

To the Chairman of the Scientific Jury
Assoc. Prof. Dr Lyudmila Kabaivanova
IMicB - BAS

R E V I E W

Dissertation thesis: "Molecular-biological studies of new bacterial glycoside-hydrolases with industrial application" for the award of the scientific degree "Doctor of Sciences" by Assoc. Prof. Dr Penka Mladenova Petrova, proposed in the professional field 4.3. Biological Sciences, speciality "Microbiology".

Reviewer: Prof. Todor Kantardjiev, MD, DSc - National Center of Infectious and Parasitic Diseases, Bulgaria

I have no co-authored
publications or conflicts of interest
of another nature within the meaning of
the ZRASRB with the applicant

Applicant's career development

The candidate for the Doctor of Science degree, Mrs Penka Mladenova Petrova, graduated from the Faculty of Biology at Sofia University "St. Kl. Ohridski" in 1994, majoring in "Biotechnological Processes", profile "Genetic and Cellular Engineering". From 1995 to 2003 he initially worked as a specialist and subsequently as an assistant at the Institutes of Molecular Biology and Microbiology at the Bulgarian Academy of Sciences. In 2004 he received a Doctor's degree from the National Academy of Sciences and since 2011 he is an Assistant Professor in the Microbial Genetics Section of the Institute of Microbiology of the Bulgarian Academy of Sciences (IMICB). Since 2013 he has been managing the Laboratory of Metagenomics and Gene Expression and since 2018 - the Department of General Microbiology at IMICB.

The relevance of the scientific problems developed in the dissertation

Many problems of the modern man related to improving the quality of life, health and longevity can be solved by developing modern branches of microbiology. The growing demands of the European consumer for consumed products are invariably leading to the production of functional foods combining value and usefulness, with research on the relationship between macro- and micro-organisms and attempts to study the human microbiome becoming increasingly relevant. Modern synbiotic preparations (synbiotics), necessarily contain probiotic lactic acid bacteria in combination with their substrates inulin and fructooligosaccharides. The study of new bacterial glycoside-hydrolase enzymes that underlie the interactions between probiotics and prebiotics is part of the current trends in the reviewed dissertation.

Another challenge in the early decades of the 21st century was the depletion of energy fossil resources and the need to seek alternative ways of synthesizing organic compounds through modern biotechnology-based on cheap and renewable natural substrates. This necessitates a further in-depth study of the genetic basis and mechanisms of action of the hydrolases enzymes responsible for the conversion of biomass into valuable organic products. As genetic diversity determines both the degree of adaptability of species and the possible applications of microorganisms in industry and medicine, it can be noted that the proposed dissertation deals with topical topics with rather unexplained and interesting aspects so far.

General data on the structure and volume of the dissertation

The dissertation is structured according to the recommendations of the NEMJ and contains a brief introduction (2 pages), a literature review focusing on the motives of the studies (107 pages), purpose and objectives are presented accurately, materials and methods are described exhaustively (28 pages), results and discussion are clearly presented, as a prerequisite for the conclusions (157 pages), the conclusions are categorical on the basis of scientific evidence, the contributions are well structured, a list of the used sources of literature and a list of publications of the author on the topic of the dissertation are presented. The total volume of the dissertation is 358 pages, and the supporting material is presented in 52 overview and explanatory text tables and 140 figures illustrating the discussed. Literary sources cited are 625 sources, of which 3 are in Bulgarian letters, 610 publications are in Latin and 11 represent databases on the Internet. More than 80% of the cited literature was

published after 2000, which speaks to the relevance of the issue and the literary awareness of the author. Even some of the articles cited are from 2019.

Assessment of the literature review, purpose and objectives

The literature review is structured for clarity in four main chapters. They are dedicated to carbohydrates, as well as substrate carbohydrate enzymes, bacterial producers of glycoside hydrolases, and applications of hydrolase enzymes in industry and medicine. The first chapter of the review looks at the structure and functions of glycans and their importance in wildlife and as prebiotics. Structural formulas of the less common polysaccharides and sialoglycans in the scientific literature are presented. Following is a detailed classification of glycoside-hydrolases and glycoside-transferase enzymes, and the inclusion of state-of-the-art databases, e.g. CAZy, which classifies enzymes by their amino acid sequences and enables evolutionary studies of enzyme families and is one of the latest advances in enzymology.

Logically, in connection with the research in the dissertation, the greatest attention and volume in the literature review is devoted to bacterial glycosidases with substrate starch: amylase, pullulanase, amylopullulanase, cyclodextrin-glucoamylase, glycosidase and substrate fructose. For all enzymes, their protein structure and their coding genes are examined, which makes the presentation very valuable and informative. Many of the enzymes are shown as good quality 3D images. Comparisons were made between the enzymes produced by different lactic acid bacteria and bifidobacteria and their probiotic potential, as well as contemporary probiotic preparations from different manufacturers and the microbial composition of the respective products in the tables.

The following section of the literature review describes the *Firmicutes* type to which all the species and results described in the Results and Discussion section belong, as well as the molecular approaches in bacterial identification and typing.

The *Bacillaceae* family (as producers of glycosidases) and lactic acid bacteria (LAB) are described in more detail - biochemical fermentation pathways, comparison of bacterial genomes produced by hydrolase enzymes.

The last chapter of the literature review deals with the application of LAB in the lactic acid fermentation of cereal-based foods and beverages.

In general, the literature review has content adequate for follow-up and discussion, and the ratio between these sections is 1: 1.5. In some places, the review is quite detailed, but

this could be justified by the need for more detailed explanations of the enzyme structures or genetic determinants.

The findings of the review outline the logic behind the goal of the dissertation: to characterize new glycoside-hydrolases and to create recombinant enzymes with improved properties and applications in industry and medicine. The tasks in the course of which the goal is to be achieved are four, with six sub-tasks. They are clearly formulated and allow for verification of their achievement.

Materials and methods

The methods used in the dissertation are divided into 1. Microbiological, 2. Biochemical, 3. Analytical and 4. Molecular-biological. They are used as classical methods for isolation of bacterial strains and biochemical tests (for the absorption of sugars, measurement of the amount of reducing sugars and protein, quantification of enzyme activities: amylase, β -fructosidase, CGT-azine, β -glucuronidase, etc., determination of pH and temperature optima of enzymes, the influence of various metal ions and EDTA on enzyme activity, determination of substrate specificity of enzymes, etc.), as well as state-of-the-art omics approaches (genomics and transcriptomics). The quantitative analysis of the galactooligosaccharides obtained was performed by liquid chromatography combined with mass spectrometry (LC-MS).

Some of the results related to gene sequencing and genomes, as well as phylogenetic analyzes, show good knowledge of bioinformatics.

Results and discussion

The results are combined with discussion due to the large volume of material presented, and this variant of the exhibition allows timely comparison of the results obtained with the world experience in the field. This chapter contains four separate sections:

- (1) Molecular-biological studies of glycoside-hydrolase enzymes in lactic acid bacteria,
- (2) Glycoside hydrolase enzymes in the genus *Bacillus*,
- (3) Molecular-biological studies of glycoside-hydrolase enzymes with sialic acid substrate

(4) Molecular studies of β -glucuronidase.

The first section describes the isolation and molecular genetic identification of a large group (115 strains) of lactic acid bacteria. For the identification of *L. delbrueckii* subsp. *bulgaricus* several molecular methods (ARDRA, pulse electrophoresis, multilocus sequence analysis) have been applied, which are applied for the first time in such a combination and contribute to the work of the field. A genetic method for distinguishing *L. casei* and *L. paracasei* species has been applied, and molecular-genetic differentiation of *Lc. lactis* subspecies has been successful.

One of the most significant scientific results in the dissertation is related to the study of the enzymes involved in the absorption of starch in amylolytic LABs (ALAB). Transcriptional analysis of the expression of genes encoding α -amylase, glycogen phosphorylase, (1 \rightarrow 4) - α -glucan branching enzyme, α -glucosidase, oligo-1,6-glucosidase and neopululanase was performed and conserved regions in the genes encoding starch-modifying enzymes. The presence and expression of genes have been demonstrated in several phylogenetically distant LAB groups - representatives of the genus *Lactobacillus*, *Pediococcus*, *Enterococcus* and *Streptococcus*. Gene expression study of *Lactococcus lactis* strain B84 was separately performed. As the strain isolated in Bulgaria is the first amylolytic representative of the species, evidence for the expression of cell-related and cytoplasmic amylase encoding genes contributes to world science.

Another type of glycoside-hydrolase enzymes of great importance for the administration of LABs as probiotics are enzymes with substrate inulin and fructo-oligosaccharides. The results of cloning and sequencing of genes encoding fructan- β -fructosidase in *L. paracasei*, purification of wall-localized β -fructosidase enzymes from the same strains are shown in the dissertation, and control of gene expression is monitored. In this connection, the transport systems related to the uptake of fructooligosaccharides by *Pediococcus acidilactici* are also considered.

The molecular study of enzymes with β -galactosidase activity was performed in *L. bulgaricus*. For the first time, synthesis of prebiotic galactooligosaccharides in yoghurt and in lactose medium has been demonstrated. The dissertation is followed by a section examining the probiotic and technological characteristics of LAB, demonstrating that they have high proteolytic activity and, therefore, the ability to synthesize peptides with potentially beneficial effects.

The second chapter of the dissertation covers the study of glycoside-hydrolase enzymes in the genus *Bacillus*. Novel and recombinant cyclodextrin-glycanotransferase

enzymes have been studied and bacillus producers of cellulose and hemicellulose degrading enzymes have been isolated. The genome of *Bacillus velezensis* 5RB strain is completely sequenced, and it is the first de novo sequenced bacterial genome by Bulgarian scientists.

Applications in medicine would find the results presented in Chapter Three of the dissertation dedicated to molecular biological studies of the gene encoding neuraminidase in *Vibrio cholerae*. Characterization of the enzyme in a non-toxigenic strain of the species enables its safe industrial production for diagnostic purposes.

The last (fourth) chapter of the results concerns studies of β -glucuronidase and its successful expression in *O. polymorpha* yeast.

The main scientific achievements of the dissertation are the following scientific contributions:

1. Genes responsible for α -glucans hydrolysis of *Lactococcus lactis* and *Lactobacillus paracasei* have been identified for the first time in the world of science. The world's first amyolytic representatives of the species *Lactobacillus sakei* and the genus *Enterococcus* were isolated. A method for rapid transcriptional analysis of genes responsible for the digestion of amyolytic lactic acid bacteria has been developed. A new PCR-based method for the detection of short thermal shock genes in *Str. thermophilus*, which enables rapid selection of strains for use in starter cultures. For the first time, the content of prebiotic carbohydrates GO3, indole-3-propionic acid and cyclic antimicrobial peptides in Bulgarian yoghurt was demonstrated.

2. A novelty in science is the demonstration of new enzymes of fructan- β -fructosidase in the probiotic species *Lactobacillus paracasei*.

3. A novel science gene and a new cyclodextrin glucanotransferase enzyme have been discovered in *Bacillus pseudocalophilus*. For the first time in the world, recombinant cyclodextrin glucanotransferase enzyme has been immobilized in magnetically modified carriers to produce cyclodextrins. Re-use of the CGT-magnetic magnetic biocatalyst indicates the possibility of producing three to four times the amount of CD in 120 minutes compared to the yield of single-use enzyme preparations.

4. Original for science is the heterologous expression of β -glucuronidase in diploid strains of *Ogataea polymorpha* yeast.

5. For the first time in the world, synthesis of enzymes hydrolyzing cellulose and hemicellulose in the species *B. safensis*, *B. toyonensis* and *B. velezensis* has been demonstrated *in vivo*. The entire genome of *Bacillus velezensis* 5RB, capable of converting lignocellulosic substrates into valuable products, has been sequenced.

6. For the first time in the world, a detailed molecular biological study of neuraminidase from a non-toxigenic strain *Vibrio cholerae* non O1 has been carried out. The sequencing and characterization of the *nanH* gene and its encoded enzyme enable the development of safe production of sialidase.

Dissertation publications and evaluation of scientometric indicators

The publications related to the topic of the dissertation are 33. Of these, two are book chapters, 18 are journal articles in journals with the academic editorial board and with an impact factor, one article is in a journal with SJR, and 4 publications are reports published by the Scientific Committee of the Forum in Proceedings of International Conferences. In most of the publications, the candidate is the first or correspondent author, which demonstrates her methodical involvement and leading role in their preparation.

The dissertation publications have been cited 281 times, proving that their author is well known and positively regarded in the scientific field and has established authority.

Conclusion

Significant results have been obtained in the dissertation in the current scientific field and with potential applications in the field of the food industry, medicine and biotechnology. Most of them are original and published in reputable impact factor magazines and have been repeatedly quoted. The applicant's scientometric performance exceeds both the minimum national requirements of the Rules For The Application of The Academic Development Law in the Republic of Bulgaria for the Degree of the Doctor of Sciences and the additional criteria for the growth of the academic staff at IMICB. In conclusion, I support the awarding of the Doctor of Science degree to Assoc. Prof. Penka Mladenova Petrova in the professional field 4.3. Biological Sciences, speciality "Microbiology".



Prof. Dr. Todor Kantardjiev, MD, Dsc

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