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Biodegradation of Hydrocarbons by an Organic Solvent-Tolerant Fungus, *Cladosporium resinae* NK-1

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Abstract A kerosene fungus of *Cladosporium resinae* NK-1 was examined for its ability to degrade individual n-alkanes and aromatic hydrocarbons by gas chromatography-mass spectrometry, and its organic solvent-tolerance was investigated by making use of the water-organic solvent suspension culture method. It grew on a wide range of solvents of varying hydrophobicities and it was found to have tolerance to various kinds of toxic organic solvents (10%, v/v) such as n-alkanes, cyclohexane, xylene, styrene, and toluene. A hydrocarbon degradation experiment indicated that NK-1 had a greater n-alkane degrading ability compared to that of the other selected strains. *C. resinae* NK-1, which could utilize 8–16 carbon chain-length n-alkanes of medium chain-length as a carbon source, could not assimilate the shorter chain-length n-alkanes and aromatic hydrocarbons tested so far. The n-alkane degrading enzyme activity was found in the mycelial extract of the organism.

Key words: *Cladosporiun resinae*, organic solvent tolerance, hydrocarbon, degradation

Microbial resistance to organic solvents has attracted attention from a number of laboratories due to the significant potential of resistant microorganisms in nonaqueous biocatalysis [2]. Apart from the biotechnological interest, the study of solvent resistance should enrich our understanding of the adaptive mechanisms that microorganisms use when challenged by extreme environmental conditions. An organic solvent present in a microbial habitat from either a natural or a man-made source is one of the worst stress factors which microorganisms can encounter, and yet they have developed an organic solvent resistance [8].

Organic solvents are highly biotoxic and can kill most microorganisms at a low concentration (0.1%) level. A few studies have shown that despite the fact that some microorganisms, including members of the genera *Pseudomonas* [14], *Achromobacter* [31], and *Norcardia* [26], can assimilate toluene, their tolerance level to the solvent is less than 0.3%. Recently, Inoue and Horikoshi [10] reported the discovery of a variant strain of *Pseudomonas putida*, strain IH-2000, which was capable of growing in a media culture containing more than 50% toluene. Moriya and Horikoshi reported that organic solvent-tolerant microorganisms isolated from deep sea sediments were numerous compared to those in land soil [18]. Several putative tolerance mechanisms have been proposed. It is known that the additional polar organic solvents are more toxic to microorganisms [1, 10, 11]. The toxicity of an organic solvent correlates negatively with the parameter log P, a quantitative index of solvent polarity [16]. Organic solvents with lower log P bind more abundantly to viable cells, and this binding ability of the organic solvent is likely to be one of the mechanisms of tolerance to organic solvents [3].

The microbial degradation of petrochemicals has attracted much interest in the development of bioremediation processes for oil-spill environments. Various studies have been made regarding the microbial degradation of hydrocarbons from ecological viewpoints [7, 27, 28] and several have dealt with the isolation of microorganisms which are capable of growing on hydrocarbons [12, 13, 17, 19, 25]. Alkanes, ranging from methane to compounds with chain-lengths of 40 or more carbon atoms, have generally been found to be degraded in both laboratory cultures and the natural environment [3, 13, 21, 30]. Some *n*-alkanes are recognized as feasible carbon sources for microbial production, such as that of biodegradable polymers [4], biosurfactants [15], and so on.

Cladosporium resinae, the kerosene fungus, is widely distributed as a component of soil microflora [22, 24] and it has often been isolated from various petroleum products. This fungus has attracted increasing amount of attention because of its ubiquitous growth on jet fuel in aircraft fuel

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systems, which often results in sludge formation and corrosion of wing components [23]. Previously [20], we isolated a fungus from contaminated kerosene samples and it was identified as *Cladosporium resinae* (NK-1) on the basis of cell and colony morphology. In this study, we investigated the ability of organic solvent tolerance and hydrocarbon-degrading ability of the fungus.

MATERIALS AND METHODS

Organisms and Media

All media were based on the salts solution of Bushnell and Haas (B-H) [6], which contains ammonium nitrate as the nitrogen source (pH 6.2, per l): 1 g of NH_4NO_3, 1 g of KH_2PO_4, 1 g of K_2HPO_4, 0.2 g of $MgSO_4 \cdot 7H_2O$, 0.02 g of $CaCl_2 \cdot 2H_2O$, and 0.05 g of $FeCl_3 \cdot 6H_2O$. The strain of *Cladosporium resinae* was coded NK-1 in our collection. It was maintained on a medium containing B-H salts solution with 2% glucose and 2% agar to maintain its ability to grow in kerosene. *C. resinae* IFO 8588 and *C. cladosporioides* IFO 6348 were used as controls for the organic solvent (OS) tolerance and hydrocarbon degradation experiments.

Organic Solvent (OS) Tolerance Experiments

The OS tolerance of the fungal strains was tested in B-H salts solution with 2% glucose. Fungal spore suspension was prepared from a 7-day-old slant in a sterile saline containing 0.05% Tween 80. After vigorous agitation, the suspension was filtered through glass wool, washed three times with sterile distilled water, and resuspended at a concentration of 10^8 spores/ml. The suspension (100 µl) was inoculated into each culture tube containing 9 ml of the above-mentioned medium and 1 ml of different kinds of OSs (10^6 spores/ml). Incubation was then carried out for two weeks at 28°C under reciprocal shaking at 120 rpm. The OS tolerance was estimated by cell growth in the presence of OS. It is important to note that the organic solvents used were of the highest quality available.

Hydrocarbon Degradation Experiments

A suspension of a hydrocarbon was prepared as follows: 1 ml (1 v/v %) of hydrocarbon was added to 100 ml of the B-H salts solution containing 1 mg of the detergent, Plysurf A210G (Daiichi Kogyo Seiyaku, Kyoto), heated in boiling water for 5 min, and then homogenized by sonication at 20 kHz for 1 min [17]. Spore suspension (10^6 spores/ml) was inoculated into 10 ml of the hydrocarbon suspension in a test tube (L-tube), followed by incubation on a reciprocal shaker at 28°C for two weeks. The hydrocarbon suspension without the spore suspension as a control was treated as mentioned above. At the end of the incubation, cultures were transferred to separating funnels for extraction and analysis of hydrocarbons. Residual hydrocarbon was extracted with chloroform/methanol (2:1 v/v). n-Eicosane was used as an internal standard. Gas chromatography-Mass spectrometry (GC-MS) spectra were measured with a Hewlett Packard 5890 II gas chromatograph that was linked to a JMS-SX102A mass spectrometer (JEOL Ltd.). A DB-1 fused silica capillary column (J and W Scientific, 15 m × 0.25 mm) was used. The carrier gas, helium, was run through the column at a rate of 50 ml/min and its temperature was programmed from 50–250°C at 10°C/min. The percentage degradation of hydrocarbons is given as the difference between the control and the inoculated samples.

Analytical Methods

In order to confirm n-alkane-degrading activity in the cell-free extract, *C. resinae* NK-1 was inoculated into 100 ml of the B-H salts solution containing dual carbon sources, glucose (1% wt/v) and hexadecane (1% wt/v), in a 500-ml shake flask, and shaken reciprocally at 120 rpm for a week at 28°C. The mycelia was harvested by centrifugation (8,000 ×g, 20 min), washed twice with H_2O, and then lyophilized. Samples (5 g) of lyophilized cells were physically disrupted with a cold mortar and pestle for 20 min, and were extracted with 20 ml of 50 mM Tris-HCl (pH 7.5) containing 0.5 mM EDTA. The homogenate was centrifuged at 18,000 ×g for 10 min, and the supernatant was used as the cell extract. Protein was measured with a Bio-Rad protein assay kit with bovine serum album as the standard [5]. One-hundred micromoles of hexadecane was added to 100 ml of 50 mM Tris-HCl (pH 7.5) containing 1 mg of detergent and treated as stated above. An appropriate amount of the cell extract was added to 2 ml of the mixture in a test tube and then incubated at 30°C for 30 min. For the control experiment, the mixture without the substrate was treated similarly. After the reaction, the remaining substrate was extracted and then determined by GC-MS, as described above.

RESULTS AND DISCUSSION

Organic Solvent Tolerance

The OS tolerance of the *C. resinae* NK-1 was compared with *C. resinae* IFO 8588 and *C. cladosporioides* IFO 6348, as control strains. Both *C. resinae* IFO 8588 and *C. cladosporioides* IFO 6348 were unable to grow in the presence of 10% kerosene, *p*-xylene, styrene, and toluene, as shown in Table 1. In contrast, *C. resinae* NK-1 tolerated various kinds of toxic OSs. The parameter log P, where P is the partition coefficient of a given solvent in the equimolar mixture of octanol and water, is used as a quantitative index of solvent polarity [9, 16]. The strain NK-1 grew in solvents where the log P values were greater than or equal to 2.8, such as toluene and styrene, since it did not grow in

Table 1. Solvent tolerance of *Cladosporium* spp.[a]

Solvent (log P_{ow})[b]	*C. resinae* NK-1	*C. resinae* IFO 8588	*C. cladosporioides* IFO 6348
n-Dodecane (7.0)	+	+	+
n-Decane (6.0)	+	+	+
n-Nonane (5.5)	+	+	+
n-Hexylether (5.1)	+	+	+
n-Octane (4.9)	+	+	+
Isooctane (4.8)	+	+	-
Cyclooctane (4.5)	+	+	-
Diphenylether (4.2)	+	+	-
n-Hexane (3.9)	+	-	-
n-Propylbenzene (3.8)	+	-	-
p-Xylene (3.1)	+	-	-
Styrene (2.9)	+	-	-
Toluene (2.8)	+	-	-
1-Heptanol (2.4)	-	-	-
Benzene (2.1)	-	-	-
Kerosene	+	-	-

[a]Organic solvent tolerance was estimated by cell growth (+, growth; -, no growth) in the presence of the organic solvent, as described in the text.
[b]Values of log P_{ow} were calculated as described in Ref. 16.

1-heptanol and benzene whose log P values are 2.4 and 2.1, respectively. Therefore, it seems that a critical point is reached between log P values of 2.8 and 2.4, at which the solvent is polar enough to prevent growth. Cells of the strain grown without the solvent thrived even in the presence of 1, 5, or 10% toluene, although the growth rate was lower (Fig. 1). A growth lag was not found, indicating that the strain grew in the toluene without any adaptation for the solvent. Furthermore, single cells of this strain formed colonies on a B-H agar medium with 2% glucose, overlaid with toluene, which remained viable for several weeks (data not shown). This indicates that solvent tolerance is a stable phenotypic property of strain NK-1.

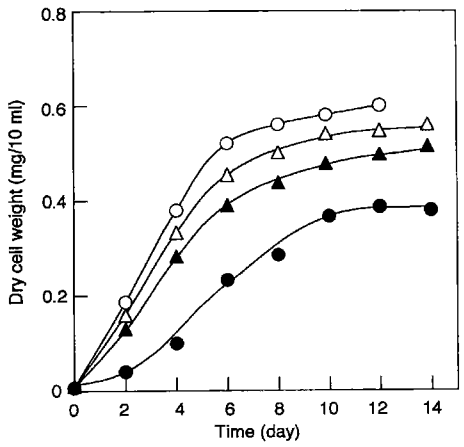

Fig. 1. Growth of the organic solvent-tolerant fungus *Cladosporium resinae* NK-1.
The isolate was aerobically grown at 28°C for 7 days in the B-H salts solution (supplemented with 2% glucose) containing 1% (△), 5% (△) and 10% (●) toluene or not containing (○). Growth was measured as the dry cell weight.

Hydrocarbon Degradation Ability

It is known that *Cladosporium* spp. could grow on *n*-alkanes from C_6 through C_{19} [29], but the use of each *n*-alkane as a single carbon source has not yet been reported until now. Therefore, fungal spore suspension was inoculated into a B-H salts solution with 1% (v/v) of each hydrocarbon [*n*-alkane (C_8-C_{16}) and aromatic hydrocarbon] as sole carbon and energy sources. Degradation of hydrocarbons by strain NK-1 was compared with the control strains. As shown in Table 2, all the strains tested could degrade *n*-alkanes as carbon sources (C_8-C_{16} or C_{10}-C_{16}). The strain NK-1 had a greater *n*-alkane degrading ability compared to that of the selected control strains. In particular, the percentage degradation of *n*-decane (C_{10}) and *n*-undecane (C_{11}) by both control strains was below 11%, whereas by the strain NK-1, it was as high as 58.0% and 48.4%, respectively. However, NK-1 showed a lower percentage degradation of *n*-decane and *n*-undecane when compared to the degradation of other *n*-alkanes such as *n*-dodecane, *n*-tetradecane, *n*-pentadecane, and *n*-hexadecane. Interestingly, all the strains tested did not degrade aromatic hydrocarbons, such as benzene, toluene, styrene, *p*-xylene, and *n*-propylbenzene. Hydrocarbon degradation is associated with assimilation. Accordingly, these results indicate that tolerance to aromatic hydrocarbons is marked off from tolerance to *n*-alkanes at least. Although

Table 2. Hydrocarbon degradation (%) by *Cladosporium* spp.

Hydrocarbons	Strains		
	C. resinae NK-1	*C. resinae* IFO 8588	*C. cladosporioides* IFO 6348
(*n*-Alkanes)			
n-C8	22.4	N.G.	N.G.
n-C9	39.5	N.G.	N.G.
n-C10	58.0	11.0	8.4
n-C11	48.4	9.7	10.6
n-C12	76.3	33.5	25.8
n-C13	56.6	30.2	35.7
n-C14	74.2	34.6	29.0
n-C15	73.7	36.9	40.3
n-C16	81.0	38.7	41.6
(Aromatic hydrocarbons)			
Benzene	N.G.	N.G.	N.G.
Toluene	N.G.	N.G.	N.G.
Styrene	N.G.	N.G.	N.G.
p-Xylene	N.G.	N.G.	N.G.
n-Propylbenzene	N.G.	N.G.	N.G.

N.G.: no growth. Degradation (%) was calculated by the following equation: 100×(Residual carbon concentration of a control - Residual carbon concentration of inoculated samples)/(Residual carbon concentration of a control).

not confirmed, breakdown products were not evident from the GC profiles. The conclusion drawn from these results was that n-alkane was assimilated to the cell material and converted to CO_2 and/or to products, which were not detectable in the GC system employed [19].

n-Alkane Degrading Enzyme Activity

The enzymes involved in the *n*-alkane degradation pathway in *Acinetobacter* sp. strain M-1 have been studied by Maeng *et al.* [17]. In their work, the *n*-alkane degrading enzyme activity found in the cell-free extract of the strain M-1 was induced during growth in a liquid medium containing *n*-alkane as a carbon source. Therefore, in order to induce the *n*-alkane degrading enzyme activity in this study, NK-1 was cultured in the B-H salts solution containing the dual carbon sources, glucose and *n*-hexadecane. The hexadecane degrading activity was found only in the cell extract. Consumption of hexadecane was observed in the reaction mixture containing the cell extract with no cofactor added, and the reaction rate was dependent on the amount

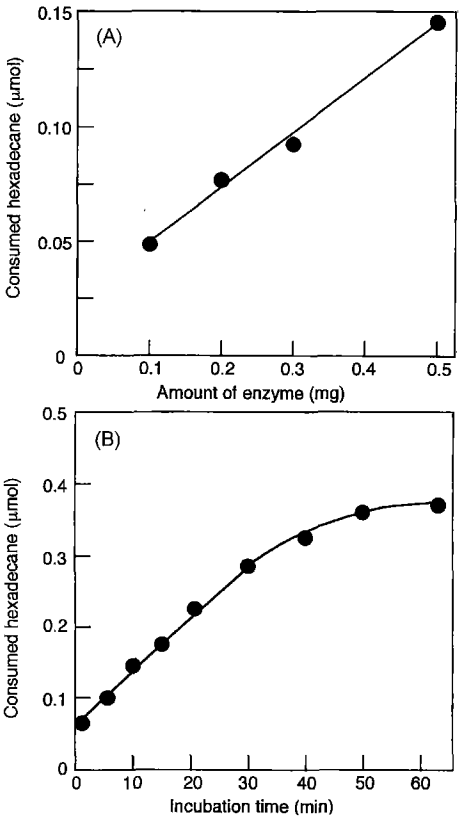

Fig. 2. *n*-Alkane degrading activity in cell extract after growth on hexadecane.

(A) Effect of enzyme concentration on activity. The reaction was carried out under standard assay conditions at 30°C for 30 min. (B) Time course of the enzyme reaction. The reaction mixture contained 2 mg (as protein) of the cell-free extract and 2 ml of 1 mM hexadecane dissolved in Tris-HCl buffer (pH 7.5). The reaction was carried out under standard assay conditions at 30°C.

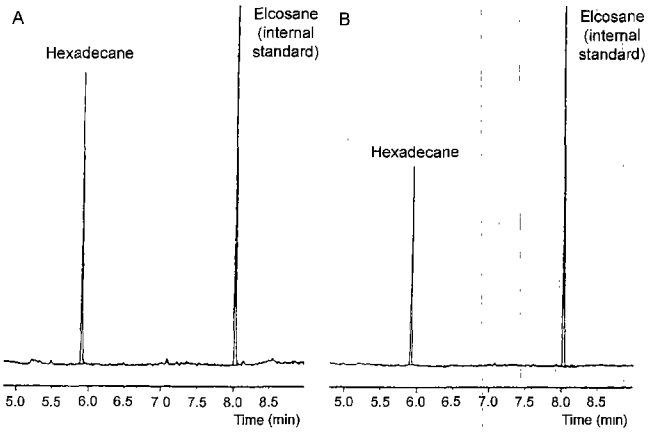

Fig. 3. Gas chromatographic profiles for hexadecane in the enzyme reaction.

The reaction mixture was composed of 2 mg (as protein) cell-free extract and 2 ml of 1 mM hexadecane dissolved in the Tris-HCl buffer (pH 7.5). The reaction was carried out under standard assay conditions at 30°C. The analysis was performed after 30 min. (A) Without any cell-free extract (control), (B) with 2 mg (as protein) of the cell-free extract.

of cell extract and incubation time (Fig. 2). Figure 3 shows gas chromatography profiles of *n*-hexadecane recovered from the enzyme reaction. During 30 min incubation at 30°C, the remaining *n*-hexadecane in the reaction mixture decreased as much as 60% when compared with the control.

CONCLUSIONS

C. resinae NK-1 was found to tolerate various kinds of toxic organic solvents. Since the OS tolerance of the fungus is also available for application of biotransformation processes which involve the use of water-insoluble organic substrates, such as biocatalysis in a water-OS two-liquid-phase system, the potential of the OS tolerance strain NK-1 should be notice. The n-alkane degrading ability of the fungus would be useful for bioremediation of oil-spills.

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REFERENCES

1. Aono, R., K. Aibe, A. Inoue, and K. Horikoshi. 1991. Preparation of organic solvent-tolerant mutants from *Escherichia coli* K-12. *Agric. Biol. Chem.* **55:** 1935-1938.
2. Aono, R., N. Doukyu, H. Kobayashi, H. Nakajima, and K. Horikoshi. 1994. Oxidative bioconversion of cholesterol by *Pseudomonas* sp. strain ST-200 in a water-organic solvent

two-phase system. *Appl. Environ. Microbiol.* **60:** 2518-2523.
3. Aono, R. and H. Kobayashi. 1997. Cell surface properties of organic solvent-tolerant mutants of *Escherichia coli* K-12. *Appl. Environ. Microbiol.* **63:** 3637-3642.
4. Anderson, A. L. and E. A. Dawes. 1990. Occurrence, metabolism, metabolic role, and industrial uses of bacterial polyhydroxyalkanoates. *Microbiol. Rev.* **54:** 450-472.
5. Bradford, M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72:** 248-254.
6. Bushnell, L. D. and H. F. Haas. 1941. The utilization of certain hydrocarbons by microorganisms. *J. Bacteriol.* **41:** 653-673.
7. Chae, J.-C., K.-J. Ahn, and C.-K. Kim. 1998. Hydrolytic dechlorination of 4-chlorobenzoate specified by *fcbABC* of *Pseudomonas* sp. DJ-12. *J. Microbiol. Biotechnol.* **8:** 692-695.
8. Ferrante, A. A., J. Augliera, K. Lewis, and A. M. Klibanov. 1995. Cloning of an organic solvent-resistance gene in *Escherichia coli*: The unexpected role of alkylhydroperoxide reductase. *Proc. Natl. Acad. Sci. USA* **92:** 7617-7621.
9. Hansch, C. and T. Fujita. 1964. ρ-σ-π analysis. A method for the correlation of biological activity and chemical structure. *J. Am. Chem. Soc.* **86:** 1616-1626.
10. Inoue, A. and K. Horikoshi. 1989. A *Pseudomonas* thrives in high concentration of toluene. *Nature* **338:** 264-266.
11. Inoue, A. and K. Horikoshi. 1991. Estimation of solvent-tolerance of bacteria by the solvent parameter log P. *J. Ferment. Bioeng.* **71:** 194-196.
12. Ju, H.-S., J. Kim, and H. Kim. 1998. Isolation of an aromatic polyhydroxyalkanoates-degrading bacterium. *J. Microbiol. Biotechnol.* **8:** 540-542.
13. Kennicutt, M. C. 1988. The effect of biodegradation on crude oil bulk and molecular composition. *Oil Chem. Pollut.* **4:** 89-112.
14. Kitagawa, M. 1956. Studies on the oxidation mechanism of methyl group. *J. Biochem.* **43:** 553-564.
15. Kosaric, N. 1993. *Biosurfactants; Production, Properties, Application.* Marcel Dekker, Inc., New York, U.S.A.
16. Leo, A. J. 1993. Calculating log P from structures. *Chem. Rev.* **93:** 1281-1306.
17. Maeng, J.-H., Y. Sakai, Y. Tani, and N. Kato. 1996. Isolation and characterization of a novel oxygenase that catalyzes the first step of n-alkane oxidation in *Acinetobacter* sp. strain M-1. *J. Bacteriol.* **178:** 3695-3700.
18. Moriya, K. and K. Horikoshi. 1993. A benzene tolerant bacterium utilizing sulfur compounds isolated from deep sea. *J. Ferment. Bioeng.* **76:** 397-399.
19. Moriya, K. and K. Horikoshi. 1993. Isolation of a benzene-tolerant bacterium and its hydrocarbon degradation. *J. Ferment. Bioeng.* **76:** 168-173.
20. Oh, K.-B., H. Hamada, M. Saito, H.-J. Lee, and H. Matsuoka. 1999. Isolation and properties of an extracellular β-glucosidase from a filamentous fungus, *Cladosporium resinae*, isolated from kerosene. *Biosci. Biotechnol. Biochem.* **63:** 281-287.
21. Oudot, J., A. Ambles, S. Bourgeouis, C. Gatellier, and N. Sebyera. 1989. Hydrocarbon infiltration and biodegradation in a land-farming experiment. *Environ. Pollut.* **59:** 17-40.
22. Parbery, D. G. 1969. The natural occurrence of *Cladosporium resinae*. *Trans. Brit. Mycol. Soc.* **53:** 15-23.
23. Parbery, D. G. 1971. Biological problems in jet aviation fuel and the biology of *Amorphotheca resinae*. *Mater. Org. (Berlin)* **3:** 161-208.
24. Parbery, D. G. 1969. *Amorphotheca resinae* gen. nov., sp. nov.: The perfect state of *Cladosporium resinae*. *Aust. J. Bot.* **17:** 331-357.
25. Park, G.-T., H.-J. Son, J.-G. Kim, and S.-J. Lee. 1998. Biodegradation of phenol by a trichloroethylene-cometabolizing bacterium. *J. Microbiol. Biotechnol.* **8:** 61-66.
26. Raymond, R. L., V. W. Jamison, and J. O. Hudson. 1967. Microbial hydrocarbon co-oxidation. I. Oxidation of mono- and dicyclic hydrocarbons by soil isolates of the genus *Norcardia*. *Appl. Microbiol.* **15:** 857-865.
27. Shin, H.-J., M.-Y. Lee, and J.-W. Yang. 1998. Biodegradation of trichloroethylene by phenol-degrading *Pseudomonas putida*. *J. Microbiol. Biotechnol.* **8:** 185-187.
28. Shin, P.-K. and E.-J. Jung. 1999. Effects of various parameters on biodegradation of degradable polymers in soil. *J. Microbiol. Biotechnol.* **9:** 784-788.
29. Teh, J. S. and K. H. Lee. 1974. Effects of n-alkanes on *Cladosporium resinae*. *Can. J. Microbiol.* **20:** 971-976.
30. Wakeham, S. G., E. A. Canuel, and P. H. Doering. 1986. Behavior of aliphatic hydrocarbons in coastal seawater: Mesocosm experiments with [^{14}C]octadecane and [^{14}C]decane. *Environ. Sci. Technol.* **20:** 574-580.
31. Worsey, M. J. and P. A. Williams. 1975. Metabolism of toluene and xylene by *Pseudomonas putida* (arvilla)mt-2: Evidence for a new function of the Tol plasmid. *J. Bacteriol.* **124:** 7-15.