Review

by Prof. Dr. Mariela Konstantinova Odjakova-Baytocheva,

Sofia University "St. Kliment Ohridski"

on the dissertation for the award of the degree of Doctor of Science

in the professional field "Biological Sciences" - 4.3, specialty "Microbiology"

Author: Assoc. Prof. Penka Mladenova Petrova, Institute of Microbiology, BAS

Subject: "Molecular-biological studies of new bacterial glycoside-hydrolases with industrial application"

General presentation of the procedure and dissertation. The presented set of materials in paper and electronic form is in accordance with the Regulations for the development of the academic staff of the Bulgarian Academy of Sciences, BAS and fulfills the criteria for obtaining the degree of Doctor of Science. Penka Petrova graduates BF at Sofia University "Kl. Ohridski ", specialty Biotechnological Processes, Genetic and Cellular Engineering in 1994. In 2003 he defended his dissertation on" Creating a gene cloning system for Streptococcus thermophilus "and obtained a Ph.D. in scientific specialty 01.06.12 - Microbiology . From 2003 to 2009 she worked at IMCB consistently as a research associate, III, II, I degree, and from 2011 she was elected as associate professor. Since 2013, she is the Head of the Gene Expression Laboratory; since 2015 she is the chairman of the MoEW Committee for GMOs (at IMicB); from 2018 she is a Head of the Department of General Microbiology and a Head of the Laboratory of Metagenomics and Gene Expression. In 2019, she was elected Director of IMCB.

Actuality of thesis. The dissertation presented is a summary of the information received from the research conducted by Assoc. Prof. Petrova related to molecular-biological characterization of new glycoside hydrolases and the creation of recombinant enzymes with improved properties and applications in industry and medicine. The study of enzyme diversity and coding genes contributes to complement the information on genetic adaptive and evolutionary mechanisms and the phylogenetic relationships between different microbial groups. On the other hand, glycoside hydrolases are one of the most important industrial enzymes with applications in the food, medicine, pharmacy and biotechnology industries. Glycosidase producers are well understood, but information about their coding genes is scarce. Although lactic acid bacteria are widely used in cereal food fermentations, lactic acid bacteria have until recently been thought to lack amylase or inulinase activity, and isolated strains with such a phenotype were very rare. The genetic underpinnings of these non-typical ICD enzyme activities are unclear, with separate genes with a small degree of similarity isolated. Today, ICDs are widely used as probiotics and in the creation of new synbiotic formulas - a combination of a probiotic strain and prebiotic carbohydrates to support its multiplication in the gastrointestinal tract. which are glycoside hydrolases synthesized by ICD. The study of the genetic and biochemical "relationships" between the probiotic strain and the prebiotic fiber would provide new information on the ability of the bacteria to absorb and potentially synthesize the fiber. Selection of ICD with amylase activity would allow the development of a new class of amylolytic probiotics with important

applications in patients with food allergies. The characterization of enzymes with amylase, β-fructosidase or β-galactosidase activity from strains of ICDs is extremely valuable in elucidating the fine symbiotic relationship between pro- and prebiotics. The structural and functional analysis of the genes and enzymes responsible for the uptake of fructo-oligosaccharides and inulin contribute to understanding the mechanism of this process, which is important in the development of synbiotic products.

Structure of the thesis. The dissertation is structured as required and contains an introduction (2 pages), a literature review (107 pages), purpose and objectives, materials and methods (28 pages), results and discussion (157 pages), conclusions, contributions, a list of the used literature sources and a list of author's publications on the topic of the dissertation. 52 tables and 140 figures are included. The cited list includes 625 sources, of which only 3 are in Cyrillic.

Research methodology. A very diverse and up-to-date methodology (microbiological biochemical, analytical and molecular biological methods) is used, which illustrates the high professional competence of Assoc. Prof. Petrova and is the basis of the high quality and value of the dissertation. The reference strains used, the composition of the culture media and the culture conditions are described in detail, as well as the classical microbiological tests used. Biochemical and analytical methods include analysis of enzyme activities, determination of the pH and temperature optimum of enzymes, enzymatic kinetics, methodologies for purification of enzyme preparations, and the like. Molecular biological methods include the preparation and purification of nucleic acids, methods for cloning and analysis of recombinant clones, sequencing and phylogenetic analyzes. High-resolution mass spectrometry in combination with liquid chromatography, RT-PCR, RAPD (Randomly amplified polymorphic DNA-PCR), MLST multilocus analysis, pulse electrophoresis, etc. were applied. The methods used are up-to-date and adequate to the planned studies.

Characterization and evaluation of dissertation work and contributions. The purpose of the dissertation is molecular and biological characterization of new glycoside hydrolases and creation of recombinant enzymes with improved properties and applications in industry and media. In order to achieve this goal, five main tasks have been set out, of which there are 6 specific sub-tasks, arranged in chronological order and reflecting the development of the scientific interests of Assoc. Prof. Petrova and her desire to develop contemporary topics. The Results and Discussion section contains 4 chapters in logical order and outlined in 157 pages.

The diversity of the enzymes and bacterial producers studied requires an interdisciplinary approach combining knowledge in microbiology, biochemistry, enzymology and molecular biology, recombinant DNA technology, transcriptomics and bioinformatics. Based on the application of new technologies in these scientific fields, the dissertation clearly demonstrates the profound theoretical knowledge, professional skills and research interests of Assoc. Prof. Petrova.

Assoc. Prof. Petrova investigates a wide range of bacterial glycosidhydrolase enzymes, characterized by modern omix technologies. The object of the study is the enzymes responsible for the breakdown or synthesis of prebiotic carbohydrates (inulin, fructooligosaccharides, galactooligosaccharides and starch) from lactic acid bacteria. The combination of the synergistic action of probiotic bacteria and prebiotic carbohydrates is based on specific molecular

interactions between bacteria and polysaccharide molecules. Current trends in the development of new bacterial preparations are directed to the use of synbiotics - drugs that contain prebiotic strains capable of consuming or producing prebiotic carbohydrates. Therefore, the characterization of LAB strains with novel or rarely manifested glycoside-hydrolases activities enables the development of novel synbiotic preparations and functional foods with novel properties and broadens the scientific knowledge of the genetic and biochemical mechanisms for the adaptation of LAB.

In the present work, dozens of bacterial species and strains are included, some of which have been used as enzyme producers and others as hosts for the heterologous expression of glycoside-hydrolases encoding genes. The activity, substrate specificity, and structure of enzymes with substrate carbohydrates in Gram-positive lactic acid bacteria and those of the Bacillus genus are examined, with the focus on research on the detection of genetic determinants that determine enzyme activity - sequencing of genes and studying mechanisms, gene expression. Some of the strains with valuable enzyme activities studied have proven potential applications in the development of biotechnology, synbiotic products and functional foods.

Using different genetic approaches (PCR amplification of 16S rDNA fragment, ARDRA, PFGE) from the created collection of 115 strains isolated from 97 fermented dairy and cereal products collected from over 40 regions across the country, it has been shown that the strains belong to 18 species of ICD as 4 of them are representatives of the typical L. d. bulgarigus. This study includes a new comprehensive study of the microflora of yogurt, boza, rye dough and more. The results show that the newly isolated strains of L. delbrueckii subsp. bulgaricus are genetically different and differ from the starter cultures of some of the most popular Bulgarian yoghurts. As a result of genetic studies, 75 rodent ICD strains and 40 lactic coca strains have been identified. .The greatest biodiversity of ICDs is observed in Rhodope yoghurts, many of which contain several types of ICDs with different morphology. For the first time, the species L. paracasei and L. rhamnosus were isolated as a concomitant microflora. The symbiotic relationship between L. delbrueckii subsp. bulgaricus and Str. thermophilus strains are best preserved in yoghurts from the villages of Central and Western Balkan.

The ability of newly isolated strains to absorb various carbohydrates was investigated. In 25 strains, triglyceride formation during lactose metabolism was demonstrated, β-galactosidase transferase activity and prebiotic galacto-oligosaccharide accumulation were shown. 115 strains of Lactobacillus, Pediococcus and Enterococcus for amylase activity were investigated and the world's first amylolytic representatives of L. sakei and Enterococcus were isolated. Glycogen phosphorylase and amylopopulanase have been shown to be irrelevant to Lc starch hydrolysis. lactis and that in the presence of glucose, ✔-amylase genes are not expressed due to catabolic repression.

4 LAB have been found that break down long-chain inulin. The genes encoding the фpy-fructosidases of L. paracasei B41 and LC1 strains were sequenced and bioinformatic analysis showed that the enzymes belonged to the cell-related β-fructosidases.

Associate Professor Peter's cloned gene (amy41) encoding amylopululanase in the L. paracasei B41 strain isolated from it and expressing it in a suitable vector for inducible expression under

the strong phage T7 promoter and expressed in E. Coli, this vector being used for the first time successfully as an expression vector, not just a cloning vector.

The ability of P. acidilactici PD3 to hydrolyze PH3, sucrose and inulin in vivo has been demonstrated. To identify genes and proteins involved in the uptake of PHOs, a P. acidilactici PD3 genomic library was constructed and two clones with increased ability to digest fructose or PHOs were selected. Gene sequencing indicates that these are the genes responsible for sugar transport.

For the first time, an analysis of the formation of POPs from L. delbrueckii subsp. Strains has been initiated. bulgaricus. The results show that several strains spontaneously form tetrasaccharides and relatively high amounts of trisaccharides, which are stable after 48 hours of fermentation. Structural analysis of POPs produced by L. delbrueckii subsp. bulgaricus 43 by LC-MS proves that the trisaccharides obtained contain β- (1 → 4) and β- (1 → 6) glycosidic bonds, with the β- (1 → 4) bound galactose unit being in a very unusual position and reported in GOP for the first time. The unique ability of the Bulgarian strains L. delbrueckii subsp. bulgaricus to form β- (1 → 4) bonds between galactosyl residues and lactose are probably related to new functional properties of GO.

The antimicrobial activity of the newly isolated ICD strains was also investigated. Three of the strains isolated from Bose (L. paracasei B41, L. pentosus N3 and L. plantarum Bom 816) are amyolytic probiotics because, in addition to their amylase activity, they exhibit high antibacterial activity against Escherichia coli HB101, Vibrio cholerae V13, Klebsiella pneumoniae G31 and Bacillus subtilis WB800N.

For the first time, the ability to synthesize indole-3-propionic acid, a potent neuroprotective antioxidant, as well as L-citrulline, used as a laxative and as a blood-plasma marker of irritable bowel syndrome, has been demonstrated. Other first-proven metabolites produced by ICD are the cyclic peptides with antibacterial activity: cyclophenylalanil-prolyl and cycloevil-prolyl.

It has been shown that the Bulgarian strains of ICD have good technological characteristics and the most promising for use in the food industry are the strains synthesizing exopolysaccharides and resistant to thermal shock and alcohols. . The cell surface hydrophobicity has been shown to be inversely proportional to the survival rate of bacteria in a medium with butanol. A method for the rapid identification of strains harboring Hsp genes has been developed. Five strains were found that contained new plasmids with hsp genes

Another successfully developed field of work is related to the isolation of new enzymes with cyclodextrin-glucanotransferase activity. The interest in these enzymes is driven by the widespread use of their cyclodextrins products in various industries as a means of 'encapsulating', transporting and storing biologically active substances. The results presented in this section have a significant contribution as they are related to the creation of one of the first recombinant CGTases. Moreover, the successful immobilization of these enzymes in natural magnetically modified carriers and the development of a new method for the re-use of biocatalysts has led to the highest cyclodextrins yields in the world, making their production many times more efficient than using natural ones enzymes. A novel gene encoding the cyclodextrin-glucanotransferase enzyme in Bacillus pseudalcaliphilus 8SB strain was cloned and

overexpressed in E. coli host BL21 (DE3). The purified recombinant enzyme has a molecular weight of 75.5 kDa, two pH optima (pH 6.0 and 8.0) and a T-optimum 60 ° C. Immobilized in magnetically modified carriers, the recombinant enzyme results in the production of 36 mg / ml cyclodextrins.

Bacterial strains that have glycoside hydrolases capable of degrading cellulose and hemicellulose, combined with the conversion of these polysaccharides into valuable products and fuels, are in demand and are of particular interest. 57 new Gram (+) strains, rod-shaped, catalase-positive bacteria were selected, with colony morphology typical of species belonging to 11 Bacillus species. The Bulgarian isolate B. velezensis 5RB was assigned to the species only after complete genomic sequencing and in silico analysis of the degree of DNA-DNA hybridization with complete genomes of each of the listed species. Some of the newly discovered bacilli species are new to our country and with atypical habitat. Bulgarian Bacillus isolates have the potential for direct hydrolysis of renewable natural substrates. The strains produce enzymes that break down cellulose, arabinoxylan, xyloglucan, branched arabinan, xylan, galactomannan, β-glucan, amylose, galactan and kurdlan. There are 225 genes responsible for the conversion and transport of carbohydrates in the genome of Bacillus velezensis 5RB. The B. velezensis 5RB strain has the genetic basis to convert cellulose, lignocellulose, starch and inulin directly into valuable low molecular weight products. Seven complete antibiotic synthesis clusters (macrolactin, bacillus, deficiency, fengycin, bacilbactin, bacillisin and surfactin) are also present.

One of the sections in the dissertation is devoted to the study of a gene (nanH) encoding the neuraminidase enzyme. For the first time, a detailed molecular biological study of the neuraminidase from a non-toxic strain of Vibio cholerae was performed. Sequencing and characterization of the nanH gene and its encoded enzyme enable the development of safe production of sialidase.

The last section is concerned with the use of ✔-glucuronidase as a model for optimizing heterologous gene expression. The gene encoding β-glucuronidase has been successfully introduced into the yeast expression system with the host Ogataea polymorpha. Increased expression levels of diploids and meiotic segregants have been demonstrated. The approach to create diploid strains carrying heterologous genes is promising for improving the productivity of O. polymorpha strains, and the positive results obtained allow us to create new strategies for increasing the levels of heterologous expression in methylotrophic yeast.

Based on the results obtained and their discussion, 18 conclusions were drawn. The contributions of the dissertation are grouped as scientific and applied scientific contributions. They reflect the most significant achievements of the dissertation and are clearly and accurately formulated.

Assessment of publications and personal contribution of the dissertation

A list of 33 scientific publications included in the dissertation is attached. Of these, two are book chapters referenced in Scopus, 18 are in IF journals (IF 25,762 overall), one is in SJR journals, 4 are full-length international conference proceedings, 5 are non-impacted journals and 3 are in proceedings of national conferences. In 16 publications Assoc. Prof. Petrova is the first author, and in 21 - correspondent. Some of the results were presented at 37 international and national scientific forums.

According to the queries in which the Web of Science Journal Citation Reports (JCR) groups Impact Factor (IF) scientific journals, 3 of them have Q1; 13 are with Q2 and 3 are with Q3. According to indicator D (publications outside the habilitation work) from Table 1 of the ППЗРАСРБ, Assoc. Prof. Petrova collects 410 points at a required minimum of 100 points, and at indicator D (quoted) 740 at a minimum of 100 points. Thus at a required minimum of a total of 350 points according to the Regional Development Agency, Assoc. Prof. Petrova has 1300 points, which exceeds the minimum national reductions. According to the additional criteria for the growth of the academic staff at IMICB, a minimum of 150 citations are required for the scientific degree of Doctor of Sciences, and the noted citations related to the dissertation publications are 281. A detailed list of authors and publications citing scientific works is attached. .

Abstract

The abstract is 114 pages long and reflects the content of the dissertation, the conclusions, contributions and publications related to it.

CONCLUSION

The dissertation contains scientific, scientifically applied and applied results, which make an original contribution to science and meet all the requirements of the Law for the Development of the Academic Staff in the Republic of Bulgaria (LDASRB), the Regulations for the application of the LDASRB and the Rules for the implementation of the LDASRB of BAS . Prof. Petrova's scientometric indicators go beyond both the minimum national requirements for the award of the Doctor of Science degree and the additional criteria for the growth of the academic staff at IMICB. The presented materials and the dissertation results fully comply with the specific requirements of the regulations of IMkB-BAS for the application of LDASRB.

The dissertation shows that Petya Petrova possesses deep theoretical knowledge and professional skills in the scientific specialty "Microbiology", demonstrating qualities and skills for conducting research with receiving original and significant scientific contributions.

As a result of the above, I am convinced of my positive assessment of the research, the results achieved and the contributions made, and I suggest that the venerable scientific jury award the Doctor of Science degree in Penka Mladenova Petrova in higher education: professional field 4.3. Biological Sciences, specialty "Microbiology".

02/29/2020 reviewer

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