STATEMENT

by **Assoc. Prof. Galina Dinkova Stoyancheva, PhD**
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regarding dissertation work for the acquisition of the educational and scientific degree
"Doctor"
professional field: **4.3. Biological Sciences,**
scientific specialty: **Microbiology**

Author of the dissertation: **Ivan Dimitrov Ivanov**, doctoral student at the Stefan Angeloff Institute of Microbiology - Bulgarian Academy of Sciences
Topic of the dissertation: **NEW PRODUCERS OF PREBIOTIC CARBOHYDRATES FROM BULGARIAN YOGURT**
Scientific supervisors: **Prof. Penka Petrova**, **PhD, DSc** Institute of Microbiology Stefan Angeloff- Bulgarian Academy of Sciences and **Prof. Kaloyan Petrov, PhD, DSc** Institute of Chemical Engineering - Bulgarian Academy of Sciences

ABOUT THE PROCEDURE:

Doctoral student Ivan Dimitrov Ivanov is directed to an official defense by Decision of the Scientific Council of the Institute of Microbiology and Immunology-BAS (Minutes No. 10/30.09.2025). According to the Order of the Director of the Institute of Microbiology and Immunology-BAS (No. I-141/01.10.2025), I am included in the Scientific Jury for the defense as an internal member, in accordance with The Act on the development of academic staff in the Republic of Bulgaria and art. 29, para. 3 of the Regulations on the conditions and procedure for acquiring scientific degrees in The Institute of Microbiology – BAS.

EVALUATION OF THE DISSERTATION:

Relevance and significance of the scientific topic. The topic of the dissertation is focused on the molecular characterization of β- galactosidase from a Bulgarian strain *of Lactobacillus delbrueckii* ssp. *bulgaricus* and its role in the synthesis of prebiotics galactooligosaccharides (GOS). The study is in line with modern trends in nutrition science and biotechnology, aimed at the development of functional foods with proven health effects. In the context of the growing interest in the microbiome and its impact on human health, the topic acquires particular relevance. The author focuses on a traditional Bulgarian product – yogurt – as a source of bioactive compounds with potential for industrial application. This connects a specific national product with global scientific priorities, such as precision healthcare and biotechnological optimization of fermentation processes. The practical significance of the work is expressed in the characterization of a new enzyme β- galactosidase with potential for implementation in the food industry.

The candidate's literary awareness and theoretical preparation. The literature review is well structured, addressing the following aspects: current knowledge on lactic acid fermentation, metabolism of *L. bulgaricus*, composition and health effects of traditional fermented products, as well as properties and biosynthesis of galactooligosaccharides. The review also includes mechanisms of probiotic and prebiotic action, industrial approaches for the production of functional ingredients, and the potential of β-galactosidases as enzymes for the production of oligosaccharides.

The overview is specific and clear, illustrated with 6 tables and 6 figures, and together with the methods and discussion of the results, is based on 218 literature sources, approximately 25% of which were published in the last 5 years.

The main purpose of the dissertation work: Molecular characterization of the *lacZ gene* and β- galactosidase from a Bulgarian strain *of Lactobacillus delbrueckii* ssp. *bulgaricus*, with a view to optimizing the in vitro synthesis of prebiotics galactooligosaccharides.

To achieve the goal, the author formulated six clearly defined tasks: collection of samples from homemade yogurts; metagenomic analysis of the microbiota; identification and comparison of lactic acid bacteria; sequencing of the *lacZ gene* from strain *L. bulgaricus* 43; heterologous expression and biochemical characterization of the enzyme β- galactosidase; assessment of the potential of the enzyme for industrial production of GOS. The tasks are logically arranged and interconnected.

Analysis of the methodological approaches to achieve the goal of the dissertation. The applied methods are well selected and suitable for solving the experimental tasks set. The dissertation is distinguished by the integration of microbiological, analytical and molecular biological techniques. Metagenomic methods were used sequencing to determine bacterial diversity, PCR and sequencing of the *lacZ gene*, heterologous expression in *E. coli* BL21(DE3), purification by liquid chromatography and functional analysis of enzyme activity. The approach provides both fundamental and applied knowledge. The author has demonstrated skills in the application of modern biotechnological methods, including the study of the influence of physicochemical factors and metal ions on enzyme activity.

Evaluation of the achieved results and contributions of the dissertation work. All tasks set have been consistently and successfully solved. Among the most significant achievements of the dissertation work is the preparation of a first-of-its-kind metagenomic profile of domestic Bulgarian yogurts from different geographical regions, which reveals not only the dominant species *L. bulgaricus* and *S. thermophilus*, but also the presence of rare and new strains, including *L. equicursoris* – established for the first time in yogurt. A strain of *L. bulgaricus* 43 with extremely high transgalactosidase activity has been identified. The *lacZ gene* has been sequenced and analyzed, and 21 unique amino acid substitutions associated with the tetrameric structure and high enzymatic activity have been identified.

Successful heterologous expression in *E. coli* BL21 (DE3) resulted in the production of recombinant β- galactosidase with an activity of over 3000 U/ ml – significantly exceeding the values published so far. The enzyme exhibits specific sensitivity to Mg^{2+}, Mn^{2+} and Ca^{2+} and demonstrates stability under optimal pH and temperature conditions. It was found that in the transgalactosylase reaction the enzyme synthesizes up to 70.9 g/L of GOS (mainly DP3), which confirms its high industrial potential.

Particularly significant is the result that the enzyme catalyzes the formation of β-(1→4) bonds between monomers in GOS, which enhances the prebiotic effect and makes it promising for the development of innovative functional foods.

The results are illustrated with 4 tables and 19 figures. The most significant results have been included in three publications with an impact factor and have been reported at three scientific forums. **The abstract** fully corresponds to the contents of the dissertation.

The conclusions are correctly formulated, and the contributions reflect the most significant results of the dissertation, namely:

1. For the first time, a metagenomic profile of domestic Bulgarian yogurts from different geographical regions has been presented, revealing both dominant and rare and novel species Lactic acid bacteria.
2. The presence of *L. equicursoris* in yogurt has been documented – a novelty in the scientific literature.
3. A strain of *L. bulgaricus* 43 with unusually high transgalactosidase activity has been identified.
4. The *lacZ* gene of the strain has been sequenced and characterized, showing 21 unique amino acid substitutions associated with a tetrameric structure and high enzymatic activity.
5. The successful heterologous expression of the recombinant β-galactosidase has led to significant industrial potential of the enzyme for the production of GOS with prebiotic properties.

Questions and recommendations. The dissertation is well structured and methodologically sound. However, some recommendations can be made for future research. The results section lacks a clear tabular comparison between β- galactosidase from *L. bulgaricus* strain 43 and the recombinant β- galactosidase obtained from *E. coli* BL21. Technical notes: the chemical element magnesium is written with the wrong symbol. Nevertheless, the presentation is scientifically sound, well-reasoned, and demonstrates in-depth knowledge in the field of microbial genetics.

Question to the doctoral student: Why do certain metal ions decrease enzyme activity?

CONCLUSION

The dissertation represents a comprehensive, original and in-depth scientific study with theoretical and practical value. The results achieved are convincing, well-argued and supported by experimental data. The work meets the requirements for a dissertation for the award of the educational and scientific degree "Doctor". This gives me reason to propose to the esteemed scientific jury to award **Ivan Dimitrov Ivanov the educational and scientific degree "DOCTOR"** in Professional field: 4.3. **Biological Sciences**, scientific specialty: **Microbiology**.

Date: 19.11.2025

(Assoc. Prof. Galina Dinkova Stoyancheva, *PhD*)