Review

From prof. Iskra Vitanova Ivanova,

of a thesis for the award of the educational and scientific degree "Doctor" (PhD)in the field of Higher education 4. Natural Sciences, Mathematics and Informatics, Professional field 4.3 Biological sciences, doctoral program (Microbiology)

Title: "Diversity and biotechnological potential of thermophilic microorganisms from Bulgarian hot springs"

Doctoral student: Nikoleta Boteva

Scientific adviser: prof. Margarita Kamburova

1. RELEVANCE AND SIGNIFICANCE OF THE PROBLEMS DEVELOPED

Less than 5% of the microbial species present in nature are "cultivable", and in extreme niches this share is even smaller. The term 'cultured micro-organisms' is relative and refers to the ability to reproduce in the laboratory the specific conditions that a niche provides for their development. The discovery of the thermophilic bacterium Thermus aquaticus in 1965 by Thomas Brock marked the beginning of a new direction in microbiology - thermophilia, which is associated with a number of fundamental and applied issues. Thermophilic bacteria are related to the group of extreme (grow optimally at 70 ° C, with a maximum of 80 ° C) or moderate thermophiles (50-60 ° C, maximum up to 70 ° C). Archaea dominate geothermal springs with temperatures above 90 ° C and belong to the hyperthermophilic microorganisms. Thermophilic microorganisms are subject of special attention because of their important place in both basic and applied science. The question of the origin of life, the molecular basis of thermophilicity and thermostability, the adaptive mechanisms of life are related with them. Of particular importance for the biotechnology industry are thermophilic enzymes (thermozymes), characterized by high temperature optimum and stability to temperature, organic solvents, detergents.

All this gives me the reason to evaluate the presented scientific study with potential for scientific achievements that have a rapid practical realization.

2. VOLUME AND STRUCTURE OF THE DISSERTATION

The dissertation is presented on 171 standard pages of text. The generally accepted scheme and the recommended ratios between the separate parts of the work are followed, as

follows: Introduction - 1 page, Literary review - 28 pages, Purpose and tasks - 1 page, Materials and methods - 13 pages, Results and discussion - 103 pages, Conclusion and contributions - 4 pages, Literature - 21 pages.

The obtained results are illustrated with 58 figures and 19 tables.

3. LITERATURE AWARENESS AND STATEMENT OF THE PURPOSE AND TASKS

The present dissertation is complex and presupposes a good knowledge of the literary sources and the methods for its solution. The doctoral student has made a thorough review of the achievements of other researchers, which she has managed to present and analyze on 29 pages in the literature review. The review presents in detail the state of the problem and proves the need to develop a dissertation thesis. A number of issues related to thermophilic microorganisms and to the biodiversity and metabolic strategies of thermophilic microorganisms are considered. Data on the molecular basis of thermophilicity and thermostability, as well as the metagenomic approach for taxonomic and functional profiling of microbial communities are presented. Also of interest are the presented metagenomic approaches for the discovery of new biocatalysts with applied potential of thermophilic microorganisms. Currently, the number of sequenced genomes of thermophilic microorganisms is 1003 and is directly related to the degree of annotation of metagenomic sequences from thermal biotopes.

The review presents information on thermostable xylanases, lipolytic enzymes, thermophilic lipases, pectin and pectinolytic enzymes. The genus Caldicellulosiruptor and its biotechnological potential are also considered. Some unresolved issues are also brought to the reader's attention. The literature review is specific, structured correctly, following the logical connection of the information. The data from the report served for the clear and correct definition not only of the goal, but also for the formulation of the tasks. Well-founded 14 experimental tasks are set to solve.

The literature (both in the review and in the whole paper) is closely related to the topic of the dissertation. The literature list includes an impressive, even for a large doctoral dissertation number of 404 titles in Latin. They are mainly from recent years. This speaks of the excellent theoretical awareness of the doctoral student and in order to find a new scientific challenge.

4. EVALUATION OF THE METHODS AND MATERIALS USED

The "Materials and Methods" section demonstrates an impressive set of methods tailored to the specific requirements of the experiments. They are modern and adequate for the realization of the dissertation. They are described precisely and in detail, fully covering the multifaceted areas of work: from classical to modern microbiological studies, including isolation and initial characterization of pure microbial cultures, isolation of total DNA strains, metagenomic analysis including: isolation of metagenomic DNA from sediment samples and anaerobic crops.

In the different stages of the work the doctoral student skillfully combines the basic microbiological approaches with modern molecular genetic methods and biochemical methods. All this allows me to give high marks to the scientific level and the excellent preparation of the doctoral student, who manages to properly combine a variety of classical with modern methods for the purposes of the dissertation, successfully solving the experimental tasks.

5. EVALUATION OF THE RESULTS OBTAINED

The aim of the present is to study the phylogenetic diversity and biotechnological potential of thermophilic microorganisms in Bulgarian hot springs. For the purpose of the present work, sediment and water samples from the Rupi basin, Levunovo, Vlasa and Mizinka springs were studied. The "Results and Discussion" section is well structured, supported by tabular and graphical material, with an appropriate interpretation of the results obtained by foreign research teams. The author consistently presents evidence on her scientific thesis, thus logically finalizing experimental work. A large volume and variety of experimental work was performed within the framework of complex microbiological and molecular genetic research.

The difficulty of creating cultivation conditions covering the various energy strategies of thermophiles defines metagenomic analysis as an extremely valuable way to study metabolism in thermophilic microbial communities. Genetic information derived from sequences or partially reconstructed genomes from different biotopes not only determines the taxonomic structure and functioning of the thermophilic community, but also provides a huge amount of coding sequences that can be cloned and functionally analyzed.

Cultivation techniques and conditions conducive to the development of relevant microbial producers are often used in the search for certain enzymes, combined with the sequencing of the culture's metagenomic DNA or the construction of expression libraries and functional screening. Techniques for enriching a microbial consortium characterized by lower

biodiversity and purposefully degrading a particular substrate is an opportunity to concentrate and recover genes encoding desired functions.

The obtained results show that moderately thermophilic, thermophilic and hyperthermophilic bacteria dominate in the studied microbial communities. The presence of thermophilic archaea has been established, as their share varies in different thermal springs refers to non-cultivated branches, indicating the degree of existence of new taxa in Bulgarian sources. Metabolic reconstruction of metagenomas from the studied sources reveals the presence of genes for enzymes from the main metabolic pathways and specific for these extreme niches such as those associated with methanogenesis, acetogenesis, hydrogenogenesis, sulfate reduction, sulfur or iron reduction. Metagenomic analysis of the xylan-enriched thermophilic consortium in the two cultures studied showed a strong predominance of extremely thermophilic anaerobes. Several draft genomes have been reconstructed from each source, referring mainly to extreme and hyperthermophilic microorganisms or unknown ones. From the reconstructed draft genome of Ca. Calescibacterium sp. is a successfully expressed gene for thermostable lipase, characterized by a high temperature optimum of 80 ° C and thermal stability up to 30 minutes at 99 ° C and 3 days at 70 ° C, as well as resistance to organic solvents. An extremely thermophilic obligate anaerobic strain described as Caldicellulosiruptor sp. 11.4 with 96% identity of the 16S rRNA gene with the closest phylogenetic neighbor C. acetigenus and a difference in a number of phenotypic properties. The author found that the cultured isolates of thermophilic anaerobes show a more diverse synthetic potential than aerobes. Partially purified polygalacturonase from the cultured anaerobic thermophile Caldicellulosiruptor sp. 11.4 has an unusually high thermal stability (80 ° C optimum temperature retaining 50% activity in the 70 ° - 88 ° C range). All results are presented and analyzed in the separate chapters of the section "Results and discussion", which logically follow the course of solving the tasks. They are summarized and discussed in the light of the published data from recent years. Both the idea and the volume of research conducted on the implementation of this task and throughout the work deserve high praise. The discussion of each experiment, the comparison of the results for the individual strains and experiments, and the comparison with the literature data, once again emphasizes the qualities of the doctoral student and the mastery of the experimental theory. With this she proves that she has completely mastered the third level of her education and is a complete experimenter. I accept the contributions made.

6. CONTRIBUTIONS AND SIGNIFICANCE OF THE DEVELOPMENT TO SCIENCE AND PRACTICE, REMARKS AND QUESTIONS

Nikoleta Boteva is a leading author in all scientific publications, as a leading researcher, which shows her creative and research activity in their development and design. The results of the dissertation are published in two journal CR ACAD BULG SCI. and in a chapter of a book. Also two participation with reports in national scientific forums and three posters in national and international forums are included in the presenting materials.

Articles:

- Boteva, N., Kambourova M. (2018) Thermophiles and their exploration for thermostable enzyme production. In: Extremophiles in Eurasian Ecosystems: Ecology, Diversity, and Applications (Egamberdieva, D., Birkeland, N.-K., Panosyan H., Li W.-J., eds). Springer Ltd. Pp. 167-186.
- Boteva, N., Birkeland, N.-K., Kambourova M. Complete nitrogen cycle driven by the thermophilic microbial community of Rupi II hot spring. CR ACAD BULG SCI, V.74 No1 pp.59-69.
- Boteva, N., Birkeland, N.-K., Kambourova M. Metagenome-assembled Genomes Related to Ammoniaoxidizing Thaumarchaeota Recovered from Near-anoxic Environment. CR ACAD BULG SCI, V.74 No3 pp.363-372.

Reports:

- Boteva, N. Diversity and Biotechnology of Extremophiles. InternationalConference 26-28 September, 2016, Sofia: METAGENOMIC ANALYSIS OF THERMOPHILIC DNAs ISOLATED FROM BULGARIAN HOT SPRINGS
- Boteva N., Kambourova M., Birkeland N-K. Diversity and biotechnological Potential of microbial communities in Bulgarian hot springs assessed by metagenomic analysis. First National Conference for PhD students 2016, held on November 1, 2016 in Plovdiv.

Posters:

- Boteva N., Kambourova M., Birkeland N-K. Biodiversity of extreme and hyperthermophilic microorganisms from Bulgarian hot springs. 10th Balkan Congress of Microbiology MICROBIOLOGY BALKANICA 2017;
- Stoilova-Disheva M., Lyutskanova D.,Boyadzhieva I., Radchenkova N., Boteva N.,Birkeland N.-K., Kambourova M. "The archaeal community in the Vlasa hot spring, in Velingrad, Bulgaria is extremely diverse and novel". 11th International Congress of extremophiles, 12-16.09.2016, Kyoto, Japan;

- Stoilova-Disheva M., Lyutskanova D.,Boyadzhieva I., Radchenkova N., Boteva N.,Birkeland N.-K., Kambourova M.Phylogenetic analysis revealed novel and unexpected structure of archaeal community in Vlasa hot spring, Velingrad, Bulgaria.Molecular Biology of Archaea 5. Focused Meeting of Microbiology Society, 1-3 August 2016, London, UK.

I do not have remarks and suggestions , because they were correctly changed in the official variant.

CONCLUSION

In conclusion, I want to emphasize that the material is dissertable. The topic is relevant, the doctoral student has mastered modern methods, the experiments are set methodically correct, the results are reliable and are a solid basis for further scientific and applied developments. Extremely original scientific and applied contributions stand out. Based on the above, I can confidently say that the peer-reviewed dissertation is an original scientific development, with theoretical and applied significance. On the basis of the collected credits the successfully envisaged educational scientific program has been implemented. The proposed dissertation is a proof that Nikoleta Boteva has developed competencies necessary for awarding the degree of doctor, including theoretical training, methodological knowledge, independence and experience in planning experiments and the ability to analyze the results.

The evaluated PhD thesis contains scientific and applied results, which represent an original contribution to science and meet the requirements of Academic Staff Development Act in the Republic of Bulgaria and Regulations of the “S. Angeloff” Institute of Microbiology BAS on the Implementation of the Academic Staff Development The PhD thesis shows that Nikoleta Ivanova Boteva has theoretical knowledge and professional skills in the scientific specialty Microbiology. Due to the above, I give my positive assessment of the research presented in the PhD thesis, summary, results and contributions, and I propose to the esteemed jury to award the educational and scientific degree “Doctor” (PhD) to Nikoleta Ivanova Boteva 4. Natural Sciences, Mathematics and Informatics, Professional field 4.3Biological sciences, doctoral program (Microbiology).

14.06.2021 г. Signature: