INFLUENCE OF GAMMA RADIATION ON THE SYNTHESIS OF SOME BIOCOMPOUNDS BY VARIOUS EXTREMELY HALOPHILIC ARCHAEA WITH USES IN BIOTECHNOLOGY

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Abstract. In this paper, the studies carried out aimed to investigate the influence of gamma radiation on the synthesis of some biocompounds of biotechnological interest in some haloarchaea strains isolated from the Bride Cave (Grota Miresei) hypersaline lake from Slănic Prahova. The studied biocompounds were carotenoid pigments: bacterioruberin, lycopene and β-carotene synthesized by haloarchaea strains. The present study analysed the effect of the gamma radiation (γ-radiation) on the growth of haloarchaea strains, as well as a possible stimulatory effect of different γ-radiation doses on the synthesis of carotenoid pigments. The hypersaline lakes are characterized by an extremely high salt concentration, high temperatures and high levels of UV radiation and γ-radiation. In these extreme habitats, haloarchaea survive and develop, showing increased tolerance to multiple stress factors in these environments. In order to survive in these extreme conditions, haloarchaea have developed remarkable resistance to UV and γ-radiation, and have a highly efficient cellular repair system by photoreactivation. Recent studies have shown that haloarchaea exhibit increased resistance to γ-radiation due to the ability of cells to repair the fragmentation that occurs in the double-stranded DNA molecule, as well as to the protective properties of carotenoid pigments in the cell membrane. Studies performed on haloarchaea of different genera: *Halobacterium, Haloarcula, Halorrubrum*, etc. have indicated that their exposure to γ-radiation is a stress factor that would increase the synthesis of bioproducts, such as carotenoid pigments. The obtained results have shown that the different irradiation doses used, between 10 Gy - 1 kGy, did not influence the development of the haloarchaea strains, the growth was also determined at values of 500 Gy and 1 kGy, depending on the tested strain. The results showed that the synthesis of carotenoid pigments (bacterioruberin, lycopene and β-carotene) was stimulated at high doses of γ-radiation (500 - 1000 Gy).

Keywords: gamma radiation, haloarchaea, biocompounds, biotechnology.

Rezumat. Influența radiației gama asupra sintezei unor biocompuși de către diferite archeae extrem halofile cu utilizări în biotehnologie. În această lucrare, studiile efectuate au avut ca scop investigarea influenţei radiaţiei gama asupra sintezei unor biocompuşi de interes biotehnologic la unele tulpini haloarchaeene, izolate din lacul hipersalin Grota Miresei, din localitatea Slănic Prahova. Biocompuşii studiaţi au fost pigmenţii carotenoizi: bacterioruberina, licopenul şi β-carotenul, sintetizaţi de tulpinile haloarchaeane. Prezentul studiu a urmărit efectul radiației gamma asupra creșterii tulpinilor haloarchaeene, precum și un posibil efect stimulator al diferitelor doze de radiație gama asupra sintezei pigmenților carotenoizi. Lacurile hipersaline sunt caracterizate printr-o concentrație extrem de ridicată de săruri, prin temperaturi crescute şi niveluri ridicate de radiaţii UV şi gama (γ). În aceste habitate extreme supravieţuiesc şi se dezvoltă haloarchaeele, care prezintă toleranţă crescută la factorii de stres multipli din aceste medii. În scopul supravieţuirii în aceste condiţii extreme, haloarchaeele au dezvoltat o rezistenţă remarcabilă la radiaţiile UV şi gama, şi posedă un sistem de reparare celulară prin fotoreactivare extrem de eficient. Studii recente au arătat că haloarchaeele prezintă rezistenţă crescută la radiaţiile γ datorată capacităţii celulelor de a repara fragmentările care se produc în molecula de ADN dublu-catenar, precum şi proprietăţilor protectoare ale pigmenţilor carotenoizi din membrana celulară. Studii efectuate asupra haloarhaeelor din genuri diferite: *Halobacterium*, *Haloarcula*, *Halorrubrum* etc. au indicat faptul că expunerea acestora la radiaţii γ reprezintă un factor de stress, care determină creşterea sintezei de bioproduşi, ca de exemplu, pigmenţii carotenoizi. Rezultatele obţinute au arătat că dozele diferite de iradiere utilizate, cuprinse între 10 Gy - 1 kGy nu au influenţat dezvoltarea haloarchaeelor, creşterea fiind determinată şi la valori de 500 Gy şi respectiv, 1 kGy în funcţie de tulpina testată. Rezultatele au evidenţiat faptul că sinteza pigmenţilor carotenoizi (bacterioruberina, licopenul şi β-carotenul) a fost stimulată la doze crescute ale radiaţiei gama (500 – 1000 Gy).

Cuvinte cheie: radiaţie gama, haloarchaee, biocompuşi, biotehnologii.

INTRODUCTION

Regarded as energy in the form of particles, radiation is non-ionizing, with lower energy and frequency, and longer wavelength (e.g., earth magnetic field, radio waves, microwaves, infrared waves, visible light and some sunrays) and ionizing, which knocks the electrons out their orbits around atoms, and has higher energy, higher frequency and shorter wavelength (e.g., alpha radiation, beta radiation, photon radiation, as gamma rays and X-rays, and neutron radiation) (***. CNSC, 2012).

Radiation is the most aggressive stressor on living organisms, outperforming all the other types of stressors and stimulating the development of specific signalling pathways to overcome its effects. Particularly, gamma rays produce water ionization (ROBINSON et al., 2011), which generates reactive oxygen species (which include hydrogen peroxide, H_2O_2, superoxide, $O_2^{\cdot-}$, and hydroxyl radicals, HO^-). These oxygen species interact with the most macromolecules, causing alteration of nucleobases and protein carbonylation, interfering with normal functions of cellular components, increasing synthesis of some biocompounds of interest and enzymes, and activating the signalling pathways to repair the damaged structures (WHITEHEAD et al., 2006).

The increase in the synthesis of some biocompounds of interest which can be used in biotechnological processes under the action of certain stressors, including gamma rays, have brought radiation-resistant microorganisms to the attention of scientists which attempted to optimize the protocols for obtaining large quantities of active biocompounds with minimal energy consumption (KISH et al., 2009).

Microorganisms resistant to gamma radiation are not only found among *Eubacteria*, but are also represented by microorganisms in the *Archaea* domain. Thus, bacteria like *Deinococcus radiodurans* can survive in the presence of high doses of gamma radiation (5000 Gy), ultraviolet radiation and desiccation (BATTISTA & RAINEY, 2001; CONFALONIERI & SOMMER, 2011). Several microorganisms in the *Archaea* domain have high levels of resistance to gamma radiation, also. Thus, the halophilic archaeon *Halobacterium* sp. NRC-1 survived at gamma radiation doses between 2500 - 5000 Gy (KOTTEMANN et al., 2005), hyperthermophilic archaea such as *Pyrococcus abyssi* exhibited 100% viability at doses between 2000 – 2500 Gy and *Thermococcus gammatolerans* showed cell growth up to 5000 Gy (TAPIAS et al., 2009; CONFALONIERI & SOMMER, 2011).

Previous studies (SHAHMOHAMMADI et al. 1997; MANCINELLI et al., 1998; KOTTEMANN et al., 2005; KISH et al., 2009; ROBINSON et al., 2011; WEBB & DIRUGGIERO, 2012; RODRIGO-BAÑOS et al., 2015; SHIRSALIMIAN et al., 2017) have shown that the presence of salts in the media (especially potassium chloride and bromides), orthophosphate, high ratios of intracellular Mn/Fe concentrations, metabolite complexes of Mn^{++}, increased synthesis of carotenoid pigments (e.g., β-carotene, lycopene, bacterioruberin, derivatives of bacterioruberin and salinixanthin) and ROS scavenger enzymes, are among the most efficient ways for the archaeal strains to overcome the damaging effects of gamma rays.

One or more of these mechanisms are present in actively growing cells, providing the archaeal cells with a higher protection that those in the stationary phase, which are more sensitive to gamma rays effects, including damages produced by ROS (KOTTEMANN et al., 2005).

The extremely halophilic microorganisms in the *Archaea* domain, also called haloarchaea, can survive and develop in habitats with extreme conditions and have increased tolerance to multiple stress factors in these environments. The hypersaline habitats inhabited by the haloarchaea are characterized by an extremely high salt concentration, high temperatures and high levels of UV and gamma radiation (DasSARMA, 2006).

In order to survive in these extreme conditions, haloarchaea have developed remarkable resistance to UV and gamma radiation, and have a highly efficient cellular repair system (McCREADY & MARCELLO, 2003; DeVEAUX et al., 2007).

Recent studies have shown that haloarchaea cells (*Halobacterium* sp. NRC-1, *Haloarcula, Halorrubrum* etc.) exhibit increased resistance to gamma radiation. Resistance has been attributed to the ability of cells to repair the fragmentation that occurs in the double-stranded DNA molecule, as well as to the protective properties of cell membrane-specific pigments (carotenoid pigments) (KOTTEMANN et al., 2005). They confer cell protection against cellular damage induced by high doses of gamma (5000 Gy) and UV radiation. Carotenoid pigments also increase cell membrane hardness, thus conferring a high degree of protection against membrane damage caused by these types of radiation (SHAHMOHAMMADI et al., 1997; ZENI et al., 2011).

Studies on haloarchaea from different genera (*Halobacterium, Haloarcula, Halorrubrum*, etc.) have indicated that these conditions specific to hypersaline habitats or culture media in which haloarchaea develop (primarily due to high concentrations of NaCl), create a protective environment against radiation. These results were obtained in studies *in vivo* on haloarchaea compared to *E. coli* cells grown under low salinity conditions (MANCINELLI, 2015). The exposure of such microorganisms to gamma radiation is a stress factor that would stimulate the increase of bioproducts synthesis, such as carotenoid pigments (which act as antioxidants), useful in various biotechnological applications (MANDELLI et al., 2012; JAN et al., 2013; ABOMOHRA et al., 2016).

In this paper, the studies carried out aimed to investigate the influence of gamma radiation on the synthesis of some biocompounds with biotechnological interest in two strains of extremely halophilic archaea isolated from the Bride Cave (Grota Miresei) Lake from Slănic Prahova County (Romania). The biocompounds synthesized by the haloarchaea strains studied in the experiments were represented by carotenoid pigments: bacterioruberin, lycopene and beta-carotene. The present study investigated the effect of gamma radiation on the growth of haloarchaea strains belonging to the *Haloarcula* genus, as well as the stimulatory effect of different doses of gamma radiation on the biologically active compounds of biotechnological interest, previously mentioned.

MATERIAL AND METHODS

Microorganisms: Two haloarchaeal strains (5/1GM and 5/2GM) isolated from the hypersaline Lake Bride Cave (Grota Miresei) located in Slănic Prahova (Prahova County, Romania) were used in this study. The 5/1GM and 5/2GM strains were preliminarily classified as belonging to the genus *Haloarcula* through polyphasic taxonomy approaches, including microbiological, biochemical and molecular methods, according to the proposed minimum standards for the description of a new taxon from the orders *Halobacteriales*, *Haloferacales* and *Natrialbales* (OREN et al., 1997). The strains showed a reddish coloration and were selected for further experiments due to the increased biosynthesis capacity of the carotenoid pigments.

Culture media and growth conditions: The strains were grown on a selective liquid culture medium for haloarchaea containing a high concentration of NaCl (250 g / L) (OREN & LITCHFIELD, 1999), under stirring conditions (150 rpm), at 37° C, for 72 hours. After this period, the cultures in the exponential growth phase were distributed in volumes of 50 ml in Falcon tubes, which were subjected to irradiation with different doses of gamma radiation.

Gamma-rays irradiation experiments: The obtained cultures were subjected to gamma radiation at average doses of 10 Gy, 100 Gy, 500 Gy and 1 kGy in order to stimulate cell growth, but also carotenoid pigments biosynthesis. In experiments the unirradiated cultures of the two haloarchaeal strains were used as a control, also. The irradiation experiments were performed using a Co-60 research irradiator (GC-5000 manufactured by B.R.I.T., India). During irradiations, the average dose rate was 0.9 Gy/s. The dose uniformity ratio (D.U.R.), defined as the ratio of maximum dose to minimum dose in the samples, was 1.259 (± 4.7 %). The doses were evaluated by means of ethanol-chloro-benzene (ECB) dosimetry system with oscillometric read-out. The doses are expressed as absorbed dose in water and their corresponding uncertainty is 3.3 %.

Following irradiation, the cultures were centrifuged (9500 rpm, 15 min. and 4^o C) and the obtained biomass was inoculated into 50 mL culture medium in Falcon tubes which were incubated under stirring conditions (150 rpm), at 37° C, for 216 hours.

The cellular growth was measured based on the spectrophotometric determination of cell density (O.D. at 660 nm) at 24-hour intervals during the incubation period (216 h), using a UV/VIS spectrophotometer (Analitik Jena Specord 210 Plus).

Carotenoid extraction and analysis: After the incubation, 10 mL culture suspensions were collected and centrifuged (9500 rpm, 15 min. and 4^o C). The harvested cells were washed with 25% NaCl solution and centrifuged in the same conditions. The pellet was then stirred in 10 mL chloroform: methanol (3:1 v / v) mixture and the carotenoid pigment extraction was performed in the dark and at 4^o C for 24 hours.

The pigment solutions in the chloroform: methanol mixture was used for spectrophotometric determination of: - the total carotenoid content at 494 nm, - bacterioruberin at 466, 495 and 528 nm, - lycopene at 448, 474 and 505 nm, - β-carotene at 425, 455 and 482 nm, using a UV/VIS spectrophotometer (Analitik Jena Specord 210 plus) (FANG et al., 2010; SUI et al., 2014; HAMIDI et al., 2017).

RESULTS AND DISCUSSIONS

The obtained results regarding the spectrophotometrically determined cell growth (as optical density at 660 nm) showed that in the case of the *Haloarcula* 5/1GM strain, the growth curves of the cultures subjected to irradiation were not significantly influenced by the tested irradiation doses (10 Gy, 100 Gy , 500 Gy and 1 kGy) compared to the growth of the non-irradiated culture. Thus, the growth of the *Haloarcula* 5/1GM strain was not inhibited by the increased doses of gamma radiation, respectively 500 Gy and 1 kGy, the growth curves recording values of 1.1556 O.D.U. (at 500 Gy) and 1.1542 O.D.U. (at 1 kGy), after 216 hours of incubation (Fig. 1, A).

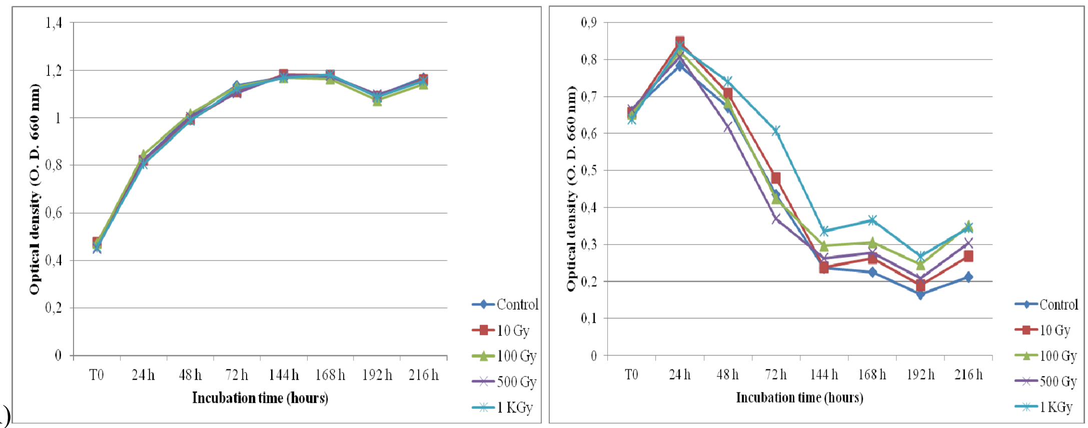
A)  B)

Figure 1. Influence of different doses of gamma radiation on the growth of the *Haloarcula* 5/1GM (A) and *Haloarcula* 5/2GM strain (B).

Regarding the growth of the *Haloarcula* 5/2GM strain, the obtained results showed a decrease of the cell density after 24 hours of incubation, both in the non-irradiated culture (control) and in the irradiated cultures with different doses of gamma radiation (10 Gy – 1 kGy). However, a slight stimulation of the growth occurred after 24 hours, at the irradiation dose of 1 kGy. Furthermore, the growth was slowed down to 0,2682 O.D.U. at 192 hours of incubation, with a small peak of increased growth at 168 h. After 192 h, the inhibition was exceeded and a small peak of growth was observed at 216 hours of incubation, reaching 0.3443 O.D.U. (Fig. 1, B).

As concerns the influence of gamma radiation on the ability of the tested *Haloarcula* strains to synthesize carotenoid pigments, the results showed that increased doses of gamma radiation (500 Gy and 1 kGy) had a stimulatory effect. Thus, the biosynthesis of carotenoid pigments by the *Haloarcula* 5/1GM strain was stimulated at the irradiation dose of 500 Gy, and high optical density values were determined for both total carotenoid content (1.5277 O.D.U.) and bacterioruberin (1.4961 O.D.U.), lycopene (1.5476 O.D.U.) and β-carotene (1.7818 O.D.U.) (Fig. 2).

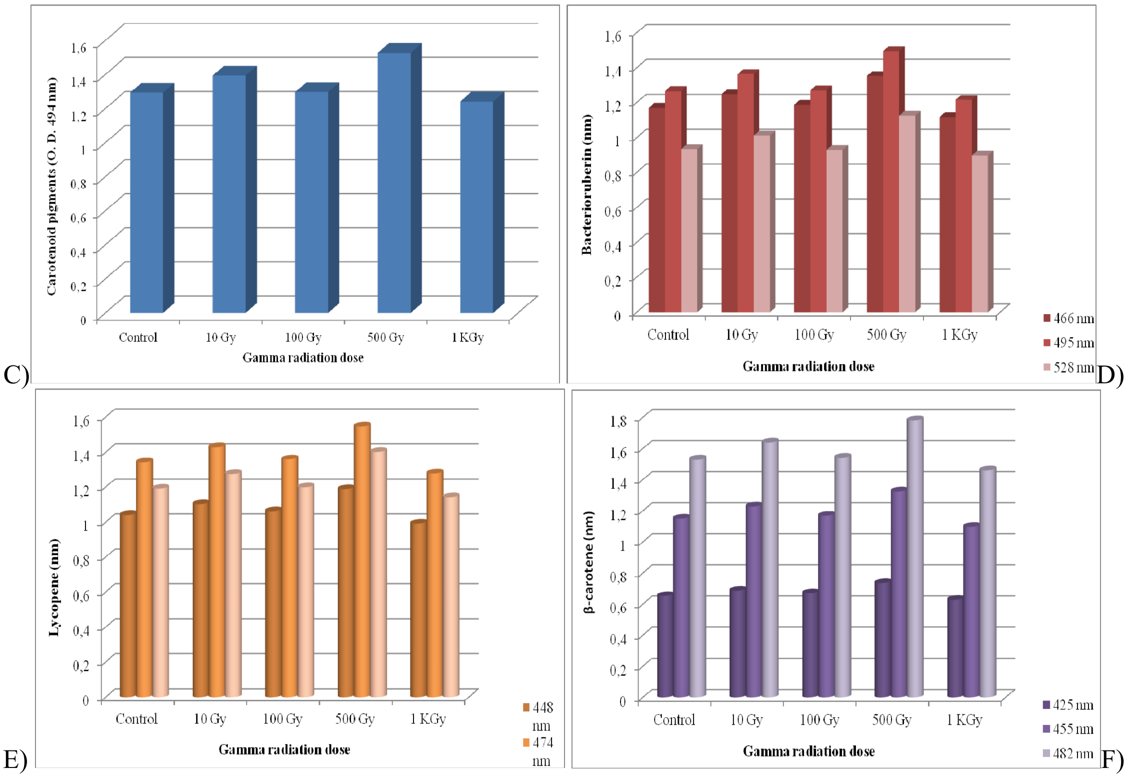


Figure 2. Production of: total carotenoid pigments (C), bacterioruberin (D), lycopene (E) and β-carotene (F) by the *Haloarcula* 5/1GM strain, after irradiation with different doses of gamma radiation.

The results obtained in the case of the *Haloarcula* 5/2GM strain showed that this strain had an increased biosynthesis capacity of the carotenoid pigments after irradiation with a high dose of gamma radiation, respectively 1 kGy. Thus, at this irradiation dose, the optical density values determined after 216 hours of incubation were 0.0664 O.D.U. for the total carotenoid pigment content, 0.0696 O.D.U. for bacterioruberin, 0.072 O.D.U. for lycopene and 0.0729 O.D.U. for β-carotene (Fig. 3).

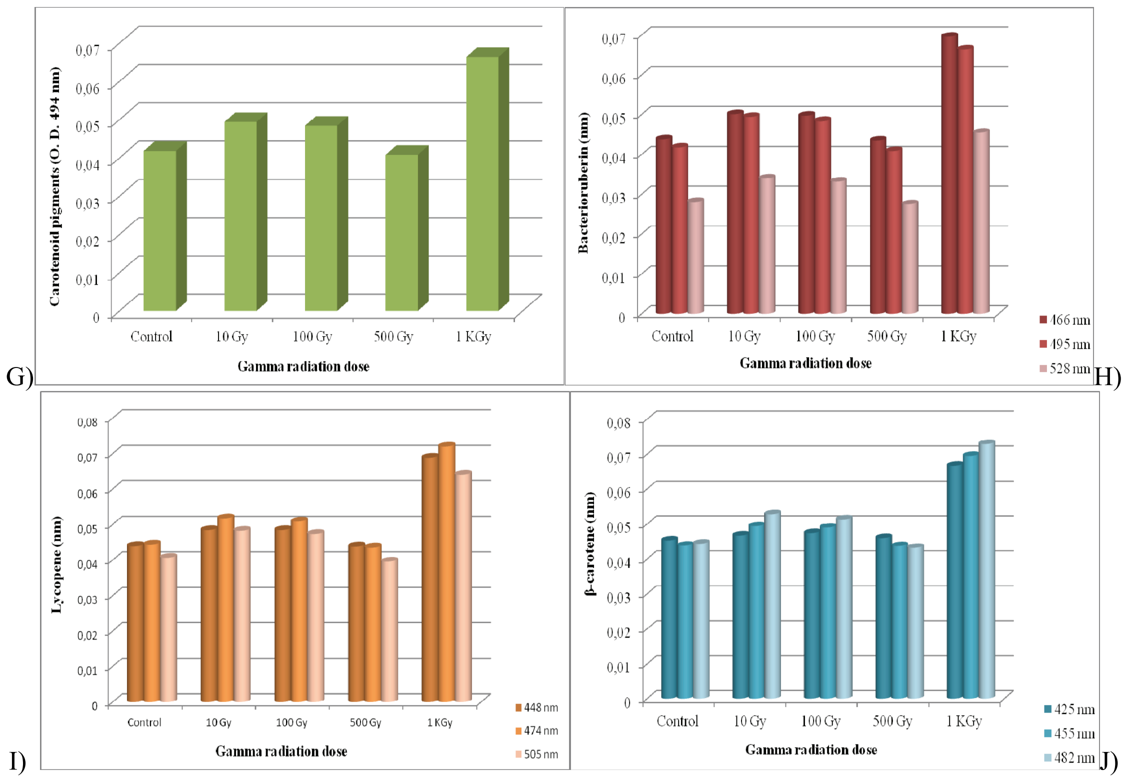


Figure 3. Production of: total carotenoid pigments (G), bacterioruberin (H), lycopene (I) and β-carotene (J) by the *Haloarcula* 5/2GM strain, after irradiation with different doses of gamma radiation.

CONCLUSIONS

The results obtained in this study revealed that the two tested *Haloarcula* strains were resistant to high doses of gamma radiation, respectively 500 Gy and 1 kGy. Furthermore, the irradiation of the strains at these doses stimulated cell growth and synthesis of various metabolic products of biotechnological interest, such as carotenoid pigments.

The results obtained in the present study are encouraging so that, in perspective, future experiments will aim to continue the research in the sense of irradiation of the strains using increased doses of gamma radiation (2 - 6 kGy), but non-lethal, in order to further stimulate the development of haloarchaea and the synthesis of biocompounds with applicative potential.

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