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Conversion of 4-Chlorophenoxyacetic Acid by the *Pseudomonas* sp. 36DCP Strain

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Abstract—The new natural 36DCP strain, which is capable of using 4-chlorophenoxyacetic acid (4-CPA) as the sole source of carbon and energy, was characterized. A culture isolated from a soil sample contaminated with chemical waste was identified according to physiological, biochemical, cultural, morphological features and analysis of the 16S rRNA gene sequence as a strain belonging to the *Pseudomonas* genus. In the batch culture, *Pseudomonas* sp. 36DCP degraded 45% of 4-CPA from the initial concentration after 5 days of incubation. Based on the identified intermediate metabolites in the culture medium (*para*-benzoquinone and hydroquinone), it was found that the conversion of 4-CPA in *Pseudomonas* sp. 36DCP proceeds through the hydroquinone pathway. This degradation pathway of monochlorinated phenoxyacetic acids has not previously been described in bacteria.

Keywords: chlorophenoxy herbicides, 4-chlorophenoxyacetic acid, *para*-benzoquinone, hydroquinone, *Pseudomonas*

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INTRODUCTION

4-Chlorophenoxyacetic acid (4-CPA) belongs to the group of chlorophenoxy herbicides widely used as plant growth regulators, most often for the treatment of tomatoes. This group also includes the 2,4,5-trichlorophenoxyacetic (**2,4,5-T**), 2,4-dichlorophenoxyacetic (**2,4-D**), and 2-methyl-4-chlorophenoxyacetic (**2M-4C**) acids, which were used as selective herbicides at the end of World War II. The large-scale use of chlorphenoxyacetic acids as agrochemicals throughout this time caused significant pollution of soil and groundwater [1]. Although chlorophenoxy herbicides are resistant to environmental exposure and can persist in the soil for a long time, there are bacteria capable of their aerobic biodegradation [2–6].

It is known that the microbial conversion of such simple (chloro)aromatic compounds can occur in two ways via the formation of various key metabolites and, as a rule, it depends on the halogenation degree of the substrate. Usually, bacteria catalyzing the aerobic degradation of phenols, as well as their mono- and dichloro derivatives, transform them to (chloro)catechols, which are further metabolized through *ortho*- or modified *ortho*-cleavage pathways [7]. Strains that are capable of converting more highly halogenated phenols hydroxylate the benzene ring in two stages with the production of intermediate products, (chloro)hydroquinones [8].

Mono- and dichlorinated phenoxyacetic acids, including 2,4-D, are mainly transformed according to the first pathways. The *Cupriavidus necator* strain (formerly *Alcaligenes eutrophus*, *Ralstonia eutropha* and *Wauteria eutropha*) JMP134 converts the above compound through 3,5-dichlorocatechol, the aromatic ring of which is then subjected to *ortho*-cleavage [2]. This pathway is peculiar to the majority of 2,4-D degraders known to date [4, 5, 9, 10]. The conversion of higher chlorinated phenoxyacetic acids proceeds according to the second metabolic variant. For example, the key metabolites of the 2,4,5-T degradation pathway by the *Burkholderia phenoliruptrix* strain (formerly *Pseudomonas cepacia*, *Burkholderia cepacia*) AC1100 are chloro derivatives of hydroquinone and hydroxyquinone [11, 12].

The goal of the work is to study the characteristics of the 4-CPA conversion by a new strain isolated from soil contaminated with chloroaromatic compounds.

MATERIALS AND METHODS

The natural bacterial strain 36DCP, which was isolated from a soil sample contaminated with chemical production waste, served as the object of study (Ufa, Russia).

Enrichment and pure cultures were obtained with the use of M9 minimal salts medium [13] of the following composition (g/L) containing 4-CPA (100 mg/L) as the sole carbon source: Na_2HPO_4—6.0; KH_2PO_4—3.0; NaCl—0.5; NH_4Cl—1.0. After autoclaving, sterile solutions of $CaCl_2$ and $MgSO_4$ were added to the medium to final concentrations of 1.0 and 2.0 mM, respectively.

The cultures were grown in conical flasks (250 mL) at a temperature of 28°C in a UVMT-12-250 thermostatic device (Elion, Russia) at 120 rpm. The growth intensity of the culture was estimated by the optical density (OD_{590}) of the cell suspension with a KFK-2 photocolorimeter (ZOMZ, Russia).

The morphometric characteristics were obtained via transmission electron microscopy on an H-300 microscope (Hitachi, Japan) at 18000 magnification (75 kV).

The cultural, physiological, and biochemical properties of the isolate were determined according to the methodological guideline [14].

Genomic DNA isolation, amplification of the partial 16S rRNA gene sequence, and sequencing were performed as described in [15]. During sequence analysis, multiple alignment and the further construction of a phylogenetic tree was performed as indicated previously [16]. A sequence of 1488 nucleotide pairs is deposited in the GenBank international database under the accession number MK072948.

The determination of the 4-CPA amount in the culture medium and extraction of metabolites were carried out according to the procedure described in [17]. The products of 4-CPA catabolism in methylated extracts were established with a Khromatek-Kristall 5000 chromatograph mass spectrometer (SKB Khromatek, Russia) based on a Finnigan DSQ II quadrupole mass spectrometric detector (ThermoFinnigan, United States). The analysis conditions included a HP-5MS capillary column 30 m × 0.2 mm and hexane as the solvent; the temperature of the injector and interface was 250°C, the initial temperature of the column was 50°C, the heating rate was 20 degrees/min, and the final temperature of the column was 250°C. The compounds obtained were identified via comparison of their mass spectra with the NIST05 spectra library. The results were processed with the Xcalibur software package.

Dioxygenase, which cleaves the aromatic ring, was inhibited according to the procedure described in [18] with minor modifications. The cells of the 36DCP strain (1.0 g) in the middle of the lag growth phase were collected by centrifugation (3630 *g*, 10 min) and resuspended in 100 mL of 0.1 M phosphate buffer (pH 7.0); 200 μL of LB medium and 100 μL of 4-CPA and 2,2'-dipyridyl were added to a final concentration of 10 mM.

RESULTS AND DISCUSSION

Gram-negative cells of the 36DCP strain had the form of straight or slightly curved sticks 0.4 × 1.6 μm in size that were capable of moving due to polar flagella. The isolate formed transparent, smooth colonies with a denser towering center after 48 h of incubation on a meat infusion agar at 28°C. The culture produced extracellular fluorescent, yellow-green pigments; on a slice of potato, it produced blue pigments. The strain was characterized by aerobic growth with oxygen as the final electron acceptor in a temperature range of 22–45°C and pH values close to neutral (6.8–7.2).

The culture used D-glucose, citrate, ethanol, and glycerol as the sole carbon source; showed catalase, lecithinase, and arginine dehydrolase activity; and reduced NO_3.

An almost complete sequence (1488 bp) of the amplification product of the gene encoding 16S rRNA was determined for the isolate. Bacteria of the *Pseudomonas* genus showed similarity to the studied culture (identity level 97.3–99.7%), and a typical representative of the species, *P. plecoglossicida* FPC951 (AB009457), turned out to be phylogenetically closest (identity level 99.7%). However, the 36DCP strain was clustered separately from the clade formed by the typical strains of *P. plecoglossicida*, *P. monteilii*, *P. taiwanensis*, *P. mosselii,* and *P. entomophila* species, although it had a common ancestor with them. At the same time, the reliability of branching within this cluster turned out to be lower than the required 50% threshold (Fig. 1). These results allowed us to identify the studied strain as *Pseudomonas* sp. 36DCP and to suggest that it may be a representative of a new type of bacteria.

In the batch culture, the *Pseudomonas* sp. 36DCP strain used 4-CPA as the sole source of carbon and energy (Fig. 2). The value of optical density (OD) of the cell suspension reached a maximum value (0.3 U) after 2 days of cultivation, while the consumption of the substrate was 10–15% per day. Further, after the unexpressed stationary phase, a decrease in cell suspension OD and, accordingly, in the substrate consumption to 3–5% was observed.

To date, the data on bacterial destruction of 4-CPA are limited to a few studies. Thus, early cultures of the strains *Flavobacterium peregrinum* and *Achromobacter* sp., grown on 2M-4C, completely oxidized 2 μM 4-CPA in 1.5 h, whereas older cultures showed less than 50% substrate oxidation even after 4 h [19].

The studied *Pseudomonas* sp. 36DCP strain was capable of 4-CPA decomposition in a fairly high concentration; moreover, it showed activity against other more chlorinated phenoxyacetic acids, namely, 2,4-D and 2,4,5-T, as well as against phenol and 2,4-dichlorophenol (unpublished data). Thus, the polysubstrate activity of the *Pseudomonas* sp. 36DCP strain can be

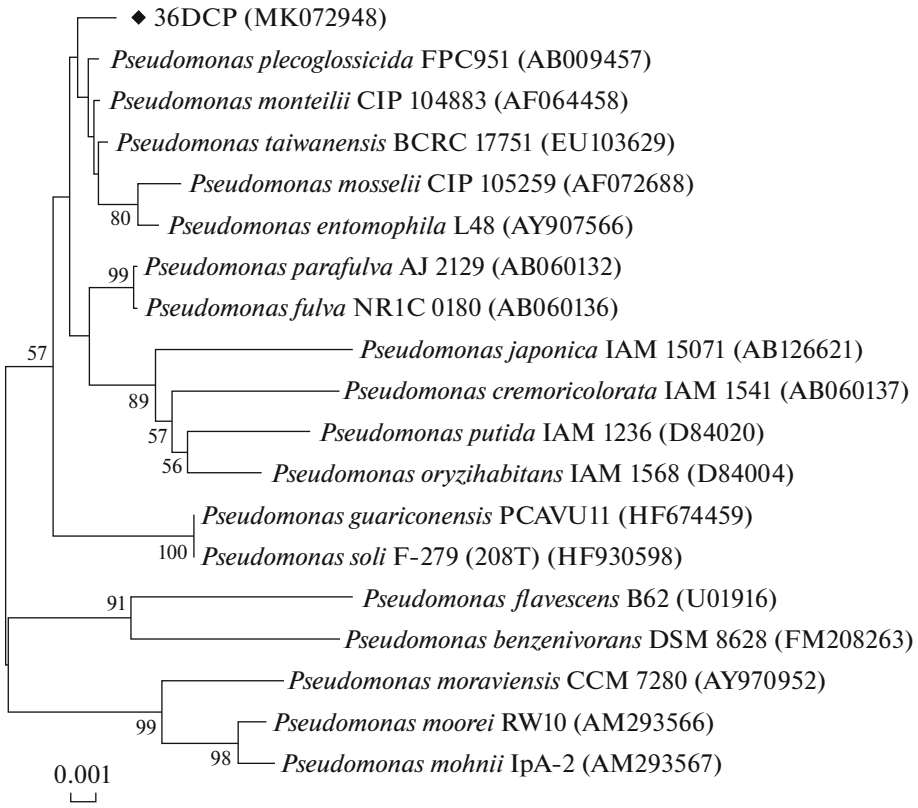

Fig. 1. Phylogenetic tree for 16S rRNA of the 36DCP strain and its homologous sequences of type bacterial species of the *Pseudomonas* genus constructed by the neighbor-joining method. The figures show the reliability of branching as calculated by bootstrap analysis (values greater than 50 are recognized as significant). The scale represents the evolutionary distance corresponding to 1 nucleotide substitution per 1000 nucleotides. The NCBI GenBank accession numbers are given in parentheses.

used to develop technologies for the purification of areas contaminated with chloroaromatic compounds.

The presence of two compounds, which are probably intermediates of the 4-CPA degradation pathway, was determined in the culture medium of the *Pseudomonas* sp. 36DCP strain. The mass spectrum was the sum of the mass spectra of two compounds identified as *para*-benzoquinone and hydroquinone. The total mass spectrum included peaks of molecular ions (M^+) with *m/z* 108 and 110 amu and peaks of fragmentation ions typical of both compounds (Fig. 3).

With the *Cupriavidus necator* JMP134 strain as an example, it was shown that, after the acetic acid residue is eliminated and the corresponding chlorophenol is formed, the aerobic degradation of mono- and dichlorinated phenoxyacetic acids proceeds according to the classical chlorocatechol pathway (Fig. 4) [2].

At the same time, the formation of hydroquinone derivatives is typical of the metabolism of more chlorinated substrates, in particular, 2,4,5-T in the *B. pheneliruptrix* AC1100 strain (Fig. 4.). The conversion is initiated as a result of the monooxygenase activity of 2,4,5-T-oxygenase, which converts 2,4,5-T to 2,4,5-trichlorophenol [20]. At the second and third stages, monooxygenase catalyzes two hydroxylations, the product of which is 5-chlorohydroxyhydroquinone. Sequential reactions of substrate reduction and oxidation then occur, resulting in the elimination of the last

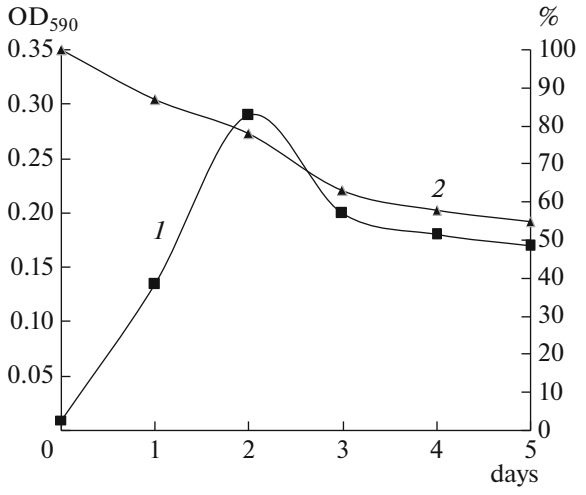

Fig. 2. Dependence of the OD_{590} values of the liquid culture (*1*) and the 4-CPA concentration (*2*) on the time of cultivation of *Pseudomonas* sp. 36DCP in the batch culture.

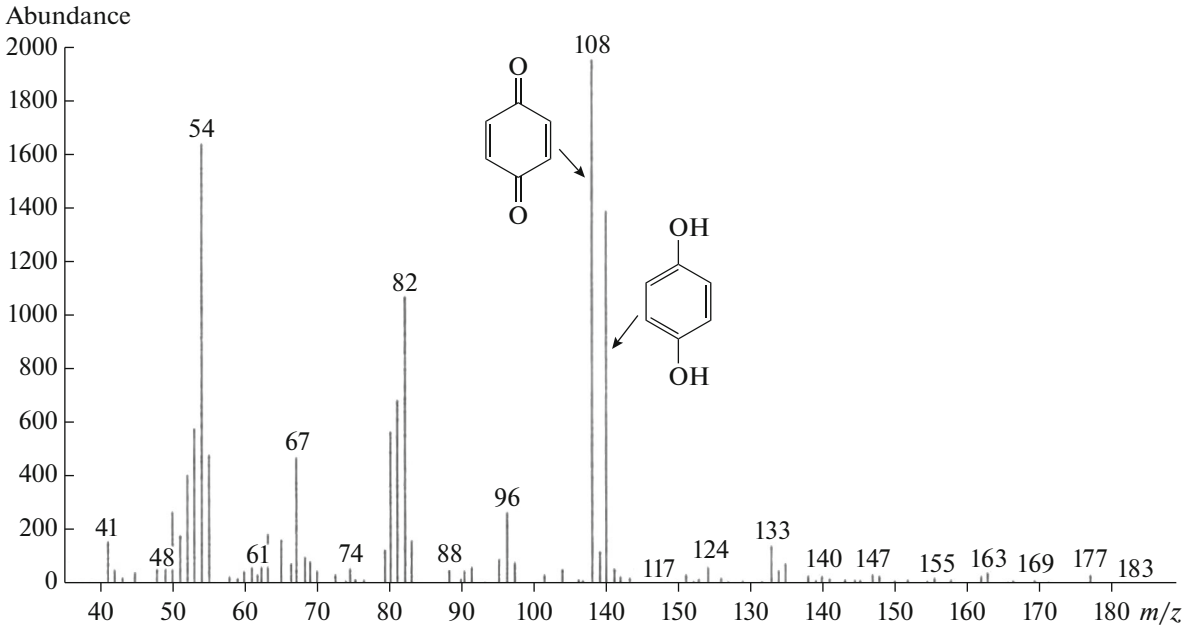

Fig. 3. Summarized mass spectrum of intermediates under the 4-CPA conversion by the *Pseudomonas* sp. 36DCP strain identified as *para*-benzoquinone (M^+ 108) and hydroquinone (M^+ 110).

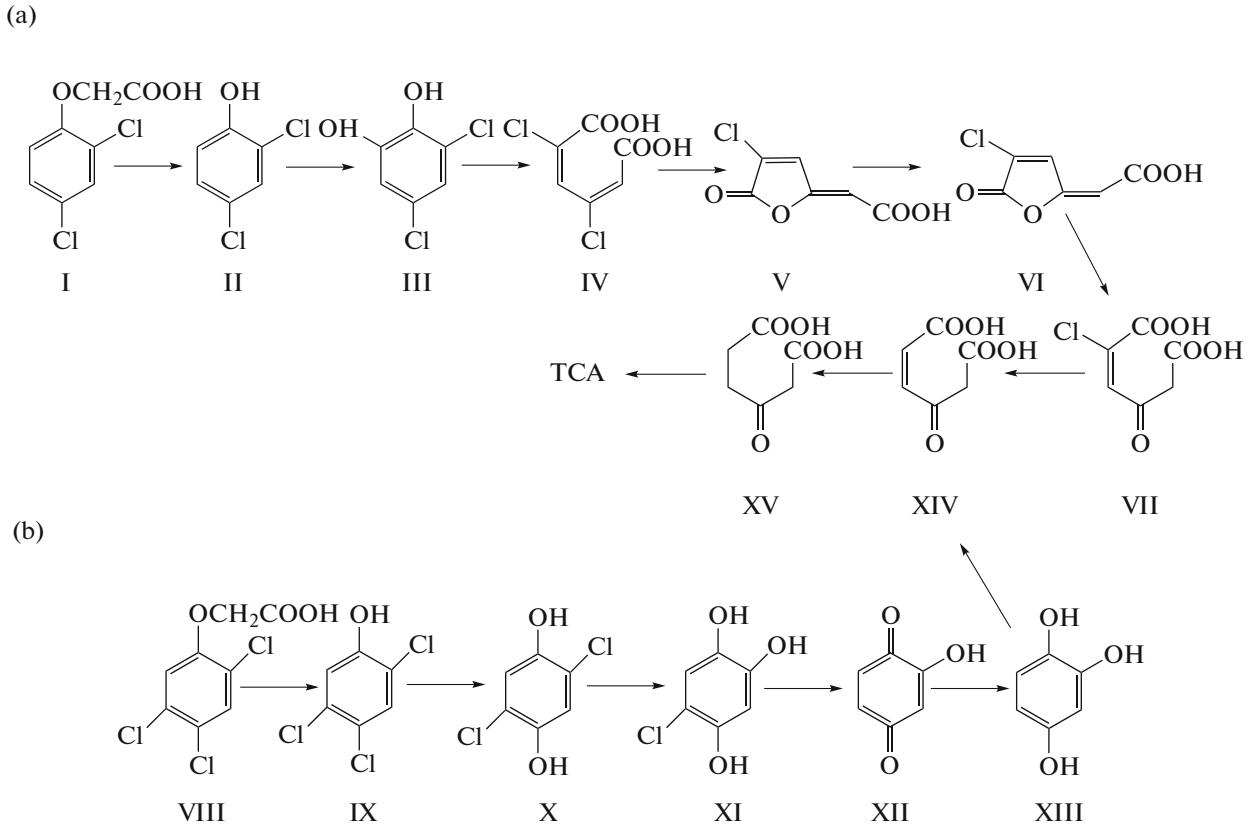

Fig. 4. Pathways of aerobic degradation of chlorinated phenoxyacetic acids in bacteria: (a) 2,4-D in the *C. necator* JMP134 strain [2], (b) 2,4,5-T in the *B. phenoliruptrix* AC1100 strain [11, 12]. I—2,4-D, II—2,4-dichlorophenol, III—2,4-dichlorocatechol, IV—2,4-dichloro-*cis*,*cis*-muconate, V—*trans*-2-chloro-dienolactone, VI—*cis*-2-chloro-dienolactone, VII—2-chloromaleylacetic acid, VIII—2,4,5-T, IX—2,4,5-trichlorophenol, X—2,5-dichlorohydroquinone, XI—5-chloro-2-hydroxy-*p*-hydroquinone, XII—2-hydroxy-1,4-benzoquinone, XIII—2-hydroxy-*p*-hydroquinone, XIV—maleylacetic acid, XV—β-ketoadipic acid, TCA - tricarboxylic acid cycle.

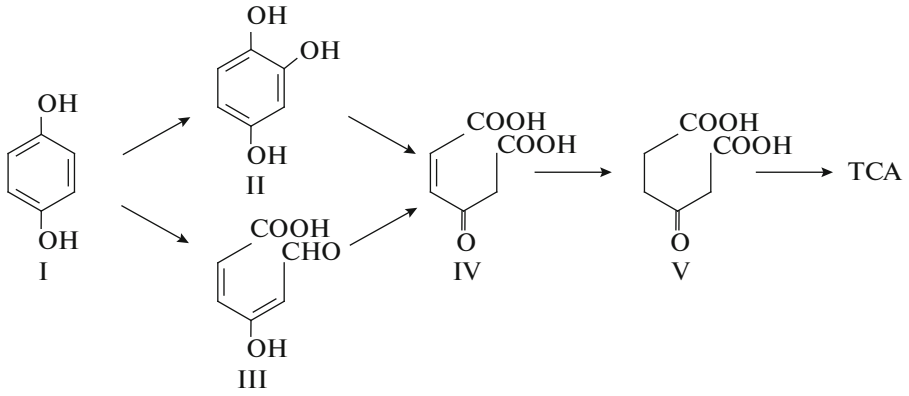

Fig. 5. Two branches of the hydroquinone aerobic degradation pathway [31]. I—hydroquinone, II—2-hydroxy-*p*-hydroquinone, III—4-hydroxymuconic semialdehyde, IV—maleylacetic acid, V—β-ketoadipic acid, TCA—tricarboxylic acid cycle.

chlorine atom and the formation of hydroxyhydroquinone, which is the substrate for the *ortho*-cleavage of the aromatic ring [11, 12]. It should be noted that the *B. phenoliruptrix* AC1100 strain was obtained by the method of so-called plasmid-associated molecular breeding [21], as a result of which the 2,4,5-T degradation pathway described above may be of a quite excessive nature and differ from the natural metabolic pathways of such compounds.

Thus, chlorophenols are products of the initiation stages of chlorophenoxyacetic acid degradation in all of the studied pathways of their metabolism. Comparative analysis suggests that chlorophenol, in which the halogen substituent is in *para*-position relative to the hydroxyl group, also initially forms as a result of 4-CPA degradation by the *Pseudomonas* sp. 36DCP strain. At the same time, the presence of hydroquinone in the culture medium shows that the further conversion of 4-CPA in the studied strain passes through the hydroquinone pathway.

It is known that both poly- and monosubstituted phenols can, via the hydroquinone pathway, be converted by bacteria. Thus, through the formation of hydroquinone derivatives of different degrees of halogenation, the metabolism of polychlorophenols proceeds, in particular, 2,4,6-trichlorophenol (2,4,6-TCP) in the aforementioned 2,4-D degrader *C. necator* JMP134 strain [22], and tri-, four- and pentochlorophenols in cultures of *Rhodococcus chlorophenolicus* PCP-I, *Rhodococcus* sp. CP-2, *Rhodococcus* sp. CG-1 and *Mycobacterium* sp. CG-2 [23, 24]. It is characteristic that the first two chlorine atoms are eliminated by hydroxylation in all cases, first in *para*- and then in *ortho*-position relative to the phenolic hydroxyl group of the substrate, to form the corresponding chloroderivatives of hydroquinone and hydroxyhydroquinone.

The initial reaction in the pathways of *para*-substituted phenol degradation, also involves the replacement of the *para*-substituent with the hydroxyl group, but the product is unsubstituted hydroquinone. In this case, an intermediate product, *para*-benzoquinone, first forms; it is then reduced to hydroquinone as a result of monooxygenase activity. Such a mechanism was established for *para*-nitrophenol degraders *Moraxella* sp., *Pseudomonas* sp. WBC-3, *Pseudomonas* sp. 1–7, *Arthrobacter protophormiae* RKJ100 [25–28]; for the 4-aminophenol degrader, *Burkholderia* sp. AK-5 [29]; and for the 4-fluorophenol degrader, *Arthrobacter* sp. IF1 [30].

Thus, the monooxygenase conversion of the proposed metabolite found in the culture medium, *para*-chlorophenol to *para*-benzoquinone (which is then reduced to hydroquinone), is most probable in the pathway of 4-CPA degradation by the *Pseudomonas* sp. 36DCP strain.

It is known that hydroquinone under aerobic conditions can be further directed through the β-ketoadipate pathway in bacteria via two different metabolic branches (Fig. 5). The first pathway includes the initial hydroxylation of hydroquinone with the formation of hydroxyhydroquinone (1,2,4-trihydroxybenzene) and the subsequent ring cleavage reaction, which was catalyzed by 1,2-dioxygenase. The second pathway of hydroquinone degradation is less common. In this case, the aromatic ring of hydroquinone is directly cleaved with the help of specific hydroquinone-1,2-dioxygenase, and the formed 4-hydroxymuconic semialdehyde is oxidized to maleylacetic acid [31].

The iron chelator 2,2'-dipyridyl was used to determine the hydroquinone pathway branch of the 4-CPA conversion in the studied strain. It is known that this compound inhibits the activity of many dioxygenases that cleave the aromatic ring [32]. There was an addition of 2,2'-dipyridyl to the culture in the lag phase, which actively increases biomass on 4-CPA as the sole source of carbon and energy. The metabolites were identified after 1 h of cultivation under the same conditions. Chromatography mass-spectrometry analysis showed the presence of only *para*-benzoquinone and

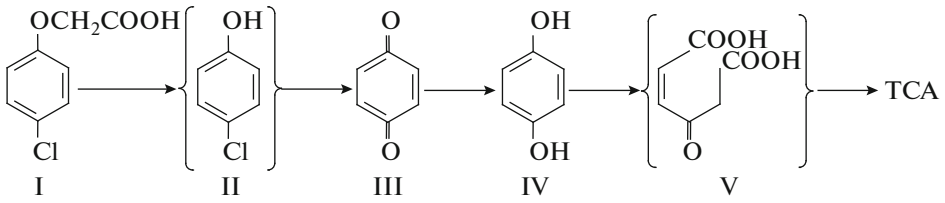

Fig. 6. Possible pathway for 4-CPA degradation in *Pseudomonas* sp. 36DCP. I—4-CPA, II—*para*-chlorophenol, III—*para*-benzoquinone, IV—hydroquinone, V—maleylacetic acid, TCA—tricarboxylic acid cycle. The proposed metabolites are in brackets.

hydroquinone in the sample. Thus, the absence of hydroxyhydroquinone in the culture medium indicates that hydroquinone is likely to be a substrate of dioxygenase, which cleaves the aromatic ring.

The direct cleavage of the aromatic ring of hydroquinone with the formation of 4-hydroxymuconic semialdehyde is known for the conversion of many environmentally significant compounds, e.g., *para*-substituted phenols (4-chloro, 4-fluoro, 4-bromo, 4-iodo, and 4-nitrophenol) in *Arthrobacter ureafaciens* CPR706 [33], lindane (γ-hexachlorocyclohexane) in *Sphingomonas* (formerly *Pseudomonas*) *paucimobilis* UT26 [34], 4-nitrophenol by strains *Moraxella* sp., *Pseudomonas* sp. WBC-3 and *Pseudomonas* sp. 1–7 [25–27], 4-hydroxyacetophenone in *Pseudomonas fluorescens* ACB [35], and alkylphenols in *Sphingomonas* sp. TTNP3 and *Sphingobium xenophagum* Bayram [36].

A metabolic pathway for the conversion of 4-CPA in *Pseudomonas* sp. 36DCP was proposed in the conducted study, which was based on the identified key metabolites in the culture medium (*para*-benzoquinone and hydroquinone) and by analogy with the other considered known biodegradation systems (Fig. 6).

CONCLUSIONS

Thus, the results suggest that the 4-CPA degradation in *Pseudomonas* sp. 36DCP begins with the transformation of the initial substrate to 4-chlorophenol, which is then directed along the pathway of *para*-substituted phenols. In this case, the monooxygenase conversion of 4-chlorophenol into *para*-benzoquinone, which is then reduced to hydroquinone, is the most probable. The latter is likely to be the substrate for further ring cleavage. It should be noted that such a pathway for the conversion of monochlorinated phenoxyacetic acids in bacteria was not previously described.

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COMPLIANCE WITH ETHICAL STANDARDS

The authors declare that they have no conflict of interest. This article does not contain any studies involving animals or human participants performed by any of the authors.

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