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Biodegradation of Phenanthrene by *Sphingomonas* sp. Strain KH3-2

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A phenanthrene-degrading bacterium was isolated from an oil-spilled intertidal sediment sample and identified as *Sphingomonas* sp. KH3-2. The strain degraded polycyclic aromatic compounds such as naphthalene, fluorene, biphenyl, and dibenzothiophene. When strain KH3-2 was cultured for 28 days at 25°C, a total of 500 ppm of phenanthrene was degraded with a concomitant production of biomass and Folin-Ciocalteau reactive aromatic intermediates. Analysis of intermediates during phenanthrene degradation using high-performance liquid chromatography and gas chromatography/mass spectrometry indicated that *Sphingomonas* sp. KH3-2 primarily degrades phenanthrene to 1-hydroxy-2-naphthoic acid (1H2NA) and further metabolizes 1H2NA through the degradation pathway of naphthalene.

Key words: Phenanthrene, *Sphingomonas*, PAH, biodegradation

Polycyclic aromatic hydrocarbons (PAHs) are ubiquitous environmental pollutants which have been isolated from air, water, soil, and marine environments (2). PAHs are components of petroleum fuels and formed during the incomplete combustion of most organic materials (3). Contaminations of the environments with PAHs and their possible exposure to human are considered to be hazardous because of their carcinogenic, mutagenic, toxic, and genotoxic properties (18). Therefore, removal of PAHs from contaminated environments is of great concern. Many microorganisms play important roles in transformation and degradation of PAHs in the environment. Therefore, informations about the metabolism of PAHs by microorganisms are useful for understanding the fate of PAHs in natural environments.

Tausson (38) isolated three strains of bacteria that attacked phenanthrene from oil-soaked soil of the Baku oil fields. These microorganisms were identified as *Bacillus phenanthrenicus bakiensis, Bacillus phenanthrenicus guricus* and *Bacterium phenanthrenicum*, and also metabolized a variety of other PAHs. Gray and Thornton (14) demonstrated later that bacteria with these potentialities were of ubiquitous occurrence in soil and marine sediments. Rogoff and Wender (31) and Colla *et al.* (6) initiated investigations towards understanding the biochemistry of these oxidative dissimilations. Pathways for the transformation of several lower molecular weight PAHs by bacteria have been proposed (5, 13, 15) and experience with land-farming of refinery wastes suggests that higher molecular weight PAHs are also biodegradable (1, 29, 35, 39). However, there is much more to be known about the physiology of PAH degraders, the kinetics of substrate and intermediate metabolism, and the strategies used by bacteria growing on such recalcitrant compounds of such low solubility.

Phenanthrene is a low-molecular weight PAH having three aromatic rings and have been used as a good model substrate in studies on biodegradation of PAHs. Two metabolic pathways have been described for the bacterial metabolism of phenanthrene (8, 22). In both pathways, phenanthrene is oxidized to 1-hydroxy-2-naphthoic acid (1H2NA) by various bacterial strains such as *Beijerinckia* B-836, *Pseudomonas putida* 119 (19), *Pseudomonas* sp. NCIB 9816, a *Nocardia* sp. (30), and other *Pseudomonas* sp. (8). 1H2NA is further metabolized through salicylate and catechol by the enzymes involved in the naphthalene pathway. Kiyohara and Nagao (22) showed that an *Aeromonas* sp. as well as various pseudomonads, vibrios and unidentified bacteria metabolized phenanthrene by an alternate pathway. In this pathway, 1H2NA was transformed to protocatechuic acid via *o*-phthalic acid (9, 10, 11, 12).

A phenanthrene-degrading bacteria, *Sphingomonas* sp. strain KH3-2, was isolated from an oil-polluted intertidal flat in this study. *Sphingomonas* is a newly classified genus, which has broad catabolic capabilities

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and therefore high potential for bioremediation and waste treatment. To investigate such potential of the KH3-2 strain, substrate ranges and optimum conditions for growth were studied and the degradation pathway was investigated through the identification of oxidation and ring fission products.

Materials and Methods

Chemicals and media

Naphthalene, phenanthrene, anthracene, pyrene, benzo(a)pyrene, chrysene, fluorene, acenaphthene, biphenyl, 1-hydroxy-2-naphthoic acid (1H2NA), phthalate, salicylate, salicylaldehyde, protocatechuate, catechol were obtained from Sigma Chemical Company (Louis, USA). All other chemicals and solvents for high-performance liquid chromatography (HPLC) were obtained from E. Merk AG (Darmastadt, Germany).

Minimal salts solution (MM2) were prepared as described by Kiyohara (24) and MM2 agar medium contained 1.5% Bacto agar (Difco). ZoBell 2216e medium was used as a complete medium (Bacto peptone 5 g, Bacto yeast extract 1 g, $FePO_4$ 10 mg , aged sea water [ASW] 750 ml, distilled water [DW] 250 ml, pH 7.0). Solid ZoBell 2216e medium contained 1.5% agar.

Enrichment and isolation of the phenanthrene-degrading bacteria

Wet sand and mud samples were collected from an oil-spilled intertidal flat in Kohyun, Goje Island, Korea. About 5 cm^3 of the sample was transferred into Erlenmeyer flasks (250 ml) containing 50 ml of MM2 medium with phenanthrene crystals (0.5%, w/v). Flasks were shaken (120 rpm) in the dark at 25°C. After 2 weeks of incubation, 1 ml of the culture was transferred into 10 ml of MM2 medium and incubated under the same conditions. Sequential transfers were performed every 2 weeks. After four transfers, a total of 14 bacterial strains were isolated on ZoBell 2216e agar medium. Each strain was inoculated in 10 ml of MM2 medium with 500 ppm of phenanthrene and incubated for 10 days at 25°C to test its ability to degrade phenanthrene. The culture media were extracted three times with 2 volumes of ethyl acetate after acidification with 6N HCl to make pH 2.5. Extracts were filtered through anhydrous Na_2SO_4 and ethyl acetate was evaporated using a rotary evaporator (Eyela, Tokyo, Japan). The remaining phenanthrene was dissolved in *n*-hexane and assayed by gas chromatograph (GC) 5890 series II (Hewlett Packard, Avondale, PA, USA) equipped with a flame ionization detector (FID) and a fused silica capillary column (15 m×0.32 mm with film thickness 0.17 mm). The oven, detector, and injector temperatures were 160°C, 280°C, and 260°C, respectively. On the basis of the GC/FID analysis, the KH3-2 strain was selected among 14 bacterial strains, and its ability to utilize phenanthrene was further tested as described by Kiyohara *et al.* (24).

Identification and characterization of the KH3-2 strain

Biochemical tests for identification of the KH3-2 strain were carried out by the methods proposed by the National Collection of Industrial Bacteria and Marine Bacteria (Aberdeen, Scotland). Whole cell fatty acids of bacteria were analysed as methyl esters by GC using Microbial Identification System (MIDI Inc., Newark, Del).

To determine the best growth conditions of the KH3-2 strain, the effects of temperature, pH, and concentration of NaCl were tested in 50 ml of ZoBell 2216e medium.

Degradation of naphthalene, phenanthrene, anthracene, pyrene, benzo(a)pyrene, chrysene, fluorene, acenaphthene, biphenyl, triphenylene, fluoranthene, dibenzothiophene, and dibenzofuran was examined as described by Kiyohara *et al.* (23).

Degradation of phenanthrene by the KH3-2 strain

Growth at the expense of phenanthrene was established by demonstrating the increase in bacterial protein, the decrease in concentration of phenanthrene, and the formation of metabolites.

After KH3-2 strain was grown in 50 ml of ZoBell 2216e medium for 2 days, the cells were harvested (12,000 × g, 15 min, 4°C) and washed twice with 50 mM phosphate buffer (pH 7.0). Cells suspended in MM2 medium was transferred to 10 ml of MM2 medium containing phenanthrene (300 ppm) to obtain a final absorbance (A_{660}) of 0.05. The culture was incubated for 10 days at 25°C in a rotary shaker (150 rpm).

Phenanthrene disappearance was determined by extracting duplicate of the cultures with ethyl acetate and assaying by the use of GC/FID as described previously. Protein concentration was measured by the use of the Bio-Rad protein assay kit II. Culture supernatants were analyzed for intermediates during phenanthrene degradation by a modified method of the Folin-Ciocalteau reaction (4). Resorcinol was used as a standard, and all values were expressed in micrograms of resorcinol equivalents (RE) per milliliter.

Identification of phenanthrene-degrading metabolites

For the determination of the phenanthrene-degradation pathway, the KH3-2 strain, in 7 L of MM2 with 0.1% phenanthrene, was incubated for 3 days at 30°C.

Metabolites and residual phenanthrene were extracted with ethyl acetate as described previously and the residue was dissolved in a small amount of methanol.

Metabolites were separated on Hewlett-Packard 1050 series II HPLC apparatus fitted with a 5 μm C_{18}-Chromospher column (15 cm × 4.6 mm, Supelco, Inc., Belllefonte, Pa, USA). Methanol and water were used as the mobile phases and the pH of the water was adjusted to 2.75 with H_3PO_4 solution. Methanol concentration was increased from 0% to 100% during the first 20 min, and maintained at 100% for 10 min. The flow rate was 1 ml/min and metabolites were detected using a UV-detector (Hewlett-Packard model 1050) at 254 nm. Metabolites were identified by comparing their retention times and absorption spectra with those of corresponding authentic standards.

For gas chromatography/mass spectrometry (GC/MS) analysis, the residue after extraction was dissolved in 250 μl acetonitrile. One-hundred μl of BSTFA (bis-trimethylsilyltrifluoroacetamide, a trimethylsilane reagent; Sigma) was added, and the reaction was carried out at 70°C for 1 h. The gas chromatograph was linearly ramped at a rate of 4°C/min from 70 to 300°C.

Results

Isolation and characterization of phenanthrene-degrading bacteria

Subculturing the samples obtained from an oil-spilled intertidal flat in MM2 medium with phenanthrene resulted in a microbial consortium enriched with phenanthrene degraders. From the enriched consortium, fourteen colonies were isolated, and an isolate, KH3-2, was selected as the strain with the highest degrading activity of phenanthrene. The KH3-2 strain produced colonies with surrounding clear zones on MM2 agar plates coated with phenanthrene after 1 week at 25°C, and degraded approximately 90% of the added phenanthrene (100 ppm) in MM2 medium in 10 days.

The strain KH3-2 formed pale yellow-colored colonies with diameters of about 1 mm on ZoBell 2216e agar medium after 2 days incubation at 25°C. The KH3-2 strain was found to be gram-negative, obligatory aerobic, non-spore forming, and motile rods. Further physiological and biochemical characteristics are shown in Table 1. According to these characteristics, the KH 3-2 strain could be classified as *Sphingomonas* sp.. To confirm the identification, whole cell fatty acid profile was analysed. Table 2 shows the GC profile obtained from fatty acid methyl ester analysis of the total lipids of the strain. The KH3-2 strain contained *cis*-octadecenoic acid as the dominant fatty acid, and the other significant fatty acids were 2-hydroxymyristic acid, *cis*-9-hexadecenoic acid, and hexadecanoic acid, which were similar to the report by Yabuuchi *et al.* (41). On the basis of these data, the KH3-2 strain was classified as *Sphingomonas* sp.

Table 1. Morphological and physiological characteristics of *Sphingomonas* sp. strain KH3-2

Characteristics	Results
Gram stain	-
Shape	rod
Motility	+
Spore formation	-
Oxidase	+
Catalase	+
O/F test(glucose)	-
Nitrate reduction	-
Indole production	-
Arginine dihydrolase	-
Urease	-
Aesculin hydrolysis	+
Gelatin hydrolysis	-
β-galactosidase	+
Phenylalanine deaminase	-
Assimilation	
glucose	+
arabinose	-
mannose	-
mannitol	-
N-acetylglucosamine	-
maltose	+
gluconate	(+)
caprate	(+)
adipate	-
malate	-
citrate	-
phenylacetate	-
Acid production	
glucose	-
glycerol	alkaline
rhamnose	+
salicin	alkaline
DNase+2.5% NaCl	+ (restricted)
Starch+2.5% NaCl	+
Tween 80+2.5% NaCl	-
Gelatin+2.5% NaCl	+
Growth at 47°C	-

Growth characteristics

The *Sphingomonas* sp. strain KH3-2 exhibited growth at temperatures from 15°C to 40°C with optimum growth at 30°C. The strain, however, did not

Table 2. Fatty acid content of *Sphingomonas* sp. strain KH3-2

Fatty acids	16:0	*trans*-16:1	*cis*-16:1	*cis*-18:1	*trans*-18:1	17:1	14:0-2OH	15:0-2OH
Content (%)	13.36	1.555	9.505	58.585	1.375	3.01	11.72	0.575

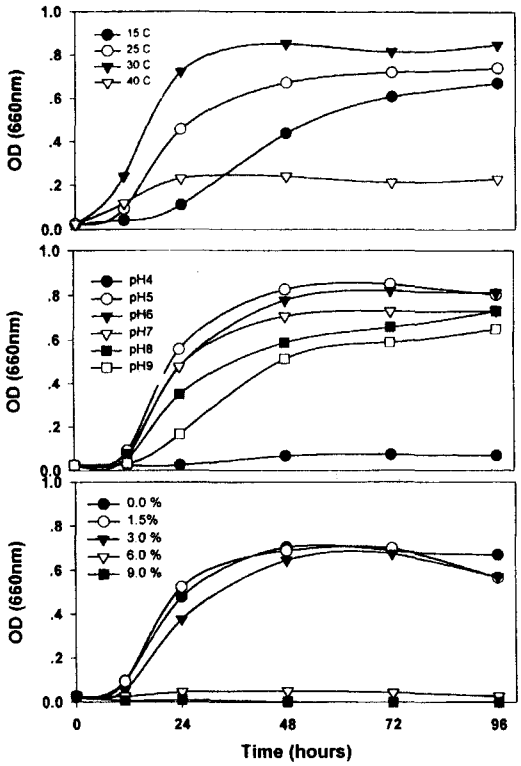

Fig. 1. Effect of temperature, pH, and NaCl concentration on the cell growth of *Sphingomonas* sp. strain KH3-2 in ZoBell 2216e medium.

show significant growth above 40°C. The strain grew on ZoBell medium at a initial pH range of 5 to 9 with an optimum at pH 5, but did not show significant growth at pH 4. The strain showed growth in the presence of 0 - 3% NaCl with an optimum of 0% to 1.5% NaCl concentration (Fig. 1).

The KH3-2 strain formed colonies with distinct clear zones on MM2 agar media sprayed with an etheral solution of phenanthrene, dibenzothiophene, or fluorene (Table 3). Also, the strain degraded over 70% of each substrate when cultured in MM2 medium with 100 ppm of phenanthrene, naphthalene or biphenyl at 25°C for 10 days.

Table 3. Degradation of aromatic hydrocarbons by *Sphingomonas* sp. strain KH3-2

PAHs	Degradation*	PAHs	Degradation*
Phenanthrene	+	Pyrene	-
Naphthalene	+	Benzo(a)pyrene	-
Fluorene	+	Chrysene	-
Biphenyl	+	Fluoranthene	-
Anthracene	-	Dibenzofuran	-
Acenaphthene	-	Dibenzothiophene	+

* Degradation of phenanthrene, naphthalene, and biphenyl was confirmed through GC/FID analysis, and degradation of fluorene and dibenzothiophene was confirmed by the presence of clear zones around colonies on MM2 medium after incubation for a week at 25°C.

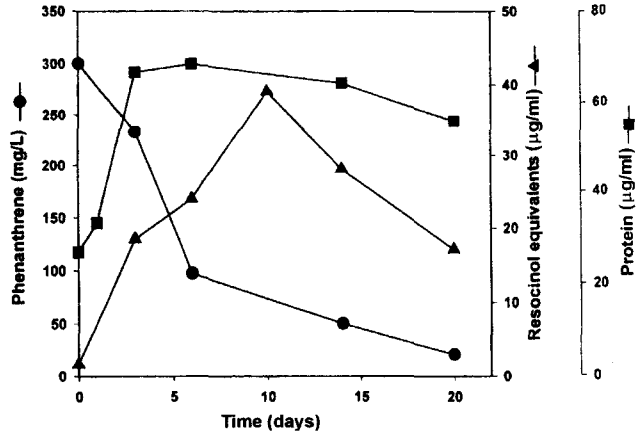

Fig. 2. Time course for phenanthrene degradation by Sphingomonas sp. strain KH3-2 grown aerobically at 25°C showing the disappearance of phenanthrene and the production of protein and Folin-Ciocalteau reactive products (expressed in resorcinol equivalents).

Degradation of phenanthrene by the KH3-2 strain

Utilization of phenanthrene as a sole source of carbon and energy by the KH3-2 strain was demonstrated by its removal from MM2 medium supplemented with phenanthrene, with a corresponding increase in bacterial biomass and appearance of metabolites. The concentration of phenanthrene decreased within 20 days from 300 ppm to a level not detected by GC/FID analysis. Bacterial biomass increased from 30 µg/ml to 70 µg/ml after 3 days of incubation. Folin-Ciocalteau reactive metabolites began to increase at the onset of phenanthrene degradation, but leveled off at a concentration of about 40 µg of resorcinol equivalents ml^{-1}. These metabolites were slowly removed from the medium over the latter half of the incubation (Fig. 2).

Concurrent HPLC analysis of KH3-2 culture extracts confirmed that only low concentrations of aromatic intermediates were formed during degradation of phenanthrene. HPLC spectra of KH3-2 culture extracts at 0, 1, 3, and 10 days are shown in Fig. 3. At the onset of phenanthrene degradation (day 1, Fig. 3), an early metabolite (Fig. 3, peak I) was produced and its retention time corresponded to that of 1H2NA. Until the 3rd day, peak I had increased, and two more polar products (peak II and III) started to appear. Peak I had diminished progressively and more intermediates appeared.

Identification of phenanthrene-degradation metabolites

To identify the forementioned metabolites, authentic intermediates were added to the culture extracts. Comparing the retention times, retention times of the

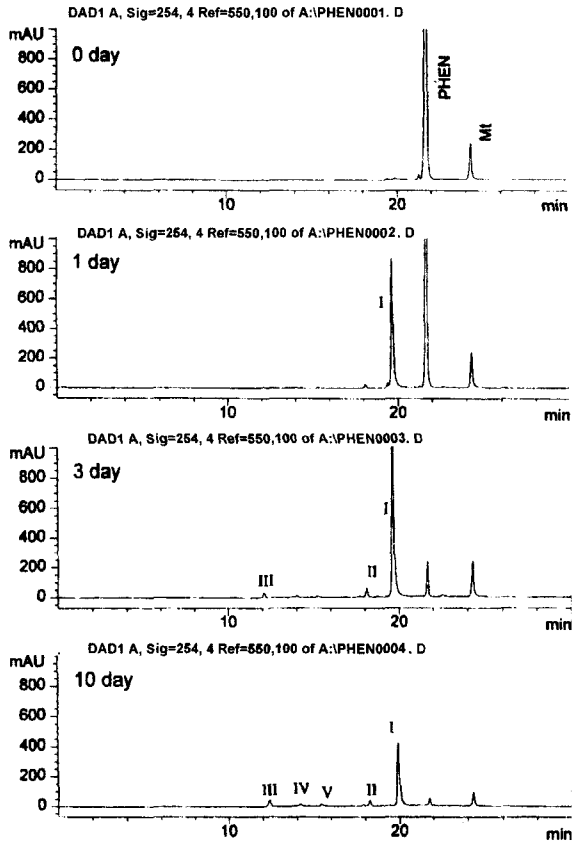

Fig. 3. High-performance liquid chromatograms of culture supernatants of *Sphingomonas* sp. strain KH3-2 at 0, 1, 3, and 10 days. Chromatograms show the disappearance of phenanthrene and the production of phenanthrene-degrading intermediates.

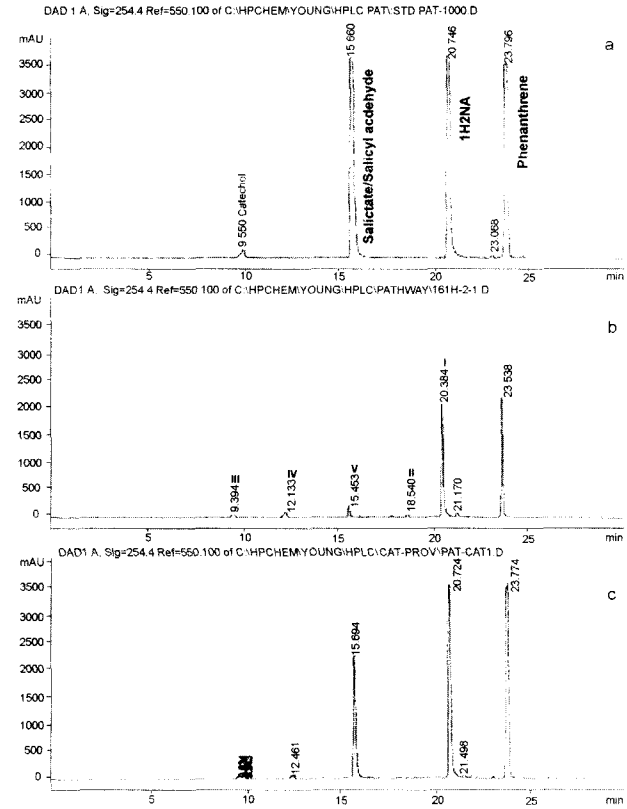

Fig. 4. High-performance liquid chromatograms of authentic intermediates (a), metabolites (b), and metabolites with authentic intermediates (c).

peaks corresponding to 1H2NA and salicylate/salicylaldehyde were overlapped with those of authentic standards (Fig. 4). Peak I was identified as 1H2NA. Salicylate and salicylaldehyde appeared to have the same retention time (peak V). Peak II, III, and IV could not be identified. The peak corresponding to the retention time of catechol was not detected by HPLC analysis. Also, the peaks corresponding to 1H2NA and salicylate/salicylaldehyde in HPLC analysis were analyzed by gGC/MS and identified as 1H2A and sa- licylate, respectively (data not shown).

Discussion

The genus *Sphingomonas* was proposed by Yabuuchi *et al.* (41) for gram-negative, aerobic, rod-shaped organisms which grow as yellow to whitish brown colonies. The members of the genus *Sphingomonas* contain sphingolipids and do not contain lipopolysaccharide; ubiquinone 10 is the main respiratory quinone of these organisms. The major cellular fatty acids are octadecanoate, 2-hydroxymyristate, *cis*-9-hexadecenoate, and hexadecanoate. Phenanthrene-degrading bacterium isolated in this study has these characteristics, and is identified as *Sphingomonas* sp. KH3-2.

Sphingomonas sp. were previously classified as *Pseudomonas*, *Flavobacterium* (28) or *Beijerinckia* (20) sp., however, these bacteria have been reclassified as *Sphingomonas* sp. *Sphingomonas* sp. are ubiquitous in soil, water, and sediments and known to degrade a variety of aromatic hydrocarbons such as dibenzo-*p*-dioxin, dibenzofuran (40), chlorinated biphenyl (37), naphthalenesulfonic acids (26), pentachlorophenol (27), toluene and naphthalene (10), biphenyl and *m*-xylene (21), and fluoranthene, pyrene, benz(a)anthracene, chrysene, benzo(a)pyrene, benzo(b)fluoranthene, dibenz(a,h) anthracene, and 1-nitropyrene (42).

Sphingomonas sp. strain KH3-2 can utilize phenanthrene as a sole source of carbon and energy. The ability was demonstrated by significant removal of phenanthrene and increase in biomass production (Fig. 2). Since many of the known phenanthrene metabolites are hydroxylated aromatic compounds, the culture supernatant fluids were analyzed for the presence of phenolic compounds by the use of the

Folin-Ciocalteau reaction. As phenanthrene (225 ppm) was degraded, intermediates expressed in resorcinol equivalents gradually increased up to 40 μg/ml, and then decreased. Resorcinol equivalents values reflect the presence of 1H2NA and other phenolic intermediates during phenanthrene degradation (4, 16). In HPLC analysis, various intermediates progressively appeared and disappeared (Fig. 3). 1H2NA and salicylate were detected in HPLC and GC/MS analysis as intermediates. These results indicate that *Sphingomonas* sp. KH3-2 primarily degrades phenanthrene to 1H2NA and further metabolizes 1H2NA through the degradation pathway of naphthalene as proposed by Evans *et al.* (8).

The end ring cleavage reactions in bacterial metabolism of naphthalene, phenanthrene, and anthracene produces pyruvate and corresponding ring cleavage products such as salicylate, 1H2NA, and 2-hydroxy-3-naphthoic acid (2H3NA), respectively. Accumulation of salicylate, 1H2NA, and 2H3NA in the culture medium during metabolism is a well known phenomenon (7, 8, 12, 16, 22, 24, 25, 31, 32). From the extensive work on naphthalene metabolism, it was found that cells utilized salicylate later as the more readily utilizable substrate (pyruvate) was exhausted (25, 34, 36). Guerin and Jones (16) approached a similar way to explain the accumulation of 1H2NA during phenanthrene degradation and degradation of 1H2NA only on depletion of phenanthrene. The results shown in Fig. 2 and Fig. 3 may be explained in the same way. The Folin-Ciocalteau reactive products were gradually accumulated until phenanthrene was almost degraded, and then started to decrease (Fig. 2). In HPLC analysis, 1H2NA (peak I), which appeared right after the degradation of phenanthrene, was accumulated in the culture medium, and started to decrease when phenanthrene was almost degraded (Fig. 3). This phenomenon may be explained by the fact that pyruvate, which was the other initial ring cleavage product of phenanthrene, served as a more favorable substrate to *Sphingomonas* sp. KH3-2 than 1H2NA.

Sphingomonas sp. KH3-2 can also utilize a variety of aromatic compounds such as naphthalene, biphenyl, fluorene, and dibenzothiophene as a carbon and energy source (Table 3). The KH3-2 strain was found to have a relatively high ability to degrade phenanthrene compared to other reported phenanthrene degraders. Seo *et al* (33) reported *Micrococcus, Arthrobacter* and *Aeromonas* degraded 50% of 1,000 ppm phenanthrene after 5 weeks. Guerin and Jones (17) reported *Mycobacterium* sp. BG1 degraded 90% of 200 ppm phenanthrene suspended in Tween 80. However, *Sphingomonas* sp. KH3-2 degraded 50% of 1,000 ppm phenanthrene after 2 weeks as shown in Fig. 5. These results indicate that *Sphingomonas* sp. KH3-2 might possess a high potential to be applied in bioremediation of environments polluted by various aromatic compounds at high concentrations.

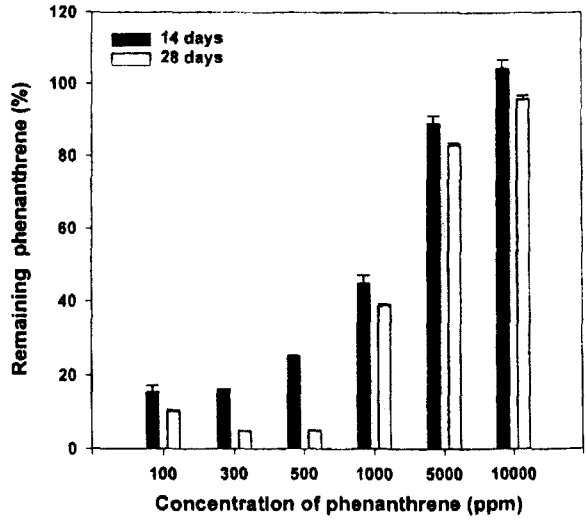

Fig. 5. Relative degradation of variable concentration of phenanthrene by *Sphingomonas* sp. strain KH3-2 grown in a MM2 at 25°C.

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