R E V I E W

of a dissertation submitted for defense under the procedure for awarding the educational and scientific degree "Doctor"
in Area of professional field 4.3. Biological Sciences (Microbiology)

Topic: **"Development of biologically active products from new natural sources"**
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The preparation of this review was made in accordance with the normative documents - Law on the Development of the Academic Staff in the Republic of Bulgaria (LAD), the Regulations for its implementation and the Recommendations of the Faculty Council of the Faculty of Biology on the criteria for acquiring scientific degrees and holding academic positions in the SU for the professional area "Biological Sciences".

1. Current state of the scientific problem and relevance of the dissertation topic

Bacterial infections in humans, animals and plants cause serious problems related not only to public health, but also to significant economic losses when they affect animals and agricultural crops. Fighting them in many cases turns out to be difficult, and this presents scientists with serious problems to solve. The most powerful means against bacterial infections in animals and humans are antibiotics, while with those in plants their use has been reduced to a few antibiotics that are now very rarely used in humans. Chemical substances are most often used against phytopathogens, which have a limited effect and lead to environmental pollution. The frequent and in many cases uncontrolled use of antibiotics as well as chemical preparations has led to an increasingly widespread resistance of pathogenic bacteria to these agents. This is a very serious problem, with which scientists from various fields of science are looking for ways to deal with it.

In recent years, a number of researchers have focused their research on the search for new antimicrobial drugs from natural sources. In this way, it is expected to avoid the complication caused by the resistance of bacteria to traditionally applied antibiotics and chemical substances, and also to limit environmental pollution. In connection with the above, I believe that the development of biologically active products from new natural sources is a serious and topical task from a scientific point of view, which is the subject of this dissertation.

2. Evaluation of the structure of the dissertation work, tasks and their compliance with the main goal

The dissertation work is structured according to the generally accepted scheme for this kind of scientific work and is composed of the following chapters: Introduction (2 pages), Literature review (46 pages), Aim and tasks (1 page), Materials and methods (20 pages), Results and discussion (62 pages), Conclusions (1 page), Contributions (1 page), List of used literature sources (477 titles) and Appendices to the dissertation work (22 pages), including additional evidentiary material (tables, figures, photos, diagrams, etc.) to the conducted experiments, recipes of used nutrient media, lists of tables and figures included in the main text of the dissertation, list of abbreviations used. The illustrative material included in the main text of the dissertation is represented by 41 tables and 22 figures. It would be more appropriate to place the list of used abbreviations at the beginning of the dissertation, and the list of used literature immediately after the end of the main text of the dissertation.

The abstract covers 55 pages of text and is prepared according to the requirements, faithfully reflecting the essence of the dissertation work.

The aim of the dissertation is research and evaluation of the antioxidant and antimicrobial activity of bioactive substances from new natural sources - products from microalgae and invertebrates (hemocyanin). Seven tasks are set, arranged in a logical sequence, through the implementation of which the goal of the dissertation should be realized. The tasks include: Isolation, identification and laboratory cultivation of promising strains of microalgae; Preparation of extracts and exopolysaccharides from selected microalgae strains; Determination of the antioxidant activity of microalgae products; Determination of the antimicrobial activity of microalgae extracts and exopolysaccharides against human and plant pathogens; Investigation of the antimicrobial activity of a combination of microalgae extracts; Determination of the antimicrobial activity of natural antibiotics in relation to phytopathogens; Determination of the antimicrobial activity of hemocyanin against human pathogens.

I believe that the doctoral student Petrova has shown skill in planning and formulating the tasks so that they correspond to the goal of the dissertation and ensure its achievement.

3. Evaluation of the methods used and compliance with the goal of the dissertation

A very wide range of diverse methods (more than 20 main ones), described in detail in the "Materials and Methods" chapter, was used for the implementation of the set tasks. They could be divided into several groups – cultivation methods - related to the isolation and cultivation of microalgae; microbiological - related to the cultivation of bacteria and determination of antimicrobial activity of the obtained extracts and substances; analytical; biochemical; molecular biological; chemical; and statistical methods. They are selected appropriately, fully correspond to the set tasks and guarantee their implementation.

4. Evaluation of the theoretical rationale and its correspondence with the experimental solutions, the obtained results and the formulated contributions

Introduction. In the introduction of the dissertation, the doctoral student provides brief and summarized information about the need to search for new means of antimicrobial protection, especially those from natural sources, emphasizing the adverse effect of the excessive and in many cases unnecessary use of antibiotics and chemical substances, which leads to the emergence of resistance among pathogens or environmental pollution. The introduction points to the idea underlying the main objective of the dissertation work.

Literature review. The overview is structured in several parts, which are mutually related and correspond to the topic of the dissertation. In the section "Natural sources of antimicrobial substances" general information is provided on the biology of microalgae and related to their use as sources of biologically active substances. In more detail, attention is focused on the microalgae studied in the dissertation. This part also presents information on other natural sources of biologically active substances such as higher plants, invertebrates and microorganisms. The second part of the review presents information on genera and species of pathogenic bacteria (test microorganisms used in the dissertation), both human and plant, their importance, methods of combating them and the need to search for alternative means. In the third part of the review, an overview of the methods for introducing natural samples into laboratory conditions is given, taking into account the peculiarities of working with microalgae isolated from the natural environment and the stages to reach an algologically pure culture. In the last part, which is the shortest, attention is paid to the therapeutic use of combinations of algae extracts with a view to studying a synergistic effect. The literature review concludes with a brief summary justifying the focus on developing antimicrobial products from natural sources such as microalgae and the species *Eriphia verrucosa* from which hemocyanin was isolated. The information provided in this chapter reflects chronologically the development of scientific knowledge on the issues under consideration, but for the most part it is up-to-date and reflects contemporary trends in the issues of the dissertation. A huge number of publications (477) are cited, of which about 38% were published between 2011 and 2022. Citing the experience of Bulgarian researchers in the search for natural sources of antimicrobial substances makes a good impression. The review is illustrated with 6 tables and 6 figures. The huge volume of cited literature is a prerequisite for acquiring good theoretical knowledge on the treated problem.

Results and discussion. In the chapter "Results and discussion" the results obtained during the development of the dissertation are presented. This chapter is structured in 7 points, which by definition do not fully cover the pre-set tasks. Items 1 and 2 of this chapter present the results of task 1, related to the isolation, cultivation and taxonomic identification of the used and newly isolated microalgae strains. There are two newly isolated cultures - one with presumed belonging to the genus *Nostoc*, and the other, designated at this stage of the research as a microalgae strain ХПВ - А1. On the basis of taxonomic studies carried out, the culture suspected of belonging to the genus *Nostoc* was identified as *Nostoc commune* (by classical

methods), and the culture designated as HPV-A1 as a member of the family *Chlorellaceae*, with the closest affinity to the genus *Muriella* (based on molecular genetic methods). As a very good approach, I want to point out the introduction of molecular methods in the identification of microalgae, which, in addition to the classical ones, greatly increase the success of this research. In item 4 of this section, the results of an analysis of the biochemical composition of two of the microalgae cultures are presented, and the total amount of proteins, carbohydrates, lipids and pigments was determined. The information obtained complements the characteristics of these microalgae and could be useful in further studies with a view to their application. This research could be formulated as a separate task, given the thesis author's opinion that such information on microalgae cultures is very important. As a result of the implementation of the second task of the dissertation work, 14 lyophilized products based on water extracts were obtained, and alcohol extracts were 3. I believe that the results of this task could be presented in more detail in the main text, instead of the reader be forwarded to the appendices.

The third task of the dissertation is the determination of the antioxidant activity of the obtained microalgae products. For this purpose, biochemical parameters characterizing the antioxidant activity of the microalgae extracts were determined, and the results are reported in item 5 of the section. As responsible for the antioxidant activity, the content of total phenols, flavonoids and TAA in *A. africanum*, *N. commune* and HPV-A1 was determined. It was established that the alcoholic extracts obtained from the biomass of the studied microalgae have the most pronounced antioxidant activity, respectively maximum amounts of phenolic and flavonoid compounds, and these indicators are the highest in the species *A. africanum*.

The fourth task of the dissertation is the determination of the antimicrobial activity of microalgae extracts against human and plant pathogens. The results obtained are presented in item 6 of this chapter. Forty four strains were used as test microorganisms, of which 28 human pathogens and 16 phytopathogens. The determination was carried out according to the classic disk-diffusion method. The antimicrobial activity of 5 extracts of *A. africanum* was determined against 25 strains of human pathogens, incl. certified and type strains. The culture medium is characterized by the highest activity, as activity was reported against 92% of the tested strains. The alcohol extract has the lowest activity. Regarding the phytopathogenic test bacteria, only the culture medium is active - against 15 out of 16 tested strains. Extracts of *N. commune* and the HPV-A1 strain were similarly tested for antimicrobial activity. Here I would like to mention that there is a certain discrepancy between the data shown in the Table. 15 and 16 and the text on page 101 (first paragraph), where it is claimed that the analyzed extracts of *N. commune* inhibit a greater proportion of the human pathogens tested. This could only be said for the low-temperature extract, which inhibited 12 (about 63%) of the 19 strains tested. For the rest of the extracts, the effect is much lower than 50% of the strains. Among the pathogenic isolates, this was reported only for two 2 strains for HT and for one strains for the ethanol extract. All tested extracts were inactive against the tested phytopathogenic bacteria, except for HT extract against *C. michiganensis*.

Lyophilized culture medium of the microalgae strain HVP-A1 at different concentrations was analyzed for antimicrobial activity against 10 phytopathogenic bacteria. Logically, the highest activity against the largest number of strains (9 out of 10 tested) was found for the most concentrated culture medium (400 mg/ml). Regarding human pathogens, 5 extracts were analyzed (CM, ExPS, HT, LT, AE) which showed weak activity and only against Gram-positive bacteria. HT and LT extract also showed activity against one of the Gram-negative isolates. However, the majority of the tested strains did not show sensitivity to the tested extracts. With the widest range of action is CM and HT extract. Four extracts (CM, HT, LT and AE) were tested against collection strains of phytopathogenic bacteria as well as isolates. Only the most concentrated version of CM has activity. The study of the effect of copper sulfate on phytopathogenic bacteria should only be commented on in a comparative aspect, in relation to its use as a control. Related to the performance of this task is the determination of the minimum inhibitory and the minimum bactericidal concentration of the microalgae extracts. The MIC and MBC of culture medium of *A. africanum* and extracts of microalgal strain HPV-A1 were determined. The MIC and MBC of both strains were found to have significantly higher values than the antibiotic gentamicin used as controls for the human pathogens and copper sulfate for the phytopathogens. In connection with this task, I would like to point out that the PhD student has developed (in co-authorship) software for measuring inhibition zones from photographic material, which I consider a methodological contribution.

As a remark, I would like to point out that in the titles of Table. 11, 13, 15, 17, 18, 21, etc.) it is incorrectly noted that the strains used are type cultures. A reference to the catalogs of the collections from which they were obtained shows that all the cultures are certified strains, but a small number of them are also type cultures. The remark would be useful in future publication of the obtained results, where the correct presentation of the data is of utmost importance.

In item 6.6. this chapter presents the results of task 5, namely the study of the combined action of microalgae extracts from the culture medium of *A. africanum* and HPV-A1 against phytopathogenic bacteria. Both synergistic and antagonistic effects of the two extracts were not found, i.e. the effect of the combination of them does not exceed that of each one individually. The results of the research on task 6 are presented in item 6.1., which should be labeled as 6.7. This research seems to me a bit out of line with the goal of the dissertation, which is to develop biologically active products from new natural sources. In this regard, I would like to point out that the two antibiotics – monensin and salinomycin were developed a long time ago, one as early as 1967, and the other a little later (in 1983). The natural sources from which these antibiotics were isolated have long been exploited as producers of various biologically active substances, such as antibiotics. These antibiotics are known for their anticoccidial action and have long been used in poultry farming, cattle breeding, etc. They have been reported to be active against viruses, Gram-positive bacteria, and some microscopic fungi. Anticancer activity of salinomycin has recently been reported. They are also known to be toxic to mammals and plant cells. However, the obtained results complement the information about their antibacterial action, since activity against Gram-negative bacteria

was found for salinomycin. I would like to point out that in the text of the dissertation, the name of the antibiotic monensin is spelled incorrectly as "monenzyme".

The results of the research related to the last task of the dissertation work are presented in item 7 of this chapter. The antimicrobial activity of the obtained native hemocyanin and 5 fractions obtained from it against 8 collection-certified human pathogens (6 bacterial and 2 yeasts strains) was investigated. Native hemocyanin does not exhibit activity against the used test microorganisms, while the 5 fractions are active only against the test bacteria. The highest activity has fraction SU1, which has a high degree of glycolysation. The most sensitive species are *B. subtilis* and *E. coli*.

Conclusions. Nine well-formulated conclusions were drawn, although specific quantitative/qualitative characteristics could have been indicated in the assessment of one or another parameter. The conclusion under number 9 is too categorically formulated, although it was made solely on the basis of *in vitro* experiments.

Contributions. PhD student Milena Petrova has formulated 5 contributions to the dissertation work. The third contribution sounds more like a conclusion, which I mentioned above is a little categorically formulated. The definition of the fourth contribution is very general and does not reveal what is new that has been achieved.

5. Critical remarks and recommendations

The critical remarks and recommendations addressed to the dissertation work are reflected in the course of evaluation of the dissertation work (in items 2 and 4).

6. Questions

1. On page 74, it is emphasized that the isolate with presumed belonging to the genus *Nostoc* is a natural isolate, i.e. not completely purified to an algologically pure culture. Is it possible in such a case to be correctly determined to species?
2. Which polysaccharides could be responsible for the antimicrobial action of the exo- and endopolysaccharide extracts and what is the possible mechanism of their action?
3. How could the higher sensitivity of the collection strains to the tested extracts compared to that of the isolates be explained?
4. How could the higher antimicrobial activity of the LT extract of *N. commune* compared to the other extracts be explained?
5. What is meant by the "bacteriostatic zone" and "zone of inhibition" that you find when testing some extracts and antibiotics by the diffusion method in agar medium, and how can bacteriostatic action be determined?

6. Given the very high MIC and MBC values of the various extracts compared to those of the antibiotics and copper sulfate used as controls, is their potential use as antimicrobial products reliable?
7. In what form do you imagine the use of the various extracts to combat phytopathogenic bacteria?
8. The lack of antimicrobial effect of native hemocyanin is unexpected for you. How do you think it could be explained?
9. Many cyanobacteria are known to synthesize toxins. Considering their application potential, are studies of the cyanobacteria used in this direction foreseen?

7. Publications in connection with the dissertation work

Milena Petrova has two publications in refereed journals with an impact factor / rank, one of which she is the first author of. They were published in Biotechnology and Biotechnological Equipment (IF:1.34; SJR- 0.376; Q3) and Oxidation Communications (IF: 0.465 and SJR = 0.216; Q3), respectively, as well as one publication in the proceedings of a scientific conference in which she is first author.

PhD Milena completed her dissertation with the financial support of two doctoral projects at the Scientific Research Fund of the University of St. Cl. Ohridski" with her participation. In addition, she has participated in two national scientific programs and one scientific project at the Scientific Research Fund of the Ministry of Education and Culture, which are not directly related to the topic of the dissertation work.

8. Correspondence of the acquired competence with the requirements of the educational and scientific degree "doctor".

In the course of her doctoral study, Milena Petrova acquired new knowledge in the specific scientific field: development of biologically active products from natural sources - microalgae and invertebrates producing hemocyanin. She acquired theoretical knowledge about basic biological features of microalgae - taxonomy, biochemical composition, etc. This knowledge, combined with the knowledge of microbiology, has contributed to the development of this dissertation.

A complex approach was used to fulfill the tasks of the dissertation. Many and varied methods have been applied - taxonomic to identify part of the microalgae cultures, microbiological, analytical, biochemical, molecular genetic, statistical, chemical, etc. I believe that applying such a wide range of methods has undoubtedly increased her methodological competence, which is important for her future career development.

Although a part of the analyzes are collective, the personal participation of Milena Petrova stands out in the overall presentation of the dissertation, which shows that she has acquired skills to present, analyze, discuss and summarize scientific results.

CONCLUSION

Based on the above, my overall assessment of the dissertation work is positive - in terms of relevance of the problem, subject of the dissertation work, tasks for its development, methodological approaches, experimental setups, obtained results, their interpretation.

My critical remarks do not detract from the scholarly merits of this work, and I believe they would be useful in future research in this area. I firmly believe that the peer-reviewed work of doctoral student Milena Petrova is an author's work that meets all the criteria for a doctoral dissertation.

In this regard, I recommend the honorable Scientific Jury, appointed by Order No. RD 38-389 / 13.07.2022 of the Rector of SU "St. Kliment Ohridski", to award Milena Nikolova Petrova the educational and scientific degree "doctor".

09.09.2020 Reviewer:

Sofia (prof. PhD Penka Moncheva)