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Enhanced Biodegradation of Total Petroleum Hydrocarbons (TPHs) in Contaminated Soil using Biocatalyst

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ABSTRACT

Biocatalytic degradation of total petroleum hydrocarbons (TPHs) in contaminated soil by hemoglobin and hydrogen peroxide is an effective soil remediation method. This study used a laboratory soil reactor experiment to evaluate the effectiveness of a nonspecific biocatalytic reaction with hemoglobin and H_2O_2 for treating TPH-contaminated soil. We also quantified changes in the soil microbial community using real-time PCR analysis during the experimental treatment. The results show that the measured rate constant for the reaction with added hemoglobin was 0.051/day, about 3.5 times higher than the constant for the reaction with only H_2O_2 (0.014/day). After four weeks of treatment, 76% of the initial soil TPH concentration was removed with hemoglobin and hydrogen peroxide treatment. The removal of initial soil TPH concentration was 26% when only hydrogen peroxide was used. The soil microbial community, based on 16S rRNA gene copy number, was higher (7.1×10^6 copy number/g of bacteria, and 7.4×10^5 copy number/g of Archaea, respectively) in the hemoglobin catalyzed treatment. Our results show that TPH treatment in contaminated soil using hemoglobin catalyzed oxidation led to the enhanced removal effectiveness and was non-toxic to the native soil microbial community in the initial soil.

Key words : Hemoglobin, Hydrogen peroxide, Soil remediation, Total petroleum hydrocarbon (TPH), Real time PCR

1. Introduction

A wide variety of soil remediation techniques are available, including soil vapor extraction, chemical oxidation with hydrogen peroxide, phytoremediation and others. Among the remediation strategies for treating TPH-contaminated soils, combining chemical and biological processes has been shown to effective (Palmroth etal., 2006), Using abiotic processes, hydrogen peroxide has been widely used to degrade xenobiotics in slurry systems requiring large amounts of hydrogen peroxide, and the generated hydroxyl radical is strongly reactive and toxic to microorganisms (Danner et al., 1973; Umezawa and Higuchi, 1989; Palmroth et al., 2006; Oh and Shin, 2013)

A wide range of pentachlorophenol (PCP), polycyclic aromatic hydrocarbons (PAHs), lipids, and fatty acids are catalytically oxidized by heme and heme proteins (Rice et al., 1983; Chen et al, 2006; Chen et al., 2009; Chung et al., 2014; Kang et al., 2015). By analogy to the peroxidase system, it was verified that adding hydrogen peroxide to soil containing heme, in which the iron is present as Fe^{+3}, can remove pentachlorophenol, crystal violet, and PAHs from contaminated soil by nonspecific catalytic reactions similar to those involving ligninase (Chen et al, 1999; Chung et al., 2014). Hemoglobin is a globular protein with an embedded four heme group (such as Fe^{+3}), and accelerates the degradation of organic compounds such as 5-aminosalicylic acid and PAHs by hydrogen peroxide (Kang et al., 2015). The reaction mechanism has been verified as hemoglobin catalyzed degradation as shown in Fig. 1 (Kang et al., 2015). Similar experiments have shown that heme without added hydrogen peroxide did not degrade crystal violet and pentachlorophenol in water and soil systems.

This experiment used contaminated soil from an industrial site contaminated with hydrocarbons, benzene, toluene, ethyl benzene and xylenes (BTEX) in the vicinity of

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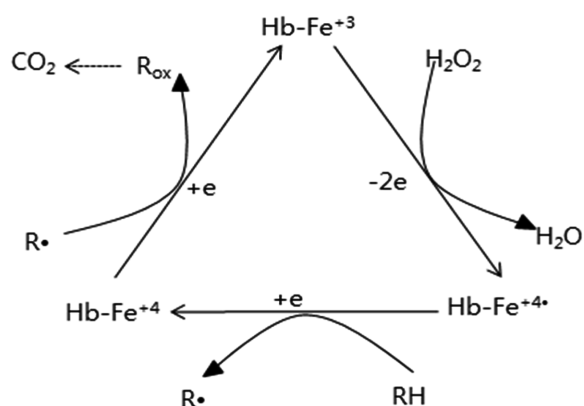

Fig. 1. Hemoglobin catalytic cycling in the presence of hydrogen peroxide and organic compounds (RH) (Kang et. al., 2015).

Pohang, Korea. The size of the Pohang industrial area is 206,094 m^2 and the area was the site for a petroleum chemical complex, with a high intensity of industrial processing industries such as carbon black and plasticizer manufacturing.

This study was conducted to evaluate the effectiveness of treating soil contaminated with TPH (total petroleum hydrocarbons). The TPH-contaminated soil was treated using hemoglobin and H_2O_2 in a soil reactor. The specific objective of the experiment was to examine the degradation of TPHs in contaminated soil using H_2O_2 only, and using added hemoglobin catalyst with H_2O_2.

2. Material and Methods

2.1. Soil sample and chemicals

TPH-contaminated soil was collected from the OCI Corp. industrial complex in Pohang, Korea. The soil was air dried and sieved using a 2 mm sieve mesh to remove any large debris or particles. The general soil texture was loamy sand and additional physical and chemical properties of the soil are given in Table 1. The initial TPH concentration was 384 ± 45.5 mg/kg.

Powdered hemoglobin was donated by Shenzhen Taier Biotechnology Co., Ltd (China) for use in the soil reactor. Hydrogen peroxide (34%) was purchased from Samchun Pure Chemical Co. Ltd. (Korea).

2.2. Laboratory reactor

The experiment was carried out in an open chamber at room temperature (25 ± 2°C) using a mixing system with speed control as shown in Fig. 2. The inner body was constructed of polyvinyl chloride with cylindrical soil reactor (40 cm long, 30 cm diameter, and 28 cm height) and a mechanical mixer with blades (mixing speed, 20 rpm).

Table 1. Physical and chemical properties of the contaminated soil (TKN = total Kjeldahl nitrogen, WHC = water holding capacity, OC = organic carbon)

Component		Unit	Pohwang soil
pH		–	8.5
WHC		%	35.0
OC		%	1.4
N	TKN	mg/kg	225
	NH_3-N	mg/kg	27
Texture	Sand	%	83
	Clay	%	15
	Silt	%	2
Fe		mg/kg	9,163

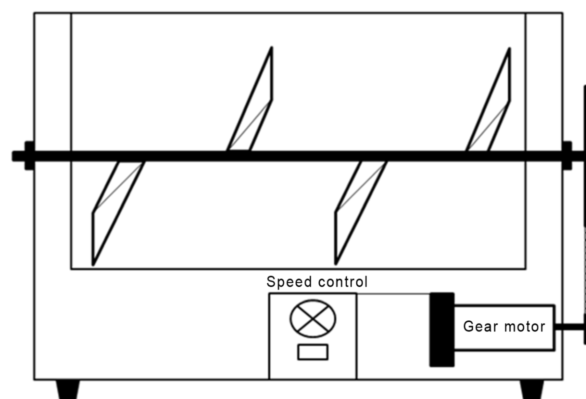

Fig. 2. Schematic diagram of mixing system with speed control (outside dimensions: 45 cm length × 45 cm height × 35 cm width, dimensions of inside mixing reactor: 30 cm diameter × 40 cm length × 28cm height).

2.3. Experimental design

The reactor was filled with 3 kg of air dried soil and mixed with 1,000 mL of 50 mM phosphate buffer (pH 7.0) and 500 mL of 5% H_2O_2 (8 g H_2O_2/kg soil) without hemoglobin catalyst (control). For both control and hemoglobin catalyst treatment, the treatments were monitored for four weeks, and the initial moisture content was maintained at approximately 30% using 50 mM phosphate buffer solution (pH 7.0).

After completion of the control experiment, 3 kg of air dried soil was again placed in the reactor and mixed with a

solution containing 10 g hemoglobin powder dissolved in 1,000 mL of 50 mM phosphate buffer (pH 7.0) and 500 mL of 5% H_2O_2 (8 g H_2O_2/kg soil) for the catalyst treatment.

The treated soil was sampled every week during the four week treatment. A representative sample was obtained by randomly collecting ten replicate soil samples and making a composite sample. The composite samples were analyzed in triplicate for soil TPH concentration.

2.4. Soil TPH analyses

The residual TPH concentration in the soil samples was analyzed following the Official Test Method for Soil Pollution in Korea (Ministry of Environment, 2009). TPH was extracted with dichloromethane (DCM) by sonication (Qsonica sonicators, USA) and quantified using gas chromatography (Agilent 6890, USA equipped with a flame ionization detector and a DB-5 column (30 m × 0.25 mm × 0.25 um). The initial oven temperature was 40°C (8 min.) and ramped up to 320°C at 12°C/min and maintained at 320°C (20 min.). Nitrogen (1.1 mL/min) was used as a carrier gas with a split ratio of 1 : 10. The injector and detector temperatures were 250 and 320°C, respectively.

2.5. Microbial community analysis

The abundance of Bacterial and Archaeal 16S rRNA gene were quantified with primer pairs Eub338-BAC515 (Nadkarni et al, 2002; Stahl and Amann, 1991) and ARC806-ARC915 (Casamayor et al., 2002; Takai et al, 2000), respectively. The real- time PCR reactions were carried out with an ABI Prism 7300 sequence detection system (Applied Biosystems). SYBR Premix Ex Taq reagent (Takara, Shiga, Japan) was used for the reactions and the experimental procedures followed the manufacturer's instructions. Thermal cycling steps were as follows: initial denaturation (95°C) for 5 min, followed by 35 cycles of denaturation (95°C) for 30 s, primer annealing for 30 s, and final extension (72°C) for 30 s. The annealing temperature for each primer pair was 55°C for *Bacteria*, and 60°C for *Archaea*. Fluorescence signals were measured during the extension step. All of the real-time PCR amplifications were performed in triplicate. The collected fluorescence signals were analyzed by using ABI Sequence Detection Software version 1.4 (Applied Biosystems).

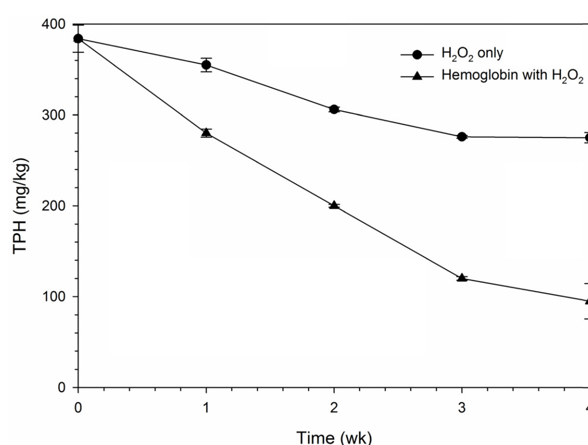

Fig. 3. Degradation of the TPH contaminated soil vs. time in the mixing reactor. Initial H_2O_2 spiked 8 g (kg soil)$^{-1}$ and hemoglobin spiked 3.3 g (kg soil)$^{-1}$.

To calculate the copy numbers of 16S rRNA gene sequences of *Archaea* and *Bacteria*, recombinant plasmid DNAs were used for generating standard curves. Purified and serially diluted DNAs from environmental clones containing 16S rRNA gene sequences were used as template DNAs for real-time PCR. Slopes of standard curves were −3.28, and −3.20 (R^2 = 0.998 and 0.994) (Hong and Cho, 2015).

3. Results and Discussion

3.1. TPH degradation in soil

The soil TPH concentrations for the control (H_2O_2 only) and treatment (with hemoglobin catalyst) during the four week experiment are shown in Fig. 3. During the experiment, soil TPH concentration decreased from 384 ± 45.5 mg/kg to 95 ± 19.5 mg/kg, resulting in an average of 75% of initial TPH removed in the hemoglobin treatment. After four weeks, the control soil had a TPH concentration of 285 ± 5.7 mg/kg, resulting in an average of 25% of initial TPH removed in the control.

During the experiment, gas bubbles were observed in soil in the reactor, which was likely to be CO_2 resulting from the mineralization of organic carbon. The relatively small decrease in soil TPH concentration in the control (H_2O_2 only) indicates a slower degradation of organic carbon. This suggests that the addition of hemoglobin to the contaminated soil enhanced the degradation of organic carbon, forming more

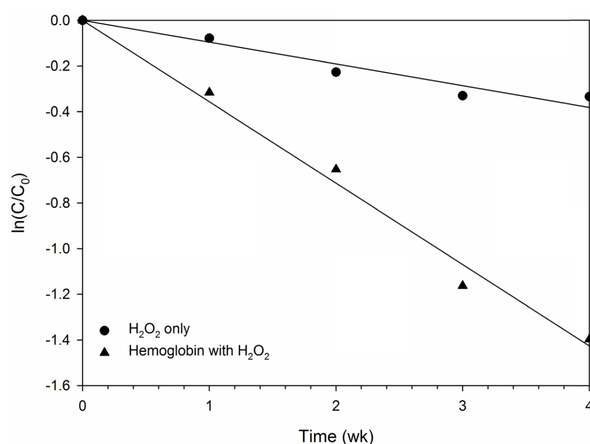

Fig. 4. Effect of hemoglobin catalyst and H_2O_2 reaction on the removal of TPH with a semi-logarithmic scale for determining the first order rate constants.

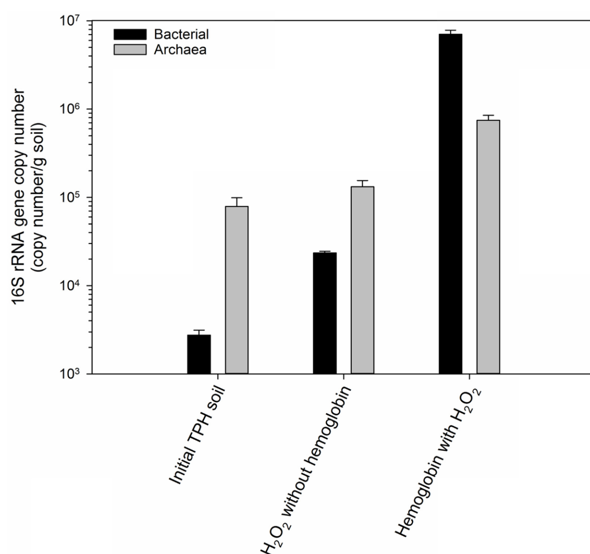

Fig. 5. 16S rRNA gene copy number after four weeks in the mixed reactor.

radicals for the TPH degradation reaction and faster TPH degradation (Fig. 1).

The first order rate constant (0.051/day) for the TPH degradation reaction in the presence of hemoglobin was about 3.5 times higher (0.014/day) than in the control (H_2O_2 only) as shown in Fig. 4.

3.2. 16S rRNA gene copy number

In order to determine the abundance of prokaryotes, real-time PCR analysis was performed with EUb338 – BAC515, and ARC806 – ARC915. The real-time PCR results (Fig. 5) showed that H_2O_2 treatment affected the abundance of prokaryotes in the soil samples contaminated with petroleum hydrocarbons. While archaeal the 16S rRNA gene copy number was 5.6 higher than the bacterial 16S rRNA gene copy number in H_2O_2-only treated soil (*Bacteria*, 2.3 $\times 10^4 \pm 0.1 \times 10^4$; *Archaea*, $1.3 \times 10^5 \pm 0.2 \times 10^5$), the *Archaea* 16S rRNA gene copy number was 9.5 times lower than the *Bacteria* 16S rRNA gene copy number in the hemoglobin treated soil (*Bacteria*, $7.1 \times 10^6 \pm 0.8 \times 10^6$; *Archaea*, 7.5 $\times 10^5 \pm 1.0 \times 10^5$). Considering that *Bacteria* is known to be more susceptible to oxidative stress caused by oxygen radicals than *Archaea*, the results suggest that the H_2O_2 treatment might have caused a more remarkable decrease in bacterial abundance than hemoglobin catalyst treatment.

4. Conclusions

The results from the laboratory soil reactor experiment show that hemoglobin catalyzed oxidation (with hemoglobin catalyst) can significantly increase the effectiveness of TPH removal in contaminated soil compared to the control (H_2O_2 only). This result confirms the expected pattern based on previous studies identifying the mechanism of the degradation reaction (Fig. 1). After four weeks of treatment, approximately 75% removal of soil TPH was obtained with H_2O_2 and hemoglobin catalyst compared to only 25% removal of soil TPH without hemoglobin catalyst.

The measured first order rate constant was 0.051/day for the treatment with hemoglobin and 0.014/day in the control, about 3.5 times higher than in the control.

The microbial communities determined as 16S rRNA gene copy number were higher as shown by the 7.1×10^6 copy number/g for *Bacteria*, and 7.4×10^5 copy number/g for *Archaea*, respectively. Therefore, the TPH removal by hemoglobin catalyzed oxidation led to the enhanced biodegradation and was non-toxic to the initial microbial community in the soil.

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