Studii şi Cercetări	Mai 2018	Biologie	27/2	49-54	Universitatea"Vasile Alecsandri" din Bacău

BIOLUMINESCENT ASSAY FOR VIABILITY OF *AZOTOBACTER SP.* FROM DIFFERENT ENVIRONMENTS

Rahela Carpa, Alexei Remizovschi

Key words: *Azotobacter sp., cell viability, ATP, total proteins*

INTRODUCTION

The ecological distribution of *Azotobacter* species is complicated and diverse environmental factors can determine their presence or absence in soil. It is proven that the climatic conditions and the characteristics of soil affect the distribution of those microorganisms (Tilakk et al., 2005) but also the organic matter content, pH and C/N ratio (Tejera et al., 2005). *Azotobacter* species are aerobic bacteria, free, with growth optimum at a neutral pH (pH 7-7,5) but it was proven that these can also grow at other pH intervals (Garrity et al., 2004).

One of the problems associated to the existence of these bacteria in the mountain soils is to prove their viability. A first step was to establish the growth conditions at the *Azotobacter* strains originating from these heterogenous soils. Then a bioluminescence protocol was chosen for the ATP content in the studied strains as an indicative of their viability.

The cell viability is correlated with the cell ATP quantity. After loosing the cell membrane integrity, in a few minutes, the cells are loosing the capacity to synthetize ATP and the endogenous ATP-ases are destroying all the remaining ATP. Thus the ATP level is decreasing promptly (Chen et al., 1994).

ATP is involved in many essential metabolic processes, and its intracellular level provides a measure of the metabolic state of a cell (Giglielmetti et al., 2008). Quantification of light emission (bioluminescence) is one of the most sensitive real-time means of assessing cell conditions, metabolism included. For these reasons, luminescent reporter systems based on insect luciferase have been successfully employed for several analytical purposes in a number of microbes (Lahtinen et al., 2007; Selbitschka et al., 2006).

Bioluminescent detection of intracellular ATP concentration could be considered as an alternative for viability assessing. All living cells accumulate a certain level of ATP to carry out metabolic processes including cell proliferation (Romanova et al., 1997). The specific intracellular ATP concentration notably decreases for a short time under stressful conditions; the level of ATP in dead cells is undetectable. The bioluminescence method based on the application of luciferase reaction is rapid, highly sensitive and simple to perform (Girotti et al., 1998). Other studies focused on contaminated soil using a method for ATP extraction from cells in the presence of oil, and the correlation of ATP data to the enumeration of *Rhodococcus sp*. cells in the oil-containing liquid media has been published (Efremenko et al., 2005).

Assessing the cell viability by luminescence is a homogeneous method for determining the number of viable cells in a culture. The ATP detection is based on a luciferase reaction. By addition to cells the CellTiter-Glo reagent accomplishes three main processes: lyses the cell membrane breaking loose the ATP, inhibits the activity of endogenous ATPases, provides luciferin and luciferase needed for measuring the cell ATP based on a bioluminescence reaction (Cree and Andreotti, 1997).

Luciferase proteins produce light in the visible spectrum (approximately 560 nm for firefly luciferase) following interaction with luciferin substrate molecules. This reaction only requires ATP and oxygen and thus can occur in any actively metabolic cell. Luciferin, a small water-soluble molecule, readily crosses cell membranes and can penetrate into virtually all tissues (Chewning et al., 2009). Light produced by the luciferase-luciferin reaction is detectable by bioluminometer.

The luminescent signal was recorded with GloMax 20/20 luminometer. The oxidation of luciferin by luciferase produces light. When ATP is the limiting factor in the luciferin oxidation reaction the light quantity produced is proportional to the ATP concentration in the sample (Fan and Wood, 2005).

$$\text{Luciferin} + \text{ATP} \rightarrow \text{Luciferyl adenylate} + PP_i$$

$$\text{Luciferyl adenylate} + O_2 \rightarrow \text{Oxyluciferin} + \text{AMP} + \text{Light}$$

Bioluminescence, emerged as an extremely useful reporter technology, provides a sensitive, non-destructive and real-time assay that allows for temporal and spatial measurement. Over the years, many studies have been done on physiological, biochemical and genetic control of bacterial luminescence (Girotti et al., 2008). Bioluminescence is coded by *luxCDABE* genes (van der Meer and Belkin, 2010). The *luxAB* genes code for the α and β subunits of the luciferase dimer, which catalyzes the

oxidation of a reduced flavin mononucleotide (FMNH2) and a long-chain fatty aldehyde in the presence of molecular oxygen, leading to the emission of blue-green light. The synthesis of the aldehyde is catalyzed by a fatty acid reductase complex that includes a reductase, a transferase, and a synthetase, coded for by the *luxCDE* genes, respectively (Yagur and Belkin, 2011).

The main aim of this study was to determine the viability of the poly-β-hydroxybutyrates producing bacteria and the bioluminescence protocol was preferred because it is fast and accurate.

MATERIALS AND METHODS

Bacterial strains and growth. The *Azotobacter* strains used in this study were obtained from different soils: humosiosol type from alpine meadow (Parâng Mountain), rendzina type from karstic meadow (Şureanu Mountain), cambic chernozem type from xeric meadow (Transylvanian Plateau-Suatu) and alluvial type from Someş flood plain. The bacterial strains from these habitats were first studied in order to find some strains with a high capacity to synthesize poly-β-hydroxybutyrates (Crpa et al., 2012).

In order to assess the optical density at the *Azotobacter* strains two culture media with different carbon sources were used: one with mannitol (modified Burk) and another with sucrose (Atlas, 2010; Carpa et al., 2014). The culture conditions were: 30 ºC temperature, *Azotobacter* optimal pH (pH=7-7,5) and continuous shaking of 150 rpm. The measurements were taken for 120 hours. The optical density was read at spectrophotometer at 600 nm.

Viability assessing methods – ATP protocol. The cell ATP quantity was measured with L/L (luciferin/luciferase) by bioluminometric method. Luminescence values are reported as the instrument's arbitrary relative luminescence units (RLU).

ATP assay kit was supplied by Promega Enliten ATP and the other reactives were prepared in our lab. Because the ATP content in cells (bacteria) was measured, the ATP was extracted with trichloroacetic acid (TCA) before the analysis. Trichloroacetic acid (TCA) is recommended because this releases ATP from cells and inactivates the ATP degrading enzymes. Because TCA inhibits the luciferase reaction, it is important to determine the minimal TCA quantity needed. Generally, TCA of 0,5% to 2,5% (final concentration) is enough to extract ATP from bacteria and eukaryotic cells (petty et al., 1995; Ronner et al., 1999). The sample must be read quickly at luminometer because ATP is fast degraded.

Total protein concentration. The total protein concentration was assessed by modified Lowry method (Hartree, 1972). The samples were centrifuged at 15000 rpm over 15 minutes at 0°C and the supernatant was analysed by Lowry method. The absorbance of the samples was read at 650 nm.

Viability testing. In order to verify if the viability assessed by measuring ATP is correct it was also tested by numbering the viable cells from the Petri dishes, stained with methylene blue. Also the plate culture method takes more time, the other method, numbering the cells on Thoma chamber, is not appropriate because both live and dead cells are numbered. For all the analyzed samples the viability was assessed by numbering the viable cells from the Petri dishes in the 40-th incubation hour, when maximal values of the relative luminescence units (RLU) were recorded.

RESULTS AND DISCUSSIONS

At all the *Azotobacter* species isolated from diverse zones growth curves were done (A), using culture media with mannitol and sucrose. There is a difference at results between the two culture media used. On mannitol lower optical density values were obtained at all the studied strains, compared with the ones on sucrose. The faster growth on the sucrose medium may be due to the fact that these strains developed a more efficient metabolization of the substances more available in the soil. Because the strains are originating from mountain soils, where the lower temperature affects more the fungi than the bacterial decomposers, the mannitol content of soil is lower. This might explain the preference for sucrose.

Furthermore, *Azotobacter* species are obligate aerobe and adapt the respiratory mechanisms in order to use different substrates. If there is mannitol in soil, from the fungal exudates or from the conifers, the mannitol-dehydrogenase is activated and thus the bacteria will use mannitol as carbon source. If there is glucose in the soil, from plant decomposition, than glucose dehydrogenase will be activated and the carbon source will be glucose. If there are both carbon sources in soil they will be used simultaneously by activating both dehydrogenases (Harris, 1994).

Figure 1A shows that the strains from the alpine zone the maximum of optical density at λ = 600 nm was reached after 80 incubation hours, being 0.9215 on sucrose medium and 0.7846 on mannitol.

One of the best markers of viability is adenosine triphosphate (ATP), which is the primary energy source for all metabolic reactions and is present in all viable cells. At these strains coming from the alpine zone the cell viability was determined by relating the relative luminescence units (RLU) to the total protein quantity (mg/ml) (Figure 1B). When using sucrose as carbon source the enhancing and diminishing of the studied ratio is swifter than on mannitol because sucrose is quicker metabolized.

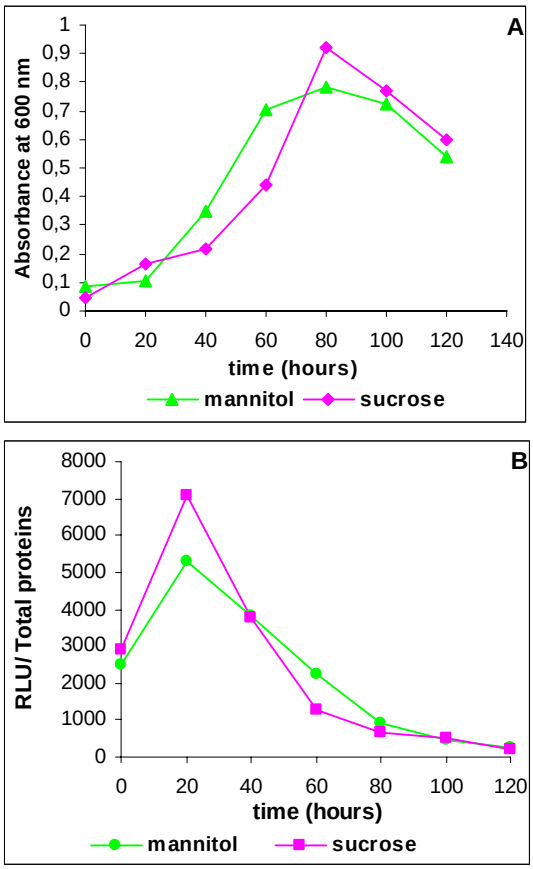

Figure 1. A. Optical density at the *Azotobacter sp.* strains from the alpine zone
B. The ratio between cellular ATP (RLU=relative luminescence units) and total protein quantity (mg/ml) in strains from the alpine zone.

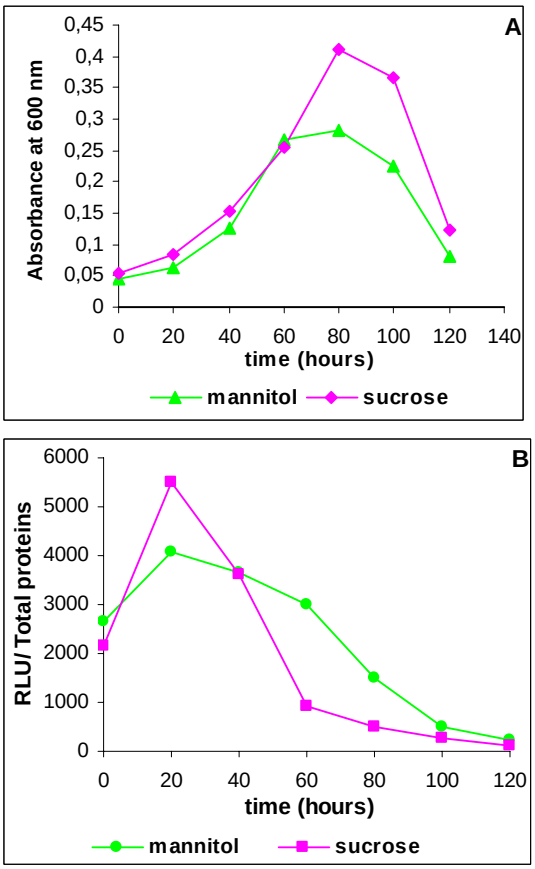

Figure 2. A. Optical density at the *Azotobacter sp.* strains from the karstic zone
B. The ratio between cellular ATP (RLU=relative luminescence units) and total protein quantity (mg/ml) in strains from the karstic zone.

At the strains from the alpine zone the obtained inclination is more reduced than in the case of zones with more favorable environmental conditions. This may be due to the fact that, in the same experimental conditions, the strains adapted to harsher environments maintain their viability longer. The ratio between RLU and protein quantity at the strains originating from the alpine zone reaches the maximum value on sucrose medium (Figure 1B), probably due to the preference of these strains for sucrose.

At the strains isolated from the karstic zone the growth was quite low, reaching the maximum after 80 incubation hours, and the values were 0.2815 on mannitol medium and 0.4121 on sucrose medium (Figure 2A). Although the growth obtained was not so great it suggests that there are *Azotobacter* strains which, being adapted to live under stress, lose their cultivability. Though they remain alive and metabolically active (Tenhami et al., 2001). However the luciferase can monitor the physiological state of all cells, because it correlates sensitively to intracellular ATP.

Regarding the viability of those strains, it was observed that, analogous to the strains from the alpine zone, the strains from the karstic zone, coming from a medium with harsh conditions, succeed in maintaining the cell metabolic activity longer at a relatively high level (Figure 2B). This dynamic is more pregnant on mannitol medium, common source of carbon in the karstic zone.

In the xeric zone the climate is more favorable to microbial activity, thus reaching a decided higher growth than the one at the other zones, with maximum values of 0.9425 on mannitol and 1.6245 on sucrose (Figure 3A). The almost double values on the sucrose medium, relative to the ones at the alpine zone strains, are due to the milder environmental conditions found here (climate, slope orientation, inclination, soil, vegetation), allowing the development of a more abundant microbiota.

At the strains from this zone the sharp decrease of the studied ratio (RLU/total protein) after 20 incubation hours (Figure 3B), due to the faster growth of proteins compared to ATP level suggests the using of other high-energy compounds.

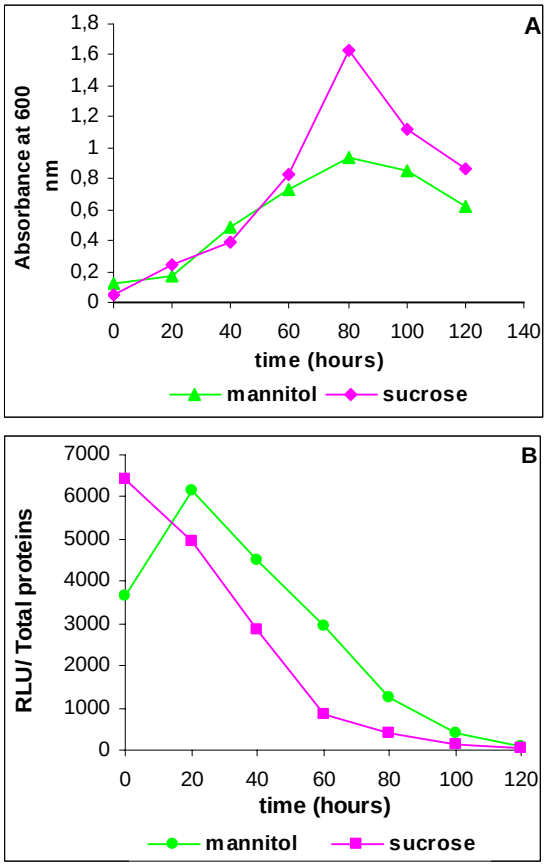

Figure 3. A. Optical density at the *Azotobacter sp.* strains from the xeric zone
B. The ratio between cellular ATP (RLU=relative luminescence units) and total protein quantity (mg/ml) in strains from the xeric zone.

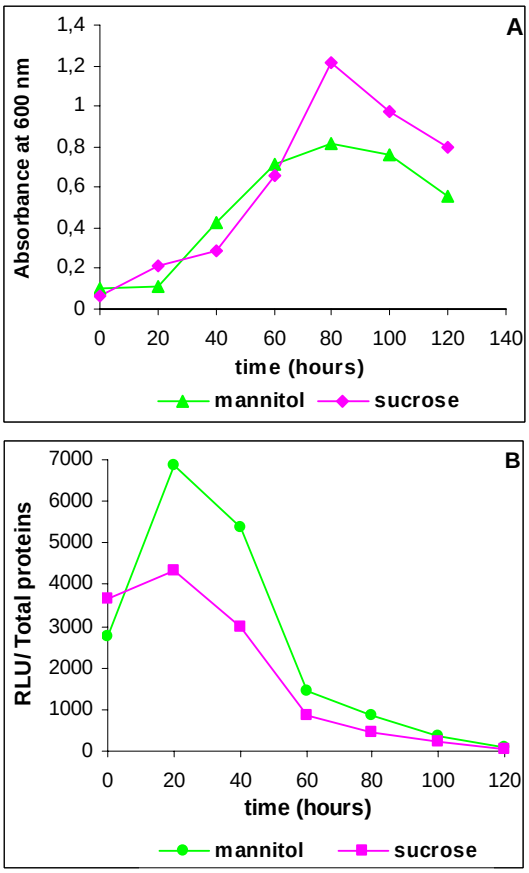

Figure 4. A. Optical density at the *Azotobacter sp.* strains from the Someş flood plain
B. The ratio between cellular ATP (RLU=relative luminescence units) and total protein quantity (mg/ml) in strains from the Someş flood plain.

At the samples from Someş flood plain the maximum values recorded were 0.8157 on mannitol medium and 1.2143 on sucrose (Figure 4A). Though there is a milder climate and more abundant vegetation, a lower growth than to the other zones was obtained, explained by the repeated floods by the river, affecting the free and aerobic *Azotobacter* species.

The lower values of RLU/proteins ratio obtained at the strains from the flood plain on sucrose medium (RLU/Total proteins= 4339) are due to the larger proteins quantity and implies the use of other high-energy compounds. Furthermore, the difference between the two carbon sources, more pregnant than to the other zones, implies that the strains from here are not very specialized in sucrose metabolization (Figure 4B).

Comparison of results obtained by bioluminescent protocol for ATP to the ones obtained by plate culture.

In order to verify if the viability established by ATP measuring is correct, the viability was also tested by numbering the viable cells on Petri dishes stained with methylene blue. In order to check the viability the viable count method was chosen.

Although this method takes more time, the other method, numbering the cells in Thoma chamber, is not appropriate because both dead and alive cells are counted. For all the analyzed samples the viability was assessed by counting the viable cells in the Petri dishes in the 40th incubation hour, when maximum values of the relative luminescence units (RLU) were reached.

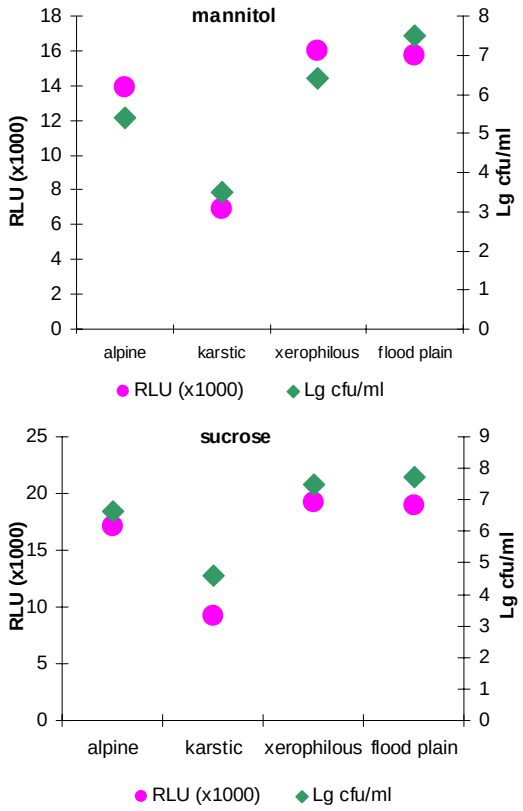

Figure 5. Cell viability in *Azotobacter* strains by bioluminometer and plate culture methods in the 40 th hour of incubation.

As shown in Figure 5, in the 40th incubation hour, at the cultures on mannitol, there is a strong correlation between the data obtained for each zone by the two methods of assessing the cell viability. At the samples on sucrose medium it is also manifest a strong similarity between the data of cell viability obtained by the two methods above. At all the bacterial strains isolated from different zones the cell viability presented very close values for the two methods of assessing the viability. By comparison with a conventional viable count method, other studies found a high correlation between ATP content and the viable count; this relationship can be applied in routine quality control to estimate viable count from the ATP content determined in a sample (Jensen et al., 2008).

Although the two viability assessing methods are very different the two data sets obtained are similar. This means that the data obtained are accurate and the methods used are efficient.

CONCLUSIONS

The cell viability was assessed in *Azotobacter* strains by bioluminometer method, the relative luminescence units (RLU) being related to the cell biomass (total proteins).

It was noticed that higher RLU values were obtained in *Azotobacter* strains originated from lower zones, with a climate more favorable to development (the ones from xeric zone and flood plain). The strains originated from higher altitudes, with harsher environment (alpine and karstic), followed. The lowest RLU values were recorded at the strains from the karstic zone.

The bioluminometer method for assessing the cell viability is an effective method, fact enforced by the results obtained using the viable count method.

ABSTRACT

Poly-β-hydroxybutyrates producing *Azotobacter* strains were isolated from zones with heterogenous environmental conditions from Romanian Carpathians and Transylvanian Plateau: alpine, karstic and xeric meadows and flood plain. These strains were grown on *Azotobacter* specific media with different carbon sources (mannitol and sucrose). In order to assess the cell viability the following parameters were determined: optical density – by spectrophotometry; viability – by measuring ATP quantity based on bioluminescence luciferase reaction but also using plate culture method of numbering the viable cells, proteins concentration – by Lowry method. The viability assessed by ATP measuring was checked by numbering the viable cells from Petri dishes stained with methylene blue.

REFERENCES

1. ATLAS R.M., 2010 - Handbook of Microbiological Media. 4rd edition, CRC Press, New York;
2. CARPA R., BUTIUC-KEUL A., LUPAN I., BARBU-TUDORAN L., MUNTEAN V., DOBROTA C., 2012 - Poly-β-hydroxybutyrate accumulation in bacterial consortia from different environments, Can. J. Microbiol., 58 (5), 660-7;
3. CARPA R., DRĂGAN-BULARDA M., MUNTEAN V., 2014 - Microbiologie Generală, Lucrări Practice, Ed. Presa Universitară Clujeană;
4. CHEN F., CUSHION M.T., 1994 - Use of an ATP bioluminescent assay to evaluate viability of *Pneumocystis carinii* from rats, J. Clinical Microbiol., 32 (11), 2791-2800;
5. CREE I.A., ANDREOTTI P.E., 1997 - Measurement of cytotoxicity by ATP—based luminescence assay in primary cell cultures and cell lines, Toxicology in Vitro, 11, 553-556;
6. CHEWNING J.H., DUGGER K.J., CHAUDHURI T.R., ZINN K.R., WEAVER C.T., 2009 - Bioluminescence-based visualization of CD4 T cell dynamics using a T lineage-specific luciferase transgenic model**,** BMC Immunology, 10, 44;

7. EFREMENKO E.N., AZIZOV R.E., RAEVA A.A., ABBASOV V.M., VARFOLOMEYEV S.D., 2005 - An approach to the rapid control of oil spill bioremediation by bioluminescent method of intracellular ATP determination, International Biodeterioration & Biodegradation, , 56, 94–100;
8. FAN F., WOOD K.V., 2007 - Bioluminescent assays for high-throughput screening, Assay Drug Dev. Technol., 5 (1), 127–36;
9. GARRITY G.M., BELL J.A., LILBUM T.G., 2004 - Taxonomic Outline of the Prokaryotes. Bergey's Manual of Systematic Bacteriology, 2nd Edition, Release 5.0, Springer Verlag, New York;
10. GIRROTTI S., FERRI E.N., FUMO M.G., MAIOLINI E., 2008 - Monitoring of environmental pollutants by bioluminescent bacteria, Analytica Chimica Acta, 608, 2-29;
11. GIROTTI S., ZANETTI F., 1998 - Wastewater and sludge: the rapid determination of microbial content by ATP bioluminescence, Annali di Chimica, 88, 291–298;
12. GUGLIELMETTI S., CIRANNA A., MORA D., PARINI C., KARP M., 2008 - Construction, characterization and exemplificative application of bioluminescent *Bifidobacterium longum biovar longum*, Int. J. Food Microbiol., 124, 285–290;
13. HARRIS J.O., 1994 - A study of adaptation in *Azotobacter*, Soil Science, Society of America, 18, 154-156;
14. HARTREE E.F., 1972 - Determination of protein: A modification of the Lowry Method that gives a linear photometric response, Analytical Biochemistry, 48, 422-427;
15. JENSEN S.E., HUBRECHTS P., KLEIN B.M., HASLOV K.R., 2008 - Development and validation of an ATP method for rapid estimation of viable units in lyophilised BCG Danish 1331 vaccine, Biologicals, 36(5), 308-14;
16. LAHTINEN S.J., JALONEN L., OUWEHAND A.C., SALMINEN S.J., 2007 - Specific *Bifidobacterium* strains isolated from elderly subjects inhibit growth of *Staphylococcus aureus*, Int. J. Food Microbiol., 117, 125–128;
17. PETTY R.D., SUTHERLAND L.A., HUNTER E.M., CREE I.A., 1995 - Comparison of MTT and ATP-based assay for measurement of viable cell number, J. Biolum. Chemilumin., 10, 29-34;
18. RONNER P., FRIEL E., CZERNIAWSKI K., FRANKLE S., 1999 - Luminometric assays of ATP, phosphocreatine, and creatine for estimation of free ADP and free AMP, Anal Biochem., 275, 208–216;
19. ROMANOVA N.A., BROVKO L.YU., UGAROVA N.N., 1997 - Comparative assessment of methods of intracellular ATP extraction from different types of microorganisms for bioluminescent determination of microbial cells, Appl. Biochem. Microbiol., 33, 306–311;
20. SELBITSCHKA W., KELLER M., MIETHLING-GRAFF R., DRESING U., SCHWIEGER F., KRAHN I., HOMANN I., DAMMANN-KALINOWSKI T., PUHLER A., TEBBE C.C., 2006 - Long-term field release of bioluminescent *Sinorhizobium meliloti* strains to assess the influence of a recA mutation on the strains' survival, Microb. Ecol., 52, 583–595;
21. TENHAMI M., HAKKILA K., KARP M., 2001 - Measurement of effects of antibiotics in bioluminescent *Staphylococcus aureus* RN4220. Antimicrob. Agents Chemother., 45, 3456–3461;
22. TILAKK K.V.B.R., RANGANAYAKI N., PAL K.K., DE R., SAXENA A.K., SHEKHAR NAUTIYAL C., MITTAL S., TRIPATHI A.K., JOHRI B.N., 2005 - Diversity of plant growth and soil health supporting bacteria, Current Science, 89, 1, 136-150;
23. TEJERA N., LLUCH C., MARTINEZ-TOLEDO M.V., GONZALES-LOPEZ J., 2005 - Isolation and characterization of *Azotobacter* and *Azospirillum* strains from the sugarcane rhizosphere, Plant and Soil, 270, 223-232;
24. van der MEER J.R, BELKIN S., 2010 - Where microbiology meets microengineering: design and applications of reporter bacteria, Nat. Rev. Microbiol., 8 (7), 511–522;
25. YAGUR-KROLL S., BELKIN S., 2011, Upgrading bioluminescent bacterial bioreporter performance by splitting the *lux* operon, Anal. Bioanal. Chem., 400, 1071-1082

ACKNOWLEDGMENTS

This work was supported by a grant PN II CNCSIS, Postdoctoral type PD-393/2010 financed by "The National University Research Council", Romania.

AUTHORS' ADDRESS

CARPA RAHELA, REMIZOVSCHI ALEXEI - Babeş Bolyai University, Faculty of Biology and Geology, Department of Molecular Biology and Biotechnology, 1 M. Kogalniceanu Str., Cluj-Napoca, 400084, Romania.

Corresponding author: CARPA R., e-mail**:** k_hella@yahoo.com