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RESISTANCE OF KARST CAVES MICROORGANISMS TO *p*-NITROCHLOROBENZENE

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The aim of this study was to determine homeostasis quantitative parameters (maximum permissible concentrations of xenobiotic and types of cell response) of microbial communities from cave clays influenced by *p*-nitrochlorobenzene. It was determined the ability of cave bacteria to transform xenobiotic. General bacteriological methods were used as well as gas chromatography-mass-spectric method. Chemoorganotrophic karst caves microbial communities isolated from Mushkarova Yama (Podolia, Ukraine) and Kuybushevskaya (Western Caucasus, Abkhazia) were highly resistant to *p*-nitrochlorobenzene. For Mushkarova Yama representative strain *Rhodococcus erythropolis* P3 the influence of *p*-nitrochlorobenzene (in concentration range 50–300 mg/l) on physiological parameters of bacterial cells was shown. The efficiency of *p*-nitrochlorobenzene degradation was proportional to the decreasing of redox potential. Thus, cave microorganisms could potentially be used to create new environmentally friendly biotechnologies, for example enterprises for wastewater treatment from nitrochloraromatic compounds.

Key words: karst caves, microbial communities, *p*-nitrochlorobenzene.

Nowadays one of the main problems in bioremediation technologies is utilization of wastewaters from organic synthesis. Xenobiotics included both nitro group, chlorine and aromatic ring are special dangerous to the biosphere. Among such compounds is *p*-nitrochlorobenzene (NCB). In Eastern Ukraine (Alchevsk and Severodonezk cities) there are several factories producing nitrochlorobenzene. Utilization of wastewaters from these enterprises is a serious problem for natural ecosystems in that area. In 2013 explosion at such plant caused a powerful release of toxic NCB and as a result, ecological disaster in Eastern region. Its toxicity is destined by the aromatic ring and attached chlorine and nitro group in the para-position. Bactericidal concentration of NCB for most soil and aquatic chemoorganotrophic microorganisms is 10 mg/l [1]. However, microbial communities of natural ecosystems [2–4] demonstrate the ability to maintain homeostasis not only in the presence of well-known bactericidal concentrations of extreme factors but even at very high. Using the word "homeostasis", in this context, means the ability of microbial communities to maintain vital activity and functioning affected by extreme factors.

The aim of our work was to determine homeostasis quantitative parameters of microbial communities isolated from Mushkarova Yama and Kuybushevskaya cave clays in the NCB concentration gradient. Homeostasis quantitative parameters included maximum permissible concentrations and types of response. In addition, it was necessary to determine ability of cave bacteria to transform xenobiotic.

Materials and Methods

The objects of study were chemo-organotrophic aerobic microbial communities isolated from caves clays and strain P3 (*Rhodococcus erythropolis*, Mushkarova Yama cave). One clay sample was collected in the farthest point from the entrance of Mushkarova Yama labyrinth cave laid in the Neogenic gypsum in Podolia, Ukraine.

Another sample was collected in the bottom hall of Kuybushevskaya vertical cave formed in the Jurassic limestones of the Western Caucasus, at the 1 km depth. After collecting both samples were stored in a refrigerator at 4 °C in sealed plastic bags.

Microorganisms isolated on agar media: Nutrient Agar (HiMedia Laboratories Pvt. Ltd.) and oligotrophic agar (OA). Concentration of organic compounds in the medium was determined by permanganate redox titration method [8]. For oligotrophic medium (OA) Nutrient Broth (HiMedia Laboratories Pvt. Ltd.) was diluted 10 times with distilled water and agarized (15 g/l).

Microbial communities quantitative parameters of resistance (CFU, colony morphotypes number and diameter) were determined in liquid and agar media with NCB. As agar media Nutrient Agar (NA HiMedia Ltd.) and oligotrophic agar (OA) were used. As liquid media Nutrient Broth (NB) and oligotrophic broth were used. In order to get concentration range 50–300 mg/l NCB aliquots of NCB alcohol solution were added in molten and cooled to 45–50 °C agar medium. Petri dishes with NCB agar medium were kept in a desiccator with silicagel for removal of condensation moisture and sterility verification. After that the plates were inoculated with microbial suspensions of samples tenfold dilutions. The agar medium without NCB was used as a sterility control. Inoculated plates were exposed during 7 days in closed desiccators with a sterile silicagel. The number of CFU, colony morphotypes and their diameter were controlling criteria of NCB resistance.

To quantify parameters of resistance of caves microbial communities we used copiocarbotrophic medium (Nutrient Agar — NA with carbon concentration is 850 mg/l) and oligocarbotrophic medium (oligotrophic agar — OA with carbon concentration 85 mg/l) due to the ability of cave microorganisms to grow in a wide range of organic compounds concentrations.

Determination of the physiological parameters of P3 strain. During the experiment we monitored pH, Eh (redox potential), optical density, the concentration of xenobiotic and gas phase composition. The optical density was determined on photocolorimeter KFK-2MP (λ = 540 nm, optical path 0.5 cm). Indexes of pH and Eh were determined using a «pH meter pH-Milivoltmeter MA-150» potentiometer. For Eh measuring we used two electrodes: measuring EVP-1 and chlorosilver comparison electrode EVL-1M3. For pH measuring — combine electrode ESK-10603/4.

Ability of P3 strain to transform xenobiotic. The strain was cultured in NCB concentration gradient (50–300 mg/l) in NB medium during three days. Concentration of NCB and chloroaniline (ClA) was determined by mass spectrometry using gas chromatography-mass-spectric system Agilent 6890N/5973inert (capillary column HP-5MS (J&W Scientific, USA)). Gas carrier — helium; initial column temperature — 150 °C; final column temperature — 250 °C; Temperature gradient — 4 °C/min; interface temperature — 280 °C; type ionization — electron impact; ionization energy — 70 eV. For this, the culture liquid was centrifuged (1700 g, 15 min). Hexane (0.5 ml) was added in the supernatant (0.7 ml), and suspended for 3 min. Next, the suspension was centrifuged (360 g, 10 min) to separate phases. Thereafter, hexane was collected (0.4 ml) and analyzed. Data processing of gas chromatography-mass spectrometry analysis was performed using the computer program ChemStation and integrated database of mass spectra NIST 02.

All experiments were run triplicate. Experimental data were analysed by statictical methods in Excel, $P \leq 0.05$.

Results and Discussion

Mushkarova Yama and Kuybushevskaya caves clay samples were collected in zones free of anthropogenic load provided no influence of anthropogenic factors on microbial communities. Maximum permissible concentrations of NCB were determined for these ecosystems microbial communities (Fig. 1).

Maximum permissible concentrations of NCB for microbial communities of Mushkarova Yama and Kuybushevskaya clays were 200 and 300 mg/l, respectively. In both cases, the number of surviving microorganisms was greater on the medium with high organic compounds concentrations (NA) comparing with the medium with a low organic compounds concentration — oligotrophic agar (OA). Perhaps this is because NCB degradation occurs in reducing way in media with high organic compounds concentrations. A high concentration of electron donors is required for reducing the aromatic ring. These electron donors are sources of carbon and energy as well.

Previously we obtained data about the resistance of microorganisms in terrestrial

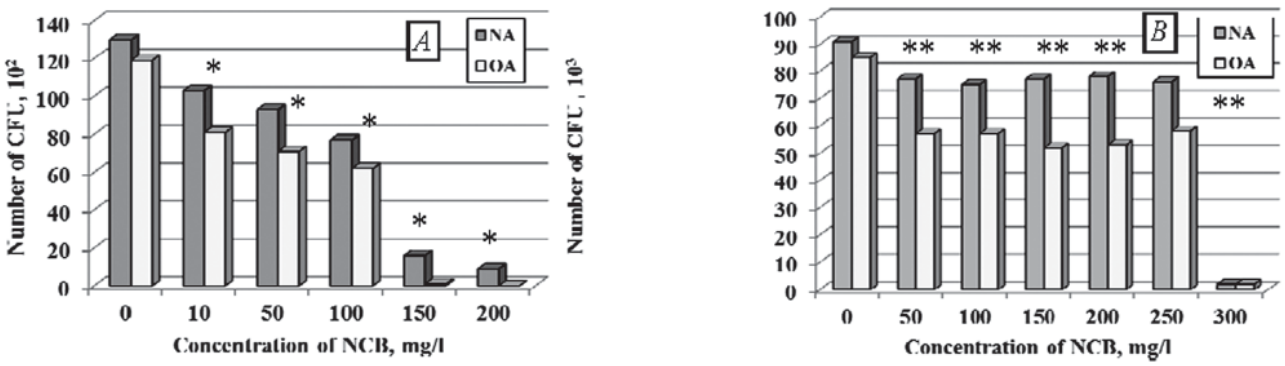

Fig. 1. **NCB effect of the CFU number *A* — Mushkarova Yama (**r* = −0.95, *p* = ≤ 0.05); *B* — Kuybushevskaya (***r* = −0.75, *p* = ≤ 0.05)**

ecosystems (Antarctic cliffs, Negev desert soil, black soil Askania Nova, the soil of the Dead Sea, etc.) to extreme factors. It was shown two types of responses on the effect of extreme factors [2, 3]. The first type of response, also called "correlative", represents inhibition of growth in increasing studied concentration range of xenobiotic. The second type of response represents absence of growth inhibition in the studied xenobiotic concentration range. Similar types of responses were shown for studied microbial communities in the concentration gradient of NCB. Thus, the correlative response (the first type of response) was characterized for Mushkarova Yama. The second type of response was observed for Kuybushevskaya karst cave microbial communities. Number of CFU of Mushkarova Yama decreased correlatively to NCB increasing up to concentration 100 mg/l. Further xenobiotic concentration increasing resulted in CFU falling off dramatically. Number of CFU of Kuybushevskaya cave decreased slightly in the concentration range of NCB 50–250 mg/l.

There are three most likely ways of nitroclorinearomatic compounds transformation: nitro group reduction, dehalogenation and aromatic ring reduction [1, 9]. Microorganisms can degrade xenobiotic by two ways: reduction to *p*-chloroaniline (*p*-ClA); reduction of the aromatic ring with subsequent cleavage of the cyclic compound. On an example of P3 strain, we determined the effect of NCB concentration gradient on bacteria physiological parameters. To assess bacteria homeostasis set of physiological parameters, or one of them (a representative) either can be used. The last is mainly used for rapid assessment. As a representative parameter we have chosen the output of carbon dioxide during P3 strain culturing in NCB concentration gradient. The yield of CO_2 decreased with increasing of the xenobiotic concentration in the medium (Fig. 2). Such regularity can be interpreted as the first type response of microorganisms on xenobiotic effect.

We compared how the concentration of carbon dioxide is reduced by increasing the concentration of the xenobiotic environment. Control — this is 0. It is the concentration of CO_2, which is synthesized in the absence of strain NCB.

We have determined P3 strain physiological parameters during 8 hours of cultivation without xenobiotic (Fig. 3) and in the presence of 300 mg/l of NCB (Fig. 4). In the control experiment (without NCB) optical density increasing and redox potential decreasing started from the first hour of cultivation. During culture growth synthesis

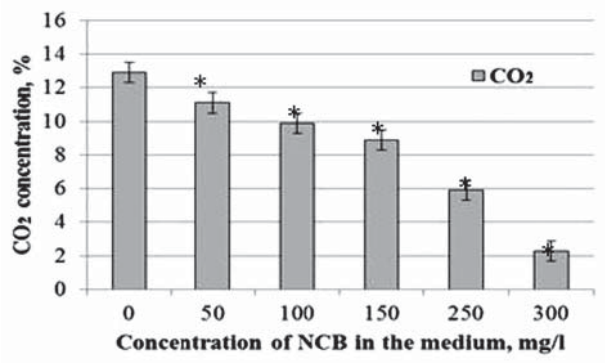

Fig. 2. **Strain *Rhodococcus erythropolis* P3 yield of CO_2 depending on NCB concentration in the medium, **r* = −0.98; *p* = 0.05**
(Cultivation time was 8 hours)

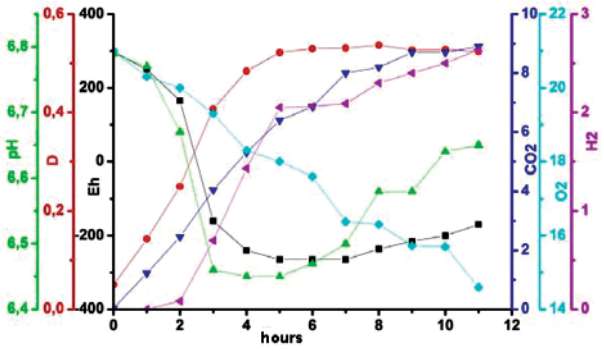

Fig. 3. **Physiological parameters of *Rhodococcus erythropolis* P3 strain during cultivating without NCB (control)**

of hydrogen was increasing and redox potential and pH were decreasing. Simultaneous synthesis of hydrogen with decreasing of redox potential and increasing the optical density of the culture fluid indicate that P3 strain was a facultative anaerobic microorganism.

Persistent organic xenobiotic NCB had a general inhibitory effect on P3 strain. The duration of the lag phase in an experiment with NCB increased in 6 times compared to control. Decreasing of redox potential was slower in the presence of NCB. Thus, the redox potential decreased to -200 mV during three hours of cultivation in the control while in the presence of NCB reached the mark 230 mV during this time. It should be noted that hydrogen synthesis in control started on the second hour of cultivation in control while in the presence of NCB — on the fifth. Hydrogen synthesis suggests the creation of low-potential anaerobic conditions where reducing destruction of the aromatic ring is possible. Decreasing of NCB concentration proportionaly to redox potential decreasing possibly indicated anaerobic reducing degradation of NCB as well as ClA. Thus, reducing of NCB associated with strain metabolic activity.

Regardless of NCB concentration in the medium during cultivation (8 hours) xenobiotic transformated into ClA. Then, apparently, the aromatic ring degradated as it was shown by the chromatographic-mass spectrometry.

We found that the correlation between the effectiveness of NCB degradation by P3 strain and initial concentration of xenobiotic was non-linear (Fig. 5).

For a visualisation of this dependence we used the indicator "effectiveness of destruction" (Ed) that displayed how many times the final concentration was less than the initial NCB concentration. For this purpose, the initial concentration of NCB was divided at final concentration in the end of three days cultivation.

Coefficient of destruction involves degradation ratio between the final and initial concentration of xenobiotic environment. For example, at an initial concentration of 50 mg/l in the medium reduced the strain concentration to 4.9 mg/L, i.e. 10 times. This corresponds to the destruction of 10 performance indicators.

Effectiveness of xenobiotic degradation was equally high (10 times) in the NCB concentration range of 50–150 mg/l. At the concentration 250 mg/l of NCB effectiveness of destruction decreased by one-third. During cultivation strain P3 decreased concentration of xenobiotic twicely in the experiment with 300 mg/l of NCB. Destruction of NCB by strain P3 was very effective in the xenobiotic concentration range of 50–150 mg/l. After 8 hours of cultivation NCB concentration decreased in 10 times. However, further increasing its concentration resulted in a reducing of degradation effectiveness. Thus,

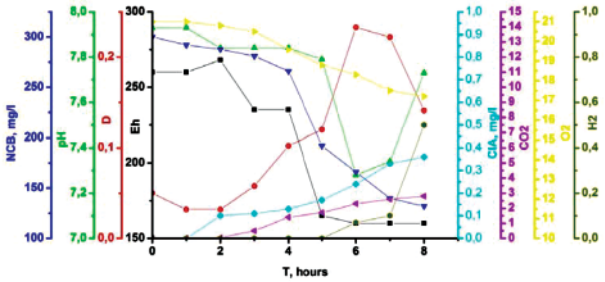

Fig. 4. **Physiological parameters (Eh, pH, optical density, O_2, CO_2, H_2 concentrations) of *Rhodococcus erythropolis* P3 strain during cultivation in the presence of 300 mg/l NCB**

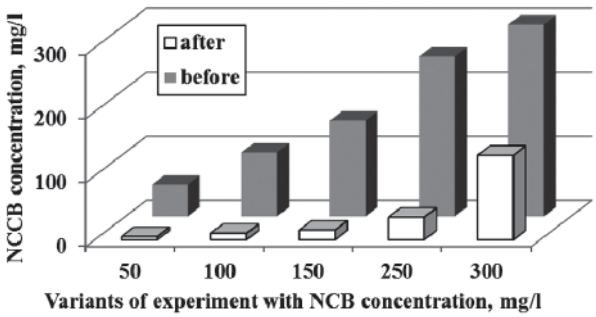

Fig. 5. **Decreasing of NCB concentration depending on its initial concentration in medium by *Rhodococcus erythropolis* P3 strain**

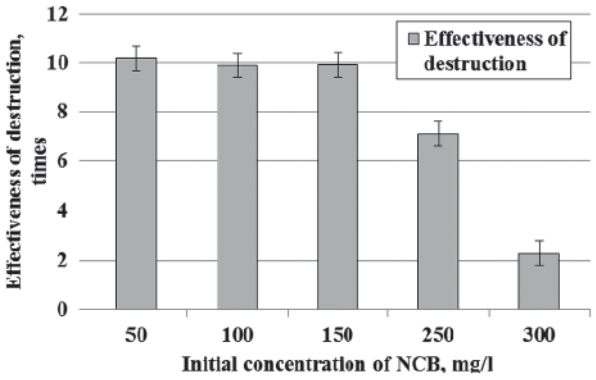

Fig. 6. **Effectiveness of NCB destruction by *Rhodococcus erythropolis* P3 strain P3 depending on the initial concentration of xenobiotic (8 hours of cultivation),** $r = 0.83$; $p = 0.05$

at the NCB concentration 150 mg/l Ed equal to 10, and at 300 mg/l — 2.

Thus, it was shown the possibility of using strain P3 in industrial purposes for organic synthesis enterprises wastewater treatment. This strain was not only highly resistant to persistent organic xenobiotic but also destoyed it. Taking into account that concentration of NCB in such wastewater does not exceed 50 mg/l, we can assume a high effectiveness of xenobiotic destruction. Moreover strain P3 potentially could be used in zones of ecological disasters when concentrations of NCB in water and soil tromendously exeed permissble concentrations.

On the example of toxic persistent organic xenobiotic NCB it was shown high adaptation ability of cave microorganisms to alien extreme factor. Chemoorganotrophic microbial communities of caves ecosystems maintained homeostasis in the presence of very high concentrations of NCB in copio- and oligocarbotrophic conditions. Representative bacterial cave strain *Rhodococcus erythropolis* was not only resistant to NCB, but also able to transform it. Transformation occured by partial reducing to ClA and apparently further destruction of the aromatic ring of the xenobiotic. NCB transformation effectiveness depended on redox potential and the initial concentration of xenobiotic and ranged between 2–10. This strain could potentially be used in new environmentaly friendly biotechnology creation for wastewater treatment of organic synthesis enterprises.

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СТІЙКІСТЬ МІКРООРГАНІЗМІВ КАРСТОВИХ ПОРОЖНИН ДО *n*-НІТРОХЛОРБЕНЗОЛУ

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Метою роботи було встановити кількісні параметри гомеостазу мікробних угруповань (максимально допустимі концентрації та типи відповіді), ізольованих із глин карстових порожнин, у концентраційному градієнті *n*-нітрохлорбензолу, а також визначити здатність бактерій карстових порожнин трансформувати ксенобіотик. Використовували загальні бактеріологічні методи та газову хромато-мас-спектрометрію. Хемоорганотрофні мікробні угруповання карстових порожнин Мушкарова Яма (Поділля, Україна) та Куйбишевська (Західний Кавказ, Абхазія) високостійкі до *n*-нітрохлорбензолу і взаємодіють з ним. На прикладі репрезентативного штаму Мушкарової Ями *Rhodococcus erythropolis* P3 показано вплив концентраційного градієнта *n*-нітрохлорбензолу (50–300 мг/л) на його фізіологічні параметри (редокс-потенціал, pH, оптична щільність, концентрація O_2, CO_2, H_2). Ефективність деградації *n*-нітрохлорбензолу пропорційна зниженню редокс-потенціалу. Таким чином, мікроорганізми карстових порожнин є перспективними для створення нових природоохоронних біотехнологій, зокрема з метою очищення стічних вод підприємств органічного синтезу.

Ключові слова: карстові порожнини, мікробні угруповання, *n*-нітрохлорбензол.

УСТОЙЧИВОСТЬ МИКРООРГАНИЗМОВ КАРСТОВЫХ ПОЛОСТЕЙ К *n*-НИТРОХЛОРБЕНЗОЛУ

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Целью работы было установить количественные параметры гомеостаза микробных сообществ (максимально допустимые концентрации и типы ответов), изолированных из глин карстовых полостей, в концентрационном градиенте *n*-нитрохлорбензола, а также определить способность бактерий карстовых полостей трансформировать ксенобиотик. Использовали общие бактериологические методы и газовую хромато-масс-спектрометрию. Хемоорганотрофные микробные сообщества карстовых полостей Мушкарова Яма (Подолье, Украина) и Куйбышевская (Западный Кавказ, Абхазия) высокоустойчивы к *n*-нитрохлорбензолу и взаимодействуют с ним. На примере репрезентативного штамма Мушкаровой Ямы *Rhodococcus erythropolis* P3 показано влияние концентрационного градиента *n*-нитрохлорбензола (50–300 мг/л) на его физиологические параметры (редокс-потеницал, pH, оптическая плотность, концентрация O_2, CO_2, H_2). Эффективность деградации *n*-нитрохлорбензола пропорциональна снижению редокс-потенциала. Таким образом, микроорганизмы карстовых полостей перспективны для создания новых природоохранных биотехнологий, в частности с целью очистки сточных вод предприятий органического синтеза.

Ключевые слова: карстовые полости, микробные сообщества, *n*-нитрохлорбензол.