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Bioremediation of Crude Oil by White Rot Fungi *Polyporus* sp. S133

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The bioremediation potential of crude oil by *Polyporus* sp. S133 pre-grown in wood meal was investigated in two separate experiment trials; liquid medium and soil. The effect of three nutrients (glucose, polypeptone, and wood meal), oxygen flow, and some absorbent on the efficiency of the process was also evaluated. Degradation of crude oil in soil was significantly increased with an addition of oxygen flow and some absorbent (kapok and pulp). The highest degradation rate of crude oil was 93% in the soil with an addition of 10% kapok. The present study clearly demonstrates that, if suitably developed, *Polyporus* sp. S133 could be used to remediate soil contaminated with crude oil.

Keywords: Bioremediation, crude oil, nutrients effect, *Polyporus* sp. S133, white rot fungi

The oil industry inevitably generates large quantities of fatty and viscous residue called oily sludge, which is formed during various production, transportation, and refining processes. Pollution caused by petroleum and its derivatives is the most prevalent and widespread environmental contaminants adversely affecting human health and posing environmental problems [5, 16]. Considering the ongoing pollution and toxicity of oil hydrocarbons, developing an effective bioremediation strategy still remains an issue. Seeding of the polluted site by hydrocarbon degraders has long been practiced as a remediation strategy, and the importance of indigenous oil-degrading bacteria has been well understood [2, 33, 39, 40]. Two of the major issues hindering rapid degradation of the oil hydrocarbons are lack of nutrients (except carbon) and poor availability of hydrocarbons. Hence, the greatest challenge for microbiologists and bioengineers in this area of study is to develop the technology that will ensure close contact of the relevant microorganisms with the hydrocarbon and adding in the deficient nutrients.

Bioremediation employs microorganisms capable of degrading toxic contaminants for the reclamation of a polluted site [4, 9]. Augmenting the contaminated site with an appropriate microorganism inoculum is a promising technique to enhance the biodegradation of hydrocarbons. Moreover, using an indigenous microorganism consortium ensures that the organisms have a higher tolerance to the toxicity of hydrocarbons and are able to withstand variations of environmental conditions [7]. Many carrier materials, mostly agricultural by-products, are used to transfer the microorganism consortium to the fields effectively. The carrier materials provide nutrients, moisture, and physical support for the increased aeration required by the microorganisms and also can lengthen the shelf-life of the microorganisms until they are applied in the field. Extended survival of the microorganisms under field conditions is essential for efficient degradation of the toxic hydrocarbons, especially of the multi-ringed aromatic hydrocarbons and the recalcitrant ones [19, 34].

White rot fungi would have a greater access to poorly bioavailable substrates, since they secrete extracellular enzymes involved in the oxidation of complex aromatic compounds like lignin and many hazardous environmental pollutants [3, 11, 24]. During the past decade, a diverse group of ligninolytic and non-ligninolytic fungi have been shown to have the ability to oxidize a wide variety of PAHs such as *Phanerochaete chrysosporium* that can degrade acenaphthene, acenaphthylene, anthracene, benzo(a)anthracene, benzo(a)pyrene, benzo(b)fluoranthene, benzo(g,h,i)perylene, benzo(k)fluoranthene, chrysene, fluorene, indeno(1,2,3-c,d)pyrene, naphthalene, phenanthrene, and pyrene [15]; *Bjerkandera adusta* that can degrade dibenzothiophene, fluoranthene, pyrene, and chrysene [38]; and *Irpex lacteus* that can degrade phenanthrene, anthracene, fluoranthene, pyrene, chrysene, benzo(b)fluoranthene, benzo(k)fluoranthene,

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benzo(a)pyrene, dibenzo(a)anthracene, benzo(g,h,i)perylene, and indeno(1,2,3-c,d)pyrene [20]. These results indicate that at least one white root fungus offers real potential for the bioremediation of a wide range of PAHs-polluted sites. Therefore, a wide range of PAH-degrading capability of other white rot fungi strains has been overlooked.

The present study aimed to investigate the ability of a new fungus isolated from hydrocarbon-contaminated soil, *Polyporus* sp. S133, to degrade crude oil in liquid medium and soil. Previously, extensive biodegradation of chrysene and phenanthrene had been revealed using this strain [13, 14]. The effects of addition of some nutrients, oxygen flow, and absorbents in the degradation process were also investigated to evaluate the optimum treatment of fungi for bioremediation in soil. This information is important for devising a fungi-based technique for treatment of crude oil pollution, especially under solid-state-like environmental conditions, which usually favor fungi growth over bacterial growth.

Materials and Methods

Fungi

Polyporus sp. S133 was isolated as previously described [13]. This strain was developed by an enrichment method using sterile wood meal. Wood meal used in this experiment was purchased from Intracawood Manufacturing, PT (Indonesia). To obtain the sterile wood meal, the wood meal was sterilized by autoclaving with shiitake nutrient at 121°C for 2 h, followed by adjusting the moisture content to 60%. They were mixed with 10 plugs of *Polyporus* sp. S133 and incubated at 25°C for 30 days.

Culture Conditions and Fungal Inoculum

Polyporus sp. S133 was selected based on its ability to degrade crude oil in a solid agar medium containing 20 ml of malt extract agar [2% (w/v) malt extract, 2% (w/v) glucose, 0.1% (w/v) polypeptone, and 1.5% (w/v) agar] with the addition of crude oil dissolved in hexane, and then incubated at room temperature for two weeks and observed daily. A single colony of crude-oil-degrading fungi was transferred to a malt extract medium containing crude oil. The malt extracts medium (MEM) contained (in g/l distilled water, pH 4.7): glucose (20), malt extracts (20), and polypeptone (1). The fungal inoculum was prepared by growing the fungus on malt extract agar plates at 25°C for 7 days. The inoculum was added to a flask containing the malt extract medium. Flasks were shaken at 120 rpm at 25°C and filtered through filter paper under sterile conditions. Mycelia were then transferred to each vial containing fresh medium.

Soil Treatment

The soil samples were collected from A Horizon (0–25 cm). They were air-dried to a water content of 0.12 g^{-1} (18°C, 72 h), sieved (3 mm), and stored at 48°C in sealed containers until used for experiment. To obtain sterile soil, the soil was sterilized through three successive autoclaving periods of 1 h each, on three consecutive days. It was followed by adjusting the moisture content to 40% of maximum water-holding capacity (WHC; 0.2 ml/g dry soil) after each autoclaving. For equilibration and biodegradation experiments, the water contents of non-treated and autoclaved soils were adjusted to 60% maximum WHC (0.31 ml/g dry soil) with sterile distilled water. To ensure the homogeneity of the treatments, soil samples were then sieved again through 3 mm mesh.

Bioremediation Assay with *Polyporus* sp. S133 in Liquid Medium

Experiments were performed in 100 ml Erlenmeyer flasks containing 20 ml of the liquid medium supplemented with various concentrations (1,000–15,000 ppm) of crude oil dissolved in hexane. Three 5 mm disk mycelia plugs of *Polyporus* sp. S133, obtained by punching out with a cork borer from the outer edge of an actively growing culture, were inoculated into a flask. Control experiments of sterilized MEM supplemented with crude oil without inoculum were also performed. All flasks were incubated at 25°C, for 30 and 60 days. All assays were conducted in triplicate.

Bioremediation Assay with *Polyporus* sp. S133 in Soil

Experiments were performed in a box containing 200 g soil and approximately 3,000 mg of crude oil. The isolated fungus pre-grown on wood meal was applied to the soil surface and then mixed thoroughly with a sterile spatula. The final concentration of crude oil in treated soils was measured at some concentration plus a specified amount of the factor to be studied: effect of nutrients addition, oxygen flow, and absorbent such as kapok and pulp. All flasks were incubated at 25°C, for 30 and 60 days. All assays were conducted in triplicate.

Quantification of Total Petroleum Hydrocarbon Contamination in Liquid Medium and Soil

Samples were collected at time zero (just before initiating the bioremediation), 30 days later, and at the end of the study (60 days after initiating the process). Total petroleum hydrocarbon (TPH) from the liquid medium flask and 10 g soil were then consecutively extracted with hexane, dichloromethane, and chloroform (100 ml each). All three extracts were pooled and dried at room temperature by evaporation of solvents under a gentle nitrogen stream in a fume hood. After solvent evaporation, the amount of residual TPH was determined gravimetrically. After gravimetric quantification, the residual TPH was fractionated into alkane, aromatic, asphaltene, and NSO (nitrogen-, sulfur-, and oxygen-containing compounds) fractions on a silica gel column. For this purpose, samples were dissolved in hexane and separated into soluble and insoluble fractions (asphalthene). The soluble fraction was loaded on a silica gel column and eluted with different solvents. The alkane fraction was eluted with 100 ml of hexane, followed by the aromatic fraction, which was eluted with 100 ml of toluene. Finally, the NSO fraction was eluted with methanol and chloroform (100 ml each). The alkane and aromatic fractions were analyzed by gas chromatography (GC-FID using Hitachi G-3000, TC-5, 30-m long wide-bore column, 0.25 mm × 0.2 μm film thickness). The injector and detector were maintained at 300°C, and the oven temperature was programmed to rise from 80°C to 240°C in 5°C/min increments and to hold at 240°C for 30 min. The flow rate was 1.5 ml/min, the interface temperature was 300°C, and the injection volume was 1 μl. Individual compounds present in the alkane and aromatic fractions were determined by matching the retention time with authentic standards.

Table 1. Characteristic of crude oil.

Density	0.9291 g/cm^3
Flash point	98°C
Flow point	25°C
Movement viscosity (50°C)	104 mm^2/s
Sulfur content	0.21 mass %
Nitrogen content	0.17 mass %
Moisture content	0.1 vol %
Residual carbon	7.59 mass %
Mineral content	0.019 mass %
Total calorie	44,470 kJ/kg

RESULTS AND DISCUSSION

Soil Analysis and Composition of Crude Oil

The soil for the bioremediation experiment was silt-loamy and light brown. The content of moisture was 14%. The pH was 7.6 at time zero and remained unchanged, whereas the water-holding capacity of the soil decreased from 60% to 52%. The crude oil contained alkane at 52% was the largest constituent of the crude oil, followed by the aromatic fraction (24%), asphalthene fraction (8%), and NSO (16%). The detailed composition of crude oil are given in Table 1 and Table 2.

Degradation of Crude Oil in Liquid Medium by *Polyporus* sp. S133

Fig. 1 shows the total petroleum hydrocarbon (TPH) in the contaminated liquid medium of several concentrations. Of all the concentrations of crude oil tested, the lowest rate of degradation of crude oil was observed at 15,000 ppm (19%). At 10,000, 7,500, and 5,000 ppm, the rates of degradation in 60 days were only 25%, 37%, and 46%, respectively. Among all the concentrations tested, 1,000 ppm was the best with 93% degradation. A general trend existed that the degradation rate decreased with the increased initial concentration of crude oil. GC analysis of the alkane fractions and aromatic fractions obtained after silica gel fractionation, of a representative sample collected from 1,000 ppm concentration, are shown in Fig. 2 and 3, respectively.

Effects of Carbon Source, Nitrogen Source, and Wood Meal on Degradation of Crude Oil in Soil by *Polyporus* sp. S133

The effects of the carbon source, nitrogen source, and wood meal on degradation of crude oil are shown in Fig. 4.

Table 2. Composition of the total petroleum hydrocarbon.

Fraction	Content (%)
Alkane	52
Aromatic	24
NSO (nitrogen-, sulfur-, and oxygen-containing compounds)	16
Asphalthene	8

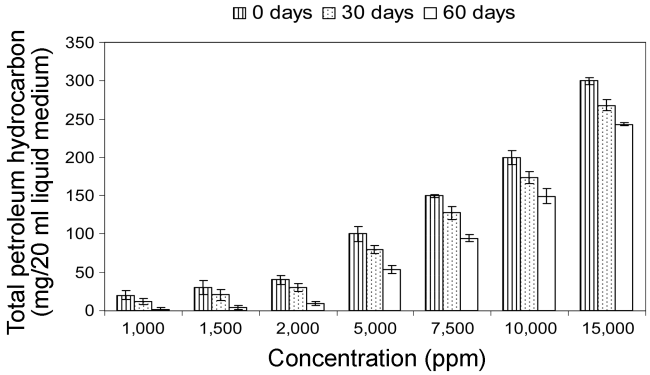

Fig. 1. Total petroleum hydrocarbon in the contaminated liquid medium of several concentrations.
Data are the means from triplicate experiments and the error bars indicate 95% confidence intervals.

Of all the treatments tested, the lowest rate of degradation of crude oil was observed at unsterile soil (not treated) (10%). For soil autoclaved and with addition of the glucose, polypeptone, and wood meal, the rate of degradation in 60 days was increased to 26%, 33%, 52%, and 43%, respectively. Degradation with sterile soil was higher than unsterile/not treated soil because autoclaving releases soluble organic carbon, presumably from humic matter, and killed biomass [6, 36]. In agreement with our study, Salonius *et al*. [36] found that autoclaving reduced the pH of a clay soil (initial pH 7.92), whereas Wolf *et al*. [41] reported no change in pH for three autoclaved soils (initial pH 6.1±7.2). The decreased pH in response to autoclaving

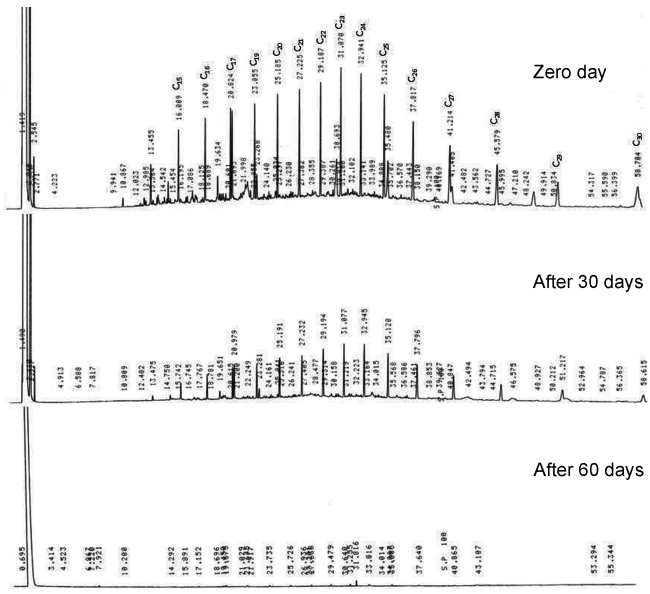

Fig. 2. Gas chromatography of a representative alkane fraction of TPH obtained from liquid culture at different times (0, 30, and 60 days).
The initial concentration of liquid culture was 1,000 ppm of crude oil.

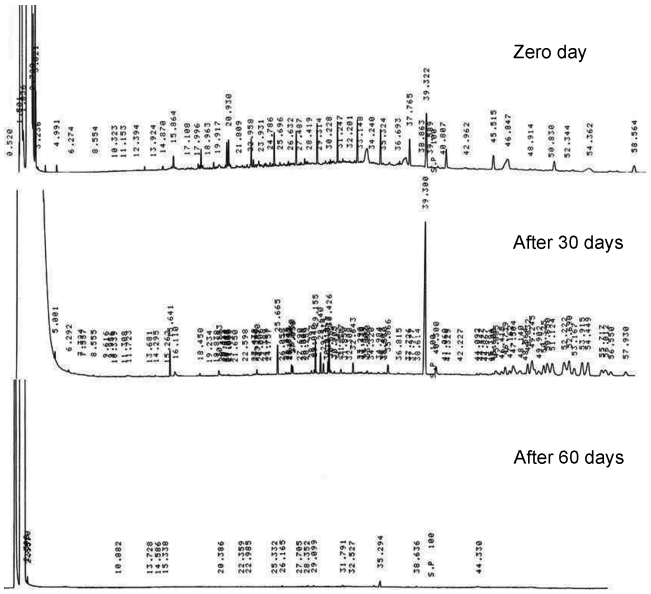

Fig. 3. Gas chromatography of a representative aromatic fraction of TPH obtained from liquid culture supplemented with 1,000 ppm of crude oil at different times (0, 30, and 60 days).
The initial concentration of liquid culture was 1,000 ppm of crude oil.

might have resulted from the solubilization of organic acids, but the magnitude of the decrease depends on the acidic buffering capacity of the soil.

On the other hand, bioremediation is based on stimulating the growth of microorganisms, primarily white rot fungi that can degrade contaminants effectively [1, 24]. When supplying oxygen (or other electron acceptors) and nutrients, microorganisms can degrade a number of soil and groundwater contaminants, including crude oil, to carbon dioxide and water [1]. The addition of a carbon source as a nutrient will increase the biomass of soil microorganisms, but may inhibit the biodegradation of contaminants and result in diauxic growth. The addition of an easily degradable carbon source, such as the wood meal, can shorten the lag time of pollutant degradation in an aqueous environment [29], and stimulate the biodegradation of pollutants in diesel-contaminated soil [30]. The observed stimulatory effect might be due to an increased microbial biomass with a capacity for hydrocarbon biodegradation.

However, other explanations such as increased bioavailability of the substrate (in soil) or changes in the microbial composition towards organisms having a higher capacity for hydrocarbon biodegradation cannot be excluded. Mechanisms of the stimulating effect from an easily accessible carbon source, such as peptone, have been demonstrated by Radwan *et al*. [32]. They found a correlation between the enhanced degradation of crude oil in desert sand soil due to peptone and an increased number of microorganisms identified as oil-utilizing microorganisms. In a study of the microbial response to organic amendments during degradation of different organic pollutants in soil, Swindoll *et al*. [37] observed that carbon sources such as glucose or amino acids inhibited mineralization of the pollutant, proposed to be the result of preferential utilization of the more readily degradable carbons. A mixture of nitrogen sources, however, shortened the lag time for degradation of crude oil.

Effects of Oxygen Flow and Some Absorbents on Degradation of Crude Oil in Soil by *Polyporus* sp. S133

The effect of oxygen flow on the degradation of crude oil by *Polyporus* sp. S133 is shown in Fig. 5. Of all the measured variables, the lowest rate of degradation of crude oil was observed in air flow (73%). After addition of oxygen flow, the rate of degradation in 60 days was increased to 67% compared with only sterile soil. Among all the treatments tested, 10% kapok was the best with 93% degradation. Addition of air treatment gives the positive result for the degradation. The key of successful biodegradation

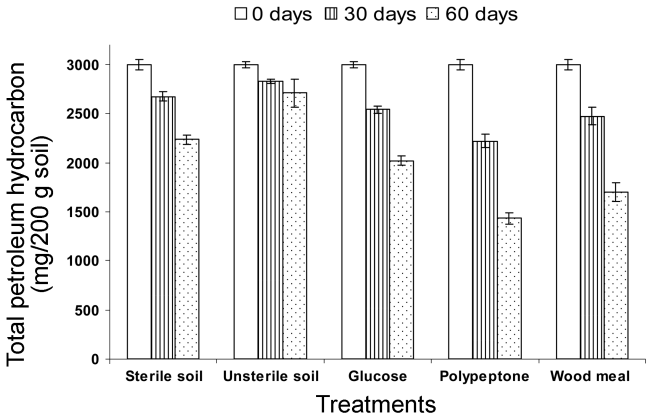

Fig. 4. Total petroleum hydrocarbon in the contaminated soil with addition of nutrients.
Data are the means from triplicate experiments and the error bars indicate 95% confidence intervals.

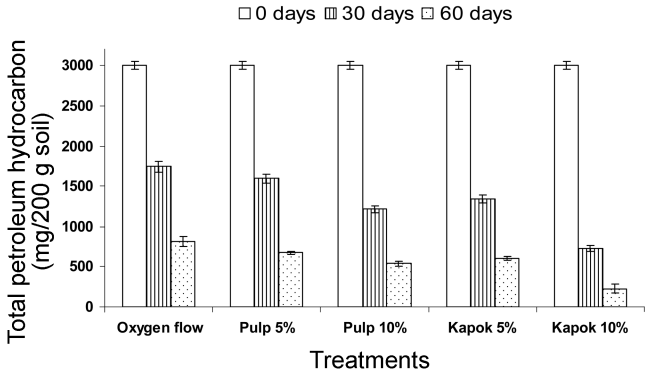

Fig. 5. Total petroleum hydrocarbon in the contaminated soil with addition of oxygen flow and some absorbents.
Data are the means from triplicate experiments and the error bars indicate 95% confidence intervals.

is to limit the input of oxygen sufficiently in order to prevent the synthesis of the aerobic biodegradative pathways for crude oil. It is possible to aerobically degrade crude oil without the production of toxic metabolic intermediates [12, 18, 28]. However, the oxygen addition had some impact on the enzymatic reactions occurring prior to the dioxygenase step in the anoxic degradation of crude oil.

The degradation of crude oil with kapok addition was high. It might be due to its high oil absorbency. Kapok has been known to have larger effective pore volume compared with polypropylene because of its hollow structure, and it also has a waxy surface that can enhance its oil adherence ability. Oils were first absorbed by interactions and van der Waals forces between the oils and the wax on the kapok fiber surface, owing to their compatibilities (both are hydrocarbons). Once the oils entered the kapok assemblies, they subsequently penetrated into the kapok lumens through internal capillary movement, and therefore, allowing more oil absorption. However, the amounts of oil absorbed and retained within the kapok assemblies depended on the oleophilicity of the kapok and the oil physical characteristics such as density, viscosity, surface tension (against air), and contact angle with the kapok surface [21]. In addition, kapok could be a promising material to accelerate biodegradation of crude oil because it is inexpensive, easy to get, gives savings in the disposal fee, and can be formed into some convenient material such as mats, pads, and non-woven sheets [10].

Regarding biological compatibility and biodecomposition, cellulose has better acceptability compared with synthetic polymers. Using waste pulp that comprises a cellulosic matrix is environmental friendly and can also reduce the price because of low cost. Cellulose, a linear homopolymer of linked-β-D-glycopyranose units (Glc) aggregated to form a highly ordered structure owing to its chemical constitution and spatial conformation [17], is the most abundantly available natural biopolymer. Its sorptive properties towards metal are also well documented [22, 23].

The present study clearly demonstrates that application of *Polyporus* sp. S133 could be used to remediate soil contaminated with crude oil. To our knowledge, crude oil-degrading bacteria that have been previously reported include species from genera *Acinetobacter*, *Marinococcus*, *Micrococcus*, *Planococcus*, *Methylobacterium*, *Rhodococcus*, and *Noccardia*, and thus *Polyporus* sp. S133 is a valuable new crude-oil-degrading bacteria. The rate of crude oil degradation observed in this study was also comparable to or greater than other studied strains [27, 35, 42]. Many agricultural residues such as wood meal, pulp, and kapok have also been found to stimulate microbial activity and degradation [26, 31], which were also observed here. However, maintenance of proper soil conditions is an essential aspect to be looked into and needs to be studied in further detail when taking up such studies, because soil conditions influence the survival of the microorganisms.

In conclusion, our results show that crude oil was degraded by *Polyporus* sp. S133 in malt extracts liquid culture. The maximum degradation rate (93%) was obtained when *Polyporus* sp. S133 was incubated in the cultures supplemented with kapok (10%) for 60 days. Furthermore, the degradation was great with the air flow, probably because the survival of the microorganism increases with the increment of oxygen in soil. The degradation rate was also affected by carbon and nitrogen sources as well as some absorbents. Thus, the capability of *Polyporus* sp. S133 to degrade crude oil can be applied for the bioremediation of PAH-contaminated environments.

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