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EXPERIMENTAL ARTICLES

Growth of *Rhodococcus opacus* on Mixtures of Monohalogenated Benzenes and Phenols

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Abstract—The growth of *Rhodococcus opacus* GM-14 on mixtures of 2-chloro- and 2-bromophenol, of 4-chloro, 4-bromo-, and 4-iodophenol, and of chloro-, bromo-, and iodobenzenes was accompanied by the consumption of the substrates and the excretion of halogen ions into the medium. During the growth on monochlorophenols, the substrates were consumed sequentially in the following order: 4-chloro-, 3-chloro-, and then 2-chlorophenol. Chlorine ions were excreted in a two-phase manner in amounts comprising 79% of the theoretical yield. The diauxic growth of *R. opacus* GM-14 can be explained by the existence in this bacterium of two modified metabolic pathways for the *ortho*- cleavage of halogenated pyrocatechols. The first pathway included 4-halogeno- or dihalogenopyrocatechols as intermediates, whereas the second pathway included 3-halogenopyrocatechols.

Key words: halogenobenzenes, halogenophenols, utilization, *Rhodococcus opacus*.

Polluted ecosystems and industrial sewages often contain combinations of xenobiotics. This stimulated the interest of researchers in microbial strains capable of utilizing xenobiotic mixtures.

Some bacterial strains are able to degrade complex mixtures of aromatic compounds [1–9], which is due to the existence of several metabolic pathways for the utilization of these compounds. For instance, *Burkholderia* sp. JS150 can grow on benzene, toluene, ethylbenzene, benzoate, oxybenzoates, chlorobenzoates, phenol, naphthalene, and their mixtures due to its ability to synthesize three different dioxygenases catalyzing the primary stages of oxidation of aromatic compounds and four dioxygenases catalyzing the cleavage of the aromatic ring of these compounds [7].

Rhodococcus opacus GM-14 is also able to utilize a wide range of aromatic compounds, including benzene (13.3 g/l), phenol (1.2 g/l), benzoate, chlorophenols (0.25 g/l), chlorobenzenes (0.5 g/l), as well as the nitro, methyl-, methoxy-, and hydroxy-derivatives of these compounds [10, 11]. The results of relevant investigations suggest that this bacterium has four oxygenases responsible for the primary stages of oxidation of aromatic compounds and three pyrocatechol 1,2-dioxygenases catalyzing the cleavage of the aromatic ring of pyrocatechol and halogenopyrocatechols (Fig. 1) [12, 13]. This prompted us to study the ability of this strain to degrade various mixtures of halogenoaromatic compounds.

The aim of the present work was to investigate the dynamics of utilization of monohalogenated benzenes and phenols by *R. opacus* GM-14 when mixtures of these compounds were added to the growth medium as carbon sources.

MATERIALS AND METHODS

Strain. *Rhodococcus opacus* GM-14 (formerly *Rhodococcus rhodochrous*) was isolated using a minimal medium with chlorobenzene as the sole carbon source [14]. The properties and cultivation conditions of this strain, as well as the composition of the minimal KSN medium, were described in detail elsewhere [10].

Cultivation conditions. The strain was grown at 28°C on a shaker in 2-l flasks sealed with Teflon stoppers. Each flask contained 200 ml of KSN medium supplemented with 5 mg/l yeast extract and the respective mixtures of aromatic compounds. The medium was inoculated with cultures grown on 1.8 mM chlorobenzene, 0.8 mM 2-chlorophenol (2-CP), and 1.5 mM 4-chlorophenol (4-CP), or on a mixture of 0.4 mM 2-CP and 0.4 mM 4-CP. To evaluate the consumption of substrates and the accumulation of halogen ions, 5-ml aliquots of the culture liquid were periodically taken with a syringe from cultivation flasks through a sealing gas-tight Teflon membrane. Growth was monitored by measuring the optical culture density at 540 nm in a Lambda 12 Perkin-Elmer spectrophotometer.

Analytical procedures. The concentration of halogenoaromatic compounds was determined by gas–liquid chromatography on a Hewlett-Packard model 6890 chromatograph equipped with an ion-capture detector

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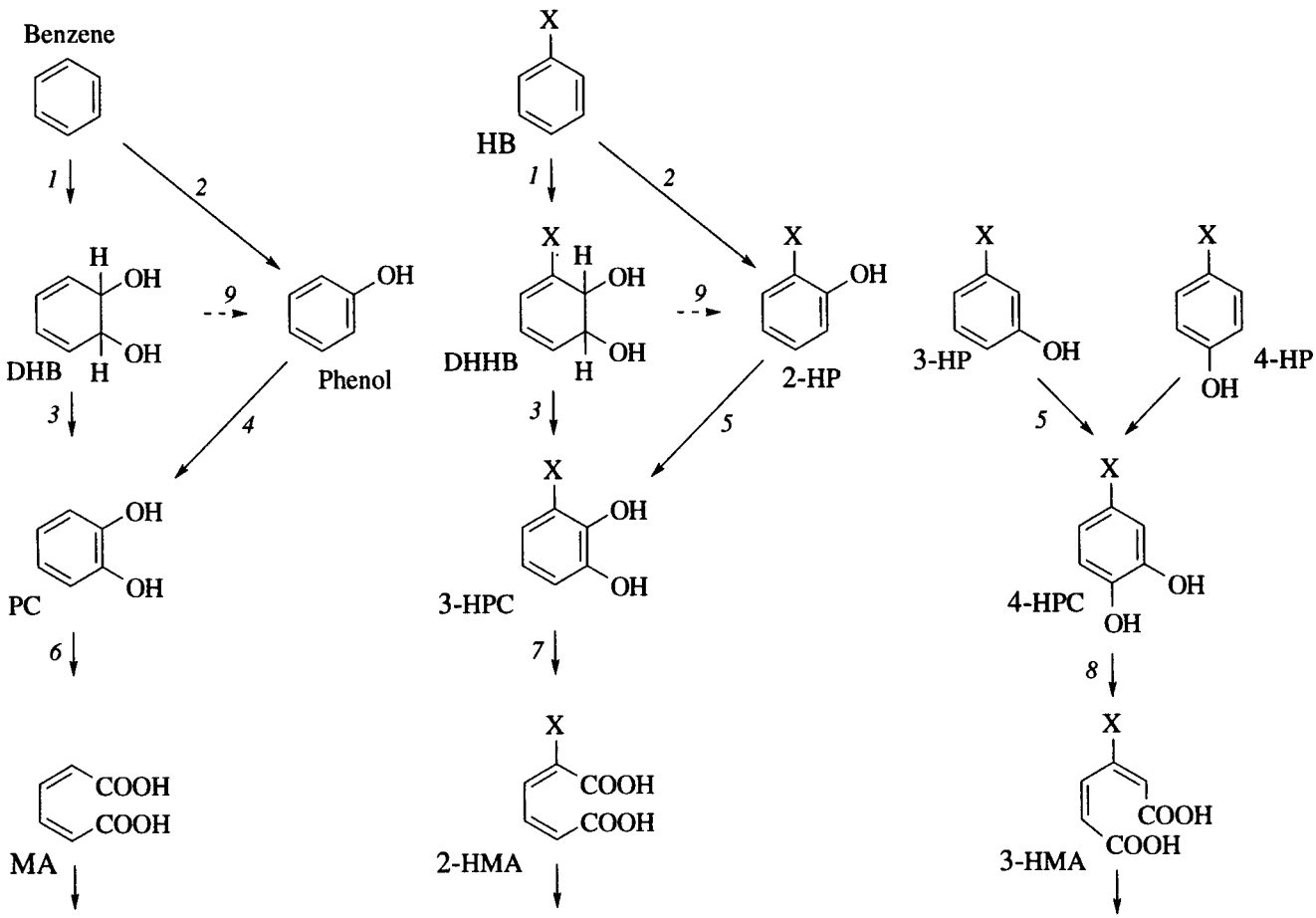

Fig. 1. Putative scheme of the primary stages of metabolism of benzene, phenol, and their monohalogenated derivatives in *R. opacus* GM-14: HB, halogenobenzene; 2-HP, 2-halogenophenol; 3-HP, 3-halogenophenol; 4-HP, 4-halogenophenol; DHB, *cis*-dihydrobenzene-diol; DHHB, *cis*-dihydrohalogenobenzene-diol; PC, pyrocatechol; 3-HPC, 3-halogenopyrocatechol; 4-HPC, 4-halogenopyrocatechol; MA, *cis,cis*-muconic acid; 2-HMA, 2- halogeno-*cis,cis*-muconic acid; 3-HMA, 3-halogeno-*cis,cis*-muconic acid; X = Cl, Br, or I; *1*, benzene dioxygenase; *2*, benzene monooxygenase; *3*, *cis*-dihydrobenzene-diol dehydrogenase; *4*, phenol monooxygenase; *5*, halogenophenol monooxygenase; *6*, pyrocatechol 1,2-dioxygenase; *7*, 3-halogenopyrocatechol 1,2-dioxygenase; *8*, 4-halogenopyrocatechol 1,2-dioxygenase; *9*, spontaneous reaction under slightly acidic conditions.

and an HP-5 capillary column [10]. Chlorine, iodine, and bromine ions in the medium were assayed using the Bergmann–Sanik method [15], the crystal violet dye [16], and an Orion 94-17B ion-selective electrode (Finland), respectively.

RESULTS AND DISCUSSION

R. opacus GM-14 grew well on a mixture of 0.3 mM 2-CP and 2-bromophenol (2-BP) (Fig. 2a), completely utilizing these compounds as carbon and energy sources within 30 h (Fig. 2b). Growth was accompanied by the nearly simultaneous consumption of halogenophenols and the accumulation of chlorine and bromine ions in the culture liquid in stoichiometric amounts (Fig. 2b). Similar results were obtained in experiments with two other mixtures: 0.25 mM 4-CP, 0.28 mM 4-bromophenol (4-BP), and 0.25 mM 4-iodophenol (4-IP) (Fig. 3) and 0.52 mM chlorobenzene, 0.5 mM bromobenzene, and 0.49 mM iodobenzene (Fig. 4). Growth was accompanied by the simultaneous consumption of component substrates in, respectively, 35 and 120 h and the accumulation of halogen ions in stoichiometric amounts (Figs. 3 and 4).

R. opacus GM-14 also grew well on a mixture of 0.39 mM 2-CP, 0.2 mM 3-chlorophenol (3-CP), and 0.39 mM 4-CP; however, in this case, substrates were consumed successively (Fig. 5): 4-CP was completely utilized in 50 h, whereas the concentrations of 2-CP and 3-CP virtually did not change within about 22 h and gradually decreased to zero over the next 39 and 45 h, respectively. A rapid oxidation of 3-CP began as soon as the concentration of 4-CP in the medium had dropped to 0.07 mM. In turn, 2-CP was rapidly oxidized after 4-CP had been completely oxidized and the concentration of 3-CP had decreased to 0.08 mM. The oxidation of 2-CP was accompanied by the transient accumulation in the medium of 3-chloropyrocatechol (3-CPC) in an amount of 0.1 mM. Chlorine ions accumulated in an amount comprising 79% of their theoret-

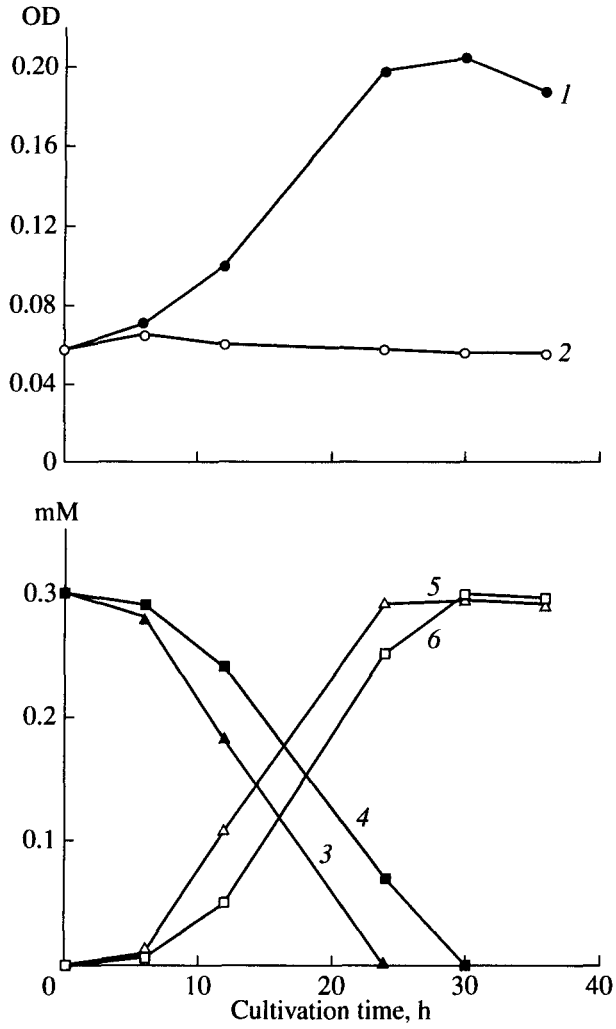

Fig. 2. Growth of *R. opacus* GM-14 on a mixture of 2-CP and 2-BP: (*1*) experiment; (*2*) control (growth without halogenophenols); (*3*) 2-CP consumption; (*4*) 2-BP consumption; (*5*) Cl^- accumulation; and (*6*) Br^- accumulation.

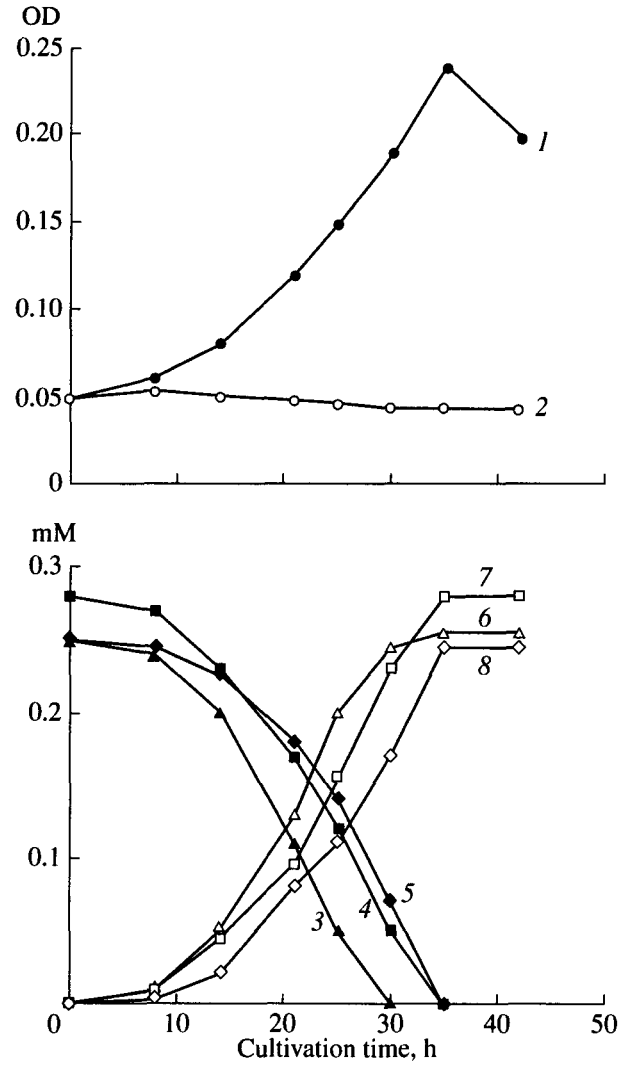

Fig. 3. Growth of *R. opacus* GM-14 on a mixture of 4-CP, 4-BP, and 4-IP: (*1*) experiment; (*2*) control (growth without halogenophenols); (*3*) 4-CP consumption; (*4*) 4-BP consumption; (*5*) 4-IP consumption; (*6*) Cl^- accumulation; (*7*) Br^- accumulation; and (*8*) I^- accumulation.

ical yield. Growth on chlorophenols was diauxic, with the first phase corresponding to the utilization of 4-CP and 3-CP and the second phase corresponding to the utilization of 2-CP.

The data obtained suggest that if a halogen substituent, no matter whether Cl, Br, or I, occurred in the aromatic ring at the same position, all the substrates were utilized simultaneously, with the concurrent accumulation in the medium of the respective halogen ions in stoichiometric amounts. However, when the growth substrate represented a mixture of phenols with the same halogen substituent (e.g., Cl) at different positions (*ortho, meta*, or *para*), substituted phenols were consumed successively. In this case, the growth of *R. opacus* GM-14 was diauxic due to the existence in this strain of two modified metabolic pathways for the *ortho*-cleavage of halogenated pyrocatechols (Fig. 1). The first pathway included 4-halogeno- or dihalogenopyrocatechol as intermediates, whereas the second pathway included 3-halogenopyrocatechol [12].

Two chloropyrocatechol 1,2-dioxygenases have recently been revealed in another *R. opacus* strain, 1cp [17]; the activities of these enzymes with 3-CPC and 4-chloropyrocatechol (4-CPC) in the extracts of cells grown on 2-CP were very close (10 and 13 U/mg protein, respectively). The activities of purified chloropyrocatechol 1,2-dioxygenases with 4-CPC were 1.7-times higher than with 3-CPC [17]. Conversely, the 3-halogenopyrocatechol 1,2-dioxygenase of *R. opacus* GM-14 grown on 2-CP, 2-BP, chlorobenzene, bromobenzene, or iodobenzene had 1.6–3.8 times higher activity with 3-CPC than with 4-CPC and was almost inactive with dihalogenopyrocatechols (unpublished data). The 4-halogenopyrocatechol 1,2-dioxygenase

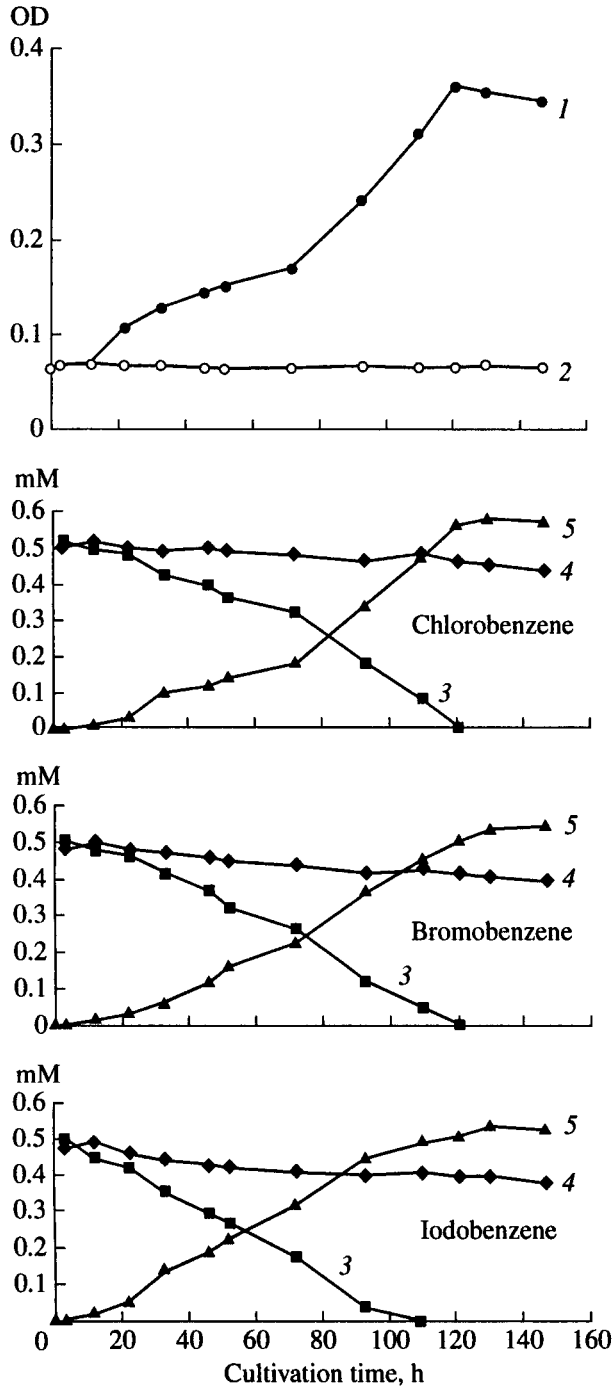

Fig. 4. Growth of *R. opacus* GM-14 on a mixture of chloro-, bromo-, and iodobenzene: (*1*) experiment; (2) control (growth without halogenobenzenes); (*3*) consumption of halogenobenzenes; (*4*) consumption of halogenobenzenes in the uninoculated medium; (*5*) accumulation of halogen ions in the experimental medium.

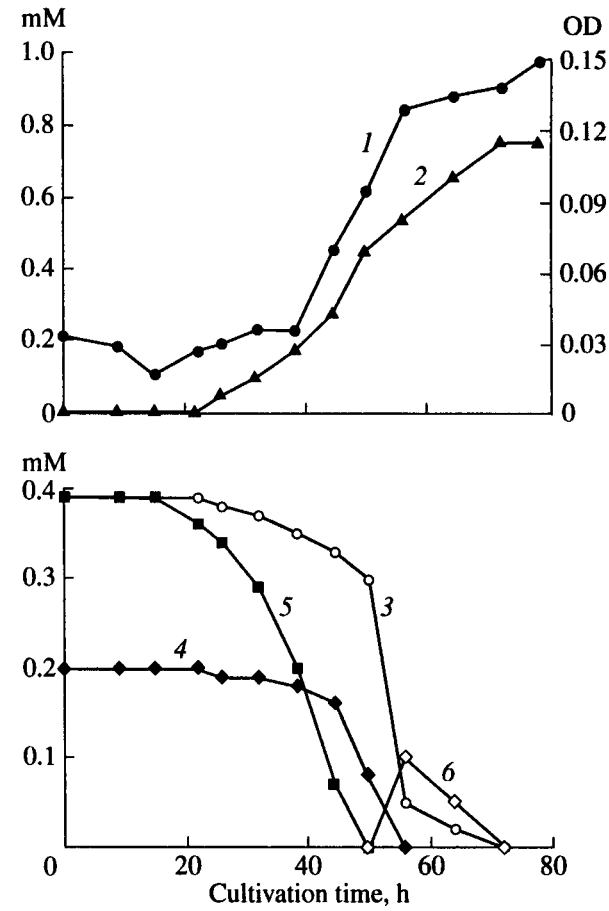

Fig. 5. Growth of *R. opacus* GM-14 on a mixture of 2-CP, 3-CP, and 4-CP: (*1*) growth; (*2*) accumulation of Cl^-; (*3*) consumption of 2-CP; (*4*) consumption of 3-CP; (*5*) consumption of 4-CP; and (*6*) accumulation of 3-CPC.

from cells grown on 3-CP, 3-bromophenol (3-BP), 4-CP, 4-BP, 4-IP, 2,4-dichlorophenol, 1,3-dichlorophenol, 1,4-dichlorobenzene, 1-bromo-3-chlorobenzene, or 1-bromo-4-chlorobenzene possessed 2–5.6 times higher activity with 4-CPC than with 3-CPC and was active with dihalogenopyrocatechols. In our opinion, the different substrate specificities of the halogenopyrocatechol 1,2-dioxygenases synthesized by *R. opacus* GM-14 may be one of the reasons for the diauxy of this strain during its growth on mixtures of halogenated aromatic compounds substituted at different atoms of the aromatic ring.

In addition to the typical muconate cycloisomerase, *R. opacus* GM-14 may also contain two halogenomuconate cycloisomerases, 2-halogenomuconate cycloisomerase and 3-halogenomuconate cycloisomerase, which differ in the substrate specificity and are responsible for the dehalogenation of halogenomuconic acids. This inference is partially confirmed by the following fact. The growth of *R. opacus* GM-14 on a mixture of 2-CP, 3-CP, and 4-CP was accompanied by the incomplete dechlorination of the substrates (Fig. 5), whereas the utilization of mixtures of monohalogenated benzenes or halogenated phenols with substituents occurring at the same atoms of the aromatic ring was accompanied by the stoichiometric accumulation of halogen ions in the culture liquid (Figs. 2–4).

Further studies of the properties, regulation, and genetic determinants of the enzymes of two modified

metabolic pathways of the *ortho*-cleavage of halogenated pyrocatechols in *R. opacus* GM-14 should essentially contribute to our knowledge of the regularities of formation of novel traits in natural microbial populations.

REFERENCES

1. Kozyreva, L.P. and Golovleva, L.A., Growth of *Nocardioides simplex* on a Mixture of 2,4,5-T and 2,4-D Herbicides, *Mikrobiologiya*, 1993, vol. 62, pp. 189–192.
2. Brunsbach, F.R. and Reineke, W., Degradation of Chlorobenzoates in Soil Slurry by Special Organisms, *Appl. Microbiol. Biotechnol.*, 1993, vol. 39, pp. 117–122.
3. Brunsbach, F.R. and Reineke, W., Degradation of Chloroanilines in Soil Slurry by Specialized Organisms, *Appl. Microbiol. Biotechnol.*, 1993, vol. 40, pp. 402–407.
4. Brunsbach, F.R. and Reineke, W., Degradation of Chlorobenzenes in Soil Slurry by a Specialized Organism, *Appl. Microbiol. Biotechnol.*, 1994, vol. 42, pp. 415–420.
5. Brunsbach, F.R. and Reineke, W., Degradation of Mixtures of Chloroaromatic Compounds in Soil Slurry by Mixed Cultures of Specialized Organisms, *Appl. Microbiol. Biotechnol.*, 1995, vol. 43, pp. 529–533.
6. Golovleva, L.A., Zaborina, O.E., and Arinbasarova, A.Yu., Degradation of 2,4,6-TCP and Mixture of Isomeric Chlorophenols by Immobilized *Streptomyces rochei* 303, *Appl. Microbiol. Biotechnol.*, 1993, vol. 38, pp. 815–819.
7. Haigler, B.E., Pettigrew, C.A., and Spain, J.C., Biodegradation of Mixtures of Substituted Benzenes by *Pseudomonas* sp. Strain JS150, *Appl. Environ. Microbiol.*, 1992, vol. 58, pp. 2237–2244.
8. Hollender, J., Dott, W., and Hopp, J., Regulation of Chloro- and Methylphenol Degradation in *Comamonas testosteroni* JH5, *Appl. Environ. Microbiol.*, 1994, vol. 60, pp. 2330–2338.
9. Puhakka, J.A., Herwig, R.P., Koro, P.M., Wolfe, G.V., and Ferguson, J.F., Biodegradation of Chlorophenols by Mixed and Pure Cultures from Fluidized-Bed Reactor, *Appl. Microbiol. Biotechnol.*, 1995, vol. 42, pp. 951–957.
10. Zaitsev, G.M., Uotila, J.S., Tsitko, I.V., Lobanok, A.G., and Salkinoja-Salonen, M.S., Utilization of Halogenated Benzenes, Phenols, and Benzoates by *Rhodococcus opacus* GM-14, *Appl. Environ. Microbiol.*, 1995, vol. 61, pp. 4191–4201.
11. Tsitko, I.V., Zaitsev, G.M., Lobanok, A.G., and Salkinoja-Salonen, M.S., Effect of Aromatic Compounds on Cellular Fatty Acids Composition of *Rhodococcus opacus, Appl. Environ. Microbiol.*, 1999, vol. 65, pp. 853–855.
12. Zaitsev, G., Uotila, J., Tsitko, I., Lobanok, A., and Salkinoja-Salonen, M., Microbial Catabolism of Halogenated Benzenes, Phenols, and Benzoates by *Rhodococcus rhodochrous* Strain GM-14, Abstracts of Papers, *7th Int. Congr. on Bacteriology and Applied Microbiology Division*, Prague, Int. Union Microbiol. Soc., Singapore, 1994, p. 533.
13. Zaitsev, G.M., Tsitko, I.V., Uotila, J.S., and Salkinoja-Salonen, M.S., Initial Steps in the Metabolism of Mono- and Dichlorinated Benzenes and Phenols by *Rhodococcus opacus* GM-14, *Environmental Research in Finland Today, Second Finnish Conf. on Environmental Sciences*, Saski, E. and Saarinen, T., Eds., Helsinki: Hakapaino Oy, 1995, pp. 205–207.
14. Zaitsev, G.M., Tsitko, I.V., Lobanok, A.G., Vazhinskaya, I.S., and Chechenin, V.P., Utilization of Halogenobenzenes by *Rhodococcus rhodochrous, Dokl. Acad. Nauk Belarus,* 1993, vol. 37, pp. 65–68.
15. Bergmann, J.G. and Sanik, J., Determination of Trace Amounts of Chloride in Naphtha, *J. Anal. Chem.*, 1957, vol. 29, pp. 241–243.
16. *Standard Methods for the Examination of Water and Wastewater*, 17th Ed., Clesceri, L.S. *et al.*, Eds., Washington: American Public Health Association, 1989.
17. Moiseeva, O.V., Lin'ko, E.V., Baskunov, B.P., and Golovleva, L.A., Degradation of 2-Chlorophenol and 3-Chlorobenzoate by *Rhodococcus opacus* 1cp, *Mikrobiologiya*, 1999, vol. 68, no. 4, pp. 461–466.