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Periodical biostimulation with nutrient addition and bioaugmentation using mixed fungal cultures to maintain enzymatic oxidation during extended bioremediation of oily soil microcosms

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ABSTRACT

New challenges are associated with crude oil biodegradation by microorganisms during extended bioremediation due to decreasing enzymatic oxidation. In the present study, strategies to maintain enzymatic oxidation during the extended bioremediation of oily soil microcosms were examined using periodic biostimulation and bioaugmentation (PBB). PBB was employed with the addition of 10 ml malt extract liquid medium (the biostimulation treatment) and 2 g pre-grown fungus in wood meal (the bioaugmentation treatment) to soil artificially contaminated with PHCs 15, 30, 60, and 90 d after the first experiment. Two kinds of fungal co-cultures: *Pestalotiopsis* sp. NG007/*Polyporus* sp. S133 (1/1) and *Pestalotiopsis* sp. NG007/*Polyporus* sp. S133/*Trametes hirsuta* D7 (1/1/1), were examined in order to compare the effects of two or three fungal strains on the crude oil degradation. The results obtained showed that PBB stimulated the biodegradation of crude oils and all fungal co-culture systems used in this study exhibited stronger enzymatic activities (C12O, MnP, and laccase) after PBB. Furthermore, PBB using three fungal strains (NG007/S133/D7) exhibited the most effective degradation, and it was possible to maintain enzymatic activities after extended bioremediation. This study offers an important strategy to remediate PHC-contaminated environments by PBB using mixed fungal cultures, specifically for extended biodegradation.

1. Introduction

Environmental pollution by oil spills has long been an important worldwide issue due to its potential to acutely and chronically damage ecosystems. The average total worldwide annual release of petroleum hydrocarbons (PHCs) was previously estimated to be 1.3 million metric tons (approximately 380 million gallons, 1 U.S. gal = 3.79 L) (National Research Council, 2003). In this report, the sources contributing to the total input were effectively categorized into four main groups: natural seep, petroleum extraction, petroleum transportation, and petroleum consumption.

The bioremediation of PHC-contaminated environments is approached using two strategies: 1) biostimulation involving the injection of nutrients or other supplementary components into the affected site in order to stimulate the degrading capacity of indigenous microorganisms (Sayara et al., 2011; Taccari et al., 2012; Abed et al., 2014), and 2) bioaugmentation involving the introduction of oil-degrading microorganisms or pre-grown microbial cultures to the affected site (Taccari et al., 2012; Wu et al., 2013).

The *Exxon Valdez* oil spill, an oil spill catastrophe from transportation due to a tanker accident in 1989, led to the first large-scale application of bioremediation using a biostimulation strategy along the shoreline. The utilization of a fertilizer (biostimulation) as a tool for the bioremediation of an oiled shoreline in Prince William Sound (PWS) achieved a total hydrocarbon loss of 25–30% within the first days to weeks after the spill (Atlas and Hazen, 2011). A large number of studies subsequently used biostimulation for the bioremediation of environments contaminated by petroleum, specifically on marine shorelines. Biostimulation has been shown to promote the growth of indigenous microorganisms

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in oil-contaminated soil and enhance the biodegradation of oil (Pritchard and Costa, 1991; Bragg et al., 1994; Venosa et al., 1996; Jackson and Pardue, 1999; Wu et al., 2016). However, when indigenous soil microorganisms are rare or not physiologically able to perform the biodegradation process, bioaugmentation, the addition of a versatile microorganism or efficient consortium to the initial indigenous microbial community of a contaminated environment, may be employed as an alternative strategy to enhance biodegradation. During the bioremediation of oil-polluted soil, previous studies reported that biostimulation was more effective than bioaugmentation (Kauppi et al., 2011; Abed et al., 2014; Andreolli et al., 2015; Wu et al., 2016). However, bioaugmentation may promote degradation during the early stage of remediation (Wu et al., 2016).

Although the addition of indigenous microorganisms may achieve the best performance by bioaugmentation, a previous study showed the similar biodegradation potential of microorganisms isolated from polluted or unpolluted soil (Chaîneau et al., 1999). The utilization of bacteria and fungi to remediate PHC-contaminated environments has been widely reported; however, fungi, because of their greater ability to access pollutants and their higher tolerance in PHC-contaminated soil than bacteria, have numerous potential advantages for use in bioremediation.

Recent studies on the bioremediation of PHCs in the field have been directed toward the utilization of biostimulation and bioaugmentation by individual or a consortium of fungi as the best alternative biodegradation strategy. Previous studies have demonstrated that this approach is successful for bioremediation (Suja et al., 2014; Lang et al., 2016; Kumari et al., 2016). We previously reported that bioaugmentation with the fungal co-culture *Pestalotiopsis* sp. NG007, an ascomycete fungus, and *Polyporus* sp. S133, a basidiomycete fungus combined with the addition of growth nutrients accelerated the biodegradation of PHCs due to their synergistic effects and the enzymatic activities produced during their interactions (Yanto and Tachibana, 2014a). The ligninolytic enzymes MnP and laccase and non-ligninolytic enzymes dioxygenases and monooxygenases in this co-culture system may have an important role in biodegradation. The ratio of NG007/S133 for the optimum degradation of PHCs is 1:1, in which the enzymatic activities of C12O, laccase, and MnP are optimally produced during degradation. However, enzymatic activities have been shown to decrease when nutrients decline periodically due to metabolism (Margesin and Schinner, 2001). In contrast, microorganisms must maintain strong enzymatic activities during extended bioremediation. Most lower molecular weight compounds such as aliphatic and aromatic fractions in PHCs are depleted during extended bioremediation. Sugiura et al. (1997) reported that fractions with a lower molecular weight in PHCs were preferentially degraded by microorganisms, resulting in a high content of asphaltenes and resins remaining in polluted soil, which is problematic for ecosystem life because of their recalcitrance to microbial degradation. Asphaltenes have complex molecular structures and their high hydrophobicities make them difficult to degrade by microorganisms (Flores and Howard, 2001). Therefore, maintaining the enzymatic activities of a fungal co-culture during extended bioremediation is considered the best approach in bioremediation.

In the present study, we developed periodical biostimulation and bioaugmentation (PBB) for oily soil microcosms with the addition of growth nutrients and a mixed fungal culture in order to investigate the maintenance of enzymatic oxidation during extended bioremediation. This study also examined the effects of two kinds of fungal co-cultures: *Pestalotiopsis* sp. NG007/*Polyporus* sp. S133 and *Pestalotiopsis* sp. NG007/*Polyporus* sp. S133/strain D7 as a new fungal co-culture for their enzymatic activities and bioremediation during PBB.

2. Materials and methods

2.1. Chemicals

Crude oil A (COA), crude oil C (COC), and asphalt were 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).

2.2. Microorganisms

Pestalotiopsis sp. NG007 and *Polyporus* sp. S133 were supplied from the Department of Applied Bioscience, the Faculty of Agriculture, Ehime University, Japan. Strain 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. 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.

2.3. Identification of the new strain D7

The genomic DNA strain D7 was extracted from a culture in MEA according to a slightly modified cetyltrimethylammonium bromide (CTAB) method (Murray and Thompson, 1980). The internally transcribed spacer (ITS) regions were amplified by PCR using the primers ITS1-F (5′-CTTGGTCATTTAGAGGAAGTAA-3′) and ITS4-B (5′-CAGGAGACTTGTACAGGTCCAG-3′) (Jellison and Jasalavich, 2000). PCR was performed in 20 μl of a solution containing 10 ng of genomic DNA, 5 pmol each of the forward and reverse primers, and 10 μl of Go Taq® Hot Start Colorless Master Mix (Promega, Wisconsin, USA). Initial denaturation was performed at 95 °C for 2 min, followed by 30–35 cycles of denaturation at 95 °C for 0.5 min, annealing at 55–72 °C, polymerization at 72 °C for 1 min, and a final extension at 72 °C for 10 min. Prior to sequencing, PCR products were purified using rAPid Alkaline Phosphatase™ (Roche, Germany) and exonuclease I (New England Biolabs, Massachusetts, USA). Purified PCR products were sequenced and BLAST searches for ITS sequences in the GenBank database were conducted. A phylogenetic tree was constructed with Mega 7 (Kumar et al., 2016). The ITS sequence obtained for strain D7 was deposited to NCBI GenBank (accession No. KX444204).

2.4. Pre-culture in liquid medium

Pestalotiopsis sp. NG007, *Polyporus* sp. S133, and strain 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.

2.5. Pre-grown in wood meal

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) shitake 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.