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INVESTIGATION OF THE PROPERTIES OF BIOENGINEERED ALPHA-D-GLUCANS, SYNTHESIZED BY MUTANT GLUCANSUCRASE URE 13-300

ABSTRACT

of a dissertation
to acquire the educational and scientific degree **„doctor”**
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All experimental work was done at the Department of "Biochemistry and Microbiology" at the Faculty of Biology and at the laboratories of Centre of technologies of the University of Plovdiv "Paisii Hilendarski".

The PhD student has published a total of 3 articles in journals referenced and indexed in the databases Web of Science and Scopus.

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The materials on the defense are provided for free access of your interests in the library of PU "Paisii Hilendarski".

Author: Stanimira Angelova Angelova

Title: Investigation of the properties of bioengineered alpha-D-glucans, synthesized by glucansucrase URE 13-300

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INTRODUCTION

Polymers are used as basic materials for the production of many everyday products, as well as for more complex materials in medicine, diagnostics and the light chemical industry. Concern for environmental protection and sustainability is pushing forward research on renewable and recyclable bio-based structures providing new or improved physicochemical properties. Among the polymers of biological origin, polysaccharides from plants, algae, fungi or microbial organisms are considered as promising alternative for the petroleum-based products. Also, the numerous enzyme sequences established and advances in the design of enzyme catalysis have led to an increased demand and supply of polymers synthesized from microbial sources.

In this regard, particularly attractive objects are α-glucans produced by different genera of lactic acid bacteria (LAB) (mainly *Streptococcus*, *Leuconostoc*, *Lactobacillus*, *Weissela*) cultivated with sucrose (Molina, M. *et al*. 2021). The group of enzymes catalyzing the synthesis of these glucans are classified in the glycoside hydrolase 70 (GH70) family. Glucan polymers differ in size, type of linkages, and degree of branching. These characteristics influence the physicochemical properties of polysaccharides (van Hijum, S. A. *et al.,* 2006). According to the type of α-glycosidic bonds in the polymers synthesized by glucansucrases, distinguished are: glucan, mutan, reuteran, dextran and alternan (Korakli, M. & Vogel, R., 2006).

Glucansucrases have domain molecular organization with five different regions in the catalytic domain having complementary functions in the course of glucan or oligosaccharide synthesis - A, B, C, IV and V. In the recent years, numerous studies have been reported with amino acid substitutions by site-directed mutagenesis, demonstrating the function of various amino acids located in proximity or around the catalytic residues of glucansucrases that are key to the enzyme activity and the type of linkages between glucose residues in the corresponding glucans. Thanks to advances in the rational design of glucansucrases to obtain tailored polymers with desirable properties, they have the potential for multiple applications in the food, medical, and cosmetic industries as value-added products.

Strain *Leuconostoc mesenteroides* URE 13, isolated from Bulgarian fermented vegetables, produces high molecular weight glucansucrase with a molecular mass about 300 kDa. The gene encoding glucansucrase URE 13-300 was successfully cloned and expressed in *Escherichia coli* BL21. The glucan synthesized by this enzyme is insoluble and contains a significant amount of α-(1→3) linkages, and the oligosaccharide products have prebiotic potential (Bivolarski, V. *et al.,* 2018). In the present work, the experiments on optimizing the synthesis of glucansucrase URE 13-300 from the recombinant strain *E. coli* BL21 and obtaining its mutant variant by site-directed mutagenesis are described. The mutated enzyme was biochemically characterized. The properties of the glucans synthesized by the wild-type and mutant enzymes were analyzed and compared.

AIM AND TASKS

The aim of the present thesis is to investigate the relationship between the structure of synthesized glucans and the substitution of the amino acid of catalytic domain 1 of glucansucrase URE 13-300 by site-directed mutagenesis.

To achieve the set goal, it is necessary to solve the following tasks:

1. To optimize the conditions for production of glucansucrase URE13-300 during cultivation by recombinant strain *E. coli* BL21 URE 13-300.
2. To study the influence of organic solvents on the transferase reaction of glucansucrase URE 13-300 in the synthesis of oligosaccharides.
3. To carry out bioinformatic analysis for detection of possible amino acid substitution sites and design of mutagenic primers.
4. To perform site-directed mutagenesis in order to obtain mutant glucansucrase URE 13-300.
5. To optimize the conditions for production of mutant enzyme, cloned in *E. coli* BL21.
6. To determine the kinetic parameters of the obtained mutant enzyme.
7. Enzymatic synthesis of polysaccharide and oligosaccharides synthesized by the mutant enzyme and determination of their structure.

MATERIALS AND METHODS

1. Microbiological methods

1.1. Bacterial strains and vectors

For expression of the starting type of glucansucrase URE 13-300, a recombinant strain Escherichia coli BL21 URE 13-300, developed at the Department of "Biochemistry and Microbiology" of University of Plovdiv "Paisii Hilendarski", containing a plasmid vector pETite N-His SUMO (Lucigen Corporation, Middleton, USA) in which the gene encoding the glucansucrase URE 13-300 was cloned. The specified vector also serves as a DNA template for introducing a point mutation by the method of site-directed mutagenesis. It was carried out using a specialized site-directed mutagenesis kit QuikChange II XL (Agilent Technologies, Cat. No. 200522). Ultracompetent cells *E. coli* XL10-Gold provided with the kit were transformed by the plasmid harboring the mutated gene. Hemocompetent *E. coli* BL21 (DE3) cells (Lucigen Corporation, Middleton, USA) were used for the expression of the mutant enzyme.

1.2. Nutritional media

2. Molecular biology methods and bioinformatics

2.1. Isolation and purification of plasmid DNA from recombinant strain *E. coli* BL21

For this step QIAprep Spin Miniprep kit (QIAGEN, Germany) was used.

2.2. Site-directed mutagenesis

2.2.1. Primer design for introducing amino acid substitution of glycine at position 449 with lysine

The following primers were used for the amino acid substitution (Bioneer, Republic of Korea) (substituted codon is underlined):

ForG449K

5'CATTAGTAGTGAGTCTAAA**AAA**ACAGATCATTTGCAAGGTGGTGCGCTC 3';

RevG449K

5'GAGCGCACCACCTTGCAAATGATCTGT**TTT**TTTAGACTCACTACTAATG 3'.

Primers were designed according to the instructions in the site-directed mutagenesis kit. After their modeling, they were checked through the software OligoEvaluator (http://www.oligoevaluator.com) for secondary structure formation.

Verification of binding of the primers to the correct locations in the forward or reverse template DNA strand was performed using the web-based software Multalin (http://multalin.toulouse.inra.fr/multalin/).

2.2.2. Site-directed mutagenesis protocol

2.2.3. PCR program, digestion of the methylated strand and transformation in ultracompetent strain E. coli XL10-Gold.

2.2.4. Growing colonies, harboring the mutation

2.2.5. Transformation of E. coli BL21 (DE3) Hi-control cells for mutant enzyme expression

2.2.6. Agarose gel electrophoresis for determination of plasmid DNA concentration

2.3. Bioinformatics and homology modelling

Comparison of the amino acid sequence of URE 13-300 and other glucansucrases was performed using the web-based tools Clustal Omega (https://www.ebi.ac.uk/Tools/msa/clustalo/), Pairwise Sequence Alignment (https://www.ebi.ac.uk/Tools/psa/) and ESPript v3.0. (https://espript.ibcp.fr/ESPript/ESPript/). Amino acid sequences of all enzymes were obtained from NCBI database (https://www.ncbi.nlm.nih.gov/). Default settings were used. Homology

model of CD1 of glucansucrase URE 13-300 was created using Swiss-Model (https://swissmodel.expasy.org) web tool. As a model template glucansucrase GtfB of *S. mutans* GS5 crystal structure was used (PDB ID: 8FJ9; Schormann, N. et al., 2023). The obtained model was optimized by energy minimization in YASARA, v22.5 program.

2.4. Sequencing primers and data evaluation

In order to confirm the correct amino acid substitution, the part of the gene, encoding URE 13-300 enzyme that was mutated was sequenced. The following primers were used (Bioneer, Republic of Korea):

F1: 5' GAACAGTTTGACGGATACGG 3'
F2: 5' TGGCTATCTAACGGCTAACAG 3'
R2: 5' GCCAACAGCATCTCATAGCC 3'

The synthesized primers and plasmid template has been sent to Macrogen Europe (https://order.macrogen-europe.com/). The sequencing results were analyzed by the web-software Benchling (https://benchling.com).

3. Biochemical methods

3.1. Enzyme activity determination

3.2. Influence of temperature, pH and substrate concentration on the glucansucrase activity

3.3. Influence of metal ions on the enzyme activity

3.4. Protein concentration determination

3.5. Tris-glycine SDS-polyacrylamide electrophoresis (SDS-PAGE)

3.6. Disruption of *Escherichia coli* BL21 URE-13 300 cells

4. Enzyme synthesis and analysis of α-glucans, synthesized by mutant glucansucrase U13M1

4.1. Synthesis reactions of modified α-glucan

4.2. Precipitation and purification of the synthesized polysaccharide

4.3. NMR analysis of α-glucan synthesized by mutant glucansucrase U13M1

4.4. Enzymatic synthesis of oligosaccharides

4.5. HPLC oligosaccharide analysis

4.6. Analysis of enzymatic reactions in the presence of maltose as an acceptor in the presence of organic solvents

RESULTS AND DISCUSSION

1. Polysaccharide and oligosaccharide synthesis by glucansucrase URE 13-300

1.1. Optimization of glucansucrase URE 13-300 production from recombinant strain *Escherichia coli* BL21 (DE3)

The gene encoding glucansucrase URE 13-300 was successfully isolated and cloned into recombinant *Escherichia coli* strain BL21 (DE3) by the research group at the Department of "Biochemistry and Microbiology". Glucansucrase was isolated from *Leuconostoc mesenteroides* URE 13 strain found in fermented Bulgarian vegetables (Bivolarski V. *et al*., 2018). The optimal conditions of secretion of glucansucrase URE 13-300 have been determined in previous studies: initial temperature of cell growth – 37 °C; after reaching optimal cell growth at an optical density (OD_{600nm}) of 0.6 to 0.8, inducer IPTG was added with a final concentration of 1 mmol/L, after which fermentation proceeded at 16 °C in a rotary shaker at 250 rpm.

Cultivation duration was investigated in order to optimize the time to achieve maximum production of glucansucrase URE 13-300. After carrying out a series of fermentations, the maximum enzyme activity was measured at the 24th hour after the addition of the inducer IPTG at a concentration of 1 mmol/L. The most pronounced increase of enzyme activity was observed between the 18th and 20th hours after the addition of IPTG – from 2.5 to 3.9 U/mg protein. The maximum values were reached between the 22nd and 24th hours, namely 4.4 U/mg protein. The optical density values in these hours varied between 1.8 and 1.9. In comparison with the OD_{600nm} values before adding the inductor, a two-fold increase was noted. When evaluating the obtained data, a gradual increase in biomass was observed in the course of the fermentation, which was comparable to gradual increase in the enzyme activity (Fig. 1). The decreased production of the enzyme between the 24th and 26th hours can be explained by the onset of the cell death phase, which is accompanied by cell lysis and release of intracellular proteases.

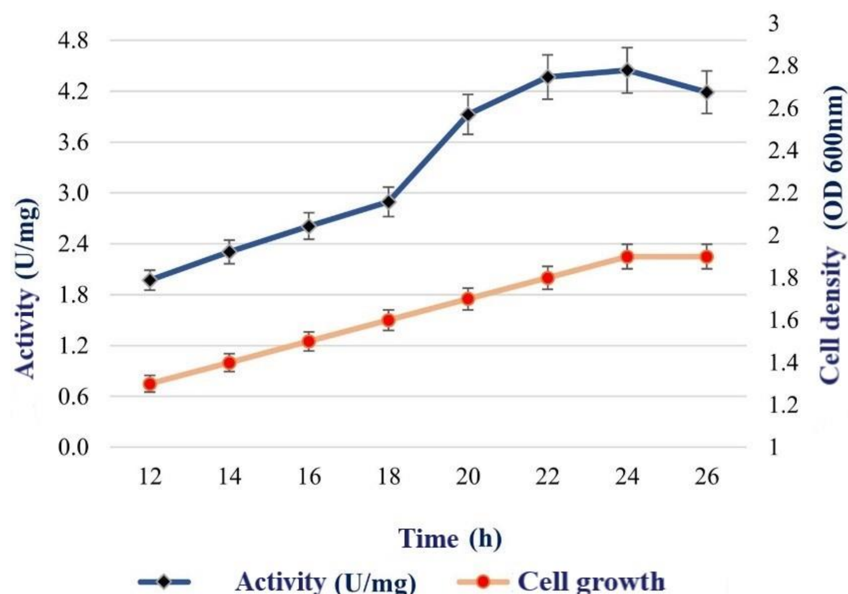

Figure 1. Investigation of the correlation between enzyme activity and cell biomass accumulation in the time course of enzyme production.

1.2. Optimization of enzymatic synthesis of α-glucan in a bioreactor

A series of reactions was performed to investigate the dynamics of glucan synthesis by glucansucrase URE 13-300. The influence of the initial activity of glucansucrase URE 13-300 on glucan synthesis was investigated by using enzyme solutions with increasing enzyme activity – 0.1 U/mL; 0.2 U/mL; 0.5/U mL and 0.7 U/mL. A comparison of the glucan fractions obtained from each reaction mixture revealed no significant change in glucan yield. Therefore, even at a low concentration of glucansucrase, an optimal amount of glucan was successfully synthesized. In subsequent studies, concentrations between 0.1 U/mL and 0.2 U/mL were used.

Enzymatic synthesis of glucans was monitored at an increasing volume of the reaction medium from 100 mL to 2000 mL (in a bioreactor) with a process duration of up to 48 hours. Reactions were performed at 30 °C and 100 rpm agitation. The obtained results show that intensive synthesis of the polysaccharide takes place during the first eight hours of the reaction. The rate of the synthesis reaction significantly decreases from this hour onwards. Between the 24th and 48th hour from the start of the reactions, only 5% of the glucan was synthesized. Taking into account the obtained results, it can be concluded that during the first 8 hours 60% of the total amount of glucan was synthesized with a substrate conversion rate of 52%. Lyophilized glucan had white crystalline mass with a cotton-like appearance.

2. Study on the application of glucansucrase URE 13-300 at carrying out enzymatic reactions in an aqueous-organic medium

In many cases, the products of transglycosylation are oligosaccharides with a branched structure and have prebiotic properties (Gangoiti *et al.,* 2018; Bivolarski *et al.,* 2018). Maltose was reported in multiple studies to be the best among a wide range of acceptors and the amount of D-glucose transferred to the acceptor depends on the acceptor: donor ratio (Mayer R. *et al*., 1981; Su D. and Robyt, 1993). Carrying out glucan synthesis by glycosyltransferases in an organic solvent medium has advantages in various cases. Many unconventional acceptors are poorly soluble in water; however, their solubility increases with the addition of organic solvents (Robyt JF, Eklund, 1983; Girard E, Legoy M.D, 1999).

In the case of glycosylation of poorly soluble acceptors such as phenolic or terpenoid compounds, organic solvents increase their solubility, therefore facilitating the interaction between the enzyme and the acceptor. Essential oil constituents are interesting targets for glycosylation due to their diverse bioactivities. Their combination with conventional antibiotics may provide alternative methods to overcome the problem of resistance of pathogenic bacteria causing food spoilage and production of toxic substances. For example, the antibacterial action and prevention of biofilm formation by essential oil components, such as carvacrol and thymol, have been tested in combination with tetracycline against oral bacteria with selective antimicrobial activity (Miladi H. *et al*., 2017).

2.1. Effect of organic solvents on the activity of glucansucrase URE 13-300

In order to establish their effect of heterogeneous media on the activity of glucansucrase URE 13-300, nine organic solvents were investigated. Experiments were performed until a 50% loss of glucanaccharase activity was reached as opposed to the activity measured in the control reaction, set as 100%. Among the studied alcohols, the addition of n-butanol or isoamyl alcohol resulted in almost complete inhibition of enzyme activity at concentrations of 5% – 99.5% and 98.5%, respectively. A drastic inhibitory effect was also observed in reactions containing 5% ethyl acetate and 5% acetonitrile, amounting to 78% and 67%

inhibition, respectively. Isopropanol and ethanol demonstrated moderate inhibitory effect with more than 50% reduction in activity at 10% and 20% alcohol concentrations. At 10% concentration of isopropanol, more than 55% inhibitory effect was determined. Experiments with octanol showed an inhibitory effect at low concentrations, however there was no inhibition observed at concentrations above 20%. This was probably due to the separation of the reaction mixture into two phases - aqueous and organic, which limits the interaction of the enzyme with the solvent. The activity of glucansucrase in the presence of DMSO decreased gradually, and at 50% concentration the enzyme retained about half of its total activity. In contrast to our results, the inhibitory effect of dimethylsulfoxide on dextransucrase B-512F and alternansucrase B-23192 was significantly higher, reaching more than 60% reduction of enzyme activity at 40% organic phase (Bertrand A. *et al.*, 2006). Therefore, the activity of glucansucrase URE 13-300 was less affected by the presence of DMSO in the enzyme reaction. Ming Miao and colleagues reported similar results in DMSO and n-hexane, where the activity of glucansucrase produced by *Lb. reuteri* SK24.003 was relatively high. Unlike URE 13-300 glucansucrase, however, the studied glucansucrase exhibited higher activity at elevated concentrations of ethanol, isopropanol, and n-butanol (Miao M. *et al.*, 2017).

The activity of glucansucrase URE 13-300 was not affected by n-hexane in all experiments performed. Of all organic solvents tested, DMSO, n-hexane and octanol showed the lowest inhibitory effect, along with 5% concentration of ethanol. Taking into account the obtained results, these organic solvents were chosen to study their influence on oligosaccharide synthesis reactions.

2.2. Effect of various organic solvents on transferase reactions in the presence of maltose as an acceptor

Four of the organic solvents analyzed in the previous subsection were selected for evaluation of their effect on the transferase reaction - DMSO, n-octanol, n-hexane and ethanol in two specific concentrations - 5% and 20%, and when ethanol was used, only the reaction at an ethanol concentration of 5% was investigated. Enzymatic assays were performed to investigate the influence of the initial concentration of sucrose and maltose on the acceptor reaction in aqueous media. Increasing concentrations of the donor substrate sucrose from 0.028 M to 0.730 M were used and at a constant ratio to the acceptor maltose – M/S=0.5. The values of K_m and V_{max} were calculated by the Michaelis-Menten equation using the non-linear regression approach (Fig. 2).

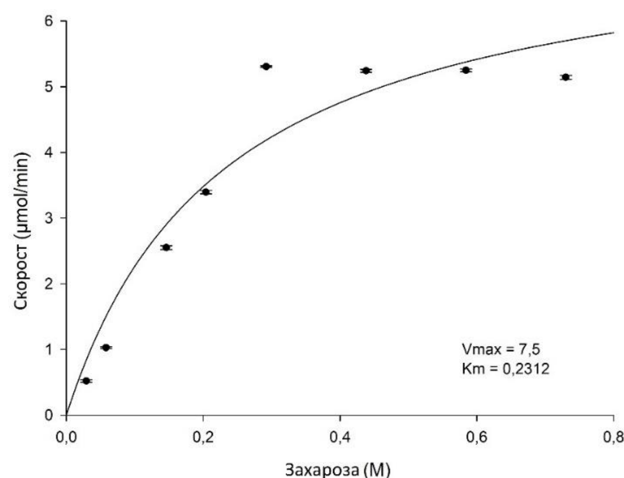

Figure 2. Influence of the concentration of sucrose in the presence of maltose (at a ratio of maltose:sucrose=0.5) on the rate of the enzymatic reaction catalyzed by glucansucrase URE 13-300.

From previous research in the lab, maltose was found to be the most suitable acceptor for the synthesis of oligosaccharides by a transferase reaction catalyzed by the studied enzyme GS URE 13-300. In the presence of maltose, an enzyme activity of 3.6 U/mg was reported, representing a 30% increase, compared to the reaction with 10% (292 mM) sucrose alone. The K_m value in the presence of maltose acceptor was 5-fold increased, therefore the substrate affinity was weaker. Similar results were obtained by Pham and co-authors, who reported an almost identical correlation using the acceptor maltose (Pham H. *et al.*, 2018). The calculated $K_m = 0.231$ M and $V_{max} = 3.6$ U/mg were determined as control values.

The kinetic parameters were also investigated in the presence of the selected organic solvents in concentrations of 5% and 20% - DMSO, n-hexane and octanol, as well as 5% ethanol. Based on the obtained results, it was found that the addition of 5% DMSO and octanol had little effect on the V_{max} values of 3.5 and 3.3 U/mg, respectively. The same trend was observed in the K_m values – 0.216 M and 0.248 M, respectively. These results correlated well with the observed inhibition of enzyme activity. However, the addition of 5% n-hexane to the reaction mixture resulted in a slight increase in enzyme activity with a simultaneous increase in the K_m value – 0.271 M of sucrose. In the presence of 20% DMSO, the K_m values of octanol and n-hexane increased between 13% and 26%, which is consistent with the observed inhibitory effect of these organic solvents.

2.3. Influence of organic solvents on the degree of polymerization of glucooligosaccharides synthesized by glucansucrase URE 13-300

A control acceptor reaction was carried out with maltose at an acceptor: donor ratio of 0.5 to follow the dynamics of oligosaccharide synthesis. For the ratio used, glucansucrase URE 13-300 is known to synthesize glucooligosaccharides (GOSs) with a degree of polymerization (DP) of 3 to 6 (Bivolarski V. *et al.,* 2018). Samples were examined at 30 minutes, 1 hour, 2 hours, 4 hours, 6 hours, and 24 hours from the start of the transferase reaction. Figure 3 presents chromatograms from 30 minutes, 1 hour and 24 hours.

As confirmed by various reports in the literature, maltose is a strong acceptor of glucose units during the synthesis of GOS by glucansucrases, which generates a series of oligosaccharide products with increased DP (Robyt J.F. & Eklund Sh. 1983). The products of each acceptor reaction serve in turn as acceptors for the production of GOS with a higher degree of polymerization. Each new fraction is less effective as an acceptor than the previous one and this leads to less accumulation of GOS with higher DP. On the other hand, the reduction of GOS with DP3 during the late stages of the reaction is due to their use as acceptors, which leads to the accumulation of DP 4 and 5 products, which are significantly less effective acceptors (Bivolarski V. *et al*., 2018). The same trend was observed in the course of transferase control reactions (Fig. 3).

Determination of the influence of the selected organic solvents on the composition and ratio between the oligosaccharides synthesized by glucansucrase URE 13-300 was carried out by analyzing samples during the transferase reaction. During the first 30 minutes of the time course, a predominant synthesis of GOS with DP 3 was detected, represented by 25% to 27% in the presence of 5% DMSO, hexane and octanol. As the reaction progressed to the 24th hour, gluco-oligosaccharide synthesis was reduced between 20% and 28%, depending on the organic solvent used, which in turn was associated with increased GOS concentrations with DP 4 to 6 (Fig. 4). The yield of oligosaccharides with DP 4, synthesized in the presence of the three solvents slowly increased by 15% from 30 min to the end of the reaction, ranging from 24 mg/mL to 28 mg/mL.

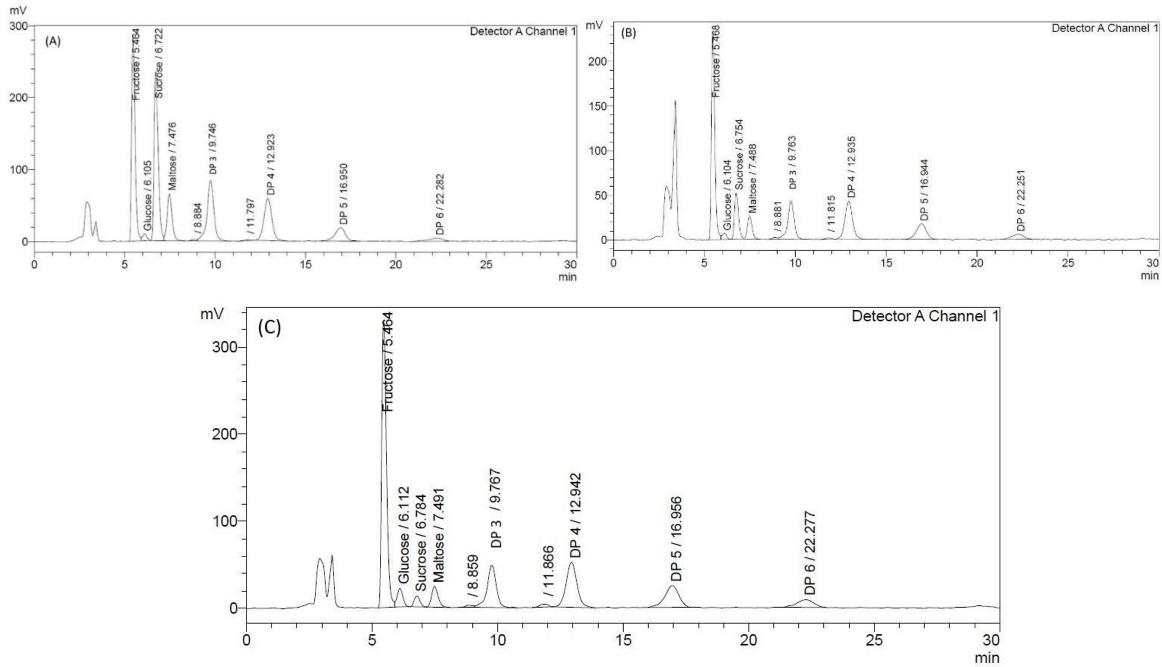

Figure 3. Oligosaccharide products profile by the transferase reaction in the presence of maltose as an acceptor at the different stages in the time course of the reaction. Chromatograms of the control sample, obtained at: (A) the first 30 min from the reaction initiation; (B) the first hour of oligosaccharide synthesis; (C) the end of the reaction – the 24th hour.

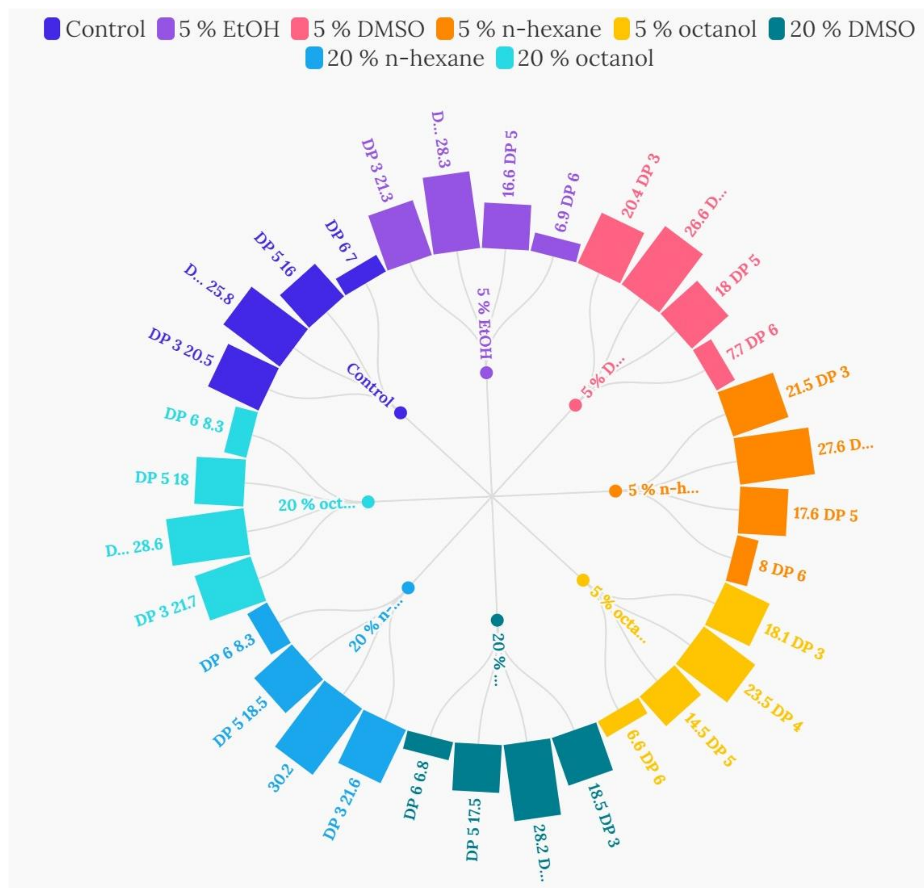

Figure 4. Overview of oligosaccharides with degree of polymerization from 3 to 6 (left to right) at the 24th hour of the transferase reaction. *Respective concentrations are presented as mg/mL.*

The most prominent shift in the dynamic of oligosaccharide synthesis was demonstrated with products with DP 5 (Fig. 3). In the presence of 5% DMSO, a peak of 20 mg/mL GOSs with DP 5 at the 6th hour of the transferase reaction is observed (shows 61% increase compared to the concentration at the 30th minute). Similar peak is observed at the reaction in 5% octanol – 19 mg/mL at the 4th hour with subsequent reduction to 14.5 mg/mL at the end of the reaction (24th hour). Obtained oligosaccharides with DP 6 gave the lowest registered yield – from 3 mg/mL at 30 min to 8 mg/mL at the 24th hour.

The obtained results of the synthesis of glucooligosaccharides in the presence of 20% organic solvents showed similar dynamics to those at 5% concentration. In reactions with 20% DMSO, oligosaccharides with DP 3 were synthesized at a concentration of 28 mg/mL at 30 min from the start of the reaction, and their amount was reduced by 34% at the end of the reaction. The concentration of oligosaccharides with DP 4, 5 and 6 increased during the course of the reaction, reaching a peak at hour 6, where the GOS fraction with DP 5 was at a concentration of 20 mg/mL. Comparable results were obtained using aqueous-organic medium with 20% hexane and 20% octanol with the maximum amount of GOS with DP 5, respectively – 21.6 mg/mL and 20 mg/mL. In both variants of the reaction, a decrease to about 18 mg/ml was determined at the end of the reaction.

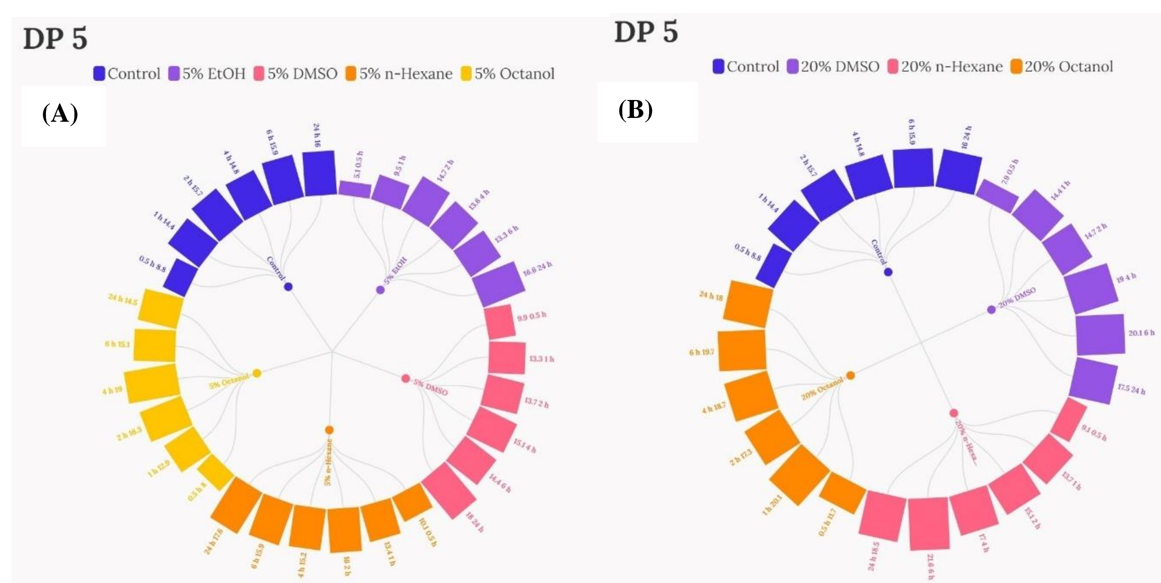

Figure 5. Dynamics of oligosaccharide synthesis with DP: *(A) in the presence of 5% organic solvents versus the control reaction; (B) in the presence of 20% organic solvents versus the control reaction.*

The presence of DMSO, regardless of its concentration, directs the reaction to the synthesis of oligosaccharides with DP 5 (Fig. 6). DP 6 products were also increased by 10%, but they had the lowest concentration in all samples tested. Comparable findings have been reported for other enzymes using carbohydrate substrates, where increased specificity towards certain oligosaccharide products was observed (Abdul Manas, NH. *et al*., 2014; Doukyu, N. *et al*., 2007). At 5% concentration of octanol, a decrease in the synthesis of all oligosaccharides was observed, which confirms the results of the analyzes of the total enzyme activity and their inhibition. Conversely, the same organic solvent at 20% concentration enhanced the synthesis of all glucooligosaccharide products.

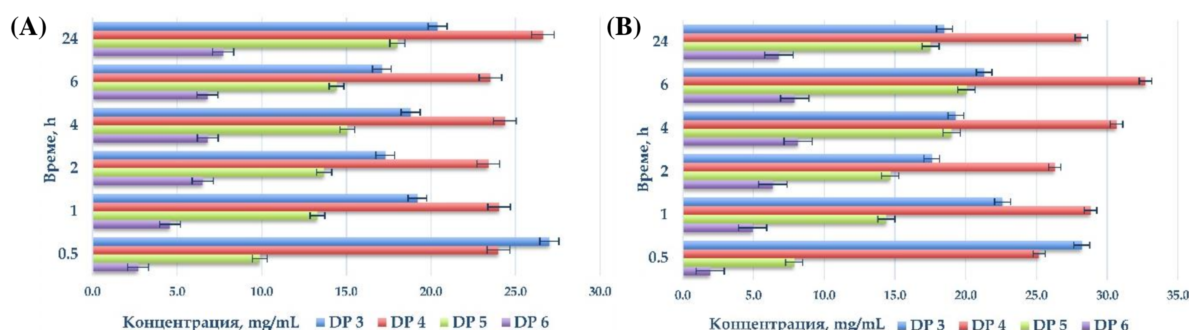

Figure 6. Dynamic changes of transferase reaction in the presence of acceptor maltose in: (A) aqueous-organic media, containing 5% dimethyl sulfoxide (B) 20% dimethyl sulfoxide.

The obtained results showed that the synthesis of oligosaccharides as a result of the transferase reaction was not significantly affected in a heterogeneous medium with 5% or 20% DMSO, n-hexane and octanol. In the indicated reactions, the synthesis of oligosaccharides with a degree of polymerization from 3 to 6 was observed, as in the control reaction without organic solvents. Therefore, regardless of the observed partial inhibitory effect of these solvents on the studied glycosyltransferase, the efficiency of the acceptor reaction was not changed in terms of the degree of polymerization of the synthesized oligosaccharides. Solvents such as n-hexane and octanol are not miscible with water, but their presence in the reaction medium leads to a redistribution of the oligosaccharides in different stages of the transferase reaction, resulting in an increase in the yield of GOS with DP 4 and 5. As a consequence, this function can to prove advantageous for tailoring the synthesis of various products.

It is known from research carried out so far that the use of biphasic systems with water-immiscible organic solvents has been successfully implemented in the glycosylation of aliphatic alcohols, monoterpenoids, aromatic alcohols and phenols. Among the advantages of biphasic systems are improved enzyme stability and easier product recovery while avoiding substrate inhibition (De Winter *et al*., 2014). In this regard, the obtained data on the dynamics of acceptor reactions catalyzed by glucansucrase URE 13-300 are a good prerequisite for tailoring the synthesis of the product after a rational or semi-rational design of mutations of key amino acid residues (Malbert, Y. *et al*., 2014; Meng, X. *et al*., 2022).

2.4. Influence of components from essential oils solubilized in 20% DMSO on the activity of glucansucrase URE 13-300

The effect of three monoterpenoid compounds from essential oils carvacrol, thymol and menthol on the total activity of glycosyltransferase URE 13-300 was investigated. The final concentrations of these compounds in the reaction mixture varied between 10 and 80 mmol/L. No inhibition of enzyme activity was detected in all reactions with carvacrol and thymol. On the contrary, a neutralization of the inhibitory effect of DMSO, which was added as a co-solvent at 20% concentration, was reported. In reactions with menthol, the inhibitory effect on the activity of GS URE 13-300 reached 28% at a concentration of 50 mmol/L, and remained unchanged when 80 mmol/L menthol was added to the enzyme reaction.

Such terpene alcohols can be released from their corresponding glycosidic precursors by enzymatic hydrolysis, resulting in the controlled release of aroma. Moreover, the anticancer, antibacterial, analgesic and anti-inflammatory activities of menthol are attractive for its introduction into value-added products (Nisar, S. 2022). Encapsulation with menthol and luteolin in microcapsules in the presence of maltodextrin and soy proteins in flavored

beverages has recently been developed (Mora-Flórez, L.S. *et al*., 2023). Detailed investigation of the prebiotic properties of GOSs synthesized by glucansucrase URE 13-300 revealed that oligosaccharide products have prebiotic potential (Bivolarski, V. *et al.,* 2018). Along with the modification of the acceptor:donor ratio, the addition of various organic solvents to the transferase reaction may prove suitable for further applications for customized products.

2.5. Conclusion

The conducted research on the dynamics of acceptor reactions with maltose, catalyzed by glucansucrase URE 13-300, defined for the first time the synthesis of oligosaccharides in the course of the transferase reaction and their kinetic characteristics. Also, the influence of organic solvents on the enzymatic activity of the studied enzyme was determined. The organic solvents DMSO, n-hexane and octanol showed relatively weak inhibitory effect up to 20% concentration in the reaction medium. Some of these solvents affected the synthesis of oligosaccharides during the transferase reaction to GOS with a higher degree of polymerization without inhibiting the transferase reaction. Glycosylation of terpenoids derived from essential oils such as menthol can be included in the modeling of value-added food additives.

3. Site-directed mutagenesis in the gene, encoding glucansucrase URE 13-300

The accumulation of data on the biochemical characterization of numerous enzyme families and the development of molecular biology in recent decades have shifted the use of the stochastic approach to a rational and semi-rational approach.

The method of site-directed mutagenesis (SDM) was developed by Michael Smith in 1978 and makes it possible to study the importance of a specific amino acid on the structure and function of the enzyme, which is an important prerequisite for studying the relationship between them (Hutchison, C.A. *et al*., 1978; Qu, G. *et al*., 2018). It involves the introduction of so-called point mutations, in which an amino acid at a predetermined location in the protein molecule is replaced by one of the other 19 amino acids. A necessary condition is the availability of a sufficiently detailed database for the structural features of the respective enzyme family. Also, understanding the enzyme catalytic mechanism affects the accuracy and success rate of rational design.

3.1. Bioinformatic analysis and design of mutagenic primers

At the first step to obtain a molecular mutant by a single amino acid substitution, a detailed bioinformatic analysis was performed. The amino acid sequence of glucansucrase URE 13-300 was compared with that of some mutated glucansucrases studied. The seven conserved motifs containing important amino acids for the binding specificity of the synthesized products were identified. Figure 7 presents motifs II, III and IV where the catalytic residues (triad) are located, respectively the nucleophile aspartate (Asp543), the acid-base catalyst glutamate (Glu581) and the transition state stabilizer – aspartate (Asp654). The analysis revealed that catalytic domain 1 possesses the highly conserved amino acid residues in these three motifs, inherent of the GH70 family. The majority of mutations performed so far affected the conserved motifs in domain A. In comparison, key amino acid substitutions at key sites in domain B, which also plays an important role in the formation of the catalytic pocket, the orientation of the acceptors in the formation of the specific glycosidic bonds and their interaction with the growing glucan chain, are less frequently reported.

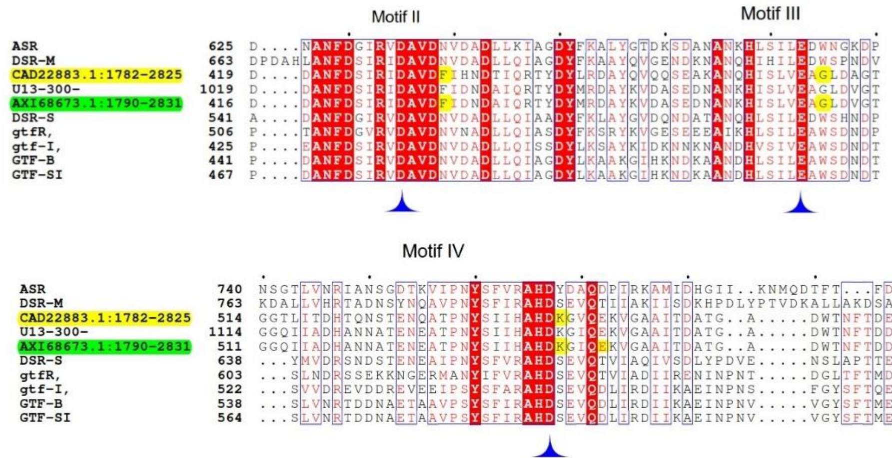

Figure 7. Multiple sequence alignment of glucansucrase URE 13-300 with other glucansucrases from GH70 family.

In a subsequent multiple sequence alignment MSA analysis, the following enzymes from GH70 family were compared with GS URE 13-300: GTF180 (AY697430.1) from *Lb. reuteri* 180; GTFR (AB025228.1) from *S. oralis* ATCC 10557; DSRS (AAD10952.1) from *Ln. mesenteroides* NRRL B-512; ASR (AJ250173.2) from *Ln. mesenteroides* NRRL B-1355, DSRE from *Ln. mesenteroides* NRRL B-1299; GTF8293 (ABJ61965) from *Ln. mesenteroides* ATCC 8293 and GTFI (AAC63063.1) from *S. downei* MFe28 (Fig. 8).

After reviewing the effects of amino acid substitution in some of these enzymes, the amino acid glycine at position 449 was chosen. This position is not strictly conserved among them, but appears most frequently in the analysis of multiple sequences. Mutansucrase GtfI has tyrosine (Tyr, Y) at this position, dextrancharase GTFR has lysine (Lys, K), and dextrancharase DSRS has serine (Ser, S).

Mutations have been carried out in some of the adjacent amino acids, proving that they determine the type of linkages in the polysaccharide products (Meng, X et al., 2014; Funane K. et al., 2005; Molina M. et al., 2019). At positions corresponding to 449 in GS URE 13-300, one mutation was found to date, in dextransucrase DSRS, which corresponds to serine at position 445. Serine is a polar amino acid and is replaced by lysine, having basic properties. In the present case, the original amino acid has amphoteric properties. The amino acid lysine was chosen for substitution, following the study model of Funane *et al*. (2000). Also, lysine is present at the corresponding position in the dextransucrase GTFR. It was interesting to follow how the substitution with an amino acid residue opposite in size and charge would affect the formation of the type of glycosidic bonds in the structure of the URE 13-300 synthesized glucans.

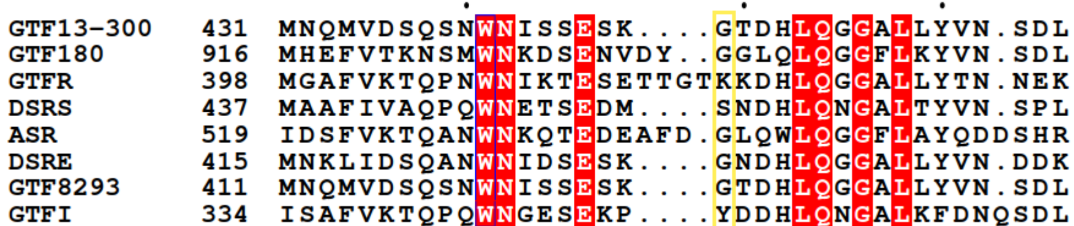

Figure 8. Multiple sequence alignment of domain B.

3.2. Homology modelling of catalytic domain 1

In order to visualize the amino acid substitution and evaluate the conformational changes, a homology model of catalytic domain 1 was built based on the solved crystallographic structure of GtfB from *S. mutans* (Schormann, N. *et al.,* 2023). The amino acid is located in the loop B1, which forms the periphery of the catalytic pocket as well as the path of the growing glucan chain. It is located spatially far from the catalytic triad, but close to the +II subsite, facilitating acceptor binding (Meng, X. *et al*., 2014). Figure 9 shows the homology model of CD1, together with the putative conformation of the enzyme before and after the amino acid substitution.

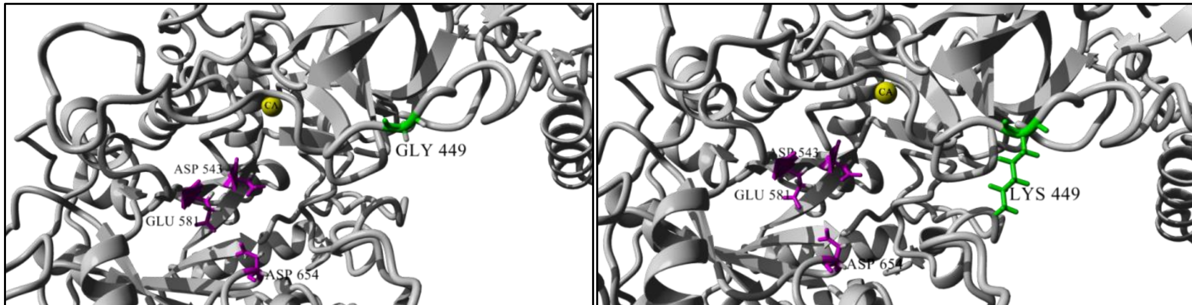

Figure 9. Homology model of catalytic domain 1 of glucansucrase URE 13-300. *Conformation of catalytic domain 1 with the original amino acid glycine (left); conformation of catalytic domain 1 with the amino acid lysine substituted (right).*

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3.3. Obtaining a mutant glucansucrase URE 13-300

3.3.1. Site-directed mutagenesis

The plasmid containing the gene, encoding glucansucrase URE 13-300 was isolated from recombinant strain *E. coli* BL21 URE 13-300 (Fig. 10).

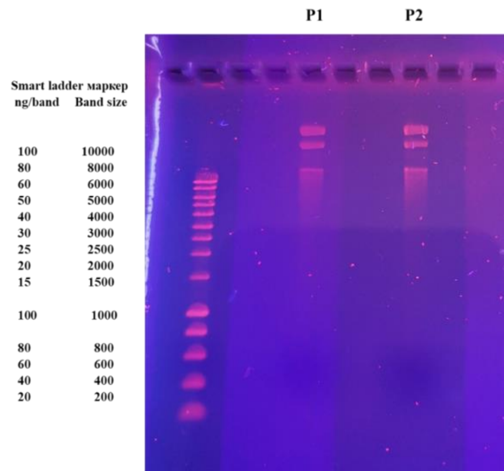

Figure 10. Agarose gel-electrophoresis for determination of isolated and purified plasmid DNA, harboring the gene, encoding glucansucrase URE 13-300. *Legend: Smart ladder – DNA standard with known molecular mass and intensity. Sample P1 u P2 – purified plasmid fractions.*

After obtaining the PCR products and digestion of the methylated (template) DNA strands, transformation was carried out in a subcloning system representing hemocompetent E. coli XL10 Gold cells. The sample containing the control plasmid was cultured on nutrient LB agar medium with ampicillin. The sample with the mutation in the gene encoding URE 13-300 was spread on two LB agar media containing kanamycin. The prepared strains were

cultured for 16 h at 37 °C. At the end of the culture time, single colonies were observed in both the control mutation and the URE 13-300 mutation (Fig. 11). The efficiency of the mutation carried out was over 95%. Single colonies of the resulting transformants were cultured in LB liquid medium with kanamycin.

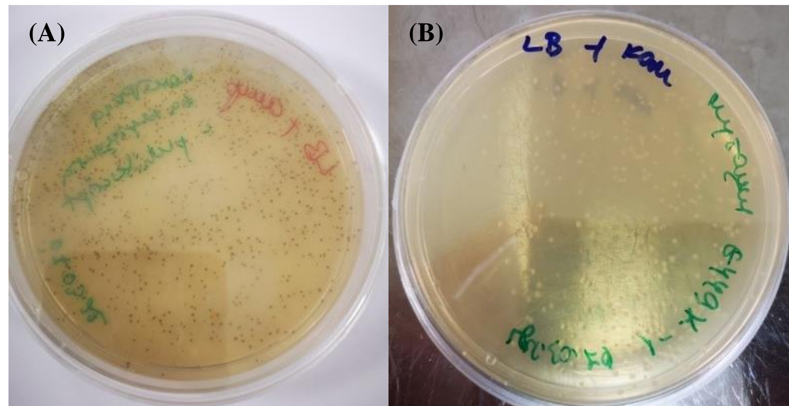

Figure 11. Control mutation (A) and mutation of URE 13-300 with primer G449K (B).

3.3.2. Isolation and purification of mutated plasmid DNA

Plasmid DNA carrying the mutant gene was isolated and purified from the cultured transformants (Fig. 12). Two of the purified plasmids were transformed into E. coli BL21 Hi-control cells to express the mutant gene (Fig. 12). Two of the new transformants were cultured in LB medium overnight.

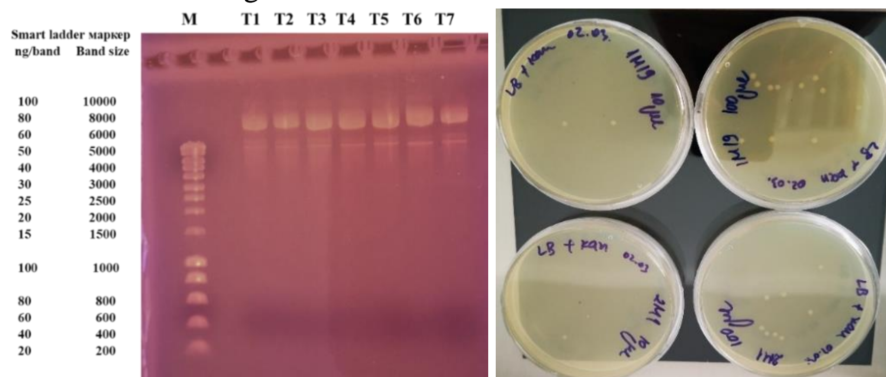

Figure 12. Purification of plasmids, harboring the mutated gene and transformed colonies of *E. coli* BL21 after transformation with plasmids T3 и T4. *Samples in the gel: M – Smart ladder standard; T1 to T7 – plasmids from transformations.*

Cultures of E. coli strain BL21 showed good growth and were grown in 5 mL of liquid TB medium, which has been shown to be more suitable for expression of the recombinant proteins (Iliev, I. *et al*., 2018). To verify polysaccharide synthesis by the mutant enzyme, both cultures were cultured on agar media containing sucrose and the inducer IPTG (Fig. 13).

Mucosal colonies were observed, which proved the production of an active mutant enzyme by the recombinant strain *E. coli* BL21. It was also observed that the amount of synthesized polysaccharide was less compared to the starting enzyme. No difference was observed between the appearance and the amount of synthesized product between the two transformants, so only one of them was selected for the following analyses. The mutant enzyme was given the name U13M1 to distinguish it from the wild-type enzyme. The two variants of the studied glucansucrase were compared by SDS-PAGE electrophoresis.

Figure 13. Screening of transformants for glucans synthesis on agar media supplemented with 20% sucrose and inducer IPTG. *Left – recombinant strain E. coli BL21 URE 13-300; right – E. coli BL21-T1; center – E. coli BL21-T2.*

Glucansucrase URE 13-300 has a confirmed molecular weight of 311.85 kDa, also confirmed by sequencing (Bivolarski, V. *et al*., 2018). From the obtained results of the performed in situ analysis, it was proved that the two forms of glucansucrase URE 13-300 showed the same bands, around 300 kDa. Substitution of only one amino acid did not lead to a visible change in the molecular weight, therefore we can conclude that no additional changes in the amino acid sequence of the enzyme occurred as a result of the mutation carried out.

3.3.3. Sequencing part of the gene, encoding glucansucrase URE 13-300, encoding the introduced mutation.

To confirm the mutation occurred and the type of amino acid substituted, plasmid DNA was isolated from *E. coli* BL21 U13M1 cells and sent for sequencing. The results obtained unequivocally showed the correct substitution of the amino acid glycine (G) with lysine (K). Alignments of the obtained sequences with the template DNA strand are shown in Fig. 14.

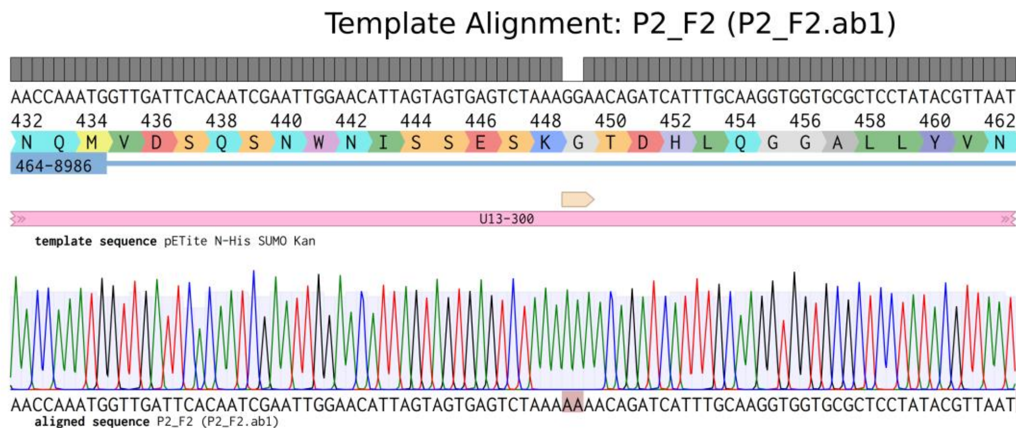

Figure 14. Results from mutated gene sequencing, encoding the substituted amino acid. *The codon AAA, for lysine is placed at the corresponding position of codon GGA, coding originally glycine, thus proving the mutation.*

4. Optimizing the conditions for producing mutant glucansucrase U13M1 and the conditions of the enzyme reaction

4.1. Optimizing conditions for producing the mutant glucansucrase U13M1

4.1.1. Influence of the temperature on the production of U13M1 mutant enzyme

The secretion of mutant U13M1 enzyme was examined at the following temperatures after the addition of inducer: 14, 16, 18, 20 and 25 °C and for a duration of 24 h after induction. The highest activity was recorded at 16 °C – 1.5 U/mL, and the lowest at 20 and 25 °C, where the activity was at the limit of detection. Close to the optimal induction temperature – 14 and 18 °C, activities of 86% and 75% of the maximum activity were reported, respectively (Fig. 15). The expression temperature was kept identical to that of the wild-type enzyme. This result is also supported by other authors, assuming that a lower temperature after induction is preferred for recombinant expression.

4.1.2. Influence of the inducer IPTG concentration on the production of mutant enzyme U13M1

Inducer IPTG concentrations of 0.3, 0.5, 0.7 and 1.0 mM were tested (Fig. 15). The results reported at the last two concentrations show that they have a comparable effect on the production of the enzyme, so both concentrations can be used. A final concentration of 0.7 mM was chosen for subsequent assays because IPTG exerts a toxic effect on the cells.

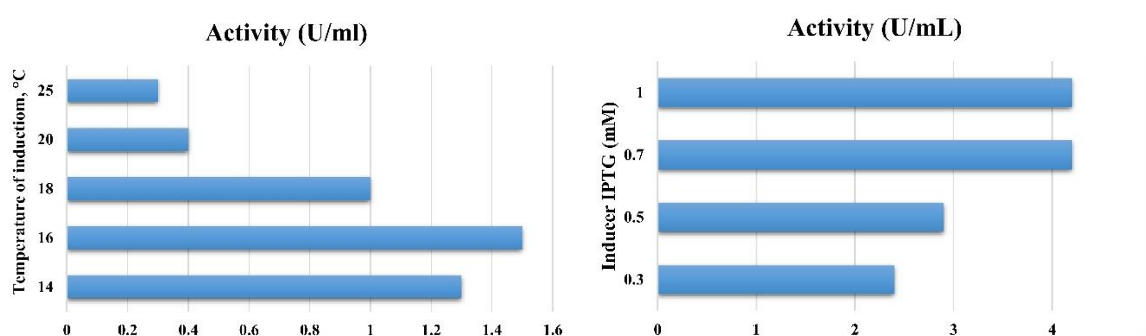

Figure 15. Influence of the temperature and inducer IPTG concentration on the production of mutant enzyme U13M1 by recombinant strain *E. coli* BL21.

4.1.3. Determination of optimal cell growth prior to addition of inducer and determination of fermentation duration

After carrying out a series of fermentations, it was found that the optimal production of the mutant enzyme was reached when the inducer IPTG was added after the cell cultures had passed the exponential growth phase. A maximum enzyme activity of 0.7 U/mg was reached at an OD_{600nm} between 1.7 and 1.8. For the duration of the recombinant expression, the highest enzyme activity was reported between the 11th and 13th hours, with values of 2.0 U/mL. After this stage, the yield decreased gradually and by the 24th hour of fermentation it had decreased by 25%. The optimal induction temperature was 16 °C.

4.2. Comparison between the expression conditions of the wild-type glucansucrase URE 13-300 and the mutant variant U13M1

When comparing the expression conditions for the two enzymes, significant differences stand out. The U13M1 mutant enzyme was successfully produced at a later stage of cell growth. Regarding the amount of inducer, approximately the same yield of U13M1 enzyme was observed at 0.7 and 1 mmol/L IPTG concentration. The temperature after induction remains the same, and the duration of fermentation is reduced from 24 hours to 13 hours. When examining the enzyme activity of mutant U13M1 under the optimal conditions determined for the starting glucansucrase URE 13-300, a significant difference was found – the highest value for enzyme activity of U13M1 was 0.7 U/mg.

4.3. Optimization of kinetic parameters of the enzyme reaction U13M1

The biochemical characteristics of the U13M1 mutant enzyme were determined to assess the effect of the amino acid substitution on the enzyme properties. The temperature optimum was determined by running reactions between 15 and 45°C. The highest enzyme activity was reported at 20°C, in contrast to the optimal temperature for the wild-type URE 13 glucansucrase, which is 30°C. At 30°C, nearly 50% decreased activity of U13M1 glucansucrase was reported. Carrying out the enzyme reaction at 40°C resulted in an almost complete loss of activity. The optimum pH values for the enzymatic reaction of the mutant variant also showed very similar differences compared to the wild-type type. The highest activity was reached at pH 6.5. When neutral buffers were used, a drastic decrease in enzyme activity was reported – more than 80% at pH 7.0 and almost complete loss of activity at pH 7.5. The activity of U13M1 in acidic buffers was also significantly reduced - about 70% lower at pH 5.5, and no enzyme activity was detected at pH 4.5. In fig. 16 the results for the enzymatic activity of the mutant enzyme are presented and compared with those for the wild-type glucansucrase URE 13-300 determined in previous studies (Iliev, I. *et al*., 2021). The starting enzyme retains its activity over a wider range of temperatures as well as having a wider pH range.

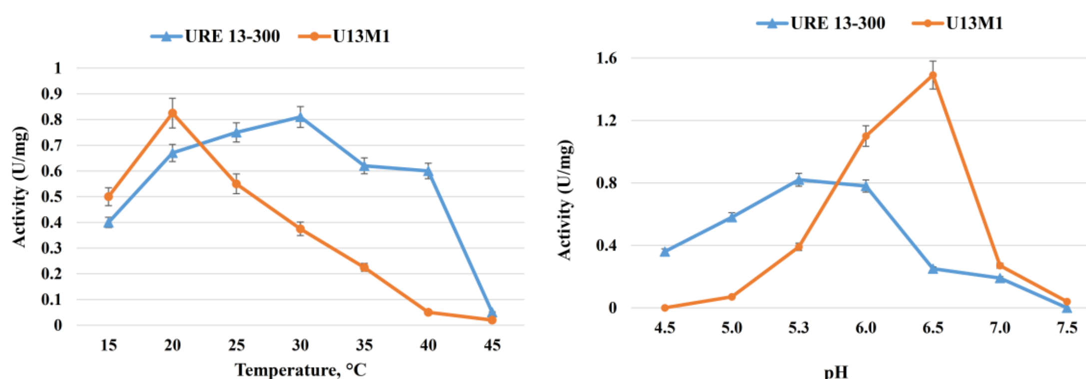

Figure 16. Optimal temperature and pH for enzyme activity of mutant glucansucrase U13M1.

4.4. Comparison of the kinetic parameters of both variants of glucansucrase URE 13-300

The kinetic parameters for the mutant variant were examined and compared with those inherent to the wild-type URE 13-300 glucansucrase (Fig. 17). Using the Michaelis-Menten equation and a non-linear regression approach, the value of K_m = 0.33 M sucrose, the enzyme reaction reached a maximum rate of V_{max} = 2.6 U/mg, and the constant representing substrate inhibition was K_i = 1.6 M sucrose. The Km value for U13M1 was increased seven-fold over the wild-type enzyme, K_m= 0.048 M, but no change in the Vmax value was reported. The affinity between substrate and enzyme is much lower in the U13M1 mutant enzyme. The optimal substrate concentration of the mutated enzyme is equal to 0.6 M sucrose, which is twice as much as compared to the original enzyme. Substrate inhibition of the mutant enzyme was observed at a sucrose concentration of 1.6 M, while the K_i of the wild-type was 0.69 M.

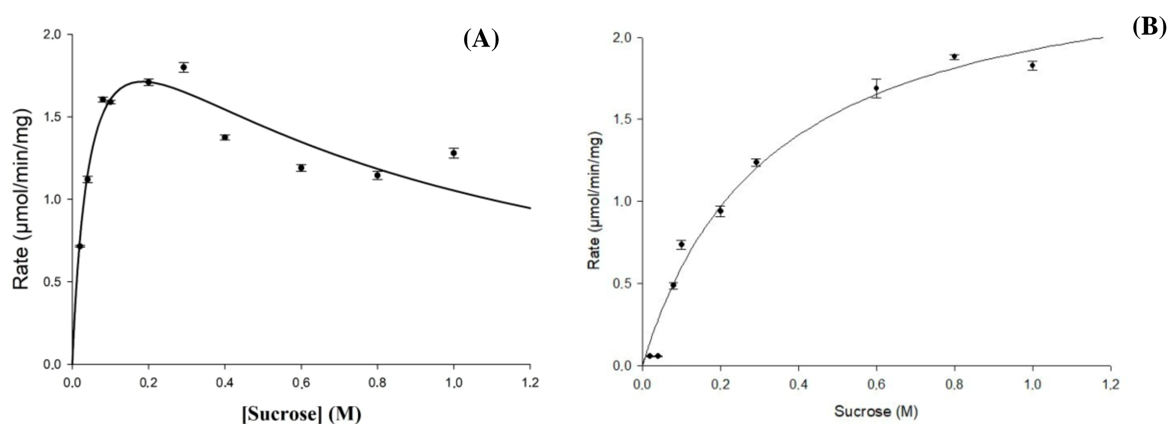

Figure 17. Comparison between kinetics of (A) wild-type glucansucrase URE 13-300 and (B) mutant enzyme U13M1 with sucrose.

4.5. Influence of metal ions on the enzyme activity of wild-type glucansucrase URE 13-300 and mutant variant U13M1

After analyzing the three-dimensional structures of the shortened forms of several glucansucrases, it was found that they include a calcium ion in the same position near the catalytic domains A and B. According to Vujičić-Žagar *et al.*, the removal of Ca^{2+} ions from the active site lead to a decrease in the enzyme activity by about 40%. This effect was likely due to compromised substrate or acceptor binding at the respective site.

The influence of exogenous Ca^{2+} ions on the enzymatic activity of the wild-type glucansucrase URE 13-300 and its mutant variant was investigated (Table 4). As reported for other glucansucrases, incubation of the enzymes with a chelating agent had a negative effect on the enzyme activities, which was overcome by the addition of calcium ions. Both enzymes were activated by Ca^{2+} ions at a final concentration of 5 mM, but the activation effect on glucansucrase URE 13-300 was twofold higher at 52% as opposed to 17% higher enzyme activity on the mutant enzyme. The study of an increased concentration of calcium ions revealed that at the highest tested concentration – 25 mM, the starting glucansucrase reached a 78% increase in activity, compared to the control reaction without the presence of Ca^{2+} ions. In comparison, the measured activity of the U13M1 mutant enzyme showed an increase of nearly 30% at a concentration of 20 mM Ca^{2+} in contrast to the corresponding control reaction. A study of the enzymatic activity of the two variants of glucansucrase URE 13-300

and with several metal ions Mg^{2+}, Fe^{2+}, Ba^{2+}, Mn^{2+} was carried out. They were shown to have a positive effect on the wild-type glucansucrase, however all showed an inhibitory effect on the mutant variant (Table 5). Taking into account all the results obtained for the enzyme activity in the presence of metal ions, the inclusion of a single point mutation G449K reduced the activation effect of metal ions on the U13M1 enzyme.

Table 1. Effect of increasing concentration of Ca^{2+} ions on the enzymatic activity of U13M1.

$CaCl_2$, mM	Relative Activity, %	
	URE 13-300	U13M1
Control	100	100
5	152	117
10	142	123
15	156	127
20	176	133
25	178	110

Table 2. Effect of different metal ions on the enzymatic activity of U13M1

Metal ions, 5 mM	Relative Activity, %	
	URE 13-300	U13M1
Control	100	100
$CaCl_2$	152	117
$MgCl_2$	110	54
$FeSO_4$	154	44
$BaCl_2$	128	32
$MnSO_4$	120	22

4.6. Conclusion

Glucansucrases with two catalytic domains are an interesting target for single point mutations. The amino acid substitution made in glucansucrase URE 13-300 is located in domain B of catalytic domain 1 of glucansucrase URE 13-300. The amino acid glycine at position 449 was replaced with lysine (Fig. 9). The effect of this substitution affected the kinetic parameters of the enzyme.

The resulting U13M1 mutant enzyme possesses a remarkable change in kinetic parameters compared to the wild-type glucansucrase URE 13-300. The Km values for the U13M1 mutant enzyme were nearly 7-fold higher than those of the wild-type glucansucrase, while maintaining an identical maximal velocity. The reduced substrate affinity we observed may be due to a change in the shape of the binding site in the mutant enzyme. Consistent with our results, mutant forms of glucansucrase GTF180-ΔN show an increased Km for sucrose. The pH optimum of U13M1 is shifted one unit higher than the wild-type enzyme – to 6.5. According to previous findings, the optimal pH of various glucansucrase was reported to be between pH 4.7 and 7.4, but often between pH 5.0 and 6.0 (Miao, M. *et al*., 2017). It has also been suggested that a more neutral pH may be necessary in different reaction media of the enzyme reaction, as it may lead to enhanced glucan synthesis as well as a change in its structure. The temperature optimum of most glucansucrase is between 30 °C and 40 °C (Kang, H.K. *et al*., 2009). After amino acid substitution with lysine, the temperature optimum of U13M1 is shifted to 20°C. Consistent with our results, glucansucrase from *Ln.*

mesenteroides 0326 has a temperature optimum of 25 °C, and its P473S/P856S double mutant variant retains nearly maximal activity at 20 °C.

From the information available in the literature, it is known that such differences in optimal conditions between the wild-type and the mutant enzyme have not been previously reported. Furthermore, the enzyme activity of U13M1 was significantly less inhibited by increasing sucrose concentrations. Glucansucrase URE 13-300 was activated by Ca^{2+} and other metal ions, similar to other glucansucrases. However, this ability was abolished by the mutation. The reason for this may be due to the mutation site proximity to the Ca^{2+} ion binding site.

5. Investigation of α-glucan and oligosaccharide synthesis by mutant glucansucrase U13M1

5.1. Structural NMR analysis of U13M1 glucan

The polymer synthesized by the glucansucrase enzyme URE 13-300 consists of 67.2% α-(1→6) linkages and 16.2% α-(1→3) linkages in the main chain, as well as 8.3% of 3,6-di - O-substituted α-D-Glcp residues at the branch points (Iliev, I. *et al.,* 2021). The nuclear magnetic resonance (NMR) results obtained from the 1H spectra of the two α-glucans showed that the ratio between α-(1→6) and α-(1→3) linkages was altered as a consequence of the mutation involved. 1H NMR spectra of polysaccharide U13M1 revealed two anomeric protons with chemical shifts at 4.96 ppm representing 90.6% α-(1→6) linkages and 5.33 ppm corresponding to 4.2% α-(1→3) linkages (Fig. 18). These results indicate a greatly reduced amount of α-(1→3) linkages present in the U13M1 polymer structure. A distinguishable signal at the base of 4.96 ppm was also observed, indicating a small amount of 3,6-di-O-substituted α-D-Glcp residues, although not as a distinct signal.

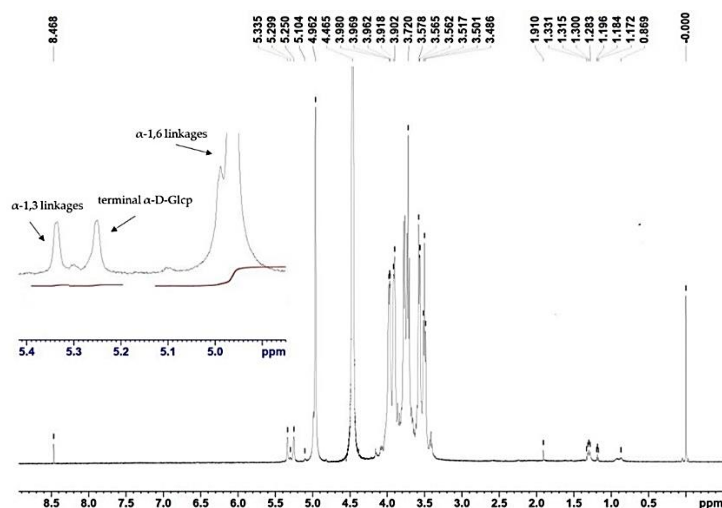

Figure 18. NMR analysis of the polymer, synthesized by mutant glucansucrase U13M1 with 20% initial sucrose concentration.

In addition to polysaccharide synthesis with the optimal sucrose substrate concentration for the U13M1 mutant, reactions were also performed with 10% sucrose. 1H NMR analysis of the polymer synthesized at 292 mM initial sucrose concentration is presented in Fig. 19. In the NMR analysis, two peaks were recorded at δH-1~4.97 ppm corresponding to 87.5% α-(1 → 6) linkages and δH-1~5.32 ppm corresponding to the presence of 12.5% α-(1 → 3) connections. The differences between the type of linkages present in the original polymer and the modified variants synthesized by mutant glucansucrase U13M1 are presented in Fig. 20.

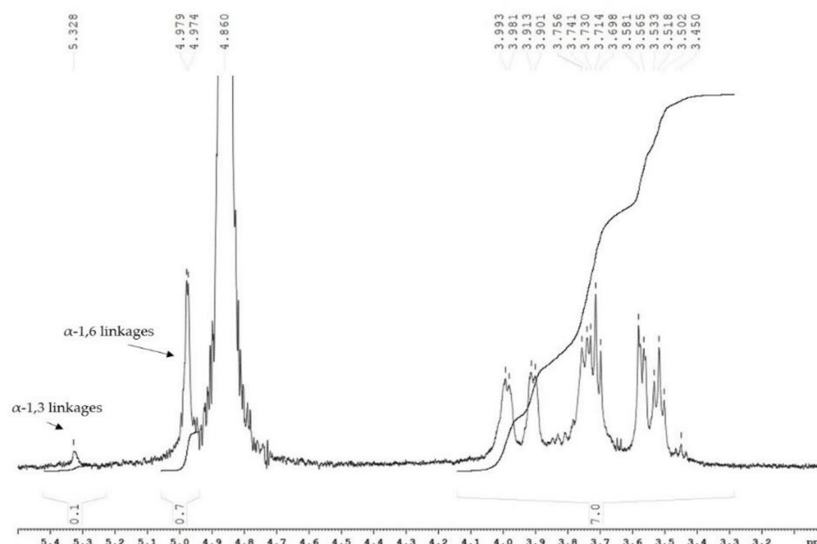

Figure 19. NMR analysis of the polymer, synthesized by mutant glucansucrase U13M1 with 20% initial sucrose concentration.

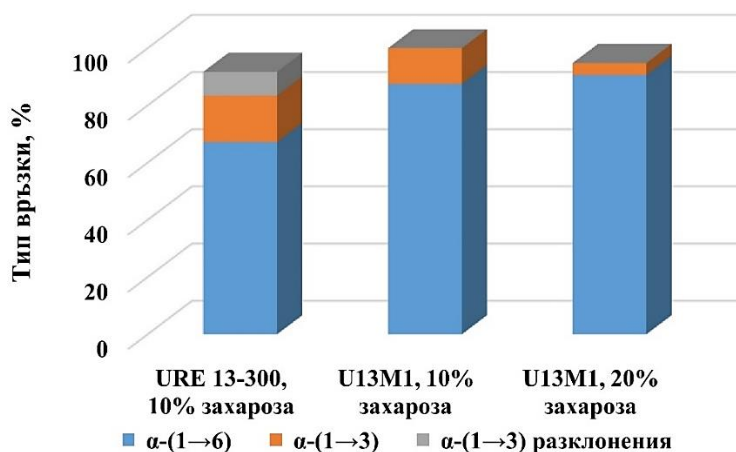

Figure 20. Comparison between the distribution of glycosidic linkages types in the α-glucan, synthesized by glucansucrase URE-300 and the modified glucans, synthesized by the mutant variant U13M1.

Mutant enzyme U13M1 synthesized α-glucan with a 23% increase in the content of α-(1→6) linkages and a corresponding decrease in α-(1→3) linkages in the main chain and also in the branches. 1H NMR spectra of the polysaccharide do not show a clear signal, detecting 3,6-di-O-substituted α-D-Glcp residues, but they are not completely absent, as they are observed at the base of the 4.96 ppm signal. The change in the ratio between α-(1→6) and α-(1→3) linkages is consistent with the results obtained by Funane *et al.* (2000). A single substitution of T350 or S445 with lysine in dextransucrase DSRS (corresponding to K344 and G449 in GS URE 13-300) leads to mutant variants synthesizing water-soluble glucans with an increased percentage of α-(1→6) linkages – up from 70% for the wild-type dextransucrase to 84% and 86%, respectively, in each of the mutant variants. Both residues are located far from the catalytic triad, but their double mutation leads to an increase in α-(1→3) linkages and even acquires the ability to include 4% α-(1→2) branch points.

Glucansucrase GTF180-ΔN from *Lb. reuteri* 180 synthesizes an α-glucan with 69% α-(1→6) and 31% α-(1→3) linkages – in the main chain and at the branch points, which corresponds to the type and ratio of linkages present in the polymer, synthesized by the wild-

type enzyme URE 13-300. Two positions in GTF180-ΔN glucansucrase – L938 and L940 – are known to be involved in shaping the acceptor substrate binding sites. Substitution of L938 with alanine, serine, and lysine (L938A/L938S/L938K) resulted in an increased amount of α-(1→6) linkages from 67% in the wild-type to 78%, 76%, and 90%, respectively (Meng, X. *et al.*, 2015). Furthermore, upon introduction of amino acids with an aromatic side chain, the ability to form α-(1→3) bonds decreased significantly upon substitution with phenylalanine (93%), and disappeared completely upon amino acid substitution with tryptophan. In comparison, the results of the present study showed that replacing the small glycine residue with the bulky amino acid lysine G449 (corresponding to G936 in GTF180-ΔN) resulted in a significant change in the ratio between α-(1→6) and α-(1 →3) links. A possible reason for this modification is that lysine may interfere with the ability to incorporate α-(1→3) linkages in the main chain and/or in the branches. The degree of branching in the modified glucan from U13M1 was greatly impaired due to the introduced mutation. Nevertheless, the polymer retained the insoluble characteristics of the polysaccharide synthesized by glucansucrase URE 13-300.

5.2. Study of the rheological properties of the modified glucan

The water-swelling behavior of both glucans produced in the enzymatic reactions with the wild-type glucansucrase URE 13-300 and the mutant variant U13M1 was determined. The water-holding capacity of U13M1 glucan was calculated to be 1125% (±7.6), representing nearly 2-fold increase, over the glucan synthesized by glucansucrase URE 13-300, namely 715% (±6.0). The insoluble polysaccharide synthesized by U13M1 formed a more uniform suspension that was not in the form of a hydrogel. High water holding capacity is usually accompanied by high water solubility due to the absorptive structure of the polymer caused by the formation of hydrogen bonds. In contrast to the results obtained in the present work, the water-soluble exopolysaccharides from *Lactococcus lactis* KC117496 and *Lb. kefiranofaciens* ZW3 had water holding capacity values of 117% and 496%, respectively (Ahmed, Z. *et al.*, 2013; Saravanan, C. *et al.*, 2016).

To investigate the rheological properties of the U13M1 polymer, its viscosity was evaluated at increasing shear rates and at different temperatures at a sample pH of 6.6 and a polymer concentration of 6% (Fig. 21). The results obtained show that the modified polymer exhibits shear-thinning behavior (decrease in apparent viscosity with increasing shear rates) like that of DSRSΔ4N or some other previously reported dextrans (Irague, R. *et al.,* 2012). The higher apparent viscosity was recorded at the lowest temperature. A temperature dependence of the viscosity of aqueous solutions of the studied polysaccharide was established.

The apparent viscosity of the original glucan showed the highest reported value of 1980 mPa.s at a temperature of 25 °C and a shear rate of 0.2 s-1. The established viscosity progressively decreases with increasing shear rate, reaching its lowest value – 750 mPa.s. This proved the shear behavior of insoluble glucan as a non-Newtonian polysaccharide. Increasing the temperatures leads to a reduction in viscosity to 623 mPa.s at 60 °C and a subsequent increase at 80 °C twice to 1220 mPa.s at the same shear rate.

In contrast to the results obtained with the native glucan, the modified insoluble glucan synthesized by mutant glucansucrase U13M1 showed a slightly higher apparent viscosity reported at 25 °C. The difference is more pronounced at a temperature of 8 °C – 3550 mPa.s at a shear rate of 0.2 s-1. Despite the lower viscosity at higher temperatures, it is noted that there is no significant difference between 25 °C and 80 °C. When the concentration of the modified insoluble glucan was reduced by a factor of two, the apparent viscosity was reduced about four times, from 2500 mPa.s to 577 mPa.s.

When evaluating the obtained results of the rheological analysis, it can be concluded that there was significant change towards a higher apparent viscosity of the modified polysaccharide as a result of the single point mutation leading to a change in its structure. This is a prerequisite for the further research of insoluble glucans with higher viscosity and their application in the food industry and as a protector of probiotic lactic acid bacteria.

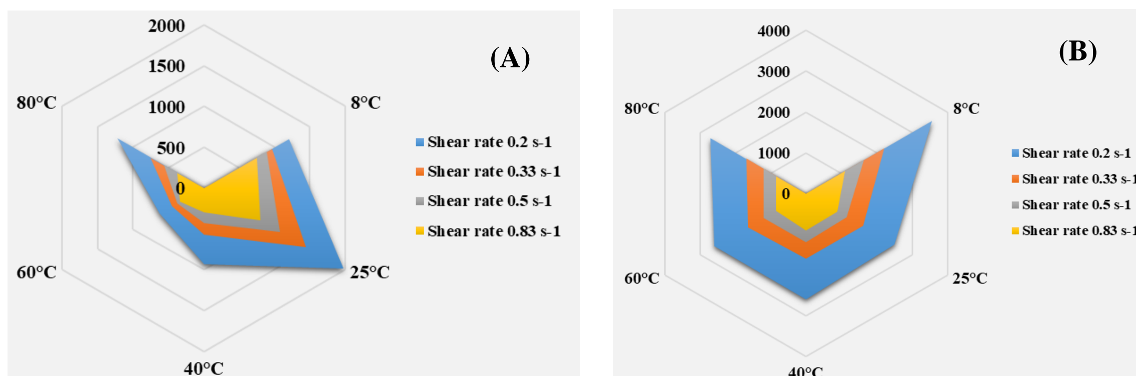

Figure 21. Influence of temperature on the apparent viscosity of the original glucan (A) and the modified glucan (B).

5.3. *Enzymatic synthesis of oligosaccharides synthesized by glucansucrase U13M1*

5.3.1. *Effect of different acceptors on oligosaccharide synthesis by glucansucrase U13M1*

In order to determine the effect of site-directed mutagenesis on oligosaccharide synthesis, a series of transglycosylation reactions were carried out in the presence of maltose, raffinose and lactose as acceptors. The type of oligosaccharides according to their degree of polymerization depends on the amount of the acceptor relative to the donor substrate. The following ratios were used for maltose and raffinose acceptors: acceptor:sucrose = 0.14/ 0.25/ 0.5/ 1/ 2/ 4 and 7. For lactose acceptor the ratios of 0.5, 1 and 2 were used.

Reactions in the presence of maltose. The highest oligosaccharide yield was reported at a ratio of acceptor:donor substrate M/S=1 – close to 118 mg/mL and ratio M/S=0.5 – 103.9 mg/mL. The lowest yield of oligosaccharides - 36.7 mg/mL was recorded in the reaction with the largest excess of maltose - M/S=7. The distribution of oligosaccharides according to their degree of polymerization depends on the ratio of the acceptor maltose to the donor of glucose residues sucrose. Oligosaccharides with DPs from 3 to 6 were synthesized through the acceptor reactions with maltose.

In all reactions, oligosaccharide fractions with DP 3 and 4 were prevailing. Oligosaccharides with DP 3 were predominant at ratios M/S=7 and M/S=4, while the remaining oligosaccharide fractions were only with DP 4. In the reactions where sucrose were in excess, the highest yield of oligosaccharides with all DP was reported at the ratio M/S=0.14. The yield of oligosaccharides with DP 3 was the highest at the ratio M/S=1 and 2 – about 50 mg/mL. The highest yield of oligosaccharides with DP 4 was achieved at a ratio of acceptor:donor M/S=1 – 56.6 mg/mL, and as a predominant oligosaccharide at a ratio of M/S=0.5.

In all analyzed reactions, oligosaccharides with degree of polymerization 5 and 6 were reported as the smallest fraction. Oligosaccharides with DP 5 are synthesized in the highest concentration at M/S=0.5 – 25.1 mg/mL. Oligosaccharides with DP 6 – 11.3 mg/mL remained the least represented in the reactions with M/S=0.14 (Fig. 22).

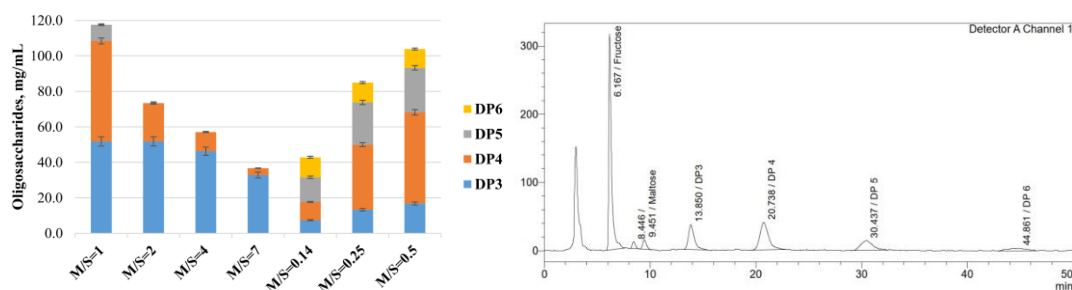

Figure 22. Distribution of oligosaccharides, synthesized by mutant U13M1 in the presence of maltose as an acceptor. HPLC chromatogram of the products at M/S=0.5 ratio.

Reactions in the presence of lactose. In the reactions carried out with lactose acceptor, predominantly oligosaccharides with DP 3 were synthesized, i.e. only one glucose residue has been added to the lactose molecule. In addition to them, a small fraction of oligosaccharides with DP 4 was synthesized - about 7 mg/mL in each of the conducted reactions. The highest total yield of oligosaccharides was reported at an equivalent ratio between acceptor and donor – 140.9 mg/mL. The distribution of oligosaccharides according to the ratio of acceptor and donor is presented in Fig. 23. Two different peaks were observed for the obtained oligosaccharides with DP 4, which proves their different structural organization.

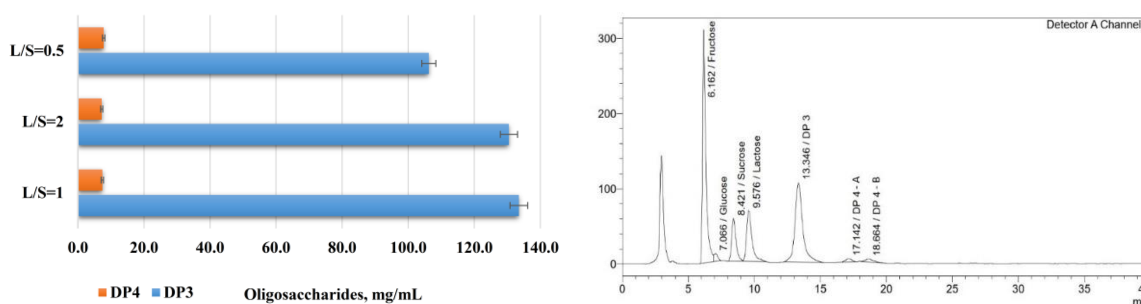

Figure 23. Distribution of oligosaccharides, synthesized by mutant U13M1 in the presence of maltose as an acceptor. HPLC chromatogram of the products at L/S=0.5 ratio.

Reactions in the presence of raffinose. During the reactions with raffinose, only oligosaccharides with DP 4 were synthesized, i.e. they contain only one glucose residue attached to the acceptor molecule. The highest yield of oligosaccharides was achieved at a ratio of raffinose:sucrose = 2 (Fig. 24). The lowest yields of oligosaccharides were obtained at ratios in which sucrose was in excess (0.14; 0.25; 0.5). The products of the reaction in the ratio R/S=7 have a concentration comparable to the previously mentioned ones. In them, a large part of the raffinose was not utilized, due to the depletion of free glucose in the medium. Other authors also reported glycosylation of raffinose. Dextransucrase DSR-S produces only GOS with DP 4, and Cote and colleagues reported the synthesis of tetrasaccharides and a minor amount of pentasaccharides from alternansucrase (Cote, G.L. *et al.,* 2009).

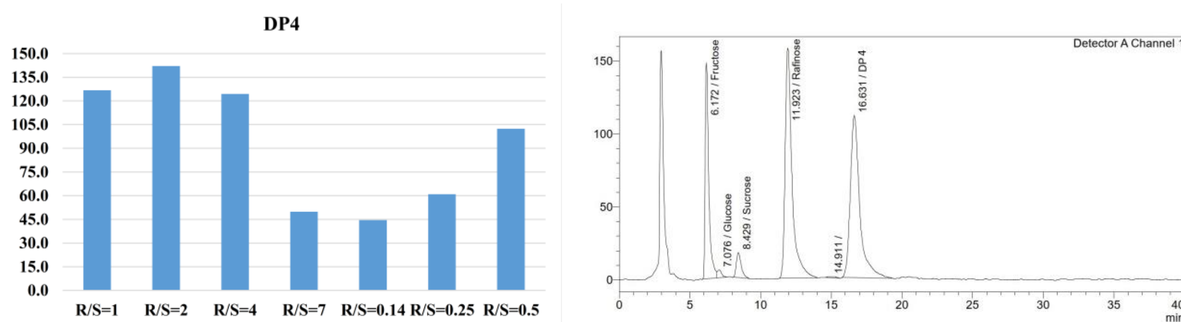

Figure 24. Distribution of oligosaccharides, synthesized by mutant U13M1 in the presence of maltose as an acceptor. HPLC chromatogram of the products at R/S=0.5 ratio.

5.3.2. Comparison between the oligosaccharide products, synthesized by the wild-type glucansucrase URE 13-300 and mutant variant U13M1

Acceptor reactions in the presence of maltose. In the reactions carried out with maltose, oligosaccharide products with a degree of polymerization of 3 to 6 were obtained, compared to those obtained from the starting glucansucrase, where oligosaccharides with DP 7 were also synthesized. Also, in both variants, the highest concentration of synthesized oligosaccharides was reported at an acceptor:donor ratio of 1. At the same ratio, glucanaccharase URE 13-300 synthesized GOS with DP 3 to 6, but the mutant enzyme synthesized mainly GOS with DP 3 and 4, as well as DP 5 at a low concentration of 9.2 mg/mL . The tendency with decreasing maltose to synthesize oligosaccharides of increasing length also remained unchanged.

When comparing the oligosaccharides synthesized by the two enzymes, it becomes clear that as a result of the mutation, the ability to synthesize oligosaccharides with a higher degree of polymerization is reduced (DP 6) or lost (DP 7). In addition, an overall decrease in the yield of total amount of oligosaccharides was observed.

Acceptor reactions in the presence of lactose. Comparing the products of the acceptor reactions with lactose, distinct differences are also found. The total oligosaccharide yield synthesized by the U13M1 mutant was nearly 15% higher than the oligosaccharides obtained from the wild-type glucansucrase. Both enzymes synthesized small fractions of oligosaccharides with DP 4, but the amount synthesized by the mutant enzyme was lower and almost equal in the three ratios tested. Subsequent analyzes of the structure of said oligosaccharides may shed light on how it has changed as a result of the mutation.

Acceptor reactions in the presence of raffinose. The obtained oligosaccharides with raffinose acceptor have a polymerization degree of 4 in both enzymes, i.e. only one glucose residue has been added to the raffinose molecule. The highest yield in U13M1 was reached at twice the concentration of raffinose (R/S=2), in contrast to the GOS obtained from glucansucrase URE 13-300, where the same result was achieved at a ratio of R/S=1. Mutant U13M1 synthesized 22% more oligosaccharides than raffinose.

5.4. Conclusion

As a result of the performed single point mutation at CD1 of glucansucrase URE 13-300, a mutant enzyme was obtained with a significant change in optimal pH and temperature values, as well as altered substrate affinity without changing the maximum reaction rate. We hypothesize that these changes in enzyme properties could shed light on possible altered conditions for the transferase reaction with the use of specific acceptors. The observed reduced catalytic potential of the mutant enzyme in the defined reaction conditions could be compensated by further optimization of the reaction parameters. Furthermore, using 20% sucrose (the optimal substrate concentration for the mutant enzyme) the polysaccharide synthesized by the U13M1 mutant enzyme showed a reduced ratio of α-(1 → 3)/α-(1 → 6) glycosidic linkages by about 30% in main chain compared to the parent glucan type. In turn, the modulated structure affects the specific physicochemical properties of the polymer and its application as a carrier of biologically active substances. In this way, it has been proven practically that a seemingly small substitution of one amino acid in a certain part of the enzyme structure can lead to a significant change in the structure of the product of the enzyme reaction, in this case polysaccharide. In addition, an increased amount of α-(1 → 3) glycosidic linkages was observed in the polymer when it was synthesized in the presence of 10% sucrose. This effect needs to be further investigated. The U13M1 polymer retained the property of insolubility in water, which is interesting in light of the increased amount of α-(1 → 6) linkages in its structure. This successful mutation of the glucansucrase URE 13-300 in its entirety, without truncation of the gene length, is a necessary basis for studying the interaction between the two catalytic domains acting as dextran sucrase and branching sucrase, respectively. With a more complete understanding of the mechanism of polymer and oligosaccharide synthesis, new polymers with improved physical properties and applications can be designed, as well as oligoglucans with modulated length and yield of targeted oligosaccharide fractions.

CONCLUSIONS

1. When optimizing the conditions for production of glucansucrase URE 13-300 in recombinant strain *E. coli* BL21 URE 13-300, a maximum production of the enzyme of 4.4 U/mg was found at a fermentation duration of 24 hours after the addition of 1 mmol/L inducer IPTG.

2. When acceptor reactions using maltose as an acceptor in the ratio M/S=0.5 in the presence of 20% organic solvents (DMSO, n-hexane and octanol) was carried out, it was found that glucansucrase URE 13-300 successfully synthesized oligosaccharides with a degree of polymerization from 3 to 6, in the following amounts: DP 3 – from 18.5 to 21.7 mg/mL; DP 4 – from 28.2 to 30.2 mg/mL; DP 5 – from 17.5 to 18.5 mg/mL; DP 6 – from 6.8 to 8.3 mg/mL.

3. As a result of the bioinformatic analysis, primers with the following sequence were designed:
   ForG449K
   5'CATTAGTAGTGAGTCTAAAAAAACAGATCATTTGCAAGGTGGTGCGCTC3';
   RevG449K
   5'GAGCGCACCACCTTGCAAATGATCTGTTTTTTTAGACTCACTACTAATG3'
   and replacing the amino acid glycine at position 449 with lysine in catalytic domain 1.

4. As a result of the performed site-directed mutagenesis, a mutant was obtained with a changed 3D structure in catalytic domain 1, expressed in a conformational change near the region connecting the acceptor molecules to the enzyme.

5. It was proved that the substitution of glycine at the 449th position with lysine changed the properties of the enzyme: the temperature optimum shifted from 30°C to 20°C, the pH optimum – from 5.3 to 6.5. The enzyme has the highest activity at twice the sucrose concentration – from 292 to 600 mM.

6. The mutant enzyme was found to have altered kinetic parameters: k_m = 0.33 M sucrose, V_{max} = 2.6 U/mg and k_i = 1.6 M sucrose compared to the wild-type enzyme.

7. It was proved that the modified glucan synthesized by mutant glucansucrase U13M1 has a changed structure, possessing nearly 70% less amount of α-(1 → 3) glycosidic bonds. The modified glucan retained its insolubility in aqueous solutions and showed altered apparent viscosity rheological properties.

8. Oligosaccharides synthesized by glucansucrase U13M1 using maltose as an acceptor retain their distribution by degree of polymerization DP 3 – 16.2%; DP 4 – 49.5%; DP 5 – 24.2%; DP 6 – 10.2% (ratio M/S=0.5). The best yield of oligosaccharides of 117.6 mg/mL was achieved at a ratio of M/S=1.

CONTRIBUTIONS

1. It was demonstrated for the first time that glucansucrase URE 13-300 can efficiently synthesize oligosaccharides in the presence of maltose as an acceptor in an aqueous-organic medium with up to 20% dimethyl sulfoxide, n-hexane and octanol, as well as in the presence of 5% ethanol.
2. For the first time mutation of glucansucrase URE 13-300 was carried out via the site-directed mutagenesis method, replacing glycine at the 449th position with lysine.
3. For the first time, site-directed mutagenesis of a glucansucrase containing two catalytic domains was performed without reducing the length of the gene, which was demonstrated to affect the overall 3D structure of the protein and its catalytic properties.
4. The possibility of obtaining a mutant glucansucrase (U13M1) which is not inhibited in the presence of twice the initial concentration of sucrose (600 mM) was demonstrated.
5. The potential of single point mutations in glucansucrase with two catalytic domains leading to the synthesis of a modified α-glucan with an altered structure that affects the physicochemical and rheological properties of its aqueous solutions has been demonstrated.

DISSERTATION RELATED PUBLICATIONS

1. **Angelova, S.**, Vasileva, T., Bivolarski, V., Iliev, I. (2023) Impact of G449 Single-Point Mutation on Glucansucrase URE 13-300 Enzyme Properties and Polysaccharide Structure. *Catalysts*, 13: 1455. https://doi.org/10.3390/catal13121455 (Q2)

2. **Angelova, S.**, Vasileva, T., Bivolarski, V., Iliev, I. Altered rheological properties of insoluble α-glucan, synthesized by mutant glucansucrase U13M1. *Acta Microbiol. Bulg.*, in press 39(4), 2023г. (Q4)

3. Peshkov, A., **Angelova, S**., Avesque, C., Iliev, I., Dimcheva, D. Determination of kinetic parameters of catalase of different origin immobilized on water-insoluble glucan synthesized by recombinant glycosyltransferase URE13-300. Ecol. Balk., in press 15(2), 2023г. (Q4)

REFERENCES

The complete literary review and complete list of references can be read in the dissertation.