

Review

by Prof. Dr. Dimitar Kadiysky, Doctor of Medical Sciences,

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Subject: Dissertation work for the award of the degree "Doctor" in Higher Education Department 4. "Natural Sciences, Mathematics and Informatics", Professional Direction 4.3 "Biological Sciences", Scientific Specialty - Biochemistry

Presented by a doctoral student in independent preparation for awarding the educational and scientific degree "Doctor" at the Institute of Experimental Morphology, Pathology and Anthropology - BAS.

on the subject:

Optimization of the production of biologically active substances from bacteria and algae and evaluation of their potential action as antitumor agents in in vitro experiments

Author: Angeliki Nikolaos Konstantinidou

Scientific consultants: Prof. Dr. Svetlozara Petkova and Assoc. Dr. Lyudmila Kabaivanova

General presentation of the procedure and the doctoral student.

By order No. RD-15 – 121 dated 19.07.2022 of the Director of the Institute of Experimental Morphology, Pathology and Anthropology with Museum - BAS, I have been appointed as a member of a scientific jury in connection with the procedure for the defense of a dissertation on the topic "*Optimization of the production of biologically active substances from bacteria and algae and evaluation of their potential action as antitumor agents in in vitro experiments*" for the acquisition of the educational and scientific degree "Doctor" in the scientific specialty - Biochemistry. The author of the dissertation work is specialist Angeliki Nikolaos Konstantinidou - doctoral student of an independent form of study at IEMPAM - BAS.

For the preparation of the review in connection with the procedure for the defense of a dissertation work with author Angeliki Konstantinidou, I was provided with a set of materials of the author, required by the Regulations for the Development of the Academic Staff in IEMPAM and in accordance with the rules for application of Law for the development of the academic staff (LDAS) in the Republic of Bulgaria and BAS. The PhD student was born in Athens, Greece, studied there and in Komotini, and graduated in Pharmacy in 2018 at the Medical University - Plovdiv as a master pharmacist. At the time of the dissertation defense procedure, she held the position of "specialist" at IEMPAM, section of Pathology. Her main knowledge, resulting from her education at the MU Plovdiv, her work as a specialist at IEMPAM and the courses she attended at the BAS Training Center, are in the field of biochemistry, organic and analytical chemistry, physical chemistry, the chemistry and analysis of bioproducts and therapeutic agents.

Actuality of the topic.

The results of Angeliki Konstantinidou's research activity are summarized in a compact scientific work, containing all the necessary sections of a dissertation - introduction, complete literature review of the topic, aim and tasks, materials and methods, results, discussion, conclusions and contributions from the achieved results.

The work on the dissertation was consulted by experienced molecular biologists and morphologists, such as Prof. Dr. Svetlozara Petkova and Assoc. Prof. Dr. Lyudmila Kabaivanova, which created an opportunity for a precise discussion and objective evaluation of the author's results.

The topic of the dissertation work is important, taking into account the increased interest in applied biomedical research for the search and promotion of new complex therapeutic approaches in malignant diseases. Screening the properties of novel bioactive compounds enriches standard classical drug approaches for cancer treatment with new possibilities. The dissertation topic is directly and indirectly related to these problems, as the goal is the search and promotion of new natural substances of bacterial and algal origin, optimization of their preparation and biomedical evaluation of these substances as potential agents possessing an antitumor effect. A basis for the search and introduction of new therapeutic agents of biological origin isolated from bacteria and algae in practice is based on their lower toxicity, and hence their greater safety compared to some of the synthetic pharmaceutical products.

Knowing the problem. Evaluation of dissertation work and contributions.

The dissertation is structured according to generally accepted requirements – introduction and literature review (21 pages), aim and objectives, materials and methods, results and discussion (33 pages), conclusions, contributions and literature. More than 100 contemporary literary sources directly related to the subject of the dissertation work are indicated in the bibliographic reference. The dissertation covers 95 pages of integral text and is illustrated with 45 figures.

The doctoral student's literary knowledge is excellent in relation to the researched problem - production of biologically active substances from bacteria and algae and antitumor capacity of these cellular products. Her awareness contributes to the precise formulation of the purpose of the research work, the tasks and the various experimental approaches in the conduct of the research. Studying the role of biologically active substances of bacterial origin applicable in biomedicine is of interest to pharmaceutical manufacturers, as it leads to the expansion of the complex of therapeutic approaches in oncology. The comprehensive review, as well as the introduction to the dissertation, emphasizes the types and properties of currently applied biologically active components from algae, mainly complex polysaccharides and pigments, important for biology and medicine. It is common knowledge that marine organisms and in general various representatives of the biocenosis and microbocenosis are sources of active secondary metabolites, some of which have long established themselves as useful therapeutic agents in health care. In most cases, it is a question of biopopulations that are easily cultivated, multiply quickly and are active producers of secondary metabolites. It is a matter of biochemical studies and biotechnological approaches to purify and characterize these products, chemically modify them and improve their bioactivity for further biomedical therapeutic application. The dissertation review evaluates heteropolysaccharides, a product of red algae that have recently attracted attention for their anticancer effects, ability to inhibit growth, stimulate the immune response, and induce apoptosis.

From a methodological point of view, an overview of the existing possibilities for the production of biologically active substances from bacteria has been made, by selecting suitable producer strains and optimizing the conditions for increased synthesis. The possibilities for characterizing and enhancing specific properties of biologically active substances related to the survival of tumor cells are considered.

In addition to summarizing the available literature data on certain bioactive substances, the author summarizes the existing methodological options related to their preparation. Attention is paid to the characteristics of surfactants and glycolipids. A review of types of microorganisms producing glycolipid biosurfactants is included. Emphasis is placed on cell immobilization methods.

Tasks naturally arising from the set goal include: optimization of the conditions for growth and development of selected bacterial and algal strains, immobilization of cells from selected strains, efforts to increase the synthesis of the sought-after biologically active substances, creation of opportunities for repeated use of the selected immobilized preparations. The developed scheme for obtaining glycolipid biosurfactants from bacteria and exopolysaccharides from algae (in vitro experiments by isolation and immobilization) facilitates the screening of the effect of the obtained glycolipids and polysaccharides on the survival of healthy and cancer cell lines, which is the final phase of Angeliki's experimental approach Konstantinidou in connection with the dissertation. It should be noted here that the methods for evaluating the results include approaches typical of both biochemical studies and cell culture methods, immunological and including morphological methods of evaluation, such as scanning electron microscope (SEM) imaging.

The main objects of the research in connection with the dissertation are products of *Pseudomonas aeruginosa* BN10, *Rhodococcus wratislavenis*. The development methodology covers multi-directional research directions: standard techniques for multiplication of *Pseudomonas aeruginosa* BN10 and *Rhodococcus wratislavenis* strains, assessment of rhamnolipid and trehalosolipid concentrations, preparation of polysaccharides from red microalgae *Rhodella reticulata* and *Porphyridium cruentum*, measurement of cytotoxic activity of rhamnolipids and polysaccharides on selected cultured cells with MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] colorimetric test. The good calibration of the experimental research approaches has helped to achieve the set goal and obtain an adequate answer to the tasks on the subject.

The results and discussion sections (including the conclusions drawn) occupy a total of 33 pages of the presentation. An essential part of the obtained results is related to the immobilization of the cells of the selected bacterial strains *Pseudomonas aeruginosa* BN10 and *Rhodococcus wratislavenis* in order to create conditions for increasing the production of glycolipids. Results regarding the kinetics of biosurfactant production are reflected and discussed. A biomedical analysis of the antitumor effect of *Pseudomonas aeruginosa* BN10 RL-1 and RL-2 products, as well as *Rhodococcus wratislavenis* trehalosolipids, was performed. Based on the data obtained, the author hypothesizes that the demonstrated difference in the action of RL-1 and RL-2 is the result of a stronger interaction with the cell surface, leading to a greater effect on cell survival, and that there is a dependence with differences in the metastatic potential of the tested breast cancer lines. For the study of the structure and visualization of the matrices used, the doctoral student applies modern morphological methods - scanning electron microscope (SEM) research, creating a 3D image of these structures. As a result of the study, the doctoral student made an optimal selection of bacterial producers of glycolipid biosurfactants - *Pseudomonas aeruginosa* BN10 and *Rhodococcus wratislavenis*, including algal producers of

exoheteropolysaccharides - red microalgae - *Porphyridium cruentum* and *Rhodella reticulata*. Through the immobilization method and the selection of a suitable matrix – polyethylene oxide cryogel matrix, the selected bacterial strain *Pseudomonas aeruginosa* demonstrated an increased synthesis of rhamnolipids - 4.6 g L⁻¹ within 7 days. The demonstrated decrease in the survival of two cancer cell lines, after administration of different concentrations of synthesized bacterial product, was most pronounced on the highly metastatic cell line MDA-MB 231. When treating the low and highly metastatic cancer cell lines with the isolated hydropolysaccharide from *Rhodella reticulata* - 75 µg/ml in the highly metastatic cell line MDA-MB231 a reduction in cell viability of up to 44% was achieved. On the other hand, as an expected result, a decrease in the vitality of the cancer cell lines was registered depending on a change in the concentration of the heteropolysaccharide. A strong effect was also found on the highly metastatic breast cancer cell line MCF-7 and almost no inhibitory effect on the normal cell line in *in vitro* experiments.

Last but not least in importance as a result is the registered dependence between the nature of the cell lines (cancerous and normal) and the effectiveness of the applied glycolipids according to chemical composition, exposure time, concentration, as well as according to the level of surface tension reduction. An important applied contribution (relevant to biotechnologies) is the achieved increase in polysaccharide production in both tested strains after immobilization in a cryogel matrix. With a good selection of the immobilization matrix, the immobilized preparations are suitable for repeated use and increase in the production of the desired product. The use of cryogel PEO, PAAm and HEC matrices in the immobilization of bacterial and algal cells by the selected producers represents an effective approach that relies on their non-toxicity, accessibility, speed and easy manipulation to increase the yield of glycolipid biosurfactants and algal heteropolysaccharides. As a final conclusion of the study, it can be assumed that the established weak cytotoxicity of the tested concentrations in normal (non-cancerous) cell lines is the best indicator of possible therapeutic applications of the investigated substances. Considering their suppressive effect against cancer cells *in vitro*, after appropriate pharmaceutical modification and testing, they may represent additional adjuncts to chemotherapy.

Assessment of publications, abstract, personal contribution of the PhD student and notes.

Angeliki Constantinidou has submitted a list of 2 publications as one printed in a refereed indexed publication registered in the second quartile (Q2) and another in the third quartile (Q3). I am convinced that the results obtained by the doctoral student are entirely her own doing, and the total number of credits earned in the educational doctoral program cover the minimum requirement of 200 points.

The abstract (autoreferat) is structured correctly, it is sufficient in volume and reflects the main results and contributions of the dissertation - it contains 56 pages and 42 figures. As an insignificant minus for Angeliki Konstantinidou's dissertation, I consider the failure to reflect in the content the merging of the results with the discussion in a common chapter (permissible by the requirements for such work). There is a similar minimal discrepancy in the autoreferat. These features in the presentation of the results in no way reduce the quality of the dissertation work.


Conclusion: The dissertation submitted for review contains scientific and scientific-practical results useful for biomedicine. It contains contributions more or less related to the improvement of therapeutic approaches in controlling uncontrolled cell proliferation in human malignancy. The research material included in the dissertation is sufficient in volume, well documented, and the work meets the requirements of the Law on the Development of the Academic Staff in the Republic of Bulgaria, the

Regulations for the Implementation of this Law and the Regulations for the Acquisition of the Doctoral Degree at IEMPAM - BAS. A substantial part of the results synthesized in the doctoral student's conclusions represent original scientific and applied contributions to science.

In connection with the above, I confidently give my positive assessment of the presented work and suggest the respected scientific jury to vote positively regarding the qualities of the presented dissertation. I propose that Angeliki Nikolaos Konstantinidou be awarded the educational and scientific degree "Doctor".

14.09.2022

Prepared the review:


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