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REVIEW

Biochemical Features of the Degradation of Pollutants by *Rhodococcus* as a Basis for Contaminated Wastewater and Soil Cleanup[1]

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Abstract—*Rhodococcus* bacteria are considered to be promising degraders of persistent pollutants and are the basis of biological preparations for contaminated wastewater and soil cleanup. Biotechnological application of this group of bacteria is based on the peculiaraties of their metabolism. This review briefly discusses the following main points:
I. Growth of *Rhodococcus* on various aromatic substrates
II. Chloro/methylcatechol transformation pathways
3-Chlorocatechol branch of the modified *ortho*-pathway
4-Chlorocatechol branch of the modified *ortho*-pathway
Modified 3-chlorocatechol branch in *Rhodococcus opacus* 1CP
Meta-cleavage of chlorocatechols
Modified pathway for methylcatechol degradation
III. Approaches to the enhancement of degradation activity
IV. *Rhodococcus*-based biopreparations
V. Prospects

Keywords: *Rhodococcus*, aromatic compounds, degradation, pathways, key enzymes, bioremediation, biopreparations.

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INTRODUCTION

Pollution of the environment with persistent toxic compounds due to anthropogenic activities is one of the most urgent problems to date. Aromatic and polyaromatic hydrocarbons and their halogenated derivatives are among the most hazardous toxins. Organic compounds including phenols, biphenyls and their derivatives are widespread in wastewaters of petroleum refineries, gas and coal production plants; productions of plastic materials, synthetic oils, fire stewing liquids, dyes, herbicides, solvents and disinfectants. All of the above compounds are chemical products with low solubility in water and can be tightly adsorbed by biological materials. The pollutants are introduced into the environment either intentionally (e.g., chloroorganic pesticides, chemical plant protectants) or as a result of accidents and disasters. About 30000 tons of various chlorophenols were used from 1934 to 1988 in Finland only. Formation of 100 to 300 g of chlorinated phenol compounds (phenols, catechols, guaiacols, syringols and vanillins) per ton of pulp during its chlorine bleaching resulted in accumulation of chlorophenol compounds on the order of microgram per liter of water and milligram per kg (dry weight) of bottom sediments in the lakes into which the wastes were discharged [1].

Hexachlorobenzene is used in a mixture with other preparations as a seed disinfectant to control the diseases of wheat, rye, buckwheat, soya, and other cereal crops; it is a component of pyrotechnical mixtures used in the defense potential of the country. Polychlorinated biphenyls (PCBs) are a group of extremely hazardous toxicants. Most PCB preparations contain 60 to 90 different derivatives. Great industrial significance of PCBs is determined by their chemical inertness, resistance to high temperatures, incombustibility, low saturated vapour pressure and high dielectric constant. They are used mainly as cooling liquids in transformers, as dielectrics in large capacitors and as components of various products: lubricants, insulating materials (in construction industry), glues, plastic materials and rubber, insecticides, paints and varnishes, etc. Considerable amounts of these toxicants got into the environment from 1.5 million tons of

[1] The article is published in the original.

PCBs synthesized in the 1927–1980s [2], and now they are found in surface and ground waters, silts and soils. PCBs bind to organic soil components, accumulate in biological tissues and interact with insoluble organic carbon in water systems. The chemical properties of polychlorobiphenyls facilitate their widespread occurrence: they are found in air, water and animal organisms.

PCBs are extremely resistant to physical, chemical and biological changes [3]. Analysis of atmospheric air over European Russia in the recent 10–15 years has shown that the background content of anthropogenic admixtures in it remains low. Still, it is reasonable to assume that the concentration decreases of the 1990s due to a decline in industrial production have already come to an end, and background pollution of the atmosphere by some contaminants, especially in the cold season, could be expected to increase [4].

Polycyclic aromatic hydrocarbons (PAHs) also pose a serious problem for the environment due to their remarkable toxicity, mutagenic and carcinogenic properties. The most widespread and toxic among these compounds are benz(a)pyrene (BP), acenaphthene and pyrene. As PAHs are both direct and side products of many industries, the level of their pollution in the environment is already high and constantly increases. According to the data by the Federal Agency for Hydrometeorology and Environmental Monitoring [4], the level of atmospheric pollution is still high. The degree of air pollution is estimated to be very high and high in 142 cities (69% of the cities under observation) and to be low only in 17%. In Russia as a whole, 38% of urban population reside in territories, where no observations of atmospheric pollution have been carried out, and 55% in cities with high and very high levels of atmospheric pollution (58.2 million people).

Chlorophenols, another group of toxic pollutants, are able to dissociate oxidative phosphorylation, disturb microsomal detoxification and influence protein and RNA syntheses. Chlorinated biphenyls are also highly toxic compounds that can act on organisms at extremely low doses and affect the liver and kidney. Their chronic effect is similar to that of chlorine derivatives of naphthalene. They cause porphyria, i.e., activate the microsomal enzymes of the liver. This property becomes more pronounced as the content of chlorine in the molecules of chlorobiphenyls increases.

An extremely important issue is how the main classes of pollutants can be degraded. Physicochemical methods either do not ensure detoxification of chlorophenols or are very expensive compared to biological methods (e.g., the electron-beam method for preparation of drinking water, considered to be one of the most efficient and reliable, requires 5 kWt-h/m^3 at the output capacity of 50 m^3/h [5]). The same is with the incineration of chlorophenols: insufficiently high temperatures inevitably lead to immeasurably more toxic polychlorodioxins, and the required high temperatures make the process very expensive.

Only enzymatic dehalogenation enables degradation products of halogenated phenols, PCBs and PAHs, not hazardous for human health and the environment. As problems of environmental protection from various pollutants remain very up-to-date, great attention is paid to isolation of native strains to use them as a basis for developing microorganisms with extended or improved biodegradabilities.

The following several trends can be distinguished between in research into bacterial degradation of persistent toxicants. All of them ultimately aim at creating efficient technologies for biological cleanup of the environment:

1. Studies of bacterial degradation pathways for different classes of xenobiotics. This stage includes isolation and identification of microorganisms from polluted sites, establishment of the transformation pathways of toxicants through identification of metabolites, isolation of enzymes, their comparative characterization in different groups of microorganisms.

2. Research into the genetic basis of the degradation of xenobiotics, mainly (chlorinated) (poly)cyclic aromatic compounds, bearing in mind, first of all, biodegradation plasmids and other mobile genetic elements enabling the strains to exchange biodegradation genes, which leads to expand the biodegradation potential of bacteria inhabiting the Earth.

3. Use of native bacterial preparations for efficient elimination of pollutions.

4. Selection of hybrid strains with extended biodegradability. Construction of plasmids that carry the genes of different pathways, making it possible to overcome the "bottlenecks" of biodegradation in decomposing persistent pollutants; protein engineering for changing the catalytic properties of particular enzymes.

5. Development of biopreparations and introduction of (hybrid) strains into the regions with high levels of pollution, enabling a decrease of local pollutions (including the critical level).

Rhodococcus bacteria are an interesting and biotechnologically promising group of microorganisms: polluted sites and water bodies are often their natural habitats. They are stable inhabitants of ecosystems even under starvation conditions; degradation of pollutants by these organisms is not affected by the presence of more accessible carbon sources. Rhodococcal cells are hydrophobic due to the aliphatic chains of mycolic acids in their cell wall, which enables them to degrade hydrophobic pollutants through adhesion in the oil/water interface. Some rhodococcal strains are psychrophilic, which is highly significant for bioremediation in cold climates. These bacteria can degrade the broadest range of aromatic compounds [6–8]. Representatives of the genus are reported to be able to

carry out various transformation reactions with potential industrial applications [9]. According to the data by Martinková et al., the number of publications and patents on nitriles and aromatic compounds transformed by rhodococci has increased significantly after 1995 [8].

The goal of this review is to give the basic ideas of metabolic potential and biochemical basis of *Rhodococcus* biodegradation activity. We discuss methods of adapting cultures to degrade high concentrations of new substrates (both individual compounds and mixtures containing various classes of substituted aromatic compounds). Ways of increasing the concentrations of xenobiotics to be degraded are presented, as well as the principles of developing highly efficient biopreparations.

1. GROWTH OF *Rhodococcus* ON VARIOUS AROMATIC SUBSTRATES

Analysis of publications describing the degradation of various aromatic compounds by *Rhodococcus* bacteria demonstrates that biodegradation activity is an inherent property of this group of bacteria. Major xenobiotic-degrading strains of the genus *Rhodococcus* and substrates they utilize are given in Table 1. Previously we have isolated more than 50 cultures from soils, based on the characteristic of growth on 3-chlorobenzoic acid (3-CBA), 2,4-D, and 4-chlorophenol (4CP). Some of them were identified by the data of 16S RNA–DNA analysis as representatives of the genus *Rhodococcus*. In the collection of E.L. Golovlev, DSc (IBPM RAS) [36], which comprises about 500 strains of nocardioform microorganisms, about 20 of the most promising cultures from the group of *Rhodococcus* growing on aromatic compounds were tested by auxanography, with main attention on chlorinated phenols and benzoates. Bacteria of the genus *Rhodococcus* were found to degrade a broad range of aromatic compounds as a sole carbon and energy source. Two strains grew on an agarized medium with toluene. Table 1 shows the data of auxanographic analysis of the bacteria. Growth of most of the tested strains was maintained on methylated substrates: *para*-cresol and *para*-toluate.

Figure 1 shows the growth curves for some of the tested rhodococci grown on benzoate, *para*-toluate and phenol. Growth on benzoate was followed by the shortest lag phase. Almost all cultures growing on *p*-toluate had a long lag phase. Strains *Rhodococcus rhodochrous* 172, *Rhodococcus* sp. 400 and *R. opacus* 6a grew on this substrate most quickly: the optical density reached a maximum in less than 48 h [61].

Selection of strains by their growth on 4-methylbenzoic acid (4MBA) and chlorinated monoaromatic compounds (2-chlorobenzoate (2CBA), 4-chlorobenzoate (4CBA), monochlorophenols (CP), 2,4-D) showed that most of the tested strains actively grew on benzoic acid and phenol (Table 1). Many cultures utilized 4MBA and chlorine-substituted substrates. All of the tested strains grew well or with average efficiency on sodium benzoate. Growth on phenol was more intensive, but not all strains effectively used it as a growth substrate (Table 1). Six strains (*R. opacus* 4a, *R. opacus* 6a, *R. rhodochrous* 89, *R. rhodnii* 135, *Rhodococcus* sp. 412, and *R. opacus* 557) were characterized by the ability to utilize CBA as a growth substrate. Nearly all strains grew well on at least one of the monochlorphenols or on 2,4-D. Growth on 4MBA was generally weaker than on phenol and benzoate: an increment of optical density by more than 0.3–0.4 units was observed only for half of the strains.

The screening results showed the following strains to have the broadest substrate specificity: *R. ruber* P25 (grew on phenol, benzoate, 3-chlorobenzoate (3CBA), 2CP, 4MBA); *R. rhodochrous* 172 and *Rhodococcus* sp. 412 (on oxidized phenol, benzoate, 4CP, CBAs, 2,4-D, 4MBA); *R. opacus* 557 and *R. opacus* 6a (phenol, benzoate, CBA, 2,4-D, 4MBA); *R. opacus* 4a (benzoate, CBA, 2,4-D, 2CP, 4MBA); and *R. opacus* 1G (phenol, benzoate, 4MBA, 4CP and 2,4D).

There were reports about a laboratory collection of microorganisms isolated from technogenic soils of the Perm Territory, Russia, which showed a degradation activity against different aromatic compounds [11, 62]. Most strains of that collection had a high degradation activity against mono- and dichlorinated biphenyls. Unique bacterial strains isolated in the course of research included *R. ruber* P25, which could degrade mono- and dichlorinated biphenyls to products of central metabolism [10, 11]. Strain *R. ruber* P25 was shown to possess unique genetic determinants responsible for decomposition properties [11]. The ability of strain *R. ruber* P25 to utilize a mixture of mono-, di-, and trichlorinated biphenyls was demonstrated [63]. Polychlorobiphenyls remain pollutants of primary importance and, until now, only a few bacterial strains capable of complete decomposition of some low-chlorinated isomers of PCB have been isolated. Nevertheless, some strains were shown to be able to cometabolize PCB when grown with biphenyl (Table 1). PCB aerobic transformation processes are reviewed in [7]. *Rhodococcus jostii* RHA1 is one of the most well-studied strains able to degrade/cometabolize a great number of aromatic compounds. The complete genome sequence of *Rhodococcus jostii* strain RHA1 is available [64]. It was shown that three large linear plasmids encode important catabolic capabilities including genes for biphenyl and alkylbenzene pathways. According to the obtained data, strain RHA1 encodes 26 peripheral aromatic pathways and 8 central aromatic pathways [64]. The number of communications on the isolation of cultures active with respect to biphenyl/PCB increased in the recent decade. Four isomers of polychlorinated biphenyl (PCB)-degrading *Rhodococcus* spp. (TA421, TA431, HA99, and K37) were isolated from a termite ecosystem and under

Table 1. Biodegradation activity of the rhodococci

Culture	Growth substrate	Reference
Rhodococcus ruber P25	Biphenyl, 2-chlorobiphenyl, 4-chlorobiphenyl, phenol, benzoate, 3-chlorobenzoate, 4-chlorobenzoate, 2-chlorophenol, *p*-toluate, *p*-hydroxybenzoate, phenanthrene, anthracene	10–13
Rhodococcus sp. B7a	Mono-, di-, trichlorobiphenyls	14
R. jostii RHA1	Biphenyl, benzoate, ethylbenzene, phthalate, terephthalate, phenylacetic acid, cometabolism of polychlorinated biphenyl and biphenyl, polybrominated diphenyl ethers and biphenyl, ethylbenzene, propane, styrene	15–18
R. globerulus P6	Biphenyl/4-chlorobiphenyl	19
Rhodococcus sp. TA421, TA431, HA99, and K37	Polychlorinated biphenyl/biphenyl	20
Rhodococcus sp. strain T104	Limonene, biphenyl	21
R. imtechensis strain RKJ300.	4-Nitrophenol, 2-chloro-4-nitrophenol, 2,4-dinitrophenol	22
R. erythropolis strains, HL 24-1 and HL 24-2	2,4-Dinitrophenol	23
Rhodococcus sp. PN1	4-Nitrophenol	24
R. opacus SAO101	*p*-Nitrophenol	25
Rhodococcus spp.	Phthalic acid esters	26
Alkali-tolerant *R. erythropolis* strains	Benzene	27
Rhodococcus sp. strain MS11	3-Chlorobenzoate, all isomeric dichlorobenzenes, 1,2,3-trichlorobenzene, 1,2,4-trichlorobenzene, 1,2,4,5-tetrachlorobenzene, *m*- and *p*-cresol	28
Nineteen strains of *Rhodococcus* spp.	Hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX)	29
R. erythropolis S-7	3-Chlorobenzoate	30
R. opacus 1CP	2,4-Dichlorophenol, phenol, benzoate, *p*-cresol, *p*-toluate, 2-chlorophenol	31–35
R. minimus 1a	Benzoate, *p*-hydroxybenzoate, *p*-toluate, ferulic acid, 2-chlorophenol, 2,4-D, anisic acid	36
R. opacus 4a	Benzoate, monochlorobenzoates, 2,4-D, 2-chlorophenol, *p*-toluate	36
R. opacus 1G	Phenol, benzoate, *p*-hydroxybenzoate, *p*-cresol, *p*-toluate, 4-chlorophenol, 2,4-D	36,37
R. opacus 6a	3-Chlorobenzoate, ferulic acid, *p*-cresol, *p*-toluate, 4-chlorophenol, 2,4,6-trichlorophenol, phenol, benzoate, 2,4-D	36, 38, 39
R. rhodochrous 172	Phenol, benzoate, *p*-hydroxybenzoate, phthalate, terephthalate, *p*-toluate, acetophenone, anisic acid, naphthalene, *p*-cresol, 2-chlorophenol, 4-chlorophenol, 2,4-D, 3-chlorobenzoate	36, 40
R. opacus 557	Phenol, benzoate, *m*-hydroxybenzoate, *p*-hydroxybenzoate, anisic acid, ferulic acid, *p*-cresol, phthalate, acetophenone, 2,4-D, 3-chlorobenzoate	36,41
Rhodococcus sp. 412	Phenol, benzoate, 4-chlorophenol, chlorobenzoates, 2,4-D	36
Rhodococcus aetherovorans, *R. opacus*	Phenanthrene, naphthalene	42
R. opacus, *R. koreensis*	*o*-Xylene	43
Rhodococcus sp. strain DK17	*o*-Xylene, benzene, phenol, toluene, ethylbenzene, isopropylbenzene	44
Rhodococcus sp. strain 19070	Benzoate, substituted benzoates, toluene, xylenes	45
R. opacus GM-14	Benzene, chlorobenzene, 1,3- and 1,4-dichlorobenzenes, phenol, 3- and 4-methylphenols, all monochlorophenols	46
Rhodococcus sp. BPG-8	1,3,5-Trihydroxybenzene	47
Rhodococcus sp. An 117 and An 213	Aniline, phenol and benzoate	48
R. opacus R7	Naphthalene, gentisate, *o*-xylene, dimethylphenols	49
R. opacus M213	Naphthalene, toluene, *m*-toluate, benzoate, *p*-hydroxybenzoate	50
Rhodococcus sp. NCIMB 112038	Naphthalene	51
Rhodococcus sp. B4	Naphthalene	52
R. erythropolis SN8	Dibenzothiophene and carbazole	53
R. erythropolis C2	Oil-degrading bacterium	54
R. baikonurensis EN3	Diesel oil	55
R. ruber, *R. erythropolis*	Mixture of N-alkane and diesel oil	56
Rhodococcus sp. strain 33	Benzene	57
R. wratislaviensis strain J3	4-Nitrocatechol, 3-nitrophenol, 5-nitroguaiacol	58
R. erythropolis AN-13	Aniline	59
Rhodococcus sp. NCIMB 13064	Haloalkane	60

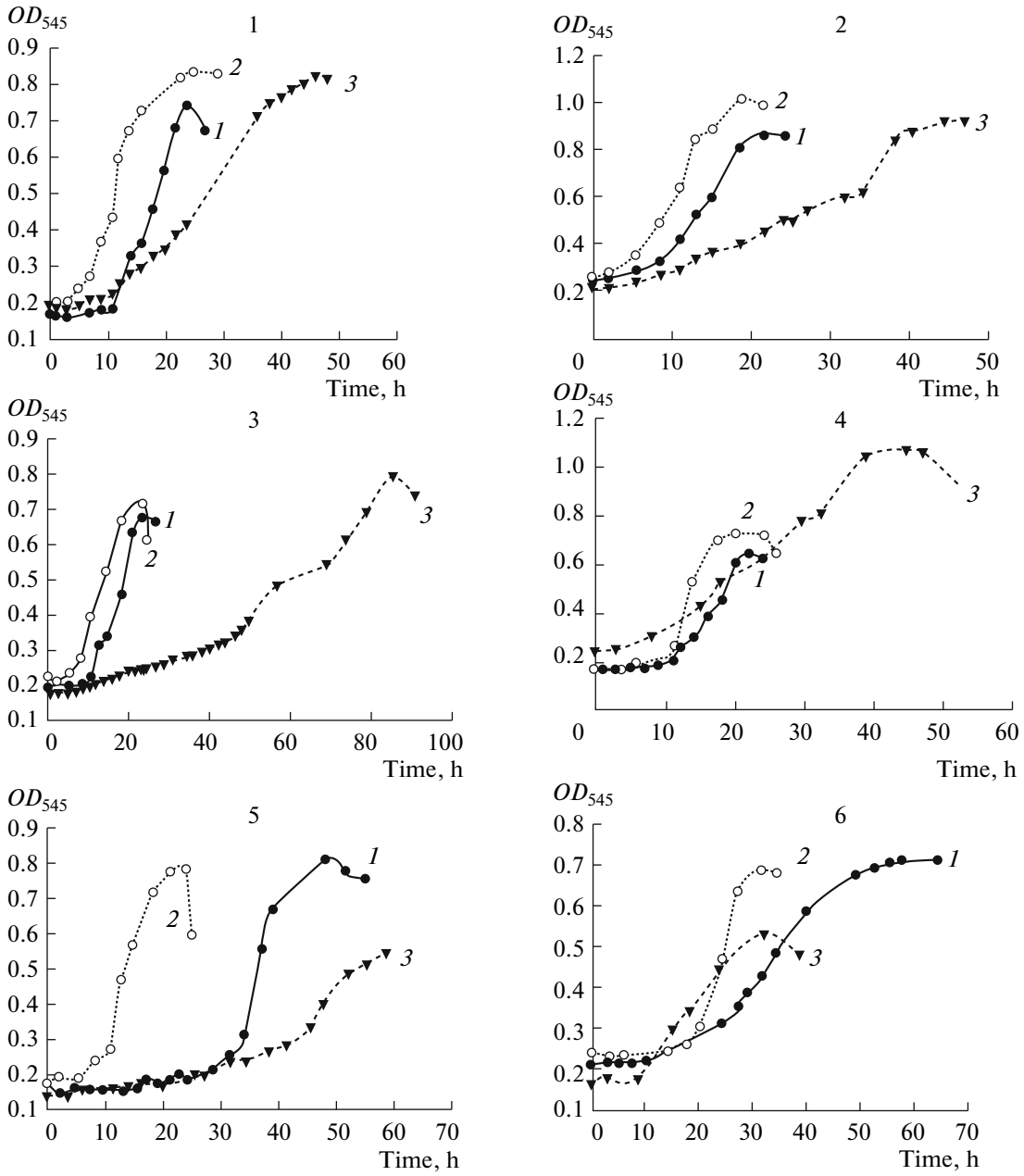

Fig. 1. Growth of the rhodococci *Rhodococcus opacus* 557 (1), *Rhodococcus* sp. 400 (2), *R. rhodochrous* 172 (3), *R. opacus* 6a (4), *R. minimus* 1a (5), *R. opacus* 1G (6) on aromatic substrates: *1*—phenol, *2*—benzoate, *3*—*p*-toluate (= 4-methylbenzoate, 4MBA).

alkaline conditions [20]. The *bph* gene cluster in strain TA421 was shown to be highly homologous to *bph* gene clusters in *R. globerulus* P6 and *Rhodococcus* sp. RHA1. The order of the genes and the sequence of the *bph* gene cluster in strain *R. rhodochrous* K37 and two other *Rhodococcus* strains HA99 and TA431 differed in these strains from those in P6, RHA1 and TA421.

Analysis of the complete nucleotide sequence of the 210-kb linear plasmid of *R. erythropolis* BD2 revealed, among other things, a *ipb* gene cluster encoding three subunits of isopropylbenzene dioxygenase, 3-isopropylcatechol dioxygenase and IPB dihydrodiol dehydrogenase [65].

When summing up the data on the degradation pathways of monoaromatic compounds by microorganisms, one can distinguish between several basic regularities of this process: (1) under anaerobic conditions, chloroaromatic compounds are degraded by reductive dehalogenation, which, however, does not always lead to complete degradation of the initial substrates [66, 67]; (2) under aerobic conditions, mono- and dihalogenated, rarely trihalogenated substrates

Table 2. The oxidation rates of catechol (100%), 4-chlorocatechol (4-CCat), 3-methylcatechol (3MCat), 4-methylcatechol (4-MCat) by rhodococcal intradiol dioxygenases

Bacteria	Growth substrate	Enzyme	Relative activity, %, with			Ref.
			4-CCat	3-MCat	4-MCat	
Rhodococcus sp. DK17	Benzene	Cat 1,2-DO	ND	9	27	44
Rhodococcus sp. An 117	Aniline, phenol, benzoate	Cat 1,2-DO	5–7	ND	60–62	48
Rhodococcus sp. An 213	Aniline	Cat 1,2-DO	6	ND	58	48
Rhodococcus erythropolis AN-13	Aniline	Cat 1,2-DO	ND	127	75	72
R. rhodochrous NCIMB 13259	Benzyl alcohol	Cat 1,2-DO	ND	79	68	73
R. rhodochrous N75	*p*-Toluate	Cat 1,2-DO	5.7	64	82	74
R. opacus 1CP	Benzoate	Cat 1,2-DO	3.1	99	88	32
	p-Toluate	Cat 1,2-DO	ND	73	89	35
		CCat 1,2-DO	113	191	253	
R. ruber P25	*p*-Toluate	Cat 1,2-DO	ND	195	117	12
R. opacus 6a	*p*-Toluate	Cat 1,2-DO	ND	138	129	39
		MCat 1,2-DO	112.6	150	282	
	4-Chlorophenol	CCat 1,2-DO	66	58	139	38
R. opacus 1CP	2-Chlorophenol	CCat 1,2-DO	50	283	270	75
	4-Chlorophenol	CCat 1,2-DO	96	208	242	76

ND – not determined, Cat 1,2-DO – catechol 1,2-dioxygenase, MCat 1,2-DO – methylcatechol 1,2-dioxygenase, CCat 1,2-DO – chlorocatechol 1,2-dioxygenase.

are converted into respective halocatechols; in this case, dehalogenation occurs after the aromatic ring of halocatechols opens up; polyhalogenated phenols are first subjected to oxidative dehalogenation to form (chloro)hydroxyhydroquinone followed by aromatic ring opening [68].

The screening of *Rhodococcus* bacteria for the ability to grow on monoaromatic compounds, which can be degraded with the *ortho*-cleavage of catechol and its derivatives, showed that phenol and benzoic acid are often degraded to form catechol, whose further degradation is catalyzed by enzymes of the classical *ortho*-pathway with narrow substrate specificity [69, 70]. It is known that 2CP, 4CP and 2,4-D can be biodegraded by enzymes of the modified *ortho*-cleavage pathway of the formed chlorinated catechols (CCat). Degradation of 3CBA and 4CBA may be also accompanied by the formation of CCat followed by their transformation by the modified *ortho*-pathway [68, 71]. Growth of strain *R. opacus* 6a on *p*-substituted substrates (4MBA, 4CP) induces the enzymes that perform the aromatic ring cleavage and belong to various types of intradiol dioxygenases different in subunit molecular masses, storage stability and substrate specificity: (1) methylcatechol 1,2-dioxygenase (MC 1,2-DO) is characterized by a broad substrate specificity and can cleave the aromatic ring of catechol (Cat), 3- and 4-methylcatechols (3MCat and 4MCat), 3- and 4-chlorocatechols (3CCat and 4CCat) and 3,5-dichlorocatechol. The best substrate for MC 1,2-DO is 4MCat; (2) 4-chlorocatechol 1,2-dioxygenase is also characterized by a broad substrate specificity, but its best substrate is 4CCat; and (3) catechol 1,2-dioxygenase (Cat 1,2-DO) with a narrow substrate specificity, active with Cat, 3MCat and 4MCat; the best substrate for it is catechol [38, 39]. Substrate specificities of other catechol 1,2-dioxygenases from various *Rhodococcus* strains are presented in Table 2.

Since the ordinary and modified transformation pathways of (chloro)catechols have been described many times, we will dwell only on their main properties.

2. CHLORO/METHYLCATECHOL TRANSFORMATION PATHWAYS

The data on the transformation pathways of substituted catechols indisputably demonstrate that microorganisms show a diversity of possible sequences and their modifications, by which the catechols formed are transformed into central metabolism intermediates. Some of these pathways, such as the *ortho*-cleavage pathways of 3-chloro-, 4-chloro- and 3,5-dichlorocatechols, may be considered as predominant and have been described in quite a number of bacteria; other pathways have been described only for a limited number of strains. However, it is not improbable that the description of such rare pathways in bacteria

reflects not their poor distribution in nature but, rather, poor knowledge of them.

2.1. 3-Chlorocatechol Branch of the Modified ortho-Pathway

Data concerning the 3-chlorocatechol *ortho*-cleavage pathway have been obtained mostly for Gram-negative bacteria and are reviewed by Pieper [7]. This pathway was among the first described for *Pseudomonas* sp. B13 and *Pseudomonas putida* [71] degrading 3-chlorobenzoate (Fig. 2). The main stages and enzymes of this pathway are shown in Fig. 2. 3-Chlorocatechol formed at the first stage is transformed under the action of chlorocatechol 1,2-dioxygenase (CC 1,2-DO) into 2-chloromuconate. Cycloisomerization of 2CM by chloromuconate cycloisomerase (CMCI) leads to the formation of *trans*-dienelactone. Then, under the action of dienelactone hydrolase (DLH), *trans*-dienelactone is transformed into maleylacetate, which is reduced to β-ketoadipate by maleylacetate reductase (MAR).

The peculiar features of the 3-chlorocatechol branch of the modified *ortho*-pathway are as follows [71, 77–87]:

1. Chlorocatechol 1,2-dioxygenase is characterized by the constant of high specificity to substrates with substituent in the *meta*-position.

2. Chloromuconate cycloisomerase is able to catalyze not only the reaction of 2-chloromuconate cycloisomerization but also 5-chloromuconolactone dehalogenation.

3. The reaction of 5-chloromuconolactone dehalogenation leads to the formation of *tranns*-dienelactone.

4. Dienelactone hydrolase is active with *trans*- and *cis*-isomers of dienelactone, though the affinity of the enzyme to *trans*-dienelactone is higher than to the *cis*-isomer.

2.2. 4-Chlorocatechol Branch of the Modified ortho-Pathway

Degradation of chloroaromatic compounds by the 4-chlorocatechol branch takes place when growth substrate oxidation results in the formation of 4-chlorocatechol (4CCat) (Fig. 2). 4-CCat is an intermediate of degradation or transformation of 3- and 4-chlorophenols by rhodococci [48]. Degradation of the 4CCat aromatic ring by CC 1,2-DO leads to the formation of 3-chloromuconate. Cycloisomerization of 3-chloromuconate via *cis*-dienelactone results in the formation of maleylacetate, which is a common intermediate of 3- and 4-chlorocatechol branches.

R. erythropolis S-7 can grow and use 3-chlorobenzoate as a sole carbon source in a temperatures range of 10–30°C with the stoichiometric release of chloride ions. The psychrotolerant ability was significant for bioremediation in low temperature regions [30].

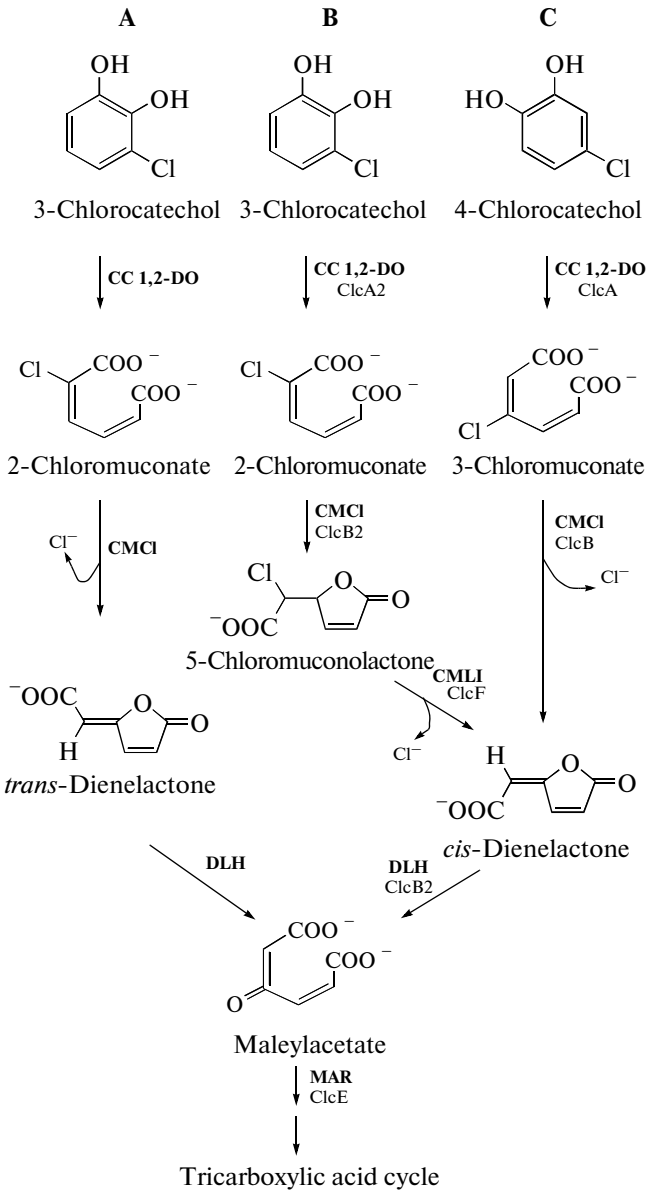

Fig. 2. Modified *ortho*-cleavage pathways of chlorocatechol degradation: (**A**) 3-chlorocatechol pathway of Gram-negative bacteria; (**B**) new 3-chlorocatechol pathway of *R. opacus* 1CP; (**C**) 4-chlorocatechol pathway of *R. opacus* 1CP.

4-Chlorophenol degradation was studied in detail for strain *R. opacus* 1CP. All enzymes of this pathway were isolated: CC 1,2-DO [76], CMCI [88], DLH [89] and MAR [90] and their encoding genes were cloned [91]. Crystals of Cat 1,2-DO were obtained from strain *R. rhodochrous* NCIMB 13259 [73] and of 4-CC 1,2-DO, from *R. opacus* 1CP [92].

The peculiarities of the enzymes of the 4-chlorocatechol branch are as follows [83–85, 93–95]:

1. Chlorocatechol 1,2-dioxygenase is able to cleave a broad range of substrates, and the dioxygenase specificity constant is higher by an order of magnitude with

the substrates having substituents in the *para*-position compared to those in the *meta*-position.

2. The best substrate for chloromuconate cycloisomerase is 3-chloromuconate. This enzyme is able to catalyze cycloisomerization of 2-chloromuconate; however, this process leads to the formation of only one compound, 5-chloromuconolactone, which is not subjected further to dehalogenation so that *trans*-dienelactone is not formed.

3. Dienelactone hydrolase is active with *cis*-dienelactone and shows but an insignificant activity with the *trans*-isomer. CMCI of the 4-chlorocatechol branch are distinguished by the ability to efficiently transform a broad range of muconates, with preference for 2,4-dichloro- and 3-chloromuconates.

It was shown that the peculiarities of substrate specificity of the enzymes of 4-chlorocatechol branch in strain *R. opacus* 1CP do not allow it to efficiently transform intermediates of 2-chlorophenol degradation. Indeed, strain *R. opacus* 1CP initially could not grow on 2-chlorophenol. However, the variant of this strain obtained after a long-time adaptation utilizes 2-chlorophenol as a sole carbon and energy source [34]. It was shown that 2-chlorophenol degradation by the new variant of the strain is mediated by an unusual set of enzymes designated as a modified 3-chlorocatechol pathway.

2.3. Modified 3-Chlorocatechol Branch in Rhodococcus opacus 1CP

The gene cluster for 2-chlorophenol degradation by the new modified *ortho*-pathway in strain *R. opacus* 1CP includes genes coding for 3CC 1,2-DO, CMCI, chloromuconolactone-isomerase (CMLI) and DLH [96]. 2-Chlorophenol degradation is accompanied by the formation of 3CCat as the main intermediate. The opening of 3CCat aromatic ring by CC 1,2-DO results in the formation of 2-chloromuconate, which is transformed into 5-chloromuconolactone under the action of CMCI. Dehalogenation of the latter with CMLI results in the formation of *cis*-dienelactone, which is a substrate for DLH. The above enzymes substantially differ in their properties from the enzymes of both the 3-chlorocatechol branch of Gram-negative bacteria and the 4-chlorocatechol branch of Gram-positive bacteria.

The peculiarities of the new modified *ortho*-pathway in strain *R. opacus* 1CP are as follows:

1. 3-Chlorocatechol 1,2-dioxygenase was able to oxidize a narrower range of substrates compared to chlorocatechol 1,2-dioxygenase of the 4-chlorocatechol pathway; however, the level of catalytic activity for all substrates was nearly the same and higher than in 4-chlorocatechol 1,2-dioxygenase; at the same time, 3-chlorocatechol 1,2-dioxygenase showed the highest affinity to 3-substituted substrates.

2. Chloromuconate cycloisomerase performed the reaction of cycloisomerization of 2-halomuconates but could not dehalogenate the halomuconolactones formed.

3. The pathways included an additional enzyme homologous to muconolactone isomerase of the ordinary *ortho*-pathway for catechol degradation and absent in the previously described modified pathways for chlorocatechol degradation (5-chloromuconolactone isomerase), which performed dehalogenation of 5-chloromuconolactone and was not active with unsubstituted muconolactone.

4. Dehalogenation of 5-chloromuconolactone results in the formation of *cis*-dienelactone but not the *trans*-isomer as in the case of the 3-chlorocatechol branch of the modified *ortho*-pathway of Gram-negative bacteria.

5. Dienelactone hydrolase of the new modified *ortho*-pathway differed in its structure and properties from analogous enzymes of other branches.

The fourth enzyme common for all branches of the modified *ortho*-pathway and for the hydroxyhydroquinol pathway is maleylacetate reductase. These enzymes are rather similar in different bacteria [97–100].

2.4. Meta-Cleavage of Chlorocatechols

Chlorine-substituted catechols are usually not degraded by the *meta*-pathway, because catechol 2,3-dioxygenase is inactivated by 3-chlorocatechol [101, 102], while *meta*-degradation of 4-chlorocatechol is unproductive due to accumulation of 5-chloro-2-hydroxymuconic semialdehyde, supposedly toxic for cells, in the culture medium [103, 104]. However, the described catechol 2,3-dioxygenase of strain *Pseudomonas putida* GJ31 degrades both 3-methylcatechol and 3-chlorocatechol by the *meta*-pathway [105]. No such pathway is known as yet for rhodococci.

2.5. Modified Pathway for Methylcatechol Degradation

The pathway of methylcatechol *ortho*-degradation was found in several bacteria of the genus *Rhodococcus* including strain *R. rhodochrous* N75 [74, 106, 107] (Fig. 3). Our studies have shown that *Rhodococcus* bacteria are generally characterized by the *ortho*-cleavage of 4MCat formed during *p*-toluate (= 4MBA) degradation, though in different rhodococci this ability is realized by different enzyme sets [35, 39]. Thus, the growth of strain *R. opacus* 1CP on 4MBA depends on simultaneous induction of the enzymes (Cat 1,2-DO and 4CCat 1,2-DO) participating in the pyrocatechol and 4CP degradation pathways; in strain *R. ruber* P25, it is the enzyme degrading methylcatechols at a high rate but not functionally adapted to these substrates; in strains *R. rhodochrous* 172 and *Rhodococcus* sp. 400, it is Cat 1,2-DO of the

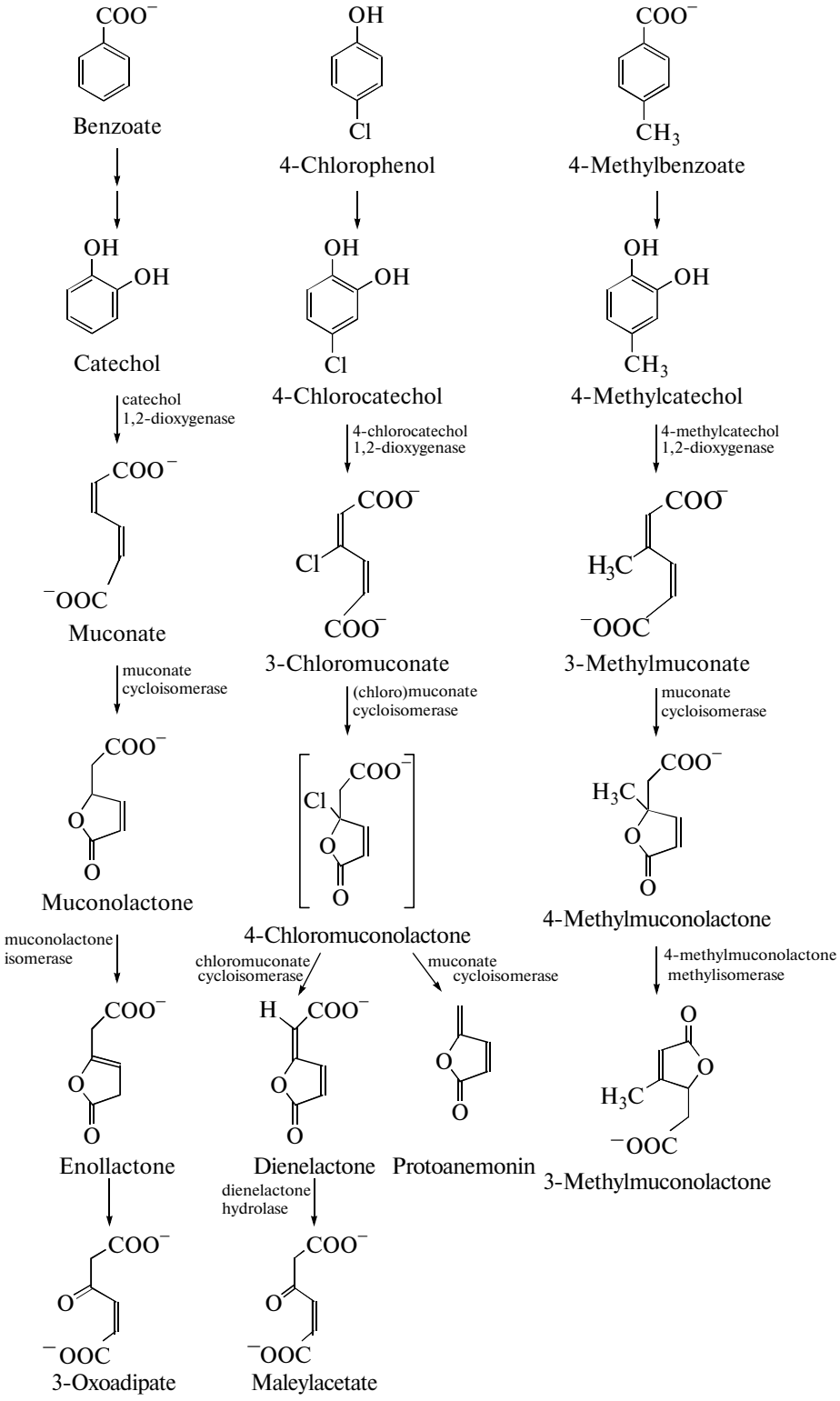

Fig. 3. Scheme of various pathways for the degradation of substituted aromatic compounds by the rhodococci. Left, the ordinary *ortho*-cleavage pathway of catechol conversion; center, a modified *ortho*-cleavage pathway of 4-chlorocatechol conversion; right, a modified *ortho*-cleavage pathway of 4-methylcatechol conversion.

common *ortho*-pathway with a narrow substrate specificity; and in strain *R. opacus* 6a it is a highly specific enzyme, methylcatechol 1,2-dioxygenase, the best substrate for which is 4MCat.

Strain *R. opacus* 6a was first adapted to growth on a mixture of 4-chlorophenol (4CP) and *p*-toluate (4MBA) taken at concentrations of 50 mg/l and 100 mg/l, respectively. The mixture ensured the growth of cells pregrown both on 4CP and on *p*-toluate; however, the cells pregrown on 4CP were characterized by lower growth indices. 4MBA was metabolized to form 4-methylcatechol (4MCat), which is usually cleaved by the *meta*-pathway [74]. The data only show the ability of the total enzyme fraction to catalyze the transformation of some or other intermediates formed in the degradation of initial substrates but do not reflect the isofunctional composition of the enzymes. The total catechol dioxygenase activity during the growth on a substrate mixture is closer to that typical of cells grown on 4MBA. Nevertheless, lower values of relative dioxygenase activity with substituted substrates do not exclude the possibility of induction of enzymes with different substrate specificity [61].

3. APPROACHES TO THE ENHANCEMENT OF DEGRADATION ACTIVITY

Strain *R. opacus* 1CP destroys 4CP, which is degraded by the modified *ortho*-pathway with 4CCat as a central intermediate, the process being mediated by the enzymes typical of this pathway [76]. Nevertheless, initially the growth of *R. opacus* 1CP on 4CP (50 mg/l) was accompanied by a substantial lag phase and a minor increase of OD_{560} to 0.2–0.3 U. Long-time cultivation of strain *R. opacus* 1CP on a medium with 4CP with repeated passages significantly changed the character of growth on the toxicant-containing medium. During the cultivation in a 10-l bioreactor, the lag phase was found to decrease to 4–5 h, the whole fermentation lasted for about 47 h, with OD_{560} increasing from 0.12 to 1.50 units and the culture summarily consumed 4 g/l of the substrate. As is known from the literature data, 4CP at a concentration above 100 mg/l has an inhibitory effect on the growth of bacterial cultures. It is due to the fact that chlorophenols are strong competitive inhibitors of chlorocatechol 1,2-dioxygenase (CC 1,2-DO), the key enzyme of their decomposition [76]. Therefore, the ability of strains *R. opacus* 1CP and *R. opacus* 6a to efficiently degrade high concentrations of 4CP enables their use for the cleanup of discharges containing this toxicant in a broad range of concentrations.

Strain *R. opacus* 1G can grow on phenol taken in concentrations up to 0.75 g/l [37]. With a phenol concentration of 0.3 g/l, OD_{545} was no more than 0.35 and residual phenol was absent in the culture medium after 12 h of incubation. At a phenol concentration of 0.75 g/l, the maximum optical density (0.7) was reached in 20 h. Intermittent introduction of phenol by 0.25–0.5 g/l resulted in culture growth to OD_{545} 0.8–1.0; total substrate consumption increased to 2 g/l.

Cell immobilization is known to have a positive influence on the resistance of cultures to toxic compounds, to contribute to bacterial culture stabilization and degradation of higher concentrations of pollutants. The observed effects are associated with providing the ability of immobilized cultures for cell–cell interactions by Quorum-sensing (QS), which allows the population to give a coordinated response to any environmental impact [108]. Besides, the planktonic forms of bacteria rarely occur under natural conditions; it is more typical of them to be in the immobilized state [109]. During the cultivation of *R. opacus* 1G in a liquid medium, culture growth was not observed at phenol concentrations above 0.75 g/l. Cell immobilization on polycaproamide fiber had a positive influence on phenol degradation: the substrate taken at a concentration of 0.5, 1.0, and 1.5 g/l was completely degraded in 24 h. Immobilized cells of strain *R. opacus* 1G under flow cultivation conditions proved to be capable of complete utilization of phenol at a concentration up to 2.2 g/l [110].

A positive influence of immobilization on different carriers has also been shown for other bacterial strains. Immobilization of *R. opacus* 6a cells on polycaproamide fiber resulted in a significant increase of 4-chlorophenol concentrations from 50–100 mg/l to 200–250 mg/l, at which the toxicant was completely degraded [61].

R. erythropolis cells acclimated to phenol were adsorbed on a Biolite ceramic support, which resulted in a marked enhancement of their respiratory activity and a shorter lag phase preceding active phenol degradation [111]. Under optimum operation conditions, immobilized cells in a laboratory-scale column reactor packed with support beads were able to completely degrade phenol in a defined mineral medium at a maximum rate of 18 kg phenol m^{-3} per day.

When phenol-acclimated cells of the same strain *R. erythropolis* UPV-1 were adsorbed on diatomaceous earth, they were able to completely degrade phenol in synthetic wastewater at a volumetric productivity of 11.5 kg phenol m^{-3} per day [112]. The authors also reported that after wastewater conditioning (i.e., dilution, pH, nitrogen and phosphorous sources and micronutrient amendments) immobilized cells were able to completely eliminate the formaldehyde present in two different industrial wastewaters obtained from local resin manufacturing companies, which contained both phenols and formaldehyde.

4. *Rhodococcus*-BASED BIOPREPARATIONS

All data available to date evidence that microbial cooperation is important in microbial degradation of organic pollutants. Analyzing the literature, we can

conclude that bacterial mixtures with different degradation potentials can be much more useful for the treatment of wastewater and polluted soils than a single bacterial strain. This is because polluted sites are often contaminated with more than one compound, pollution, as a rule, is of a complex nature and the specificity of particular bacteria does not make it possible for them to decompose complex mixtures of xenobiotics. Pollutants that may be present as mixtures are, e.g., BTEX compounds, chlorobiphenyls, pesticides, chemicals used for wood preservation (chlorobenzenes, chlorophenols and polyaromatic hydrocarbons). A mixture of selected hydrocarbon-degrading strains, *Acinetobacter* sp., *Pseudomonas* sp. and *Rhodococcus* sp., was used to eliminate 90% of a preparation of total hydrocarbons, which consisted of benzene, toluene, ethylbenzene, xylenes (BTEX) and heavy aliphatic hydrocarbons, in 15 days in the presence of nutrients [113].

It was assumed that cooperative interaction of two strains, *Rhodococcus* sp. CDT3 and *Pseudomonas* sp. PBM11, could contribute to the decomposition of the cypermethrin as well as eliminate the residues of the intermediate products [114].

Strains *R. rhodochrous, Gordonia sputi, Pseudomonas putida* were the basis for the cleanup of exhaust gases from a cable factory's coil-wire varnishing division among the microflora settling the pilot biofilter bed [115]. Isolated and identified bacteria metabolized phenol at a high rate (about 14 to 42 g m^{-3} per hour).

Crude oil, being a widespread fuel and energy source, may cause large-scale pollutions. Effective measures for purification of soils polluted with petroleum products and fuel-processing wastes are based on the application of pollutant-decomposing strains. It has been shown that the efficiency of such measures depends both on the biodegradation potential of decomposer preparations and on the conditions (salinity, temperature, pH) under which these preparations are used. The preparations for oil-polluted soil recovery developed at the Institute of Biochemistry and Physiology of Microorganisms RAS are based on bacteria of the genera *Pseudomonas* and *Rhodococcus*: *Rhodococcus* sp. X5, *Rhodococcus* sp. S67, *Pseudomonas* sp. 142 (pNF142) and *Pseudomonas putida* BS3701 (pBS1141, pBS1142). These biopreparations are based on psychrotrophic halotolerant microorganisms degrading oil at low temperatures and salt concentrations up to 10% [116–121]. Experiments on purification of soils contaminated with complex pollutants (diesel fuel, gas condensate, black oil) showed that the content of residual hydrocarbons decreased within one month by 99.6% due to simultaneous introduction of a biopreparation and a mineral fertilizer [116]. The authors studied the influence of the method of cell drying on the maintenance of the maximum activity of oil-degrading biopreparations and showed that contact drying had advantages over lyophilization expressed by a greater amount of living cells [122].

Strains classified as *R. erythropolis* and *Arthrobacter* spp. performed desulfurization of dibenzothiophene-containing soil [123].

PROSPECTS

The review shows the following approaches to efficient purification of wastewaters from pollutants: adaptation of strains capable of degrading aromatic compounds to growth on new substrates by intermittent introduction of the toxicant, control of the pH level, increase of the amount of toxicant introduced; adaptation of strains to utilization of a mixture of aromatic compounds present in contaminated wastewaters; direct introduction of single cultures or consortia of microorganisms into soil and wastewaters; immobilization of cells of highly active strains on different carriers to accelerate the degradation processes.

Rhodococci can utilize a wide range of pollutants as the sole carbon and energy sources: unsubstituted aromatic compounds and those carrying different substituents, diesel oil, carbazole, dibenzothiophene, limonene, triazine (RDX), etc. This process is accompanied by the induction of the key enzymes with various properties, providing the strains with the ability to degrade isomeric substrates efficiently. Occurrence of several enzymes with various affinities and catalytic activities in a single strain enables it to adapt for growth on new substrates, which supports the prospects of using *Rhodococcus* bacteria for nature conservation. Besides, the presence of isofunctional enzymes indicates the general "mobility" of microbial biodegradation systems ensuring their rapid adaptation to new nutrient sources.

An important role in expanding the *Rhodococcus* utilization activity is played by the transfer of genetic material between various representatives of this group of bacteria and closely related groups. Among other things, this process is provided for by the presence of linear plasmids, carrying biodegradation genes, in rhodococcal cells. The exchange of this genetic material expands the degradation potential of both particular microorganisms and the group as a whole. Therefore, the approach based on the use of mobile genetic elements for the transfer of particular (groups of) genes among the microbial population is an advanced trend for expanding the degradation potential.

The ability of rhodococci to synthesize surfactants, little investigated until now, is also biotechnologically promising. Along with a broad degradation potential, the occurrence of various surfactants enables these bacteria to adapt readily to the decomposition of hydrophobic substrates.

Ever greater attention of investigators has been recently attracted by systems providing for the communicative functions of bacteria. A rather advanced approach in this respect is the use of chemical

homologs of compounds involved in cell–cell interactions for changing/expanding the metabolic activity of bacteria. This, together with production of hybrid strains by the method of mosaic assembly [124] makes it possible to produce highly active bacterial preparations, which could be successfully used for the cleanup of both polluted soils and wastewaters.

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