REVIEW
by **Prof. Dr. Genoveva Atanasova Nacheva**
of the dissertation of **Ivan Dimitrov Ivanov**
on the topic "**New Producers of Prebiotic Carbohydrates from Bulgarian Yogurt**",
submitted for the award of the educational and scientific degree "**Doctor**" in Professional Field 4.3. "Biological Sciences," scientific specialty "Microbiology"

1. General Information

By order of the Director of the "Stefan Angelov" Institute of Microbiology at the Bulgarian Academy of Sciences No. I-141/01.10.2025, I have been appointed as a member of the academic jury for the defence procedure of the dissertation of **Ivan Dimitrov Ivanov**. The set of materials submitted by Ivan Ivanov includes the following documents:

- Application to the Director of the Institute of Microbiology, BAS, for initiation of the defence procedure
- Curriculum Vitae
- Copy of diploma for completed higher education (Master's degree)
- Order for enrolment in doctoral studies
- Certificates of passed exams/doctoral minimums in the specialty
- Transcript of earned credits during the training
- Report on fulfilment of the minimum national requirements according to the Law on the Development of the Academic Staff in the Republic of Bulgaria (LDASRB)
- Protocol from the seminar for the preliminary defence
- Order for change of the dissertation title
- Order for removal from doctoral studies with the right to defend
- Preliminary review from the preliminary defence
- Dissertation
- Abstract
- List of publications and copies thereof
- List of conference participations related to the dissertation
- List of citations
- Declaration of originality of the results

2. Brief Biographical Information about the Doctoral Candidate

Ivan Ivanov is a graduate of Trakia University in Stara Zagora. In 2004, he obtained a Master's degree in Veterinary Medicine with the qualification of Veterinary Physician. From 2005 to 2012, he worked as an assistant at the Institute of Animal Science in Kostinbrod. Since 2020, he has been an assistant at the Genetics Laboratory, Department of General Microbiology at the "Stefan Angelov" Institute of Microbiology, Bulgarian Academy of Sciences. At the beginning of 2021, he was enrolled as a full-time doctoral student in the same department under the supervision of Prof. Dr. Penka Petrova from the Institute of Microbiology, BAS, and Prof. Dr. Kaloyan Petrov from the Institute of Institute of Chemical Engineering, BAS. He was discharged with the right to defend his dissertation at the end of 2023.

3. Relevance of the Topic

Galactooligosaccharides (GOS) are key prebiotics that support gut and immune health by stimulating beneficial bacteria. They are an important component of functional nutrition and are used both in natural foods and as dietary supplements. Natural sources of GOS include milk

and dairy products, legumes, certain vegetables such as onions, garlic, leeks, broccoli, and cereals. Market demand for GOS is expected to grow steadily in the coming years, driven by the trend toward preventive nutrition and increased attention to the microbiome. The prospects for discovering new sources of GOS in Bulgarian yogurt are promising, as the rich diversity of lactic acid bacteria in traditional starter cultures can be harnessed for targeted production of prebiotic compounds.

In this context, the research conducted by Assistant Ivanov is timely and has both theoretical and practical significance. Recent metagenomic studies show that more than 100 bacterial species are present in various commercial brands of yogurt, including some that are not yet fully characterized. This opens up opportunities for identifying new strains capable of synthesizing GOS, with even greater diversity expected in homemade yogurts. Biotechnological enhancement of starter cultures through strain selection or genetic improvement is another important aspect of GOS production. This could lead to the development of innovative functional dairy products that combine tradition with modern biotechnology and strengthen Bulgaria's position as a leader in prebiotic foods.

3. Characteristics and Evaluation of the Dissertation

The dissertation consists of 120 pages, illustrated with 27 figures and 13 tables, and includes 218 references in Latin script, approximately 40% of which are from the last 10 years.

The *Literature review* spans 44 pages and comprises four main sections and conclusions. The first two sections are dedicated to lactic acid bacteria, lactic acid fermentation, and traditional Bulgarian fermented dairy products. The characteristics and metabolism of the two strains typical for Bulgarian fermented products—*Lactobacillus delbrueckii* ssp. *bulgaricus* and *Streptococcus thermophilus*—are examined. A detailed overview of traditional Bulgarian products is provided, describing the types, production technology, distinctive qualities and composition, and health-beneficial metabolites such as amino acids, peptides, antioxidants, and exopolysaccharides. The information is well-structured and clearly written, making it an engaging read. I would recommend that the author publish this section as a separate review article in Bulgarian, which would help raise public awareness about the composition, technology, and qualities of Bulgarian fermented dairy products.

The other two sections are dedicated to oligosaccharides, and more specifically to galactooligosaccharides (GOS), focusing on their structure, biosynthesis, properties as prebiotics, and biotechnological production. The detailed literature review and analysis of the cited sources have enabled the doctoral candidate to identify his research niche and to clearly and precisely formulate the *aims and objectives* of his dissertation. The aim of the dissertation is *the molecular characterization of the lacZ gene and β-galactosidase from a Bulgarian strain of Lactobacillus delbrueckii ssp. bulgaricus, with the goal of optimizing the in vitro synthesis of prebiotic galactooligosaccharides (GOS)*. In light of the presented literature data, I find the stated aim to be appropriate. The six defined objectives, which pertain to the methodological investigations necessary to achieve the aim, are also well-aligned.

The "*Materials and Methods*" section spans 12 pages of the dissertation and provides a comprehensive description of the materials and methods used, allowing for the reproduction of the experiments without the need for additional methodological literature. It includes all microbiological, biochemical, and molecular biology techniques such as liquid chromatography with mass spectrometry (LC/MS), PCR, sequencing preparation and bioinformatic analysis,

cloning, enzyme purification, and others. This section demonstrates that the doctoral candidate employs a wide range of biochemical, analytical, molecular biology, and bioinformatic methods, as well as advanced and sophisticated scientific equipment—one of the core objectives of doctoral training in the respective professional field.

The results of the conducted research and their discussion are presented in the "*Results and Discussion*" section, which covers 31 pages. It is illustrated with 17 figures and 4 tables.

This section can be thematically divided into two subsections. In the first, Ivanov presents his results from the analysis of microbial communities isolated from four geographic regions of Bulgaria with endemic microflora, obtained through metagenomic analysis. The raw sequencing data have been deposited in GenBank at NCBI. Here, the doctoral candidate presents a substantial volume of results concerning the species diversity of bacteria in the selected samples. It is shown that yogurt from the Rhodope region is the richest source of *L. bulgaricus*, which is characteristic of Bulgarian yogurt. In addition to the main strains *L. bulgaricus* and *S. thermophilus*, newly identified accompanying strains—previously unknown in traditional starter cultures—have been analysed, indicating the high biodiversity of homemade yogurts. The identified microbial communities contribute to improved organoleptic properties and production of metabolites with positive effects on human health. The results of these studies have been published in international journals ranked in Quartile 2 and have received a high number of citations, which attests to their quality.

The second subsection is dedicated to the results obtained from sequencing the *lacZ* gene of *L. bulgaricus* strain 43, which demonstrated unusually high transgalactosidase activity, its heterologous expression, and the molecular characterization of the β-galactosidase enzyme. The nucleotide sequence of the gene has been deposited in GenBank at NCBI. The structure of the protein and the amino acids involved in the formation of the active site and substrate binding have been analysed. The gene was cloned into *E. coli* strain BL21(DE3) under the control of a T7 inducible promoter. Conditions for optimal expression were established, and the enzyme was partially purified using affinity chromatography. The optimal conditions for enzymatic activity—such as temperature, pH, and ionic composition—were investigated. Mass spectrometry analysis revealed that the GOS produced by the β-galactosidase from *L. bulgaricus* 43 consist of three monomers linked by β-(1→4) bonds, which is atypical for *L. bulgaricus*. Successful heterologous expression of the enzyme was achieved, showing very high activity—over 3000 U/mL, approximately 2010 U/mg—and in vitro synthesis of 70.9 g/L of trisaccharides. These studies are undoubtedly of contributory significance, as the cloned and characterized β-Gal from *L. bulgaricus* 43 is a promising producer of GOS in industrial quantities. The results have been published in *International Journal of Molecular Sciences*, a Quartile 1 journal, which is indicative of their quality.

In connection with these studies, I have the following question: Does the doctoral candidate intend, in his future work, to develop a methodology for the purification of β-Gal from *L. bulgaricus* 43, and if so, what does he consider the appropriate strategy?

The *conclusions* logically follow from the results of the conducted research. They are formulated concisely and clearly, with the undoubtedly most significant contributions of the doctoral candidate being: i) the new data on the accompanying microflora, as well as the presence of a rare lactic acid bacterium (*L. equicursoris*), identified for the first time in Bulgarian yogurts; and ii) the heterologous expression and characterization of β-Gal from *L. bulgaricus* 43, which demonstrates strong potential for industrial application.

It is worth noting that the dissertation is written with expertise in precise and clear scientific language, and no typographical errors are present.

4. Publications Related to the Dissertation

As previously noted, **three publications** related to the dissertation have been published in **Quartile 1 and 2 journals, and they have been cited multiple times.** The doctoral candidate is the first author of one of them. The publications fully reflect the results of the dissertation. The findings have also been presented through posters and oral presentations at six scientific forums both in Bulgaria and abroad. With this scholarly output, Assistant Ivanov fully meets—and exceeds—the requirements of the Law on the Development of the Academic Staff in the Republic of Bulgaria (LDASRB) and the internal regulations of the Bulgarian Academy of Sciences for awarding the educational and scientific degree "Doctor."

5. Dissertation Abstract

I have reviewed the Dissertation Abstract and find that it adequately reflects the content and achievements of the dissertation.

Conclusion

In his dissertation, Assistant Ivan Ivanov sets ambitious goals, which he successfully achieves. During its development, the doctoral candidate acquired substantial theoretical and practical knowledge and skills in various fields—microbiology, biochemistry, molecular biology, and bioinformatics—and obtained results that open new avenues for future research. The experiments were conducted using modern methodology and advanced experimental techniques, leading to results published in three scientific articles. Through his scientific achievements, Ivanov presents himself as a mature researcher who is capable of formulating scientific problems, successfully addressing them, and accurately interpreting the results obtained. The dissertation fully meets the requirements of the Law on the Development of the Academic Staff in the Republic of Bulgaria (LDASRB), its implementing regulations, and the internal regulations of the Bulgarian Academy of Sciences for awarding the educational and scientific degree "**Doctor**." Based on all of the above, I confidently recommend to the esteemed Academic Jury ***that Ivan Ivanov be awarded the educational and scientific degree "Doctor" in Professional Field 4.3. "Biological Sciences," scientific specialty "Microbiology."***

Sofia, 10.11.2025 Referee:

/Prof. G. Nacheva/