Biodegradation of buried crude oil in soil microcosm by fungal co-culture

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Abstract. Crude oil pollution in soil is a major environmental problem over the world. After long-term contamination, crude oil can be buried in the soil and leaving the toxic and persistence compounds. Treatment of buried crude oil in soil is a major challenge. In this study, buried crude oil in soil (20 cm-depth) was treated by the co-culture of three fungal strains (Pestalotiopsis sp.NG007/Polyporus sp. S133/Trametes hirsuta D7 with composition 1/1/1 and pre-grown in wood meal) placed on the top or bottom layer of the soil. Biodegradation behavior of crude oils were analyzed at different depth interval (0–5 cm, 5–15 cm, and 15–20 cm) with constant oxygen diffusion. The results showed that both position of fungal co-culture could degrade crude oil 63 – 92% after 30 days. Placing the co-culture in the bottom layer position of the soil demonstrated more effective degradation for all the depth interval than that of the top layer position. During the biodegradation, the co-culture produced oxidative enzymes such as catechol 1,2-dioxygenase, catechol 2,3-dioxygenase, laccase, manganese peroxidase, and lignin peroxidase with significant activities. This study offers an important strategy to remediate crude oil contaminated soil, specifically buried crude oil.

1. Introduction
Exploitation of crude oil continues to grow and plays a significant role in the modern industry. Some protocols and regulations have governed the use of crude oil, but unconsciously crude oil becomes a source of contaminants in the environment and ecosystems around the world [1,2]. The average global crude oil spread is around 9.1 million tons per year and is predicted will continue to grow [3]. Contamination of crude oil can occur in aquatic and terrestrial environments. Oil spills cases in Indonesia were recorded in 1999, about 4000 barrels of oils had contaminated sea in Cilacap - Central Java; in 2009 occurred in Manyar, Gresik - East Java; in 2014 occurred in Indramayu, West Java (along 7 km of coastline was polluted by crude oil) and 200 barrels of crude oil contaminated the swamp river in Kab. Siak – Riau.

Crude oils that contaminated the aquatic and terrestrial environments exhibit unique characteristics. In order to achieve an appropriate target in the bioremediation of crude oil, understanding these
characteristics is important. Since the crude oil contaminated soil, spilled oil is generally slow-moving or static. The oil collects in depressions or against natural and man-made barriers, usually, the size of the affected area is small and it is easy to define the location and amount of surface oil, only light oils spread to form a thin layer; often considerable pooling of oil weathering slows considerably after approximately 24 hours [4]. Besides, soil characterization, enzymes involved during biodegradation, and metabolites are major factors in the bioremediation of crude oils [5].

Degradation of crude oil in contaminated soil has been reported widely. Strain Polyporus sp. S133 degrades by 26% crude oil 15000 ppm in soil [6]. Bacillus sp. degrade crude oil (80-89%) from the initial concentration of 5000 ppm for 5 days [7]. Etreptomyces sp. ERI-CPDA-1 has the ability to extract diesel oil by 98% from an initial concentration of 1000 ppm for 7 days [8]. Dietzia cinnameno, Hoyosella altamirensis and Vibrio alginolyticus bacteria isolated from the Gulf of Kuwait are able to degrade crude oil by 39% (initial concentration of 1000 ppm) for 14 days [9]. However, research on degradation of buried crude oil in soil is still limited. After long-term contamination, crude oil can be buried in the soil and leaving toxic and persistence compounds. Treatment of buried crude oil in the soil is a major challenge. Therefore, degradation of buried crude oil in soil (20 cm-depth) is important to be investigated. This study uses the co-culture of three fungal strains placed on the top or bottom layer of the soil to degrade crude oil. Biodegradation behavior was analyzed at different depth intervals of the soil.

2. Materials and methods
Crude oil was obtained from Taiyo Petroleum Co., Ltd. (Japan). Agar, malt extract, glucose, silica gel C-200, polypeptone, and other solvents were purchased from Wako Pure Chemical Industries, Ltd. (Japan). Pestalotiopsis sp. NG007 and Polyporus sp. S133 were previously reported to have the ability to degrade crude oil [10–12]. T. hirsuta D7, a basidiomycete, was newly isolated from a fungal body that grew on rotten wood near the River Pakning in the peat swamp forest Bengkalis, Riau, Indonesia [13]. All three fungi were cultured on malt extract agar (MEA) medium containing 20 g L⁻¹ malt extract, 20 g L⁻¹ glucose, 1 g L⁻¹ polypeptone, and 20 g L⁻¹ agar.

2.1. Pre-culture in liquid medium
Pestalotiopsis sp. NG007, Polyporus sp. S133, and T. hirsuta D7 were individually pre-cultured in 20 mL malt extract (ME) liquid medium containing 20 g L⁻¹ ME, 20 g L⁻¹ glucose, and 1 g L⁻¹ polypeptone. The pH of the medium was adjusted to 4.5. Strains were pre-cultured under dark conditions at 25°C for 7 d. Each culture was homogenized at 5000 rpm for 10 min before being applied to soil artificially contaminated with crude oils.

2.2. Pre-grown in wood meal
The wood meal was obtained from Daigo Mokuzai Co., Ltd. (Osaka, Japan). Several 5-mm disks of an actively growing strain: NG007, S133, or D7 in MEA medium as described in section 2.2, were transferred to 3-h autoclaved wood meal (100 g) combined with 10% (w/w) glucose, 15% (w/w) shiitake nutrient (a kind of nutrient for fungal growth purchased from Showa Sangyo Co. Ltd., Tokyo, Japan), and 60% (v/w) distilled water, and then incubated for approximately one month. Pre-grown cultures were maintained in a cool room at 4°C prior to use.

2.3. Penetration test of individual fungus
Fungi NG007, S133, and D7 were individually placed on the top or bottom layer of soil (containing 10% w/w glucose and 60% v/w distilled Water). The ability of the fungus to penetrate the soil with 20 cm depth was investigated every day for 17 days.

2.4. Experimental design
Soil consisting of 10% w/w glucose and 60% v/w distilled water (non-slurry soil) was artificially contaminated by crude oil to a final concentration of 1000 mg kg⁻¹ soil. The artificially contaminated
soil was mixed until it was homogenous then was placed in cylinder flask 2 L with 20 cm-depth. Pre-grown fungi NG007, S133, and D7 were mixed and added to soil with composition 1/1/1 and final total concentration of 10% w/w of the artificially contaminated soil. In this experiment, the fungal co-culture was placed on the top or bottom layer of soil. During incubation, oxygen diffusion was applied with constant flow rate. The soil was divided into 3 depth intervals (0–5 cm), (5–15 cm), and (15–20 cm), respectively from the top layer of soil. Degradation of crude oil was investigated after 15 and 30-day incubation in each interval. Abiotic control was performed with no addition of fungal co-culture into soil mixture containing 1000 mg kg⁻¹ crude oil.

2.5. Chemical analysis of remaining PHCs
Description of the biodegradation analysis of crude oils and their fractions (aliphatic, aromatic, resin, and asphaltene) was provided in our previous study [10]. The crude oil that remained in each depth interval of the soil mixture was consecutively extracted (taken about 30 gram from each depth interval) using 100 ml n-hexane, 100 ml dichloromethane, and 100 ml chloroform. After evaporation, the total biodegradation of crude oil was analyzed using the gravimetric method. n-Hexane was used to separate asphaltene from the PHCs. The n-hexane-insoluble fraction that remained in the filter paper after filtration mostly consisted of asphaltene. The weight of asphaltene was analyzed by the gravimetric method. n-Hexane-soluble fractions were further separated to obtain aliphatic, aromatic, and resin fractions using purification on a silica gel (C-200) column eluted with 100 ml of n-hexane, 100 ml of toluene, and 200 ml of chloroform: methanol (1:1 v/v), respectively. The aliphatic and aromatic fractions were subjected to gas chromatography (GC-FID Shimadzu 2014) with a TC-5 capillary column (30 m, id x 0.25 mm x 0.25 µm). The carrier gas, helium, was delivered at a constant flow rate of 1.5 ml min⁻¹ with a column pressure of 100 kPa and an interface temperature of 280°C. The column temperature was started at 60°C, increased at 10°C min⁻¹ to 280°C, and then maintained at 280°C for 10 min. The injection volume was 2 µl and the injector temperature was maintained at 280°C. Aliphatic and aromatic biodegradation was calculated in the post-run analysis with a calculated minimum area of 100 using the following equation:

\[ B = \frac{\sum A_0 - \sum A_1}{\sum A_0} \times 100\% \]  (1)

where \( B \) (%) is the biodegradation of the aliphatics or aromatics; \( \sum A_0 \) is the total area of the aliphatic or aromatic fraction from the abiotic control; \( \sum A_1 \) is the total area of the aliphatic or aromatic fraction after treatments.

2.6. Enzymatic assay
The enzymatic activities of catechol 1,2-dioxygenase (C120), catechol 2,3-dioxygenase (C23O), laccase, manganese peroxidase (MnP), and lignin peroxidase (LiP) were assayed using a UV-Vis spectrophotometer (Shimadzu UV-1600, Japan). C12O activity was assayed by the method described by Nakazawa and Atsushi [14]. C23O activity was assayed as described by Nozaki [15]. Laccase activity was measured as described by Leonowicz and Grzywnowics [16]. MnP activity was determined based on the oxidation of 2,6-dimethoxyphenol (2,6-DMP) by the MnP system to form a quinine dimer [17]. LiP activity was determined by the oxidation of veratryl alcohol to form veratryl aldehyde at 310 nm [18]. All experiments were performed at 25°C. Activities were expressed as units per gram wet soil matter (U g⁻¹), in which one enzyme unit (U) was defined as that forming 1.0 µmol of the product per minute under the assay conditions.

3. Results and discussion

3.1. Composition of crude oil
Crude oil used in this study was composed 49.3% aliphatic, 34.0% aromatic, 8% resin, and 8.67% asphaltene with the API gravity number 17.9 [10]. The aliphatic fraction contains C12 to C30 with the
majority of middle carbon numbers and the aromatic fraction contains several polyaromatic hydrocarbons (PAHs) such as anthracene (ANT) and phenanthrene (PHE) [12]. Based on the API gravity number, the crude oil sample was categorized as heavy crude oil.

3.2. Penetration of fungal growth
Penetration of individual fungal culture NG007, S133 and D7, respectively were investigated in 20 cm-depth of soil contaminated with crude oil. Each culture was placed in the top or bottom layer of the soil. As shown in figures 1.a and 1.b, all the fungi placed in the bottom layer grow faster than that of in the top layer. The penetration of mycelial fungi in the soil was influenced by the type of fungi, moisture content of the soil and type and percentage of bulking agents [19]. This study found that placement of the fungi also affected the ability of fungi to penetrate the soil. For individual fungal strain, NG007 demonstrated a faster growth compared with strain D7 and S133, respectively. Strain NG007 was only able to reach a depth of downward penetration as far as 14.7 cm after 17 days of incubation when it was placed in the top position. However, when it was placed in the bottom position, strain NG007 was able to cover the entire upper layer in 5 days. These phenomena were also found in strain D7 and S133 which was able to grow until the upper layer in 13 and 15 days respectively when they were placed in bottom position figure 1.b. For fungal co-culture, the growing penetration rate detected in the soil was lower than that of all the single strain. The fungal co-culture covered all the soil within 15 and 25 days when they were placed in the top or bottom position, respectively. This might be caused by the time-need interaction of all the fungal strains in the co-culture at the initial stage of growth.

![Figure 1](image1.png)

**Figure 1.** Penetration depth of individual fungal growth placing in the top layer (a) or bottom layer (b), and mixture of fungal co-culture growth (c). Symbol in Figure 1.a and 1.b (Pestalotiopsis sp. NG007 (○), *T. hirsuta* D7 (△), and *Polyporus* sp. S133 (□)). Symbol in Figure 1.c (top layer (●) and bottom layer (▲))

3.3. Biodegradation of crude oil
Long-term contamination by crude oil allows the components of crude oil to settle in the soil for a long time. Some fractions like aliphatic and aromatic may move to the bottom layer easily than the higher fractions (resin and asphaltene). This will lead to the increasing concentration of aliphatic and aromatic fractions in the bottom layer. Aliphatic and aromatic are generally more susceptible to biodegradation than resin and asphaltene. Therefore, the degradation results for TPH were generally
lower from top to bottom (figure 2.a). In addition, placing the fungal co-culture in the bottom position made the biodegradation enhanced in all the layer because of the higher activities of enzyme produced.

The study found that the ability of fungi to degrade crude oil was influenced by the distance of the crude oil sample from the fungi and the growth ability of the fungi to penetrate the soil. The efficiency of degradation of crude oil in a distance 20 cm from the initial fungi decreased 9.8 and 26.8% when the fungi were placed in the bottom and top of the soil, respectively. Besides, because of the oxygen-dependent growth, some fungi preferred to grow in an upward direction than to penetrate down the soil.

The fungal co-culture degraded 63–92% crude oil in the soil for 30 days. All the fractions in crude oil could be degraded by the fungal co-culture with a degradation rate 42–90% for aliphatic fraction, 86–98% for aromatic fraction, 85–100% of asphaltene fraction, and 55–93% of resin fraction, respectively. Strain NG007, D7, and S133 had been reported to be able to degrade PAHs and crude oils [10]. A combination of all these fungi has been reported had a beneficial effect in total petroleum hydrocarbons (TPHs) degradation. This study supports previous results that the fungal co-culture is also able to degrade buried crude oil in the soil to a depth of 20 cm.

**Figure 2.** TPH degradation (a) and fractions degradation (b) of crude oil by mix culture of NG007/D7/S133 1/1/1 in soil for 15 and 30 days at different interval of depth 0–5 cm (1), 5–15 cm (2), and 15–20 cm (3).
3.4. Enzyme activities

Crude oil is a complex mixture that contains aliphatic, aromatic, resin and asphaltene compounds. The co-culture NG007/S133/D7 1/1/1 had been reported able to degrade crude oil by the combination of enzymes produced by the co-culture. In this study, during the incubation of the co-culture in soil buried crude oil, several oxidative enzyme such as C12O, C23O, lac, MnP and LiP were detected (table 1). Dioxygenase enzymes such C12O and C23O had been reported initiating the degradation of aliphatic fractions in crude oil. Also, ligninolytic enzymes such as lac, MnP and LiP may contribute to the acceleration of crude oil biodegradation by attacking the aromatic, resin and asphaltene fractions. Placement of the initial position of the co-culture affected the degradation and enzyme production in different depth interval of soil. Generally, the activities decreased when the distance of soil from the initial place of co-culture increased. For example, when the co-culture was placed in the top position, activities of enzymes generally decrease from top to bottom layer.

| Depth (cm) | Bottom | Top |
|-----------|--------|-----|
|           | C12O   | C23O | Lac | MnP | LiP | C12O | C23O | Lac | MnP | LiP |
| 1 (0 - 5) | 135    | 20   | 103 | 31  | 30  | 248  | 49   | 1185| 225 | 26  |
| 2 (5 - 15)| 120    | 29   | 163 | 14  | 13  | 73   | 29   | 141| 92  | 15  |
| 3 (15 - 20)| 145  | 49   | 1924| 129 | 40  | 103  | 33   | 123| 17  | 30  |

4. Conclusions

Both positions of fungal co-culture degraded the crude oil 63–92% after 30 days. The co-culture placed in the bottom layer position of the soil demonstrated more effective degradation for all the soil depth interval than the top layer position. During the biodegradation of the crude oil, the co-culture produced several oxidative enzymes such as catechol 1,2-dioxygenase, catechol 2,3-dioxygenase, laccase, manganese peroxidase, and lignin peroxidase with significant activities indicating the important role of these enzymes during the degradation. This study offers an important strategy to remediate crude oil contaminated soil, specifically for soil buried crude oil.

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