R E V I E W

of the dissertation of **Assoc. Prof. Dr. Penka Mladenova Petrova** on "Molecular-biological studies of new bacterial glycoside-hydrolases with industrial application" for the scientific degree "Doctor of Sciences", proposed in the professional field 4.3. Biological Sciences, specialty "Microbiology"

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Hydrolases underlie the living organisms catabolism. Although they are one of the best studied groups of enzymes as a mechanism of action, the development of modern omix-technologies enables the broaden of the knowledge of their structure and genetic diversity. The glycoside hydrolases produced by the bacteria are the cause of the wide substrate spectrum of these microorganisms and hence, their distribution in all known habitats. The relevance of the research is related to the applications of the strains-producers and the enzymes themselves in the various branches of the food industry (in the manufacturing of bread, meat and sausages, beer, wine, dairy products, modification of food proteins, clarification of juices and processing, and the development of biotechnologies for the production of metabolites from cheap and renewable natural sources.

DISSERTATION DESCRIPTION

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 the author's publications on the topic of the dissertation. The total volume of the dissertation is 358 pages, and the documentary materials include 52 tables and 140 figures. The list of references includes 625 sources, of which only 3 are in Cyrillic. Despite its large volume, the dissertation is easy to read thanks to the extremely good linguistic and scientific style and the negligible number of typographical and spelling mistakes.

LITERATURE REVIEW

The literature review is detailed, thorough and comprehensive. The first chapter deals with the structure and functions of carbohydrates: mono-, di-, polysaccharides and sialoglycans. The second part of the review is devoted to a review of the structure and function of enzymes with substrate carbohydrates - hydrolases and transferases, with a more detailed look at the classes of enzymes under study in the dissertation: amylase, amylopululanase, CGTase, fructosidase, and bacterial glycosidases with substrate cellulose and hemicellulose. In addition to the general characteristics of the enzymes, the review presents their structure, their coding genes and bioinformatic data on the conserved regions and the catalytic center of the enzymes.

Chapter three of the literature review describes the bacterial producers of glycoside-hydrolase enzymes, molecular approaches for bacterial identification and typing, and reviews the major taxonomic groups included later in the presentation: lactic acid bacteria (LAB) and the genus *Bacillus* as producers. Since new enzymes with amylase activity in lactic acid bacteria have been searched for in the dissertation, the last part of the literature is devoted to the application of LAB in fermentation of cereal-based foods and beverages with functional claims,

and the probiotic properties of LAB are discussed. The literature review concludes with a summary of the current state of the studies and logically follows the stated goal - molecular biological characterization of new glycoside hydrolases and development of recombinant enzymes with improved properties and industrial applications. To achieve this goal, four main tasks and six sub-tasks have been identified, which are clearly formulated and adequate to the objective pursued.

MATERIALS AND METHODS

The section includes three subsections. The first subsection "Microbiological methods" describes in detail the reference strains used, the composition of the culture media and the culture conditions, and the classical microbiological tests used. Biochemical and analytical methods include analysis of enzymatic activities, determination of the pH and temperature optimum of enzymes, kinetic constants, and methodologies for purification of enzyme preparations. Molecular biological methods describe procedures for 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 was used for the analysis and identification of metabolites. The methods used are up-to-date and adequate to the planned studies.

RESULTS AND DISCUSSION

Nearly a century after the discovery of *L. delbrueckii* ssp. *bulgaricus* the research of Assoc. Prof. Petrova is the first, which includes a comprehensive study of the microflora of traditional Bulgarian foods. In general, this chapter describes the isolation of new bacterial strains producing glycoside hydrolases belonging to 14 different enzyme families as two of the enzymes under study, in addition to hydrolases, also exhibit transferase activity. The results obtained are reported and discussed in 5 separate points, including a brief conclusion.

The first item in the Results and Discussion chapter is the most comprehensive and presents data on glycoside-hydrolases enzymes with α-glucans and fructans substrates produced by lactic acid bacteria. It involves the isolation, identification and study of the metabolism of carbohydrates in lactic acid bacteria (LAB). A unique collection of 115 strains has been created, isolated from 97 fermented dairy and cereal products collected from over 40 regions across the country. The strains belong to 18 species of LABs, with the largest (42) being representatives of the typical *L. delbrueckii* ssp. *bulgaricus*. For the species and subspecies identification of the strains, Assoc. Prof. Petrova has applied a variety of molecular genetic methods. To distinguish the sole representative of *Lc. lactis* subsp. *lactis* and subspecies *cremoris* ARDRA analysis was applied. The result was confirmed by an independent method, a species-specific PCR of the glutamate decarboxylase gene. Similarly, ARDRA and another independent method, RAPD, were applied to distinguish *L. paracasei* from *L. casei*. However, for most strains, these methods are not sufficiently discriminatory for their species determination, therefore, the 16S rRNA gene is sequenced. In the part concerning the identification of the strains, Assoc. Prof. Petrova pays particular attention to 12 of the newly isolated strains *L. delbrueckii* ssp. *bulgaricus* by comparing them with Domlyan yogurt isolates and Danon's “Na baba”. A combination of three methods, RAPD, MLST, and PFGE was used to demonstrate the uniqueness of these 12 strains and their difference from the starter cultures of some of the most popular yoghurts on our market. Other more interesting findings from the species characteristics of the isolates are: 1. The greatest variety of LABs is observed in the Rhodope yoghurt, which contains several types of LABs; 2. For the first time the species *L. paracasei* and *L. rhamnosus* have been isolated as a concomitant

microflora in the yogurt, and 3. The classic "symbiotic" partner of *L. delbrueckii* ssp. *bulgaricus* in Bulgarian yogurt *Str. thermophilus* is today largely displaced by the less pretentious but no less useful *Pediococcus acidilactici*.

A unifying characteristic of all LABs is their ability to form lactic acid from glucose. However, glucose can be the end product of the breakdown of various di- and polysaccharides and Assoc. Petrova examines the newly isolated strains for their ability to absorb 15 different carbohydrates. She finds that they all absorb lactose, glucose and galactose, and almost all of them are fructose and cellobiose, an observation that is confirmatory. In a study of lactose degradation metabolites, 25 strains were found to form triglycerides, demonstrating the rare transferase activity of β-galactosidase and the accumulation of prebiotic galacto-oligosaccharides. Further, Petrova's attention is focused on the strains that are capable to convert starch (amylolytic LABs, ALAB). They are 37 of all 115 and represent mainly the genera *Lactobacillus*, *Pediococcus* and *Enterococcus*. The strains were tested for amylase activity - intracellular, cell-bound and extracellular, and data obtained for lactobacilli confirm the observations of other authors. Assoc. Prof. Petrova isolated the world's first amylolytic representatives of *L. sakei* and *Enterococcus*. The highest extracellular amylase activity was measured for the Bulgarian strain *L. paracasei* B41 isolated from Boza "Bomax".

According to the Japanese KEGG database, seven genes encoding amylolytic enzymes are present in the genomes of ALAB: α-amylase (*amy1*), glycogen phosphorylase (*glgP*), (1,4) -α-glucan branching enzyme (*glgB*), α-glucosidase (*agl*), oligo-1,6-glucosidase (*malL*) and neopululanase (*dexC*). To study the expression of these genes in newly isolated Bulgarian ALAB, Assoc. Prof. Petrova generates specific primers to amplify the 7 genes and use them as DNA probes for the hybridization of total RNA. In most cases, a good correlation was found between the type and strength of gene expression, amylase activity and its cellular compartmentalization. All lactobacilli strains were found to express the seven analyzed genes except *L. sakei* 3/30, which expresses only 3 of them.

In this regard, it is noted that not only in this particular case, but in general, a large part of the glycoside hydrolases in lactobacilli are still unexplored, which underscores the relevance and purpose of the dissertation.

Particular attention was given to the only *Lactococcus* representative, able to degrade starch (B84). Assoc. Prof. Petrova aims to find the chromosomal determinants responsible for the amylolytic properties of this strain. Interestingly, in order to create PCR primers for 4 amylolytic genes [(cytoplasmic (*amyL*) and extracellular (*amyY*) α-amylases, amylopopulanase (*apu*) and glycogen phosphorylase (*glgP*)], the genomes of two strains were used The *L. lactis* and *L. cremoris* subspecies, which do not exhibit amylase activity, were subsequently shown to be specific to the 4 analyzed genes, but express only one of them (*apu*). The expression was also examined by reverse transcription (RT-PCR) of the 4 genes when culturing the strain in a glucose or starch medium Transcription of only two α-amylase genes was detected upon starch-only growth. From this result, two important conclusions were drawn, namely that glycogen phosphorylase and amylopululanase have no bearing on the hydrolysis of starch from *Lc. lactis* and that in the presence of glucose, the α-amylase genes are not expressed due to the catabolic repression phenomenon. While the second conclusion is largely confirmatory, the discovery of the genetic bases of amylase activity contributes to understanding the process of hydrolysis of starch in lactococci.

Particular attention is paid to the *L. paracasei* B41 strain, which is interesting in that it exhibits the highest extracellular amylase activity among the tested ALABs. Assoc. Prof. Petrova describes the cloning and heterologous expression of an *amy41* gene encoding amylopululanase

in the *L. paracasei* B41 strain. The gene is sequenced and its bioinformatic analysis reveals the presence of a consensus *cre*-element in the promoter region responsible for the catabolic repression phenomenon. The presence of this element agrees well with the observed lack of gene transcription when strain B41 is cultured in glucose medium. In subsequent experiments, the gene was cloned into a suitable vector for inducible expression under the strong phage T7 promoter and expressed in *E. coli*. It is emphasized that in this dissertation, this vector was used for the first time successfully as an expression vector, not just as a cloning vector. After optimization of the fermentation conditions, the maximum enzyme activity in the culture medium was reached and it was three times higher than that of the original strain B41. Bioinformatic analyzes reveal the greatest similarity to the *amy41* gene with translated proteins of other *L. paracasei* strains as well as those of *L. casei* and *L. rhamnosus* (up to 99% identity). Interestingly, despite the high homology, none of these lactobacilli strains exhibit amyolytic properties. To this comment I have the following Note: It is more likely that "mutations with a positive sign in the amy41 gene" (p. 187) did not lead to the synthesis of the active enzyme, and conversely, mutations in the protein-coding sequences of the rest lactobacilli have "inactivated" the enzymes.

The first point of the Results and Discussion chapter discusses further glycoside hydrolases with β-fructans substrate. In the collection of newly isolated LABs, 4 were found to degrade long-chain inulin. The interest in these strains is driven by the fact that inulin is a prebiotic, the strains have a probiotic effect, and the mechanisms of inulin and fructooligosaccharides (FOS) uptake are not fully understood. The genes encoding the fructosidases of *L. paracasei* B41 and LC1 strains have been sequenced and the bioinformatic analysis was performed. Assoc. Petrova's assign the enzymes to the cell-bound fructans-β-fructosidases, and from the presence of an amino acid substitution at their active center, it was coincluded that they represent new enzymes. The literature lacks information on purified β-fructosidases from *L. paracasei* and this niche is filled by Assoc. Petrova, who undertakes their purification. Isolation of cell-bound proteins is problematic, as is their expression in *E. coli*, which is why the enzymes are purified from their natural producers. Due to the problematic purification of the protein anchored in the cell wall, Assoc. Prof. Petrova concludes that the use of whole cells with inulinase activity as "microbial factories" for the production of inulin metabolites is more promising. The substrate specificity of both enzymes was investigated and inulinase of strain B41 was found to exhibit equally high affinity for short-chain FOS and bacterial levan, weaker for inulin and extremely weak for sucrose. The inulinase of the other strain LC1 has the highest affinity for levan, followed by that of inulin and FOS, and does not bind sucrose, which confirms other researchers' observations of the substrate specificity of β-fructosidase enzymes from other lactobacilli species. Note: On page 206, the differences in substrate specificity of the two inulinases are commented to be due to "differences in the sequences of their coding genes". It is more accurate to state that these differences are due to differences in the amino acid sequences of the two enzymes and to indicate what those differences are. Both inulinase producing strains also exhibit amylase activity. For this reason, Assoc. Prof. Petrova examines the transcription of inulinase and amylopululanase genes when strains grow in inulin and starch media. It concludes that the two genes are under common control, most probably by the cis-located *cre*-element in them. This is confirmed by the fact that both genes are not expressed in the presence of glucose in the medium. The results obtained are the first report on the simultaneous hydrolysis of mixed substrates by LAB.

Very rare is the ability of *Pediococcus* species to ferment prebiotics, and so far no data are available on the uptake of FOS or inulin by *P. acidilactici*. For this reason, Assoc. Prof. Petrova focuses her attention on the isolated PD3 strain of this species, which utilizes FOS. Studies have

been undertaken to identify the genes and proteins involved in the uptake of FOS. After development of genomic library in *E. coli* and its screening, 2 clones with increased ability to absorb FOS were obtained. Their sequencing indicates that these are not genes for hydrolytic enzymes, but rather those responsible for the transport of sugars, which sheds light on the mechanism of FOS uptake by *P. acidilactici*. It is important to emphasize that due to its ability to fully ferment fructose, this strain is promising for its application as a probiotic in cases of fructose malabsorption leading to irritable bowel syndrome.

Beta-galactosidases are enzymes that break down milk sugar to glucose and galactose. Prof. Petrova's interest, however, is directed to another activity of these enzymes, namely trans-glycosylase, which leads to the synthesis of galacto-oligosaccharides (GOS) with valuable probiotic properties. The analysis of the final metabolites of β-galactosidase activity in 10 L. bulgaricus strains showed GOS formation when the strains were cultured in milk and / or in lactose medium. The synthesis of GOS and their structure were demonstrated by high resolution mass spectrometry preceded by liquid chromatography. The samples analyzed derived from yogurt after 48 hours of fermentation. The result shows that the synthesized by strain 43 GOS are tri- and tetrazaccharides, with the last galactose residue being linked to a lactose molecule. The synthesis of five times higher amount of GOS was reported, compared to literature (GOS in Spanish yogurt). This is also the first communication on the formation of GOS by *L. bulgaricus*. For the first time, a β-(1,4) bound galactose unit in the GOS was also detected.

At the end of the first section of the Results and Discussion chapter, some of the probiotic and technological characteristics of LAB are discussed. These characteristics are important for the practical application of the newly discovered by Prof. Petrova LAB, as many of them have the potential for industrial integration. An examination of cell-free supernatants from the culture medium in which LABs have been cultivated for antimicrobial action confirms the fact that Bulgarian strains isolated from Boza have probiotic activity and strains from yoghurt show impressive activity against Gram-negative pathogens. It has also been shown that a large proportion of lactobacilli and most lactic acid cocci secrete extracellular proteases with high proteolytic activity. The synthesis of amino acids and cyclic antimicrobial peptides by LAB was investigated. Synthesis of indole-3-propionic acid by LAB, which is a potent neuroprotector, and the presence of two antimicrobial compounds in yogurt, cyclophenylalanil-prolyl and cyclo-leucyl-prolyl, have been demonstrated for the first time. The production of exopolysaccharides (EPS) by LAB is a desirable quality since it imparts a higher viscosity and a denser texture to the products. The strains of *L. bulgaricus* are rated as very good producers of EPS and the best are strains of the accompanying microflora (*L. fermentum / Leuc. mesenteroides*). Another important technological feature is the resistance of LAB strains to industrial stress. *Str. thermophilus* was used as the model, because in industrial production of yogurt it is subjected to heat treatment up to 50°C-70°C. The heat shock proteins (Hsp) were used as markers. A method for the rapid identification of strains harboring *hsp*-genes has been developed that would allow rapid screening of large industrial collections. 49 strains of the species were checked by colonial PCR for the presence of *hsp* genes. Five strains were found that contained new plasmids with *hsp* genes. One of the strains was tested for survival in heat shock conditions and showed higher resistance compared to the plasmid-cured strain. A further study of stress resistance caused by organic solvents was conducted and it was shown for the first time that the hydrophobicity of the cell surface was inversely proportional to the survival rate of bacteria in a medium with butanol.

The second item in the Results and Discussion chapter refers to glycoside-hydrolases enzymes produced by the *Bacillus* genus. Cyclodextrin-glucanotransferases (CGT-ases) have been considered as representatives of hydrolases that degrade β-glucans. As the name of these

enzymes shows, in addition to hydrolases, they also have transglycosylase activity leading to the synthesis of cyclodextrins (CDs) with many industrial applications. By sequencing a fragment of the 16S rRNA gene of two alkalophilic *Bacillus* strains that secrete high levels of CGTase, the strains were assigned to *B. pseudalcaliphilus*. The enzyme of one of the strains (8SB), purified from the culture medium, has a high specific activity, which makes it a valuable producer of mainly β-dextrin. The 8SB strain CGTase gene (*cgt*) was sequenced and the deduced amino acid sequence of the enzyme was compared to that of other *Bacillus* species CGTase. Due to the low degree of homology, it was concluded that the *cgt* gene of strain 8SB encodes a novel, previously unrecorded enzyme. Secreted expression of the gene in *E. coli* was achieved and after optimization of the fermentation conditions over ten times higher enzyme activities were achieved than the original ones, with a particular γ-CGTase activity of 1275 U / ml. For the first time, such high values for this enzyme have been described in the literature, making the selected *E. coli* clone promising for industrial production of γ-dextrin. The recombinant enzyme is purified by a combination of ultrafiltration, adsorption on starch and gel filtration. Immobilization is an approach that allows the re-use of enzymes as biocatalysts and Assoc. Petrova undertakes immobilization of the enzyme on various carriers loaded with ferrofluid nanoparticles. The best results for the conversion of starch to CD by the action of the immobilized enzyme are obtained by using haloiside as a nanocarrier, which also has the advantage that the enzyme enters the interior of the carrier. Further experiments were conducted to reuse immobilized CGTase during 6 consecutive 20-min reactions, demonstrating the possibility of 3 to 4 times higher CD yields per 120 min than the yield of single-use enzymes. The best results are obtained with an enzyme immobilized on magnetically modified washed algae with no α-CD in the final product.

Enzymes with the β-glucans substrate, and in particular cellulose and hemicellulose degraders, have been studied as representatives of the *Bacillus*-derived glycoside hydrolases. This part of the research is justified by the key place of biotechnology in the world economy, directed to alternative raw materials sources for the production of valuable products and fuels. Bran and embryos *e.g.* is a grain waste in the mill industry that is a rich and renewable source of polysaccharides suitable for the production of enzymatic products. Prof. Petrova's research focuses in particular on the ability of *Bacillus* strains to degrade lignocellulosic substrate. To this end, she collects 57 strains belonging to 11 species of the genus. Species determination was performed by sequencing the 16S p RNA gene, and in cases where this approach was found to be non-discriminatory, by polyphase taxonomy methods. One of the strains was designated species *B. velesenzis* only after complete genomic sequencing. Some of the identified bacilli species have been newly discovered in our country, such as *B. safensis*, which so far has been isolated only from the Odyssey spacecraft and the Gujarat desert, and has been found here at the Sofia Waste-water purification station. The strains were tested for their ability to display 12 different hydrolase activities. 15 of the strains were shown to exhibit cellulase activity. Strains that break down hemicellulose substrates have also been identified. Following sequencing, 9 genes for cellulose hydrolyzing and modifying enzymes and 8 genes for hemicellulose conversion enzymes were detected in the genome of *B. velesenzis* 5RB. Similar genes have been found in other *B. velesenzis* strains and the results are confirmatory. Bioinformatic genome analysis also indicates the presence of a complete set of genes from the metabolic pathway for the synthesis of butanediol and is the first report of such a metabolic pathway in the species *B. velesenzis*. 7 more operons have been discovered for the production of antibiotics, which would allow the industrial application of the strain for fermentation under non-sterile conditions.

THIRD POINT OF THE RESULTS AND DISCUSSION chapter presents studies on glycoside-hydrolase enzymes (sialidases or neuraminidases) with sialic acid substrate. These enzymes are a well-known virulence factor for some viruses and pathogens and are widely used in biochemical and medical research. However, the industrial isolation of the enzyme from pathogenic micro-organisms poses a certain health risk to production participants. Assoc. Prof. Petrova sees the only alternative in the use of non-pathogenic strains, but until the beginning of her study there is no information on neuraminidase from a non-pathogenic source. It uses as such a Bulgarian isolate of *Vibrio cholerae* (non-O1 / 13) with an intact neuraminidase gene (*nanH*) and purifies the enzyme from the culture medium. The resulting preparation is enriched about 500 times in enzyme activity. In addition, the neuraminidase gene is sequenced, and all amino acid residues necessary for the enzymatic action of the enzyme are found in the deduced amino acid sequence of the enzyme. In this part of the dissertation, Assoc. Prof. Petrova reports the results of the world's first molecular biological study of neuraminidase from non-pathogenic *Vibrio cholerae*.

THE FOURTH POINT OF THE "RESULTS AND DISCUSSION" chapter reports results related to the use of the glycoside-hydrolases enzyme β-glucuronidase as a model for optimizing heterologous gene expression in methylotrophic yeast *Ogataea polymorpha*. Until now, haploid strains of the species have been used for this purpose and diploid strains have been avoided. According to Assoc. Prof. Petrova, this is due to the misconception that the diploid phase of this species is very short and unstable. Therefore, it aims to investigate the effect of yeast ploidy on the expression of *E. coli*-encoded glucuronidase. For this purpose, various autotrophic mutants of the *O. polymorpha* strain were used as hosts. In her studies, Assoc. Petrova used a plasmid with a cloned β-glucuronidase gene provided by her other researchers. After transformation of the plasmid into yeast, the transformant with increased β-glucuronidase activity were selected and crossed with another haploid strain to produce diploid. Analysis of the meiotic segregants of this diploid shows stable integration of the bacterial gene into the yeast genome. Four of the meiotic segregants with high enzyme activity were cross-linked in all possible combinations and three hybrid diploid strains were selected. Diploids have been found to exhibit up to ten-fold higher enzyme activities, indicating that the approach for generating diploid yeast strains is promising for enhancing the efficiency of heterologous gene expression.

In chapters V and VI of the dissertation, Assoc. Prof. Petrova formulated, respectively, 18 conclusions and 12 scientific and applied contributions, which I emphasized during the analysis of the chapter "Results and Discussion". I will only mention here that the conclusions have a solid experimental basis and the contributions are precisely formulated. The “Abstract” book is compliant and in a compact format of 188 pages, and reflects the main content of the dissertation.

SCIENTOMETRIC INDICATORS

The total number of author's publications in the full text connected with the subject of the dissertation is 33. Of these, two are book chapters referenced in Scopus, 18 publications are in journals with IF, 1 in a journal with SJR, 4 publications are in proceedings of international conferences in full text, 5 are in non-impacted journals and 3 are in national conference proceedings. In 16 of the publications Assoc. Prof. Petrova is the first author, and in 21 - corresponding. The articles related to the dissertation have a total IF of 25.762 and have been cited a total of 281 times. According to indicator G (publications outside the habilitation work) from Table 1 of the Rules For The Application of The Academic Development Law in the Republic of Bulgaria, 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, Assoc. Prof. Petrova collects 1300 points, which exceeds almost 4 times the minimum national requirements. According to the additional criteria for growing the academic staff at IMicB, a minimum of 150 citations are required for the Doctor of Sciences degree. As I have already mentioned, only the articles on the topic of the dissertation have been cited 281 times, and according to the reference of Assoc. Petrova and in other databases they are a total of 693. Total IF is 47.875, compared to required minimum of 25.

CONCLUSION

Assoc. Prof. Petrova presents us with a monolithic, large-scale and in-depth study conducted with the latest microbiological and molecular-genetic methods. A large number of original and significant scientific results with application in practice have been obtained. Most of the research has been published in refereed international journals with IF and has found wide reverberation among the scientific community, as evidenced by the number of citations, over 200, of dissertation articles. Prof. Petrova's scientometric indicators go beyond both the minimum national requirements for “Doctor of Sciences” degree and the additional criteria for the growth of the academic staff at IMicB. All this gives me reason to convince my positive vote on the award of the Doctor of Science degree to Assoc. Prof. Penka Mladenova Petrova in the professional field 4.3. Biological Sciences, specialty "Microbiology".

02/28/2020

Sofia

Reviewer:
/ Prof. R. Mironova /