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Biodegradation of Aromatic Hydrocarbons and Phenols by Bacteria Isolated from Caspian Waters and Soils

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Abstract—Experimental studies have been carried out on the degradability of monoaromatic hydrocarbons (benzene, toluene, and ethylbenzene) and phenols (phenol, pyrocatechol, hydroquinone, tetrachloropyrocatechol) by bacteria isolated from coastal waters and soils of the Absheron peninsula at the Caspian Sea. It has been shown that the degradation of pyrocatechol, in particular, by *Pseudomonas* sp. bacteria occurs with the cleavage of the aromatic ring in two directions (in the *meta*- and *ortho*-positions), and that of tetrachloropyrocatechol involves the cleavage of the aromatic ring in the *ortho*-position and the formation of tetrachloromuconic acid. By reversed-phase high-performance liquid chromatography, more than ten individual compounds were detected and identified in the biodegradation products under the test conditions. It has been also shown that mainly linear functional compounds formed by aromatic ring opening in the *meta*- or *ortho*-position are found in the case of pyrocatechol and hydroquinone.

Keywords: biodegradation, phenols, aromatic hydrocarbons, microorganisms, microbial synthesis, liquid chromatography

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It is known that aromatic compounds are widely distributed in the nature [1–4] and they are major environmental pollutants [5–7]. Of all approaches to the problem of removal of aromatic pollutants from environment, their microbial degradation is the most promising. Destructor microorganisms catabolize aromatic substances, transforming them into hydroxylated derivatives with the subsequent opening of the benzene ring and the formation of numerous easily utilized substrates [1, 8–12]. The biodegradation products formed are of interest from the standpoint of research in the basic principles of utilization (biodegradation) of aromatic compounds and prospects for their use in biotechnologies of environmental remediation. The study of microbial degradation of petroleum hydrocarbons, including phenolic compounds, is important for the development of methods for bioremediation of the environment from crude oil and phenolic contaminants [13–15], so we have continued research in this area [13, 16, 17].

The aim of the present work is to study the products of biodegradation of some monoaromatic hydrocarbons (benzene, toluene, and ethylbenzene) and phenols (phenol, pyrocatechol, tetrahloropyrocatechol, and hydroquinone) by bacteria isolated from water and soil of the Absheron peninsula at the Caspian Sea.

EXPERIMENTAL

The Stolbunov's medium was used to isolate the bacteria [18]. The salinity of the medium was adjusted to 13%. Well-known microbiological methods were used for isolation of the bacteria on plain agar (PA) agarized media [19]. The isolated bacteria on the basis of their morphological, physiological and biochemical characteristics were attributed to the *Micrococcus, Bacillus, Pseudomonas, Arthrobacter, Mycobacterium, Acinetobacter, Aeromonas, Vibrio, Sarcina*, and *Alcaligenes* genera according to the Bergey's manual [20].

The intensity of the biodegradation process was indirectly assessed by the accumulation of the biomass of bacteria growing on individual aromatic and phenolic compounds during a month. In this experiment, 1–2 day cultures of microorganisms grown on the PA were used as the inoculum. The cells were washed off with 10 mL of distilled water; then, 5 mL of the resulting suspension was placed in flasks with 0.1 L of a nutrient medium containing benzene, toluene, phenol, pyrocatechol, or tetrahloropyrocatechol as the

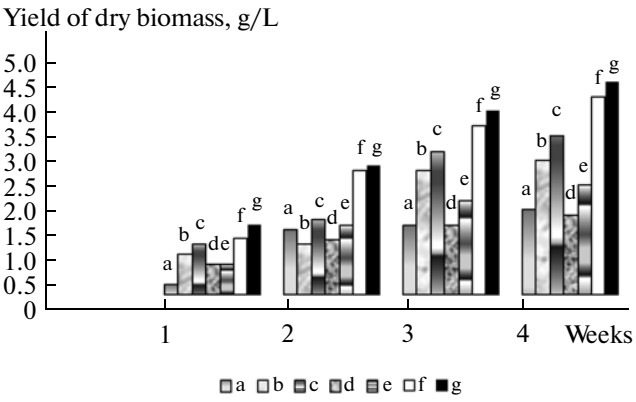
sole carbon source. In all the experiments the cultures were incubated at a temperature of 28°C. Further, the dynamics of the bacterial population and processes of biodegradation of aromatic compounds added to the medium in concentrations of 100, 300, and 500 mg/L were studied. After incubation, the biodegradation products were extracted with an equal volume of chloroform from the culture medium freed from cells by filtration.

Products of the degradation of aromatic and phenolic compounds were investigated by reversed-phase high-performance liquid chromatography (HPLC). Samples of initial and residual (after biodegradation) aromatic compounds were analyzed by the NMR and IR spectroscopic techniques as well [21–23].

Chromatographic investigations were carried out on a Kovo high-performance liquid chromatograph (Czech Republic) with an UV spectrophotometric detector operating at a wavelength of $\lambda = 254$ nm. Two 3.3×150 mm columns packed with the Separon SGX-C18 reverse stationary phase (particle size 7 μm; temperature, 20–25°C) were used. Eluent, methanol : water (75 : 25 vol. %). Mobile phase flow rate, 0.3 mL/min. The components were identified by comparing the retention parameters for a standard mixture and biotransformation products. Standard solutions with a concentration of 1–1.5 mg/mL were prepared in the methanol : water (75 : 25% vol.%) eluting system by the conventional procedure [23, 24]. The structural composition of the products of biodegradation of individual hydrocarbons and phenolic compounds was determined by the methods of infrared spectroscopy (UR-20) (thin layer) in the spectral range 4000–700 cm^{-1} and ^1H NMR spectroscopy (Tesla BS-487B spectrometer with an operating frequency of 80 MHz; solvent, CCl_4; internal standard, hexamethyldisiloxane (HMDS)). Control tests (without introducing biodestructor bacteria) were performed for all the experiments.

RESULTS AND DISCUSSION

The experiments showed that all investigated aromatic hydrocarbons and phenolic compounds served as the only carbon source for the growth of each of the isolated group of bacteria. Of the isolated bacteria strains, 47% grew on benzene; 54%, on ethylbenzene; 43%, on toluene; 100%, on phenol and pyrocatechol; 70%, on hydroquinone; and 13%, on tetrachloropyrocatechol. The accumulation of biomass in the medium with admixed aromatics (benzene, toluene, ethylbenzene) and phenolic compounds (phenol, pyrocatechol, hydroquinone, tetrachloropyrocatechol) at a concentration of 500 mg/L is shown in the figure. As can be seen in the figure, the accumulation of biomass for the *Pseudomonas* sp. strain occurred more intensely upon utilization of phenolic compounds than with aromatic ones, with the biomass varying from 1.6 g/L to 4.5 g/L.



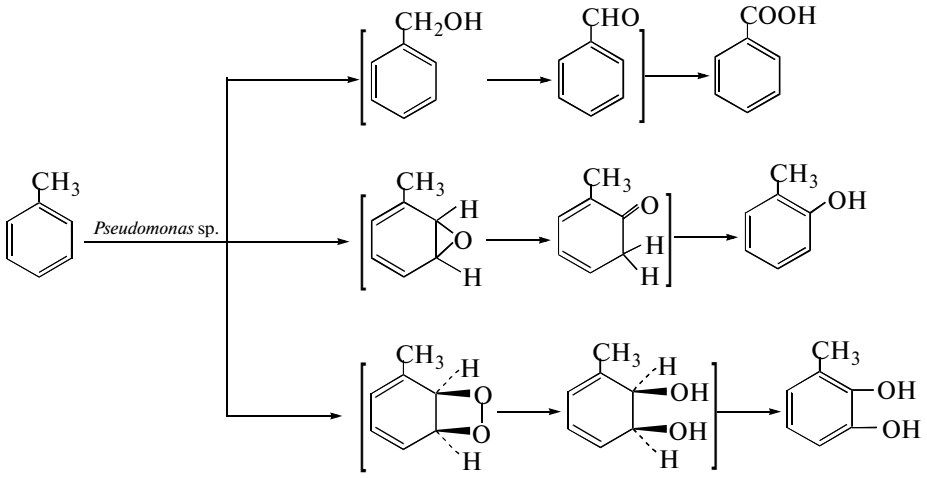
The yield of *Pseudomonas* sp. biomass upon growth on aromatic ((a) toluene, (b) benzene, (c) ethylbenzene) and phenolic ((d) hydroquinone, (e) tetrachloropyrocatechol, (f) pyrocatechol, (g) phenol) compounds.

The results show that the degradation of the test compounds by the action of the above mentioned microorganisms is accompanied by the transformation of the compounds to corresponding phenols, carboxylic and phenolcarboxylic acids, polyhydric phenols, benzoquinones, as well as to small quantities of polymers. Note that the identification of the biotransformation products by HPLC analysis was carried out at the final step of the microbiological synthesis with the formation of individual organic compounds.

As follows from the chromatographic data, starting from the oxidation of the aromatic ring the degradation of *benzene* after 6 days results in the formation of pyrocatechol and phenol in amounts of 30% and 60%, respectively.

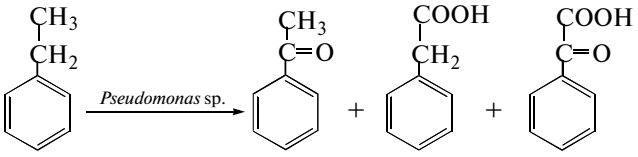
Along with the chromatographic determination data, the IR and ^1H NMR data were examined; they confirmed the structure of the products. The IR spectra of the benzene biodegradation products exhibited absorption bands in the region of 3590–3650 cm^{-1} typical of the hydroxyl group along with the bands characterizing the phenyl ring. The chemical shifts of aromatic ring and hydroxyl protons in their ^1H-NMR spectra were detected at $\delta = 6.25–7.2$ ppm and $\delta = 7.65–9.00$ ppm, respectively, [21, 22].

In the case of *toluene* degradation, basically three individual compounds were detected; *o*-cresol (50%), benzoic acid (30%), and 2,3-dihydroxytoluene (10%) prevailed in the products. The conversion occurred through the formation of intermediates; and, thus, based on the known theoretical concepts, we can propose the following scheme of toluene biodegradation by the action of *Pseudomonas* sp.:



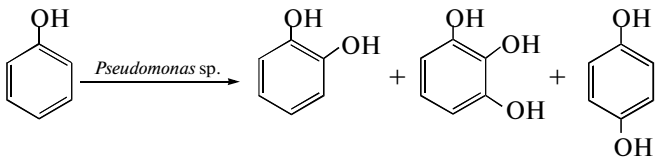
The IR spectra of the toluene biodegradation products displayed absorption bands characteristic of hydroxyl (3590–3650 cm^{-1}) and carboxyl (1680–1700 cm^{-1}) groups along with the Ar–CH_3 bands. The ^1H NMR spectrum of the degradation products displayed signals at δ 2.1–2.8 ppm, δ 7.70–9.00 ppm, and δ 9.5–10.5 ppm characteristic of the protons of the Ar–CH_3, Ar, Ar–OH, and Ar–COOH groups.

In [13], we showed that ethylbenzene is converted into acetophenone and phenylacetic acid by the action of micromycetes under co-oxidative conditions. In contrast, experiments in this study have shown that the degradation of ethylbenzene by the action of *Pseudomonas* sp. bacteria yields benzoylformic acid (30%) in addition to acetophenone (28%) and phenylacetic acid (45%), according to the following scheme:



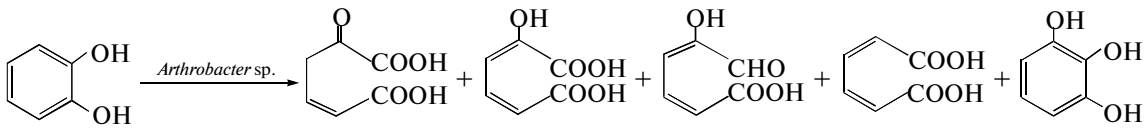
Thus, the transformations of toluene and ethylbenzene under the conditions developed in this work occur in two directions simultaneously, involving the oxidation of the side chain and the aromatic ring. Despite the fact that the degradation of toluene via the mixed mechanism is known from the literature [8], the composition of the oxidation products in this case differs from the one found previously. Thus, in [12, 13] it was shown that toluene degradation occurs in different directions, depending on the microorganism species. Interestingly, the compounds formed in the prior experiments [13, 16, 17] were not detected in this study. This suggests that the same bacteria cultured under different conditions and isolated from different sources may differ in the mechanism of action.

The chromatographic analysis of *phenol* biodegradation by the action of phenol-digesting bacteria showed that phenol under the test conditions is transformed into polyhydric phenols: pyrocatechol (40%), pyrogallol (20%), and hydroquinone (10%). Among the phenol biotransformation products (ca. 15%), a polymer fraction with a relatively diffuse band was detected; the fraction corresponds to oligophenylene with a molecular weight of ~3500.



It was found that unlike aromatic hydrocarbons and phenol investigated in the study, the degradation in the case of *pyrocatechol* and *hydroquinone* biotransformation by *Pseudomonas* sp. bacteria proceeded mainly via the route involving aromatic ring opening in the *ortho*- and *meta*-positions. By analysis of the chloroform extracts of pyrocatechol and hydroquinone, linear compounds relating to muconic acid

and its various derivatives were successively revealed. Thus, 2-keto-*cis,cis*-muconic acid (10%), 2-hydroxy-*cis,cis*-muconic acid (20%), semialdehyde of 2-hydroxy-*cis,cis*-muconic acid (20%), *cis,cis*-muconic acid (40%) and pyrogallol (5%) are formed in the case of pyrocatechol, as shown in the following scheme:



But in the case of hydroquinone, 3-keto-*cis,cis*-muconic acid (25%), 4-hydroxy-*cis,cis*-muconic acid (35%), semialdehyde of 4-hydroxy-*cis,cis*-muconic acid (30%), and hydroxyhydroquinone (4%) are formed.

The IR spectra of the pyrocatechol and hydroquinone biodegradation products showed the absorption bands characteristic of the aromatic ring, the double bond (1600–1660 cm^{-1}), and the carboxyl group (1700–1725 cm^{-1} and 3560–3650 cm^{-1}). In this case, the absorption bands of the aldehyde group are found at 1685 cm^{-1}. In their ^1H NMR spectra, double-bond protons appear at δ 6.44–6.8 ppm ($J = 9.3$ Hz). Protons of the carboxyl group appear as a singlet at δ 9.5–10.5 ppm; protons of the aromatic ring are identified as a multiplet at δ 6.25–7.25 ppm, and those of the aldehyde group are manifested as a singlet at δ 9.25 ppm.

The further study showed that unlike pyrocatechol, *tetrachloropyrocatechol* undergoes biodegradation only in one direction to form tetrachloromuconic acid (yield up to 13%), the structure of which was also confirmed by IR and ^1H NMR data. The IR spectrum of the acid exhibits absorption bands at 1720 cm^{-1} due to the carboxyl group in addition to the absorption bands of the C–Cl (650 cm^{-1}) and C=C– (1625 cm^{-1}) bonds. Signals at δ 7.80–7.90 ppm typical of the carboxyl protons were found in the ^1H NMR spectrum.

The onset of the transformation of the test compounds by the isolated bacteria in all cases was observed as early as after 6–7 days. The formation of intermediates lasted for 3–4 days. During this time, the chromatograms of the substrates showed not only a contact time-dependent decrease in the intensity of their UV signals, but also a simultaneous growth in the intensity of signals due to biotransformation products. Note that these data relate only to the final stage of the biotransformation of the test substrates, whose composition remained stable for a certain period of time (20–25 days). This period of time is essential for the treatment of the individual compounds produced. It was found that their complete utilization until the complete disappearance of the corresponding signals in the chromatograms takes 30 days or less.

Thus, the biodegradation of aromatic hydrocarbons (benzene, toluene, ethylbenzene) and phenols (phenol, pyrocatechol, hydroquinone, tetrachloropyrocatechol) by bacteria of the *Pseudomonas* sp. genus isolated from coastal waters and soils of the Absheron peninsula in the Caspian Sea has been studied. More than ten individual compounds, biodegradation products, were discovered and identified by reversed-phase liquid chromatography in the biodegradation products of the test compounds. Of these products, only the compounds detected in the case of pyrocatechols, which degrades via aromatic ring opening in the *meta*- and *ortho*-positions, were found to be linear. It is shown that unlike the case of pyrocatechol, the decomposition of tetrachloropyrocatechol occurs only in the *ortho*-position with the formation of tetrachloromuconic acid. The results can be used in fine organic synthesis for the production of practically useful functional compounds (acids, ketones, aldehydes, etc.) [25].

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