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VIABILITY AND PHENOTYPIC HETEROGENEITY OF *RHODOCOCCUS RHODOCHROUS* CNMN-Ac-05 IN THE PRESENCE OF FULLERENE C₆₀

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Introduction. In recent years, due to wide applications of nanotechnologies in various fields, the safety of nanomaterials has become a pressing issue. Fullerene C₆₀ 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 C₆₀ 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 C₆₀ in concentrations of 1-25 mg/L fullerene C₆₀ stimulated the growth of *R. rhodochrous* by 2.4-2.8 times. As the concentration of fullerene C₆₀ 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 C₆₀ the rhodococci population remained homogeneous, being composed of 100% S type colonies. The increase of fullerene C₆₀ 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 C₆₀ 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: fullerena C₆₀, rodococi, viabilitate, heterogenitate fenotipică.

VIABILITATEA ȘI HETEROGENITATEA FENOTIPICĂ A TULPINII *RHODOCOCCUS RHODOCHROUS* CNMN-Ac-05 ÎN PREZENȚA FULERENEI C₆₀

Introducere. Siguranța nanomaterialelor devine din ce în ce mai actuală, având în vedere utilizarea tot mai intensă a nanotehnologiilor în diferite domenii. Fullerena C₆₀ nu este o excepție. Cercetările privind activitatea vitală a microorganismelor și interacțiunea lor cu nanoparticulele are importanță majoră atât pentru fiecare microorganism în parte, cât și pentru ecosistem în totalitate.

Material și metode. Fulerena C₆₀ a fost achiziționată de la Sigma-Aldrich. Obiectul cercetării a servit tulpina *R. rhodochrous* CNMN-Ac-05. Numărul de celule bacteriene viabile a fost estimat prin unități formatoare de colonii (UFC). Caracterile morfologice ale coloniilor de rodococi au fost descrise conform metodei microbiologice uzuale.

Rezultate. S-a stabilit că fullerena C₆₀, în concentrații de 1-25 mg/L, stimulează creșterea tulpinii *R. rhodochrous* de 2,4-2,8 ori. Odată cu creșterea concentrației până la 50-100 mg/L, multiplicarea și creșterea rodococilor a scăzut cu 29,5% și, respectiv, 38%. Tulpina de rodococi crescută în prezența a 1-10 mg/L fullerena C₆₀ a rămas omogenă, populația fiind alcătuită din 100% colonii de tip S. Mărirea concentrației de fullerena C₆₀ a dus nu doar la scăderea numărului de celule, dar și la apariția coloniilor de tip R, până la 1,3% din populație.

Concluzii. Fulerena C₆₀ în concentrațiile 1-100 mg/L nu are efect toxic evident asupra tulpinii de rodococi. Concentrația optimală este de 10 mg/L. Concentrațiile mai mari de 25 mg/L duc la disocierea populației și la scăderea semnificativă a numărului CFU, dar nu și 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 C₆₀ 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 C₆₀ 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 C₆₀ 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 C₆₀ is determined by a number of factors, such as particle size, organic matter and ionic strength of the medium, fullerene C₆₀ concentration and time of exposure, bacterial growth conditions, the age of C₆₀ 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 possibility 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 C₆₀ along with the rhodococcal strain in bioremediation procedures.

MATERIAL AND METHODS

Chemicals. Fullerene C₆₀ 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 distilled water.

Determination of the effects of fullerene C₆₀. Bacterial biomass was resuspended in distilled 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 $\lambda=540$ nm, with subsequent recalculation to cell dry weight according to the calibration curve. Colloidal aqueous suspension of fullerene C₆₀, 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 C₆₀ in following concentrations (mg/L): 1, 10, 25, 50, and 100. The PAS medium contained (g/L): 4.35 K₂HPO₄, 1.7 KH₂PO₄, 2.1 NH₄Cl, 0.2 MgSO₄, 0.05 MnSO₄, 0.01 FeSO₄ 7H₂O, and 0.03 CaCl₂ 2H₂O. 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 C₆₀ 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 C₆₀ 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.

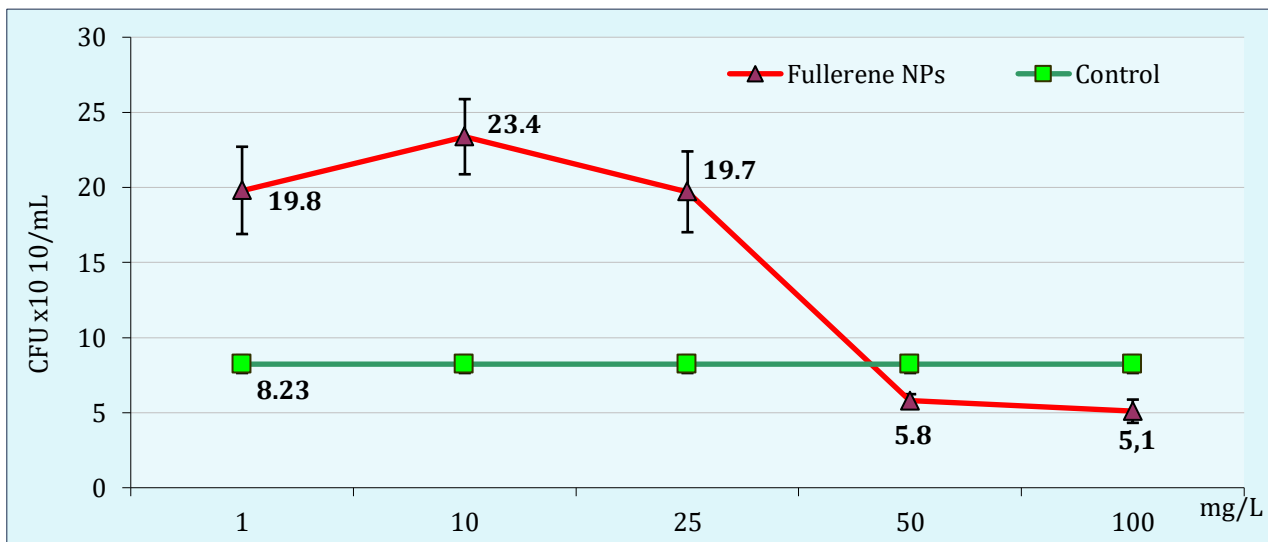


Figure 1. Influence of fullerene C₆₀ on the viability *R. rhodochrous* CNMN-Ac-05.

In addition to cell multiplication, the phenotypic modification of *Rhodococcus* colonies in the presence of fullerene C₆₀ 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 C₆₀ 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 C₆₀ remained homogeneous, being composed of 100% S type colonies, similar to the control sample. Moreover, cultivation in the presence of 25 mg/L fullerene C₆₀, 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 C₆₀ 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 C₆₀.

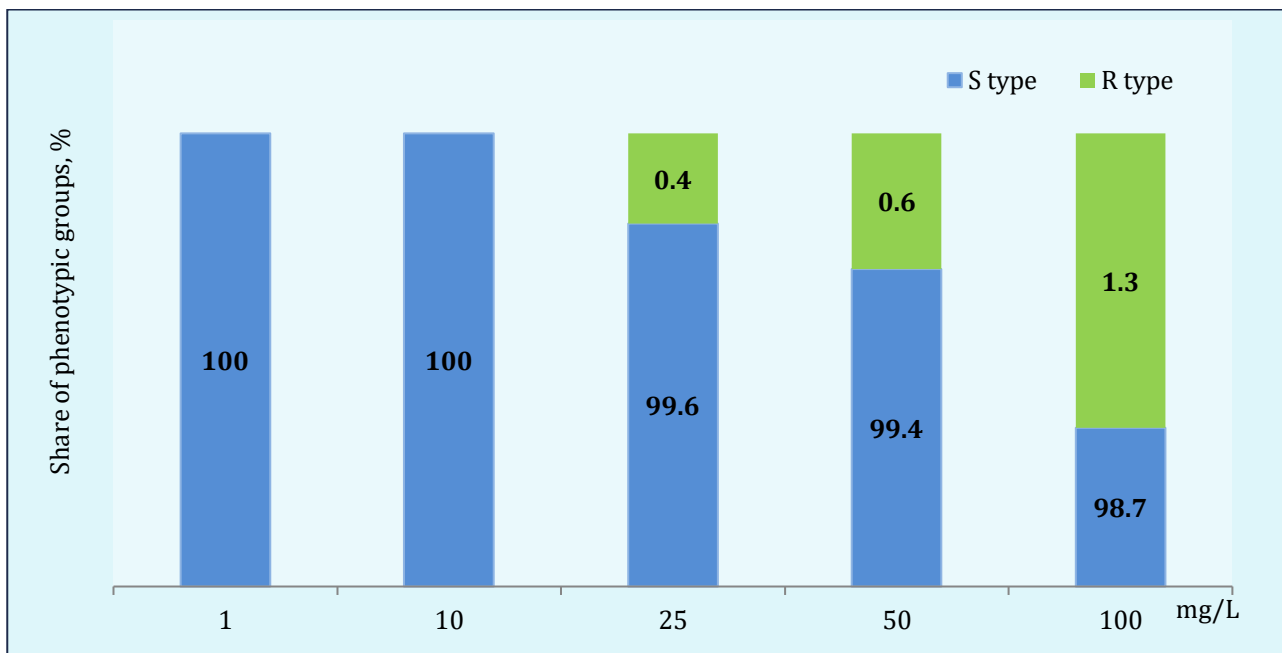


Figure 3. Dissociation of *R. rhodochrous* CNMN-Ac-05 grown in the presence of fullerene C₆₀.

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 C₆₀ 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 C₆₀. This phenomenon was confirmed by the experimental variants, when rhodococci were grown in the presence of 50 and 100 mg/L of fullerene C₆₀. 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 C₆₀.

CONCLUSIONS

1. The fullerene C₆₀ in tested concentrations did not display any obvious toxic effects on *R. rhodochrous* CNMN-Ac-05 cells. The addition of fullerene C₆₀ 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 C₆₀ concentrations resulted in lower growth activity and higher phenotypic heterogeneity of the strain.

CONFLICT OF INTERESTS

The authors declare no conflict of interest.

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