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Pathways of α-methylstyrene Oxidation by *P. aeruginosa* DS-26

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Abstract

Today in world practice the biotechnological methods of cleaning of environment from oil and oil products are widely used. They based on the use of microorganisms-destructors that are able to utilize hydrocarbons.

The microbial object of the research was bacterial strain *P. aeruginosa* DS-26. This bacterial culture was isolated from sewage water of rubber industry. This strain was able to grow in a medium, which contained α-methylstyrene. This compound, which is widely used as an active component in many industries, has a strong toxic effect on living organisms including humans.

Different types of microbial metabolism play an important role in the hydrocarbon degradation process in the environment Metabolism of α-methylstyrene by *P. aeruginosa* DS-26 characterized by variety and in depth of carried out transformations. The analysis of the obtained data allowed offering two ways of oxidation of α-methylstyrene. The first way, primary, was accompanied by formation of products of the direct hydroxylation of aromatic ring - cis-2.3-dihydroxy-1-isopropenyl-6-cyclohexene and 3-isopropyl-catehol which are later metabolized by meta-pathway with formation of keto-acids. The next, secondary pathway came true through oxidation of α-phenylpropionic acid and 4-methylbenzene alcohol with formation of acetophenone as a final product.

There are many researches that are devoted to study biochemical conversion of oil hydrocarbons. In order to utilize specific compounds as the only one source of carbon, microorganisms have to actively maintain a specific system of enzymes. Investigation of α-methylstyrene degradation by *P. aeruginosa* DS-26 enzymes has revealed specific enzymes that oxidize aromatic hydrocarbons. Investigation results of the studying strain enzymes can be confirmation of the proposed above scheme of primary α-methylstyrene metabolic pathway oxidized by *P. aeruginosa* DS-26.

Introduction

Petroleum and petroleum industry have especially considerable significance because of its tremendous contribution to Kazakhstan economy. However, crude oil extraction, transportation and refinery have negative impact on environment since oil processing produce a lot of harmful compounds that pollute environment.

Today in world practice the biotechnological methods of cleaning of environment from oil and oil products are widely used. They based on the use of microorganisms-destructors that are able to utilize hydrocarbons of oil [1, 2].

Bioremediation is widely applied biotechnological methods of liquidation of hydrocarbon contamination of environment [3, 4]. Engineering bioremediation technologies assumes utilization of living organisms, particularly microorganisms, which have an ability to degrade various xenobiotics including hydrocarbons [5, 6].

Different types of microbial metabolism play an important role in the hydrocarbon degradation process in the environment. In environmental conditions these microorganisms are affected by other bacterial populations, which are due to their heterogeneity and the presence of interconnected metabolic pathways are able to biodegrade hydrocarbon pollutants. This is the one of the main ways to save the environment. However, biological degradation has rationale if complete mineralization, decomposition and detoxification occur.

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It is generally acknowledged that microorganisms can relatively easy convert aromatic hydrocarbon molecules. Moreover, according to many authors, the most important role in the oxidizing process belongs to bacteria genus *Pseudomonas* [7, 8].

There are many researches that are devoted to study biochemical conversion of oil hydrocarbons [9]. In order to utilize specific compounds as the only one source of carbon, microorganisms have to actively maintain a specific system of enzymes.

Last years, great attention has been paid to the study microbial metabolic pathways of alkyl-substituted benzenes, aromatic compounds that are the main contaminant in the industrial waste [10, 11]. Various metabolic pathways of chemical compounds , which are based on a benzene ring, requires consideration of the sequence of reactions that begin with degradation of the benzene ring and finish with accumulation of primary metabolism compounds.

Materials and Methods

α-Methylstyrene (AMS), $C_6H_5(CH_3)C{=}CH_2$ – is an alkyl-benzene derivative, is colorless or slightly yellow liquid with specific sharp smell. AMS is used to produce sterilized drying oils, sterilized alkyl resin, and especially synthetic rubber of butadiene-methylstyrene type. This compound, which is widely used as an active component in many industries, has a strong toxic effect on living organisms including humans.

The microbial object of the research was bacterial strain *P. aeruginosa* DS-26. This bacterial culture was isolated from sewage water of cooperative association «Karbid» rubber industry in Temirtau town. This strain was able to grow in a medium, which contained AMS.

The growth of *P. aeruginosa* DS-26 in aerobic conditions was performed in 500 ml Erlenmeyer flasks with 100 ml of liquid medium E-8 the following composition: KH_2PO_4 – 0.7 g; $(NH_4)_2HPO_4$ – 1.5 g; NaCl – 0.5 g; $MgSO_4$ – 0.8 g; H_2O – 1.0 L; agar – 20 g. The process of aeration was fulfilled on an air shaker (200 r/min) and at the temperature 30 ºC. AMS, as the sole source of carbon, was directly introduced to the growth medium in concentration from 2.0 to 6.0 g/l.

Electron-microscopic investigations were conducted on the electron microscope JEM-100B. Samples were stained with 1% solution of phosphorwolfram acid and spurred chromium.

The rate of the culture growth in the liquid synthetic medium was nephelometric controlled on photoelectric colorimeter-60 with the wavelength 540 nm [12].

For identification of AMS oxidation products, bacterial cells were precipitated from cultural liquid by centrifugation, then, cultural liquid was acidified to pH 2.0 by 0.1 M HCL and extracted three times with diethyl ether. Ether then was evaporated on a rotary evaporator at the temperature 20 ºC, and precipitate was dissolved in ethanol.

Qualitative analysis of AMS and its metabolites was conducted by thin layer chromatography (TLC) on silicagel sheets (Silufol IU-254) in solvent mixture: benzene: dioxane: acetic acid 90:10:2 (system 1) and benzene: ethylacetate 4:1 (system 2).

Identification of the oxidation products of AMS was performed with UV- and infrared (IR)-spectroscopy techniques, nuclear magnetic resonance (NMR) spectroscopy and chromatography-mass spectrometry. UV-spectrums were registered on the spectrophotometer "Specord UV-VIS" in 96% ethyl alcohol in range 200-350 nm. IR-spectrum was determined on infrared spectrophotometer UR-20. NMR-spectrum was analyzed on WM-400 device.

Chromato-mass spectrometric analysis was conducted on LKB-2091 appliance. For the analysis of acids samples treated with diazometan.

Quantitative assay of AMS was performed spectrophotometrically on spectrophotometer "Specord UV-VIS" with wavelengths from 240 nm to 300 nm and with a calibration curve. Also, the amount of AMS was quantified by the gas chromatography method on GGD chromatograph from Pye Unicam firm.

Cell free extracts that was on exponential phase of the growth were used to indicate enzymes oxidative activity of an aromatic ring. To receive cell free extracts cells grown on liquid medium E-8 in the presence of the AMS and aromatic acids. Then the cells were centrifugation, washed three times 0.05 phosphatic buffer with pH of 7.5. Suspension, containing 1 g cells on a wet weight, basis in 2 ml of buffer solution and destroyed on the ultrasonic disintegrator during 3 minutes at 18-20 kHz and a temperature of +40 ºC. The supernatant was used to determine the activity of enzymes.

Activity of the following enzymes were analyzed in accordance with conventional techniques: p-hydroxybenzoate hydroxylase, catechol 1.2-dioxygenase (1.2-CTD), catechol 2,3-dioxygenase (2.3-CTD), protocatechuate 3.4-dioxygenase (3.4-PCD), gentisiate-1.2-dioxygenase (GDO-1.2-do), homogentisate dioxygenase (HGD) [13-16]. Activity of all enzymes was expressed in nanomoles of reaction products that were produced in 1 min.

Results and Discussion

The strain *P. aeruginosa* DS-26, which can thrive on E-8 medium with studying aromatic hydrocarbon, was chosen to investigate AMS oxidation (Fig. 1).

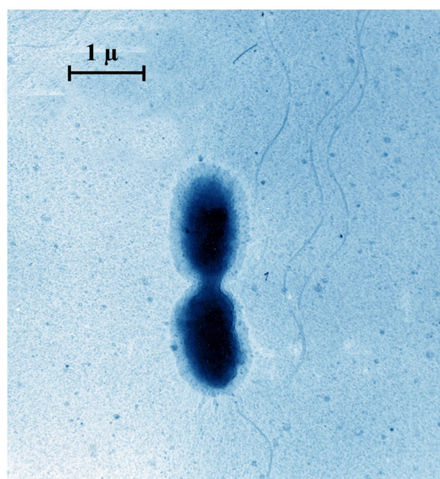

Fig. 1. Electron-microscopy image of *P. aeruginosa* DS-26 cell.

Initially, before investigating metabolic pathways of the isolated strain, it was necessary to study oxidation of AMS in the liquid synthetic medium in dynamics.

According to the results in the Fig. 2a, bacterial culture achieves the stationary phase of the growth at 24 hours in concentration of AMS 2 g/l and at 48 hours – 4 g/l wherein the toxic compound was absent in the growth medium (Fig. 2b).

The strain was characterized by the active growth on AMS concentration 6 g/l although in this concentration the growth decreased – longer lag phase, but active exponential phase of the growth. Laboratory investigations revealed that the optimal growing concentrations of AMS for the studying strain were 2-4 g/l. At these concentrations, the growth dynamics and accumulation of by-products by substrate oxidation were analyzed.

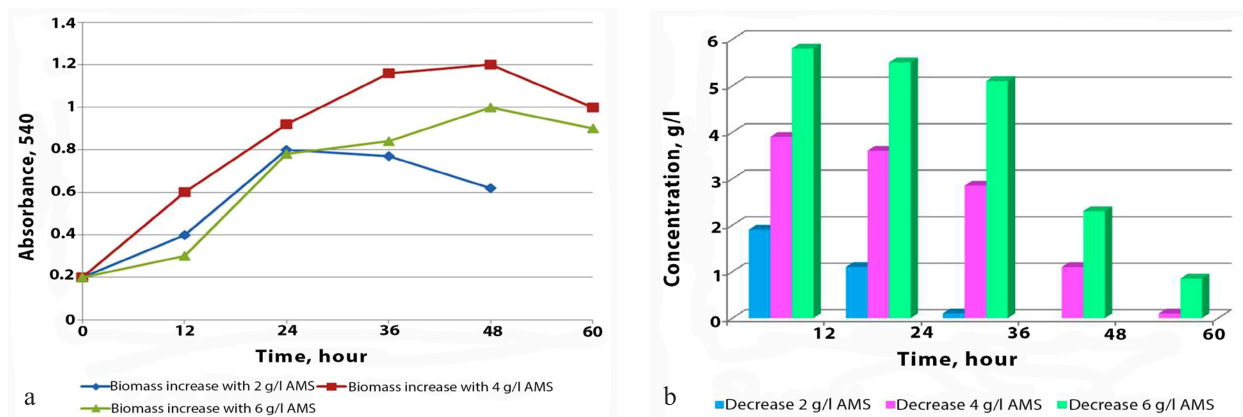

Fig. 2. Growth dynamics (a) and AMS oxidation (b) by bacterial strain *P. aeruginosa* DS-26.

Up to this time, microbial metabolic pathways of mono-aromatic compounds had been well studied. Degradation of benzene derivatives begins with one of the main three reactions – monooxygenation and dioxygenation of an aromatic ring or oxidation of the side chain if it is available.

Results of the study showed that in the growing culture with 4 g/l of AMS, the cultural medium had 11 metabolites, which were distinguished by retain time, displayed features of detecting reagents, and UV-spectrum (Table 1).

Compound 1 had maximum absorbance of UV-light at 280 nm. Methyl ether of the product had the molecular ion peak with m/e 164, and ionized molecule fragmentation gave an intensive peak with m/e 105 (M^+-$COOCH_3$) and with m/e 77 (M^+-$COOCH_3$, -C_2H_2) (Fig. 3).

Obtained data allowed to establish the structure α-phenylpropionic acid, which amount gradually decreased.

Table 1

UV-spectrum and TLC characterization of AMS bioconversion products by *P.aeruginosa* DS-26

AMS bioconversion products	Rf		Absorbance maximum, nm
	System 1 (benzene: dioxane: acidic acid)	System 2 (benzene: ethylacetate)	
Compound 1	0.54	0.47	280
Compound 2	0.63	0.55	230
Compound 3	0.72	0.62	242
Compound 4	0.62	0.74	270
Compound 5	0.3	0.35	300
Compound 6	0.75	0.69	212
Compound 7	0.24	0.4	232
Compound 8	0.75	0.70	215
Compound 9	0.8	0.85	260

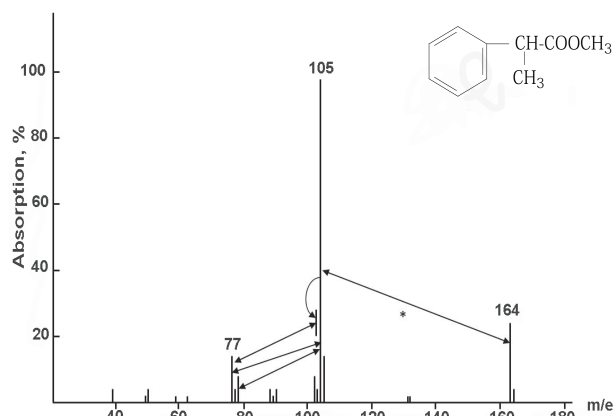

Fig. 3. Mass-spectrum of methyl ether of α-phenylpropionic acid – the product of AMS oxidation by *P.aeruginosa* DS-26.

Compound 2 was not accumulated in cultural liquid, and its small amounts were detected only after 8-10 hours from the beginning of the cultivation. This substance, according to mass-spectrum data, had molecular weight 122 atomic unit. The cleavage of the CH_3-group resulted in a molecular ion that formed the fragment with m/e 79. The loss of two hydrogen atoms of this fragment led to generation phenyl-cation with m/e 77 (Fig. 4).

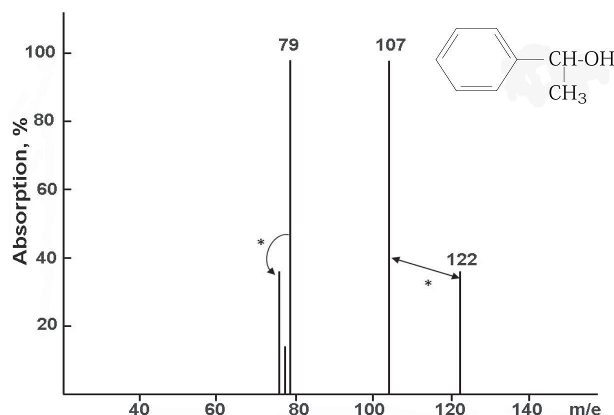

Fig. 4. Mass-spectrum of α-methylbenzene alcohol – *P. aeruginosa* DS-26 oxidized product of AMS.

Based on the mass-spectrum determination, compound 2 was identified as α-methylbenzene alcohol.

Compound 3 was accumulated in cultural liquid after 12 hours, and, up to the stationary phase, was detected in very small quantities. UV-spectrum of the product in ethanol suggested that an aromatic ring was preserved (Table 1).

In mass-spectrum of the 3rd compound, the molecular ion peak was observed with m/e 120 a.u., and main fragments m/e 105 (M^+-CH), m/e 77 (M^+-CH_3, -CO). This type of decay is typical for acetophenone (Fig. 5). Acetophenone mass- and UV-spectrum standards exactly coincided with those of the 3rd compound.

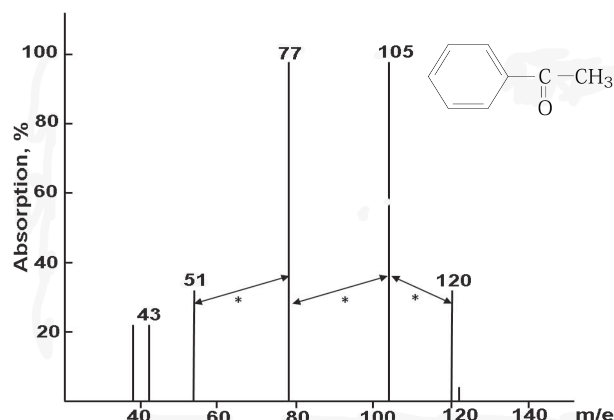

Fig. 5. Mass-spectrum of acetophenone – the product of AMS oxidation by *P.aeruginosa* DS-26.

Compound 4 had maximum absorbance at 270 nm. Mass-spectrum of the methyl ether product had the molecular ion peak at m/e 162, the intensive fragment m/e 103 (M^+-$COOCH_3$) and m/e 77 (M^+-$COOCH_3$, -C_2H_2) (Fig. 6). The way of the 4th compound decay completely matched with the spectrum of α-phenylacryl acid, which was detected at trace amounts in the cultural medium. Probably, this is because α-phenylacryl acid was formed as a result of dehydrogenation of α-phenylpropionic acid.

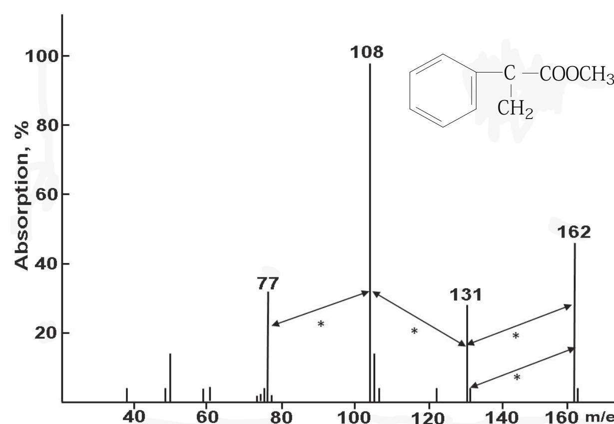

Fig. 6. Mass-spectrum of α-phenylacryl acid – the product of AMS oxidation by *P.aeruginosa* DS-26.

Compound 5 had absorbance maximum at 300 nm. Mass-spectrum of this compound had the peak

of a molecular ion at m/e 152; the ionized molecule fragmentation demonstrated intensive peaks at m/e 121 (M^+ CHOH) and m/e 134 (M^+-OH) (Fig. 7).

From IR-spectroscopy spectrum of product 5 demonstrated bending vibrations of aryl CH-bonds, which are typical for mono-substituted benzene derivatives. Also, the fact that absorption spectrum were at 707 and 750 cm^{-1}, 3038, 3070 and 3095 cm^{-1} suggested that this chemical was mono-substituted benzene intermediate. Absorption maximum at 3640, 3620, 3600 and 3580 cm^{-1} indicated the presence of primary and ternary hydroxyls.

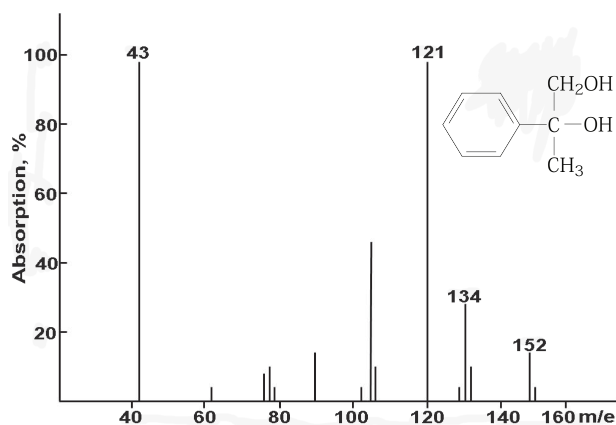

Fig. 7. Mass-spectrum of 1,1-phenylmethylglycole – the product of AMS oxidation by *P. aeruginosa* DS-26.

In NMR-spectrum, methyl group (-CH_3) has singlet in 1.48 m.d., 5 aromatic protons – single-protons duplets in 7.2 m.d., single-protons triplets in 7.31 m.d., 7.46 m.d. and 7.48 m.d.

Obtained physicochemical data allowed to conclude that the 5th compound was 1,1-phenylmethylglycole.

This product was formed after 6 hours at the beginning of exponential phase, and its amount reduced gradually.

Compound 6 was formed after 12 hours of cultivation. This substance was found in a very small amount in the stationary phase. Mass-spectrum of the product had the molecular ion peak at m/e 134, ionized fragments at m/e 119 (M^+-CH), 94, 91, and 77. Decay pattern consistent with the presence of the hydroxyl group, whose location was not identified in the molecule. Received data assumed the monohydroxyl AMS derivative structure – oxy-isopropenylbenzene.

Compound 7 is polar, and when this metabolite was treated with benzidine, it rapidly turned to brown color. Absorption maximum of UV-light was at 232 nm. Mass-spectrum of product 7 had the peak of the molecular ion at m/e 154, and fragmentation of the ionized molecule had the following values of m/e: 136, 121, 110, 95 and 67 (Fig. 8). Methylation of the product showed equivalence and spatial proximity of hydroxyl groups but, also, suggested non-aromatic nature of the substance.

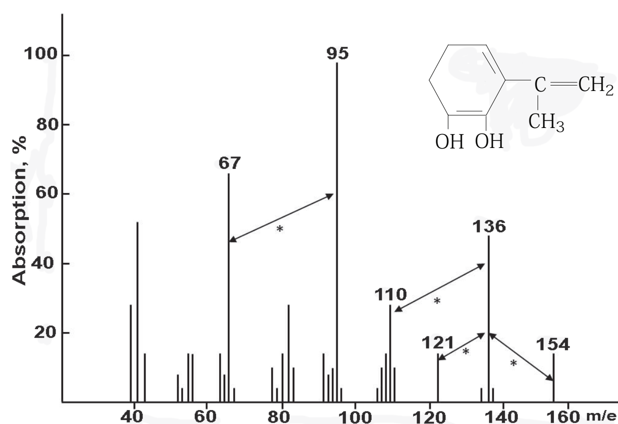

Fig. 8. Mass-spectrum of 2,3-dihydroxy-1-isopropenyl-6-cyclohexene – the product of AMS oxidation by *P. aeruginosa* DS-26.

IR-spectrum of the 7th compound denoted valence vibration at 3625 and 3575 cm^{-1}, which assumed two hydroxyls. Absorbance at 1610, 1640, 3050 and 3100 cm^{-1} suggested two double bonds (-CH), and following absorbance values 2830, 2875, 2930 and 2955 cm^{-1} indicating methyl and methylene groups (-CH_3, -CH_2). Also, there were specific absorption bands for an aromatic ring.

Based on the obtained data, the 7th compound was identified as 2,3-dihydroxy-1-isoprenyl-6-cyclohexene.

It should be noted that this *P. aeruginosa* DS-26 oxidized particular AMS metabolite was an unusual degradation product of aromatic compounds, which are characterized by formation of substituted ortho-diphenols, which, in turn, are substrates for benzene degrading enzymes.

Compound 8 was found in the exponential phase of the growth after 8-10 hours of cultivation. This metabolite absorbed UV-light at 215 nm. When benzidine is applied on silica sheets, compound 8 displayed intensive brown dots. Also, product mass-spectrum demonstrated the intense peak of the molecular ion at m/e 134, and fragmentized ion at m/e 119, 94, 91, and 77 that indicated hydroxyl group. According to this information, metabolite was defined as oxy-iso-propylbenzene.

Compound 9 was accumulated after 20 hours of cultivation in trace amounts. Product ion mass-spectrum had the peaks at m/e 152, and peaks of fragmentation were at m/e 139, 109, 81, and 77.

The decay pattern indicated that CH_3-group and two carbon dioxide molecules gradually disappearing from molecular ion. This fact confirmed the presence of OH groups. In addition, the fact that there is a fragment (M^+-H_2O) assumes ortho-position of these groups. Based on mass-spectrometry assay information, compound 9 was identified as 3-isopropylcatehol.

It should be pointed out that, aliphatic keto-acids accumulated at the beginning of cultivation (products 10 and 11), and these metabolites were not detected later. Mass-spectrum of the 10th product had the molecular ion peak at m/e 154, 117, 69, 59, and 41 with low intensity (Fig. 9).

IR-spectrum of the product was consistent with spectra of non-saturated aliphatic acids, which, probably, is the result of 3-iso-propylkatehole degradation. According to mass-, IR-spectrometry, and TLC chromatography, non-saturated aliphatic keto-acid had the following structure:

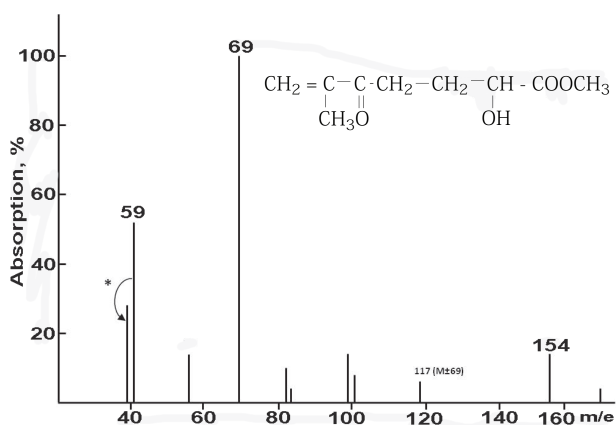

Fig. 9. Mass-spectrum of methyl ether of aliphatic keto-acid – the product of AMS oxidation by *P. aeruginosa* DS-26.

At the same time, accumulation of α - phenyl-acrylic acid and α - phenyl-methylglycol confirmed the presence of minor AMS oxidation pathways, which can be explained by the presence of enzymatic systems that catalyze aromatic compounds and have broad substrate specificity.

Detailed investigations of AMS products degradation allowed determining the sequence of catabolic reactions. This research has indicated two biochemical pathways, one of which is primary, when formed metabolites are involved in the central metabolism, and secondary with accumulation of compounds that are not catalyzed later.

Based on received data, *P. aeruginosa* DS-26 oxidized AMS metabolism can be summarized in the following diagram (Fig. 10).

Thus, investigation of AMS aerobic biodegradation process by *P. aeruginosa* DS-26 strain has indicated that metabolic conversions are oxidation reactions and have branched catabolic sequence – primary and secondary pathways.

As it can be seen from Fig. 10, the primary pathway of AMS oxidation accumulates products that were formed as a result of direct hydroxylation of an aromatic ring, cis-2.3-dihydroxy-1-isoprenyl-6-cyclohexene and 3-isopropyl-pyrokatehine. Then these intermediates are metabolized by meta-pathway with formation keto-acids. Secondary in the significance pathway is catalyzed through α-phenylpropionic acid and 4-methylbenzene alcohol with accumulation acetophenone as a final product.

The primary enzymes that oxidize an aromatic ring have been studied in order to confirm obtained data from the assay of AMS intermediates oxidized by *P. aeruginosa* DS-26 strain.

In extracts of cells in the exponential phase of *P. aeruginosa* DS-26 growth on various substrates the following oxidative enzymes were detected: p-hydroxybenzoate hydroxylase (PHBH), catechol 1.2-dioxygenase (1.2 CTD), catechol 2.3-dioxygenase (2.3-CTD), protocatechuate 3.4-dioxygenase (3.4-PCD), gentisiate-1.2-dioxygenase (GDO – 1.2 do), homogentisate dioxygenase (HGD) (Table 2).

Table 2

Activity of *P. aeruginosa* DS-26 key enzymes that are involved in oxidation of an aromatic ring

Strain	Substrate	Activity, nanomoles/min/mg protein					
		1.2-CTD	2.3-CTD	PCC-3.4-do	HGD	GDO-1.2-do	PHBH
P. aeru - ginosa DS-26	AMS	151	0	0	2.0	-	104
	Biphenyl	420	0	0	1.8	-	-
	Toluene	350	20	0	-	-	-
	Benzoate	19.4	0	0	0	0	0

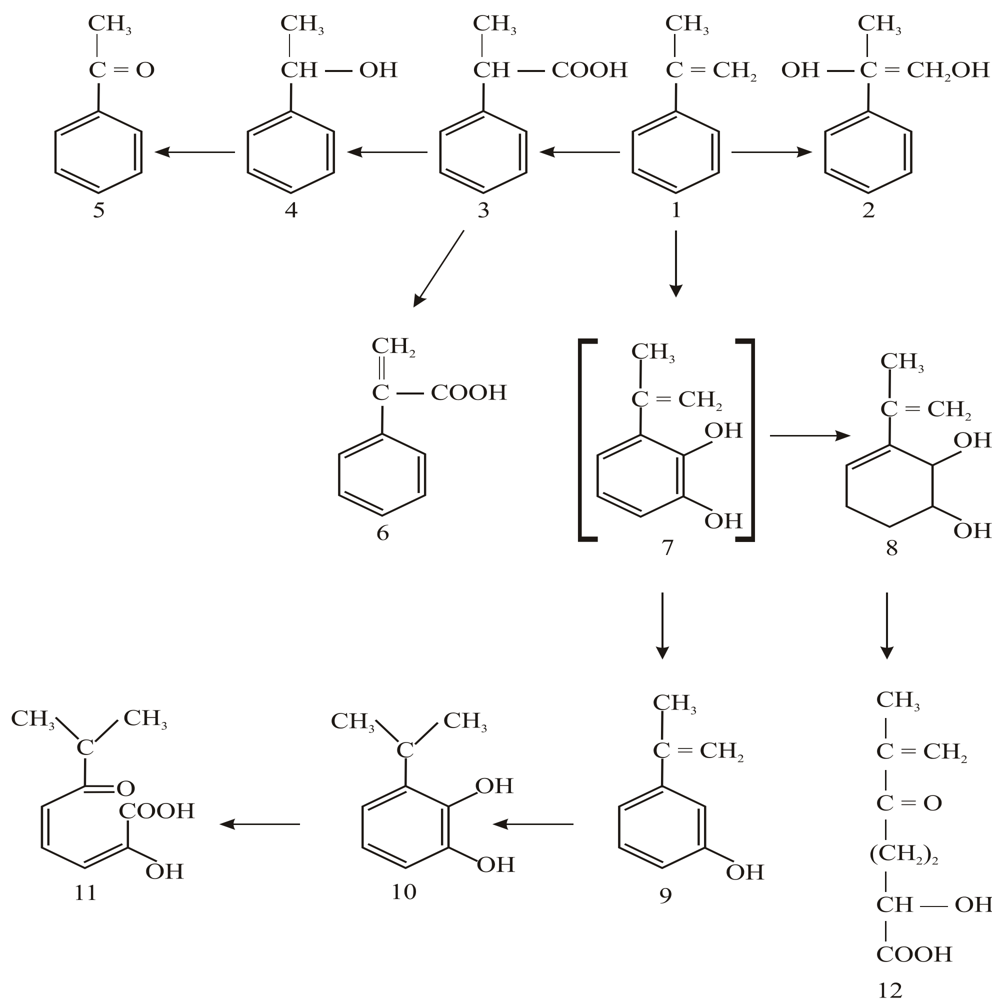

Fig. 10. Pathways of AMS oxidation by *P. aeruginosa* DS-26: 1 – AMS; 2 – 1.1-phenylmethylglycol; 3 – α-phenylpropionic acid; 4 – methylbenzene alcohol; 5 – acetophenone; 6 – α-phenylacryl acid; 7 – hypothetical product; 8 – (cis)-2.3-dihydro-oxi-1-isopropenyl-6-cyclohexene; 9 – (oxy)-isopropenylbenzene; 10 – 3-isopropylcatehol; 11-12 – aliphatic keto-acids.

Enzymatic assay results given in Table 2 elucidate pathway reactions of AMS oxidation because none of the known dioxygeneses catalyze AMS except meta-pyrocatechase, which levels is considerably high in *P. aeruginosa* DS-26 strain when this bacteria are grown in this substrate.

Thus, enzyme activity assay results have confirmed earlier assumed metabolic pathways of aromatic hydrocarbon – AMS, which is one of the most common industrial pollutants.

To sum up, aerobic AMS degradation by *P. aeruginosa* DS-26 strain had been oxidative metabolic reactions. In addition, these reactions have divided into two catabolic pathways – primary and secondary. In the primary pathway, an aromatic ring – cis-2.3-dihydroxy-1-isoprenyl-6-cyclohexene and 3-isopropyl-pyrocatehine – undergoes direct hydroxylation, and then formed metabolites catalyzed by meta-pathway into keto-acids; whereas, the secondary pathway results in acetophenone as a final product through oxidized α-phenylpropionic acid and 4-methylbenzene alcohol intermediates.

Thus, it is shown that metabolism of AMS by *P. aeruginosa* DS-26 characterized by a variety and in depth the carried out transformations. For investigation of way of microbiological degradation of AMS it was distinguished and identified 11 products of its oxidation differing on a chemical structure. The analysis of the obtained data allowed offering two ways of oxidization of AMS. The first way, primary, was accompanied by formation of products of the direct hydroxylating of aromatic ring - cis-2.3-dihydroxy-1-isopropenyl-6-cyclohexene and 3-isopropyl-pyrocatehol which are later metabolized by meta-pathway with formation of keto-acids. The next, secondary pathway came true through oxidation of α-phenylpropionic acid and 4-methylbenzene alcohol with forming acetophenone as a final product.

Investigation of α-methylstyrene degradation by *P. aeruginosa* DS-26 enzymes has revealed spe-

cific enzymes that oxidize aromatic hydrocarbons. In conclusion, investigation results of the studying strain enzymes can be confirmation of the proposed above scheme of primary AMS metabolic pathway oxidized by *P. aeruginosa* DS-26.

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