REVIEW

by **Prof. Maria Bogomilova Angelova, DSc**, The Stefan Angelov Institute of Microbiology, Bulgarian Academy of Sciences

on a dissertation submitted to a Scientific Jury, formed by order № RD-38-354/10.07.2023 of the Rector of Sofia University "St. Kliment Ohridski" for obtaining the educational and scientific degree "Doctor" in Professional direction: 4.3. Biological Sciences (Microbiology)

Author of the dissertation: **Polya Galinova Marinovska**

Topic of the dissertation: **Quiescent state in yeast Saccharomyces cerevisiae – a model for studying toxicological and stress response**

Supervisor: **Assoc. Prof. Ventsislava Petrova**, **PhD**

Relevance and significance of the PhD thesis subject

First of all, I want to note that the presented dissertation is multidisciplinary and is aimed at the most serious challenges of today - environmental protection, drug resistance, and oxidative stress, to which the aging process should be added. Each of them is the object of increasing interest in scientists due to the complex mechanisms of control, due to unexplained problems, and their extraordinary impact on the health and social life of people. Collected in one place, they characterize the relevance of the development, delineate a broad and unexplored niche that covers both scientific and applied aspects, and are a prerequisite for original contributions. Another merit of the dissertation should be added here - the use of the yeast species *Saccharomyces cerevisiae* as an object of research. As is known, *S. cerevisiae* is one of the first living organisms used in human life, they are an efficient "cellular factory" for the production of biologically active substances and a suitable model for elucidating the molecular mechanisms underlying a number of biological processes. Their unique genetic characteristics and eukaryotic nature allow the multiplication of the resulting biological dependencies in higher eukaryotes. As proof of the relevance of the topic, I would like to note that the International Journal of Molecular Sciences (IF 5.6; Q1) will publish at the end of 2023 a special issue entitled "Stress Response Research: Yeast as Models".

The main theoretical focus of the research is related to gaining new knowledge about the relationship between the phases of the life cycle in *S. cerevisiae* and the characterization of the cellular response against stress stimuli. In a scientific-applied aspect, the dissertation proposes a new, alternative model for analysis, on the basis of which predictive information for toxicological response in higher eukaryote organisms can be obtained, useful in the development of new drugs.

Knowledge of the subject

The dissertation is constructed in a traditional academic form with relevant sections. It is written in 229 standard computer pages and illustrated with 61 figures and 5 tables. The literature review is based on 575 scientific publications, it is focused, covers a wide range of aspects related

to the studied problem, and reflects its contemporary level. In 45 pages, Marinovska very clearly and accessibly introduces readers to the theory of all forthcoming research and demonstrates her strong expertise in the field. In the end, a subsection "Conclusion" is presented that highlights the unresolved issues and argues the need for investigations. In my opinion, this is a very good idea; the text and the 13 figures included in the review, make it easier for the reader to appreciate the relevance of the thesis.

Based on this in-depth analysis, the aim of this thesis is derived: to investigate the applicability of *S. cerevisiae* cells at different life cycle phases as a eukaryotic model for cellular responses to toxic and stress agents. In my opinion, it corresponds to the relevance of the problem and highlights the innovative nature of the dissertation. The aim is clear, well formulated, and unifies the lines of experimental work. For its realization, 8 specific, interrelated, and logically following tasks have been formulated, which include all mandatory stages of such a study. Already here one can see the serious amount of work set before the PhD student.

Methodology of the study

The section “Materials and Methods” demonstrates a very broad range of methods tailored to the specific requirements of the experiment. They are both routine and advanced, microbiological, biochemical, molecular-biological, bioinformatic, etc. The preparation of cell fractions, including ultracentrifugation, microscopic analyses, the assessment of different types of abiotic stress, the work with ultrasound and gravitational force, the analysis of stress biomarkers and free-oxy radical (ROS) concentration, etc. are impressive. I would like to highlight the molecular genetic approaches used to study the effect of stress stimuli on DNA changes, as well as the bioinformatic methods used for sequence selection, the programs used for multiple sequence comparison, the analyses of intracellular localization of enzymes, and the recognition of mitochondrial, nuclear, and peroxisomal proteins. The methods are described in great detail (in some places even with redundant details) and can be reproduced. All are sufficient grounds for reliability and precision.

Characterization and evaluation of the dissertation and contributions

The section "Results and discussion" in the dissertation of Polya Marinovska outlines a serious scientific study, which is illustrated with 48 figures and 5 tables. The study is distinguished by its large scale and clearly expressed logical sequence. The main part of the experimental work is related to obtaining a population of G_0 cells from the haploid strain *S. cerevisiae* BY4741 from the collection of EUROSCARF Frankfurt. This strain, the subject of much research, was chosen with the presumption that the results obtained in the thesis could be compared with those previously published. The conditions for yeast cell differentiation were established and the populations are characterized. The compounds menadione, H_2O_2, ibuprofen, and zeocin were selected for the

evaluation of the toxicological impact. Following the mandatory survival study of three subcellular fractions (Log, Q, and NQ) of the model strain upon challenge with the test compounds, the LD_{50} concentration for each was determined. This concentration is the basis for the following experiments. In the second stage, the cytotoxicity of the model compounds was analyzed by determining the level of the main biomarkers (ROS, oxidatively damaged proteins, and lipid peroxidation) and the level of antioxidant protection through the amount of total glutathione. In my opinion, the selected criteria allow for a correct assessment of the degree of stress in the examined cells - Log, Q, and NQ. In addition to the evidence of the effect of zeocin, DAPI fluorescence images are presented of the changes in mitochondrial DNA from cells in the exponential and stationary phase, as well as the accelerated generation of ROS with Rhodamine 123 staining. The author's effort to characterize most fully the cytotoxic effects of each compound on individual subcellular fractions based on a large number of literature sources should be emphasized here.

Following the assessment of the cytotoxicity, as a logical extension, the genotoxicity of menadione and zeocin was investigated by molecular genetic studies on their effect on DNA double-strand breaks (DSBs). The author compared spontaneous and induced DSBs in Log, Q, and NQ cell populations of *S. cerevisiae* strain BY4741. The repair capacity of zeocin treatment was calculated to be highest in Log-growing cells (60 min for damage repair).

To characterize the model, Marinovska studied the influence of different physical factors on the survival of cell populations of the model strain. I would like to emphasize the importance of the conducted experiments in achieving the set goal. All these factors (temperature, pH, osmotic stress, UV, ultrasound, gravitational and mechanical force) were applied to Log, Q, and NQ cell populations with time or concentration variation. It was determined which cells were most resistant to the respective impact. In the discussion, the author searches for logical explanations against the background of a defense complex regulated at the level of morphology, at the gene or biochemical level, at the level of a cell differentiation program, or tolerance to the respective factor. Some specific elements of the cellular response against oxidative stress (e.g. the presence of reserve carbohydrates) are also noted. I would recommend some further consideration of the relationship between these physical factors and the generation of oxidative stress.

The bioinformatic analysis of the strategies of *S. cerevisiae* for entering cells into a resting state sounds like a logical conclusion to the section. *In silico* data were obtained on the presence of the *TOR1* and *TOR2* genes, encoding the synthesis of two PIK (phosphoinositide)-related protein kinases, which are involved in the regulation of a number of cellular processes. The possibilities for targeting these kinases to peroxisomes, to the nucleus, or to the vacuole have been interpreted in great detail. The data in the dissertation confirm a published opinion in the literature that these are peripheral membrane proteins whose C- and N-termini are located on the inner side of the membrane and are associated with the response to stress and the state of rest. Bioinformatic analysis

of the genome of the model strain showed the presence of the genes *TRK1*, *TRK2*, and *TRK3*, which encode the proteins Trk1p, Trk2p, and Trk3p. The labeled proteins make up the catalytic subunits of cAMP-dependent protein kinase (PKA), promoting vegetative growth in accordance with the availability of nutrients in the cell. Data on the intracellular localization of the proteins involved in the PKA metabolic pathway were obtained and the differences and similarities with the human data were demonstrated. With the same methods, the PKC1 gene encoding the synthesis of serine/threonine protein kinase C (1151 aa) and the SNF1 gene encoding the yeast homolog of the human AMP-activated protein kinase (AMPK) were identified. Convincing data were obtained for the amino acid sequence, the percentage of homology with the human genome, and the probability of localization in the cell. The evidentiary material from this subsection and especially the tables, allows its correct perception by the reader.

The presentation of the results is combined with a skillful discussion at all stages of the study. A thorough and professional discussion of the data has been made, using appropriate literature sources. This creates an impression of confidence and commensurability with what other authors have published. The discussion also demonstrated the serious experimental work of the doctoral student and her deep knowledge in this field. It makes a very good impression that, in addition to comparing the results with what has been achieved by other authors, the doctoral student is looking for new explanations and correct proposals for a mechanism of action.

At the end of the dissertation, a section "Conclusion" was created, which very informatively summarizes the obtained data, and their significance and presents a Physiological profile for the response to stress of *S. cerevisiae* cells located in different phases of the cell cycle - proliferative, G_0 and stationary non-proliferative phase. Moreover, the shaped figure (Fig. 61) provides a visual insight into the stress response characterization of the Log, Q, and NQ subcellular populations at the morphological, biochemical, and molecular levels. This way of conclusion makes it easier for the reader to orient himself very precisely in the study and perceive the achievements in it. Here I would like to emphasize the role of the research supervisor, Assoc. Prof. Ventsislava Petrova, who is one of the few specialists in the field of the doctoral thesis and, in my opinion, contributed to the excellent presentation of the material.

I want to emphasize with pleasure the very good layout of the dissertation, the tight scientific style in which it was written, the correct reflection of the results in tables and figures, as well as their professional presentation.

In my opinion, the conclusions are a logical consequence of the experimental data and provide the necessary information about the value of the research conducted. I also accept the wording of the contributions and want to emphasize their importance in a theoretical and scientific-applied aspect. Essentially, they refer to the following:

1. The goal of the dissertation was achieved – the applicability of *S. cerevisiae* cells in different phases of the life cycle as a eukaryotic model for cellular response to toxic and stress agents was proven. For the first time, a comparative Functional Profile has been developed, which enables further theoretical and scientific-applied studies.

2. New knowledge was obtained regarding the adaptive response of *S. cerevisiae* cells in different phases of the cell cycle to a wide range of abiotic stress stimuli.

3. New information was obtained about the genotoxic effect of the drugs menadione and zeocin on the DNA of yeast cells.

4. The high redox status of cells from the G_0 population of *S. cerevisiae*, which, like cancer cells, rapidly generate ROS, was characterized.

5. With modern bioinformatics approaches, the genes responsible for the G_0 phase of the yeast cell cycle and their homology with those in humans have been characterized.

6. Valuable information was obtained regarding the lower level of susceptibility of metabolically inactive eukaryotic cells to antitumor agents.

7. The metabolic characterization of G_0 cells makes it possible to use them as a model for the study of:

- the toxicological mechanisms of harmful substances in animals and humans;
- the resistance to chemical and physical stress for application in industry.

Critical remarks, recommendations, and questions

In addition to the remarks noted above in the text, I have the following recommendations for Marinovska's dissertation:

1. The articles included in the dissertation should not be part of the bibliography.

2. I recommend that the author should start the appropriate subsection in the section “Results and Discussion” with her own results and then present their discussion. Long preambles characterize some of the passages as better suited to the sections “Literature Review” and “Materials and Methods”.

The mentioned remarks are of a technical character and do not in the least detract from the value of the dissertation. I mark them with the presumption that I will be useful for the future work of the doctoral student.

I have the following questions for PhD student Polya Marinovska:

1. What are the characteristics of G_0 cells that make them an effective model for studying toxicological and stress response?

2. Besides double-strand DNA breaks, what other damages can zeocin exposure cause at the genetic level

3. In what direction can the work on the presented problem be continued based on the obtained theoretical and applied results?

Assessment of publications and personal contribution of the PhD student

Data from the dissertation have been included in 2 scientific articles and 6 presentations to scientific forums. The journal articles have been printed in the journal BioRisk with IF 1.167 and quartile Q2. The results have been presented at 4 international and two national forums. This gives me reason to believe that Polya Marinovska's results have become known to our and the international scientific community. In all articles and 5 forum presentations, the PhD student is ranked first, which proves her significant contribution to the development of the thesis.

Conclusion

In conclusion, I would like to emphasize that the PhD student has fulfilled the requirements ZRASRB and the additional criteria of the SU “St. Kliment Ohridski” for obtaining the educational and scientific degree "Doctor". The material presented by Polya Marinovska is dissertable, the topic is relevant and offers a contemporary level of an important issue for theory and practice. The conducted experiments are methodologically correct, the obtained results are reliable and are a solid basis for further scientific and applied studies. A great deal of experimental work has been carried out, and the problem posed has been studied in a multifaceted and detailed way at a modern level, significant contributions have been made, and suggestions for application in practice have been formulated. To this characteristic of the dissertation I would like to add that, in my opinion, Polya Marinovska comes out of the PhD training as a well-trained specialist in the field of microbiology and molecular biology, has mastered a large number of modern methods, has gained experience in interpreting scientific data. All of this qualifies her as a young scientist, a worthy competitor to colleagues from foreign laboratories.

On the basis of the analysis made and the proven growth of the PhD student, I give my high evaluation for its defense and recommend the members of the Scientific Jury to award **Polya Galinova Marinovska** the educational and scientific degree "Doctor" in Professional direction 4.3. Biological Sciences (Microbiology).

22. 09. 2023

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/Prof. M. Angelova, DSc/