EVALUATION REPORT

On the PhD thesis for acquisition of the educational and scientific degree "Doctor of Philosophy", professional direction 4.3. Biological sciences, doctoral program - Microbiology
Thesis Author: Vladislava Georgieva Dishliyska, "Stefan Angelov" Institute of MicrobiologyThesis title: CATALASE FROM ANTARCTIC FUNGI: ROLE IN ANTIOXIDANT PROTECTION, REGULATION, AND PROPERTIES
Scientific supervisor: Assoc. Prof. Ekaterina Tsankova Krumova, PhD, Institute of Microbiology "Stefan Angelov", Bulgarian Academy of Sciences
Evaluator: Assoc. prof. Lyudmila Simova-Stoilova, PhD, Institute of Plant Physiology and Genetics, Bulgarian Academy of Sciences

Concerning the procedure: Doctoral student Vladislava Dishliyska is directed to official thesis defense by Decision of the Scientific Council at the Institute of Microbiology-BAS (Protocol No. 7/30.07.2024). According to an Order of the Director of the Institute of Microbiology (No. I-107/31.07.2024), I am included in the Scientific Jury for the defense as an external member, in accordance with the respective Lows, Regulations for the implementation and the Regulations for the terms and conditions for acquiring scientific degrees at the Institute of Microbiology, BAS.

EVALUATION OF THE THESIS:

Relevance and significance of the scientific subject. Recently, there has been an increased interest in organisms adapted to live in extremely cold habitats - most commonly bacteria, archaea and fungi. The harsh conditions in the Antarctic include, in addition to low temperature, high levels of dissolved oxygen, increased UV radiation, insufficient nutrients, a highly variable photoperiod, frequent alternating freeze-thaw cycles and all this leads to severe oxidative stress, but little is known about the adaptation of organisms to such living conditions. Fungi isolated from extremely cold habitats produce temperature-sensitive enzymes that are characterized by conformational flexibility, increased catalytic turnover, and high efficiency at low temperatures. Cold-active catalase is such an enzyme, but there is only scarce information about its role in the survival of extremophiles. Besides the scientific interest, temperature-sensitive enzymes may also have the potential for biotechnological application in some industrial sectors such as e.g. to remove hydrogen peroxide from milk after cold pasteurization, in the textile and pharmaceutical industries and others. In this regard, the presented thesis is relevant and significant both from a scientific-fundamental and from a practical point of view.

Awareness on the current state of knowledge on the problem and theoretical preparation of the candidate. The literature review is targeted and well structured, including the following aspects: characterization of Antarctica as extreme climatic conditions, adaptive mechanisms of microorganisms to low temperatures and classification as psychrophilic, psychrotolerant and mesophilic, oxidative stress and relation between cold and oxidative stress, the leading role of antioxidant enzymes as the first line of defense and detailed characterization of catalase enzymes, features of temperature-sensitive enzymes, filamentous fungi as an experimental model system, classification and morphology of Antarctic fungi and morpho-physiological changes after exposure to extremely low temperatures. The review is concise, specific and clear, illustrated with 7 figures and, together with the methods and discussion of the results, is based on 305 literature sources. In conclusion, an outline of key unsolved issues is formulated that logically lead to clearly defined aims and tasks of the dissertation. The excellent awareness and theoretical preparation of the doctoral student makes a good impression.

The aim of the thesis is to study the involvement of the antioxidant enzyme catalase in the mechanisms of adaptation to low-temperature stress in filamentous fungi isolated from extremely cold habitats (Antarctica). **The set tasks** follow a logical scheme, including an initial characterization of a large number of strains of filamentous fungi isolated by Antarctic soil, in terms of growth and biomass accumulation at different temperatures, and intracellular and extracellular catalase activities. Based on this information, model strains should be selected for the elucidation of the role of catalase in low-temperature stress, for isolation, sequencing and expression of catalase genes, and purification and characterization of temperature-sensitive catalase from a selected producer strain.

Analysis of the methodological approaches to achieve the goals of the thesis. The applied methods are well selected and suitable for solving the set experimental tasks. Classic microbiological, biochemical and molecular genetic methods are combined. All methods are described briefly but clearly and with the necessary details. Changes in cell morphology were followed by light and electron microscopy. Biochemical methods were applied to determine enzyme activities, the amount of protein and oxidative damage to proteins and lipids, concentrations of glucose, glycogen and trehalose, level of generated free oxy-radicals. Molecular biology methods were used to isolate and sequence the genes encoding catalases; their gene expression was estimated in normal and stressed conditions by Real-Time PCR. A temperature-sensitive catalase enzyme from *P. Griseofulvum* has been isolated and purified combining different techniques - ultrafiltration, Q-sepharose column chromatography, hydrophobic phenyl-sepharose chromatography. A correct statistical processing of the experimental results was performed. The ample set of classical and modern methods learned by the doctoral student during her thesis elaboration will be useful for her in the future scientific research.

Evaluation of the achieved results and contributions of the dissertation work. All assigned tasks are sequentially and successfully solved. Initially, a broad screening was done for optimal growth temperature, biomass accumulation and extracellular and intracellular catalase activities (61 strains of Antarctic filamentous fungi from the mycological collection of the Institute of Microbiology, BAS). The strains are assigned to the respective temperature classes (psychrophilic, psychrotolerant and mesophilic). From the total number of strains tested, 19 with higher catalase activity were selected, which were studied in cultivation conditions of deep culture at different temperatures. The temperature optimum for catalase activity was found to coincide with the optimum growth temperature of most strains. A good extracellular catalase activity was established for 8 of the strains studied, from which 2 model strains with stable catalase activity under different growing conditions were selected – one psychrotolerant (P. *griseofulvum* P29) and one mesophilic (P. *chrysogenum* P27). This routine time consuming work is important and necessary for all subsequent tasks.

The two model strains were used for studying the changes in growth, development and sporulation as a result of exposure to cold stress. It was found that the generation of reactive oxygen species and the oxidative damage of proteins and lipids under low temperature stress are more pronounced in the mesophilic strain, especially the oxidative stress has been increased in the mitochondrial fraction. In the mesophilic strain, a higher basal level of the two reserve carbohydrates trehalose and glycogen was found, compared to the psychrotolerant strain, and in stress conditions an additional accumulation of glycogen and trehalose was detected, in connection with their protective effect on lipids and proteins. Under stress, a strong increase in superoxide dismutase activity was observed for the mesophilic strain, while there was a greater increase in catalase activity in the psychrotolerant strain.

Based on these results, the strain *Penicillium griseofulfum* P29 was determined as a better and promising producer of cold active catalase enzyme, that is why this strain has been used for analysis of the morphological and ultrastructural changes occurring under cold stress conditions. It was established that the main damages in the ultrastructure of the cells affect the mitochondria and the cell wall, with an indication of impaired processes of export and/or import of macromolecules or disorders in the processes of cell membrane renewal. The ultrastructural changes corresponded to the biochemical findings concerning mitochondria. The development of oxidative stress under conditions of low temperature stress has been confirmed, as well as the key role of the antioxidant enzymes under cold stress, and more specifically the importance of catalase enzymes.

In the task of identifying the genes encoding for proteins with catalase activity, initially were studied the catalase gene sequences available in the public databases. In the published complete sequence of the species P. griseofulvum, (GenBank GCA_001561935.1), 5 catalase genes have been identified. By means of specific primers, the presence of these genes in P. griseofulvum strain P29 was proven and the corresponding genes were sequenced - cat 1 encodes a bifunctional catalase-peroxidase enzyme with an expected molecular weight of 82 kDa, cat 2 to cat 5 encode monofunctional heme-containing enzymes with expected molecular weights 80, 55, 52, 52 kDa, respectively. The individual catalase enzymes have different structure, localization and functions. The expression of catalase genes in P. *griseofulvum* P29 under normal conditions and under low temperature stress was compared and 4 of the 5 catalase genes were found to have statistically significant increased expression at 10°C, with the largest difference observed in the cat1 gene. The obtained complete sequences have been deposited in GenBank (https://www.ncbi.nlm.nih.gov/genbank/).

In order to characterize the catalase enzyme with the most essential role in low-temperature stress in *P. griseofulvum*, the next necessary steps included optimization of the conditions for its production, followed by isolation and purification of the enzyme from the spent medium. The yield of the enzyme was increased by optimizing the concentration of glucose in the medium, the amount of inoculum, the temperature and the amount of dissolved oxygen. Controlling the amount of dissolved oxygen in the fermentation medium is a hitherto undescribed approach to improve the biotechnological production of catalase from filamentous fungi. A purification scheme was developed for secreted catalase, involving concentrating of the cell-free extract and sequential application of ion-exchange and hydrophobic chromatographic techniques. According to data from SDS-PAGE, the purified enzyme was with molecular mass of 45.5 kDa per subunit and about 182 kDa for the holoenzyme. The enzyme was active in a wide temperature range of 5 - 70°C, with an optimum for activity at 20°C and with a pH optimum between 4.0 and 6.0.

The results are illustrated with 12 tables and 23 figures. The most substantial part of them was included in two publications with an impact factor and reported at two scientific forums. The abstract of the thesis completely corresponds to what is stated in the dissertation.

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

1. New evidence for the involvement of the enzyme catalase in the adaptation to low-temperature stress of filamentous fungi isolated from Antarctica.
2. New data on the distribution of intracellular and extracellular catalase in Antarctic fungi from different thermal classes.
3. An efficient method developed for the purification of temperature-sensitive catalase from *P. griseofulvum*, an enzyme with a temperature optimum of 20°C and a pH optimum of 6.0.
4. Five genes encoding catalases in *Penicillium griseofulvum* P29 were identified and their complete nucleotide sequences were deposited in a database.

Questions. I have a few questions concerning the thesis

1. About 90% of the catalase activity is intracellular - in the cytoplasm and peroxisomes, and very rarely such activity is found extracellularly - what is its function? Is it an active secretion? Is there any relation between extracellular and intracellular catalase activities?
2. To which of the five established catalase genes of P. *Griseofulvum* the purified enzyme product belongs? Is any further been done to establish the AA sequence of the purified enzyme?
3. It becomes clear that thermo-sensitive catalase has a great potential for practical application in the textile, food and pharmaceutical industries - what are the next steps in this direction?

Recommendation: The CV is very poor in terms of information. Vladislava Dishliyska graduated with a master's degree in biotechnology at the University of St. Cl. Ohridski", began work in the Institute if Microbiology in 2005, assistant since 2007 - that's all. There is no information in the CV about publications, citations, participation in projects, and there are such. According to Scopus author Vladislava Dishliyska - there are 14 articles in the period 2021-2024, citations in total 27, h index 4 – a pretty good scientific activity. The articles on catalase are 4, and two of them are on *Penicillium griseofulvum* - on the thesis topic. The remaining 10 publications are on other topics and on projects developed by the group, which speaks of active participation and contribution to the common work, good cooperation and productive teamwork of the doctoranl student. These are qualities that should be reflected in the CV. My recommendation is to pay attention to the CV in the future, which is like a business card for the scientists.

CONCLUSION. Based on the above, I consider that the presented PhD thesis is an original scientific development with theoretical and applied significance, it meets all the conditions of the Law on the Development of the Academic Staff in the Republic of Bulgaria, the Regulations for its application and the Regulations of the Institute of Microbiology - BAS. This gives me grounds for an overall high assessment of the presented work, on the basis of which I confidently propose to the respected scientific jury to award Vladislava Georgieva Dishliyska the educational and scientific degree "DOCTOR" in professional direction 4.3. Biological sciences, doctoral program – Microbiology.

На основание
чл. 2 от ЗЗЛД

Sept 10, 2024

(assoc. prof. L. Simova-Stoilova)