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学位論文全文に代わる要約
Extended Summary in Lieu of Dissertation

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学位論文題目：
Title of Dissertation

Biodegradation of Polycyclic Aromatic Hydrocarbons (PAHs) and Crude Oil by Saline-Alkaline Tolerant Fungi
(海水およびアルカリ性耐性菌による多環芳香族炭化水素(PAHs)および原油の生分解)

学位論文要約：
Dissertation Summary

Polycyclic aromatic hydrocarbons (PAHs) are persistent organic compounds with two or more aromatic rings in various structural configurations (linear, angular, and cluster arrangements). PAHs constitute a large and diverse class of organic compounds (Juhasz and Naidu, 2000; Speight and Arjoon, 2012). The chemical properties, and hence the environmental fate, of PAHs depend on molecular size (i.e., the number of aromatic rings and the pattern of ring linkage). Because PAHs are insoluble and chemically stable, their degradation depends on the ability of microbes to introduce oxygen into the rings, which has the effect of increasing both the solubility and chemical reactivity of PAHs (Gadd, 2001; Speight and Arjoon, 2012). Molecule stability and hydrophobicity are two primary factors that contribute to their persistence in the environment. In addition, the solubility of PAHs in aqueous media is very low, which affects degradation of these compounds and can lead to accumulation in ecosystems. The main sources of PAH contamination are natural oil seeps, refineries, oil storage, accidental oil spills, and municipal and urban waste water. These processes generate large volumes of unwanted sludge that contains PAHs and is detrimental to the surrounding ecosystems, especially marine environments and coastal areas. Recent studies on PAH contamination have focused on these areas.

PAHs are a minor constituent of crude oils; however, they are among the most toxic to plants and animals. They are widely distributed as environmental contaminants that have detrimental biological effects, and are toxic, mutagenic, and carcinogenic (Haritash and Kaushik, 2009). PAHs can enter the human body through inhalation, ingestion, and skin contact, and exposure to them has been linked to skin, lung, liver, intestinal, and pancreatic cancers (Doyle and Doyle, 2008). Crude oil contains more than 30 PAH compounds and 16 of them have been selected as priority pollutants by the United States Environmental Protection Agency (USEPA). The degradation of PAHs in crude oil has become one parameter to measure the success of bioremediation of petroleum-related spills. However, PAHs have been recognized as recalcitrant compounds to degrade. For example, in the case of the

Exxon Valdez oil spill, PAHs have persisted at some beaches in Prince William Sound (Alaska, USA) for more than 20 years after the beaches became contaminated by oil. Generally, PAHs with five or more rings are not easily degraded and may persist in the environment for long periods (Speight and Arjoon, 2012; Boufadel et al., 2016). The structure of four common PAHs found in nature can be seen in Figure 1. These PAHs were used as model compounds in this study.

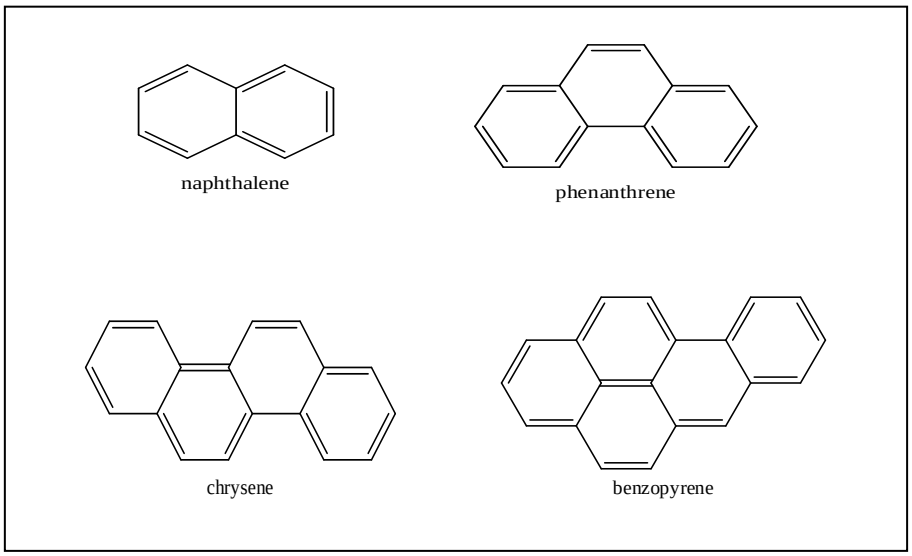

Figure 1. The structure of common polycyclic aromatic hydrocarbons (PAHs)

The need to remediate sites contaminated with PAHs and crude oil has led to developing new technologies that emphasize the detoxification destruction of the contaminants rather than the conventional method of disposal. Bioremediation is one removal technology, which can be defined as a method that employs living organisms, most often microorganisms, plants, or both, or products produced from living organisms to degrade, detoxify, or sequester toxic chemicals present in natural waters and soils (Arun and Eyini, 2011). Bioremediation shows greater potential as a solution to organic pollutant contamination, such as that by PAHs, than physical or chemical treatments. It is inexpensive, completely destroys organic pollutants (via mineralization to produce carbon dioxide and water as the final products), and can be used as an in situ treatment (Isikhuemhen et al., 2003). Bioremediation is also supported by the biodiversity of microbes in nature, such as white rot fungi (WRF), which can degrade many hazardous xenobiotic compounds. WRF are well-known producers of ligninolytic enzymes [laccase (Lac), manganese peroxidase (MnP), and lignin peroxidase (LiP)], which can degrade many pollutants such as PAHs (Johannes and Majcherczyk, 2000; Ding et al., 2008; Hadibarata and Kristanti, 2012). However, most studies have focused on the degradation of PAHs by WRF under non-saline rather than saline conditions because limited

numbers of saline-tolerant basidiomycetes have been identified and examined. On the other hand, saline-tolerant WRF and their extracellular ligninolytic enzymes potentially have environmental applications in coastal areas because they degrade a broad array of environmental pollutants such as high molecular weight PAHs (HMW-PAHs).

In the present study, 82 potential WRF screened from nature were investigated in an attempt to identify fungi with the potential to degrade PAHs and tolerate salinity and high pH. Decolorization of Remazol brilliant blue R (RBBR), an industrially important dye, was used as a pre-screening method. Based on the results of the investigation, only 12 of the 82 screened fungi decolorized RBBR, and only 5 of the 12 (Table 1) decolorized RBBR under saline-alkaline conditions with different levels of tolerance. The five fungi decolorized RBBR from blue to orange at various decolorization rates: 15–100% under non-saline conditions at pH=4.5 (NSC) and 0–75% under saline conditions at pH 8.2 (SC). Benzo[a]pyrene (BaP, 5-ring PAH) was degraded by the five best fungi at various rates, and the ability to degrade BaP differed significantly between the NSC and SC cultures. Of the five strains of WRF screened, the SM46 isolate exhibited the highest degradation rate of BaP under both conditions (Figure 2). BaP is one of the most recalcitrant PAHs, having low water solubility and high resonance energy, and is toxic to some organisms (Juhasz and Naidu, 2000). Additional stresses such as saline-alkaline conditions for some organisms may inhibit their ability to degrade BaP. In a culture of SM46 and SM27, salinities up to 20 g l^{-1} did not affect fungal growth. However, BaP degradation was not affected up to a salinity of 20 g l^{-1}, but decreased at 35 g l^{-1}. In the present study, we found that salt tolerance in a submerged culture of the fungi was higher than that of S03, S04, and SM04, and it was also higher than that of two other terrestrial-derived WRF, *Phanerochaete chrysosporium* and *Trametes versicolor* (Kamei et al., 2008).

Table 1. Decolorization rates (%) of the five best white rot fungi in RBBR-containing czapek-dox at pH 4.5 or czapek-dox under saline conditions of 0–35 g l^{-1} and pH 8.2 (data are for the first seven days of growth)

	Salinity of sea salt (g l^{-1})			
Fungal strain	0	10	20	35
S03	96±3.0	61±4.8	26±1.5	0
S04	51±0.3	15±3.0	0	0
SM04	52±1.2	10±2.2	0	0
SM27	100±0.0	95±5.7	72±2.4	45±4.5
SM46	100±0.0	98±3.6	75±3.0	52±2.0

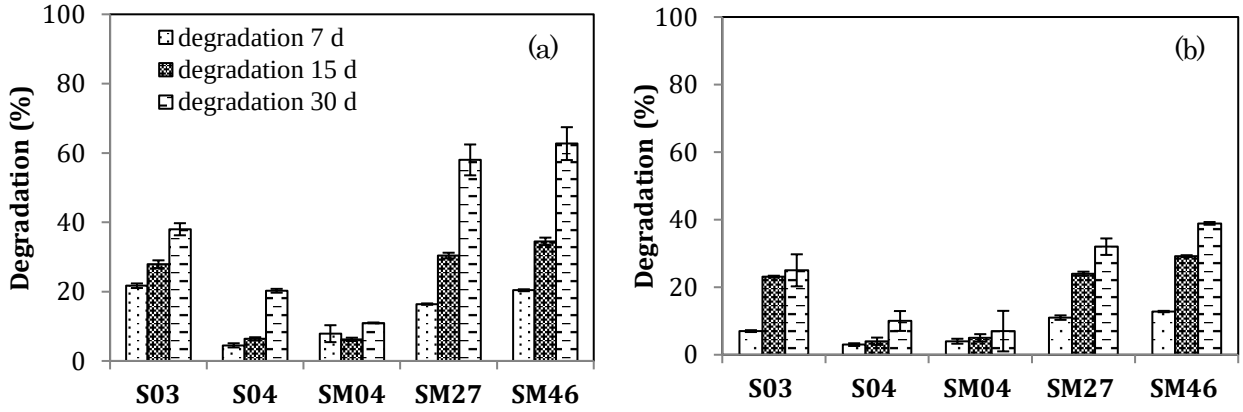

Figure 2. Degradation of BaP in a liquid medium at pH 4.5 (a) and saline conditions at pH 8.2 (b) by five strains of WRF for 7, 15, and 30 days

$MnSO_4$ as an inducer improved the degradation rate and enzyme expression. MnP and LiP activity also increased by 7-fold and 5-fold, respectively. SM46 degraded BaP (38–89% over 30 days) in an acidic environment (pH 4.5) and under saline-alkaline stress conditions (pH 8.2). Investigating the metabolites produced revealed BaP-1,6-dione as the main product, indicating the important role of ligninolytic enzymes in initializing BaP cleavage. The other metabolites detected (naphthalene acetic acid, hydroxybenzoic acid, benzoic acid, and catechol) may have been ring fission products (Figure 3). In the present study, the formation of four compounds with lower molecular weights than that of BaP-1,6-dione indicates that the ring fission process occurred via meta-cleavage, decarboxylation, and hydroxylation. The role of non-ligninolytic enzymes requires further study. Carbon dioxide and water may be formed as final products (Kotterman et al., 1998; Haritash and Kaushik, 2009).

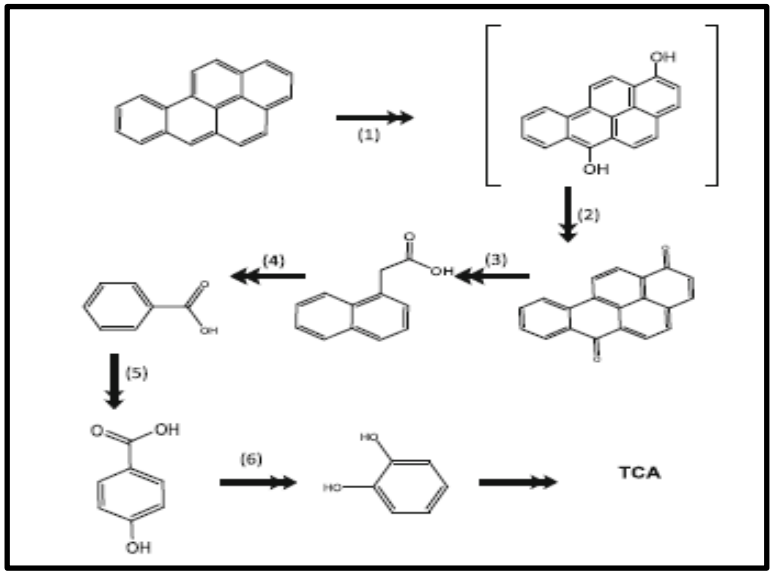

Figure 3. Proposed pathway for the degradation of BaP by *Bjerkandera adusta* SM46 under saline-alkaline stress conditions. (1) hydroxylation, (2) oxidation of hydroxyl group, (3) ring fission, (4) subsequent meta-cleavage, (5) hydroxylation, and (6) decarboxylation

According to molecular gene analysis and morphological characteristics, SM46 was similar (99–100%) to *B. adusta* (GenBank accession number: KU055648). The four other WRF strains were identified as *Trametes*

versicolor S03, *Ganoderma gibbosum* S04, *Microporus subaffinis* SM04, and *B. adusta* SM27 (GenBank accession numbers: KU055649, KU055650, KU055646, and KU055647, respectively) (Andriani et al., 2016).

In the present study, we also found that using an enhanced culture system (ECS) for the WRF, by adding $MnSO_4$ as an enzyme inducer and Tween 80 (T-80), increased BaP biodegradability and induced the ligninolytic system during the degradation process. This system can also be used in various extreme conditions such as saline-alkaline stress to alleviate PAH contamination in seawater (Andriani et al., 2017). Based on contour plot analysis of $MnSO_4$ and T-80 in biodegradation, the optimum condition (degradation rate more than 40%) at 15 days was obtained at a concentration for $MnSO_4$ of > 0.25 mM and for Tween 80 of 0.10–0.65% (Figure 4a). The optimum condition (degradation more than 84%) at 30 days was obtained at a concentration for $MnSO_4$ of 0.4–0.9 mM and for T-80 of 0.45–0.70% (Figure 4b). The condition improved degradation up to 87% at 30 days. Adding $MnSO_4$ and T-80 increased BaP degradation 24% compared with control, but a high concentration of T-80 (more than 0.8%) seems to have decreased degradation to less than 60%.

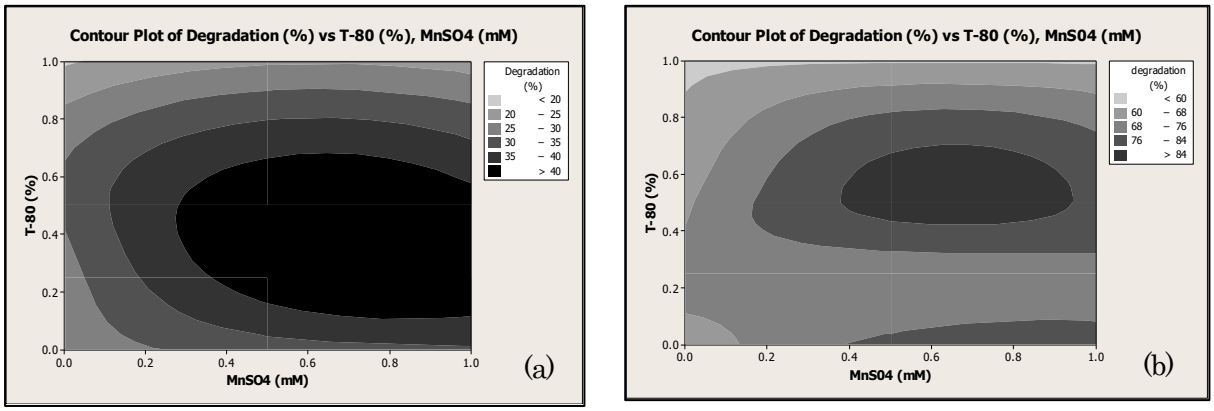

Figure 4. Contour plot of the degradation of BaP versus $MnSO_4$ and T-80 for 15 days (a) and 30 days (b)

In the culture treatment, $MnSO_4$ has two important roles during the degradation process by the fungus. The first is to induce the catabolic system of the fungus to produce a more specific ligninolytic enzyme such as MnP. For the second, MnP functions as a redox mediator in the enzymatic reaction of BaP and MnP. T-80 is a non-ionic surfactant and emulsifier derived from polyethoxylated sorbitan and oleic acid and is often used in foods. T-80 has been reported not only to enhance hydrophobic-pollutant solubility but also to increase LiP production by WRF (Lestan et al., 1994; Yanto and Tachibana, 2014). Adding T-80 can increase the oxygen mass transport coefficient between the growth medium and the interior of the fungal cell (Baldrian et al., 2005). Behnood et al. (2014) also found that adding T-80 can reduce interfacial tension (IFT) and increase the solubility of hydrophobic pollutants such as crude oil (Yanto and Tachibana, 2014).

In this study, we applied the ECS condition for BaP degradation under saline-alkaline stress. At pH 4.5, the ECS condition significantly increased (at a 95% level of significance) MnP and LiP production by 13-fold and 5-fold, respectively (Figure 5a). Enhancing the production of these enzymes stimulated more BaP removal in the culture system, improving degradation to 87% at 30 days. The ECS condition increased BaP removal by about 25% at pH 4.5, but it inhibited laccase production 8-fold at day 15. This phenomenon may be due to the interaction of the $MnSO_4$ and Tween 80 with a self-inhibiting laccase in the medium. Under a saline condition (pH 8.2), the BaP degradation rate increased significantly up to 82% at day 30. Moreover, production of ligninolytic enzymes under saline-alkaline stress (non-ECS) was lower than under an acidic condition. The saline-alkaline condition reduced BaP removal by the fungus. However, adding $MnSO_4$ and T-80 to the culture alleviated the inhibition effect of saline-alkaline stress. The degradation of BaP under ECS conditions was 44% higher than the control treatment (Non-ECS). Laccase and MnP increased 37-fold and 43-fold at day 15, respectively (Figure 5b). However, there was no significant enhancement of LiP under the saline condition. The ECS treatment under a saline condition (pH 8.2) had a greater effect than under an acidic condition (pH 4.5). The fungal intracellular mechanism with $MnSO_4$ and Tween 80 under saline-alkaline stress by *B. adusta* SM46 requires further investigation. Kamei et al. (2008) reported that the ability of a fungus to produce ligninolytic enzymes under a saline condition is affected by a specific saline-dependent regulation of the enzymes.

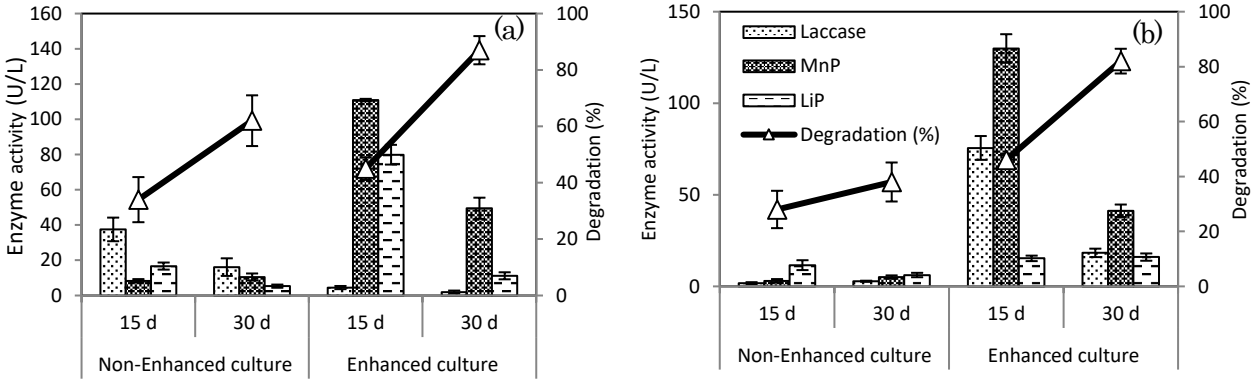

Figure 5. Comparison of degradation rate and ligninolytic enzyme production at pH 4.5 (a) and saline pH 8.2 (b)

In a further investigation, we examined the use of several lignocellulosic materials (kapok fibers, rice straw, pulp waste, and wood meal) as WRF-immobilizing agents to degrade four PAHs with different aromatic rings: naphthalene (NAP), phenanthrene (PHE), chrysene (CHR), and benzo[a]pyrene (BaP). Lignocellulosic materials, as natural substrates for WRF, can support fungal growth and induce the ligninolytic system, and, as a consequence, pollutant degradation (Dzul-Puc et al., 2005; Mohammadi and Nasernejad, 2009; Rubilar et al., 2011; Sari et al.,

2014). Of the four lignocellulosic materials studied, rice straw was selected as the most suitable support based on fungal growth, ligninolytic enzyme production, and degradation rate of PAHs after inoculation with *B. adusta* SM46. Rice straw-immobilized *B. adusta* (RSIB) showed faster growth and colonization, and increased laccase (Lac), manganese peroxidase (MnP), and lignin peroxidase (LiP) activity. The optimum granule size of rice straw as an immobilizing agent for *B. adusta* was 840 μm.

The capability of WRF to produce different enzymatic systems has been shown to depend on cultivation conditions (Rubilar et al., 2011; Rivera-Hoyos et al., 2013). In the present study, we inoculated RSIB on PAH-contaminated sea sand and seawater. The results showed that RSIB could degrade all the PAHs tested in the sea sand. The recovery of each PAH from incubated biologically inactive control sea sand differed. The coefficients of variation between controls after 15 and 30 d did not exceed a maximum of 10%, except for CHR (12%), the degradation of which was somewhat obscured by strong abiotic losses, primarily by absorption, extraction, and volatilization during the incubation (Silva et al., 2004; Sari et al., 2014). LMW-PAHs were degraded the most by RSIB at 94% for NAP and 70% for PHE, whereas HMW-PAHs were degraded by 55% for CHR and 63% for BaP after 30 d. NAP was degraded the most, with 69 and 94% being degraded after 15 and 30 d, respectively. CHR was the most difficult to degrade by the fungus at only 16% after 15 d and 55% after 30 d. Mean degradation rates were found to differ among the PAHs tested in the order of NAP > PHE > BaP > CHR.

Different PAHs affected the ligninolytic enzymes produced by RSIB. Three ligninolytic enzymes were detected in all PAH treatments with different activity ranges. The highest Lac, MnP, and LiP activities of 9, 5.3, and 5.4 U g^{-1} after 15 d, respectively, were observed with PHE as the pollutant. NAP, which had the lowest molecular weight with only two aromatic rings, was efficiently degraded despite low ligninolytic enzyme activity. The degradation rate of PAHs by microorganisms is affected not only by degrading-related enzyme production but also by the physicochemical characteristics of PAHs such as molecular weight, ring aromatic stabilization, ionization potential (IP), and solubility (Eibes et al., 2006; Hadibarata et al., 2009). A similar trend of PAH degradation was also found in the PAH-contaminated seawater. Additionally, the decreasing trend of PAH concentration over time in the different medium treatments can be described using first-order kinetics, which had high correlation coefficients ranging from 0.90 to 0.99, as shown in Table 2. Moreover, $t_{1/2}$ (half time) of PAH removal varied greatly among the treatments ranging from 5.31 to 46.31 days, and PAH degradation was faster in the seawater for the LMW-PAHs, whereas degradation of the HMW-PAHs was faster in the sea sand. This phenomenon might correlate with the high solubility of the LMW-PAHs in the seawater, which contributes to accelerating the degradation.

Table 2. Comparison of PAH degradation rate in sea sand and seawater

Medium	PAH	Average PAH degradation rate ($mmol\ l^{-1}\ d^{-1}$)	First order kinetics		
			$k\ (d^{-1})$	r^2	$t_{1/2}$
sea sand	NAP	0.0122	0.0942	0.9924	7.36
	PHE	0.0065	0.0401	0.9444	17.28
	CHR	0.0040	0.0270	0.9026	25.67
	BaP	0.0042	0.0333	0.9197	20.81
seawater	NAP	0.0127	0.1304	0.9398	5.31
	PHE	0.0089	0.1049	0.9728	6.61
	CHR	0.0026	0.0149	0.9957	46.51
	BaP	0.0040	0.0314	0.9999	22.07

Crude oil is a major source of PAH contamination in the environment. The method of removing PAHs from the environment cannot be considered separately from the ability to degrade crude oil as a complex mixture. Because crude oil consists of different fractions with various chemical and physical properties, degrading crude oil by using an organism consortium has been reported to be more effective compared with using a single organism. For degradation by bioremediation of crude oil-contaminated sea sand, five fungi including *B. adusta* SM46 capable of growing on the BaP-containing saline malt agar medium (pH 8.2) were selected. The co-culture of *Pestalotiopsis* sp. NG007 and *Polyporus* sp. S133 at a ratio of 25/75 was found to be the most suitable fungal co-culture for both the degradation of crude oil and enzyme activities. Crude oil [heavy oil C (HOC), asphalt, heavy oil A (HOA)] in crude oil-contaminated sea sand was degraded efficiently by the fungal co-culture. Periodically adding malt extract (10%) as a nutrient source, Tween 80 (0.5%) as a surfactant, and a mineral mixture of $MnSO_4$ and $CuSO_4$ (1 mM) as enzyme inducers to the crude oil-contaminated sea sand enhanced enzymatic activities during bioremediation even after 120 d, by contributing to a new generation of mycelia. The nutrient biostimulation enhanced enzymatic activities, leading to greater degradation of TPH (total petroleum hydrocarbons) than the control treatment (without nutrient biostimulation).

Periodic biostimulation improved the biodegradation rates of HOC, asphalt, and HOA during extended bioremediation compared with control and the malt extract only treatment. Treatment with the fungal co-culture of NG007/S133 enhanced biodegradation rates by 31 and 39% for asphalt at concentrations of 15,000 and 30,000 ppm, respectively. The degradation rates of HOC increased more than 19% at both concentrations of 15,000 and 30,000 ppm (Figure 6). The degradation rates of HOA were the highest among the three crude oils used in the experiment with rates of more than 95 and 93% at concentrations of 15,000 and 30,000 ppm, respectively. The low

concentration of crude oil (in the HOC and HOA treatments) was almost completely degraded by the fungal co-culture. These results indicated that the degradation of crude oil by the fungal co-culture was strongly influenced by the type of crude oil. HOA, a light crude oil, was more easily degraded than heavier crude oil types such as HOC and asphalt.

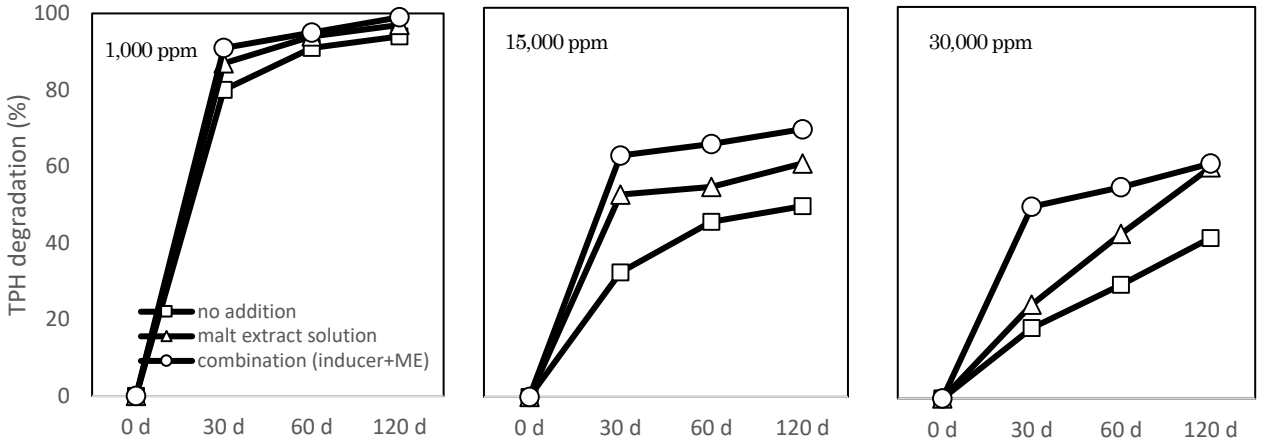

Figure 6. Degradation of heavy oil C with combination nutrient amendment at three concentrations: 1,000, 15,000, and 30,000 ppm

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