OPINION

of Prof. Penka Angelova Moncheva, PhD, Sofia University “St. Cl. Ohridski ”, Faculty of Biology
on the dissertation submitted for defense against a scientific jury, formed by order No. I-127 / 23.12.2019 of the Director of the Institute of Microbiology "Stefan Angelov" at the Bulgarian Academy of Sciences for obtaining a scientific degree "Doctor of Science" in the area of higher education 4. Natural sciences, mathematics and informatics”, professional field 4.3. Biological Sciences
Author of the dissertation: Assoc. Prof. Dr. Penka Mladenova Petrova
Dissertation title: "Molecular-biological studies of new bacterial glycoside-hydrolases with industrial application"

The dissertation is focused on molecular biological studies of glycoside-hydrolase enzymes with bacterial origin. The research in this dissertation follows three main lines. One of them, which I think is the main one, involves the study of enzymes responsible for the breakdown or synthesis of prebiotic carbohydrates by lactic acid bacteria, which are of great use in the food industry and medicine. The development of synbiotics plays an important role in the treatment and prevention of gastrointestinal tract problems in humans and reflects current trends in medicine, nutrition and nutritiology. The development of such preparations requires isolation of strains of lactic acid bacteria having such activities. The second line of this research is related to the isolation of enzymes with cyclodextrin-transferase activity from representatives of the genus *Bacillus*. The interest in these enzymes is based on the widespread use of their products - cyclodextrins, in various fields of human activity. The third line of research is related to the study of a *nanH* gene encoding neuraminidase in a non-toxic strain of *Vibrio cholerae*, which reveals the possibility of the first industrial production of sialidase by a safe producer. Such study is done for the first time. The thesis treats and questions relating to the study of biochemical and genetic potential of newly-isolated strains of bacteria from the genus *Bacillus* in the synthesis of cellulose and lignocellulose degrading enzymes for the production of important products from renewable natural resources. The dissertation research is thematically relevant.
The dissertation is structured classically in seven sections - Literature Review, preceded by an introduction, Objectives and Tasks, Materials and Methods, Results and Discussion, Conclusions, Contributions, References. It includes a list of the author's publications related to the dissertation. I think that the section "Literature Review" is informative, purposeful, and adequate to the topic of the dissertation and characterizes the author as a scientist with high theoretical competence in the scientific field of the dissertation. The Literature review is focused on four major issues: Glycan structure, function and application; Enzymes having glycans as a substrate - classification and general characteristics; Bacteria producing glycoside hydrolase enzymes; Use of lactic acid bacteria in lactic acid fermentation of food and beverages based on cereals. 625 literature sources are cited, 44% of which, over the last ten years. The thesis aims at molecular-biological characterization of new glycoside hydrolases and the production of recombinant enzymes with improved properties and applications in industry and medicine. There are 4 main tasks to accomplish this goal, two of which consist of several sub-tasks. I think the sub-tasks are very broadly defined and could be differentiated. Various methods have been used in the development of the dissertation, which can be classified into three categories - microbiological, analytical-biochemical and molecular biological methods. The vast array of methods is impressive and includes both classical and modern analytical and molecular-biological methods. This section demonstrates the high methodological level at which the tasks have been fulfilled, which ensures reliable and reproducible results. The Results and Discussion section contains the results obtained and their discussion. The section is illustrated with 109 figures and 31 tables. The results of all the tasks are presented. The results of the first task include identification of newly isolated LAB strains producing different glycoside-hydrolase enzymes. The identification of the bacteria is performed by a wide range of molecular biological methods that allow the differentiation between closely related species and subspecies, as well as strain differentiation within a species. Seventy-five rod-shaped and forty cocci LAB strains are identified at species and subspecies level. The strains are characterized by a fermentation profile, a way of converting lactose with determining the type of metabolites from its degradation. The results of the study of enzymes related to starch uptake in strains exhibiting amylolytic activity are presented. Transcriptional analysis of starch uptake is made - for representatives of the genus *Lactobacillus*, *Pediococcus*, *Entercoccus* and *Streptococcus* and for strains of the species *Lactococcus lactis*.

Based on the established high extracellular amylase activity established for the strain B41 of *L. paracasej*, subsp. *paracasei amy*41 gene responsible for the amylase activity of the strain is identified, sequenced and the sequence is deposited in the NCBI database. In order to determine the conditions for the expression of this gene, the effect of different carbohydrates is studied. Cloning and expression of the gene in *E. coli* strain is performed. Study on glycoside-hydrolase enzymes with substrates inulin and fructooligosaccharides among the LAB strains is performed. Cloning and sequencing of the genes encoding fructan-β-fructosidase in *L. paracasei* B41, and production and purification of β-fructosidase enzymes from *L. paracasei* strains are achieved. The biochemical properties of cell-bound β-fructosidases in strains of the same species are investigated. Results of studies of LAB enzymes with β-galactosidase activity are presented with a view to its application in trans-glycosylation reaction of lactose for the synthesis of galactoligosaccharides, which are probiotic oligosaccharides. It is shown that seven strains of *L. delbrueckii* subsp. *bulgaricus* synthesize GOS in milk or lactose medium, and three of them synthesize GOS in the largest amounts. Probiotic characteristics of 33 strains of the genus *Lactobacillus* and of 24 spherical cell-shaped LAB strains are investigated. It is shown that almost all of them had antibacterial activity against three Gram-negative bacterial species, when native supernatant are tested, due primarily to the synthesized lactic acid. Ten strains have activity against three or fewer test-bacteria, when neutralized supernatants are tested. In addition, the proteolytic activity of the strains as well as the synthesis of indole-3-propionic acid, tryptophan, citrulline, as well as some other metabolites with human health benefits are examined. Several strains belonging to different species have been shown to be highly active in the synthesis of one or the other of the abovementioned substances. In view of potential application, some technological characteristics of several LAB strains, such as the biosynthesis of exopolysaccharides, the synthesis of stress proteins under conditions of industrial stress, stress to organic solvents, and in particular butanol, have also been investigated. Without neglecting the research involved in examining several probiotic and technological characteristics of LAB, I think they are a little outside the main focus of the dissertation. Another part of the dissertation combines research on glycoside-hydrolase enzymes in bacteria of the genus *Bacillus*. Results are presented for novel and recombinant cyclodextringlucantransferases, which catalyze production of cyclodextrins that have various industrial applications. This study starts by identification of two *Bacillus* strains producing CGT, followed by biochemical characterization (molecular weight of the purified enzyme, temperature and pH optimum and stability) of CGT from one of the strains - *B. pseudalcaliphilus* 8SB. The gene encoding the CGT in the strain is sequenced and the sequences are deposited in the NCBI gene bank. The gene is cloned into *E. coli* DH5α and heterologous expression is achieved, and the recombinant enzyme is partially purified. This part of the dissertation also includes results of a more technological nature related to the future application of the enzymes as biocatalysts, namely immobilizing them on magnetically modified particles of different nanocarriers. Enzyme preparations of varying degrees of purity are used in these experiments. The highest amount of cyclodextrins is obtained using purified recombinant CGT immobilized on washed algae. Newly isolated and identified strains of the genus *Bacillus* have been analyzed for glycoside-hydrolase enzymes degrading lignocellulosic substrates. The genome of the most promising strain *Bacillus velezensis* 5RB has been sequenced, and it has found genes for a wide range of enzymes related to carbohydrate metabolism, which reveals the potential of the strain for future use. The third major part of the dissertation presents the results of molecular biological studies of glycosidhydrolase enzymes with sialic acid as a substrate (neuraminidases, sialidases). In this study, neuraminidase is obtained, purified and characterized from a non-pathogenic strain of *Vibrio cholera*. The *nanH* gene of the producer strain is isolated, sequenced and compared with the genes of toxigenic strains of *Vibrio cholerae*. The complete nucleotide sequence of the gene of the Bulgarian strain has been deposited in the NCBI gene bank. Molecular studies of β-glucuronidase from *E. coli* are included in the dissertation. Heterologous expression of the gene coding the enzyme in *O. polymorpha* has been performed. At the end of this section the author assesses what has been obtained in relation to the future application of the strains in various fields. Based on the results obtained, 18 conclusions are drawn. Most of them are well formulated and logically derived from the results obtained. I would like to point out that conclusions 1 and 2 are rather the results obtained, than conclusions. The dissertation is a contribution to characterization of bacterial glycoside hydrolases with molecular methods. Some of the results are reported for the first time, which gives originality of the study. I think the most important are the scientific contributions from 1 to 5, and from the applied ones – from 1 to 3 and 5.

Assoc. Prof. Petrova has 33 scientific papers related to the dissertation, of which 2 chapters in books, 18 publications in Impact factor journals, 1 - in Impact rank journal, 4 scientific papers from participation in

international scientific forums, printed in full text, and 8 publications in non-Impact Factor / Impact rank journals. The total impact factor of these works is 25,762, and they have been cited in total of 281 times in various databases. Assoc. prof. Petrova's scientometric indicators are in accordance with all requirements for the degree of “Doctor of Science”, significantly exceeding some of them.

Conclusion:

The evaluated doctoral dissertation combines the results of studies covering a period of 15 years, which are thematically related to the study of glycoside-hydrolase enzymes synthesized by different producers with modern molecular genetic methods. The subject of the dissertation is up-to-date. Extensive and in-depth research has been carried out at a high methodological level. Despite the heterogeneity of the research regarding the producers and the enzymes synthesized by them, Assoc. Prof. Petrova has very skillfully combined them, which is possible due to the sustainability of her scientific topics, which has been elaborated during this considerable period of time. The dissertation presents Assoc. Prof. Penka Petrova as an erudite scientist in the field of microbiology and molecular biology in the study of microbial glycoside-hydrolase enzymes. The results of these studies have been reported through 33 scientific papers in prestigious scientific journals and have been recognized by the scientific community by the citations received. The dissertation is a contribution in the relevant scientific field, and has originality and innovativeness.

On the basis of the above, as well as in view of the Rules for the Implementation of the Law for the Development of Academic Staff in the Republic of Bulgaria and that of the Stefan Angelov Institute of Microbiology at BAS, I believe that the presented work meets the requirements for a doctoral dissertation. My assessment of the thesis is completely positive, and I strongly recommend the Scientific Jury to award the scientific degree "Doctor of Science" in the professional field 4.3. Biological Sciences of Assoc. Prof. Dr. Penka Mladenova Petrova.

04/03/2020 Signature:

Sofia (Prof. Dr. Penka Moncheva)