drum.

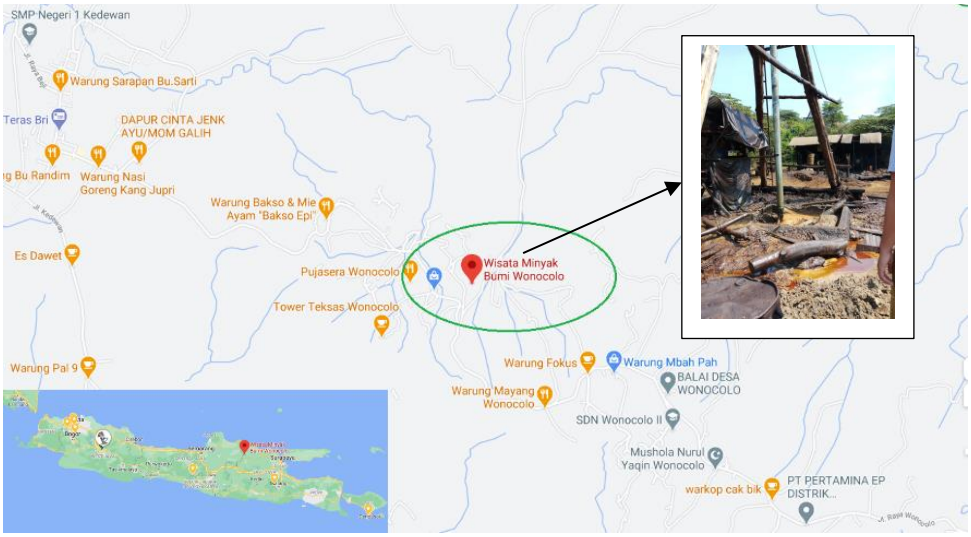

Figure 1. Map for the sampling location of oil-contaminated soils

2.3. *Marasmielus palmivorus* Culture Preparation

Potato Dextrose Agar (PDA) medium was used to inoculate pure culture of *Marasmiellus palmivorus* fungi, then incubated at 25°C for 7 days. Furthermore, fungi were grown in the preconditioned medium before being cultured using the solid-state fermentation (SSF) technique. The precondition medium consists of 1000 grams of wood powder, 5% (w/w) rice bran, 2.5% (w/w) lime, and 1% (w/w) NPK fertilizer. The SSF technique was carried out with the same medium as the preconditioned medium, then incubated at 25°C for 14 days.

2.4 Crude Laccase Enzyme Extraction and Activity Assay

Crude laccase was extracted using a 200 mM sodium phosphate buffer of pH 6 with the agitation of 150 rpm at 25°C for 120 minutes. One liter of sodium phosphate buffer was prepared by mixing 880 mL 200 mM NaH_2PO_4 (molecular weight = 137.99 g/mol) with 120 mL 200 mM Na_2HPO_4 (molecular weight = 268.03 g/mol). The crude laccase extract was isolated by centrifugation at 5,000 rpm for 20 minutes. The supernatant was stored at 4°C for further experiments.

The laccase activity was assayed using 2,2'-azinobis (3-ethylbenzthiazoline)-6-sulfonic acid (ABTS) (Sigma-Aldrich, purity 98%) in the 0.5 mM acetate buffer pH 4.5 as the substrate. The comparison between crude laccase extract and ABTS was 1: 1 with a total volume of 0.5 mL. ABTS oxidation is determined by spectrophotometer (Thermo Scientific, Genesys 10s UV-Vis) at a wavelength of 420 nm (εM = 36,000 M^{-1}/cm). One unit of laccase activity shows the amount of enzyme that can oxidize 1 µmol ABTS ml/minute. Laccase activity is calculated using Equation **(1)**.

$$\text{Laccase Activity (U/L)} = \frac{\text{Vt}}{\varepsilon \text{ x d x Vs}} \text{ x } \frac{\Delta\text{Abs}}{\text{t}} \text{ x Df x Cf} \quad \textbf{(1)}$$

Where U is enzymatic Activity (µmol/min/l), Vt is total volume of reaction (mL), ε = extinction coefficient ABTS at 420 nm (36,000 M^{-1}/cm), d is path length of the cuvette (1 cm), Vs is total volume of crude extract (mL), ΔAbs = delta absorption, t is incubation time (minute), Df = dilution factor (100×), and Cf is correction factor (10^6 µmol/mol)

2.5 Protein Assay

Protein concentration of crude laccase was measured using Bradford reagent (Coomassie dye). A standard protein curve was made using bovine serum albumin (BSA) as a reference with a concentration of 0 to 0.1 mg/mL. The absorbance value was obtained using a spectrophotometer (Thermo Scientific, Genesys 10s UV-Vis) at 595 nm. The laccase-specific activity was determined using Equation **(2)**.

$$Spesific\ activity\ of\ laccase\ (U/mg) = \frac{Laccase\ Activity\ U/mL}{Protein\ Concentration\ U/mg} \quad \textbf{(2)}$$

2.6 Oil Removal Determination

The laccase crude extract was mixed with oil-contaminated soil in a 250 ml Erlenmeyer flask with specific activity (5, 10, and 22 U/mg). Then, samples were collected at 0, 6, 12, 18, 24, 30, and 36 hours. Blank experiments were done using oil-contaminated soils without adding laccase extract. Before the analysis, oil contaminated soils were centrifuged at 1000 rpm for 10 min. The soil will be tested to find the oil removal. In addition, the supernatant will also be tested to find out the percentage of oil recovery.

2.7 Analysis and Measurements

Total petroleum hydrocarbon (TPH) was analyzed using the gravimetric method using APHA 5520-F standard. Ten (10) grams of soil were mixed with n-hexane (ratio 2:1) in an Erlenmeyer. Then the mixture was stirred for 10 minutes. Then the mixture was decantated until the solid and supernatant separated. The supernatant was filtered with Whatman No. 1 and put into a vial bottle. The dry weight of filter paper was taken after the supernatant was dried in the fume hood.

Saturates, asphaltenes, resins, and aromatic (SARA) were analyzed using chromatographic techniques with sequential petroleum leaching processes by organic and mixed solvents. Oil was weighed first, weighing 0.5 – 1 gram, then eluted with n-hexane and filtered with Whatman paper no. 1 to get the asphaltic fraction. Maltenes (saturates, aromatic, and resins) compounds were separated by column chromatography containing silica gel measuring 70-230 mesh. To get the saturate fraction, eluted with n-hexane, which produced a yellow-greenish color. The aromatic fraction is obtained by toluene which will produce a yellowish-brown color. The resin fraction is obtained with a toluene mixture: methanol (9: 1) which is brown.

The qualitative analysis of oil compounds was analyzed using Gas Chromatography-Mass Spectroscopy (GC-MS). The oil samples were prepared using n-hexane solvents. Then analyzed by the helium gas type Gas Chromatography-Mass Spectrometry (GC-MS). The oven temperature is 70°C, and the HP-5MS column measures 0.25 mm x 30 m x 0.25 µm with an initial temperature setting of 290°C and a maximum temperature of 350°C.

2.8 Oil Characterization

The characterization of the oil-contaminated soils is shown in Table 1. The initial TPH concentrations were 4% for soil A (lightly contaminated) and 17% for soil B (heavily contaminated). Moreover, SARA analysis indicated the difference in oil fraction between soil A and soil B. Soil A showed the presence of saturated fraction (53.21%) followed by aromatic oil fraction (41.82%). On the other hand, soil B indicated the presence of four oil fractions, including saturated fraction, aromatic fraction, asphalt fraction, and resin fraction, with the percentage 50.95%, 32.38%, 7.42%, and 3.25%, respectively. Colloidal instability index (CII) was used as a benchmark for determining the instability of colloidal systems in the oil, where both samples showed CII > 0.9, which indicates the precipitation and aggregation phenomena of the colloid (Joonaki et al., 2019). The colloid's precipitate and aggregate would negatively affect the oil recovery (Sun et al., 2014).

Table 1. Initial oil characterization

Samples	TPH (%)	Saturate (%)	Asphaltene (%)	Resin (%)	Aromatic (%)	CII[a]
Soil A	4	53.21	-	-	41.82	1.27
Soil B	17	50.95	7.42	3.25	32.38	1.64

[a]CII = (saturates + asphaltenes)/(resins + aromatics). CII > 0.9 unstable and CII < 0.7 stable

2.9 Enzymatic Activity Optimization

Two variables that may significantly affect oil removal from soil were tested using central composite design (CCD) to find their optimum levels. The details of CCD experiments are listed in Table 2. The experimental data were analyzed by response surface methodology (RSM), and the relationship between the independent variables has been explained by the second-order polynomial model (Equation (3)).

Table 2. Central composite design matrix for two variables (e.g., reaction time and laccase concentration)

Run	Designed Factor	
	Reaction time (hour) - X_1	Laccase concentration (U/mg) - X_2
1	18	10
2	18	10
3	0	10
4	18	0
5	18	10
6	18	10
7	0	0
8	18	22
9	36	10
10	36	0
11	36	22
12	18	10
13	0	22

$$Y = a_0 + \sum a_i X_i + \sum a_{ii} {X_i}^2 + \sum a_{ij} X_i X_j \quad (3)$$

Where Y is the response (TPH removal from oil-contaminated soil); Xi and Xj are independent variables in input values, a_o is the model intercept, a_i is the linear coefficient, a_{nd} the quadratic coefficient

a_{ij} is the interaction effect. Experimental design, regression analysis, and response surface graph were performed using the statistical software StatEase DesignExpert ver. 12 (trial version).

3. Result and Discussion

3.1 TPH Degradation from Contaminated Soil

The laccase from *Marasmiellus palmivorus* was tested to degrade the TPH. In parallel, blank experiments were done in the absence of laccase. The control experiment for both soils showed the removal of TPH from oil-contaminated soil is up to 47% (Figure 2). The relatively high removal of TPH from oil-contaminated soils was due to indigenous soil microorganisms, which can degrade the oil and the possibility of volatile oil (Mori et al., 2015). All samples containing laccase demonstrated a higher ability to remove TPH than in the absence of laccase. Whereas the highest removal for soil A and soil B were around 52% (laccase = 22 unit/mg, reaction time = 12 hours) and 60% (laccase = 5 unit/mg, reaction time = 36 hours), respectively (Figure 2). The slightly improved removal of TPH observed by adding laccase suggests that the enzyme has actively participated in the remediation process. The synergetic effect of indigenous microorganisms and laccase on the soil leads to the higher removal of oil (Polyak et al., 2018; Wu et al., 2008). Therefore, it indicated the enzymatic activity of laccase to degrade oil on contaminated soil, and the optimization of laccase degradation is needed to achieve the best combination performance.

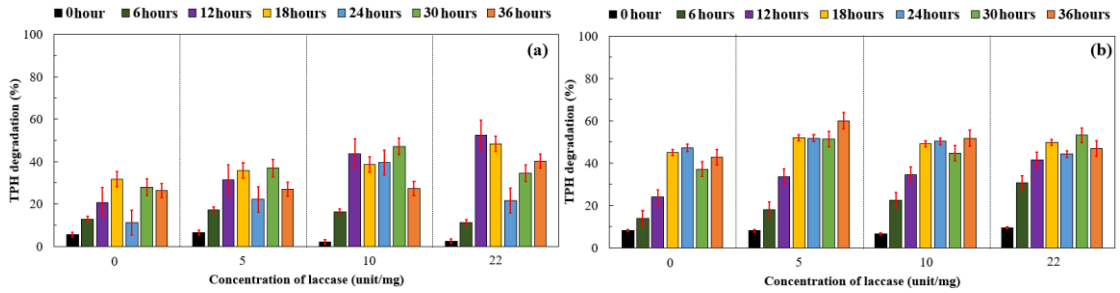

Figure 2. TPH degradation from (a) soil A and (b) soil B under different concentration of laccase (0, 5, 10, and 22 unit/mg) and various reaction times (0, 6, 12, 18,24, and 36 hours).

Two parameters for laccase degradation performance of TPH (e.g., laccase concentration and reaction time) were selected. The interaction between these factors and the value of optimal levels was studied using CCD (Table 3). Moreover, two second-order polynomial model equations were generated to predict TPH degradation on soil A (Equation (**4**)) and soil B (Equation (**5**)).

Table 3. Central composite design matrix for two variables with experimental and predicted values of TPH degradation

Run	Designed Factor		TPH Degradation (Experiment)	TPH Degradation (Prediction)	Residual	R^2	R^2adj
	Reaction time (hour) - X_1	Laccase concentration (U/mg) - X_2					
			Soil A				
1	18	10	38.68	38.34	0.34	0.98	0.97
2	18	10	38.68	38.34	0.34		
3	0	10	2.03	1.88	0.15		
4	18	0	31.70	36.36	-4.66		
5	18	10	38/68	38.34	0.34		
6	18	10	38.68	38.34	0.34		
7	0	0	5.62	3.84	1.78		
8	18	22	48.46	45.52	2.94		
9	36	10	27.43	29.29	-1.86		

Run	Designed Factor		TPH Degradation (Experiment)	TPH Degradation (Prediction)	Residual	R^2	R^2adj
	Reaction time (hour) - X_1	Laccase concentration (U/mg) - X_2					
10	36	0	26.24	23.36	2.88		
11	36	22	41.19	41.20	-1.01		
12	18	10	38.68	38.34	0.34		
13	0	22	2.39	4.32	-1.93		
			Soil B				
1	18	10	49	49.1	-0.1	0.99	0.99
2	18	10	49	49.1	-0.1		
3	0	10	6.57	9.39	-2.82		
4	18	0	44.96	45.41	-0.45		
5	18	10	49	49.1	-0.1		
6	18	10	49	49.1	-0.1		
7	0	0	8.13	6.22	1.91		
8	18	22	49.83	48.86	0.97		
9	36	10	51.79	48.45	3.34		
10	36	0	42.79	44.24	-1.45		
11	36	22	46.96	48.84	-1.88		
12	18	10	49	49.1	-0.1		
13	0	22	9.44	8.52	0.92		

$$Y = 38.74 + 14.10X1 + 54.58X2 + 4.34X1X2 - 22.75X1^2 \quad (4)$$

$$Y = 49.28 + 19.58X1 + 1.73X2 - 20.18X1^2 \quad (5)$$

The adequacy of the above models has been checked using analysis of variance (ANOVA) and only significant coefficients (p-value < 0.05) that included in the model generation. Moreover, for both models, the regression model's R2 (correlation coefficient) value is > 0.95, indicating that the experimental data fitted well with the quadratic model. The three-dimensional and contour plots for TPH degradation for soil A and B as a function of reaction time and laccase concentration are also shown in Figure 3. This analysis detected the maximum TPH degradation for both soils. Based on the model prediction, the optimum laccase concentration was 21.37 units/mg, and reaction time was 26.23 hours, providing a maximum TPH degradation of around 48.57%. On the other hand, the maximum TPH degradation for soil B could reach around 54.1% with an optimum laccase concentration of 12.37 units/mg and a reaction time of 28.28 hours. The higher removal of TPH from soil B is due to the highly polar SARA fraction (i.e., the presence of asphaltene and resin) compared to soil A (Kucharzyk et al., 2018).

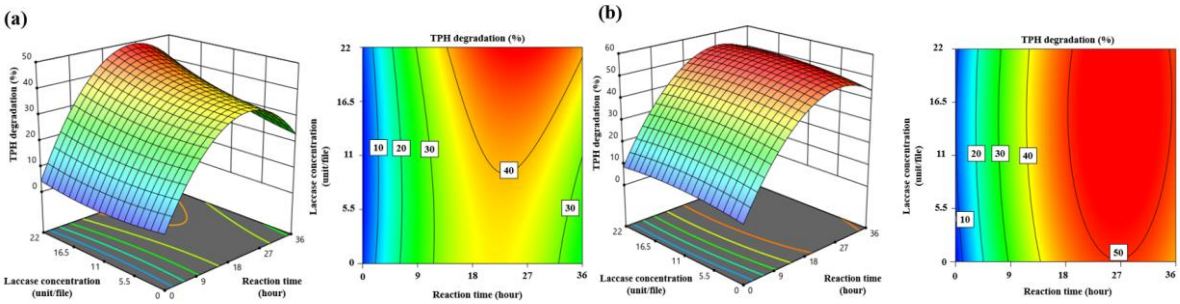

Figure 3. Three-dimensional and contour plots of the two second-order polynomial analysis representing the combination of laccase concentration, reaction time, and resulting TPH degradation for (a) soil A and (b) soil B.

3.2 Changes in Hydrocarbon Composition

According to the GC-MS analysis (Figure 4), there were 45 compounds for soil A before treatment with laccase. After enzymatic remediation with laccase, there were around 35 compounds left. The most dominant compound for soil A (i.e., before and after treatment) is pentadecane 2,6,10,14-tetramethyl. In the case of soil B, GC-MS detected around 62 and 57 compounds before and after the laccase treatment, respectively. Further analysis indicated that soil B's most dominant compound is the same as soil A (i.e., pentadecane 2,6,10,14-tetramethyl). This compound is also known as pristane (C19). The occurrence of pristane in crude oil is occasionally abundant, and it is used as a recalcitrant biomarker. The presence of pristane in high concentration after enzymatic remediation indicated that the crude oil used in this study could be categorized as a less biodegradable substance.

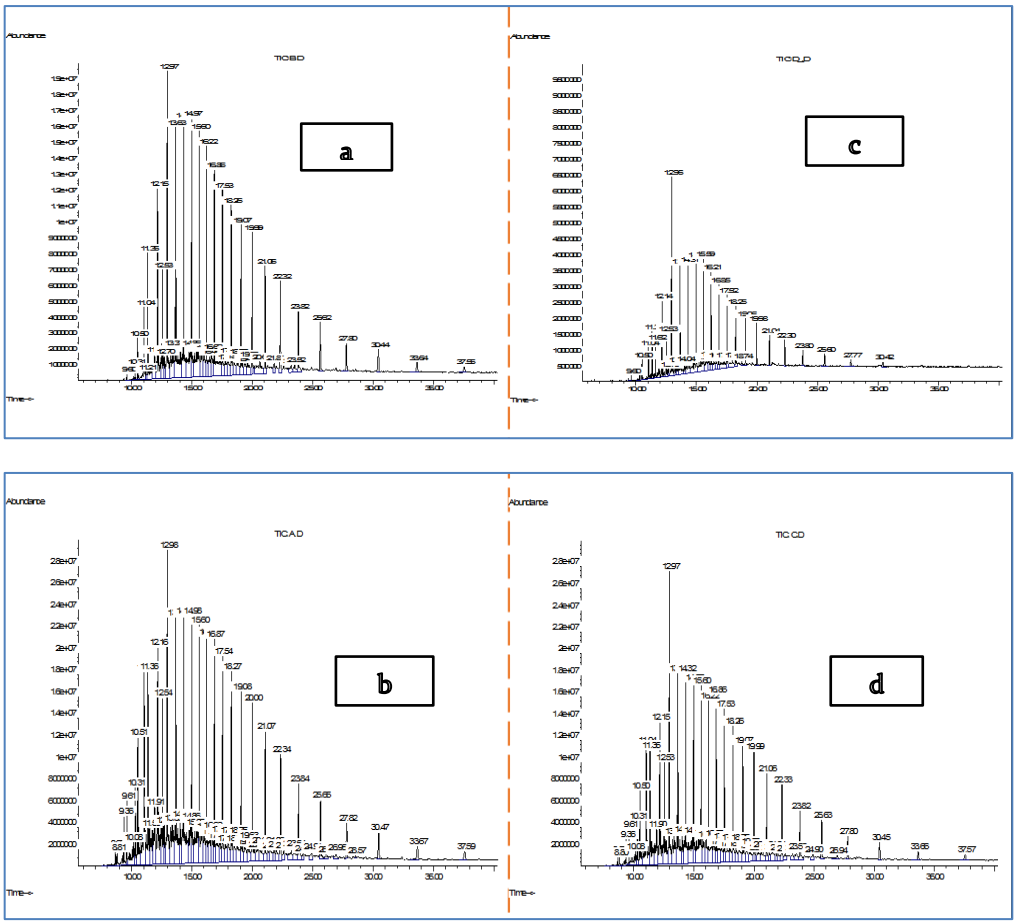

Figure 4. Analysis of GC-MS for (a) soil A before treatment, (b) soil B before treatment, (c) soil A after laccase treatment, and (d) soil B after laccase treatment.

3.3 Oil Recovery

Instead of fully mineralizing, it was found that the long-chain Hydrocarbons were oxidized and degraded into shorten chain hydrocarbons, especially aromatic compounds, by adding laccase enzyme. Therefore, oil binding in the form of aromatic compounds with soil matrix was loosened, and as a result, these aromatic compounds were released as recovered oil. The effect of laccase addition on the oil recovery is shown in Figure 5. Samples containing laccase all exhibited higher TPH recovery than in the absence of laccase (i.e., control experiment). The highest TPH recovery for soil A and B were 9.89% (laccase = 10 unit/mg, reaction time = 30 hours) and 10.1 % (laccase = 5 unit/mg, reaction time = 12 hours), respectively.

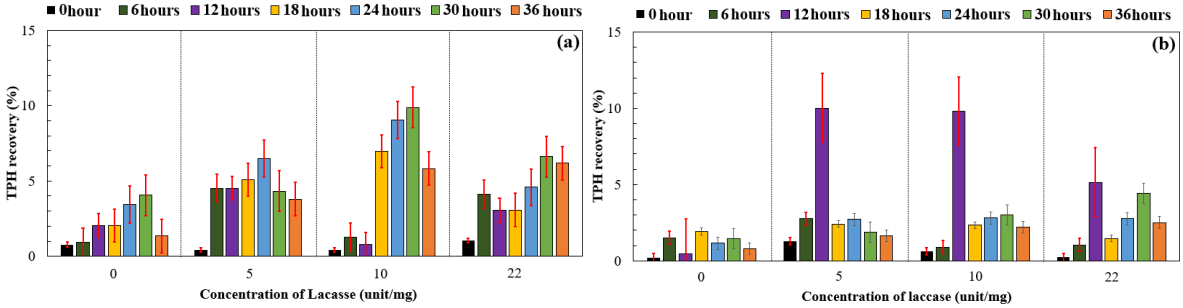

Figure 5. TPH recovery from (a) soil A and (b) soil B under different concentration of laccase (0, 5, 10, and 22 unit/mg) and various reaction times (0, 6, 12, 18,24, and 36 hours)

Furthermore, the results of recovered oil fraction indicated the shift of oil characterization (Table 4). The aromatic fraction has become the leading oil fraction for both soils, followed by the small amount of saturated fraction. The disappearance of asphaltenes and resin fraction in soil B indicated the contribution of laccase to degrade polar SARA fraction (Kucharzyk et al., 2018). Furthermore, CII values after laccase recovery tend to decrease (CII < 0.7), which indicates stable oil formation (i.e., no precipitation and no aggregation), which can be quickly recovered (Joonaki et al., 2019; Xu and Lu, 2011).

Table 4. SARA fraction of recovered oil

Samples	Saturate (%)	Asphaltene (%)	Resin (%)	Aromatic (%)	CII
Soil A	9.7	-	-	86.98	0.11
Soil B	2.56	-	-	91.16	0.03

4. Conclusion

Laccase enzyme extracted from *Marasmiellus palmivorus* was successfully used for the enzymatic remediation and oil recovery from two different characteristics of oil-contaminated soils. The presence of laccase and indigenous soil organism led to the increment of TPH degradation and oil recovery. The polarity of the SARA fraction showed a positive effect on TPH degradation, where laccase preferentially degraded the high polarity SARA fraction on the soil. Moreover, laccase showed the capability for oil recovery by decreasing the CII that makes the oil stable and easy to recover from the soil. Finally, the optimization of laccase performance for TPH degradation was performed under realistic conditions (i.e., using natural oil-contaminated soils). Overall, the enzymatic remediation using laccase could provide a promising alternative technology. Future studies should optimize and emphasize more on the utilization of laccase in oil recovery

Acknowledgment

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List of Abbreviation

TPH: Total Petroleum Hydrocarbon; PDA: Potato Dextrose Agar; SSF: Solid State Fermentation; NPK: Nitrogen Phosphate Kalium; ABTS: 2,2'-azinobis (3-ethylbenzthiazoline)-6-sulfonic acid; GC-MS; Gas Chromatograph – Mass Spectrophotometer; SARA: Saturates, Asphaltenes, Resins, and Aromatic; CII: Colloids Instability Index; HDPE: High-Density Polyethylene; APHA: American Public Health Association; CCD: Central Composite Design; RSM: Response Surface Methodology; BRIN: Badan Riset & Inovasi Nasional.

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