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Biodegradation of Heavy C Oil by *Alcanivorax* sp. a1 Strain Isolated from Recovered Bunker Oil Spilt in the *"Solar I"* Accident

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Key words: *Alcanivorax,* alkanes, polycyclic aromatic hydrocarbons (PAHs), alkylated PAHs, biodegradation

Abstract

The genus *Alcanivorax* is known as a petroleum hydrocarbon degrader and primarily contributes to bioremediation process of hydrocarbon pollution in petroleum-contaminated marine environments. In the present study, biodegradability of heavy C oil constituents by *Alcanivorax* sp. a1 strain, isolated from bunker oil recovered from the *"Solar1"* oil spill accident offshore the Guimaras Island, the Philippines in 2006, was investigated. The isolate showed remarkable growth in UPFe medium supplemented with heavy C oil. Reduction of the hydrocarbon constituents including *n*-alkanes, polycyclic aromatic hydrocarbons (PAHs), and alkylated PAHs were observed in the culture, suggesting their biodegradation by the isolate a1.

Hydrocarbons are major components in crude oils, accounting for 50-98% of the total components.[1)] The hydrocarbons include saturated hydrocarbons such as *n*-alkanes, *iso*-alkanes and *cyclo*-alkanes, which are main constituents in crude oils, and aromatic hydrocarbons such as monocyclic hydrocarbons and polycyclic aromatic hydrocarbons (PAHs), most of which are potentially mutagenic or carcinogenic. Hydrocarbons are released into marine environments via natural seepage of petroleum, accidental or deliberate release, and biological processes. Among them, although it accounts for only one eighth of the oil discharged into aquatic environments,[1)] tanker oil spill accidents have caused severe damage to the marine coastal environments, including mass mortality of marine animals and reduction in population sizes and community diversity of intertidal or subtidal organisms. The introduced petroleum is removed from the coastal areas through physical and chemical processes including washing-out by waves and tides, evaporation, and dissolution as well as human actions like booming, skimming, wiping, and application of dispersants or solidifiers.[1)]

In addition, diverse microorganisms are known to play a significant role to remove petroleum, particularly non-volatile components, from polluted marine environments;[2)] hydrocarbonoclastic bacteria, which degrade petroleum hydrocarbon constituents including alkanes and PAHs, are widespread in marine environments and successively dominate in oil-contaminated intertidal area.[3)] Among them, limited numbers of genera *Alcanivorax, Marinobacter, Thallassolituus, Cycloclasticus* and *Oleispira,* which are obligately hydrocarbonoclastic, are most prevalent.[4)]

The genus *Alcanivorax* is known as a petroleum hydrocarbon degrader; this group utilizes alkanes, one of the petroleum constituents, as sole carbon and energy sources.[5)] *Alcanivorax* becomes dominant in petroleum-contaminated marine environments and primarily contributes to bioremediation processes of hydrocarbon pollution,[6)] although it inhabits at very low or undetectable levels before pollution events.[4)] So far, five species, *A. borkumensis, A. venustensis, A. jadensis, A.*

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dieselolei and *A. balearicus* have been isolated, and their biodegradability of alkanes as well as other carbon sources is documented.[5,7-9)]

In the present study, *Alcanivorax* sp. a1, isolated from recovered bunker oil spilt in the *"Solar1"* accident offshore the Guimaras Island, the Philippines in 2006, was cultivated with heavy C oil, and its biodegradability of main oil constituents including *n*-alkanes, PAHs, and alkylated PAHs was monitored as well as the bacterial growth.

Materials and methods

Bacterial cultivation

Alcanivorax sp. a1 was inoculated into separate 100 mL Erlenmeyer flasks containing 50 mL of UPFe medium (37.5 mg of urea, 0.58 mL of 100 mM KPO_4 buffer (pH 7.5), and 1 mg of iron(III) ammonium citrate in 50 mL of seawater) with 5 μL of heavy C oil. The initial bacterial number of each flask was adjusted to OD_{600} = 0.003. The flasks were incubated at 30˚C with orbital shaking at 150 rpm. Three of the 15 flasks were subjected to hydrocarbon extraction in triplicate every week until the day 28. Bacterial cell numbers in the flasks were estimated by the most probable number method with modified Zobell seawater medium.[10)]

Extraction of hydrocarbon substances

Extraction and quantification of hydrocarbon substances were carried out based on the method described previously.[11)] Petroleum hydrocarbons were extracted from 40 mL of the each culture flask with 10 mL of *n*-hexane:dichloromethane (3:1). Excess water was removed by anhydrous sodium sulfate. The extracts were concentrated with nitrogen gas flow to 1 mL and transferred to a column packed with 3% silica gel suspended in water. The flow-through fractions obtained by washing the column with *n*-hexane and the adsorbed fraction eluted by 3:97 mixture of acetone:*n*-hexane were concentrated with nitrogen gas flow to 1 mL. Both the fractions were mixed and subjected to gas chromatography-mass spectroscopy (GC-MS).

Analysis of hydrocarbon substances by gass chromatography-mass spectroscopy

The extracted substances were quantified in a gas chromatograph-mass spectrometer (Agilent, Palo Alto, CA) with 0.25 mm (ID) x 30 m (length) x 0.25 μm (film thickness) DB-5 5% phenylmethylsiloxane capillary column. The temperature was programmed as follows: 1 min at 60˚C, ramp to 190˚C at 15˚C min^{-1}, ramp to 220˚C at 2˚C min^{-1}, and ramp to 300˚C at 5˚C min^{-1}. The mass selective detector was operated in the SIM mode.

In order to analyze hydrocarbon constituents, the heavy C oil was dissolved into *n*-hexane at a final concentration of 1.0 μg mL^{-1} and subjected to GC-MS.

Results and Discussion

Growth of *Alcanivorax* sp. a1

For bacterial cultivation, UPFe medium with 0.1 mg L^{-1} of heavy C oil was used; this medium contains petroleum hydrocarbons as the carbon sources. Urea was also added as a supplementary organic nutrient to expect the enhancement of petroleum hydrocarbon biodegradation.

The isolate showed remarkable growth in the first 7 days of cultivation to reach maximum at 2.52 x 10^8 cells mL^{-1} (Fig. 1), suggesting its capability to metabolize petroleum hydrocarbons such as alkanes and PAHs as well as urea as carbon sources. The cell number decreased subsequently, probably due to exhaustion of the carbon sources available to the isolate.

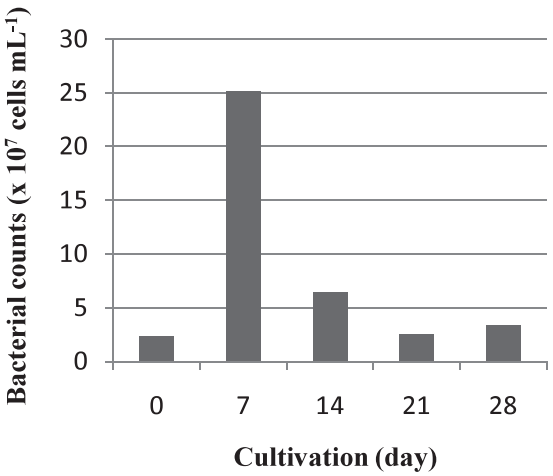

Fig. 1. Bacterial counts of *Alcanivorax* sp. a1 cultivated in UPFe medium supplemented with 0.1 mg L^{-1} heavy C oil.

Bioegradation of *n*-alkanes by *Alkanivorax* sp. a1

Alcanivorax sp. a1 showed high biodegradability of *n*-alkanes (Fig. 2A); total n-alkanes in the culture decreased to approximately 10% of the initial concentration in the first 7 days as the bacterial number increased. Among the *n*-alkanes, C_{16}-C_{22}, C_{25} and C_{27} (the number in C_n indicates the number of aliphatic carbons) were significantly reduced on the day 7 as

compared with *n*-alkane composition of the heavy C oil (Figs. 2B and 2C), suggesting that the isolate is able to incorporate and metabolize *n*-alkanes with long chain length preferentially. By contrast, C_9-C_{13} remained almost constant or rather increased over the experimental period (Figs. 2C-F). It presumably results from the biodegradation of the long-chain alkanes to provide short-chain alkanes, but their inefficient biodegradation by the bacterium. The bacterial growth was consistent with these presumptions; the isolate grew markedly until the day 7 by utilizing long-chain alkanes, but their exhaustion caused decrease of the bacterial cell number and it was kept at a low level after the day 14.

Numbers of aerobic biodegradation pathways of *n*-alkanes have been reported in various bacteria.[12] In each pathway, the first step is oxidation of *n*-alkanes by monooxygenase, which is a determinant of alkane chain length available for bacteria. *A. borkumensis* carries a gene set for alkane degradation including *alkB* (alkane monooxygenase), *alkG* (rubredoxin), *alkH* (alcohol dehydrogenase) and *alkJ* (alcohol dehydrogenase),[13] and can metabolize C_6-C_{20} *n*-alkanes.[14] *A. dieselolei,* which is the closest relative of *Alcanivorax* a1, was also able to utilize *n*-alkanes as the sole carbon sources, ranging in chain length from C_5 to C_{36}, which were wider than that of *A. borkumensis,* although its growth on C_5-C_7 was weak.[8] It is suggested that *Alcanivorax* sp. a1 possesses the same pathway as *A. borkumensis* and degrade long-chain *n*-alkanes favorably.

It is intriguing that C_{26} were less biodegradable than C_{16}-C_{22}, C_{25} and C_{27}. It implies that the first enzyme of an alkane metabolism pathway of the isolates, possibly alkane hydroxy-

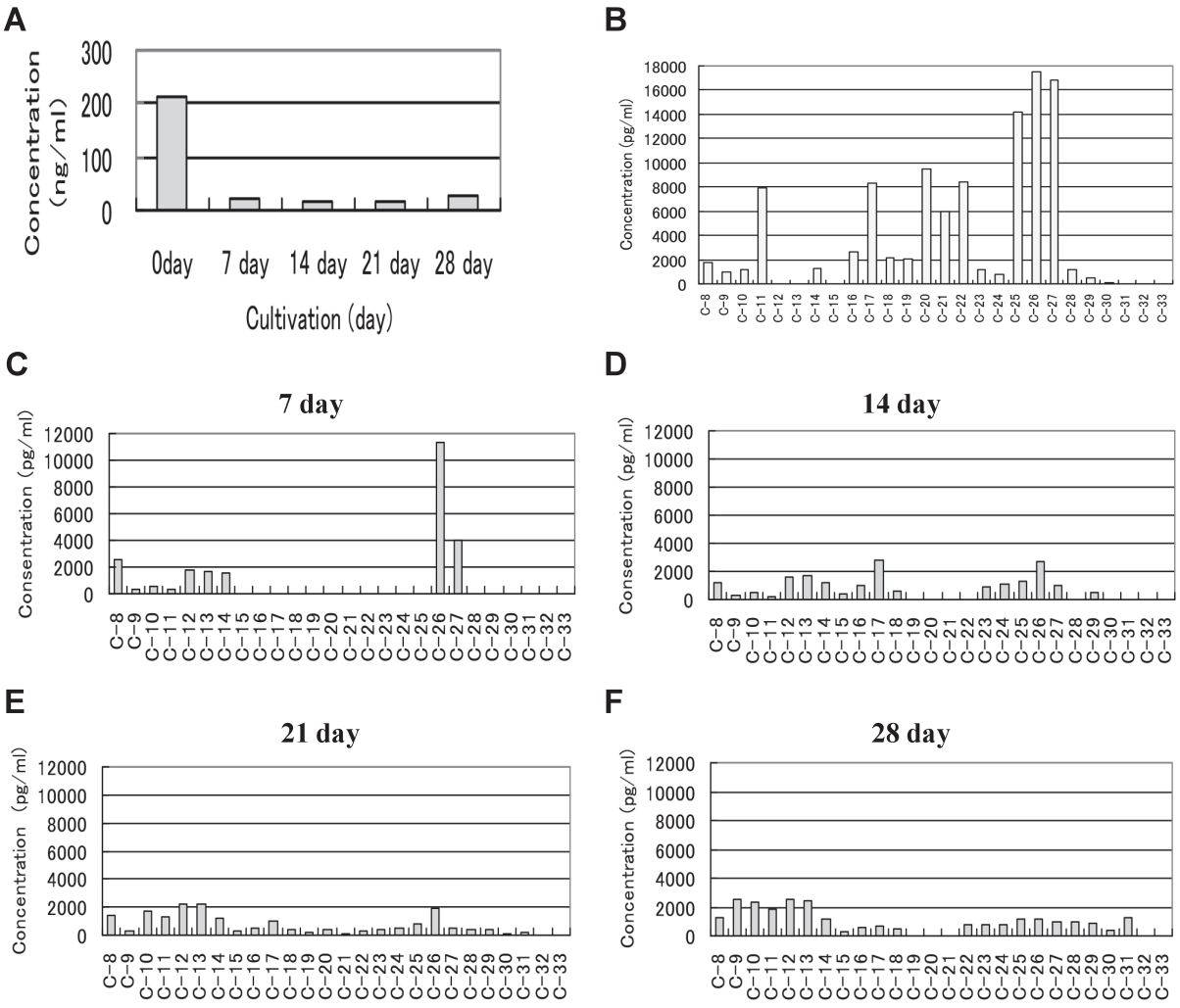

Fig. 2. Change of *n*-alkane concentration in the *Alcanivorax* sp. a1 culture. A, change of the total *n*-alkane concentration in the bacterial culture; B, concentration of C_8-C_{33} *n*-alkanes in the heavy C oil; C-F, change of each *n*-alkane concentration in the bacterial culture on the day 7, 14, 21 and 28. Number in C_n indicates the number of aliphatic carbons in *n*-alkanes.

lase AlkB, has lower affinity to C_{26}, and that conversion of C_{26} to shorter alkanes is the rate-limiting step of the biodegradation. In addition, C_{28}-C_{31} rather increased over the experimental period, possibly due to emulsification and consequent solubilization by biosurfactant secretion, which is also reported on *A. borkumensis* SK2,[5,15] but low biodegradation activity of these long-chain alkanes.

Biodegradation of PAHs by *Alcanivorax* sp. a1

Changes of total PAHs in the bacterial culture were different from ones of the total *n*-alkanes (Fig. 3A); concentration of the total PAHs remarkably increased in the first 7 days, suggesting that the biosurfactant production by *Alcanivorax* sp. a1 made the PAHs emulsified. Then, the isolate would have incorporated and metabolized the emulsified PAHs intracellularly, leading to the gradual decrease of total PAHs during the following 3 weeks.

The isolate biodegraded naphthalene and phenanthrene much more effectively among the 18 PAHs analyzed (Figs. 3B-3F). It has been becoming evident that many bacteria have the tools to degrade two- or three-ring PAHs including naphthalene, phenanthrene and anthracene aerobically.[16,17] A metabolic pathway of the low-molecular-weight PAHs is constituted by upper and lower pathways. The upper pathway converts naphthalene, phenanthrene and anthracene to salicylic acid, 1-hydroxy-2-naphthoic acid and 2-hydroxy-3-naphthoic acid, respectively. The lower pathway introduces them into the tricarboxylic acid (TCA) cycle. The first step of the upper path-

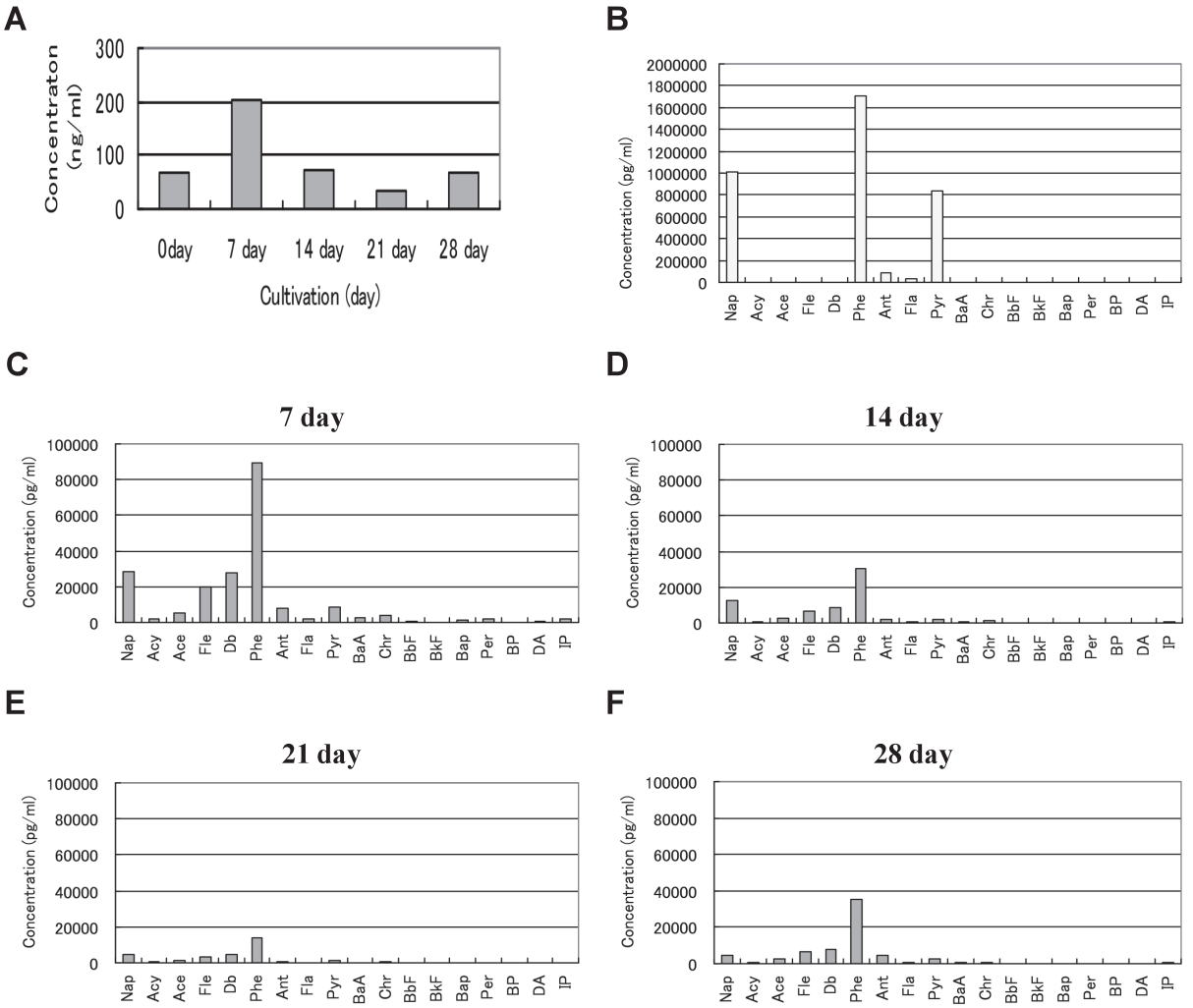

Fig. 3. Change of PAH concentration in the *Alcanivorax* sp. a1 culture. A, change of the total PAH concentration in the bacterial culture; B, concentration of PAH constituents in the heavy C oil; C-F, change of each PAH concentration in the bacterial culture on the day 7, 14, 21 and 28. Nap, naphthalene; Acy, acenaphthylene; Ace, acenaphthene; Fle, fluorene; Db, dibenzothiophene; Phe, phenanthrene; Ant, anthracene; Fla, fluoranthene; Pyr, pyrene; BaA, benzo[a]anthracene; Chr, chrysene; BbF, benzo[b]fluoranthene; BkF, benzo[k]fluoranthene; Bap, benzo[a]pyrene; Per, perylene; BP, benzo[g.h.i]perylene; DA, dibenzo[a.h]anthracene; IP, indeno[1.2.3-c.d]pyrene.

way is oxidization of PAHs by dioxygenases, which produce dihydroxy-PAHs. Gene clusters involved in the aerobic PAH-degrading pathways has been elucidated: *nah* and *pah* in *Pseudomonas* spp.[18,19] and *phn* in *Barkholderia* sp.,[20] *Alcaligenes faecalis,*[21] and *Cycloclasticus* sp. (Kasai et al., 2003).[22] In addition, degradation of alkyl-aromatic hydrocarbons including *n*-alkylbenzenes by *Alcanivora* sp. MBIC 4326 was also reported by Dutta and Harayama.[23] However, there is hitherto no evidence to clarify the mechanism by which the isolate a1 degrades PAHs.

Biodegradation of alkylated PAHs by *Alcanivorax* sp. a1

In order to understand degradability of alkylated PAHs by *Alcanivorax* sp. a1, concentration of alkylated PAHs in the bacterial culture, including C_2-C_4 alkylnaphthalene, C_1-C_4 alkyldibenzothiophene, C_1-C_3 alkylpyrene, C_1-C_3 alkylfluorene, C_1-C_4 alkylphenanthrene, and C_1-C_4 alkylchrysene (the number in C_n indicates the number of methyl groups bound to aromatic rings) were analyzed (Fig. 4). It is notable that there were decreases in the concentration of the alkylated PAHs; the concentrations on the day 14 declined to less than half on the day 7. However, ratios among C_1-C_4 alkylated PAHs remained steady comparatively (Fig. 5), although a tendency was observed that C_3- and C_4-alkylated PAHs gradually decreased during the experimental period. It can be speculated from these results that alkylated PAHs were degraded by cleaving their aromatic rings rather than the substituted methyl groups, possibly similar to the same mechanism as nonsubstituted PAH degradation. In addition, the cleavage of aromatic rings by the bacterial isolate was not be affected by existence/absence of alkylation.

Conclusion

In the present study, biodegradation of *n*-alkanes as well as PAHs and alkylated PAHs by *Alcanivorax* sp. a1 were speculated. Such features are notable from bioremedial points of view, since *Alcanivorax* spp. are known to play a major role in restoration of oil-polluted marine environments by assimilating petroleum-derived alkanes exclusively,[6] but few reports has been made at PAH and alkylated PAH biodegradation by this genus so far. Besides, microbial degradation of alkylated PAHs, which are one of the major constituents of crude oil and tend to remain in oil-polluted environments for a long time, has not been well understood yet. In order to obtain the direct evidences, more detailed investigation including enzymatic activities and genetics involved in non-substituted and

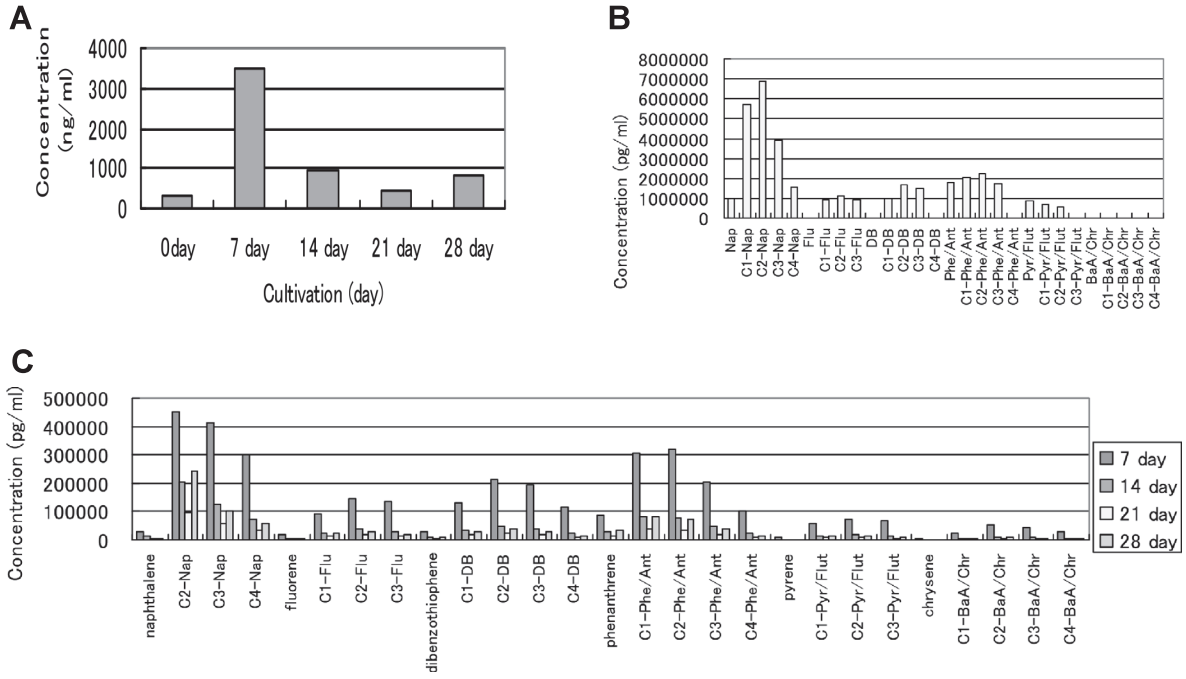

Fig. 4. Change of alkylated PAH concentration in the *Alcanivorax* sp. a1 culture. A, change of the total alkylated PAH concentration in the bacterial culture; B, concentration of alkylated PAH constituents in the heavy C oil; C, change of each alkylated PAH concentration in the bacterial culture on the day7, 14, 21 and 28. Abbreviations of the PAHs are the same as Fig. 3. Number in Cn indicates the number of methyl groups bound to the aromatic rings. Phe/Ant, Pyr/Flut, and BaA/Chr are the sums of the concentration of phenanthrene and anthracene, pyrene and fluoranthene, and benzo[a[anthracene and chrysene, respectively.

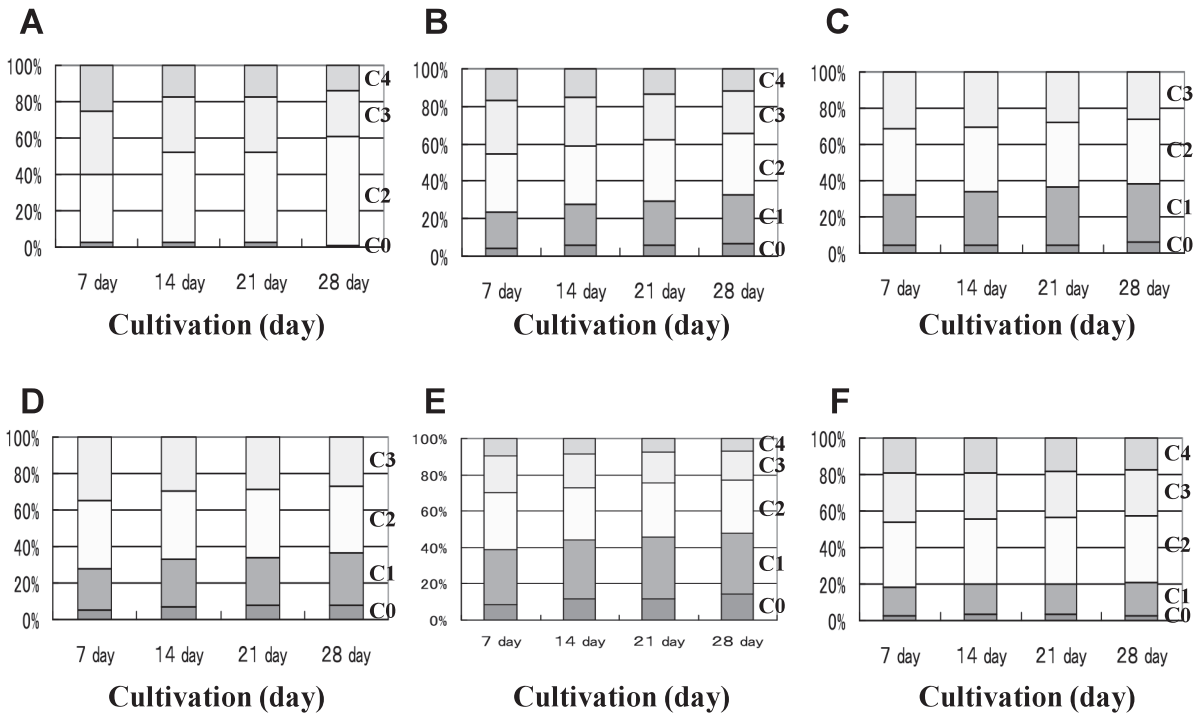

Fig. 5. Change of alkylated PAH ratios in the *Alcanivorax* sp. a1 culture. A, alkylnaphtharene; B, alkyldibenzothiophene; C, alkylpyrene; D, alkylfluorene; E, alkylpenanthrene; F, alkylchrysene. Number in Cn indicates the number of methyl groups bound to the aromatic rings.

methyl-substituted PAHs would be necessary.

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