Thesis for the Degree of Master of Science

TPH removal of the landfarming process using indigenous microorganisms in the diesel contaminated soil

by

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February, 2012

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토착미생물을 이용한 토양경작법의 디젤 오염토양 내 TPH 제거

Advisor : Prof. Minhee Lee

by
Minho Park

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A dissertation

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CONTENTS

List of Figures

List of Tables

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Abstract

Batch experiments using indigenous and commercialized adventive microorganisms were performed to investigate the feasibility of the landfarming process for the diesel contaminated soils. In batch experiments, TPH removal efficiencies of commercialized microorganisms were compared with those of indigenous microorganisms in landfarming process. The study area was located in 'Camp Hialeah', Busan, Korea, which had been used for US military army since 1950. Soils were sampled by using a backhoe at two sites (A and C site) for the experiments and their TPH concentrations were determined as 2,881 ㎎/㎏ and 5,715 ㎎/㎏, respectively, which were higher than Korea Soil Pollution Warning Limit (500 ㎎/㎏). The main pollutant in the study area was determined to a diesel. Indigenous microorganisms having high TPH degradation activity were isolated from the A and C site for the experiments. By 16S rRNA gene sequence analysis, they were identified as *Arthrobacter* sp., *Burkholderia* sp., *Cupriavidus* sp. and *Bacillus* sp., which were used for the landfarming experiments. Two kinds of commercialized solutions cultured with adventive microorganisms were also used for the

experiments. Each solution was named as "Oilbug 1010" (from BioSaint Inc., Korea) and "Penazyme" (from Life Science of Suwon University, Korea), respectively. Various landfarming conditions such as the amount of microorganism, water content and the temperature were applied to experiments for the bioavailability change.

In case of soils without additional microorganisms (on the natural attenuation condition), 35 % of initial TPH was removed from the soil by living indigenous microorganisms in contaminated soil for 20 days of the landfarming. When commercialized microorganisms were added into the soil, the average TPH removal efficiencies using "Oilbug 1010" and "Penazyme" were 66 %, and 57 %, respectively, which were higher than that without additional microorganisms. When indigenous microorganisms isolated from the contaminated soil were added into the soil, the average TPH removal efficiency increased up to 77 % after 31 days in the landfarming process. Results suggested that TPH biodegradation efficiency by using isolated indigenous microorganisms was higher than those by using commercialized adventive microorganisms and only by the natural attenuation. According to the average calculation of the biodegradation rates for adventive microorganism and monospermous indigenous microorganism, the remediation goal would reach within 28 ('O' microorganism) and 26 days (*Bacillus* sp.), respectively. Consequentially, it was demonstrated that the landfarming process adding isolated indigenous microorganisms isolated from the contaminated soils is the successful process to remove TPH from the diesel contaminated site.

Key words: TPH removal, landfarming process, indigenous microorganisms, diesel contaminated soil

CHAPTER I. INTRODUCTION

Soil and groundwater contamination resulted from hydrocarbon pollutants seriously threatens the earth environment and human being (Whang et al., 2008). Among hydrocarbon pollutants, diesel fuel is the complex mixture of alkane series and aromatic compounds that are regularly reported as major soil and groundwater contaminants leaking from underground storage tanks (USTs) and pipelines or released in accidental spills (Gallego et al., 2001). Since 1990s, the accidental diesel fuel spills and unexpected leakages from USTs have occurred in military camps in Korea, and they have incurred serious environmental problems (Gu et al., 2007).

There are many processes to remediate diesel contaminated soils and groundwater. Among a number of physical and chemical remediation processes, ex-situ bioremediation processes including slurry-phase remediation, composting, treatment-bed remediation, biopile and landfarming were frequently applied for oil contaminated sites (EPA, 1993). Landfarming process using the biodegradation of microorganisms is cost-competitive, effective on organic compounds and also simple to design (Alexander et al., 1999; Rittmann et al., 2001). Most of all, landfarming process includes spreading excavated contaminated soils and stimulating aerobic microbial activity with the aeration and the addition of nutrients and water (Watanabe et al., 2001). Efficiencies of landfarming process can be promoted by biostimulation (introducing nutrients and oxygen into the soil) or bioaugmentation (through inoculation of an enriched microbial consortium into soil) (Richard and Vogel, 1999; Barathi and Vasudevan, 2001; Seklemova et al., 2001). In the field, it was reported that the bioaugmentation adding

adventive microorganisms into the soils was less promising for the landfarming process (Frankenberger, 1992). However, the degradation of organic pollutants in contaminated soils could be enhanced by indigenous microorganisms (Hong et al., 2010; Karamalidis et al., 2010). This study is to evaluate TPH (Total Petroleum Hydrocarbon) removal efficiency for the landfarming process adding indigenous microorganisms into the contaminated soil through the batch experiments.

CHAPTER II. OBJECTIVE

The main objectives in this study are to investigate TPH removal efficiency of the landfarming process adding indigenous microorganisms to the diesel contaminated site, and to determine the optimum operation conditions for the landfarming such as water content, temperature and population of microorganism.

This study was divided into three main experimental objectives.

1. To evaluate TPH removal efficiency of the landfarming process adding two commercialized cultured solution with adventive microorganisms for diesel contaminated soils

2. To evaluate TPH removal efficiency of the landfarming process adding indigenous microorganisms in diesel contaminated sites

3. To determine the optimal operation conditions of the landfarming process by using indigenous microorganisms, applying for the real site

CHAPTER III. BACKGROUND

3.1 Outline of the research area

3.1.1 History of the study area

The study area is located in 'Camp Hialeah', Busan, Korea, which had been used for US military army since 1950. In 2006, 'Camp Hialeah' was closed and the ownership of the real property and facility was transferred to Korean government. (http://www.globalsecurity.org/military/facility/camp-hialeah.htm., 2011). From result of investigation in 2006 and 2009, it was reported that the 'Camp Hialeah' was partially contaminated with organic pollutants leaking from USTs, and the pollutants were mostly used to heat the facility and building. The main pollutants were considered diesel and motor oil and about 76,000 m^3 of soils were contaminated in 'Camp Hialeah' (BEC Inc., 2010).

3.1.2 Geological characteristics

The study area is located at the southeastern part of the Gyeongsang basin and mainly consists of Cretaceous sedimentary rocks and the latter Cretaceous intrusive rocks (Fig. 1). The NNE-SSW trending Dongnae fault zone is developed across the middle part of the study area. Sedimentary rocks named Icheonri formation is the lowest bedrock and is composed of sandstone, siltstone, sandy shale, and shale (Son et al., 1978). The andesitic and igneous rocks intruded over the Icheonri formation (Chang et al., 1983). The andesitic rocks are dominant at the eastern part of the Dongnae fault and are composed of andesitic volcanic breccias, andesite and tuffaceous sediments (Paik et al., 1996; Kim et al., 2004). At the western part, the igneous interpenetrated andesitic rocks are dominant and are mostly composed of biotite granite (Cho et al., 2007). These bedrocks were covered by Quaternary deposits mainly along the Dongnae fault.

Research site is located within the alluvium (at the northwestern part of the study area) and is surrounded by Icheonri formation and biotite granite. The site area was covered by artificially filled soils, which were the mixture of silt, mud and sand in 2 m of depth (Kim et al., 2011).

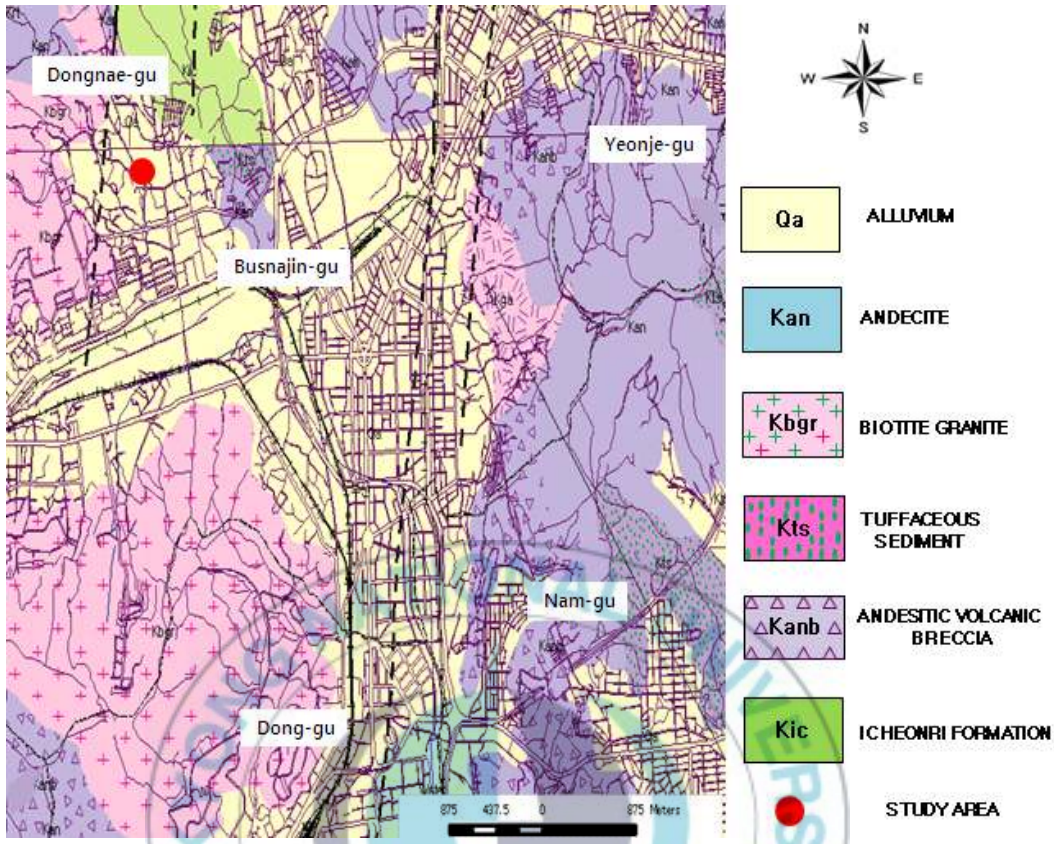


Fig. 1. Geological map in the study area (from Korea Institute of Geoscience and Mineral resources, 2002).

3.2 Properties of organic pollutants in the study area

3.2.1 Diesel and total petroleum hydrocarbon (TPH)

Diesel is an oil fuel used in diesel engines such as industrial machines and transportation vehicles and is produced from the fractional distillation of crude oil between 200 °C and 350 °C at atmospheric pressure, resulting in a mixture of carbon chains that typically contain between 8 and 21 carbon atoms per molecule (EPA, 1996; Yu et al., 2007). Diesel is composed of about 75 % of saturated hydrocarbons such as paraffins, isoparaffins and cycloparaffins and 25 % of aromatic hydrocarbons including naphthalenes and alkylbenzenes (Senn and Jonson, 1987). The average chemical formula for common diesel fuel is $C_{12}H_{23}$, ranging approximately from $C_{10}H_{20}$ to $C_{15}H_{28}$ (Lee et al., 1992; EPA, 1997). Diesel combustion exhaust is a major source of atmospheric soot and fine particles, which is a fraction of air pollution implicated in human heart and lung damage (http://www.epa.gov/region1/eco/diesel/health_ effects.html, 2011).

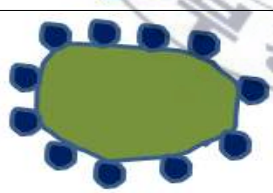
Total petroleum hydrocarbon (TPH) is classified by four main components such as paraffins, isoparaffins, naphthenes and aromatic series and these compounds were composed hundreds of organic materials and come from crude oil (Hajššlovā et al., 1998). Most of oil fuels including gasoline, jet fuels, diesel and bunker fuels belong to TPH (Zhou et al., 1995; Liebeg et al., 1999; Vasudevan., 2001).

3.2.2 Interaction between organic pollutants and soil matrix

When organic pollutants spill over the ground, they can infiltrate into the subsurface and finally exist as three different phases (aqueous, gaseous and non-aqueous phase liquids (NAPLs)). Although most components of diesel are presented as NAPLs, some fraction would dissolve into water and other would be gaseous phases in soil pores (EPA, 1996). The interaction between organic pollutant and soil matrix is generally controlled by sorption, complexation and precipitation (Tan, 2000).

The general meaning of 'sorption' is used to indicate the process in which the solutes such as ions, molecules and compounds are divided between the soil particle surface and liquid phase. Physical adsorption occurs when the contaminants are attracted to the soil component surfaces because of the untreated charges of the soil particles. Chemical adsorption occur by chemical bond. In concrete adsorption, ions transpierce the coordination shell of the structural atom and are bonded by covalent bonds through oxygen ion and hydroxyl ion groups to the structural cations (Volkering et al., 1995). The interaction by complexation and precipitation is also occurred by the inorganic contaminants. Organic contaminants like petroleum hydrocarbons are adsorbed physically due to hydrophobic forces on the soil surface. Physical forms of organic pollutants in soil matrix are shown in Table 1 (Volkering et al., 1998).

Table 1. Various physical forms of organic pollutants in soil matrix (from Volkering et al., 1998)

Various forms of organic pollutants in soil matrix	
	Separated (free phase) liquid blobs
	Absorption into soil
	Liquid film
	In soil macro pores (in liquid phase in pores)
	Adsorption onto soil
	In soil micro pores (solid and liquid)

3.3 Principle of bioremediation mechanisms

3.3.1 Ex-situ Landfarming process

Bioremediation is well known for the pre-environmental process for oil contaminated sites and its technology is actually applied in many contaminated sites. Bioremediation offers several advantages, which can be done on site, are often less expensive and site disruption is minimal. It is also can be coupled with other physical and chemical remediation processes. Bioremediation has also its limitations. Some chemicals are not amenable to the biodegradation, for instance, heavy metals, radionuclides and some chlorinated compounds (Boopathy, 2000). Ex-situ bioremediation processes were more developed than in-situ bioremediation processes, because that in-situ remediation technologies such as bioventing and biodegradation are mostly limited by the space of treatment (EPA, 1993; Jørgensen et al., 1999).

Landfarming process is one of the most widely used Ex-situ bioremediation process and its typical operation process in Fig. 2. Landfarming process used heavy equipment to excavate contaminated soils, which were paved in a wide space. Water, nutrients and microorganisms were injected and layered soils were periodically tilled for the aeration (EPA, 1993).

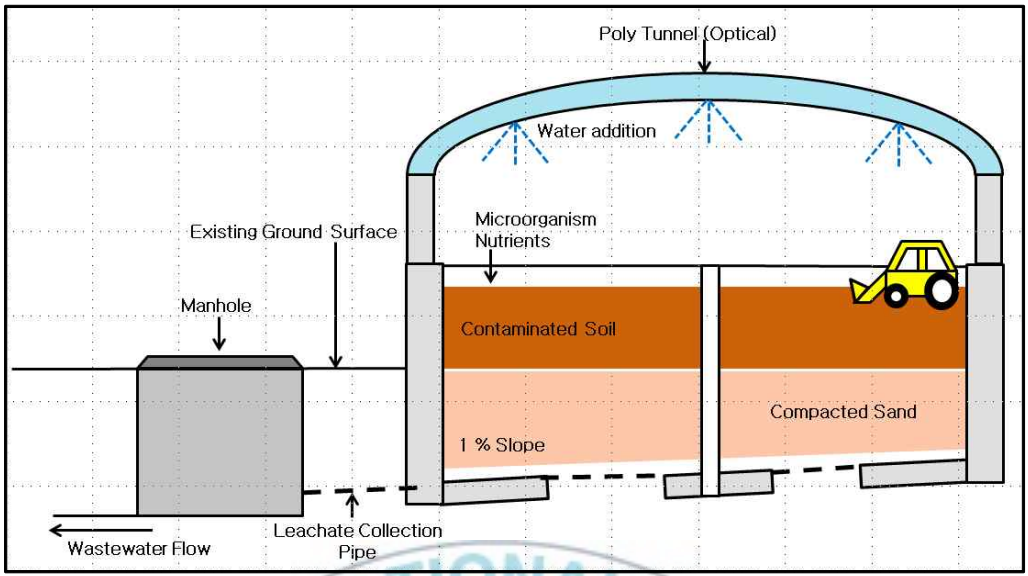


Fig. 2. The schematic of the typical landfarming process.

3.3.2 Biostimulation and bioaugmentation

TPH removal in the landfarming process is commonly achieved by using biostimulation and bioaugmentation methods (EPA, 1996; Alexander et al.,1999; Whang et al., 2008). Biostimulation is the method controling parameters such as nutrients, cation exchange capacity (CEC), water content, temperature and soil pH to accelerate indigenous microbial decomposition. Table 2 shows the critical environmental parameters for microbial activity (Sims et al., 1984; Huddleston et al., 1986; Paul et al., 1989). Nutrients such as nitrogen and phosphorus are necessary to degrade the organic pollutants. When these nutrients are deficient in soils, the biodegradation of oil contaminated soil is accelerated by adjustment of carbon/nitrogen/phosphorus ratios in soil (Jobson et al., 1974; Jamison et al., 1975; Dibble et al., 1979). Continuous maintenance of high soil water content in soil is also extremely

important and the proper water content in soil for the biodegradation is about 12 to 30 % by weight. Either too little or too much soil water content is noxious to microbial activity (EPA, 1993). Temperature and soil pH also affect the bioavailability of microorganism. The suitable ranges of temperature and soil pH are 15 ~ 40 ℃ and 6 ~ 8, respectively. (Atlas et al., 1988; EPA, 1993).

Bioaugmentation is the method adding adventive or indigenous microorganisms such as a oil-degrading microbial solution which is artificially cultivated (Atlas et al., 1981; Thomassin-Lacroix et al.,2002). Each method is independently used in contaminated sites, but the combined method may be used to increase TPH removal efficiency of the landfarming process.

Table 2. Critical parameters to control the microbial activity

Environmental parameters	Optimum value
Nutrient (C:N:P)	100:10:1
Water content	12 ~ 30 % by weight
Temperature	15 ~ 40 ℃
Soil pH	6 ~ 8

3.3.3 Mechanisms of TPH removal by microorganism

The success of bioremediation process for TPH removal, depends on the accurate site characterization, the quantitative evaluation of the migration pathway and the application of the optimal remediation mechanism in the contaminated site. Migration pathway of organic contaminants in soil is shown in Fig. 3 (EPA, 1989).

In bioremediation process, the biochemical reactions resulting in microbial degradation include dechlorination, hydrolysis, cleavage, oxidation, reduction and dehydrohalogenation. Organic pollutant is degraded by the enzyme of microorganism, and consequently it is decomposed to inorganic form such as carbon dioxide (CO_2), water (H_2O) and chlorine ion (Cl^-), respectively (Dragun, 1988, EPA, 1996; Alexander et al., 1999).

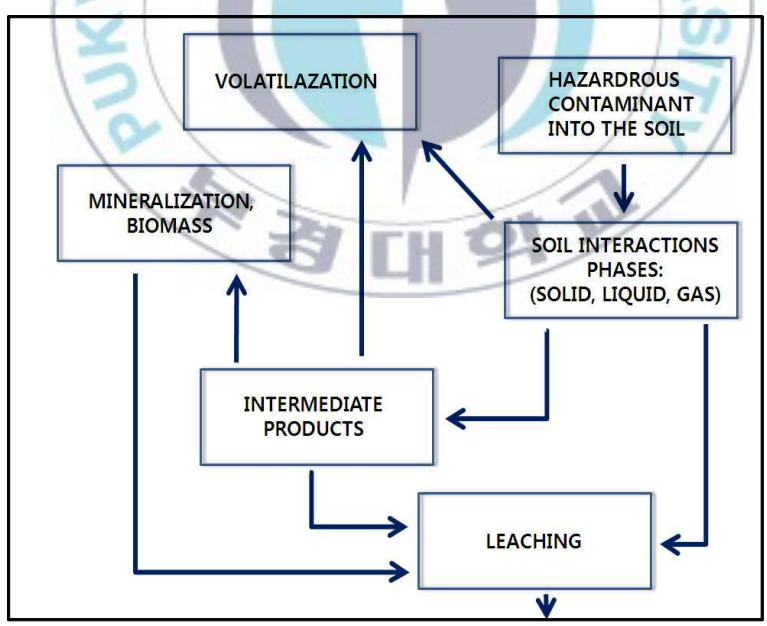


Fig. 3. Pathway of organic contaminants in soil.

The main reactions and their reaction processes deriving biochemical degradation were listed below :

(1) Dechlorination reaction : Dechlorination is used to certain degradation of chlorinated organic compounds by chemical reduction with release of inorganic chloride ions. Reaction (1) shows the dechlorination process (Mohn et al., 1992; Smidt, 2004).

$$CCl_4 \rightarrow HCCl_3 \rightarrow H_2CCl_2 \quad (1)$$

(2) Hydrolysis reaction : Hydrolysis is a chemical process in which a water molecule is added to a substance resulting in the split of that substance into two parts. One fragment of the target molecule gains a hydrogen ion (H^+) from the split water molecule. The other portion of the target molecule collects the hydroxyl group (OH^-) of the split water molecule. Reaction (2) shows the hydrolysis process (Arup et al., 1995).

$$RX + H_2O \rightarrow ROH + H^+ + X^- \quad (2)$$

(3) Cleavage reaction : In organic pollutants, combination of reciprocal carbons is divided or terminal carbons are wandered by cleavage chemical mechanism. Reaction (3) shows the cleavage process (Jaworski et al., 2006).

$$RCOOH \rightarrow RH + CO_2 \quad (3)$$

(4) Oxidation reaction : The oxidation reaction basically removes electrons from organic pollutants and is activated by two pathways. As one is heterolytic or polar reactions. An electrophilic agent attacks an organic material and removes an electron pair leading to the formation of oxidized product. The another pathway is a homolytic or free-radical reaction. In case of aromatic compounds, oxidation reaction is launched from hydroxylation. Reaction (4) shows the oxidation process (EPA, 1989).

$$RCH_3 \rightarrow RCH_2OH \rightarrow RCHO \rightarrow RCOOH \quad (4)$$

$$CH_3CHCl_2 + H_2O \rightarrow CH_3CCl_2OH + 2H^+ + 2e^-$$

(5) Reduction reaction : In contrast to oxidation reaction, the reduction reaction obtains electrons into organic pollutants. A nucleophilic hydrogen agent attacks an organic material and removes chlorine ions. Reaction (5) shows the reduction process (Lionel et al., 2006).

$$CCl_4 + H^+ + e^- \rightarrow CHCl_3 + Cl^- \quad (5)$$

(6) Dehydrohalogenation reaction : This reaction is similar to dechlorination reaction. Hydrogen ion and chlorine ion are broken loose from organic compounds. Reaction (6) shows the dehydrohalogenation process (Christos et al., 1984).

$$CCl_3CH_3 \rightarrow CCl_2CH_2 + HCl \quad (6)$$

CHAPTER IV. MATERIALS AND METHODS

4.1 Experiment to measure soil properties

4.1.1 Sampling of contaminated soil and TPH analysis

From investigation reports in 2006 and 2009, two sites (A and C site) at 'Camp Hialeah' were serious contaminated by diesel (Fig. 4 and 5). Soils were sampled by using a backhoe for the experiments and were transported to laboratory at 4 ℃. Soil samples were pretreated for TPH concentration analysis according to Korean Soil Pollution Standard Analytical Process (Korea Ministry of Environment, 2009). Soil samples were sieved with No. 10 sieve (2 ㎜ in diameter) and about twenty grams of soils were disintegrated into small particles, placing in a porous cellulose thimble filter. The thimble filter was placed in an extraction chamber, which was connected above a flask containing the dichlomethane (CH_2Cl_2) and below a condenser. The flask was heated on a hot plate at 120 ℃ for 24 hr and the solvent evaporated and moved up into the condenser where it was converted into a liquid that trickled into the extraction chamber containing the soil sample. The extraction chamber was designed so that when the solvent surrounding the sample exceeded a certain level, it overflowed and trickled back down into the boiling flask (Fig. 6).

At the end of the extraction process, the flask containing the solvent and TPH were removed. The solvent in the flask was then evaporated into 2 ㎖ and the mass of the remaining lipid in 2 ㎖ is measured by GC (Hewlett

Packard, Agilent 6890 Plus) with a flame ionization detector (FID) (Fig. 6). A capillary DB-1 TPH column (30 m in length, 0.32 ㎜ in diameter, and 0.25 ㎜ in film thickness; purchased from Agilent Co., USA) was used to measure TPH concentration in the residual solution. An auto-injector was used to inject 2 ㎕ (split ratio of 3:1) of the solution and the temperature of the inlet was 290 ℃. The initial oven temperature was 70 ℃ (5 min hold) and increased to 300 ℃ at a rate of 8 ℃/min (24 min hold). The carrier gas was nitrogen (99.99 % purity) and its velocity in a column was 1.5 ㎖/min. The method detection limit (MDL) of this method was 10 ㎎/㎏ for total petroleum hydrocarbons (TPH).

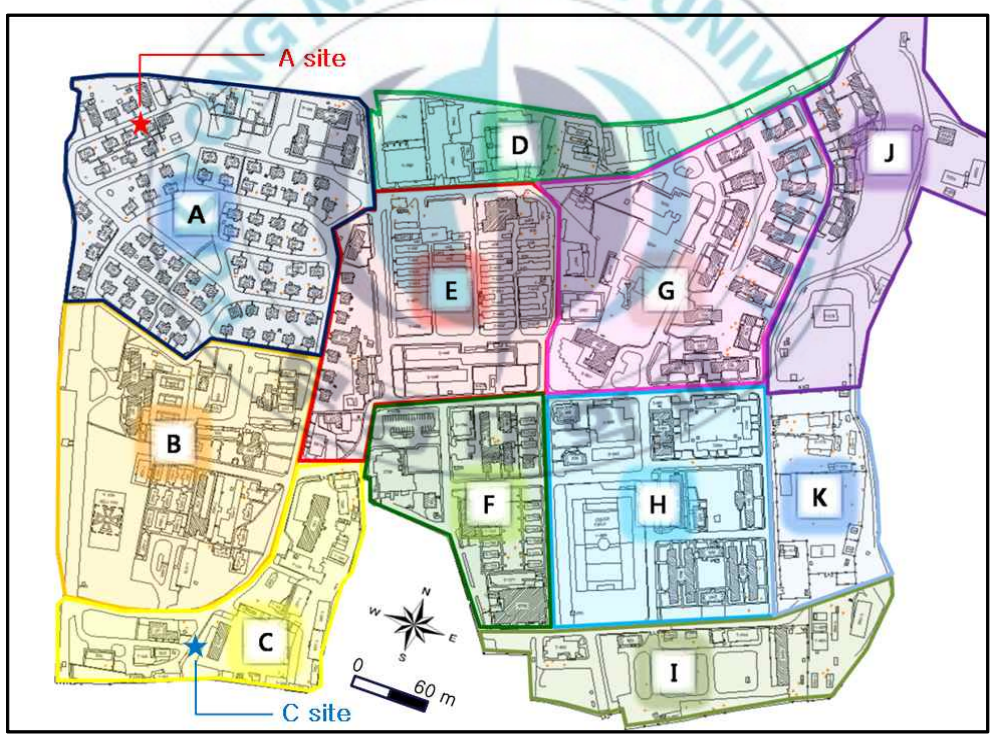


Fig. 4. Location of soil sampling sites for the research in 'Camp Hialeah'.

(a) Photograph of A site

(b) Photograph of C site

Fig. 5. Photographs of soil sampling sites in 'Camp Hialeah'.

(a) Soil samples into the thimble filter

(b) Setting the Sohxlet extract facility

Fig. 6. Sohxlet extraction process for TPH concentration analysis in soils.

Fig. 7. GC/FID (Hewlett Packard, Agilent 6890 Plus with Flame Ionization Detector) with a auto injector (Agilent 7683 series) for TPH analysis.

4.1.2 Physicochemical characteristics of soil

Several analyses were performed to identify physical and chemical properties of contaminated soil for the experiments. Physical properties such as particle distribution, bulk density and water content were measured through Korean Soil Pollution standard analytical process (Korea Ministry of Environment, 2009).

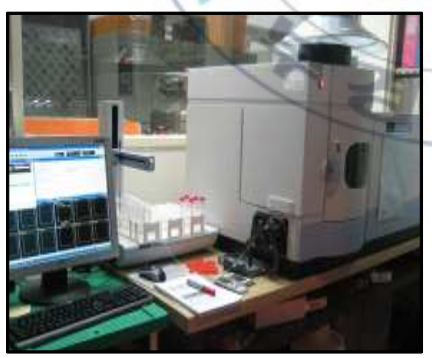
For the chemical properties, pH of soils were measured by an electrometer (Istek, 815PDC) and their principle components were analyzed by X-ray fluorescence spectrometer (XRF : Shimadzu, XRF-1700). Total organic carbon contents (TOC) and heavy metal concentrations of soil were measured by TOC meter (Shimadzu, TOC-vcph) and ICP/OES (Perkin Elmer, Optima 7000DV), respectively. The concentration of nitrogen and phosphorus in soils were also analyzed on ultraviolet-visible spectrometer (UV-VI : JASCO, V-670) for the biodegradation (Fig. 8).

(a) Automatic sieves for soil particle distribution

(b) pH meter (Istek, 815PDC)

(c) XRF (Shimdzu, XRF-1700)

(d) TOC meter (Shimadzu, TOC-vcph)



(e) ICP/OES (Perkin Elmer, Optima 7000DV)

(f) UV-spectrophotometer (JASCO, V-670)

Fig. 8. Analytical equipments for physico-chemical properties of soils.

4.2 Microbiological analysis

4.2.1 Isolation and identification of indigenous microorganisms

In order to identify indigenous microorganisms from contaminated soils taken in A and C site, 1 g of soil sample was mixed with 9 ㎖ of sterile distilled water. Each diluted solution of 0.1 ㎖ was plated onto an oil medium plate, which contained the following constituents (per 1 ℓ): 2 g diesel, 3.32 g $Na_2HPO_4 \cdot 12H_2O$, 1 g KH_2PO_4, 0.2 g ㎎ $SO_4 \cdot 7H_2O$, 0.01 g $CaCl_2$, 0.02 g $FeSO_4 \cdot H_2O$, 1 g NH_4NO_3, 2 g $(NH_4)_2NO_3$, 0.5 g Tween 20 (a kind of surfactants) and 19 g agar powder (Schmealenberger et al., 2001; Clarridge, 2004). Each diluted solution on an oil medium plate was cultivated at 30 ℃ for 3 days in an incubator and representative colonies on the oil medium plate were identified through sequencing by using the 16S rRNA gene method (Munson et al., 2004; Petti et al., 2005). All culture processes were conducted aseptically in the clean bench and isolated microorganisms were stored as liquid cultures containing with 10 % glycerol at -70 ℃ before the experiment.

4.2.2 Enumeration of indigenous microorganisms

Counting the total indigenous bacteria in the contaminated soil indicates whether the soil involves a healthy indigenous microbial population capable of supporting bioremediation (Song et al., 1990). The enumeration analysis of indigenous microorganisms was performed by using the most probable number (MPN) method (Mark et al., 1980; Stevens et al., 1995; Haines et al., 1996). Isolated microorganism suspension from cultivated in nutrient broth (NB) medium was diluted with 9 ㎖ of sterile distilled water and 0.1 ㎖ of each dilution was plated onto a nutrient agar (NA) medium (Becton, Dickinson and Company, Sparks, USA), which consisted of 5 g of peptone, 3 g of beef extract and 15 g of agar per 1 ℓ. Plated dilutions were cultivated at 30 ℃ for 1 - 2 days in an incubator. After cultivation, the estimation of microorganism populations was determined by counting the dilution series on each NA medium (Lennette et al., 1974).

4.2.3 SEM analysis of isolated indigenous microorganisms

SEM (Scanning Electron Microscope) is one of the representative equipment to visualize the surface of substrates and materials. SEM analyses for the indigenous microorganisms in contaminated soils were conducted to evaluate the structure of microorganisms.

For the image analysis, indigenous microorganisms, cultivated on each NA medium at 30 ℃ for 1 - 2 days were shifted on glass slide. Pre-fixation was performed at 4 ℃ for 1 hour with 2.5 % of glutaraldehyde. After pre-fixation, rinsing process was conducted at 4 ℃ with pH 7.4 phosphate buffer solution and post-fixation at 4 ℃ for 1 hour was conducted with 1 % osmium tetraoxide (OsO_4). After post-fixation, samples were dehydrated in order of ethanol concentration increase at 4 ℃ for 5 minutes (50 % - Absolute ethanol). Post-fixed samples were substituted by isoamyl acetate for 15 minutes and it was replicated for 2 times. After the substitution process, samples were dried with CPD (Critical Point Dryer). Dried samples were fixed to SEM stubs using either a colloidal silver adhesive paint or epoxy resin. Gold coating was carried out in a sputter coater for 2 rain at 1.3 kV (approximately 600 ~), immediately prior to observation at 10 kV in a scanning electron microscope (Carl Zeiss, DSM 940A, Germany).

4.3 Landfarming batch experiment using commercialized microorganism cultivated solutions

4.3.1 Experiments with different TPH concentrations using commercialized microorganism cultivated solutions

Batch experiments using commercialized microorganism cultivated solutions were performed to investigate TPH removal efficiency by the landfarming process. Two kinds of commercialized solutions were used for the experiment and they consist of commercialized microorganism cultivated solution, named "Oilbug 1010" (from BioSaint Inc., Korea) and "Penazyme" (from Life Science of Suwon University, Korea), respectively. Physical and chemical properties of each commercialized microorganisms were shown in Table 3. In order to evaluate the TPH removal efficiency of each commercialized solution, three different soils having different initial TPH concentrations (S1 : 1,787 ㎎/㎏, S2 : 2,901 ㎎/㎏, and S3 : 5,715 ㎎/㎏) were used.

In the plastic petri dish (150 ㎜ in diameter × 20 ㎜ in depth), about 300 g of TPH contaminated soil was mixed with each commercialized microorganism cultivated solution at different ratio (for "Oilbug 1010" 0.5 ㎖ and 1.0 ㎖ of solution (O1 and O2) per 100 g soil; for "Penazyme", 0.1 ㎖ and 0.2 ㎖ of solution (P1 and P2) per 100 g soil) (Table 4). In batch test with "Oilbug 1010" microorganism, nutrients solution offering from "BioSaint lnc." was added into each reactor with the same amount of commercialized microorganism solution. As the recommendation of Suwon University, nutrients such as nitrogen and phosphorus were not added in the experiment

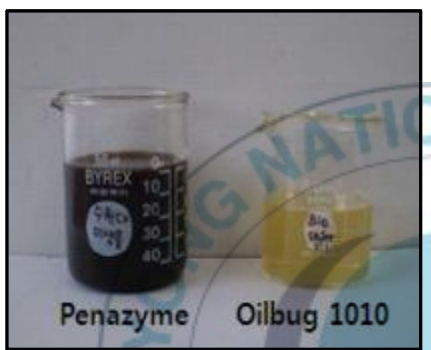
with "Penazyme". Water content of 10 % wt and 20 ℃ of temperature maintained for the experiments. Water content decreased due to the vaporization and the distilled water was added into the batch reactor to maintain a constant water content and the artifical soil mixing for aeration in reactor was conducted every 48 hours (Fig. 9). Soil samples for the monitoring of TPH biodegradation using commercialized microorganism cultivated solution were taken from each batch reactor at 4 times (3, 5, 10 and 20 days during the experiment). After the sohxlet extraction pretreatment method, TPH concentrations of soils were measured by GC/FID to calculate TPH removal efficiency for each reactor.

Table 3. Physical and chemical properties of each commercialized solution

	Chemical components	Physical characteristics and effect	Contents (wt %)
"Oilbug 1010"	$(NH_4)_2SO_4$	Nutrients for efficiency increase	22.5
	Na_2HPO_4	Nutrients for efficiency increase	23.3
	KH_2PO_4	Nutrients for efficiency increase	11.3
	S1 (The trade secret)	Oil-degrading microorganism	The trade secret
	S2 (The trade secret)	Oil-degrading microorganism	The trade secret
"Penazyme"	Sarsapogenin	Removal of NH_3	20.0
	Spirostant	Surface tension decrease	20.0
	Humic acid	Removal of heavy metals	10.0
	Zymogen	Decompositon of organic matter	10.0
	Zyme-Leaven	Yeast fungus	10.0
	Parigenin	Vitalization of microorganism	10.0
	Glycine	Neutralization	5.0
	Laminarin	Vitalization of microorganism	5.0
	Sarsaponin	Removal of NH_3 and H_2S	5.0
	Proenzyme	Decompositon of organic matter	4.0
	Quzyme	Vitalization of microorganism	1.0

Table 4. Various conditions of batch experiments by using commercialized microorganisms cultivated solution

Initial TPH conc. of soil sample (㎎/㎏)	Types of commercialized microorganism	Amount of microorganism cultured solution and nutrients solution applied in experiment (per 100 g soil)
1,787 (S1)	Oilbug 1010	0.5 ㎖ + 0.5 ㎖ (N, P) (O1)
2,901 (S2)		1 ㎖ + 1 ㎖ (N, P) (O2)
5,715 (S3)	Penazyme	0.1 ㎖ + No addition (N, P) (P1)
		0.2 ㎖ + No addition (N, P) (P2)



(a) commercialized microorganism solutions

(b) Injection of microorganism solution to the batch reactor

(c) Soil mixing in the reactor

(d) Cultivation of the batch reactor

Fig. 9. Photographs of the batch experiment process using commercialized microorganisms cultivated solution.

4.3.2 Experiments for the effects of temperature and water content on the landfarming process

Batch experiments were performed to investigate the effect of various water content and temperature change on TPH removal efficiency for bioremediation. Initial TPH concentration of the soil was 2,989 ㎎/㎏ and "Oilbug 1010" solution from previous batch experimental results was used.

In plastic petri dish (150 ㎜ in diameter × 20 ㎜ in depth), about 300 g of soil were mixed with 0.5 ㎖ of "Oilbug 1010" solution and 0.5 ㎖ of nutrients (N and P) per 100 g soil. Various conditions for water content (5 %, 10 % and 20 %) and temperature (20 ℃, 30 ℃ and 40 ℃) were applied for the experiments (Table 5). The water content for experiment decreased due to the vaporization and the distilled water was added into the batch reactor to maintain each constant initial water content (5 %, 10 % and 20 %) and soil mixing for aeration was conducted every 48 hours. Soils were sampled at 5, 10 and 20 days (3 times) and then were analyzed on GC/FID to calculate TPH removal efficiency on various conditions.

Table 5. Various water content and temperature conditions applied for the experiment

Initial TPH conc. of soil (㎎/㎏)	Water content (%)	Temperature (℃)
2,989	5	20
	10	30
	20	40

4.4 Landfarming batch experiments using indigenous microorganisms

The one of the main objectives in this research is to compare TPH removal efficiencies of commercialized microorganism cultivated solutions with those of indigenous microorganisms in landfarming process. From results of indigenous microorganisms isolation from contaminated soils, 4 types of indigenous microorganisms (3 types in A site and 1 type in C site) were identified for the experiment. Each isolated microorganism named as "A-1", "A-2", "A-3" and "C-1", respectively. The mixture of four microorganisms was named as "T-1". They were spreaded on 100 ㎖ NB (nutrient broth) medium in a 250 ㎖ flask and were cultivated by using constant temperature shaker (30 ℃) at 100 rpm for 24 hours. NB medium consisted of 5 g of peptone and 3 g of beef extract per 1 ℓ. In order to maximize the efficiency of batch experiment using indigenous bacteria, essential nutrients such as $(NH_4)_2PO_4$ and KH_2PO_4 for bioavailability were added to the batch reactor. The ratio of C:N:P in solution was determined at 100:10:1. In this experiment, contaminated soil at C site was used and the initial TPH concentration of soil was 3,819 ㎎/㎏.

In plastic petri dish (150 ㎜ in diameter × 20 ㎜ in depth), approximately 300 g of soil were mixed with different amounts of each cultivated indigenous microorganism solution (0.1 ㎖ and 0.2 ㎖ of each bacteria solution (I1 and I2) per 100 g soil). The ratios of soil and indigenous microorganism solutions were 1000:1 and 500:1 (I1 and I2), respectively. Various water content (10 % and 20 %) and temperature (20 ℃, 30 ℃ and 40 ℃) conditions were applied for the experiments (Table 6). The

water content in the reactor decreased due to the vaporization and the distilled water was also added into the batch reactor to maintain the constant initial water content (10 % and 20 %). Soil in the reactor was mixed for the aeration every 48 hours. For the analysis of TPH concentration, soil samples were taken from each reactor at 3, 5, 10, 17, 24 and 31 days.

Table 6. Various conditions for batch experiments by using indigenous microorganisms

Types of indigenous microorganism	Amount of indigenous microorganism solution (per 100 g soil)	Water content (%)	Temperature (℃)
Arthrobacter sp. (A-1) *Burkholderia* sp. (A-2) *Cupriavidus* sp. (A-3) *Bacillus* sp. (C-1)	0.1 mℓ (I1)	10	20 30
Mixed microorganisms (A-1 + A-2 + A-3 + C-1 : T-1)	0.2 mℓ (I2)	20	40

CHAPTER V. RESULTS AND DISCUSSION

5.1 Results of the analyses for soil properties

Physical and chemical properties of soils were measured and their results were shown in Table 7. Soil at A site was composed of 93.9 % of Sand, 5.9 % of Silt and 0.2 % of Clay. For soil at C site, its mass ratio was 95.7 % of Sand, 4.0 % of Silt and 0.4 % of clay. Their soil textures were plotted on the soil textural diagram presented by United States Department of Agriculture (USDA) and the soil textures in study area were found to be "Sand" (Fig. 11). The pH of A site soil was 6.65 (weak acid) and pH of C site soil was 8.29 (weak base), suggesting that pH conditions of soils were available for the application of bioremediation process. Bulk density of soils at A and C site were 1.50 g/㎤ and 1.18 g/㎤, respectively. Water content of soils in A and C site were low (2.59 % and 1.19 %, respectively) and their TOC (total organic carbon contents) were 2.39 % and 0.66 %, respectively. From results of inorganic matters (total nitrogen and phosphorus) analysis, T-N and T-P at A site were 4.80 and 0.06 ㎎/㎏ and soil at C site were 5.26 and 0.15 ㎎/㎏, respectively. Results of principle component analysis for soils in study area were shown in Table 8. The major components for soils in A and C site were SiO_2 (silicon oxide), Al_2O_3 (aluminium oxide), Fe_2O_3 (ferric oxide), CaO (quicklime oxide) and K_2O (potassium oxide) in order. Table 9 shows initial TPH and heavy metal concentration of soils. TPH concentrations of soils at A and C site were determined as 2,881 ㎎/㎏ and 5,715 ㎎/㎏, respectively, which were higher

than Korea Soil Warning Limit (KSWL of TPH : 500 ㎎/㎏). Pb concentration at C site soil exceeded KSWL (KSWL of Pb : 200 ㎎/㎏). Soil samples were contaminated with both TPH and Pb. Typical GC peaks of contaminants in soils were shown in Fig. 11. From results of GC analysis, the main pollutants in the study area were determined to diesel mostly and motor oil.

Table 7. Results of the analysis for soils properties

Sampling site	Size distribution (%)			pH	Bulk density (g/㎤)	Water content (%)	TOC (%)	T-N (㎎/㎏)	T-P (㎎/㎏)
	Sand	Silt	Clay						
A site	93.9	5.9	0.2	6.65	1.50	2.59	2.39	4.80	0.06
C site	95.7	3.9	0.4	8.29	1.18	1.19	0.66	5.26	0.15

Table 8. Results of principle component analysis by using XRF for soils

Main components	Mass distribution ratio (wt %)	
	Soil at A site	Soil at C site
SiO_2	52.96	71.84
Al_2O_3	21.19	10.11
Fe_2O_3	8.67	2.58
K_2O	1.94	2.87
CaO	1.63	3.90
MgO	1.34	0.37
TiO_2	0.82	0.39
Na_2O	0.26	1.27
MnO	0.24	0.07
P_2O_5	0.12	0.06
LOI	10.16	5.93
Total	99.35	99.39

* LOI : Loss on ignition

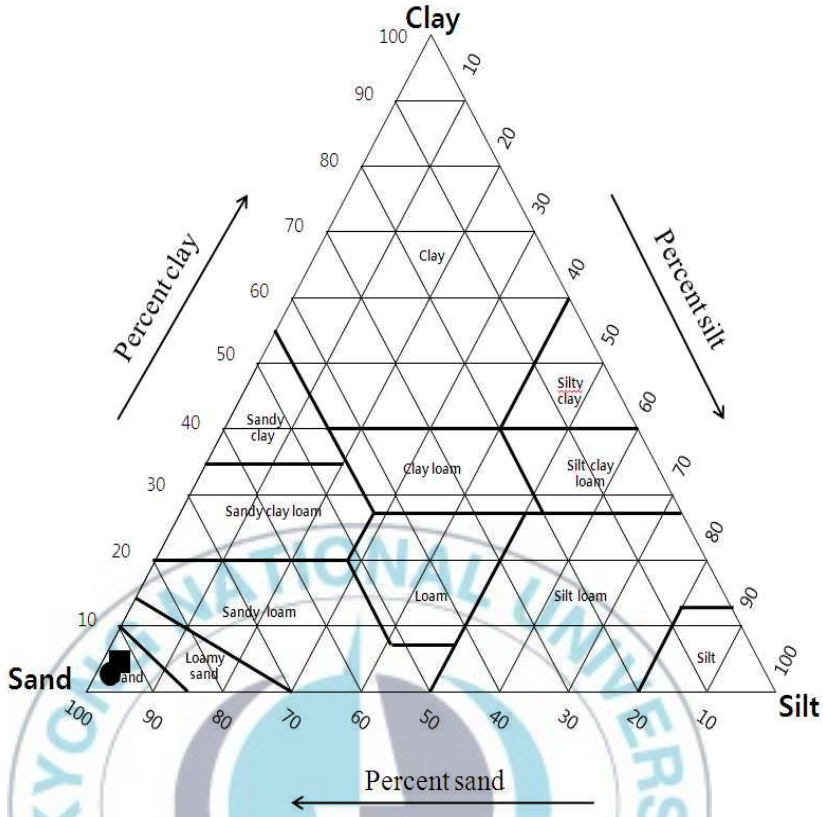
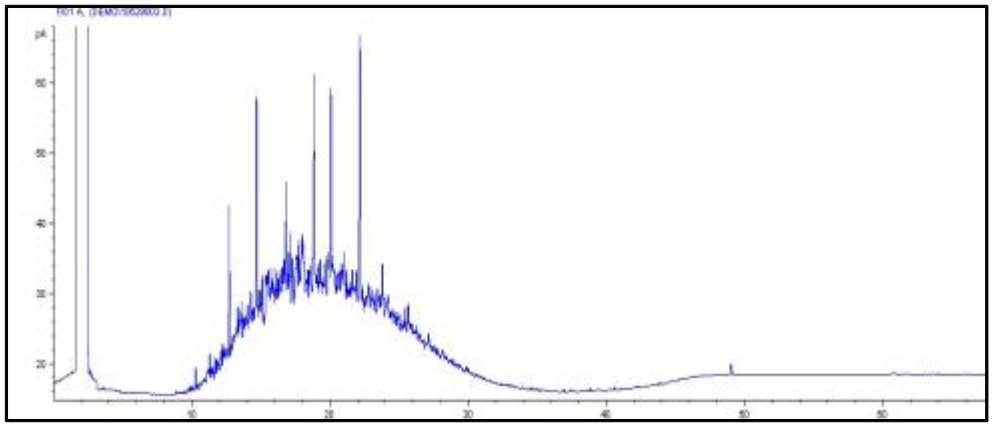


Fig. 10. Soil textures for soils at study area (●: A site soil, ■: C site soil).

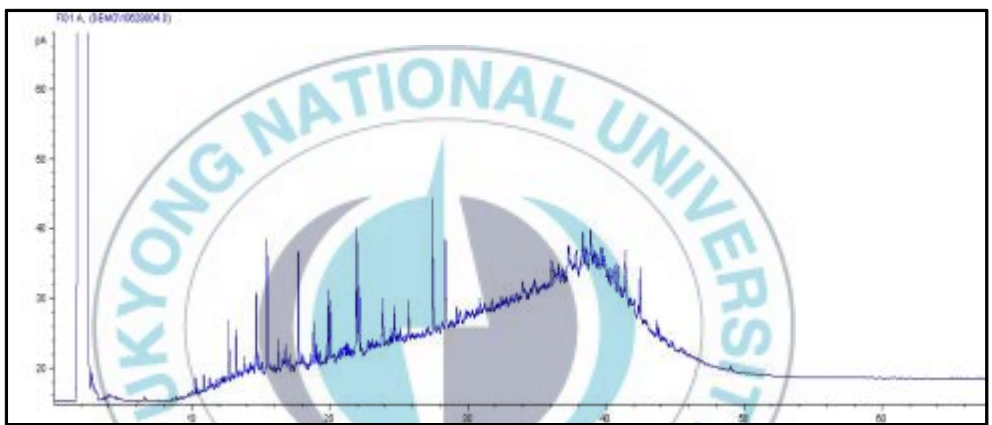
Table 9. Results of initial TPH and heavy metal concentration of soils

soil sampling site	Average concentration (㎎/㎏)			
	TPH	Cd	Zn	Pb
A site	2,881	2.31	48.90	161.75
C site	5,715	N.D.	244.38	288.99
KSWL	500	4	300	200

* KSWL : Korea Soil Warning Limit



(a) Typical GC peak of TPH for A site soil



(b) Typical GC peak of TPH for C site soil

Fig. 11. Typical GC peaks of TPH contaminants in soils for the study.

5.2 Results of microbiological analysis

5.2.1 Isolation and identification of indigenous microorganisms

From results of microbiological isolation and identification analyses, total four indigenous microorganisms were isolated from contaminated soils at A and C site. Fig. 12 shows phylogenetic trees of four isolated microorganisms. Isolated indigenous microorganisms were recognized as *Arthrobacter* sp., *Burkholderia* sp., *Cupriavidus* sp. and *Bacillus* sp.. From results of phylogenetic trees, all of isolated microorganisms were recognized as new species.

Characteristics of these microorganisms were summarized below.

Description of *Arthrobacter* sp.

: Recently, it has been discovered that several species of *Arthrobacter* can reduce hexavalent chromium, which can cause severe irritations to humans, and they are also known to degrade agricultural pesticides (Julie et al., 1980).

Description of *Burkholderia* sp.

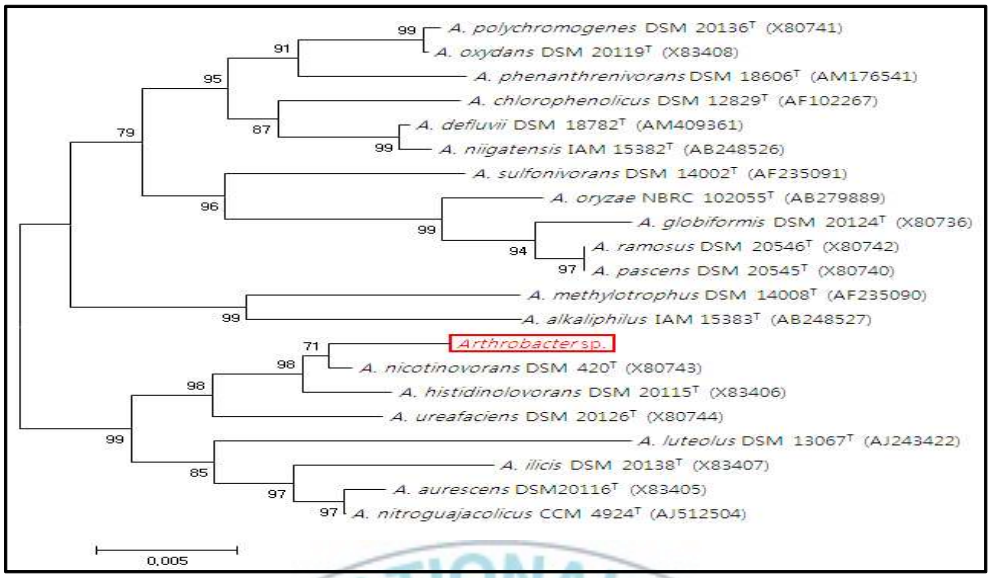
: *Burkholderia* sp. is renowned for its ability to degrade chlororganic pesticides and polychlorinated biphenyls (PCBs) (Chen et al., 2008).

Description of *Cupriavidus* sp.

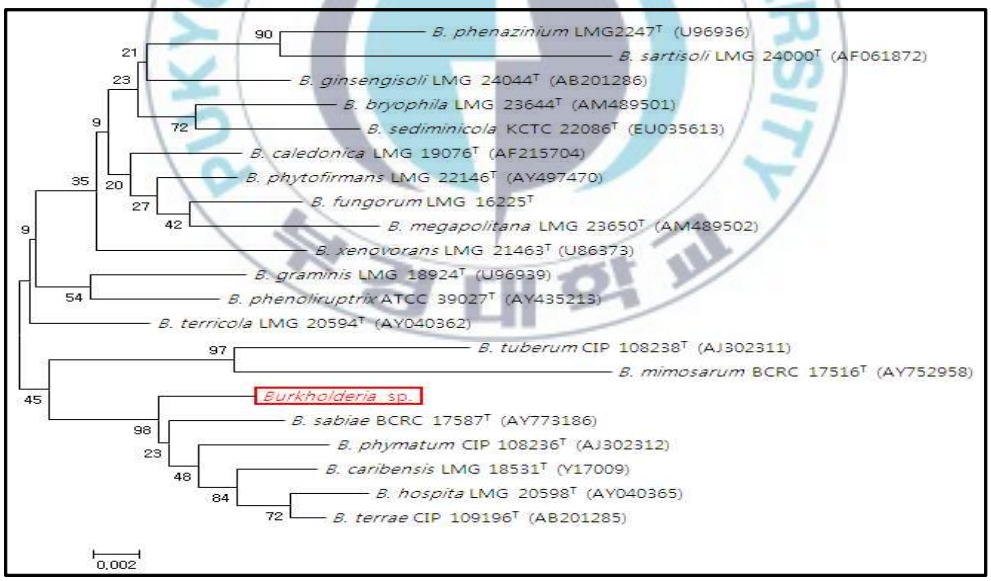
: *Cupriavidus* sp. is used as energy sources such as chlorinated benzene, chlorinated toluenes and 1,2,4,5-tetrachlorobenzene (Peter et al., 2004).

Description of *Bacillus* sp.

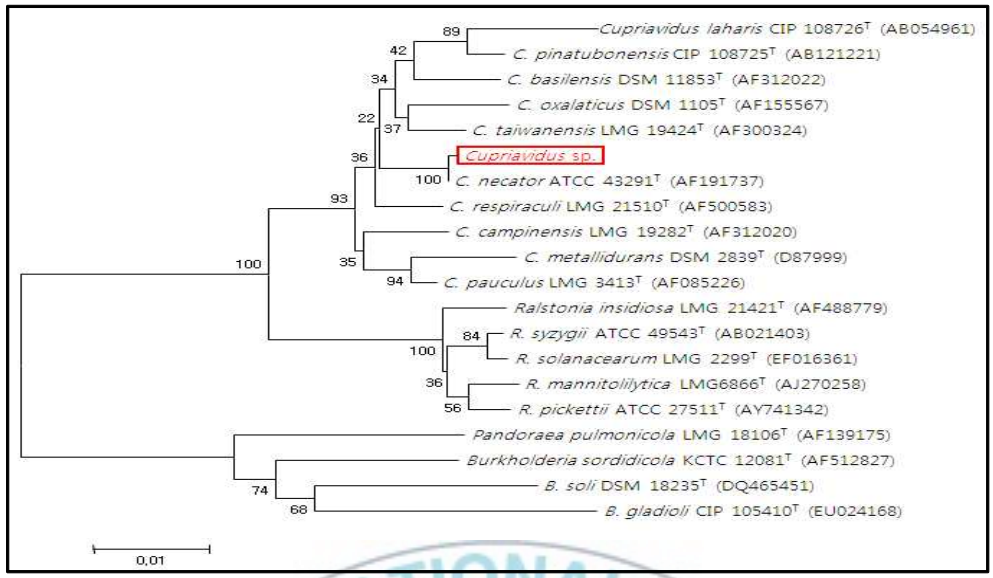
: *Bacillus* sp. has been successfully used for the remediation contaminated soils (Farhadian et al., 2008) It was also known to use BTEX (benzene, toluene, ethylbenzene and xylene) as carbon sources (Gallego et al., 2001).



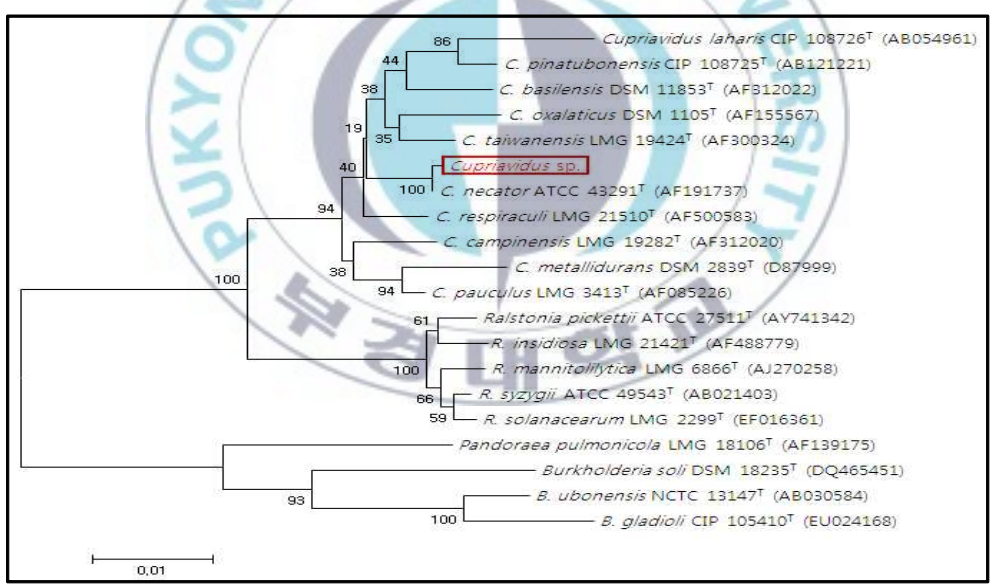
(A) *Arthrobacter* sp. from soil at A site



(B) *Burkholderia* sp. from soil at A site



(C) *Cupriavidus* sp. from soil at A site



(D) *Bacillus* sp. from soil at C site

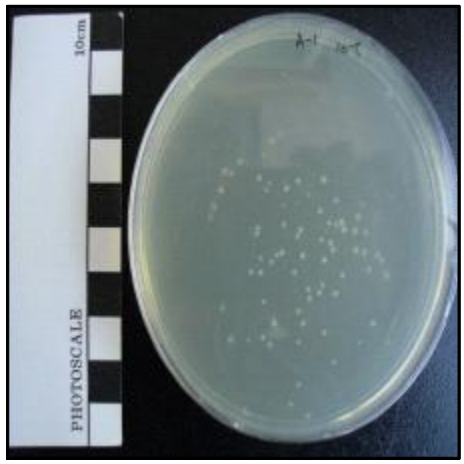
Fig. 12. Phylogenetic trees of four isolated microorganisms from the contaminated soils at A and C site.

5.2.2 Enumeration of indigenous microorganisms

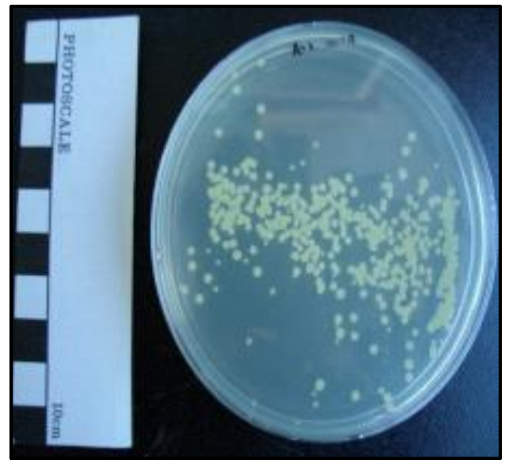
For the adaptation of microbial population to chemical pollutants, three mechanisms such as induction and derepression of enzymes, genetic changes and selective enrichment were applied or used. It was reported that the population of microorganism must be more than 10^3 CFU/㎖ for the application of these biomechanisms (Alexander, 1999). A low microbial population and inadequate microbial diversity can adversely affected the bioremediation efficiency (Alexander, 1999; Joseph et al., 1990). The concentration of each indigenous microorganisms is shown in Table 10 and Fig. 13. Results suggested that the microbial population in soil is abundant to apply the landfarming process.

Table 10. Concentrations of microorganisms in contaminated soils (measured by most probable number (MPN) method)

Indigenous microorganisms	Concentrations of microorganisms in soil (CFU/㎖)
Arthrobacter sp. (A-1)	7.6 X 10^6
Burkholderia sp. (A-2)	2.1 X 10^6
Cupriavidus sp. (A-3)	1.6 X 10^7
Bacillus sp. (C-1)	1.1 X 10^6

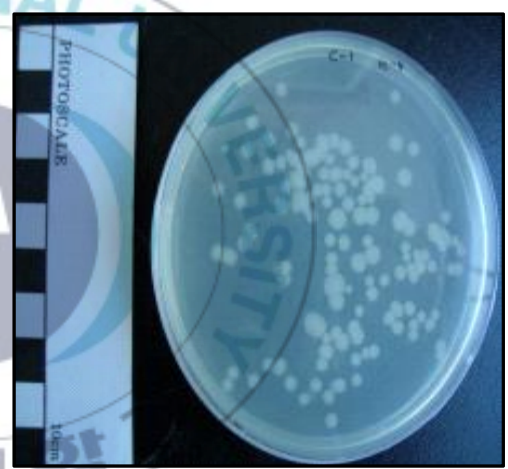


(A) *Arthrobacter* sp.



(B) *Burkholderia* sp.

(C) *Cupriavidus* sp.



(D) *Bacillus* sp.

Fig. 13. Photographs of isolated microorganisms colonies cultivated on the plate for the enumeration by using most probable number (MPN) method.

5.2.3 SEM analysis of isolated indigenous microorganisms

SEM images of indigenous microorganisms were taken to investigate their structures and compositions (Fig. 14). Four indigenous microorganisms were rod type, but their sizes and shapes were various.

Characteristics of *Arthrobacter* sp.

: *Arthrobacter* sp. is aerobic, non-spore-forming and gram-positive bacteria which change morphology during growth from pleomorphic rods when young to coccoids as they age (Julie et al., 1980).

Characteristics of *Burkholderia* sp.

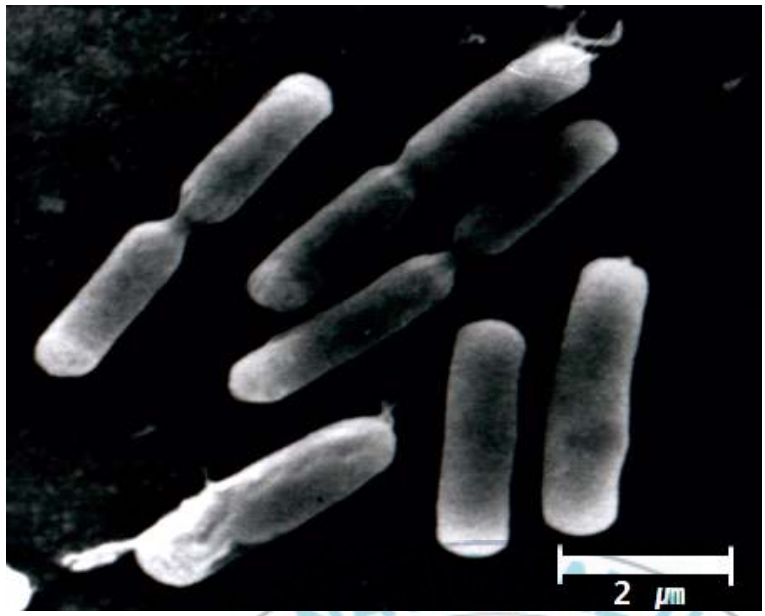
: *Burkholderia* sp. refers to a group of virtually ubiquitous gram-negative, motile and aerobic rod-shaped bacteria including both animal/human and plant pathogens as well as some environmentally important species (Chen et al., 2008).

Characteristics of *Cupriavidus* sp.

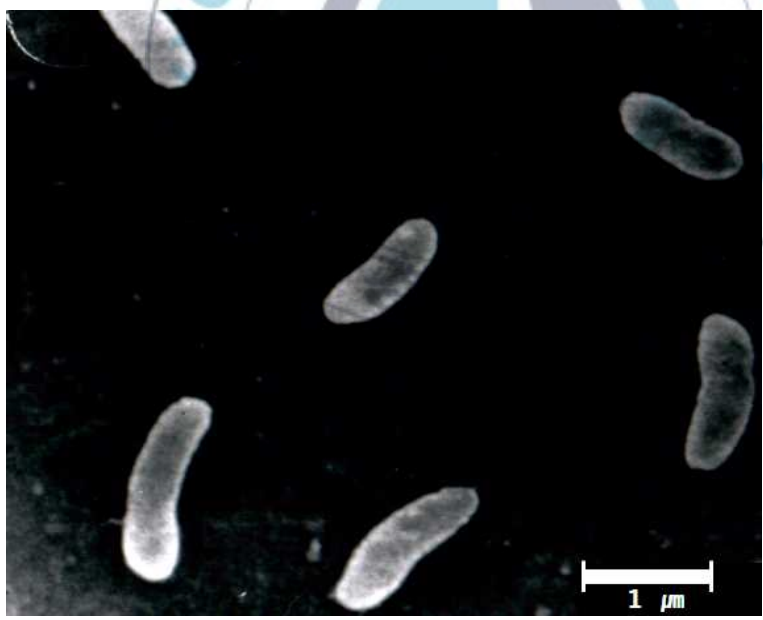
: *Cupriavidus* sp. is a genus of bacteria that includes the former genus Wautersia. It is a non-obligate bacterial predator of various gram-negative and gram-positive soil bacteria and fungi (Peter et al., 2004).

Characteristics of *Bacillus* sp.

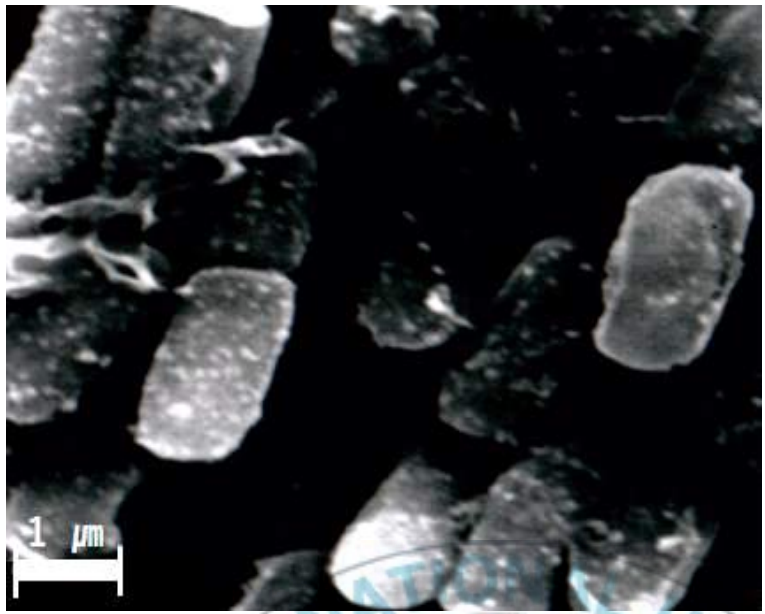
: *Bacillus* sp. is a genus of Gram-positive rod-shaped bacteria and a member of the division Firmicutes (http://en.wikipedia.org/wiki/*Bacillus*, 2011).



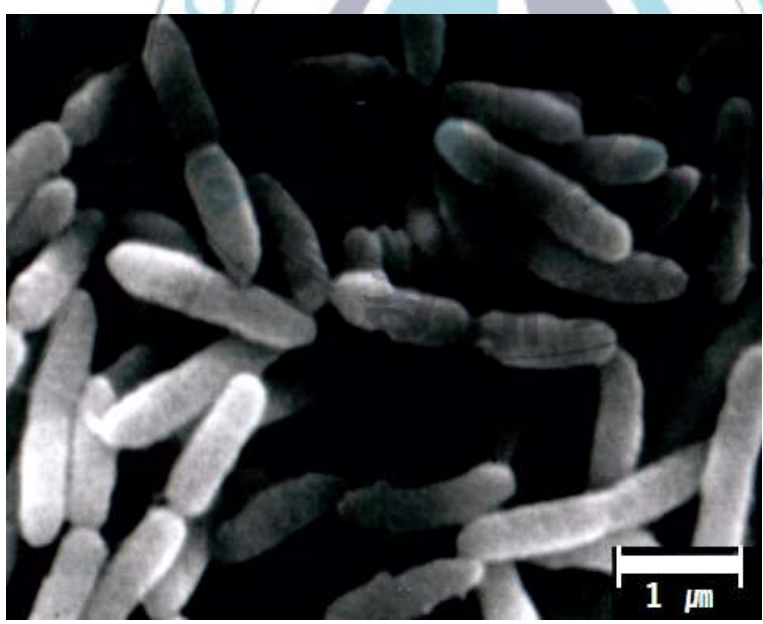
(A) *Arthrobacter* sp.



(B) *Burkholderia* sp.



(C) *Cupriavidus* sp.



(D) *Bacillus* sp.

Fig. 14. SEM images of isolated four indigenous microorganisms.

5.3 Results of landfarming batch experiment using commercialized microorganism cultivated solutions

5.3.1 Experiments with different TPH concentrations using commercialized microorganisms cultivated solutions

Results of batch experiments using commercialized microorganisms ("Oilbug 1010" and "Penazyme") are shown in Fig. 15. For the control soils without additional microorganisms (S1-control, S2-control and S3-control in Fig. 15), 44 %, 35 % and 26 % of initial TPH were removed from the soil for 20 days by the landfarming, suggesting that the biodegradation of TPH by the indigenous microorganisms in the control soils occurred in the reactors. TPH removal efficiency decreased with increase of initial TPH concentration of soil (S1 → S3), suggesting that it is hard to remediate the soils having high TPH concentration (more than 1,700 mg/kg) by using only natural attenuation. TPH removal efficiencies of soils treated by commercial microorganisms of "Oilbug 1010" was 65 % and 61 %, respectively when "Oilbug 1010" was used, suggesting that TPH removal efficiency increase more than two times by adding commercialized microorganisms. Even all of two microorganisms were available to degrade TPH from the soil, "Oilbug 1010" was more effective to remove TPH from the soil than "Penazyme". From results, TPH removal efficiency incerased with the increase of the reaction time, but it was not dependent on the amount of microorganism solution injected to the reactor (O1 vs. O2 and P1 vs. P2) (See Table 4 for the detail conditions of the amount.). By using commercialized microorganisms for 20 days, TPH removal efficiency increased but it did not

reach to the remediation goal (final TPH concentration of treated soil < 500 mg/kg) except 'S1-O1' soil (Fig. 15(a)).

In order to investigate the effect of "Oilbug 1010" for soils having different initial TPH concentration, the degradation kinetics were studied (Horvath, 1972; Perry, 1979). For calculation of constants (k), coefficient of determination (r : a measure of the proportion of variability in a data set that is accounted for by a statistical model), half-life and time (day) to reach the remediation goal, the second-order reaction equation (1) was used (Table 11).

$$\frac{dC}{dt'} = -k \times [C]^2 \qquad (1)$$

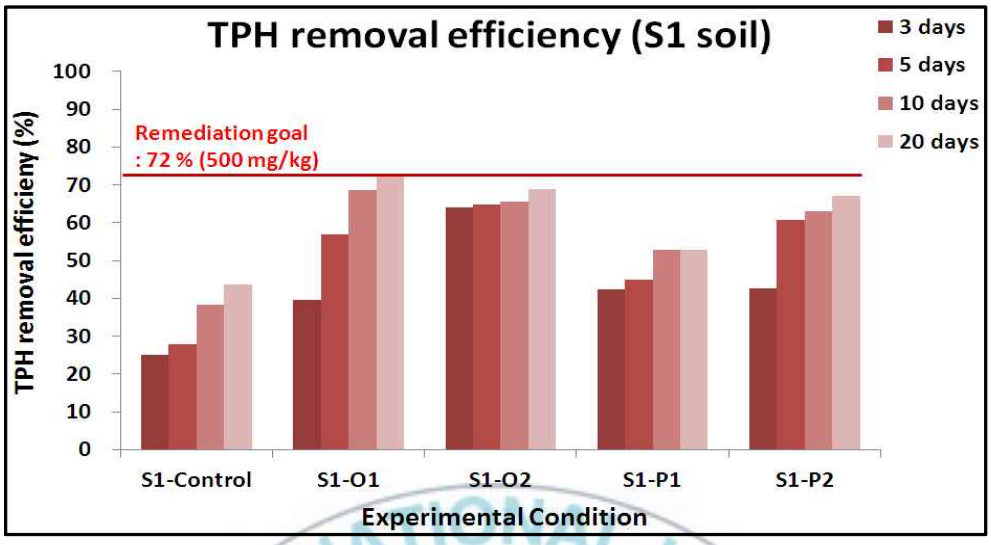
where,

k = biological degradation constant

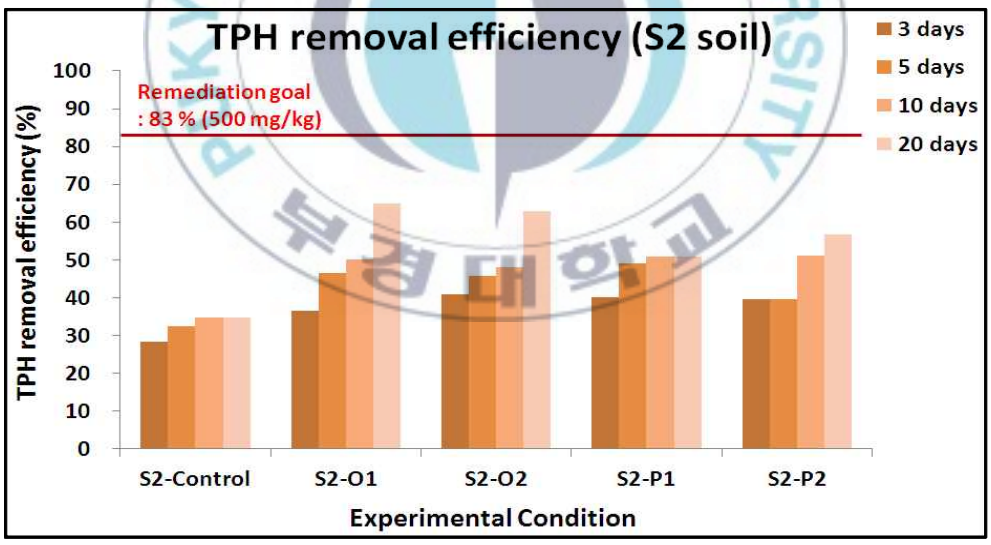
C = remaining TPH concentration at any time

t = time period

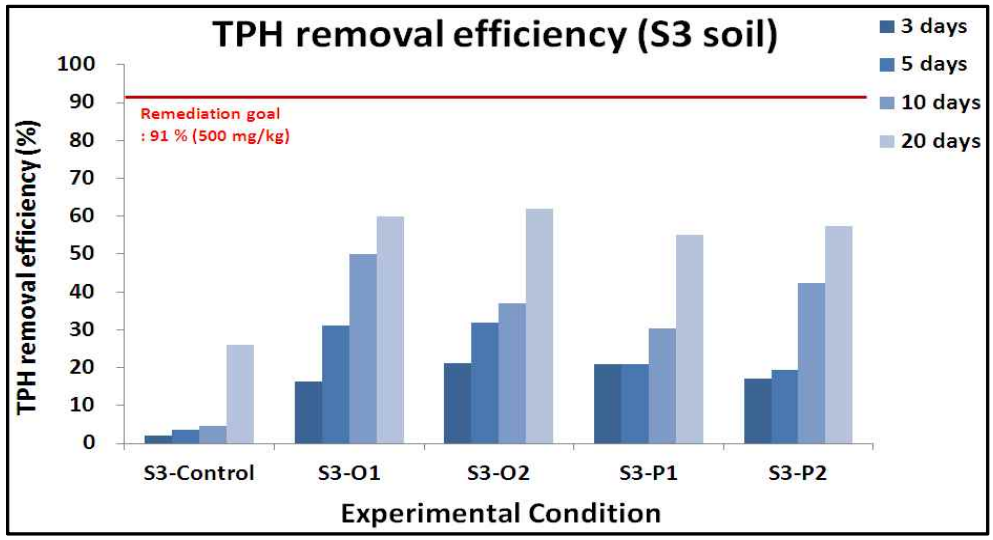
From the batch experiment using "Oilbug 1010" for injecting 0.5 ㎖ of microorganism cultivated solution (O1), the TPH degradation constants of S-1, S-2 and S-3 were 0.09×10^{-3}, 0.03×10^{-3} and 0.01×10^{-3}, respectively. According to the calculation, the degradation rate of "Oilbug 1010" will reach the remediation goal within 16 (S-1), 55 (S-2) and 183 (S-3) days, respectively. These results suggested that half-life and the day reached the remediation goal by using "Oilbug 1010" increased with the increase of initial TPH concentration.



(A) TPH removal efficiency using commercialized microorganism cultivated solutions for S1 soil (Initial TPH concentration : 1,787 ㎎/㎏)



(B) TPH removal efficiency using commercialized microorganism cultivated solutions for S2 soil (Initial TPH concentration : 2,901 ㎎/㎏)



(C) TPH removal efficiency using commercialized microorganism cultivated solutions for S3 soil (Initial TPH concentration : 5,715 ㎎/㎏)

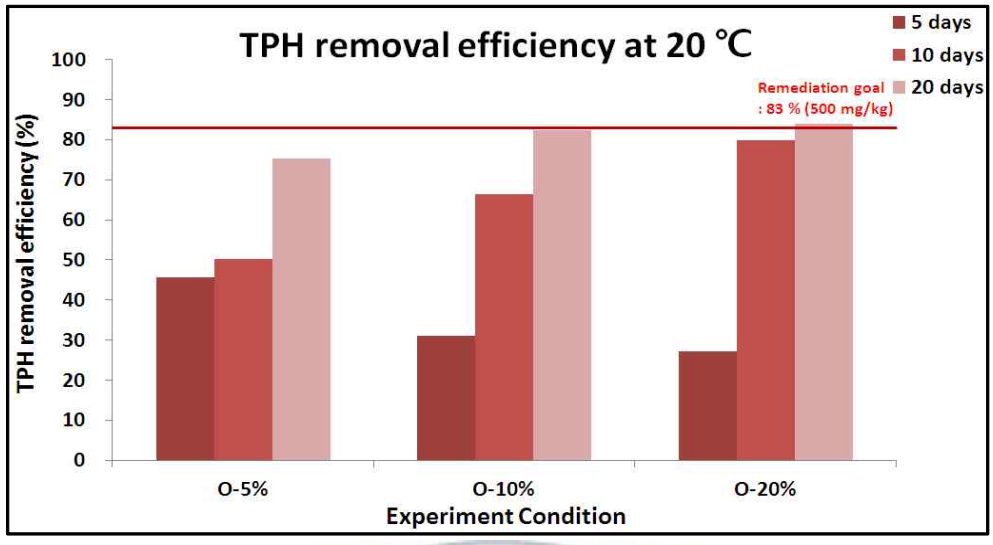
Fig. 15. Results of batch experiments using "Oilbug 1010 ('O')" and "Penazyme ('P')" at different soils (S1, S2 and S3).

Table 11. Second-order reaction rate constants, half-life and time reached the remediation goal (500 ㎎/㎏ of TPH) using 'Oilbug 1010' with soils having different initial concentrations

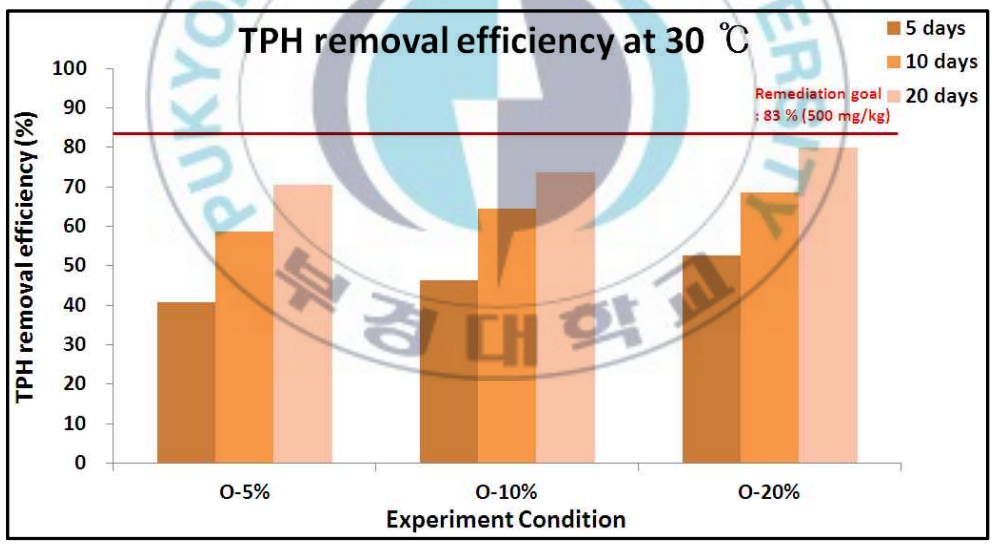
Experiment condition	k (day^{-1})	r	Half-life (day)	Day to reach the remediation goal
S1-O1	0.09×10^{-3}	0.82	6.22	16.00
S2-O1	0.03×10^{-3}	0.88	11.49	55.18
S3-O1	0.01×10^{-3}	0.96	17.00	182.50

5.3.2 Experiments for the effects of temperature and water content on the landfarming process

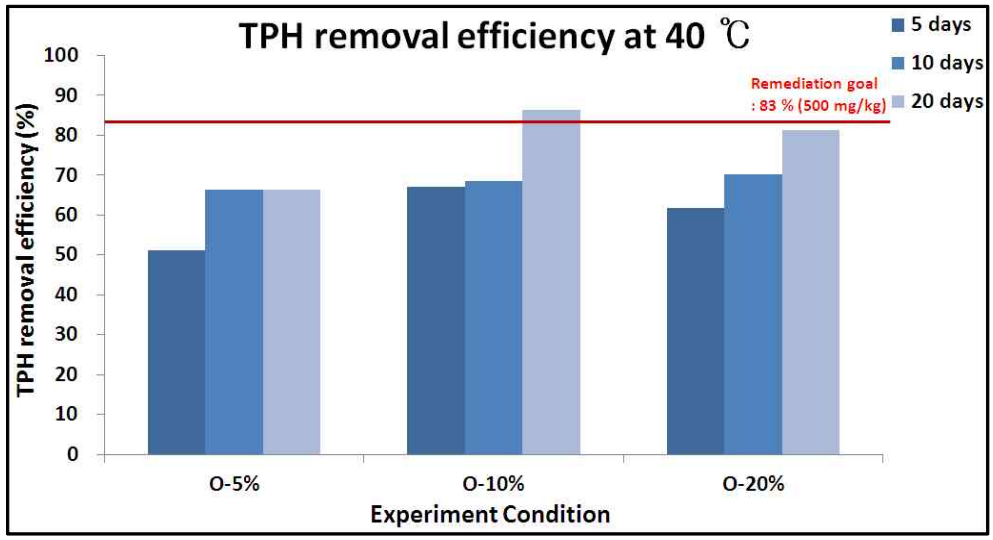
The effect of temperature and water content on the biodegradation ability in landfarming was investigated in batch experiments and their results are shown in Fig. 16. Temperature and water content are most important parameters to control the bioactivity of microorganism (Dibble et al., 1979; Atlas et al., 1981). From results, TPH removal efficiency increased with the increase of water content for 20 days at all of temperature conditions. However, the temperature was less positively correlated with TPH removal efficiency at 20 ~ 40 ℃ range. In order to investigate the effect of "Oilbug 1010" by various temperature, the degradation kinetics was studied (Table 12). From the batch experiment using "Oilbug 1010" for injecting 0.5 ㎖ of microorganism cultivated solution (O1) with 10 % water content, the TPH degradation constants of 20 ℃, 30 ℃ and 40 ℃ were 0.07×10^{-3}, 0.05×10^{-3} and 0.01×10^{-2}, respectively. From the calculated degradation rate of "Oilbug 1010" with 10 % water content, the remediation goal would reach within 24 (20 ℃), 33 (30 ℃) and 17 (40 ℃) days, respectively. These results suggested that the available temperature and water content conditions for landfarming process by using "Oilbug 1010" are > 20 ℃ and > 10 %, respectively.



(A) TPH removal efficiency using "Oilbug 1010 ('O')" solution at 20 ℃ (water content : 5 %, 10 % and 20 %)



(B) TPH removal efficiency using "Oilbug 1010 ('O')" solution at 30 ℃ (water content : 5 %, 10 % and 20 %)



(C) TPH removal efficiency using "Oilbug 1010 ('O')" solution at 40 ℃ (water content : 5 %, 10 % and 20 %)

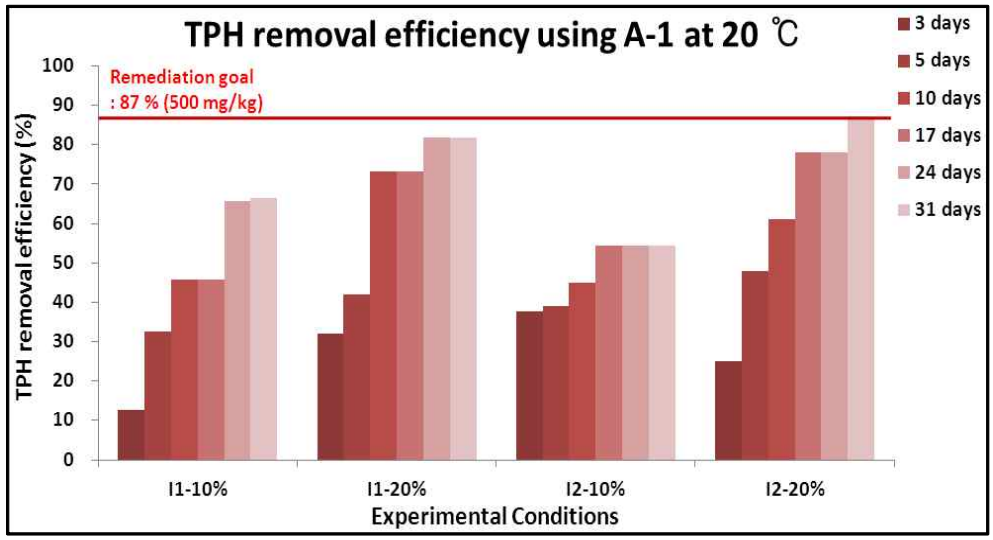
Fig. 16. Results of TPH removal efficiency using "Oilbug 1010" at various temperature and water content conditions.

Table 12. Second-order reaction rate constants, half-life and time reached the remediation goal of biodegradation using 'Oilbug 1010' by each conditions

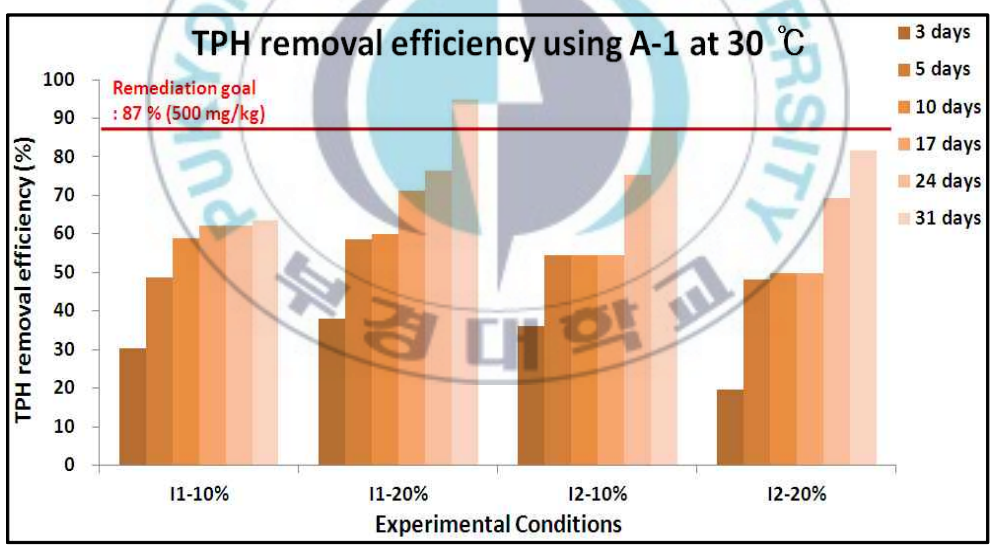
Experiment condition	k (day^{-1})	r	Half-life (day)	Day to reach the remediation goal
O - 10 % - 20 ℃	0.07×10^{-3}	0.96	4.78	23.79
O - 10 % - 30 ℃	0.05×10^{-3}	0.98	6.69	33.31
O - 10 % - 40 ℃	0.01×10^{-2}	0.96	3.35	26.65

5.4 Results of landfarming batch experiment using indigenous microorganisms

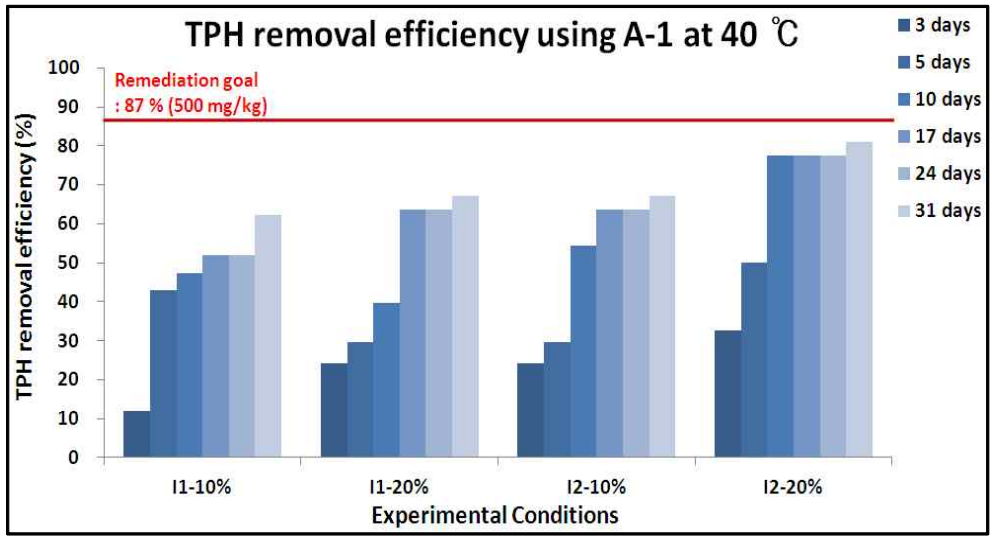
Batch experiments to investigate TPH removal efficiency of landfarming using indigenous microorganisms were performed for 31 days. Results for *Arthrobacter* sp. (A-1) at various conditions are shown in Fig. 17. TPH removal efficiency of landfarming with 20 % of water content was higher than that with 10 % of water content. At 20 ℃ and 30 ℃, the biodegradation of *Arthrobacter* sp. (A-1) was more effective that at 40 ℃. The highest TPH removal efficiency was 94 % while 0.1 ㎖ of cultivated solution was injected (I1) into the soil at 30 ℃ with 20 % of water content (Fig. 17 (B)), which was much higher than that without microorganism and also higher than those with commercialized microorganisms. Results suggested that the use of *Arthrobacter* sp. (A-1) in landfarming reach TPH remediation goal (500 mg/kg or 87 % of removal efficiency) for the contaminated soils at C site.



(A) The use of *Arthrobacter* sp. (A-1) at 20 ℃ with various microorganism concentrations and water contents



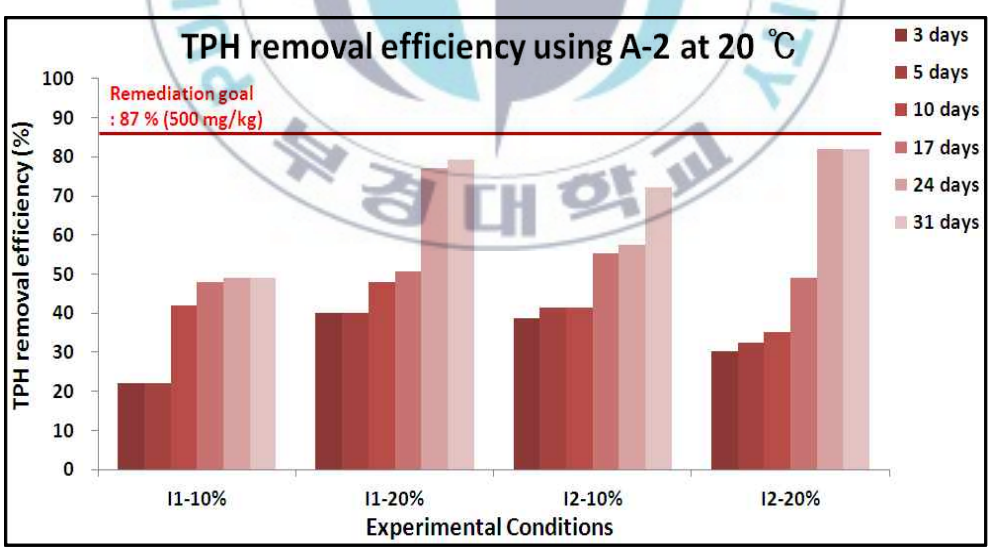
(B) The use of *Arthrobacter* sp. (A-1) at 30 ℃ with various microorganism concentrations and water contents



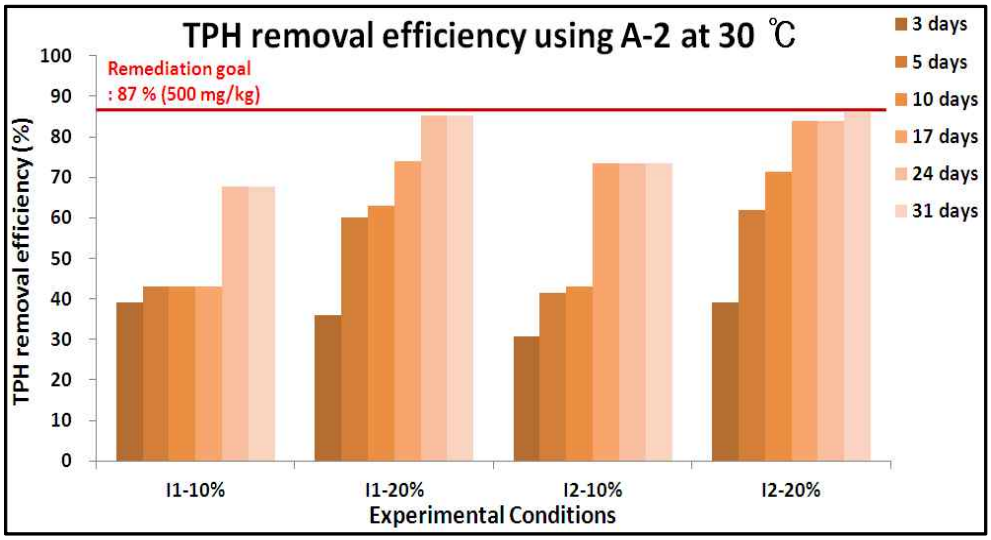
(C) The use of *Arthrobacter* sp. (A-1) at 40 ℃ with various microorganism concentrations and water contents

Fig. 17. Results of TPH removal efficiency using *Arthrobacter* sp. (A-1) at various conditions for landfarming.

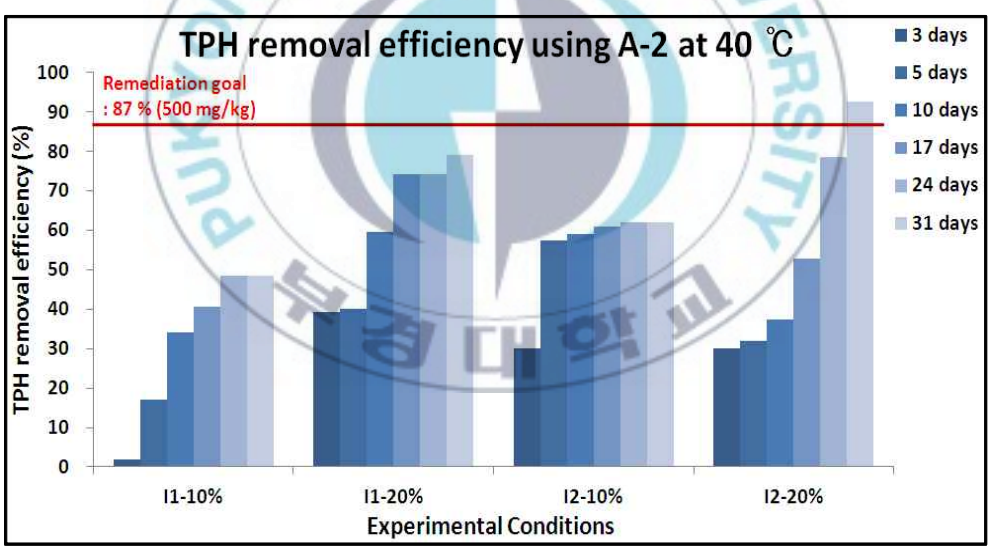
Results of experiments using *Burkhoderia* sp. (A-2) at various conditions are shown in Fig. 18. TPH removal efficiency of *Burkhoderia* sp. (A-2) with 20 % of water content was higher than that with 10 % of water content at all temperature conditions and it also increased with the increase of amount of cultured solution (I1 → I2). When 0.2 ㎖ of *Burkhoderia* sp. (A-2) solution was used, at 40 ℃ and with 20 % of water content, TPH removal efficiency reached to 93 % which was higher than the remediation goal (87 %) (Fig. 18 (C)).



(A) The use of *Burkhoderia* sp. (A-2) at 20 ℃ with various microorganism concentrations and water contents



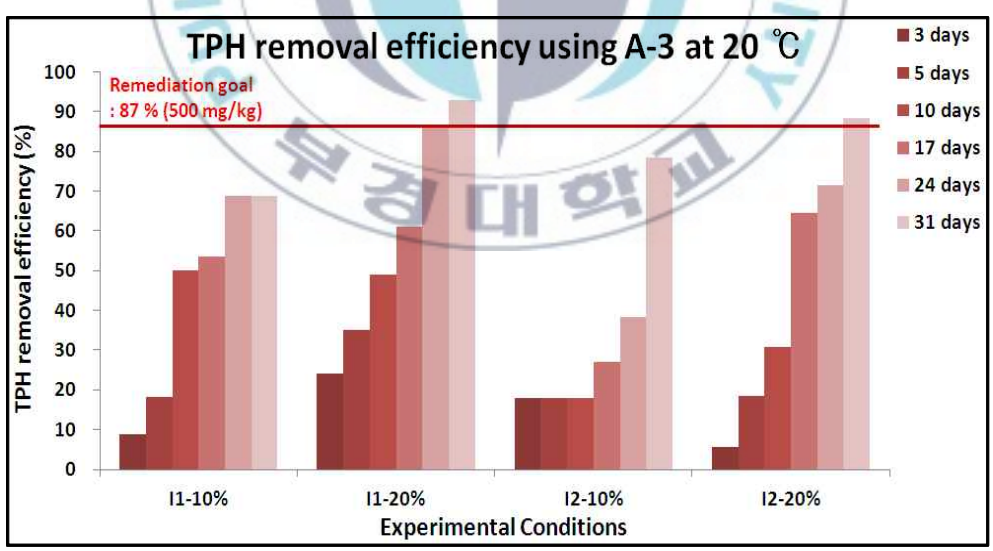
(B) The use of *Burkhoderia* sp. (A-2) at 30 ℃ with various microorganism concentrations and water contents



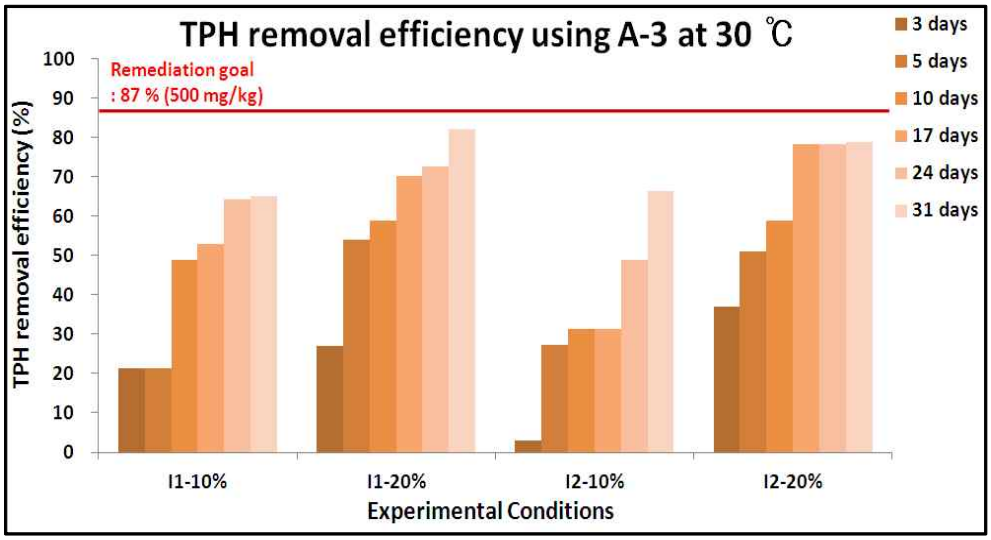
(C) The use of *Burkhoderia* sp. (A-2) at 40 ℃ with various microorganism concentrations and water contents

Fig. 18. Results of TPH removal efficiency using *Burkhoderia* sp. (A-2) at various conditions for landfarming.

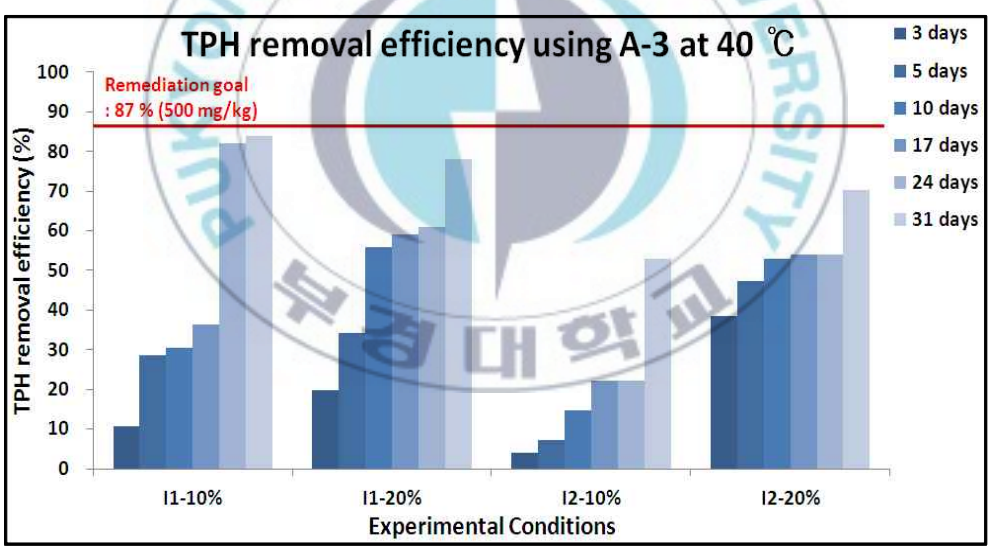
Results of experiments using *Cupriavidus* sp. (A-3) are shown in Fig. 19. TPH removal efficiency in landfarming increased with the addition of *Cupriavidus* sp. (A-3) solution but TPH removal efficiency of landfarming was outstanding at low temperature (20 ℃). In contrast with previous results using *Arthrobacter* sp. and *Burkhoderia* sp., TPH removal efficiency was not dependent on the amount of the microorganism culture solution added into the reactor. The maximum efficiency of *Cupriavidus* sp. (A-3) was 93 % by adding 0.1 ㎖ of cultured solution (I1) at 20 ℃ and with 20 % of water content by injection (Fig. 19 (A)).



(A) The use of *Cupriavidus* sp. (A-3) at 20 ℃ with various microorganism concentrations and water contents



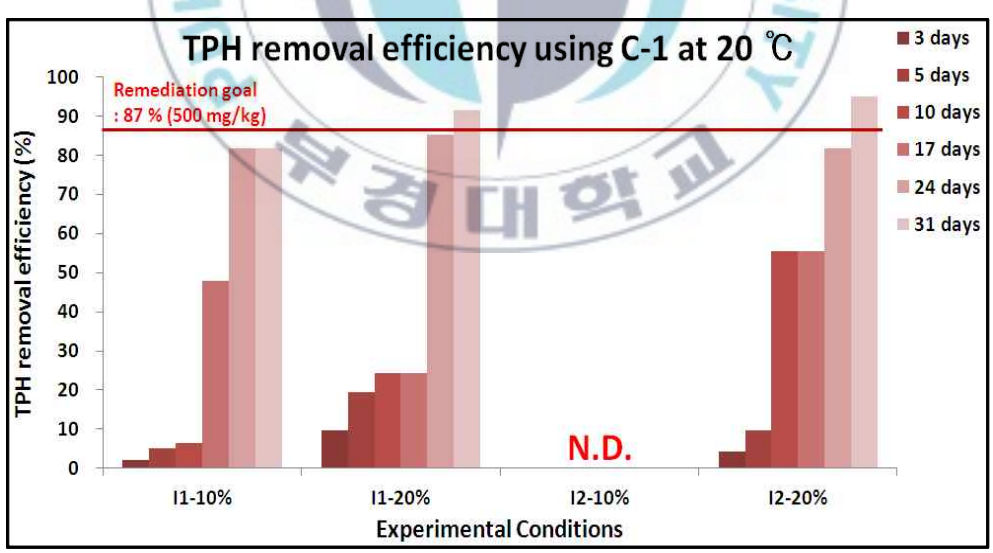
(B) The use of *Cupriavidus* sp. (A-3) at 30 ℃ with various microorganism concentrations and water contents



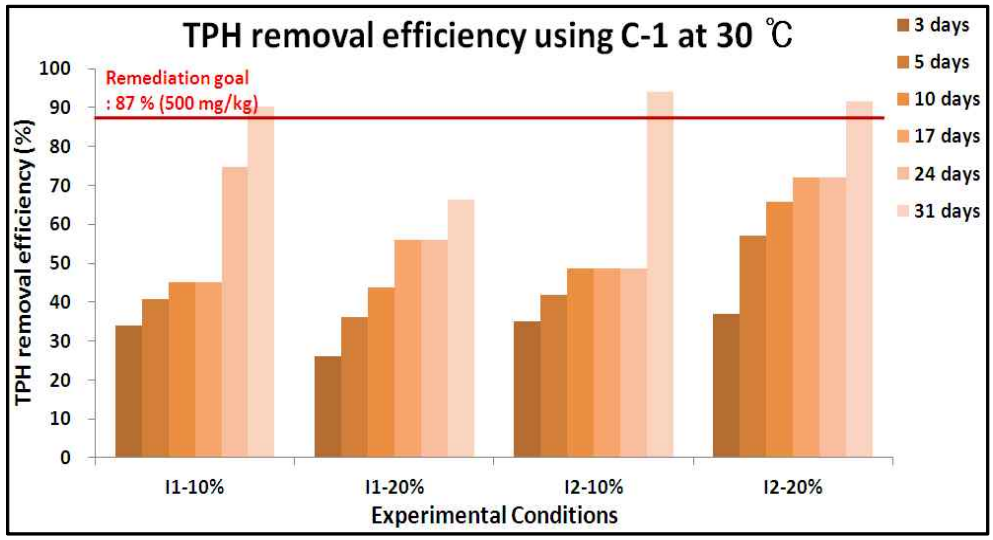
(C) The use of *Cupriavidus* sp. (A-3) at 40 ℃ with various microorganism concentrations and water contents

Fig. 19. Results of TPH removal efficiency using *Cupriavidus* sp. (A-3) at various conditions for landfarming.

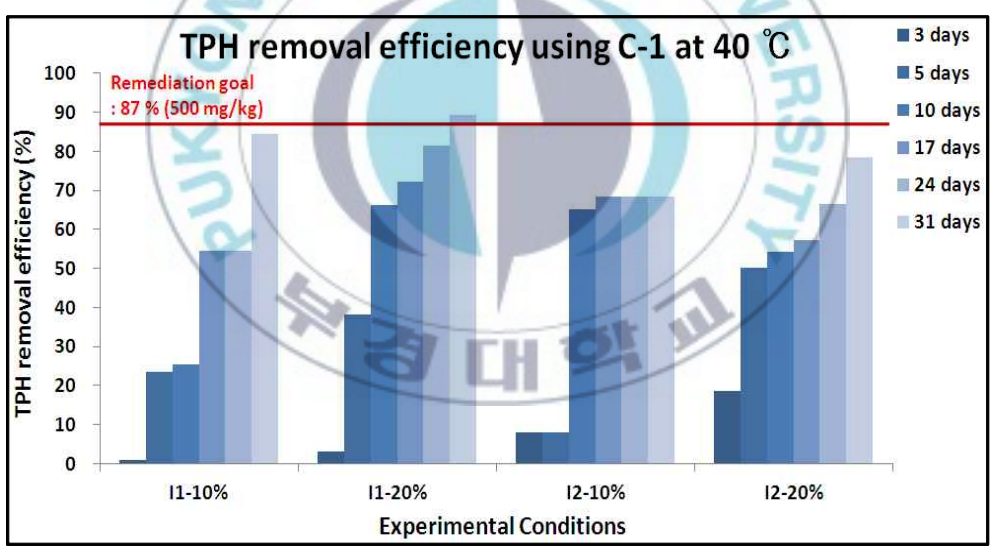
Fig. 20 shows the results of experiments using *Bacillus* sp. (C-1) on various conditions. TPH removal efficiency of landfarming using *Bacillus* sp. (C-1) for 31 days were higher than those using three other indigenous microorganisms at the same landfarming conditions. At 30 ℃, by adding of 0.2 mℓ of cultured solution per 100 g of soil, TPH removal efficiency reached to the remediation goal (87 %) for 31 days.



(A) The use of *Bacillus* sp. (C-1) at 20 ℃ with various microorganism concentrations and water contents



(B) The use of *Bacillus* sp. (C-1) at 30 ℃ with various microorganism concentrations and water contents



(C) The use of *Bacillus* sp. (C-1) at 40 ℃ with various microorganism concentrations and water contents

Fig. 20. Results of TPH removal efficiency using *Bacillus* sp. (C-1) at various conditions for landfarming.

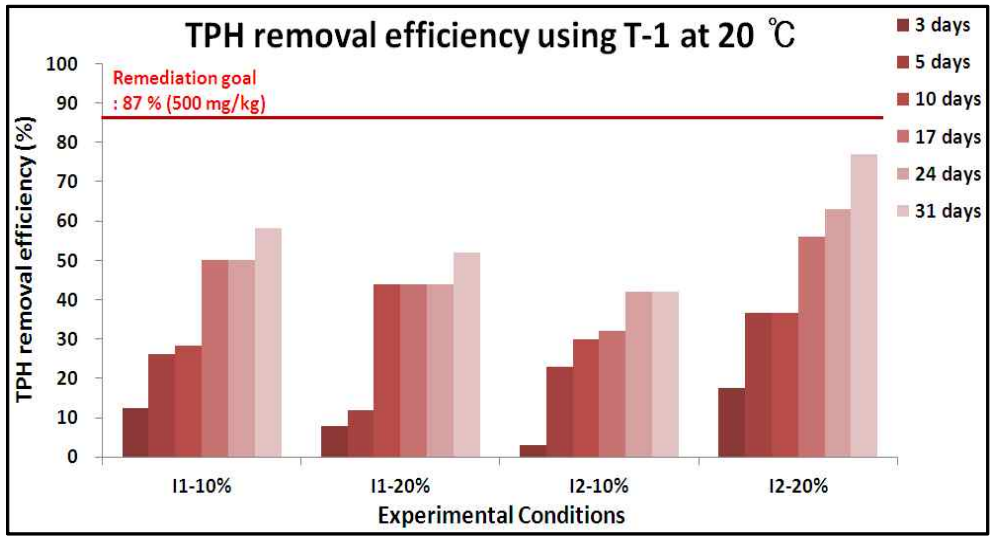
Results of batch experiment using mixture of 4 indigenous microorganisms (T-1 : A-1 + A-2 + A-3 + C-1) are shown in Fig. 21. TPH removal efficiency for T-1 increased with the increase water content but did not depend on the amount of cultured solution. At 30 ℃, the highest TPH removal efficiency (82 %) was occurred in landfarming with 20 % of water content (Fig. 21 (B)). These results suggested that TPH biodegradation efficiency by using a mixture of indigenous microorganisms was lower than that by using monospermous indigenous microorganism.

The degradation kinetics of indigenous microorganisms were studied (Table 13). For calculation of constants (k), coefficient of determination (r), half-life time (day) to reach the remediation goal, the first-order reaction equation (2) was used (Rittmann and McCarty, 1980).

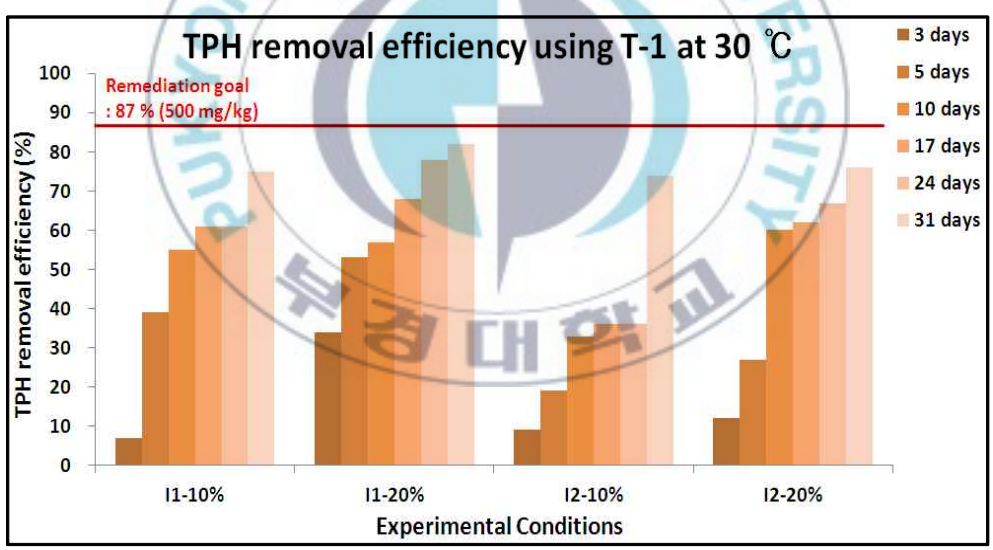
$$\frac{dC}{dt} = -k \times C \qquad (2)$$

where,
k = biological degradation constant
C = remaining TPH concentration at any time
t = time period

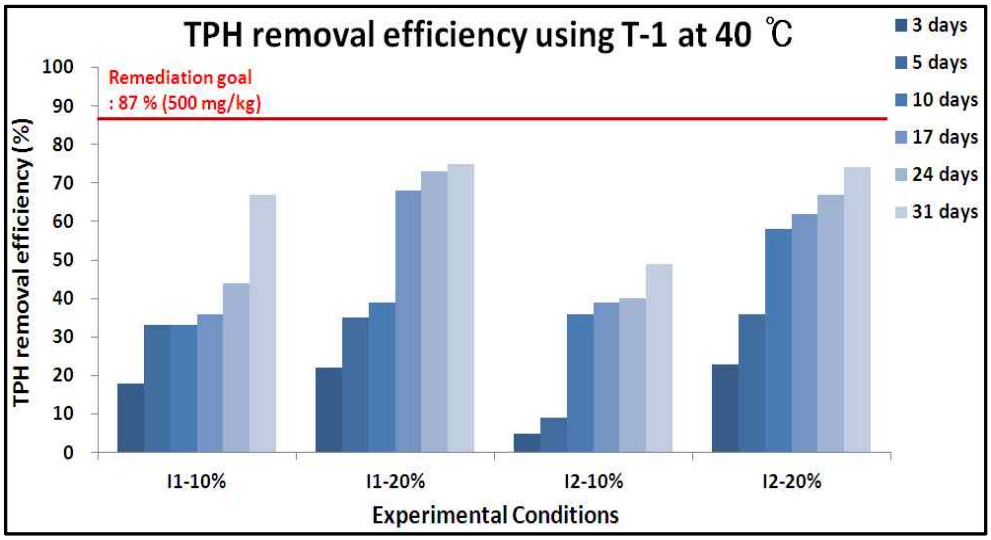
From the batch experiment using indigenous microorganisms, the TPH degradation constants of A-1, A-2, A-3, C-1 and T-1 were 0.08, 0.07, 0.08, 0.08 and 0.06, respectively. According to the calculation of the degradation rates for A-1, A-2, A-3 and C-1, the remediation goal would reach within 30 days.



(A) The use of mixed microorganisms at 20 ℃ with various microorganism concentrations and water contents



(B) The use of mixed microorganisms at 30 ℃ with various microorganism concentrations and water contents



(C) The use of mixed microorganisms at 40 ℃ with various microorganism concentrations and water contents

Fig. 21. Results of TPH removal efficiency using mixed indigenous microorganisms at various conditions for landfarming.

Table 13. First-order reaction rate constants, half-life and time reached the remediation goal of biodegradation using indigenous microorganisms by effective conditions

Microorganisms (Experiment condition)	k (day^{-1})	r	Half-life (day)	Day to reach the remediation goal
A-1 (I1 - 30 ℃ - 20 %)	0.08	0.87	8.57	25.13
A-2 (I2 - 40 ℃ - 20 %)	0.07	0.90	9.68	28.40
A-3 (I1 - 20 ℃ - 20 %)	0.08	0.96	8.74	25.64
C-1 (I2 - 20 ℃ - 20 %)	0.08	0.90	8.70	25.51
T-1 (I1 - 30 ℃ - 20 %)	0.06	0.84	11.11	32.58

TPH removal efficiencies of the landfarming using indigenous microorganisms were compared with those using commercialized adventive microorganisms. The average removal efficiency for each landfarming experiment is shown in Fig. 22. In case of soils without additional microorganisms, the average removal efficiency was 35 % by living indigenous microorganisms in contaminated soil for 20 days. When commercialized microorganisms were used, average TPH removal efficiencies using "Oilbug 1010" and "Penazyme" cultured solutions were respectively 66 %, and 57 % which were higher than that without additional microorganisms for 20 days. From results using an isolated indigenous microorganism (*Bacillus* sp.) from the contaminated soil, average TPH removal efficiency increased to 85 % after 31 days by landfarming process. Results suggested that TPH biodegradation efficiency by using isolated indigenous microorganism (*Bacillus* sp.) was higher than those by using only natural attenuation and commercialized microorganisms. Consequentially, it was demonstrated that the landfarming process using indigenous microorganisms from the contaminated soils can be an effective process to remove TPH from the diesel contaminated site.

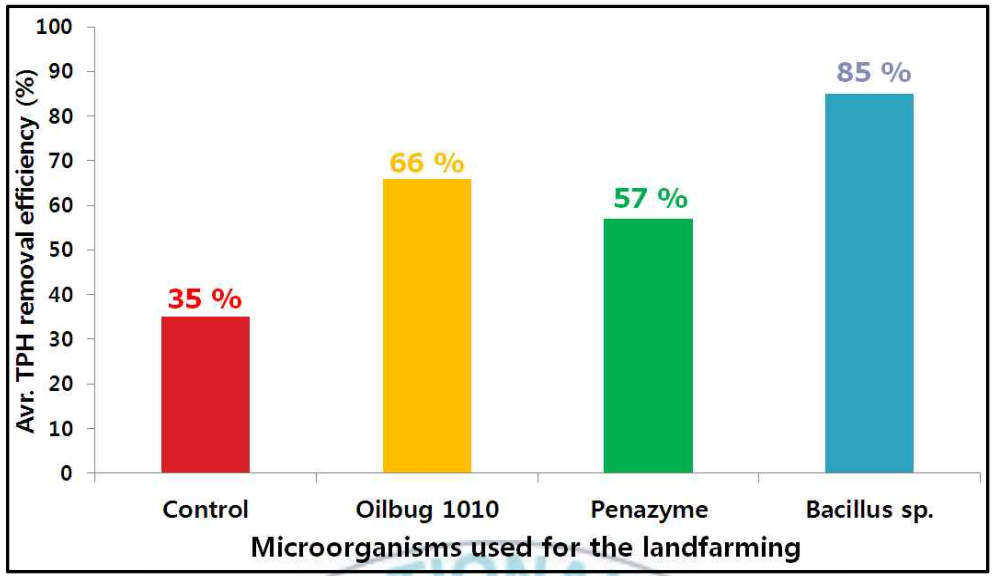


Fig. 22. The comparison of average TPH removal efficiency for landfarming in batch experiments.

CHAPTER VI. CONCLUSION

Landfarming batch experiments using indigenous microorganisms were performed to remediate TPH contaminated soils. To compare TPH removal efficiencies using indigenous microorganisms with those of commercialized microorganisms, batch experiments were repeated at various environmental conditions. Conclusions derived from this study were listed below.

1. The main pollutants of soil samples in A and C site were identified as diesel mostly and motor oil. The average of TPH concentrations in A and C site were determined as 2,881 ㎎/㎏ and 5,715 ㎎/㎏, respectively which exceeded Korea Soil Warning Limit (TPH : 500 ㎎/㎏).

2. Indigenous microorganisms were isolated from A and C site and were also identified by 16S rRNA gene sequence. They were found out as *Arthrobacter* sp., *Burkholderia* sp., *Cupriavidus* sp. and *Bacillus* sp., respectively. From results of phylogenetic trees analysis, all of isolated microorganisms were discovered as new species.

3. For the batch experiments without additional microorganisms (control soils), 44 %, 35 % and 26 % of initial TPH were removed from the soils having three different initial TPH concentrations, suggesting that it is very hard to remediate the soils having high TPH concentration (more than 1,700 mg/kg) by using only the natural attenuation.

4. For the batch experiments using commercialized microorganisms, the average TPH removal efficiencies using "Oilbug 1010 (BioSant Inc., Korea)" and "Penazyme (Life Science of Suwon Uni,, Korea)" were 66 % and 57 %, suggesting that TPH removal efficiency using "Oilbug 1010" cultured solution was more higher than that using "Penazyme" cultured solution.

5. For the batch experiments using "Oilbug 1010" microorganism cultivated solution by various environmental conditions, more than 86 % of TPH removal efficiency with 10 % water content and at 40 ℃ was removed. When "Oilbug 1010" cultured solution was used at 20 ℃ and with 10 % water content, TPH removal efficiency reached to remediation goal (83 %).

6. From the results of experiment using four indigenous microorganisms, the average TPH removal efficiencies were 76 %, 73 %, 77 % and 85 %, respectively. Generally, TPH removal efficiencies increased with the increase of water content at 30 ℃. By contrast, *Cupriavidus* sp. was more adapted to low temperature. Especially, TPH removal efficiency by using *Bacillus* sp. with 10 % water content and at 30 ℃ was more than 92 %.

7. From results of experiment using mixture of isolated indigenous microorganisms, the average TPH removal efficiency was 67 %, suggesting that TPH biodegradation efficiency by using the mixture of indigenous microorganisms is lower than that by using monospermous indigenous microorganism.

From this study, the landfarming process using isolated indigenous microorganisms from the contaminated soils can be an effective process to remediate the contaminated site.

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토착미생물을 이용한 토양경작법의 디젤 오염토양 내 TPH 제거

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요 약 문

외부에서 배양하여 상품화된 유류분해 미생물과 유류오염 토양 내 토착미생물을 이용하여 대표적인 생물학적 공법인 토양경작법의 유류제거 효율 실험을 실시하였다. 실험에 사용된 토양은 부산에 위치한 미군부대 내 디젤로 오염된 두 지역에서 채취하였다. 외부 유류분해 미생물 제재로는 B 사의 'O' 미생물과 S 대학 미생물학과에서 제공받은 'P' 미생물을 이용하였으며, 토착미생물은 오염토양에서 분리한 미생물 중 오일 배지에서 활성도가 좋았던 균주들을 배양하여 사용하였다. 선택된 균주는 16S rRNA sequencing 방법을 이용하여 동정한 결과 *Arthrobacter* sp. (A-1), *Burkholderia* sp. (A-2), *Cupriavidus* sp. (A-3), *Bacillus* sp. (C-1)으로 각각 나타났다. 플라스틱 페트리 디쉬 (지름 150 ㎜ x 높이 20 ㎜) 에 오염토양과 각각의 미생물 제재를 농도별로 주입하고 다양한 온도와 수분함량을 적용하여 실험을 실시하였다. 미생물을 추가하지 않은 Blank 실험 결과, 평균 35 % 의 TPH 제거효율을 나타내었다. 외부 유류분해 미생물을 추가한 배치 실험 결과, 평균 TPH 제거효율은 각각 66 % ('O' 미생물) 와 57 % ('P' 미생물) 로 나타났으며, 특히 'O' 미생물을 이용하였을 경우

Blank 실험 결과보다 약 2 배 정도 제거효율이 높았다. 오염토양 내 서식하는 토착미생물을 이용한 배치 실험 결과, 평균 TPH 제거 효율은 77 %로 가장 높게 나타났다. 실험 결과를 바탕으로 한 분해 반응상수 값을 이용하여 목표농도 (TPH : 500 ㎎/㎏) 에 도달하는 반응시간을 계산해 본 결과, 외부 유류분해 미생물 ('O' 미생물) 의 경우 평균 27.92 일이 걸리는 것으로 나타났으며 토착미생물인 *Bacillus* sp. 를 적용하는 경우 평균 25.51 일이 걸리는 것으로 나타났다. 외부 유류분해 미생물과 오염토양 내 토착미생물을 이용한 토양경작법의 배치실험 결과로부터 계산 되어진 각 미생물에 대한 분해 반응상수 값들을 비교해 보았을 때, 토착미생물인 *Bacillus* sp. 을 이용한 실험 결과가 가장 좋은 것으로 나타났으며, 이는 오염토양 내 서식하는 토착미생물이 유류오염 환경에 잘 적응하여 다른 외부 미생물을 이용하였을 때 보다 TPH 분해능이 높다는 것을 의미한다. 본 연구를 통하여 유류오염 토양에 토양경작법을 적용할 경우, 오염토양 내 토착미생물을 배양하여 재 주입 방법의 TPH 제거 효과가 매우 높다는 것을 입증하였다.

주제어 : TPH, 토양경작법, 토착미생물, 토양 복원, 디젤 오염