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Microbial Degradation of Crude Oil by Fungi Pre-Grown on Wood Meal

Tony HADIBARATA[1,2] and Sanro TACHIBANA[1]

[1]*Department of Applied Bioscience, Faculty of Agriculture, Ehime University, 3-5-7 Tarumi, Matsuyama, Ehime 790-8566, Japan*
[2]*Laboratory of Pulp, Paper and Environment, Department of Forest Product Technology, Mulawarman University, Kampus Gunung Kelua, Jl. Ki Hajar Dewantara No. 1, Samarinda 75119, Indonesia*

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Abstract—The white rot fungus *Polyporus* sp. S133 collected from petroleum-contaminated soil was tested for its ability to grow and degrade crude oil, obtained from petroleum industry. The ability of *Polyporus* sp. S133 pre-grown on wood meal to degrade crude oil was measured. Maximal degradation (93%) was obtained when *Polyporus* sp. S133 was incubated in 1000 ppm of crude oil for 60 days, as compared to 19% degradation rate in 15000 ppm. Increased concentration of crude oil decreased the degradation rate.

Keywords: crude oil, degradation, white rot fungus, *Polyporus* sp. S133

INTRODUCTION

Annual worldwide consumption of petroleum hydrocarbons was estimated to be of the order of 10^{12} US gallon (Prince, 1993). Much of it travels by water and at some instances some amount of oil inevitably spills from tankers and pipelines. Some of spilled crude oil or the washings of storage tanks pollute the beaches by the formation of tar balls and render them unusable. Oil refineries generate huge volume of oily sludge during the refining of crude oil. Oily sludge is carcinogenic and a potent immunotoxicant (Propst *et al*., 1999). Improper disposal and handling of oily sludge contaminates soil and may pose a serious threat to groundwater. Bioremediation offers a promising means to reclaim such contaminated soil (Bartha, 1986; Bragg *et al*., 1994). Bioremediation employs microorganisms capable of degrading toxic contaminants (Bossert and Bartha, 1984; Eriksson *et al*., 1995). Augmenting the contaminated site with an appropriate inoculum of microorganisms is a promising technique to enhance the biodegradation of hydrocarbons.

Moreover, using an indigenous microorganism consortium ensure that the organisms have a higher tolerance to the toxicity of hydrocarbons and are resistant to variations in the environment (Dibble and Bartha, 1979). Many carrier

materials, mostly agricultural byproducts, are used to transfer the microorganism consortium to the fields effectively. The carrier materials provides nutrients, moisture, and physical support for the increased aeration needed by the microorganisms, and also assist in extending the survival 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 and the recalcitrant hydrocarbons (Lal and Kanna, 1996). The purpose of this study was to investigate the crude oil degradation by the white rot fungus *Polyporus* sp. S133 in liquid medium.

MATERIALS AND METHODS

Development of fungi

A fungus that can degrade oily sludge was developed from a hydrocarbon-contaminated soil by the enrichment method using wood meal. Wood meal was autoclaved at 121°C for 2 hours with shiitake nutrient and moisture content was adjusted to 60%. 10 plugs *Polyporus* sp. S133 was added to the wood meal and incubated for 30 days.

Culture conditions and fungal inoculum

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

Experimental design

Experiments were performed in 100-ml Erlenmeyer flasks containing 20 ml of liquid medium plus some concentration of crude oil dissolved in hexane. As the strains have different growth rates, the period of incubation was varied from 5 to 7 days in order to obtain similar radial growth and to minimize variation in the starting inoculums. Mycelial plugs of a selected fungus were cut from the outer edge of an actively growing culture on an inoculum plate. Three 5-mm disks obtained by punching out with a cork-borer from the outer edge of an actively growing culture of a particular fungus were inoculated into a flask containing 20 ml of liquid medium supplemented with 1 mM of substrate. The flasks were incubated at 25°C. Growth and substrate consumption were determined at 7-day intervals. One set of inoculated flasks was incubated stationary. All media were

Table 1. Characteristics of crude oil from Taiyo Oil Company, Shikoku Brach.

Sample name	T-0513 0.3 HP C Fuel
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	44470 kJ/kg

Table 2. Composition of the total petroleum hydrocarbon.

Fraction	Content (%)
Alkane	52
Aromatic	24
NSO	16
Asphalthene	8

sterilized by autoclaving at 121°C for 20 minutes. Control experiments were performed by incubating crude oil in autoclaved cultures (121°C for 20 min) and by incubating MEM with crude oil without an inoculum. All assays were conducted in triplicate. All remaining flasks were incubated for 15 and 30 days.

Quantification of total petroleum hydrocarbon contamination

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 hydrocarbons (TPH) from liquid medium flask and 10g 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 (asphalthene) fractions. 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 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

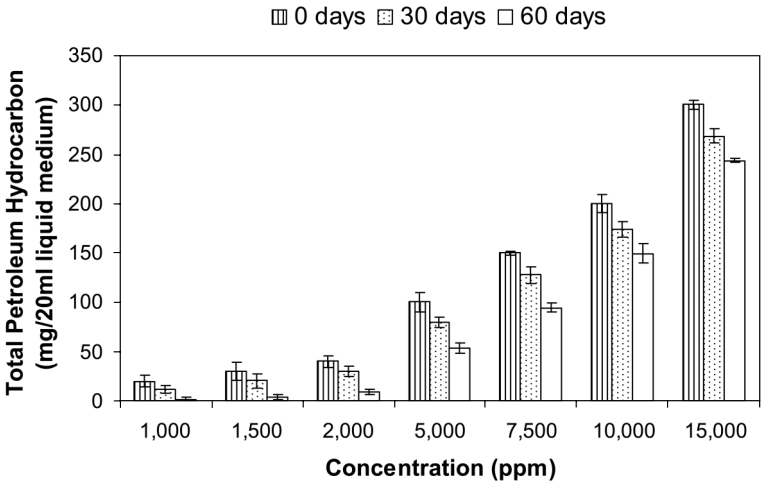

Fig. 1. Total petroleum hydrocarbons in the contaminated liquid medium of several concentrations.

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 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, interface temperature was 300°C, and injection volume was 1 μl. Individual compounds present in the alkane and aromatic fractions were determined by matching the retention time with authentic standards.

RESULTS AND DISCUSSION

Soil analysis and composition of crude oil

The soil for the bioremediation experiment was silt-loamy and light brown in color. The moisture content 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 was collected from Taiyo Oil Company, Matsuyama branch and contained 52% alkane, the largest constituent of the crude oil, followed by aromatic fraction (24%), asphalthene fraction (16%), and NSO (8%). The detailed composition of crude oil is given in Tables 1 and 2.

Degradation of crude oil in liquid medium by Polyporus sp. S133

Figure 1 shows the total petroleum hydrocarbons (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 15000 ppm (19%). At 10000, 7500, and 5000 ppm the rate of degradation in 60 days was only

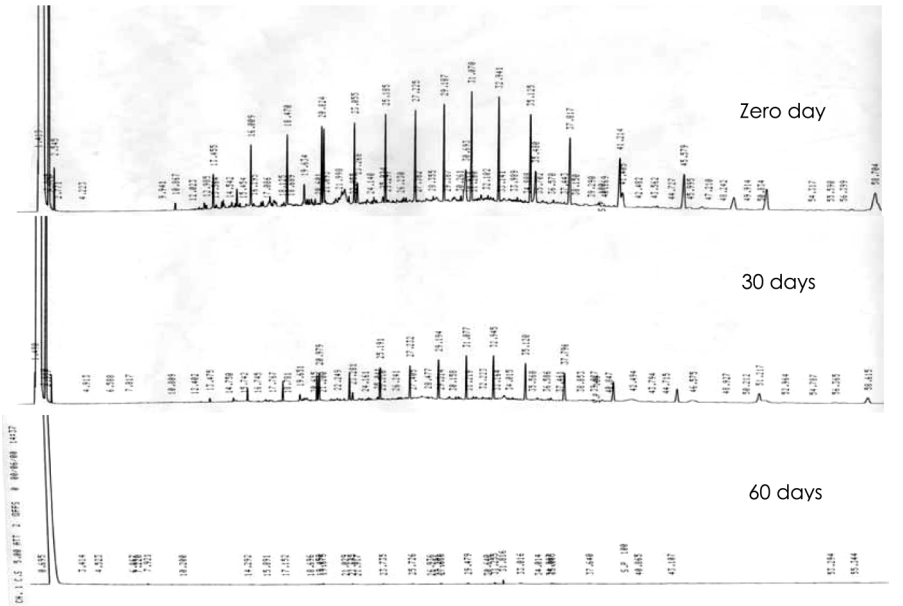

Fig. 2. Gas chromatography of a representative alkane fraction of TPH obtained from liquid culture at different times.

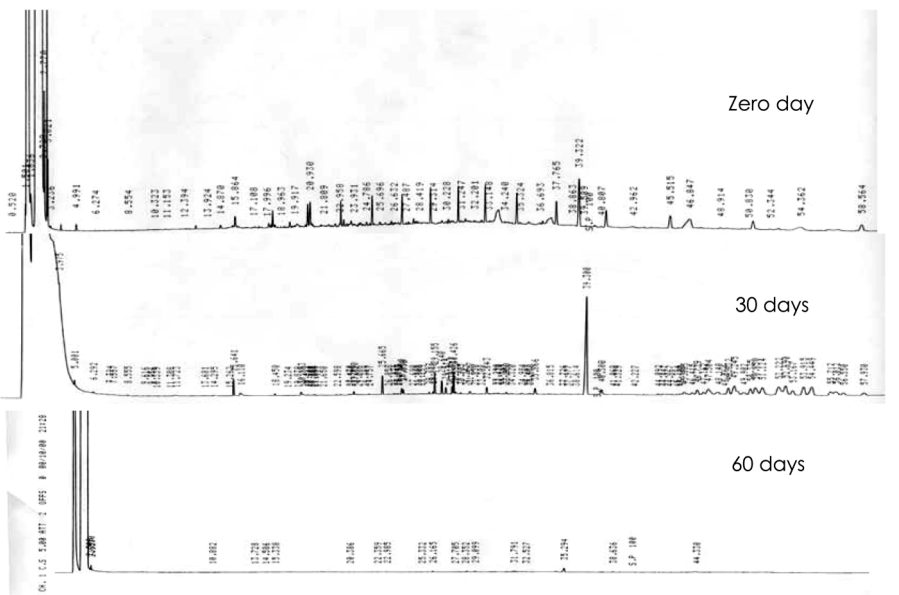

Fig. 3. Gas chromatography of a representative aromatic fraction of TPH obtained from liquid culture at different times.

25, 37, and 46 %. Among all the concentrations tested, 1000 ppm was the best with 93% degradation. Increased concentration of crude oil decreased the degradation rate. GC analysis of alkane fraction and aromatic fraction, obtained after silica gel fractionation, of a representative sample collected from liquid medium is shown in Figs. 2 and 3.

Bioremediation is a cheap and easy method to reduce oily sludge contamination. This approach does not require the soil to be moved from its site, thus reducing any chance of secondary contamination. Using microbial inoculants is a common practice, which enhances the rate of biodegradation (Eriksson *et al*., 1995; Lal and Khanna, 1996). The present study clearly demonstrates that, if suitably developed, application of a carrier-based indigenous microorganism can be used to remediate soil contaminated with crude oil. 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. Fungi show tremendous diversity and adaptability in utilization of different organic molecule as a carbon source; however their abilities to degrade a specific hydrocarbon as a source of energy and/or biomass may differ. The chemical composition of a crude oil may also be a factor in determining the type of fungi, which may grow on it (Davies and Westlake, 1979).

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T. Hadibarata (e-mail: thadibrata@fahutan.unmul.ac.id) and S. Tachibana