

REVIEW

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

on a dissertation for awarding the educational and scientific degree "Doctor"

by Field of higher education 4. Natural sciences, mathematics, and informatics;

Professional direction 4.3. Biological Sciences; PhD program Biochemistry

Author of the dissertation: Stanimira Angelova Angelova

Topic of the dissertation: INVESTIGATION OF THE PROPERTIES OF BIOENGINEERED ALPHA-D-GLUCANS, SYNTHESIZED BY MUTANT GLUCANSUCRASE URE 13-300

Supervisor: Prof. Ilia Iliev, PhD, Paisii Hilendarski University of Plovdiv

1. General description of the presented materials

By order No. PD 21-2465 dated 18.12.2023 of the Rector of the Plovdiv University "Paisii Hilendarski" (PU), I have been appointed as a member of the scientific jury to ensure a procedure for the defense of a dissertation on the topic: "Investigation of the properties of bioengineered alpha-d-glucans synthesized by mutant glucansucrase URE 13-300" for obtaining the educational and scientific degree "Doctor" in the field of higher education 4. Natural sciences, mathematics, and informatics; Professional direction 4.3. Biological Sciences; PhD program Biochemistry. The author of the dissertation is Stanimira Angelova Angelova, a PhD student in the full-time form at the Department of Biochemistry and Microbiology with the scientific supervisor Prof. Dr. Ilia Nikolov Iliev from Plovdiv University "Paisii Hilendarski".

The set of materials presented by Stanimira Angelova is in accordance with Art. 36 (1) of the Regulations for the Development of the Academic Staff of the PU and includes all the required documents. The PhD student submitted 3 scientific publications, participated in 6 scientific forums, and 5 research projects (1 with international funding). All of them are related to the developed doctoral thesis. The number of scientific articles corresponds to the requirements of the Law on the Development of Academic Staff in the Republic of Bulgaria (LDASRB) and the Regulations of the PU.

2. Brief biographical data about the PhD student

The PhD student Stanimira Angelova received her higher education at the Faculty of Biology of PU. In 2014 she graduated with a Bachelor's degree in Molecular Biology, and in 2018 she obtained a Master's degree in "Biopharmaceutical Biochemistry". After that she worked successively as a customer service organizer at Retail Direct Ltd., as a chemist-analyst at Biovet AD, Sofia, and as an analyst at Bio Biovet in Peshtera/Huvepharma. Her scientific career began in May 2020, when she was appointed as a researcher at the Centre of Technology at PU. The entire training of the doctoral student and the work before the start of the doctoral studies are aimed at obtaining knowledge and experimental skills in the field of molecular biology and biochemistry, a

basis for her further scientific career. For the period from 01.03.2020 to 01.05.2023 Stanimira Angelova is a full-time PhD student at the Department of Biochemistry and Microbiology of PU.

3. Actuality of the topic and appropriateness of the set goals and tasks

First of all, I would like to point out that the presented thesis is multidisciplinary and addresses the most serious challenges of today - the creation of new materials for products of medical and industrial importance. To this must be added their significant ecological effect. In recent years, biopolymers have emerged as a sustainable alternative to traditional plastics. Advances in this field open new directions for the rational engineering of bacteria for targeted production of biopolymers. Knowledge of the molecular and regulatory mechanisms that underlie these processes is constantly growing. This provides powerful tools for engineering bacteria capable of efficient production of biopolymers and modified polymers with unique properties for a specific application with high economic and environmental value. The global bioplastics and biopolymers market has demonstrated significant growth. It is projected to reach \$31 223 million in 2027.

Taken together, these effects characterize the relevance of the thesis, outline a broad and unexplored niche that encompasses both scientific and applied aspects, and are a condition for original contributions. Moreover, Stanimira Angelova had the chance to work in the school of Prof. Iliev, which is a prerequisite for a modern level of research and the growth of a qualified scientist in the modern field of biochemistry.

The main theoretical orientation of the research is related to obtaining new knowledge on the synthesis of new biopolymers by bioengineering approaches, as well as evidence of the relationship between the structure of the enzyme and the structure of the product of the enzymatic reaction. In the scientific and applied aspect, the thesis proposes a new, alternative model for the production of alpha-d-glucans based on mutant glucansucrase with a perspective for application.

4. Knowing the problem

In my opinion, the presented doctoral dissertation is a large-scale scientific study, which respects its in-depth theoretical analysis, modern methodological approaches, and logically connected experiments, each of them with a serious evidential value. The dissertation is constructed in a traditional academic form with appropriate sections. It is written on 157 standard computer pages and illustrated with 37 figures, 12 appendices, and 5 tables. The literature review is formed on the basis of 175 scientific publications, it is targeted, covers a wide range of aspects related to the studied problem, and reflects its current level. In 32 pages, Angelova very clearly and accessible introduces the readers to the theory of all forthcoming research and demonstrates her strong expertise in the field. It introduces the reader to the characterization of bacterial glucansucrases, their structure and functional organization, and the catalytic mechanism of glucan synthesis. Attention is paid to the mutations of the genes encoding glucansucrases of the GH70 family, the structure of glucans and oligosaccharides, and their properties. An important part of the review is the subsections

on the design of glucansucrases for the preparation of polysaccharides and oligosaccharides with controlled chain length, as well as the design of glucansucrases and branching sucrases for the glycosylation of non-natural acceptors. The review concludes with data on the application of alpha-glucans and oligosaccharides produced by the glucansucrases of GH70 family.

Based on this in-depth analysis, the aim of the present thesis was derived - to investigate the relationship between the structure of the synthesized glucans and the changes of the amino acid sequence of catalytic domain 1 of glucansucrase URE 13-300 by site-directed mutagenesis. In my opinion, the aim corresponds to the relevance of the problem and highlights the innovative characteristics of the dissertation. It is clear, well formulated, and unifies the strands of experimental work. For its realization, 7 specific, interrelated, and logically following tasks have been formulated, which include all mandatory stages of such research. Already here one can see the serious amount of work that the doctoral student has to do.

5. Research methodology

The section Materials and Methods demonstrates a very wide range of methods tailored to the specific requirements of the experiment. They are both routine and state-of-the-art in the fields of microbiology, biochemistry, molecular biology, bioinformatics, etc. Of note are the methods for conducting site-directed mutagenesis, which involves designing primers and performing PCR to replace the glycine codon with the lysine codon; transforming into *E. coli* strain BL21 (DE3) Hi-control for expression of the mutant gene; bioinformatics analysis and homology model building. SDS-PAGE, NMR, and HPLC analyses of α -glucans and oligosaccharides were used. The methods are described in great detail and can be reproduced. All of them are sufficient grounds for credibility and precision. This section once again emphasizes the innovative character of the dissertation and its high level.

6. Characterization and evaluation of the dissertation work

The section "Results and discussion" outlines an in-depth scientific study, realized at a high methodical level. The work is distinguished by a clearly expressed logical sequence, by a large number of purposefully conducted experiments, and by the competent analysis of the obtained results. The first and mandatory part of the experimental work is related to optimizing the expression of the gene encoding glucansucrase URE 13-300 in terms of the main characteristics of the fermentation process – biomass, duration, importance of the inducer, and activity of the synthesized enzyme. The obtained remarkable increase in enzyme activity is the basis for the next stage, in which optimization of the conditions for enzymatic synthesis of α -glucan in a bioreactor was achieved. A detailed study of the enzymatic reaction in an aqueous-organic medium follows. Results were obtained for the effect of 9 organic solvents at different concentrations. Of interest is the conclusion that the recombinantly produced glucansucrase URE 13-300 is inhibited by lower alcohols with low molecular weight, in contrast to the native type of enzymes. After the selection of suitable solvents,

data were obtained on their influence on the transferase reactions in the presence of maltose as an acceptor. The kinetic parameters of the reaction were determined and a 30% increase was demonstrated, compared to the reaction in the presence of sucrose alone. The kinetic characteristics of the enzyme reaction in the presence of the selected organic solvents in the appropriate concentrations are also presented. This part includes extensive evidentiary material in the text and the appended Appendix #1.

The next stage includes the mandatory characterization of the enzymatic reaction through the influence of organic solvents on the degree of polymerization of the resulting glucooligosaccharides. The compositions of the resulting oligo-saccharide mixtures were determined by HPLC analysis. I would like to note that the analyses were carried out during the transferase reaction, which emphasizes the level of reliability. These data are very clearly demonstrated in Fig. 11, which offers an overview of oligosaccharides according to their degree of polymerization. Here we must add the extremely detailed presentation of the dynamics of the synthesis of oligosaccharides with DP 5 in the presence of two different concentrations of organic solvents and their informative presentation in figures. With these experiments, it is possible to increase the yield of glycosylated acceptor substrates with increased water solubility. The possibility of glycosylated terpenoids and essential oils being included in the composition of nutritional supplements with probiotic, antimicrobial, and anti-inflammatory properties is discussed.

The PhD student presents evidence of successful site-directed mutagenesis in the gene encoding glucansucrase URE 13-300, which is a major achievement of the thesis. The experiments were conducted according to all the rules of the methodical approach used. Through bioinformatics analysis, the amino acid sequence of glucansucrase URE 13-300 was proven and the conservative motifs responsible for the specificity of the connections in the synthesized products was determined. Based on these results, a homology model of catalytic domain 1 was created and a concept for the conformation of the enzyme before and after the amino acid substitution was proposed. A plasmid containing the gene for expression was isolated from a recombinant strain *E. coli* BL21 URE 13-300 and cultures carrying the target mutation were obtained. The author presents the necessary evidence in confirmation of the mutation that occurred - SDS-PAGE analysis and the sequence of that part of the gene encoding glucanucrase URE 13-300, in which the mutation was introduced. As a result of the conducted experiments, a functioning glucansucrase U13M1 was obtained, which can successfully synthesize polymer from sucrose in the appropriate medium.

Also of interest are the studies on optimizing the conditions for the synthesis of mutant glucansucrase U13M1 and the conditions of the enzyme reaction. The parameters for efficient yield of the enzyme and its application were studied in detail. A remarkable change in the kinetic parameters of the mutant enzyme U13M1 compared to the original type of glucansucrase was demonstrated. The activity of the U13M1 mutant enzyme is significantly more resistant to inhibition

by high sucrose concentrations, which is an important requirement for its use in real conditions. It should be noted that similar data have not been reported in the literature.

As a logical end of the section, the author presents the preparation of α -glucans and oligosaccharides with mutant glucansucrase U13M1, as well as the data on their structure. Using NMR spectroscopy and rheological analysis, the differences between the two enzymes were established, which concern the formation of binding sites with the acceptor substrate and the high dynamic viscosity of the modified polysaccharide.

The presentation of the results is combined with a skilful discussion at all stages of the development based on appropriate literature sources. This section also demonstrates the serious experimental work and deep knowledge of the field of the PhD student. It is noteworthy her approach to discuss in great detail the results of the study, to compare the results with the data of other authors. At the end of each subsection, Stanimira Angelova forms a conclusion that very informatively summarizes the obtained data, what is new in them, their importance for achieving the set goal, and the possibility of being a basis for further theoretical and applied research.

I want to emphasize with pleasure the excellent 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 conducted research.

7. Contributions and significance of the study for science and practice

I accept the formulation of the contributions and I would like to emphasize their importance in theoretical scientific and applied aspects. Essentially, they relate to the following:

1. The aim of the thesis has been achieved - by site-directed mutagenesis, a mutant with a changed 3D structure in catalytic domain 1 was obtained for the first time, and data on the relationship between the structure and properties of the mutant glucansucrase were obtained.

2. New knowledge regarding the synthesis and properties of bioengineered alpha-d-glucans by mutant glucansucrase was obtained.

3. For the first time, the possibility of effective oligosaccharide synthesis with a maltose acceptor in an aqueous-organic medium with glucansucrase was demonstrated.

4. A functional mutant glucansucrase (U13M1), more resistant to high sucrose concentrations was obtained. The enzyme can successfully synthesize a polymer from sucrose in the appropriate medium.

5. New information regarding the potential of single point mutations in glucansucrase with two catalytic domains, as well as their importance for the synthesis of modified α -glucan with altered structure and improved physicochemical and rheological properties was obtained.

6. The successfully generated glucansucrase URE 13-300 mutation without shortening the gene length is a theoretical basis for studying the interaction between the two catalytic domains acting as dextransucrase and branching sucrose, respectively.

7. The author proposed a method for:

- glycosylation of acceptor substrates, poorly soluble in water but with improved water solubility and bioavailability. They can be applied in the food industry and as new formulas in pharmacy and cosmetics.
- glycosylation of terpenoids, derivatives of essential oils, with antimicrobial, analgesic and anti-inflammatory properties. They can be included in the modelling of dietary supplements.

8. Evaluation of publications on the dissertation work

The data from the dissertation are included in 3 scientific papers and 6 participations in scientific forums. The papers are published in renowned specialized journals: Catalysts - IF 3.9 and quartile Q2; Acta Microbiologica Bulgarica - quartile Q4 and Ecolgia Balkanica - Q4. Reports have been presented at 4 international and 2 national forums. It is an indication that the results have become available to our and the international scientific community. In two of the articles, Stanimira Angelova is in 1st place and in 1 – in 2nd place, in all 6 reports she is in 1st place. This proves her significant contribution to the development of the thesis.

Here I would like to add that the PhD student participated in 5 research projects, 1 of which was internationally funded, 1 – Centre of Competence; 1 was funded by FNI and 2 were funded by PU "Paisii Hilendarski", which gave her the opportunity to increase the skills necessary to develop of the dissertation. Stanimira Angelova's specialization at the Centre for Synthetic Biology at the Department of Biotechnology of the University of Ghent, Belgium contributed to this.

9. Abstract

The abstract has been prepared according to the requirements of the Regulations of PU "Paisii Hilendarski", it correctly and sufficiently reflects the obtained results, conclusions, and contributions of the work. The PhD student presents the abstract in Bulgarian and in English. They are identical in content and layout.

10. Critical remarks and recommendations

I have no critical remarks about the presented dissertation, it meets the set goal, as well as the requirements of the "Paisii Hilendarski" PU Regulations. It is prepared in an academic style and at a high methodical level. I have the following recommendations:

1. The literary review should be designed and published as a review article.
2. A patent or utility model application should be mad

CONCLUSION

In conclusion, I would like to emphasize that the presented doctoral dissertation is an original scientific study of an innovative idea, respectable with its scale and research depth. The dissertation contains scientific, scientific-applied, and applied results, which represent an original contribution to science and meet all the requirements of LDASRB, the Regulations for its Implementation, and the relevant Regulations of PU "Paisiy Hilendarski".

The material presented by Stanimira Angelova is dissertable, the topic is current and offers a modern level of an important issue for theory and practice. The conducted experiments are methodically correct, the obtained results are reliable, and are a solid basis for further scientific and applied developments. A great deal of experimental work has been carried out, and the problem has been studied in many ways and in detail at a modern level, and significant contributions have been made, both theoretical and applied. To this characteristic of the dissertation, I would like to add that, in my opinion, Angelova leaves the doctoral program as a well-trained specialist in the field of biochemistry and molecular biology, she has mastered a large number of modern methods, and she has gained experience in interpreting scientific data. All of this qualifies her as a scientist worthy of competing with colleagues from foreign laboratories.

Based on the analysis made and the proven growth of the doctoral student, I propose to the respected members of the scientific jury, formed by order No. PD 21-2465 of 18.12.2023 of the Rector of Plovdiv University "Paisii Hilendarski" to award Stanimira Angelova Angelova the educational and scientific degree of "Doctor" in scientific field 4.3 Biological Sciences, PhD program Biochemistry.

15. 02. 2024
Sofia

Reviewer:.....
/Prof. M. Angelova, DSc/