OH&RM ONE HEALTH & RISK MANAGEMENT

VOL. 2, ISSUE 3
2021

RESEARCH ARTICLES – ARTICOLE DE CERCETARE – ARTICLES DE RECHERCHE – НАУЧНЫЕ СТАТЬИ

BIOLOGICAL SCIENCES

VIABILITY AND PHENOTYPIC HETEROGENEITY OF *RHODOCOCCUS RHODOCHROUS* CNMN-Ac-05 IN THE PRESENCE OF FULLERENE C_{60}

Olga POSTOLACHI, Inna RASTIMESINA, Valentina JOSAN

Institute of Microbiology and Biotechnology, Republic of Moldova

Corresponding author: Olga Postolachi, e-mail: oleseap@yahoo.com

DOI: 10.38045/ohrm.2021.3.01 CZU: 579.87.083.13:615.281.9

Keywords: *fullerene C_{60}, rhodococci, viability, phenotypic heterogeneity.*

Introduction. *In recent years, due to wide applications of nanotechnologies in various fields, the safety of nanomaterials has become a pressing issue. Fullerene C_{60} is not an exception. Research on the activity of microorganisms and their interaction with nanoparticles is of major importance, both for microorganisms and for the ecosystem as a whole.*

Material and methods. *Fullerene C_{60} powder was purchased from Sigma-Aldrich. The object of study was R. rhodochrous CNMN-Ac-05 strain. The number of viable bacterial cells was estimated by colony-forming units (CFU). The morphological features of the rhodococci colonies have been described according to the usual microbiological method.*

Results. *It was established that fullerene C_{60} in concentrations of 1-25 mg/L fullerene C_{60} stimulated the growth of R. rhodochrous by 2.4-2.8 times. As the concentration of fullerene C_{60} increased up to 50-100 mg/L, the multiplication and growth of rhodococci decreased by 29.5% and 38% respectively. In the presence of 1-10 mg/L fullerene C_{60} the rhodococci population remained homogeneous, being composed of 100% S type colonies. The increase of fullerene C_{60} concentration led both to the decrease in the CFU number and to the appearance of R type colonies, up to 1.3% of population.*

Conclusions. *Fullerene C_{60} in concentrations 1-100 mg/L had no obvious toxic effect on the rhodococci strain. The optimum concentration is 10 mg/L. The concentrations higher than 25 mg/L led to the dissociation of rhodococcal population and diminution in the CFU counts, but not to the total inhibition.*

Cuvinte cheie: *fulerena C_{60}, rodococi, viabilitate, heterogenitate fenotipică.*

VIABILITATEA ŞI HETEROGENITATEA FENOTIPICĂ A TULPINII RHODOCOCCUS RHODOCHROUS CNMN-Ac-05 ÎN PREZENŢA FULERENEI C_{60}

Introducere. *Siguranţa nanomaterialelor devine din ce în ce mai actuală, având în vedere utilizarea tot mai intensă a nanotehnologiilor în diferite domenii. Fullerena C_{60} nu este o excepţie. Cercetările privind activitatea vitală a microorganismelor şi interacţiunea lor cu nanoparticulele are importanţă majoră atât pentru fiecare microorganism în parte, cât şi pentru ecosistem în totalitate.*

Material şi metode. *Fulerena C_{60} a fost achiziţionată de la Sigma-Aldrich. Obiectul cercetării a servit tulpina R. rhodochrous CNMN-Ac-05. Numărul de celule bacteriene viabile a fost estimat prin unităţi formatoare de colonii (UFC). Caracterele morfologice ale coloniilor de rodococi au fost descrise conform metodei microbiologice uzuale.*

Rezultate. *S-a stabilit că fulerena C_{60}, în concentraţii de 1-25 mg/L, stimulează creşterea tulpinii R. rhodochrous de 2,4-2,8 ori. Odată cu creşterea concentraţiei până la 50-100 mg/L, multiplicarea şi creşterea rodococilor a scăzut cu 29,5% şi, respectiv, 38%. Tulpina de rodococi crescută în prezenţa a 1-10 mg/L fulerena C_{60} a rămas omogenă, populaţia fiind alcătuită din 100% colonii de tip S. Mărirea concentraţiei de fulerenă C_{60} a dus nu doar la scăderea numărului de celule, dar şi la apariţia coloniilor de tip R, până la 1,3% din populaţie.*

Concluzii. *Fulerena C_{60} în concentraţiile 1-100 mg/L nu are efect toxic evident asupra tulpinii de rodococi. Concentraţia optimală este de 10 mg/L. Concentraţiile mai mari de 25 mg/L duc la disocierea populaţiei şi la scăderea semnificativă a numarului CFU, dar nu şi la inhibare totală.*

INTRODUCTION

Fullerenes are an allotropic form of carbon with many synthesized modifications. Due to its structure, composed of 60 carbon atoms, fullerene C_{60} are good heat and electricity conductor that possess an excellent tensile strength. These properties make it to be a unique functional material for electronics and optics, energy, biochemistry, and molecular medicine. More recently, fullerenes have been used for bioremediation of environment contaminated with polyethylene (1), pesticides (2, 3), or other xenobiotics of different nature (4).

Wide applications use of nanotechnologies in various fields, including the field of remediation and reduction of environmental contamination, the safety of nanomaterials has become a current issue. Regarding the action of fullerene C_{60} on living cells, scientists observed some effects: on the one hand, the antioxidant action of fullerene was shown (5, 6, 7), on the other hand the antimicrobial activity was demonstrated (6, 8-11). For example, a nano-composite containing fullerene was effective in degrading pesticides Imidacloprid, Isoproturon and Malathion, but at the same time, it was used for inactivating *Pseudomonas aureus*, *Staphylococcus aureus*, *Pseudomonas fluorescens*, *Bacillus subtilis* and *Escherichia* coli bacteria (2).

Numerous studies of fullerene C_{60} have revealed that its biological activity is rather complex and multilateral, due to several factors, such as tropism for cell membranes, due to its lipophilicity, interaction with free radicals, and fullerene's ability to transfer energy to the oxygen molecule and convert it to single oxygen (7, 12, 13). It has been shown that the toxicity of fullerene C_{60} is determined by a number of factors, such as particle size, organic matter and ionic strength of the medium, fullerene C_{60} concentration and time of exposure, bacterial growth conditions, the age of C_{60} used, and the bacterial species tested (5, 8, 14).

Over time, it has been shown that the advantages of immobilized microorganisms, compared to free cells, are the enhanced stability of the biocatalyst and posibility of recovering and reusing microorganisms. Moreover, immobilization could also protect the cells and thus increase the tolerance to high concentration of pollutants (15, 16). Voznyakovskii, et al. (2020) (17) demonstrated the possibility of using microorganisms immobilized on carbon structures for eliminating the consequences of contamination with petroleum products.

The *Rhodococcus rhodochrous* CNMN-Ac-05 strain was retrieved from the stock of our laboratory, which is a destructor of benzothiazole and its metabolites (18). The purpose of our research was to evaluate the effects of fullerene on the viability and phenotypic heterogeneity of *R. rhodochrous* CNMN-Ac-05 in order to estimate the possibility of using fullerene C_{60} along with the rhodococcal strain in bioremediation procedures.

MATERIAL AND METHODS

Chemicals. Fullerene C_{60} powder (purity 98%) was purchased from Sigma-Aldrich. The particle size is approximately 0.7 nm in diameter.

Bacterial strain and culture conditions. *Rhodococcus rhodochrous* CNMN-Ac-05 was deposited within the National Collection of Non-Pathogenic Microorganisms of the Republic of Moldova, being able to degrade benzothiazoles and persistent organic pollutants (18). *R. rhodochrous* was grown in 100 mL portions of Tryptic soy (TS) broth (Sigma-Aldrich) in 300 mL Erlenmeyer flasks incubated at 28°C and 200 rpm. The cells were harvested over 36 h of culture and centrifuged at 6.000 rpm for 20 min. The bacterial pellet was washed first with a NaCl solution (0.8%) and then with distillated water.

Determination of the effects of fullerene C_{60}. Bacterial biomass was resuspended in distillated water (pH 7.2) to prepare cell suspension 6 mg/mL (1.4 mg cell dry weight/mL). The concentration of the cell biomass was determined spectrophotometrically by measuring the optical density of culture at λ=540 nm, with subsequent recalculation to cell dry weight according to the calibration curve. Colloidal aqueous suspension of fullerene C_{60}, 2 mg/mL was prepared on ultrasonic cleaner at 50 kHz for 5 min. For experiments, 5 mL of bacterial cells suspension was added in 250 mL Erlenmeyer flasks containing 95 mL of medium PAS and fullerene C_{60} in following concentrations (mg/L): 1, 10, 25, 50, and 100. The PAS medium contained (g/L): 4.35 K_2HPO_4, 1.7 KH_2PO_4, 2.1 NH_4Cl, 0.2 $MgSO_4$, 0.05 $MnSO_4$, 0.01 $FeSO_4$ $7H_2O$, and 0.03 $CaCl_2$ $2H_2O$. pH adjusted at 7.2. Inoculated flasks were

incubated in a rotary shaker (180 rpm) at 28°C for 24 h. After a serial dilution, the 50 µL of suspension was spread on agar plates with TS medium; afterwards the plates were incubated at 28°C for 96 h until the bacterial colonies appeared. The number of viable bacterial cells was estimated by colony-forming units (CFU) inoculated on agar plates.

The morphological features of the rhodococci colonies were described according to Egorov method (19), by using a magnifying glass (8-fold magnification).

Statistical analysis was performed via MS Excel. All results were expressed as means of three individual replicates ±CI (confidence intervals). All the differences were considered statistically significant at $P<0.05$.

RESULTS

The results of the action of fullerene C_{60} on the viability of *R. rhodochrous* CNMN-Ac-05 are shown in Figure 1. These results indicated that the concentration of the test substance was of major importance. The optimal concentrations for rhodococcal cell multiplication were 1-25 mg/L. At these concentrations, the growth of the strain was stimulated by 2.4-2.8 times compared to the control samples. The highest CFU count value was obtained at a concentration of 10 mg/L. However, an increase in the fullerene C_{60} concentration up to 50 and 100 mg/L, obviously decreased the rhodococci capacity of multiplication and growth. The values of the CFU number were lower by 29.5% and 38.0% compared to the control samples.

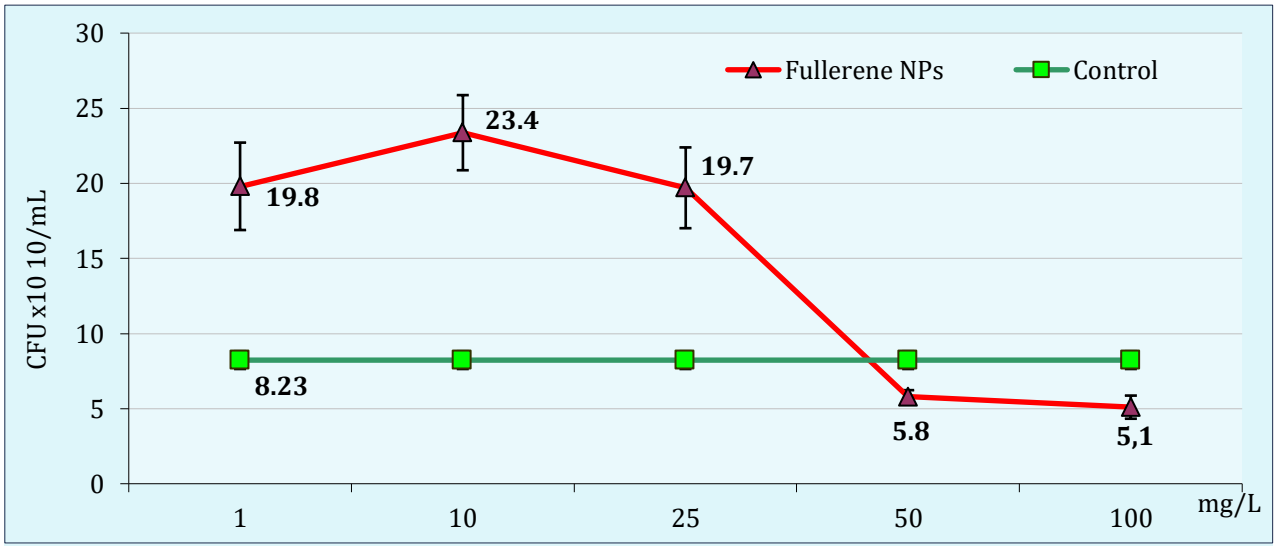


Figure 1. Influence of fullerene C_{60} on the viability *R. rhodochrous* CNMN-Ac-05.

In addition to cell multiplication, the phenotypic modification of *Rhodococcus* colonies in the presence of fullerene C_{60} was described. Macroscopically observable features and colony types of *R. rhodochrous* CNMN-Ac-05 are presented in Table 1 and Figure 2.

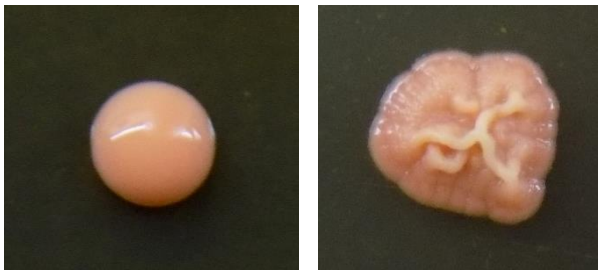
Basic morphological features of *R. rhodochrous* CNMN-Ac-05 colonies were similar to the S type (smooth). Thus, cultivation on TS medium without fullerene (control), resulted in the formation of 100% S type colonies. The colonies of S type were dominant in all the experimental variants, regardless of fullerene C_{60} concentration, ranging between 98.7-100% (fig. 3).

The population of *R. rhodochrous* cultivated in the presence of 1 and 10 mg/L of fullerene C_{60} remained homogeneous, being composed of 100% S type colonies, similar to the control sample. Moreover, cultivation in the presence of 25 mg/L fullerene C_{60}, under conditions of active multiplication, resulted in phenotypic dissociation of strain into two types of colonies, S (99.6%) and R (0.4%) types.

The further increase of fullerene C_{60} concentration (50-100 mg/L) caused both a substantial decrease in the cell number and an increase in the phenotypic heterogeneity of the rhodococci. The highest percentage of R type cells – 1.3% was observed at the concentration of 100 mg/L.

Table 1. Types of colonies formed by *R. rhodochrous* CNMN-Ac-05.

Morphological features	Colony type	
	S	R
Form	Round	Irregular
Size, mm	1.0-4.0	1.0-4.0
Margin	Entire	Undulate
Elevation	Convex	Convex
Surface	Smooth and glistening	Rough and dull
Color	Pink	Pink
Opacity	Opaque	Opaque



Type S **Type R**

Figure 2. Types of colonies formed by *R. rhodochrous* CNMN-Ac-05 cultivated in the presence of fullerene C_{60}.

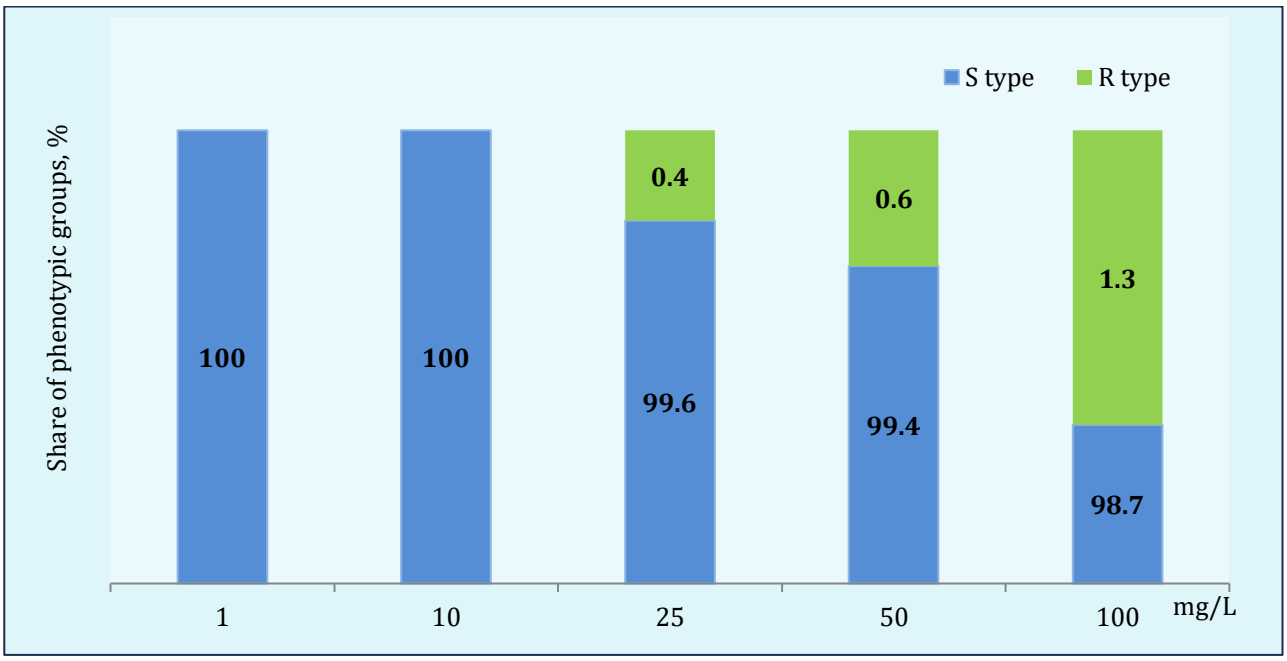


Figure 3. Dissociation of *R. rhodochrous* CNMN-Ac-05 grown in the presence of fullerene C_{60}.

DISCUSSIONS

The genus *Rhodococcus* belongs to the phylum and class *Actinobacteria*, the order *Actinomycetales*, and the family *Nocardiaceae*. Rhodococci possess a broad catabolic diversity and numerous enzymatic capabilities. They are able to use a wide range of organic compounds as sole sources of carbon and energy for growth, and that makes them well-equipped for biotransformation and biodegradation of xenobiotic compounds (15, 20, 21).

Since rhodococci are nocardioform bacteria, the phenotypic heterogeneity is quite common among them. According to Goodfellow et al. (22), rhodococci colonies may be mucoid, rough or smooth, as well as a pigmented, yellow, orange, red, or colorless buff and cream. The splitting of a homogeneous rhodococci population into variants with different morphological, physiological, biochemical, and genotypic properties was observed by many researchers (23-26).

Phenotypic heterogeneity refers to the phenomenon when individual cells within an isogenic population, that have a uniform genetic background, can nevertheless display differences in phenotype (27, 28). Non-genetic variations, that exist within an isogenic population, benefit the population through division of labor and improving the ability to exhibit a high level of metabolic activity. Therefore, phenotypic heterogeneity allows bacterial populations to

improve their ability to adapt to changing environments (29, 30).

In the present study, the concentration-toxicity ratio of fullerene C_{60} to rhodococcal cells was clearly established. In case of concentrations of 1 and 10 mg/L, the population of rhodococci was homogeneous and grew very actively, whereas starting with 25 mg/L, R type colonies appeared. Since typical S colonies are produced only under optimal cultural conditions, the appearance of R type colonies indicates a stressful condition, caused by the presence of increased concentrations of fullerene C_{60}. This phenomenon was confirmed by the experimental variants, when rhodococci were grown in the presence of 50 and 100 mg/L of fullerene C_{60}. The number of R type colonies increased in these variants, additionally to the decrease in the CFU count. Similar results were obtained by Sah et al. (2010) (1) that reported a moderately decrease in the CFU counts of bacterial strains at the concentration of 100 mg/L fullerene C_{60}.

CONCLUSIONS

1. The fullerene C_{60} in tested concentrations did not display any obvious toxic effects on *R. rhodochrous* CNMN-Ac-05 cells. The addition of fullerene C_{60} in concentrations up to 25 mg/L, stimulates the growth and multiplication of rhodococcal cells. The optimum concentration was 10 mg/L.
2. Concentration increases beyond 25 mg/L caused a dissociation of the rhodococcal population, expressed by the appearance of R type colonies, as well as a significant decrease of the CFU counts (29.5-38.0%), no total inhibition occurs. Higher fullerene C_{60} concentrations resulted in lower growth activity and higher phenotypic heterogeneity of the strain.

CONFLICT OF INTERESTS

The authors declare no conflict of interest.

ACKNOWLEDGEMENTS

The study was performed within the Moldova-Belarus Bilateral Project 19.80013.51.07.09A/BL.

REFERENCES

1. Sah A, Kapri A, Zaidi MG, Negi H, Goel R. Implications of fullerene-60 upon in-vitro LDPE biodegradation. *J Microbiol Biotech.* 2010; 20(5):908-16.
2. Raizada P, Sudhaik A, Saini AK, Singh P. *Graphitic carbon nitride base metal free nanocomposite for pesticide degradation and bacteria disinfection*. India Patent IN201811023694. 27 Dec 19.
3. Jampílek J, Kralova K. Potential of nanoscale carbon-based materials for remediation of pesticide-contaminated environment. In: Abd-Elsalam KA. *Carbon nanomaterials for agri-food and environmental applications*. Elsevier Inc.; 2020, p. 359-399.
4. Ajiboye TO, Kuvarega AT, Onwudiwe DC. Recent strategies for environmental remediation of organochlorine pesticides. *Applied Sciences*. 2020; 10(18):6286. doi:10.3390/ app10186286
5. Tong Z-H, Bischoff M, Nies LF, Carroll NJ, Applegate B, Turco RF. Influence of fullerene (C60) on soil bacterial communities: aqueous aggregate size and solvent co-introduction effects. *Scientific Reports. 2016;* 6:28069. doi: 10.1038/ srep28069
6. Lawrence JR, Waiser MJ, Swerhone GD, Roy J, Tumber V, Paule A, et al. Effects of fullerene (C60), multi-wall carbon nanotubes (MWCNT), single wall carbon nanotubes (SWCNT) and hydroxyl and carboxyl modified single wall carbon nanotubes on riverine microbial communities. *Environ Sci Pollut Res Int.* 2016; 23(10):10090-102.
7. Emelyantsev S, Prazdnova EA, Chistyakov V, Alperovich I. Biological effects of C60 fullerene revealed with bacterial biosensor-toxic or rather antioxidant? *Biosensors.* 2019; *9*:81. doi:10.3390/bios9020081
8. Lyon D.Y, Brown D.A, Alvarez PJJ. Implications and potential applications of bactericidal fullerene water suspensions: effect of nC60 concentration, exposure conditions and shelf life. *Water Science & Technology*. 2008; 57(10):1533-8.
9. Johansen A, Pedersen AL, Jensen KA, Karlson U, Hansen BM, Scott-Fordsmand JJ, Winding A. Effects of C60 fullerene nanoparticles on soil bacteria and protozoans. *Environ Toxicol Chem.* 2008; 27(9):1895-903.
10. Kolosnjaj J, Szwarc H, Moussa F. Toxicity studies of fullerenes and derivatives. *Adv Exp Med Biol*. 2007; 620:168-180.
11. Spohn P, Hirsch C, Hasler F, Bruinink A, Krug HF, Wick P. C60 fullerene: a powerful antioxidant or a damaging agent? The importance of an in-

depth material characterization prior to toxicity assays. *Environ Pollut.* 2009; 157(4):1134-9.
12. Piotrovsky LB, Eropkin MY, Eropkina EM, Dumpis MA, Kiselev OI. Mechanisms of biologic action of fullerenes-Dependence on aggregate state. *Psychopharmacol Biol Narcol.* 2007; *7*:1548-1554.
13. Galvan YP, Alperovich I, Zolotukhin P, Prazdnova E, Mazanko M, Belanova A, Chistyakov V. Fullerenes as Anti-Aging Antioxidants. *Curr Aging Sci*. 2017; 10(1):56-67.
14. Lyon DY, Adams LK, Falkner JC, Alvarez PJJ. Antibacterial activity of fullerene water suspensions: effects of preparation method and particle size. *Environ. Sci. Technol.* 2006; 40:4360-4366.
15. Hou J, Liu F, Wu N, Ju J, Yu B. Efficient biodegradation of chlorophenols in aqueous phase by magnetically immobilized aniline-degrading *Rhodococcus rhodochrous* strain. *Journal of Nanobiotechnology*. 2016; 14:5. doi: 10.1186/s12951-016-0158-0
16. Yoshimoto S, Ohara Y, Nakatani H, Hori K. Reversible bacterial immobilization based on the salt-dependent adhesion of the bacterionanofiber protein AtaA. *Microbial Cell Factories.* 2017; 16:123. doi.org/10.1186/s12934-017-0740-7
17. Voznyakovskii AP, Novikova, II, Voznyakovskii AA, Boikova IV, Neverovskaya AYu. 2D Nanocarbons as the matrix for immobilized microbial preparations. *Tech. Phys.* 2020; 65:1384-1390.
18. Cincilei A, Tolocichina S, Rastimesina I, Delort A-M, Besse-Hoggan P, Sancelme M. Removal of aqueous benzothiazoles using immobilized bacteria. *Proceedings of 34th AMOP Technical Seminar on Environmental contamination and Response*. 2011; 43(17):74-82.
19. Egorov NS. *Practical Work on Microbiology. Textbook*. Moscow: Publishing house of Moscow. University press: 1995. 307 p.
20. De Carvalho CCCR. Adaptation of *Rhodococcus* to organic solvents. In: Alvarez HM. *Biology of Rhodococcus*. *Microbiology Monographs*. Springer-Verlag Berlin Heidelberg; 2010. 16:109-132.
21. Kuyukina MS, Ivshina IB. Application of *Rhodococcus* in bioremediation of contaminated environments. In: Alvarez HM. *Biology of Rhodococcus*. *Microbiology Monographs*. Springer-Verlag Berlin Heidelberg; 2010. 16:231-262.
22. Goodfellow M, Alderson G, Chun J. Rhodococcal systematics: Problems and developments. *Antonie van Leeuwenhoek*. 1998; 74:3-20.
23. Kolomytseva MP, Solyanikova IP, Golovlev EL, Golovleva LA. Heterogeneity of *Rhodococcus opacus* 1CP as a response to stress induced by chlorophenols. *Appl Biochem Microbiol*. 2005; 41(5):474-479.
24. Stancu MM. Response of *Rhodococcus erythropolis* strain IBB_{Po1} to toxic organic solvents. *Braz J Microbiol*. 2015; 46(4):1009-18.
25. Konovalova EI. Solyanikova IP. Golovleva LA. Degradation of 4-chlorophenol by the strain *Rhodococcus opacus* 6a. *Microbiology*. 2009; 78(6):805-807.
26. Iwabuchi N, Sunairi M, Anzai H, Nakajima M, Harayama SH. Relationships between colony morphotypes and oil tolerance in *Rhodococcus rhodochrous. Appl Environ Microbiol*. 2000; 66(11): 5073–77.
27. Ackermann MA. A functional perspective on phenotypic heterogeneity in microorganisms. *Nat Rev Microbiol*. 2015; 13:497-508.
28. Grote J, Krysciak D, Streit WR. Phenotypic heterogeneity, a phenomenon that may explain why quorum sensing does not always result in truly homogenous cell behavior. *Appl Environ Microbiol*. 2015; 81(16):5280-89.
29. Abda EM, Krysciak D, Krohn-Molt I, Mamat U, Schmeisser C, Förstner KU, Schaible UE, et al. Phenotypic heterogeneity affects *Stenotrophomonas maltophilia* K279a colony morphotypes and β-lactamase expression. *Frontiers in microbiology*. 2015; 6:1373. doi: 10.3389/fmicb. 2015.01373
30. Calabrese F, Voloshynovska I, Musat F, Thullner M, Schlömann M, Richnow HH, Lambrecht J, et al. Quantitation and comparison of phenotypic heterogeneity among single cells of monoclonal microbial populations. *Frontiers in microbiology*. 2019. doi: 10.3389/fmicb. 2019.02814

Date of receipt of the manuscript: 29/11/2020
Date of acceptance for publication: 06/04/2021

Olga POSTOLACHI, ORCID ID: 0000-0002-2240-7376
Inna RASTIMESINA, ORCID ID: 0000-0002-3303-4771
Valentina JOSAN, ORCID ID: 0000-0002-6971-7638