J. Microbiol. Biotechnol. (2006), **16**(8), 1306–1310

JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY

A New Intermediate in the Degradation of Carbofuran by *Sphingomonas* sp. Strain SB5

PARK, MYUNG RYEOL[1], SUNWOO LEE[2], TAE-HO HAN[3], BYUNG-TACK OH[4], JAE HAN SHIM[1], AND IN SEON KIM[1]*

[1]*Division of Applied Bioscience and Biotechnology, Institute of Agricultural Science and Technology, College of Agriculture and Life Sciences, Chonnam National University, Gwangju 500-757, Korea*
[2]*Department of Chemistry, College of Natural Science, Chonnam National University, Gwangju 500-757, Korea*
[3]*Division of Plant Biotechnology, College of Agriculture and Life Sciences, Chonnam National University, Gwangju 500-757, Korea*
[4]*Department of Environmental Sciences and Biotechnology, Hallym University, Chuncheon, Gangwon-do 200-702, Korea*

Received: January 7, 2006
Accepted: March 3, 2006

Abstract *Sphingomonas* sp. strain SB5 could degrade carbofuran and carbofuran-7-phenol to a hydrolytic product, 2-hydroxy-3-(3-methlypropan-2-ol)phenol, and several red metabolites. However, the chemical structures of the red metabolites have largely remained unidentified. In this study, we identified the structure of one of the red metabolites as 5-(2-hydroxy-2-methyl-propyl)-2,2-dimethyl-2,3-dihydro-naphtho[2,3-6]furan-4,6,7,9-tetrone by using mass spectrometric and NMR (^{1}H, ^{13}C) analyses. It is suggested that the red metabolite resulted from condensation of some metabolites in the degradation of 2-hydroxy-3-(3-methlypropan-2-ol)phenol, a hydrolytic product derived from carbofuran. To our knowledge, this is the first paper to report a red metabolite in bacterial degradation of the insecticide carbofuran.

Key words: Carbofuran, pesticide, *Sphingomonas* sp. strain SB5

Sphingomonas sp. strain SB5 is a carbofuran degrader capable of degrading carbofuran and carbofuran-7-phenol by hydrolysis at the furanyl ring [8]. This microorganism could degrade carbofuran and carbofuran-7-phenol to a number of metabolites including 2-hydroxy-3-(3-methlypropan-2-ol)phenol and several red intermediates [8]. To date, the chemical structures of the red intermediates have largely remained unknown. In this study, we investigated one of the red metabolites by mass spectrometric and NMR analyses.

Sphingomonas sp. strain SB5 (SB5 hereafter) was grown in mineral salt media (MSM) containing carbofuran as the sole carbon source, as described previously [8]. The cultures were centrifuged at 8,000 ×*g* for 10 min to remove microbial cells. The supernatant was extracted twice with two volumes of dichloromethane. The organic phase was dehydrated over anhydrous sodium sulfate and evaporated to dryness in an evaporator at 40°C. The dried extract was dissolved in a solvent mixture of chloroform and ethyl acetate (1:1, v/v) and subjected to silica gel column chromatography. A chromatographic column (25 mm i.d.× 50 cm length) was slurry-packed with 20 g of silica gel (70–230 mesh, Sigma, U.S.A.) in chloroform. The sample was carefully added to the column, and the column was washed with 100 ml of the above solvent mixture, followed by washing with 100 ml of ethyl acetate. These fractions were discarded. The column was finally eluted with 150 ml of a solvent mixture of ethyl acetate and methanol (1:1, v/v), and this fraction was used for characterization of

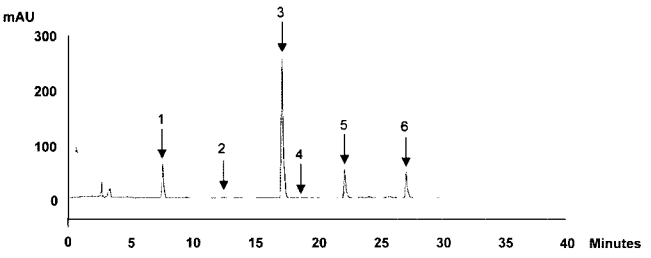

Fig. 1. HPLC chromatogram of carbofuran metabolites extracted from cultures.
The cultures were incubated with carbofuran for 24 h and extracted with dichloromethane, followed by HPLC analysis. The arrow symbols represent the time when metabolite M-1 (1), an intermediate with a $(M+H)^+$ at *m/z* 165.4 (2), carbofuran-7-phenol (3), carbofuran (4), and red metabolites (5, 6) were detected, respectively. The peak of carbofuran is not shown, because it was completely degraded.

*Corresponding author
Phone: 82-62-530-2131; Fax: 82-62-530-2139;
E-mail: mindzero@chonnam.ac.kr

a red metabolite of carbofuran. The above eluate was evaporated to dryness in an evaporator at 40°C and dissolved in methanol for prep-HPLC analysis. The prep-HPLC was a Dionex P680 dual pump (Dionex, U.S.A.) equipped with a Dionex PDA-100 photodiode array detector (PDA) and the HPLC column was a μ-Bondapak C18 preparative stainless

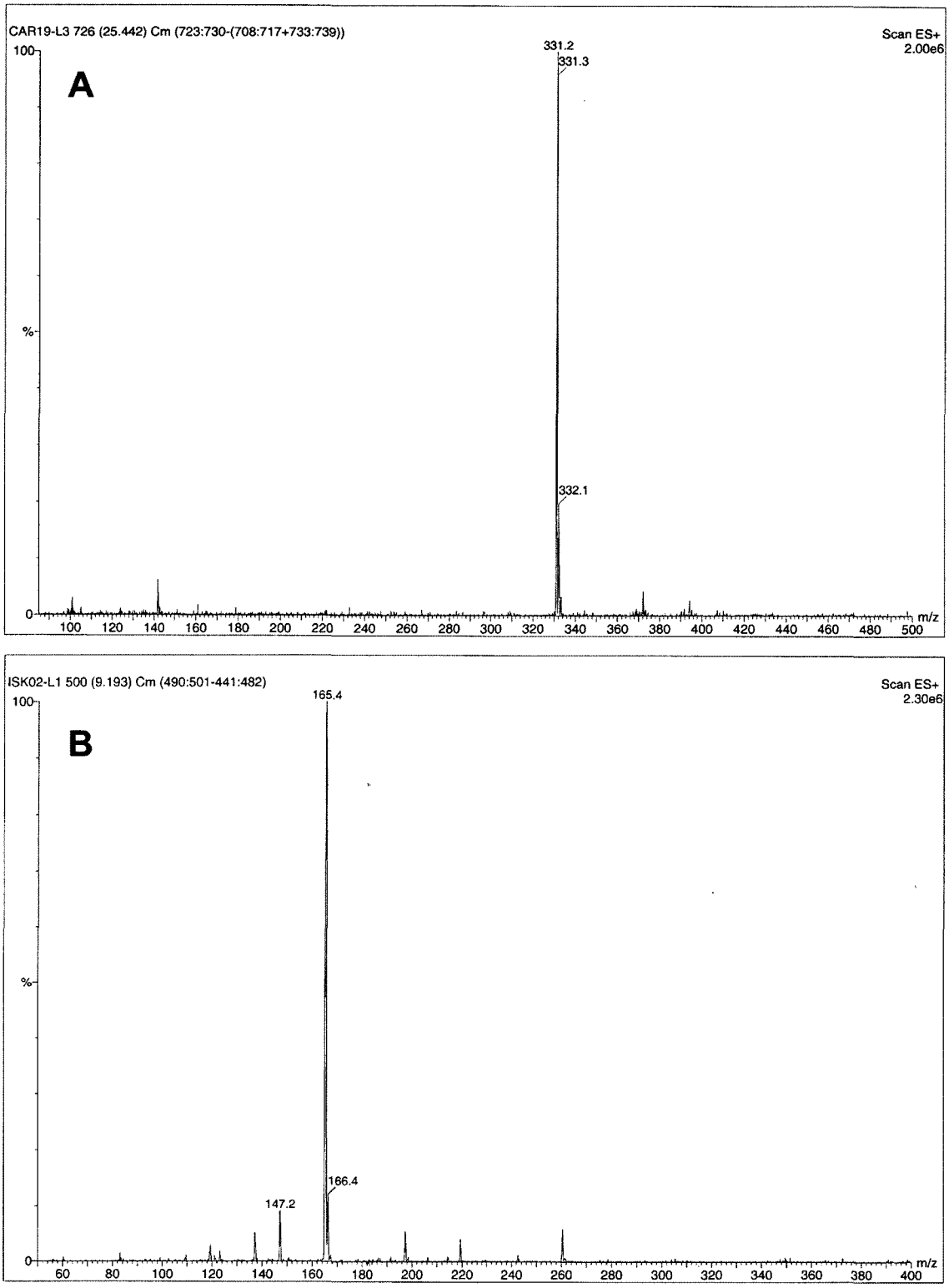

Fig. 2. Positive ion LC/MS spectra of the isolated red metabolite (**A**) and an intermediate with a $(M+H)^+$ at *m/z* 165.4 (**B**).

column (7.8 mm i.d.×300 mm length, 10 μm film thickness). The HPLC mobile phase consisted of 30% (v/v) aqueous acetonitrile (solvent A) adjusted to pH 2 by addition of trifluoroacetic acid (TFA) and 60% (v/v) aqueous methanol (solvent B). Isocratic elution of samples was carried out with 100% (v/v) solvent A for 5 min, and linear gradient elution with 100% (v/v) solvent B for 25 min. A column clean-up stage was used, holding at 100% (v/v) solvent B for a further 10 min, followed by equilibration at 100% (v/v) solvent A for 15 min. The column was eluted with the mobile phase at 2 ml/min. Peaks at retention time of 22.3 and 27.5 min on HPLC chromatograms were collected without the leading edge and the tailing end of the peaks, respectively (Fig. 1). The peak fractions were concentrated to a water residue in an evaporator at 40°C, and the water residue was extracted with two volumes of dichloromethane. The organic phase was dehydrated over anhydrous sodium sulfate and evaporated to dryness in an evaporator at 40°C. One red metabolite at retention time of 27.5 min was chosen for further study. The metabolite was further purified by repetitive silica gel column chromatography in the manner as described above, so that the red metabolite had a single spot on Merck 25 aluminum TLC plates (silica gel 60 F_{254}, 20×20 cm) in a solvent system of dichloromethane-ethyl acetate-acetic acid (85:15:1, v/v/v). The isolated metabolite was used for mass spectrometric and NMR analyses.

Carbofuran is one of the pesticides belonging to the *N*-methylcarbamate class of insecticides and has been extensively used to control pests in rice paddies in Korea since 1975. The intensive use of carbofuran could increase the possibility of environmental exposure to this pesticide. The technology for degradation of environmental contaminants such as pesticides and other aromatics by using microorganisms has been suggested to be one of the useful strategies in environmental remediation [1, 5, 6, 12]. The possibility of environmental exposure to carbofuran prompted study on the biodegradation of carbofuran and its metabolites, because carbofuran is known to exhibit extreme toxicity in mammal. Most studies on the microbial degradation of carbofuran have demonstrated hydrolysis at the moiety of *N*-methylcarbamate linkage and hydroxylation at the ring structure. Metabolites of carbofuran by microbial hydrolysis and oxidation include carbofuran-7-phenol, 3-hydroxycarbofuran, 4-hydroxycarbofuran, 3-ketocarbofuran, 3-hydroxycarbofuran phenol, and 3-ketocarbofuran phenol [2, 3, 7, 10, 11]. SB5 has been reported to degrade carbofuran and carbofuran-7-phenol to 2-hydroxy-3-(3-methlypropan-2-ol)phenol and several red intermediates including two major red metabolites and one minor pink metabolite [8]. In the present study, we characterized one of the red metabolites by mass spectrometric and NMR analyses.

LC/MS analysis identified a red metabolite by a $(M+H)^+$ peak at *m/z* 331.2 (Fig. 2A). This *m/z* 331.2 value was significantly higher than the molecular weights of carbofuran (220 g/mol) and carbofuran-7-phenol (164 g/mol), suggesting that the red metabolite isolated would be a condensation product of some metabolites. Kim *et al.* [8] have recently shown 2-hydroxy-3-(3-methlypropan-2-ol)phenol (M-1 hereafter) as a biological metabolite derived from hydrolysis of carbofuran at the furanyl ring by SB5. To investigate if the metabolite M-1 was involved in the formation of the red metabolite with a $(M+H)^+$ peak at *m/z* 331.2, M-1 was isolated and tested for its degradation by SB5. For this purpose, SB5 cells grown on carbofuran for 12 h were washed twice with MSM. Optical density of the washed cells was adjusted to approximately 1.0 (OD_{600}), and they were incubated with M-1 at a final concentration of 7.7 μmol/l in 100 ml of MSM. When the isolated M-1 was incubated with SB5 cells, the disappearance of M-1 was indicated by the appearance of red color in the mixture, suggesting that M-1 would correlate with the appearance of red metabolites. An intermediate with a $(M+H)^+$ peak at *m/z* 165.4 was also observed during the incubation of M-1 with SB5 (Fig. 2B). The intermediate with a $(M+H)^+$ peak at *m/z* 165.4 varied by the appearance of the red metabolite with a $(M+H)^+$ peak at *m/z* 331.2. One might wonder if the intermediate with a $(M+H)^+$ peak at *m/z* 165.4 was carbofuran-7-phenol, because the $(M+H)^+$ value of carbofuran-7-phenol was *m/z* 165.2, which is close to *m/z* 165.4. However, the HPLC retention time (12.7 min) of the intermediate with a $(M+H)^+$ peak at *m/z* 165.4 was different from that (17.5 min) of carbofuran-7-phenol (Fig. 1), suggesting that the intermediate with a $(M+H)^+$ peak at *m/z* 165.4 was not carbofuran-7-phenol. These findings suggested the possibility that the red metabolite is the condensation product of M-1 and an intermediate.

To investigate the chemical structure of the red metabolite with a $(M+H)^+$ peak at *m/z* 331.2, the metabolite was subjected

Table 1. NMR spectral data of the isolated red metabolite in CD_3OD.

Position	δ_H (mult, *J*, 500 MHz)	δ_C (125 MHz)
2-Me	1.52(6H, s)	28.56
2	-	93.01
3	2.89 (2H, s)	41.08
3a	-	120.29
4	-	178.09
4a	-	125.94
5	-	166.22
6	-	184.69
7	-	174.46
8	7.17 (1H, s)	123.15
8a	-	139.00
9	-	185.84
9a	-	164.15
1'	3.04 (2H, s)	44.91
2'	-	86.79
2'-Me	1.48 (6H, s)	28.51

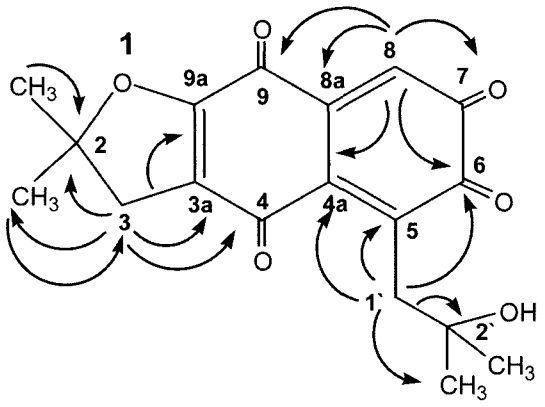

Fig. 3. Chemical structure and C-H correlations from the HMBC spectrum of the isolated red metabolite with a $(M+H)^+$ peak at *m/z* 331.2.

to ^{1}H- and ^{13}C-NMR analyses and Table 1 shows the NMR data of the isolated red metabolite. The ^{1}H-NMR (500 MHz, methanol-d_4) spectrum of the red metabolite exhibited one olefinic methine proton at δ_H 7.17 (s), two methylene protons at δ_H 3.04 (s) and 2.89 (s), and four methyl protons at δ_H 1.52 (×2, s) and 1.48 (×2, s). ^{13}C-NMR (125 MHz, methanol-d_4) and DEPT spectra of the compound showed 16 carbons that could be classified into four methyl carbons at δ_C 28.51 (×2) and 28.56 (×2), two methylene carbons at δ_C 41.08 and 44.91, two saturated oxygenated quarternary carbons at δ_C 86.79 and 93.01, one olefinic methine carbon at δ_C 123.15, five olefinic quarternary carbons at δ_C 120.29, 125.94, 139.00, 164.15, and 166.22, and four carbonyl carbons at δ_C 174.46, 178.09, 184.69, and 185.84. The mode of binding between proton and carbon atoms was established from an HMQC spectrum. The COSY spectrum did not show any relationship among the protons. On the basis of the LC/MS and NMR analyses, the molecular formula of the red metabolite was suggested as $C_{18}H_{18}O_6$, and C-H correlations of the metabolite could be clarified by an HMBC experiment (Fig. 3). These data demonstrated the existence of a naphtalenetetraone structure, in which 2 and 3 carbons of a 1,4-benzoquinone moiety and 5 and 6 carbons of a 1,2-benzoquinone moiety were combined to be the same ones. In addition, the position of the oxygen of the furan ring was connected at C-9a, not at C-3a.

Based on the present observations, a possible pathway for degradation of carbofuran by SB5 is shown in Fig. 4. In brief, carbofuran was degraded first to carbofuran phenol. Carbofuran-7-phenol was degraded to give a hydrolytic product, M-1. In the pathway, the intermediate 2,2-dimethyl-2,3-dihydro-benzofuran-4,7-one might combine with M-1 to give a red metabolite, 5-(2-hydroxy-2-methyl-propyl)-2,2-dimethyl-2,3-dihydro-naphtho[2,3-6]furan-4,6,7,9-tetrone.

The fact of the appearance of a red color correlating with disappearance of carbofuran and carbofuran-7-phenol has earlier been reported by others [4, 8, 9]. In the present study, we identified for the first time one of the red metabolites in the degradation of carbofuran by SB5. It should be pointed out, however, that further study is required to identify the chemical structures of additional red metabolites, because carbofuran was found to be degraded to several red metabolites by SB5.

Acknowledgments

This study was supported by a grant (No. F00004) from the Korea Research Foundation, Republic of Korea.

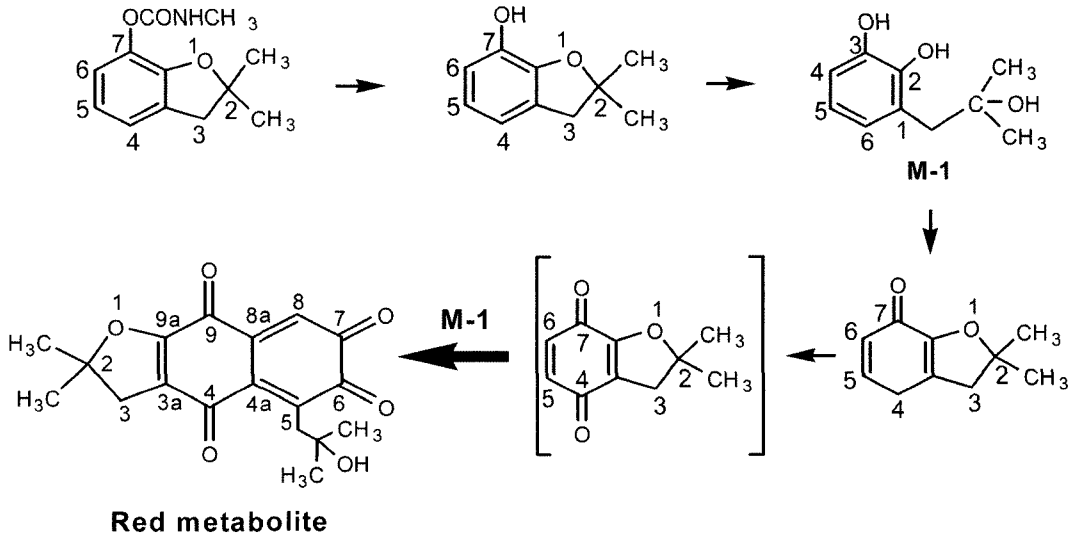

Fig. 4. Proposed degradation pathway of carbofuran by *Sphingomonas* sp. strain SB5.

REFERENCES

1. Ahn, T.-S., G.-H. Lee, and H. G. Song. 2005. Biodegradation of phenanthrene by psychrotrophic bacteria from Lake Baikal. *J. Microbiol. Biotechnol.* **15:** 1135–1139.
2. Chaudhry, G. R., A. Mateen, B. Kaskar, M. Sardessai, M. Bloda, A. R. Bhatti, and S. K. Walia. 2002. Induction of carbofuran oxidation to 4-hydroxycarbofuran by *Pseudomonas* sp. 50432. *FEMS Microbiol. Lett.* **214:** 171–176.
3. Feng, X., L. T. Ou, and A. V. Ogram. 1997. Plasmid-mediated mineralization of carbofuran by *Sphingomonas* sp. CF06. *Appl. Environ. Microbiol.* **63:** 1332–1337.
4. Head, I. M., R. B. Cain, and D. L. Suett. 1992. Characterization of a carbofuran-degrading bacterium and investigation of the role of plasmids in catabolism of the insecticide carbofuran. *Arch. Microbiol.* **158:** 302–308.
5. Kim, J. D., S. H. Shim, and C. G. Lee. 2005. Degradation of phenanthrene by bacterial strains isolated from soil refinery fields in Korea. *J. Microbiol. Biotechnol.* **15:** 337–345.
6. Kim, Y. M., K. Park, J. H. Choi, J. E. Kim, and I. K. Lee. 2004. Biotransformation of the fungicide chlorothalonil by bacteria glutathione s-transferase. *J. Microbiol. Biotechnol.* **14:** 938–943.
7. Karns, J. S., W. W. Mulbry, J. O. Nelson, and P. C. Kearney. 1986. Metabolism of carbofuran by a pure bacterial culture. *Pestic. Biochem. Physiol.* **25:** 211–217.
8. Kim, I. S., J. Y. Ryu, H. G. Hur, M. B. Gu, S. D. Kim, and J. H. Shim. 2004. *Sphingomonas* sp. strain SB5 degrades carbofuran to a new metabolite by hydrolysis at the furanyl ring. *J. Agric. Food Chem.* **52:** 2309–2314.
9. Ogram, A. V., Y. Duan, S. L. Trabue, X. Feng, H. Castro, and L. T. Ou. 2000. Carbofuran degradation mediated by three related plasmid systems. *FEMS Microbiol. Ecol.* **32:** 197–203.
10. Ramanand, K., M. Sharmila, N. Singh, and N. Sethunathan. 1991. Metabolism of carbamate insecticides by resting cells and cell-free preparations of soil bacterium, *Arthrobacter* sp. *Bull. Environ. Contam. Toxicol.* **46:** 380–386.
11. Topp, E., R. S. Hanson, D. B. Ringelberg, D. C. White, and R. Wheatcroft. 1993. Isolation and characterization of an N-methylcarbamate insecticide-degrading methylotroph bacterium. *Appl. Environ. Microbiol.* **59:** 3339–3349.
12. Woo, S. H. and J. M. Park. 2004. Biodegradation of aromatic compounds from soil by drum bioreactor system. *J. Microbiol. Biotechnol.* **14:** 435–441.