

Efficiency Assessment of Biological Samples for Destruction of Phenol Compounds

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Abstract – The issues of soil contamination in production territories, solid waste landfills and urban environment are discussed by scientists of many countries. The problem of rehabilitation of such territories is considered relevant, and thus requires the corresponding technologies. Regarding phenol compounds the paper describes the recovery options of phenol degraders and its derivatives; suggests using the consortia of bacterial destructors although without the results of their application in real conditions for remediation of the polluted soil. In this study the native saprophyte strain *P. putida* SU12 with strong desctuctive potential is selected from the consortium of destructors of chronically polluted soils. The in vitro studies of the adaptation of a strain destructor to remediation conditions of chernozem leached in the impurity mode with phenol compounds is conducted. Biological product samples are obtained for phenol destruction in liquid (microcapsules) and solid (sorber) phases. The dynamics of phenol content change in soil samples from Terengulsky district of Ulyanovsk region in the presence of samples of biological products proves its considerable destruction. The biological product is perspective for field tests since bacteria-destructors quickly adapt to natural conditions of the polluted soil in the presence of soil microflora without losing their destructive properties. Modeling of sorption-biological technology of rehabilitation of soils polluted by phenol compounds is carried out and the scheme of application of the selected biological product sample in natural field conditions is developed.

Keywords – remediation of soils, phenol compounds, biodestructor, *Pseudomonas putida*, biological product, sorber

1. INTRODUCTION

One of the relevant tasks of modern biotechnology is the creation of biological products on the basis of xenobiotic strains-destructors recovered from native microflora to solve

various tasks related to the rehabilitation of polluted soils as a result of increased technogenic burden [1–5].

Phenol compounds represent the most widespread composed class of secondary compounds with various biological activity. Phenol compounds belong to the class of stable organic antiseptics with strongly expressed toxic action. The main sources of phenol pollution are paper and pulp makers, wood processing and oil-processing industries. Phenol compounds change the soil-forming processes, chemical and physical properties of soil [6-7]. Besides, strong soil pollution with phenol compounds leads to significant changes of microbiocenosis and decreases the activity of microbiological processes since autochthonous microflora is usually inhibited under high intoxication of soil. In such cases the process of soil self-cleaning is almost completely oppressed, and it is possible to restore such soils only through compulsory measures.

In the course of compulsory bioremediation, biological products based on targeted strains-destructors get to polluted soil. There are standard ways to receive such strains: selection and with the use of targeted genetic modifications [8–10]. From the environmental point of view, the selection method with the recovery of destructors from native microflora of chronically polluted soils is considered the most expedient [11–12]. Such strains have stable destructive potential, which is genetically fixed in generations. However, many authors apply a “stressful” method to receive specific biodestructors in case of artificial laboratory pollution of soils with targeted xenobiotic. In this case targeted selection takes place already at the pollution stage when weak forms of microorganisms do not manage to adapt to high concentrations of pollutant and die, while the strains with high pollutant destruction survive.

Despite various sources of origin of strains-destroyers, the recovery technology of pure cultures from natural consortia withstanding high pollution doses via the method of accumulative liquid culture is widely discussed in literature [1, 5, 7, 13–14]. Considering the relevance and demand for biological products for bioremediation of soils polluted by phenol compounds we analyzed the literature data on strains-destroyers and tested biotechnological aspects of such strains recovered from chronically polluted soils in Terengulsky district of Ulyanovsk region and from soil samples with experimental pollution.

The use of various carriers for immobilization of biodestructors seems quite relevant. Similar studies of phenol are described in work of Carabajal M., et al. (2016). But we failed to find data on the use of such combined biological products for biodestruction of phenol in soil conditions. Therefore, in our study we used findings of domestic authors on perspective sorbents for immobilization of bacteria-destroyer and conducted research on sorption-biological technology of soil remediation systems.

II. METHODS AND MATERIALS

The paper studies the culture of a destructor *P. putida* SU12 in agarized elective medium with phenol content in the amount of 270 mg/l received via standard reseeded from liquid to dense elective medium with gradual increase of phenol concentration [15]. This culture grown via the technique suggested by the authors was analyzed from a comparative aspect [16]. The initial culture *P. putida* SU12 was sowed in a test tube with 5–10 ml of semi-nutrient medium and placed on a rotor shaker at a temperature of 28–30°C at the rotation speed of 220 rpm during one day. The grown accumulative culture served the inoculum for sowing in 200 ml of liquid phenol-containing elective medium in Erlenmeyer flask. The inoculum was placed in the concentration of cells in medium 8–10x10⁷ kl/ml and was grown within 7 days on a rotor shaker, after which the concentration of cells and residual phenol in a cultural liquid was defined.

The destructive activity of a strain *P. putida* SU12 in liquid-phase medium was studied in carbon-free mineral medium M9 [17] with gradually increasing amounts of phenol equivalent PNEC (elective medium).

The concentration of bacterial cells was measured in cultural liquid on spectrophotometer SF-102 at wavelength $\lambda = 630$ nanometers in a ditch of 10 mm (OP₆₃₀) and defined by the calibration curve expressing the dependence of optical density on the number of cells determined via turbidity standard BAK-10 (LLC ORMET).

The concentration of phenol compounds was defined on mass-spectrometer Agilent Technologies (GH 7820A, MS 5975). Sample preparation and extraction were carried out according to Methodical instructions 4.1.1062-01. Chromato-Mass-Spectrometry of semi-volatile organic compounds in soil and industrial and consumption waste. [18]. The degree of phenol biodegradation was counted as the difference between initial and final content of hydrocarbon in a sample to initial HC content expressed in percentage points.

The surface morphology of sorbents was studied via scanning electronic microscope TESCAN MIRA II LMU. The specific surface area and porosity (micro, mesoporosity) of sorbents were measured via low-temperature adsorption of nitrogen on a high-speed analyzer of gas sorption Quantachrome NOVA 4200e by Quantachrome (USA). The Barrett-Joyner-Halenda (BJH) method was used to study the volume of pores and their size distribution.

The microscopic study of samples of microcapsules was conducted visually on a microscope Mikmed-5 (by LOMO) at lens magnification by 40 and 100 times.

The quantitative analysis of the element structure of used bentonite sorbents was made on a power dispersive x-ray fluorescent spectrometer EDX-720 (SHIMADZU, Japan) via the calibration curves and fundamental parameters.

The X-ray phase analysis of samples was carried out using DRON-4 diffractometer with BSV-6 tube. PCPDFWIN database, v. 2.02, 1999 of the International Center for Diffraction Data (JCPDS) was used to analyze diffractograms.

The specific surface area, porosity, pore volume, size distribution of granulated forms of sorbents were defined via low-temperature adsorption of nitrogen on gas sorption analyzer Quantachrome NOVA 4200e by Quantachrome (USA) with the software for complex processing of experimental data.

The pore volume and definition of their size distribution was studied via the Barrett-Joyner-Halenda (BJH) method. Desorption or adsorption isotherm in the pressure range of 0.967–0.4 P/Po served the basic data for BJH calculations.

The granules were received by roasting of bentonite powder at different temperatures (550, 800 °C) with organic components, on the equipment of the LISSKON Scientific and Production Enterprise (Saratov) [19].

Statistical processing was carried out via the Excel statistics package (MS Office 2007). All experiments were repeated three times.

III. RESULTS

Earlier from soils chronically polluted by phenol compounds on the ground in Terengulsky district of the Ulyanovsk region we recovered the consortium of strains-destroyers of phenol compounds [15], from which the strain *P. putida* SU12 capable to use phenol as the only source of carbon and energy and not demonstrating pathogenicity [16] was selected and identified as a model.

Standard selection methods to increase the destructive activity of this strain included reseeded of culture from liquid to dense elective medium with gradual increase of phenol concentration. As a result, the resistance of *P. putida* SU12 on mineral dense medium to phenol reached the concentration of 270 mg/l of phenol, which is equivalent to 2000 PNEC (PNEC of phenol makes 0.136 mg/kg of soil), or conditional doses. However, in liquid elective medium the degree of phenol destruction was not too big and made about 70 mg/l (500 PNEC, or 500 conditional doses) within 9 days. Further increase of phenol concentration in liquid medium was

oppressing both the growth of this strain and its destructive activity. Therefore, it was planned to carry out in vitro strengthening of the destructive potential of phenol strain-destroyer *P. putida* SU12 via cyclic selective method gradually increasing phenol concentration in liquid-phase medium in combination with the intermediate stage of enrichment culture. The scheme of cyclic gradual increase of phenol concentration in liquid-phase medium in combination with intermediate stage of enrichment culture was developed and applied. As a result, within 7 days *P. putida* SU12 is able to reduce phenol concentration in liquid medium by 92.6% of initial concentration of 340 mg/l (2500 conditional doses earlier called as the maximum allowable concentration). The strain is kept in dense medium with phenol concentration of 700 mg/l, i.e. 5000 conditional doses that demonstrates its good resistance to high phenol concentrations [20]. In laboratory soil systems experimentally polluted by phenol up to 500 conditional doses when *P. putida* SU12 was introduced in the concentration of 10^8 cells into 1g of air-dry soil, on the 30th day the residual amount of phenol made 49% of its initial content.

The identification of this strain was confirmed with nucleotide sequences of 16S rRNA gene fragments in the Laboratory of Molecular Biology of the All-Russian State Center for Quality and Standardization of Medicines for Animals and Feed. The comparative analysis of the received sequences was carried out via BI databases (GenBank, EMBL, DDBJ, PDB). The homology with sequences of *Pseudomonas putida* 100% is found (GenBankacc. No. AB513735, JF911376, etc.).

Many authors highlighting pure cultures or consortia position them as strains-destroyers [5, 8, 10]. However, it is not always possible to apply such strains to actual recultivation of polluted soils since they can be inefficient in soil in the conditions of interspecies competition and influence of biotic and climatic factors. In this regard, the authors consider it necessary to study the ability of recovered strains to adaptation and manifestation of destructive properties within the experiment or in chronically polluted soil to confirm the status of a destroyer of the specified culture. Besides, the practical application of destroyers for bioremediation of soils is only possible after production of biological products from them. As a rule, combined biological products, in which bacteria-destroyers are immobilized on carriers-sorbents with high accumulating capacity, are used: vermiculite, sawdust, peat, straw, activated coal and others, which increases the viability and activity of bacteria thus improving the quality of cleaning [12, 14]. In this regard, we selected a sorbent-carrier as an abiotic component of a future biological product sample. Microcapsules – porous granules of polyurea and bentonite granules were chosen for pilot studies. The cell immobilization technology of phenol strain-destroyer on microcapsules was tested and the sample of combined biological product representing a liquid-phase system from a suspension of bacteria with the concentration of 10^8 C/ml in the balanced solution of mineral salts from 0.1% and microcapsules in the ratio from 97.5 to 2.5 wt. %, respectively was developed (sample 1).

Bentonite granules of various modification and different degree of fraction dispersion from 0.5 to 2 mm, according to GOST P 51641-2000 were also used as a matrix for a combined biological product [21]. The morphology of the granulated sorbent surface was studied, the specific surface area and porosity (micro, mesoporosity), volume of pores and their size distribution were determined. The scanning electronic microscopy of the granulated sorbent structure detected different voids optimal for bacteria occupation. The method of immobilization (introduction of ready porous materials, natural settling of carrier pores with bacteria) of bentonite granules was selected empirically by incubating the granules with the suspension of strain-destroyer cells in the balanced solution of mineral salts from 0.1% of carbohydrate with 10^9 concentration of cells in 1 ml of a mix. The combined granulated biological product (sample 2) represented a solid-phase system from bentonite granules with the basic structure of a destroyer.

The adaptation of *P. putida* SU12 and the designed biological product samples to remediation conditions of chernozem leached in the impurity mode by phenol compounds was studied in vitro.

To control the ability of bacterial cells it is the most effective to use phenol as the only source of carbon, bacterium from different biological products: from the culture of a destroyer grown in the minireactor on the 2nd day in working cultivation (F11), from encapsulated option of a biological product (F12), from reseeded of medicine on the basis of a sorbent in liquid nutrient medium (F13), from initial culture in the liquid medium with phenol (F14) and in dense medium (F15) were streak-inoculated in dense minimal medium M9 containing phenol in various concentrations. After incubation in thermostat under standard conditions the visual definition of existence or lack of growth was carried out within 3 days.

It is found that bacteria from all options of medicine kept the ability to growth in dense medium (Fig. 1) containing 10 maximum allowable concentrations (1.36 mg/l) and 100 maximum allowable concentrations of phenol (13.6 mg/l). For quantitative assessment of destructive ability of biological product prototypes in relation to each other the gain of biomass was defined by the change of OP_{630} of a cultural liquid and by the change of phenol concentration. To exclude the effect of imposing the processes of phenol self-disintegration on biodestruction processes, the control sample was placed. The M9 medium from 0.2% of glucose of 100 ml in volume without bacteria containing the same concentration of pollutant and cultivated in the same conditions as prototypes served the control sample (Tab. 1). It is established that the cultures of biological product cells in a liquid phase and inoculated on a sorbent had the greatest gain of biomass and destructive activity: decrease in concentration at F11 – by 69.4 %, at F13 – by 88.0 %.

To check the ability of prototypes of a biological product to utilize pollutants in the conditions closer to natural, we conducted laboratory tests with the model pollution of soil. Since under natural conditions the soils polluted by phenol compounds have native microflora, in laboratory conditions it was necessary to consider its possible contribution to pollutant

destruction. To obtain the objective data on the destructive ability of prototypes of a biological product, sterile and unsterile soils were used during creation of model systems in laboratory studies.

TABLE I. CHANGE OF PHENOL CONCENTRATION IN THE CULTIVATION MEDIUM OF BACTERIA-PHENOL DESTRUCTORS

Figure 1. Growth of destructor bacteria from different options of a biological product on agarized medium containing different phenol concentrations: a-b) 10 maximum allowable concentration of phenol; c-d) 100 maximum allowable concentration of phenol Table 1. Change of phenol concentration in the cultivation medium of bacteria-phenol destructors Studied options	Initial concentration of phenol, mg/l	Final concentration of phenol, mg/l	OP ₆₃₀ initial seeding	OP ₆₃₀ final seeding
Control	13.10	4.50	-	-
FI1	12.10	3.70	0.07	0.35
FI2	13.70	2.60	0.06	0.32
FI3	13.46	1.60	0.07	0.83

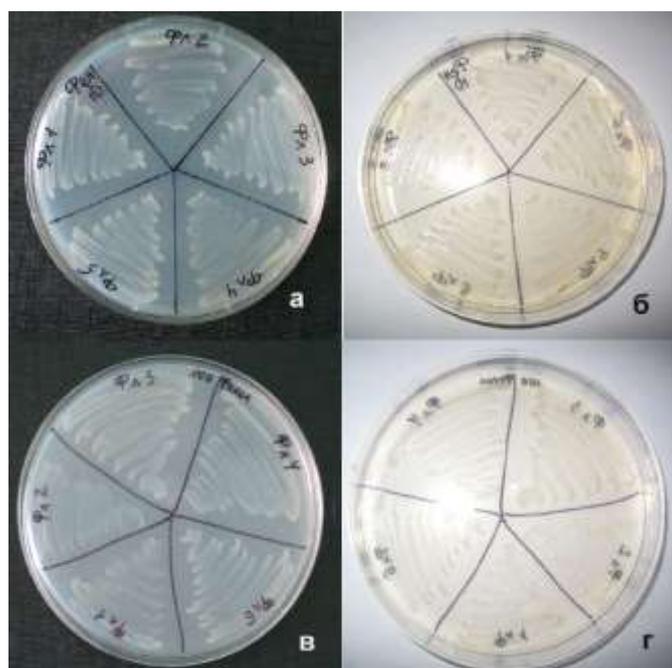


Fig. 1. Growth of destructor bacteria from different options of a biological product on agarized medium containing different phenol concentrations: a-b) 10 maximum allowable concentration of phenol; c-d) 100 maximum allowable concentration of phenol

The samples of sterile soil were received during sterilization of soil samples (4 flasks with 100 grams of chernozem leached from the control platform in Terengulsky district of Ulyanovsk region) in the autoclave at 0.5 atm within 30 minutes. Then, phenol was introduced as a solution in the amount equal to the final concentration in soil corresponding to 100 maximum allowable concentrations in these samples, and kept for three days at natural lighting and room temperature to get a uniform distribution and good adsorption of a pollutant in soil. In three days we recovered samples to

define the initial concentration of phenol (0 days). Then the prototypes of a biological product were placed in soil in flasks named “sterile test” (T_{st}) to final concentration of bacteria in 10⁸ C/gram of soil. A flask with soil where 20 ml of M9 sterile medium was added from 0.5% glucose was used as a sterile control sample (C_{st}).

Nonsterile soil samples (4 flasks by 100 grams of the same soil) were left open for free gas exchange in the course of the experiment. Besides, phenol was added to final concentration of 100 maximum allowable concentrations, and samples were recovered in three days to define the initial concentration of a pollutant (0 days). Then biological product prototypes were placed in soil in flasks named “nonsterile test” (T_{nst}) to final concentration of bacteria in 10⁸ C/gram of soil. Soil where 20 ml of M9 sterile medium was added from 0.5% glucose was used as nonsterile control (C_{nst}).

Flasks with all samples of soil were cultivated at natural lighting and room temperature within 21 days. The concentration of phenol in samples was defined on 7, 14 and 21 days from the moment of defining the initial concentration of pollutants (0 days) by gas-chromatographic method. Table 2 shows the study of destructive ability of a biological product sample in a liquid phase (with microcapsules) in soil polluted by phenol.

TABLE II. CHANGE OF PHENOL CONCENTRATION IN MODEL SOILS IN THE RESENCE OF A BIOLOGICAL PRODUCT SAMPLE IN A LIQUID PHASE

Studied model soils	Phenol concentration (initial concentration of 100 MAC), mg/kg			
	0 days	7 days	14 days	21 days
C sterile	12.8	12.4	12.8	11.5
T sterile	12.8	10.5	7.8	3.9
C nonsterile	12.1	11.9	10.4	8.8
T nonsterile	11.8	8.9	8.4	2.7

Figure 2 shows the study of destructive ability of a biological product sample in a solid phase (with sorbent) in soil polluted by phenol.

Minor decrease of nonsterile control sample is noted in the analysis of dynamics of phenol concentration in soil, while in sterile control sample the phenol was almost unchanged. In this regard, it is possible to assume that some native microorganisms have some ability to phenol destruction

In soil samples with destructors the concentration of phenol decreased rather intensively.

However, the comparison of phenol concentration decrease in both options of soils (sterile and nonsterile) showed significant differences. The destructors introduced into soil with already existing native microflora subjected phenol to destruction as effectively as the sterile soil. The reduction of phenol concentration taking into account natural losses within 21 days made: in a sterile sample – 59.3 %, in nonsterile sample – 49.8 %.

Analyzing the obtained data on the dynamics of phenol concentration in soil samples in the presence of biological product samples, it is possible to draw the conclusion on the

destruction of a pollutant in significant quantities. At the same time the substantial contribution to destruction from native microflora is noted.

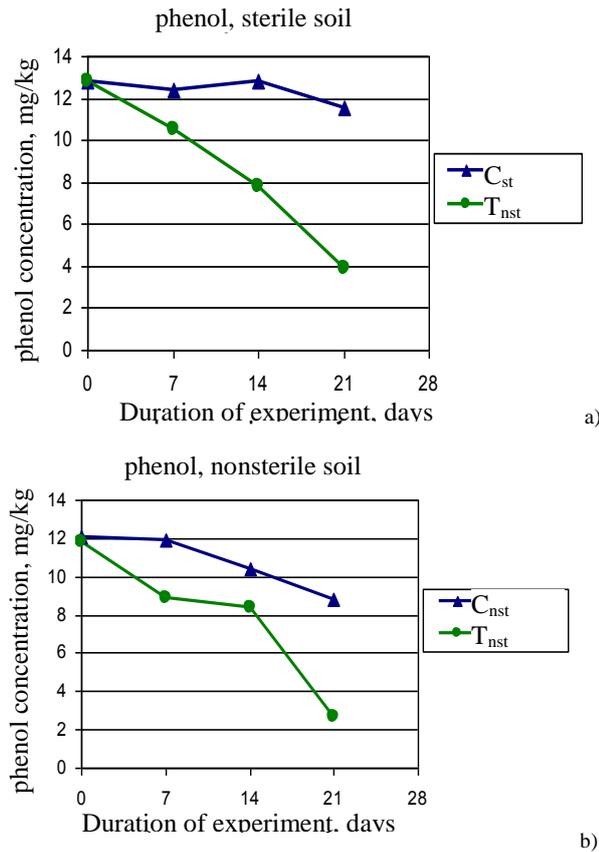


Fig. 2. Dynamics of phenol concentration in soil with a biological product sample in a solid phase (with sorbent): a) destruction of phenol in sterile soil; b) destruction of phenol in nonsterile soil

IV. CONCLUSION

The developed and applied selection scheme of *P. putida* SU12 allowed considerably strengthening its natural destructive potential. This strain does not demonstrate any factors of pathogenicity and is native for Ulyanovsk region. The set of these features makes it possible to position it as a true strain-biodestructor of phenol with excellent biotechnological potential as a biological component in a complex biological product.

The biological product samples developed and tested in vitro in sterile and nonsterile soils for phenol destruction can be considered perspective for field tests since bacteria-destructors can quickly adapt to natural conditions and joint activity with soil microflora without losing their destructive abilities.

The study is performed under financial support of the grant of the Russian Foundation for Basic Research (No. 16-44-732050).

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