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# Investigation of the process of biodegradation of aromatic hydrocarbons and phenols by a method of reversed-phase high-efficient liquid chromatography

**VELIYEV MAMMAD GUSEINALI[1, 2], Salmanov Mamed Ahad[2, 3], Bektashi Nazim Rauf[2, 3], Aliyeva Saida Rafael[2, 3], Gasanova Arzu Rasul[2, 3]**

[1]Laboratory "High-unsaturated compounds", Sumgayit, Azerbaijan
[2]Institute of Polymer Materials of Azerbaijan National Academy of Sciences, Sumgayit, Azerbaijan
[3]Institute of Microbiology of Azerbaijan National Academy of Sciences, Baku, Azerbaijan

**Email address:**
mveliyev@mail.ru(V. M. GUSEINALI), aliyeva_saida_bio@yahoo.com(V. M. GUSEINALI)

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**Abstract:** By a method of reversed-phase high-efficient liquid chromatography (HELC) the dynamics of the process of biodegradation of monoaromatic hydrocarbons (benzene, toluene, ethylbenzene) and phenols (phenol, pyrocathechin, hydroquinone, tetrachlorpyrocatechin) by bacteria isolated from coastal waters and soils of Absheron peninsula of the Caspian Sea has been investigated. It has been shown that in the selected conditions, in particular, by bacteria *Pseudomonas sp*. the symptoms of the products of biotransformation are appeared approximately on sixth day of the exposition. In this case a full formation of the obtained products takes place in 3-4 days and the individual compounds a composition of which remains stable during 25 days are appeared. The used method allowed to detect and to identify in composition of the products of biodegradation more than 15 individual compounds being the products of direct oxidation of aromatic ring and side chain.

**Keywords:** Biodegradation, Aromatic Hydrocarbons, Liquid Chromatography

## 1. Introduction

The aromatic compounds [1-4] widely spread in nature, including aromatic hydrocarbons containing in oil, in products of its decomposition, vegetative objects, in water, soil, air medium are priority pollutants of the environment [5-7]. The approaches to solution of the problem of purification of the environment from the aromatic compounds are known quite a lot, but the most perspective is their microbiological degradation. The microogaisms-destructors catabolize aromatic substances converting them into hydroxylated derivatives with the subsequent opening of benzene ring and formation of numerous easily utilized substrates [1, 8-12]. The forming products of biodegradation are of interest from the point of view investigation of basic principles of utilization (biodegradation) of the aromatic compounds and perspectives of their use in biotechnologies of purification of the environment.

The study of dynamics of the process of microbiological degradation of the oil hydrocarbons, including phenol compounds has an important value for development of methods of biological purification of the environment from oil and phenol pollutions [13-17].

## 2. Experimental

The investigations was conducted using active oil oxidizing strains of bacteria (Bacillus sp. 15 and Pseudomonas sp.4) isolated from waters of the Absheron peninsula of the Caspian Sea[18]. As a unique sources of carbon benzene, toluene, phenol, pyrocatechin, tetrachlorpyrocatechin was added in the microbiological medium in concentration 100, 300, 500 mg/l. The cultures were incubated at temperature 28ºC in all experiments. After incubation the medium was filtrated from the cells and biodestruction products were extracted by equal volume of chloroform.

The products of biodestruction of the aromatic and phenol compounds were investigated by method of Reversed-Phase High-Efficient Liquid Chromatography (HELC). The

analyses of samples of initial and residual after biodestruction of the aromatic compounds were also carried out by the NMR- and IR-spectroscopic methods [21-23] for definition of their structure.

The chromatographic investigations were carried out on high-efficient liquid chromatograph of firm «Kovo» (Czechia), with UV-spectrophotometric detector, with working wavelength λ=254 nm. Two columns by size 3.3·150 mm, filled by reversed immobile phase «Separon SGX-C18», by size of particles 7 mcm, medium temperature 20-25ºC were used. Eluent – methanol:water (75:25 rev.%). The mobile phase rate – 0.3 ml/min. The identification of components was carried out by comparison of retention parameters of standard mixture and products of biotransformation. The standard solutions with concentration 1-1.5 mg/ml were made in eluting system methanol : water (75:25 rev. %) according to the standard method [23, 24]. The structural composition of biodegradation of the individual hydrocarbons and phenol compounds was determined by the methods: IR-spectroscopy (UR-20) (thin layer) in the range of the spectrum 4000-700 cm-1; for spectra NMR1H - apparatus «Tesla BS - 487B» with working frequency 80 mHz in CCl4 with use of hexamethyldisiloxane (HMDS) as the internal standard. For all experiments the control experiments (without making – bacteria-biodestructors) have been carried out.

## 3. Results and Discussion

The conducted experiments showed that degradation by *Bacillus sp. 15* and *Pseudomonas sp.4* of the studied compounds is impact by their transformation to the corresponding phenols, carboxylic and phenol carboxylic acids, polyatomic phenols, to benzoquinones and also in small quantities – to polymers.

The results of the chromatographic investigations of the products of biodestructions prepared in the process of degradation of the aromatic compounds by the strain of *Pseudomonas,* are presented in Fig.1. It should be noted that the identification of the products of biotransformation has been carried out on final stage of the microbiological synthesis continuing about 25 days and accompanying by formation of the individual organic compounds.

As seen from Fig.1 (curve a) a degradation of benzene beginning by oxidation of aromatic ring in six days, on the tenth day of the exposition is completed with the formation of pyrocatechin (peak 1) and phenol (peak 2) 30% and 60 % respectively. But in a case of toluene (fig 2, curve b) in this mode of degradation there have been fixed basically 3 individual compounds dominating by quantity among which were: o-cresol (50 %) and benzoic acid (30 %) (peaks 2 and 3), and also 2,3-dihydroxytoluene (10%) (peak 1).

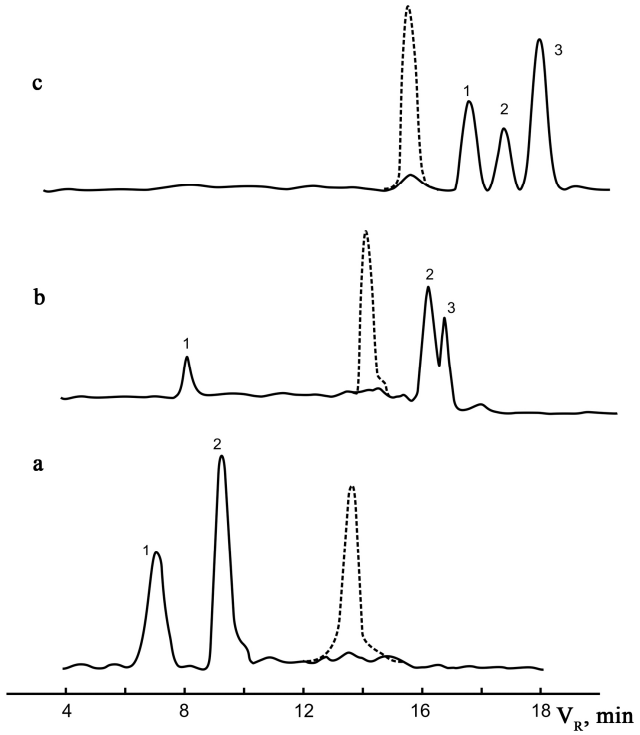



***Fig 1.*** *Chromatographic curves of the products of biodegradation of benzene (a), toluene (b) and ethylbenzene (c): a) Pyrocatechin (1), phenol (2). b) Dihydroxytoluene (1), o-xylene (2), benzoic acid (3). c) Acetophenon (1), phenylacetic acid (2), benzeneformic acid (3). The initial products – benzene, toluene and ethylbenzene are shown by dotted lines. Here and in the other figures the numbers of peaks are shown in brackets.*

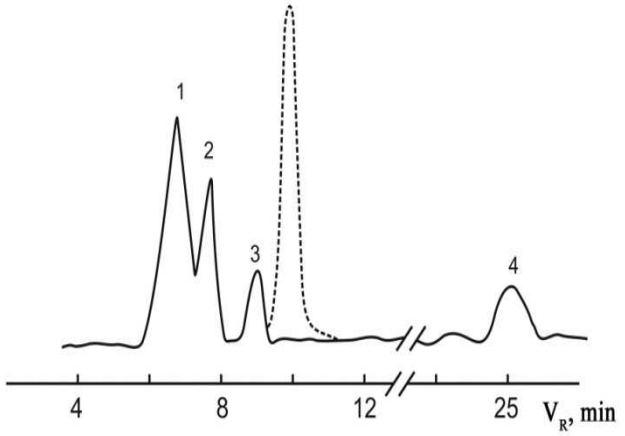
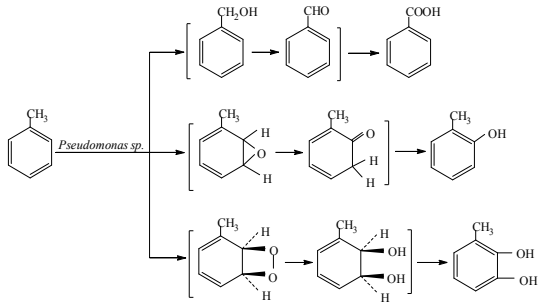


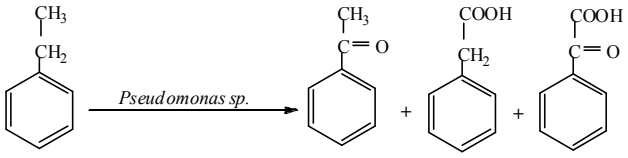

***Fig 2.*** *Chromatographic curves of the products of biodegradation of phenol.Hydroquinone (1), pyrocatechin (2), pyrogallol (3), oligophenylene (4), (dotted lines – initial phenol).*

Undoubtedly, a transformation of these compounds takes place with formation of the intermediate substances and, consequently on the basis of known theoretical concepts, a scheme of biodegradation for ex., toluene under action of *Pseudomonas sp.* can be presented as follows:




Along with the chromatographic analyses the IR- and NMR$^1$H spectroscopies confirming structure of the products of biodegradation of the aromatic hydrocarbons have been carried out. In the IR-spectra of the products along with the absorption bands characteristic for phenyl ring the absorption bands in the field of 3590-3650 cm$^{-1}$ characteristic for hydroxyl group have been also detected. In their NMR$^1$H spectra the chemical shifts of protons of the aromatic ring and hydroxyl group have been detected at δ=6.25-7.2 ppm and δ=7.65-9.00 ppm, respectively [21, 22]. In the IR-spectra of the products of biodegradation of toluene along with the absorption bands characteristic for Ar-$CH_3$ , the bands characteristic for hydroxyl (3590-3650 cm$^{-1}$), and carboxyl (1680-1700 cm$^{-1}$) groups have been also detected. In the NMRP$^1$H –spectrum of the products of degradation the signals in the fields of δ=2.1-2.8 ppm., δ=7.70-9.00 ppm. and δ=9.5-10.5 ppm characteristic for protons Ar-$CH_3$, Ar-OH and Ar-COOH groups have been found.

It has been shown in work [13] that ethylbenzene under action of micromycetes in cooxidative conditons is converted into acetophenon and phenyl acetic acid. The investigations showed that a degradation of ethylbenzene under action of bacteria of a kind *Pseudomonas sp.* proceeds with formation besides acetophenon (28%) and phenyl acetic acid (45%) (fig. 2, curve c, peaks 2 and 3), benzoyl formic acid (30%) (peak 1) on scheme:




Thus, in the developed conditions a transformation of toluene and ethylbenzene takes place simultaneously on two directions both with oxidation of side chain and aromatic ring. In spite of the fact that a degradation of toluene on mixed mechanism is known from literature [8], nevertheless, the products of oxidation in this case are differed from previously detected ones. For ex., it has been shown in work [12, 13] that depending on view of microorganisms a degradation of toluene takes place in various directions. It is interesting to note that the forming compounds in experiments previously carried out by the authors [13, 16, 17] have not been fixed in this work. This suggests that the same bacteria cultivated in various conditions and isolated from various sources on mechanism of action can be differed.

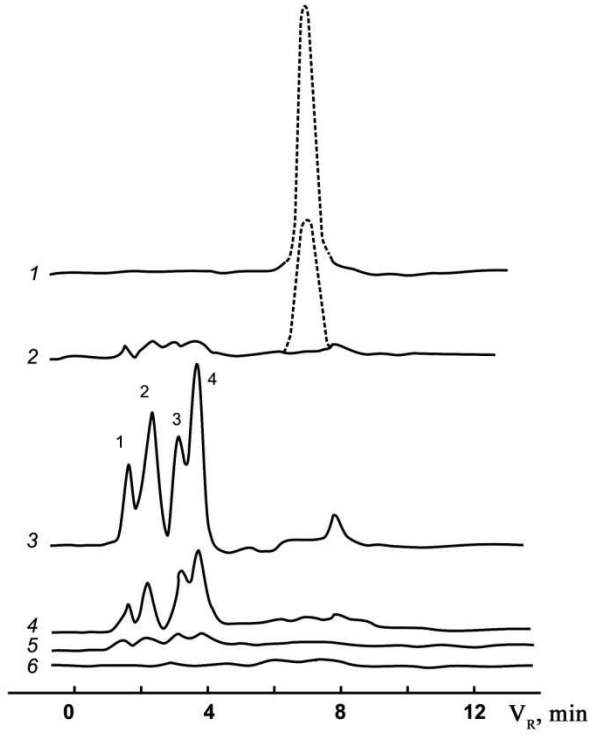
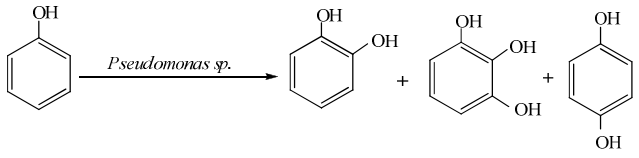



***Fig 3.*** *Chromatographic curves of the products of biodegradation of pyrocatechin depending on exposition time. Curves: 1 – initial pyrocatechin; 2-6 – exposition time – 6-7, 10-25, 26, 28 and 30 days respectively. Peaks 1-5: 2-keto-cis, cis-muconic acid (1), 2-hydroxy-cis, cis-muconic acid (2), polyaldehyde 2-hydroxy-cis, cis-muconic acid (3), cis, cis-muconic acid (4), pyrogallol (5), (dotted lines – initial pyrocatechin).*

The analysis of biodegradation of phenol under action of phenol-acquiring bacteria by the chromatographic methods showed that (fig.3) a phenol in the investigated conditions is transformed to polyatomic phenols: to pyrocathechin (40%) and to pyrogallol (20%), and also to hydroquinone (10%) (peaks 2, 3 and 1). Among the products of biotransformation of phenol (about 15 %) the polymer fraction with comparatively washed band corresponding to oligophenylene with molecular mass ~3500 (peak 4) has been fixed:




It has been established that unlike aromatic hydrocarbons and phenol investigated in work in a case of biotransformation of pyrocatechin and hydroquinone by bacteria of a kind *Pseudomonas sp.* the process of degradation proceeded mainly on third way with opening of the aromatic ring in ortho- and meta-positions. During analysis of chloroform extracts of pyrocatechin and hydroquinone consecutively 4 and 3 linear compounds relating to muconic acid and its various derivatives have been revealed. In Fig.4 the chromatographic curves of dynamics of change of the process of biodegradation of

pyrocatechin depending on exposition time are in detail presented. As follows from figure an intensity of peak of the initial pyrocatechin (curve *1*) on the sixth day of exposition is noticeably decreased and on chromatogram of extract the traces of peaks apparently corresponding to the individual compounds of biodestruction are appeared (curve *2*). It has been established that after ten days of exposition a formation of the products of destruction is fully completed (curve *3*) and the peaks of the following compounds by maximum intensities are fixed : – 2-keto-cis, cis-muconic acid (10%), 2-hydroxy – cis, cis-muconic acid (20%), polyaldehyde 2-hydroxy cis, cis-muconic acid (20%), cis, cis-muconic acid (40%) and pyrogallol (5%) (peaks 1-5). It should be noted that in this case a stability of composition of these compounds is kept to 25 days. But the next days (to a month) an utilization of the forming compounds by sharp decrease of intensity of signals up to their full disappearance on chromatogram takes place (curves *4-6*).

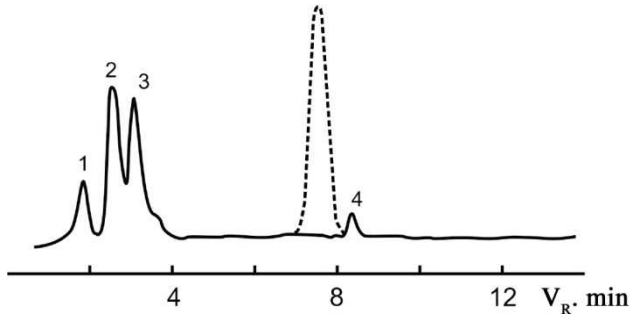
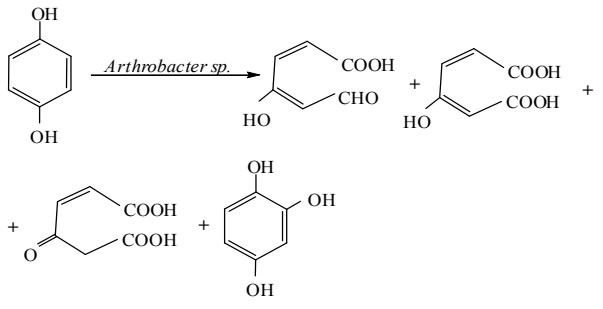


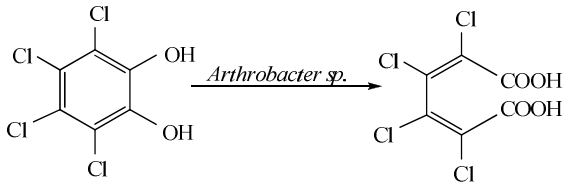

***Fig 4.*** *Chromatographic curves of the products of biodegradation of hydroquinone 3-keto- cis, cis-muconic acid (1), 4-hydroxy-cis, cis-muconic acid (2), polyaldehyde 4-hydroxy-cis, cis-muconic acid (3), hydroxyhydroquinone (4), (dotted lines – hydroquinone).*

In a case of hydroquinone are formed: 3-keto- cis, cis-muconic acid (25%), 4-hydroxy- cis, cis-muconic acid (35%), polyaldehyde 4-hydroxy-cis, cis-muconic acid (30%) and hydroxyhydroquinone (4%) (fig.5, peaks 1-4), as is presented on the following scheme:




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

The investigations showed that unlike pyrocatechin a biodegradation of tetrachlorpyrocatechin proceeds only in one direction with formation of tetrachlormuconic acid with yield to 13%.




The structure of terachlormuconic acid has been also confirmed by data of the IR- and NMR$^1$H-spectroscopy. In the IR-spectrum of last one along with the absorption bands of C-Cl (650 $cm^{-1}$) and C=C (1625 $cm^{-1}$) bond the absorption bands at 1720 $cm^{-1}$ characteristic for carboxyl group have been detected. In NMR$^1$H-spectrum the signals in the field of σ =7,80-7,90 ppm for protons of carboxyl group have been found.

It should be noted that the beginning of the process of transformation of the investigated compounds isolated by bacteria, as noted above, in all cases was observed in 6-7 days, a formation of intermediate products was continued for 3-4 days. During this time on chromatograms of the initial compounds depending on exposition time their signals fixed by UV-detector disappear completely.

Thus, application of the method of reversed-phase high-efficient liquid chromatography for definition of the products of biodegradation of the aromatic hydrocarbons and phenols– by the selected microorganisms helped to identify more than 15 individual compounds. . The linear compounds among them were only compounds fixed in a case of pyrocatechin and hydroquinone a degradation of which took place with opening of aromatic ring in meta- and ortho-positions. It has been shown that unlike pyrocatechin and hydroquinone, in a case of tetrachlorpyrocatechin a decomposition proceeds only on ortho-position with formation of tetrachlormuconic acid. The prepared results can be used in thin organic synthesis for preparation of practically useful functional compounds (acids, ketones, aldehydes, etc) [25]. The developed conditions of reversed-phase HELC open the prospect of further improvement of the method of identification of aromatic hydrocarbons and phenols in the process of biodegradation.

# 4. Conclusions

The use of a method of phase high-efficient liquid chromatography helped to study the dynamics and direction of the process of biodegradation of aromatic hydrocarbons and phenols. Besides that, the application of this method favored identification of the products of biodegradation, a structure of which has been confirmed by the spectral

methods (IR- and NMR[1] H). The prepared results can have an important value for development of biopreparations on purification of the environment from oil and phenol pollutions.

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