REPUBLIC OF TÜRKİYE
YILDIZ TECHNICAL UNIVERSITY
GRADUATE SCHOOL OF SCIENCE AND ENGINEERING

CHARACTERIZATION OF GLUCANSUCRASE FROM *Leuconostoc mesenteroides* YTU-40 AND PRODUCTION OF DIFFERENT OLIGOSACCHARIDES BY ACCEPTOR REACTIONS

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MASTERS OF SCIENCE THESIS

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July, 2024

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A thesis submitted by Sevda DERE in partial fulfillment of the requirements for the degree of **MASTER OF SCIENCE** is approved by the committee on 23.07.2024 in Department of Food Engineering, Program of Food Engineering.

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Sevda DERE

Signature

This work was supported by the the project no “FBI-2022-5143 of YTU Coordinatorship of Scientific Research Projects”

ACKNOWLEDGEMENTS

First of all, I would like to express my deepest gratitude to everyone who supported and guided me throughout my academic life. I would like to thank my advisors, Prof. Dr. Enes DERTLİ and Prof. Dr. Osman SAĞDIÇ, for their unwavering support throughout my graduate studies. Their knowledge and experience have guided me, and I believe the valuable information they provided will greatly benefit me in my future professional life.

I am also deeply grateful to Assoc. Prof. Hümeyra İSPİRLİ who offered her guiding knowledge and experience in my studies, and Assist. Prof. Fatih BOZKURT, who sincerely assisted me with his technical knowledge throughout my laboratory studies. I extend my sincere thanks to Assoc. Prof. Yunus Emre TUNÇİL for allowing me to conduct *in vitro* fecal fermentation studies in his laboratory, generously sharing his expertise, and contributing to my research. I also thank Dr. Zuhal ALKAY for her invaluable support in conducting these analyses. I am grateful to Prof. Dr. Rabia ÇAKIR and Betül MUTLU for their support in carrying out cell culture studies.

Furthermore, I would like to acknowledge Yıldız Technical University Scientific Research Projects Coordination for supporting this study under project code FBI-2022-5143, and TÜBİTAK for supporting me through the 2210-A Domestic General Master's Scholarship Program.

I would like to thank my colleagues Kader KORKMAZ EDİŞ, Naciye Afranur KANIBOL, Duygu ÖZMEN, Öznur SAROĞLU and Selma KAYACAN ÇAKMAKOĞLU for the moral support they gave me throughout my studies and for making this process more enjoyable.

Finally, I extend my heartfelt thanks to my mother and brother for their endless support, belief, and sincerity.

Sevda DERE

TABLE OF CONTENTS

LIST OF SYMBOLS

α	Alpha Glycosidic Bond
~	Approximately
δ	Bending vibration
β	Beta Glycosidic Bond
C	Carbon
CO_2	Carbondioxide
$^{\circ}C$	Degrees Celsius
D_2O	Deuterium oxide
H	Hydrogen
μ	Micro
N	Nitrogen
Ni	Nickel
%	Percentage
V	Volt
H_2O	Water

LIST OF ABBREVIATIONS

aa	Aminoacid
Amp	Ampicillin
ATCC	American Type Culture Collection
BLAST	Basic Local Alignment Search Tool
Bp	Base pair
BSA	Bovine Serum Albumin
Caco-2	Colon Adeno Carcinoma Cell-2
CAZy	Carbohydrate-Active enZYmes
DMEM	Dulbecco's Modified Eagle Media
DNA	Deoxyribonucleic acid
DNS	Dinitrosalicylic Acid
DP	Degree of Polymerization
ELISA	Enzyme-Linked ImmunoSorbent Assay
EPS	Exopolysaccharide
F	Fructose
FBS	Fetal Bovine Serum
g	Gram
G/Glc	Glucose
Gal	Galactose
GC	Gas Chromatography
GH70	Glycosidic Hydrolase 70
GRAS	Generally Recognized as Safe
GS	Glucansucrase
GTF	Glycosyltransferase
h	Hour
HePS	Heteropolysaccharide
His-tag	Histidine-tagged
HoPS	Homopolysaccharide
HPLC	High Performance Liquid Chromatography
IL	Interleukin
IPTG	Isopropyl-Isopropyl-β-D-1-thiogalactopyranoside
K	Kelvin

kDa	Kilodalton
L	Leucrose
LAB	Lactic Acid Bacteria
LB	Luria Bertani
LC-MS	Liquid Chromatography-Mass Spectrometry
LDS	Lithium Dodecyl Sulfate
LIC	Ligation Independent Cloning
mg	Milligram
mL	Milliliter
MRS	DE MAN, ROGOSA and SHARPE
nm	Nanometer
NMR	Nuclear Magnetic Resonance
OD	Optical Density
PCR	Polymerase Chain Reaction
ppm	Parts Per Million
QPS	Qualified Presumption of Safety
SCFA	Short-Chain Fatty Acid
SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
Sec	Second
SOC	Super Optimal Medium with Catabolic Repressor
TBE	Tris Borate EDTA
TLC	Thin Layer Chromatography
TNF	Tumor Necrosis Factor
UV	Ultraviolet

LIST OF FIGURES

LIST OF TABLES

ABSTRACT

Characterization of Glucansucrase from *Leuconostoc mesenteroides* YTU-40 and Production of Different Oligosaccharides by Acceptor Reactions

Sevda DERE

Department of Food Engineering
Master of Science Thesis

Supervisor: Prof. Dr. Osman SAĞDIÇ
Co-supervisor: Prof. Dr. Enes DERTLİ

Glucansucrases (GS) are enzymes that enable the synthesis of α-glucans and various oligosaccharides using sucrose as the main substrate and are members of the glycoside hydrolase 70 (GH70) family. The presence of this enzyme has been demonstrated in many strains of lactic acid bacteria belonging to the genera *Leuconostoc, Lactobacillus* and *Weissella*. Glucan production is the basic reaction of GSs and glucans with different structures (dextran, alternan, mutan and reuteran) are produced depending on the amount of α-(1,6), α-(1,4) and α-(1,3) bonds in the structure. Another reaction catalyzed by GSs is acceptor reactions, which enable the synthesis of oligosaccharides by transferring the glucose released after the breakdown of sucrose to an acceptor sugar in the environment. Both the glucans produced by these enzymes and different oligosaccharides have various functional properties, including various prebiotic and immunomodulatory activities. Within the scope of this research, the glucansucrase gene was investigated in *L. mesenteroides* YTU-40 isolated from bee pollen and cloned and expressed in *Escherichia coli* BL21. In the next step, this enzyme was characterized, and the production of glucans and different oligosaccharides was carried out under *in vitro* conditions. The glucan was structurally characterized by NMR and its monomer units were revealed by HPLC. The ability of this enzyme to catalyze acceptor reactions was demonstrated by using maltose, melibiose, raffinose and cellobiose as acceptor sugars. These reactions were observed by TLC and the reaction products were characterized by NMR and LC/MS analyses. In order to evaluate the prebiotic potential of glucans and oligosaccharides, *in vitro* fermentation was carried out with colon microorganisms, and short-chain fatty acid production was monitored. Finally, these immunomodulatory effects were determined by measuring TNF-α, IL-12, IL-10 and IL-4 cytokine levels in the Caco-2 cell line (human colon adenocarcinoma, ATCC HTB-37). As a result, the ability of this enzyme to synthesize glucans and different oligosaccharides *in vitro* was demonstrated and it was revealed that these structures exhibited different prebiotic and immunomodulatory properties.

Keywords: Glucansucrase, glucan, oligosaccharide, prebiotic, immunomodulator.

ÖZET

Leuconostoc mesenteroides YTU-40'tan Glukansükrazın Karakterizasyonu ve Akseptör Reaksiyonları ile Farklı Oligosakkaritlerin Üretimi

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Glukansükrazlar (GS), ana substrat olarak sükroz kullanılarak α-glukanların ve farklı oligosakaritlerin sentezini sağlayan enzimlerdir ve glikozit hidrolaz 70 (GH70) ailesinin üyeleridir. Bu enzimin *Leuconostoc*, *Lactobacillus* ve *Weissella* cinslerine ait birçok laktik asit bakteri suşunda varlığı gösterilmiştir. Glukan üretimi GS'lerin temel reaksiyonu olup yapıdaki α-(1,6), α-(1,4) and α-(1,3) bağ miktarına bağlı olarak farklı yapılardaki glukanlar (dekstran, alternan, mutan ve reuteran) üretilir. GS'lerin katalizlediği bir diğer reaksiyon sükrozun parçalanmasından sonra açığa çıkan glikozu ortamdaki alıcı bir şekere aktararak oligosakkaritlerin sentezini sağlayan akseptör reaksiyonlarıdır. Bu enzimler ile üretilen glukanların ve farklı oligosakkaritlerin prebiyotik ve immunomodulatör aktivite başta olmak üzere çeşitli fonksiyonel özellikleri bulunmaktadır. Bu araştırma kapsamında, arı poleninden izole edilen *L. mesenteroides* YTU-40'da glukansükraz geni araştırılmış, *Escherichia coli* BL21'de klonlanmış ve eksprese edilmiştir. Sonraki aşamada bu enzim karakterize edilmiş ve *in vitro* koşullarda glukan ve farklı oligosakkaritlerin üretimi gerçekleştirilmiştir. Üretilen glukan NMR ile yapısal olarak karakterize edilmiş ve monomer birimleri HPLC ile açığa çıkarılmıştır. Bu enzimin akseptör reaksiyonları katalizleme yeteneği ise alıcı şeker olarak maltoz, melibiyoz, rafinoz ve sellobiyoz kullanılarak gösterilmiş, bu reaksiyonlar TLC ile gözlemlenmiş ve NMR ve LC/MS analizleri ile reaksiyon ürünleri karakterize edilmiştir. Glukan ve oligosakkaritlerin prebiyotik potansiyellerinin değerlendirilmesi amacıyla da kolon mikroorganizmaları ile *in vitro* fermentasyonu gerçekleştirilmiş ve kısa zincirli yağ asidi üretimi izlenmiştir. Son olarak bu yapıların immunomodulatör etkileri Caco-2 hücre hattı üzerinde (insan kolon adenokarsinomu, ATCC HTB-37) TNF-a, IL-12, IL-10 ve IL-4 sitokin seviyelerinin ölçülmesiyle belirlenmiştir. Sonuç olarak bu enzimin *in vitro* koşullarda glukan ve farklı oligosakkaritleri sentezleme yeteneği ortaya koyulmuş ve bu yapıların farklı prebiyotik ve immunomodulatör özellikler sergilediği gösterilmiştir.

Anahtar Kelimeler: Glukansükraz, glukan, oligosakkarit, prebiyotik, immünomodülatör.

1
INTRODUCTION

1.1 Literature Review

1.1.1 Lactic Acid Bacteria and Exopolysaccharide

Lactic acid bacteria (LAB), which include many genera such as *Lactobacillus*, *Lactococcus*, *Pediococcus*, *Streptococcus*, *Leuconostoc* and *Weissella,* are bacteria that produce energy and lactic acid by fermenting carbohydrates in foods. Lactic acid bacteria play a role in food fermentations, especially dairy products, and are found in many food matrices such as meat, fruits, vegetables and grains. In addition to their role in the formation of taste and aroma in foods, the regulation of texture, and as a starter culture, they have many benefits for human physiology and the immune system [1, 2].

Most probiotic bacteria, which are called "living microorganisms that provide health benefits to the host when administered in sufficient amounts," are members of the LAB [3]. While it is known that these bacteria must reach the host intestine alive to show their positive health-related effects, studies have proven that the metabolites of these bacteria can also show these effects, and the production of these metabolites has gained importance [4]. The important metabolites produced by LAB during fermentation are short-chain fatty acids, various organic acids such as lactic acid, bacteriocins, amino acids, neuroactive molecules, vitamins, oligosaccharide and exopolysaccharide (EPS) [5]. These compounds generally serve as a nutrient source for microflora, a protector for the intestinal epithelial barrier, and an inhibitor against pathogenic bacteria. It has also been reported in previous studies that they have antioxidant, immunomodulator, antitumor, anticancer and neurotransmitter properties [6]. Among these metabolites, exopolysaccharides have attracted increasing attention due to their various functional properties demonstrated in recent years.

EPS are long-chain polysaccharides, basically consisting of repeating monomers such as glucose, fructose, galactose and rhamnose, in a linear structure or in a branched structure with different degrees of branching. They are structures that are secreted into the environment of bacterial cells during their growth without being permanently attached to their surfaces and their synthesis is carried out by a group of enzymes called glycosyltransferases (GTFs) [7-9]. The functions understood so far are that it creates biofilms for colonization and acts as a protector against various vital risks in cells, such as preventing dehydration by keeping cells from losing water, preventing phage attacks, and phagocytosis [9, 10]. The fact that LAB have generally safe (GRAS) and qualified presumption of safety (QPS) status makes the EPS produced from them safe for food applications, and it is important to investigate their functional properties [4]. EPS define a group with wide structural diversity and are divided into two subgroups, homopolysaccharides (HoPS) and heteropolysaccharides (HePS) according to the monomer diversity in their structures. HoPS are polymer structures containing a single monomer such as glucose and fructose; HePS contain repeating units of at least two different monomers in their structure [7].

Although HoPS and HePS structures are different, the biosynthesis pathways of different microorganisms are extremely similar. In general, HoPS are synthesized outside the cell through the enzyme secreted by the microorganism, while HePS are synthesized inside the cell. The genetic sequence responsible for producing HePS contains more than one glycosyltransferase, whereas HoPS is synthesized extracellularly from sucrose by only one enzyme, such as glucansucrase and fructansucrase enzymes [8]. The biosynthesis pathway of HoPS is generally two-stage, and in the first stage, polymerization is completed by forming repeating units of monomer units forming the substrate, for example, as a result of glucansucrase enzyme activity, and then the polymer is released into the extracellular environment [12].

Figure 1.1 shows the classification and bond structures of the homopolymeric EPS [11]. According to the monosaccharide composition of HoPss, it can be divided into 3 subgroups: glucan, fructan and galactane, depending on whether it contains glucose, fructose and galactose, respectively. Most studies on homopolysaccharides focus on glucans. Glucans with different bonds are produced depending on the amount of α-(1,6), α-(1,4) and α-(1,3) in the structure. According to their chemical bonds, these structures are dextran (α-1,6), mutan (α-1,3), reuteran (α-1,4 and α-1,6) and alternan (α-1,6 and α-1,3) [13].

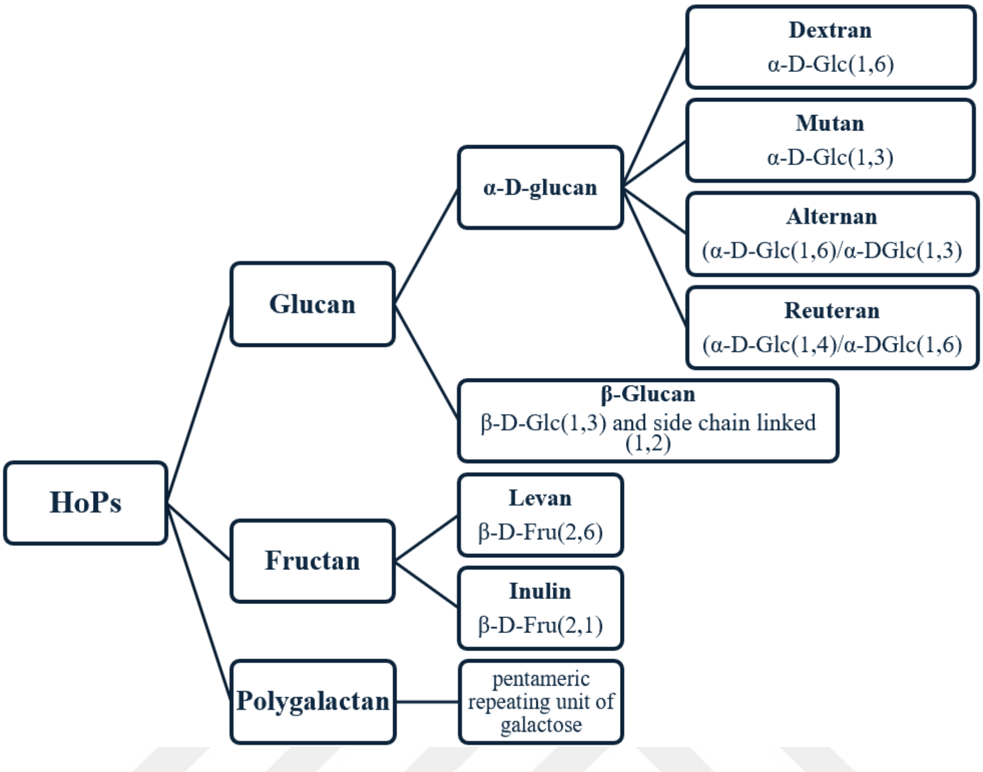

Figure 1.1 Homopolymeric exopolysaccharide (EPS) types and structures [11]

1.1.1.1 Dextran

Dextran is a type of glucan, where 50-97% of the glycosidic bonds in its basic structure are formed by α-1,6 bonds and may contain different amounts of α-(1,2), α-(1,3), α-(1,4) bonds (Figure 1.2) [14].

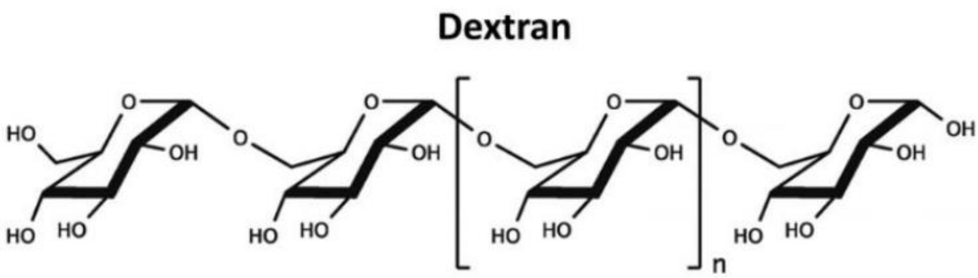

Figure 1.2 Structures of dextran, mainly containing α-1,6 bonds [14]

Dextrans are α-glucans produced from sucrose with different degrees of branching as a result of the activities of dextransucrase (EC 2.4.1.5) enzymes. Its production was first demonstrated in *Leuconostoc* strains, and *L. mesenteroides,* which is also used in commercial dextran production, are well-known dextran producers [15]. Studies have also shown that many *Lactobacillus, Weissella and Streptococcus* strains have the ability to produce dextran, and it has been revealed that the physical and chemical structures of those produced by different microorganisms are different For example, the water solubility capacity of these compounds depends on their size, branching levels, and the number of bonds in the structure. The size of these glucans varies depending on the amount of enzyme and substrate present in the environment for biosynthesis. Linear dextran containing high amounts of α-(1,6) is highly soluble in water. Commercial dextran produced by *L. mesenteroides* NRRL B-512F, contains 95% α-(1,6) and 5% α-(1,3) bonds. On the contrary, the structure of the *L. mesenteroides* NRRL B-742 strain contains 50% α-(1,6) and 50% α-(1,3) bonds [16, 17]. The dextran structure in the *L. mesenteroides* BI-20 strain isolated from bee pollen by Yilmaz et al. [18] was clarified by NMR analysis, and it was reported that the structure of branched dextran contains 80% α-(1,6) bonds and 20% (1,3) bonds. It has been reported that higher amounts (96-97%) of α-(1,6) are found in *Weissella* strains with demonstrated dextran production ability [19]. It was stated that in the dextran structure produced by *W. confusa* H2 strain, there were 96.6% of α-(1,6) bonds in the main chain and 3.4% of α-(1,3) branching [20].

Dextran, which has functions in both the food industry and non-food uses, is also used in medicine as a cholesterol reducer and as column material in chromatographic separation technologies. Dextran is currently used as thickening, stabilizing and gelling agent in foods and affects the rheological properties of food systems [9, 12]. It contributes to aroma and consistency when used in bakery, confectionery and sauce products. They helped preserve flavor by delaying oxidation in dairy products such as meat and cheese. It has the potential to be used in biofilm formation and packaging materials to be biodegradable and prevent moisture loss. In addition, its positive effects on improving human health and its prebiotic potential have also been investigated. Particularly low molecular weight dextrans have been shown to function as prebiotics [15, 21].

1.1.2 Glucansucrase

Glucansucrases (GSs) belong to the glycoside hydrolase 70 (GH70) enzyme family and are involved in synthesizing polysaccharides and oligosaccharides using sucrose as a substrate. These enzymes are typically responsible for the production of various glucans and are known as dextransucrases, mutansucrases, reuteransucrases, and alternansucrases [20]. Figure 1.1 in section 1.1.1 shows the α-D-glucans that these enzymes are responsible for producing. According to CAZy (http://www.cazy.org), a database that studies carbohydrate biosyntheses and the enzymes involved, this enzyme was encoded and characterized in more than 60 bacteria mostly belonging to the *Leuconostoc*, *Streptococcus*, *Lactobacillus* and *Weissella* genera, and their structures were elucidated. Despite LAB having similar glucansucrases, the resulting polysaccharides and oligosaccharides may exhibit different functional and technological properties [17].

The presence of glucansucrase enzyme activity in bacteria is understood by the formation of slime-type viscous structures in agar to which sucrose is added. This ability of new glucan producers in LABs can be demonstrated in a short time, but the sequences, structure, and characterization of the glucansucrases responsible for production are not frequently performed. The glucan synthesis ability of bacteria found in food matrices such as pickles, sourdough, fruits and vegetables, dairy products, meat products, grains, and bee products, where LAB are dominant, has been demonstrated [22-25].

Glucansucrase is an enzyme secreted outside of the cell and induced by sucrose, the presence of dextransucrase and sucrose alone in the reaction medium is sufficient for dextran formation [26]. In general, one of the basic methods of obtaining bacterial glucan is microbial fermentation with the relevant substrate. These methods are complex, costly, and often difficult to obtain high yield products. *In vitro* enzymatic synthesis of these structures has provided the opportunity to work with low-cost substrates, sustainable production, and high yield. It includes the stages of growing bacteria under appropriate conditions (substrate, pH, temperature, etc.) and enzyme isolation. Biotechnological developments have enabled the synthesis pathway of glucans and GS enzymes to be elucidated. Heterologous expression of the GS gene encoded in LAB on *E. coli* and extraction of the recombinant enzyme enable *in vitro* glucan production. Thus, high-efficiency glucan production can be achieved instead of costly and complex methods based on fermentation [27, 28].

In general, glucansucrases have a molecular weight of 160 kDa. While the optimum operating temperature is between 30-37 °C, 5.0-6.0 pH is the optimum operating conditions [29]. When the structures of GSs are examined, it is shown that they consist of 4 basic areas. The structure includes a (1) signal peptide, an (2) N-terminal region consisting of highly variable amino acids, a (3) catalytic region common to GS with a high sequence similarity, and a (4) C-terminal domain consisting of tandem repeats involved in the binding of glucans [28, 30, 31]. Figure 1.3 represents the basic structural characterization of glucansucrase of the *L. mesenteroides* NRRL B-512F strain used in commercial dextran production [28].

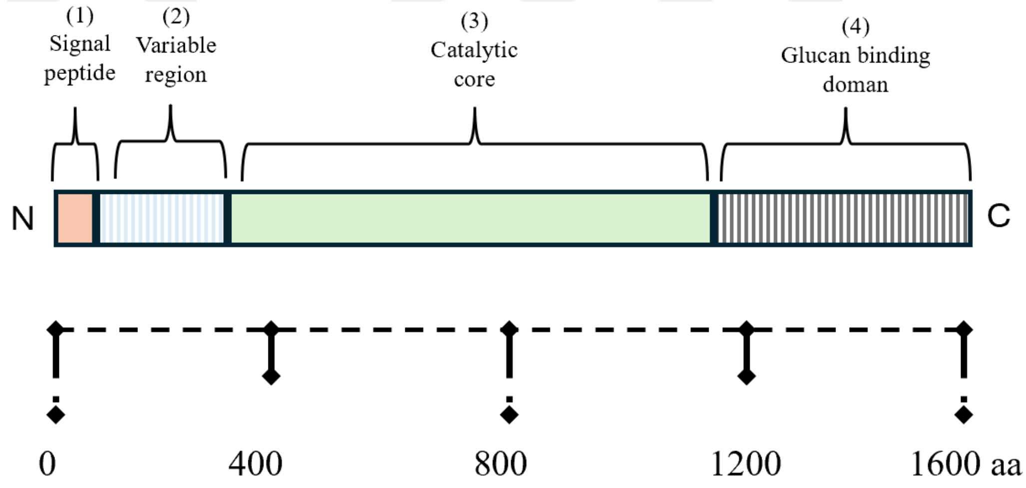

Figure 1. 3 Basic structural characterization of glucansucrase of the *L. mesenteroides* NRRL B-512F [28].

The structure of *L. mesenteroides* NRRL B-512F represents a typical example of the chemical structure commonly found in GS enzymes. There are 35-38 amino acids in the signal peptide (1) region. This is followed by a highly variable region (2) consisting of 140-261 amino acids, where the number of conserved amino acids is very low. It has been reported that the differentiation of this region among GSs may be a sequence specific to the enzyme. The catalytic core (3) contains approximately 900 amino acids and shows high similarity among glucansucrases as the region primarily responsible for glucan production [30, 31].

1.1.2.1 Mechanism of Glucansucrase

In the mechanism of glucansucrases, the first step of the reaction is the breaking of the α-glycosidic bonds between glucose and fructose, which form sucrose. This enables 3 different reactions to occur: hydrolyzation (1), acceptor reactions (2) and polymerization (3). The first reaction is hydrolysis, where water molecules act as acceptors, and sucrose is broken down into glucose and fructose. The second reaction results in the transfer of glucose units from the medium to an acceptor. The most basic mechanism is the polymerization mechanism, in which the energy released as a result of the breakdown of sucrose in the environment is used to bind glucose units, and the growing glucan chain acts as an acceptor [32, 33].Three different reactions catalyzed by glucansucrases are shown in Figure 1.4 [24].

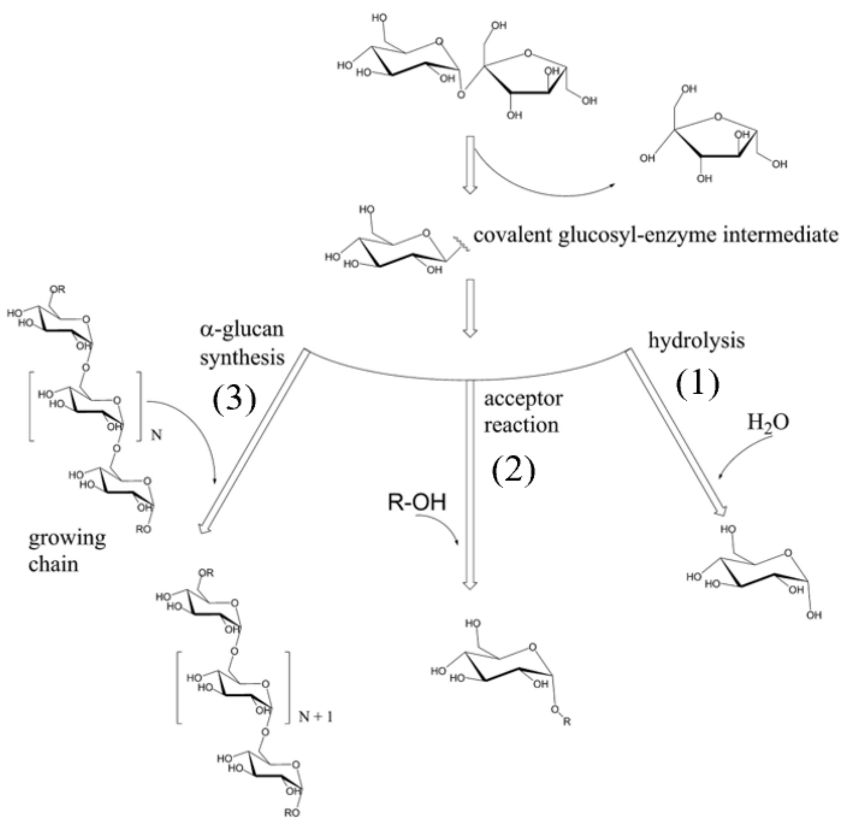

Figure 1. 4 Reactions catalyzed by glucansucrases [24]

Two alternative mechanisms have been proposed for glucan formation: α-retaining double displacement (1) and reducing end elongation (2). In the first stage, the fructose α(1→2)-glycosidic bond in the sucrose structure is broken down and released, and a glucosyl enzyme intermediate product is formed, which is covalently bonded to the catalytic nucleophile via the β-glycosidic bond. The covalently attached glucosyl group is transferred to the non-reducing end of a growing glucan chain, leading to the reformation of the α-glycosidic bond [34].In the second mechanism, there are two sucrose binding sites that act as nucleophiles. Initially, these nucleophilic sites attack two sucrose molecules, creating two covalent glucosyl-enzyme intermediates. One glucosyl intermediate C-6 hydroxyl group then attacks the C-1 of the other glucosyl intermediate, forming an α(1→6) glucosidic bond and an isomaltosyl intermediate. The newly freed nucleophilic site then attacks another sucrose molecule, forming a new glucosyl-enzyme intermediate. This symmetric and alternating action of the two sucrose binding sites leads to the elongation of the glucan chain by its reducing end [29,31].

In the formation of branched products, the GS enzyme also catalyzes branching reactions. Examining the substrates and enzymes of all these mechanisms together may help elucidate the mechanisms. Parameters such as the number and location of donor and acceptor subregions, glycosidic bond types, degree of branching, etc. may play a role in the differences between the products formed [31].

1.1.2.2 Oligosaccharide Synthesis with Acceptor Reaction

Another important reaction catalyzed by GS enzymes is acceptor reactions, which allow the formation of different oligosaccharides depending on the acceptor substrate present in the reaction [35]. Oligosaccharides are low molecular weight structures with degrees of polymerization generally between 3-10 [36].

In recent years, interest in oligosaccharides with different structures has increased because they show various health benefits to humans and are described as prebiotics. Oligosaccharides that have gained importance in this sense are fructooligosaccharides, galactooligosaccharides, xylooligosaccharides and glycooligosaccharides [37]. The natural sources of these oligosaccharides are various foods and biomass sources, and production methods include extraction, polymer hydrolyzation, enzymatic, and chemical methods. Unlike enzymatic methods, other methods require longer steps, and a wide variety of by-products are formed in the reaction environment. One of the methods that has been frequently used in the production of new oligosaccharides in recent years is the use of recombinant GS enzymes. The use of glucansucrases in oligosaccharide synthesis stands out as a low-cost, environmentally friendly, and efficient method [38-41].

In the reaction, firstly, the fructose α-(1,2)-glycosidic bond in the sucrose structure is broken down and released, the glycosidic part is transferred to the acceptor substrate in the environment, and the α-anomeric configuration is preserved [42]. Studies have revealed that these enzymes can produce different oligosaccharides in high yields by using acceptor molecules such as glucose, maltose, cellibiose, raffinose, mellibose, lactose, mannose, gentibiose, etc. as an effective acceptor sugar molecule [29, 43, 44]. One of the most comprehensive studies on this subject examined the products formed as a result of the reactions of the Glucansucrase E81 enzyme with a wide variety of acceptors such as maltose, raffinose, lactose, cellobiose, mannose, melibiose and gentiobiose [41, 44-49]. It has been shown that raffinose-derived oligosaccharides are not digested *in vitro* and DP4 oligosaccharides have a prebiotic effect on *Bifidobacterium.* Mannose acceptor oligosaccharides, on the other hand, could reach 7 degrees of polymerization and did not cause induction of anti-inflammatory and pro-inflammatory cytokines tested under *in vitro* conditions. The products of melibiose receptor reactions consist of DP3-4 oligosaccharides, and these structures have shown immunomodulatory functions by triggering the production of anti-inflammatory and pro-inflammatory cytokines. Based on these results, it can be inferred that the changing structures in the reaction of these oligosaccharides, depending on their acceptor types, have different biological and functional properties [44, 46, 47].

The functional properties of oligosaccharides produced by glucansucrase enzymes have provided them with potential for use in many industries. Currently, it is known that in addition to the previously mentioned prebiotic effects, it also has immune system regulating properties. They have a wide range of applications in the food, nutraceutical, pharmaceutical, feed, and cosmetic industries [24]. While it can be used in foods as stabilizers, thickeners, etc., it is also added to develop functional food products [28].

Low molecular weight acceptors (glucose, maltose, gentibiose, etc.) derived oligosaccharides are structures that contain at least one more D-glucopyranosyl group than acceptor carbohydrates. It is suggested that the most ideal receptor for the formation of gluco-oligosaccharides is maltose [42]. In the study conducted by

Hu et al. [42] acceptor reactions were established using glucansucrose produced by *Leuconostoc citreum* SK24.002, and malto-oligosaccharides with polymerization degrees varying between 3-5 and shown to have prebiotic effects were produced. DP3, oligosaccharides promoted the growth of probiotic bacteria, and DP4 oligosaccharides showed human health-improving effects by increasing the production of short-chain fatty acids.

Various parameters (time, temperature, donor/acceptor concentration, etc.) and yields are important in the oligosaccharide reactions of GSs. The sizes, polymerization degrees, and reaction efficiencies of glucansucrase-derived oligosaccharides depend on the enzyme used and the substrate concentration in the medium [46]. In the study conducted by Bivolarski et al. [50] the ability of the glucansucrase URE 13–300 enzyme to produce oligosaccharides through acceptor reactions was demonstrated using maltose, raffinose, and lactose. In the reaction, malto-oligosaccharides with polymerization degrees 3-7 were synthesized. The highest oligosaccharide yield for each acceptor was obtained when the donor and acceptor reacted at the same rate. It was observed that when the ratio of maltose/sucrose in the reaction increased, the ratio of maltooligosaccharides with a degree of polymerization greater than 3 decreased. When this ratio decreased, the production of maltooligosaccharides with polymerization degrees of 4, 5, 6 and 7 increased. On the other hand, it has been shown that reactions using raffinose are not affected by the donor and acceptor ratio and produce only DP4 products. This is important in showing the effect of the concentration and structure of the acceptor molecule on the products. In addition, the reaction time for oligosaccharide synthesis *in vitro* directly affects the oligosaccharide yield. As a result of changing parameters and reactions with different glucansucrases, oligosaccharide structures and their functional properties vary [51].

1.2 Objective of the Thesis

Compounds with various physiological and functional properties are produced by lactic acid bacteria. Among these compounds, exopolysaccharides are compounds with different chemical structures produced by the glucansucrase enzyme activity of lactic acid bacteria. Glucans constitute a significant part of these structures, and the most well-known is dextran. Synthesizing these structures *in vitro* is important for elucidating their chemical structures and investigating their functional properties. The first aim of the study is to reveal the glucansucrase gene encoded in *Leuconostoc mesenteroides* YTU-40, clone it in *E. coli* BL21 and then express the enzyme. In addition, it is aimed at producing glucan and different oligosaccharides as a result of two basic reactions of this enzyme by breaking down sucrose *in vitro*. Finally, it was aimed at elucidating the structures with various chemical characterization analyses and investigating the changes in the prebiotic potentials and immunomodulatory effects of different structures.

1.3 Hypothesis

Due to the wide variety and complex structures of the products produced in the reactions catalyzed by glucansucrases, studies are needed to fully understand the mechanisms, elucidate the structures and determine their functional properties. Therefore, the expression of glucansucrases is important for the efficient production of compounds with many functional properties with cheap substrates. This study aims to draw a road map for the detection, cloning, and expression of glucansucrase in YTU-40, a strain of *L mesenteroides*, one of the important representatives of lactic acid bacteria. In this regard, demonstrating potential compounds that can be produced *in vitro* and characterizing the glucan and different oligosaccharide structures produced may shed light on future studies. In addition, as a result of the analyses to be carried out in this research, it is aimed at understanding the prebiotic functions of these structures and revealing their immunomodulation abilities.

2
MATERIAL AND METHODS

2.1 Material

2.1.1 Bacterial Strain, Media and Growth Conditions

The *Leuconostoc mesenteroides* AP-40 strain used in this study was isolated from bee pollen as part of the study conducted by Ispirli and Dertli [52] and was shown to have glucansucrase activity within the scope of the project numbered FBI-2022-5143, supported by Yıldız Technical University Scientific Research Projects Coordination Unit, and all its genome has been sequenced. (This strain was recorded in Yıldız Technical University Food Engineering culture collection as *L. mesenteroides* YTU-40). This strain was grown in Man, Rogosa, and Sharpe (MRS) medium at 30°C, stock solutions containing 20% glycerol were prepared and stored at -80°C throughout the study.

E. coli BL21 and *E. coli* TOP10 strains were used as expression and cloning vectors in the transformation, respectively, and were grown in Luria Bertani (LB, 10.0 g/L tryptone, 5.0 g/L yeast extract, and 10,0 g/L NaCl) Broth medium at 37°C, 200 rpm and under aerobic conditions. LB media containing 100 µg/mL ampicillin were used to ensure the selectivity of the plasmid.

SOC (super optimal broth with catabolite repression, 20.0 g/L tryptone, 5.0g/L yeast extract, 4.0 g/L glucose, 10 mM NaCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$ and 2.5 mM KCl,) medium was used to grow *E. coli* strains after transformation.

All strains were obtained from the culture collection of Yıldız Technical University, Department of Food Engineering.

2.1.2 Primers, Plasmids and Kits

Bacterial genomic DNA of *L. mesenteroides* YTU-40 was extracted using the genomic DNA kit (Thermo Scientific, USA).

The aLICator Ligation Independent Cloning (LIC) and Expression Kit 3 (Thermo Scientific, USA) was used to clone the GS40 gene from *L. mesenteroides* YTU-40.

This kit enables ligation independent cloning equipped with the IPTG-inducible vector pLATE31 as shown in Figure 2.1.

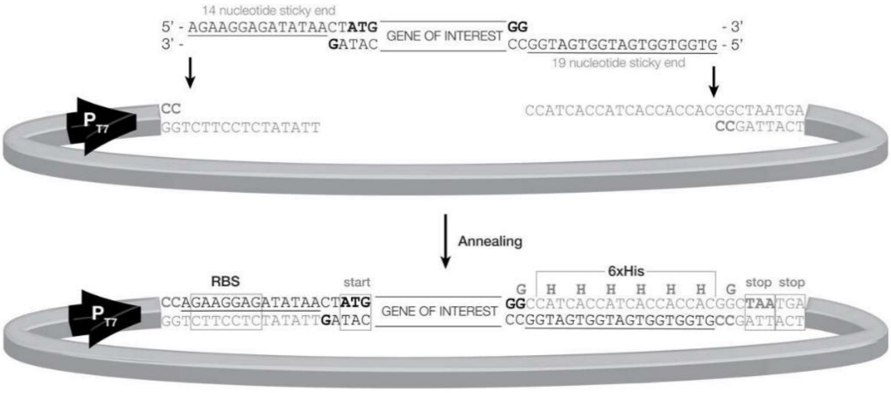

Figure 2.1 The pLATE31 expression vector

The primers used for the cloning of the GS40 gene from *L. mesenteroides* YTU-40 strain are as given below. Primers were obtained from Sentegen (Ankara, Turkey).

GS40_LIC_F 5'- AGAAGGAGATATAACTATGtataaatctgggaaaatgttag-3’

GS40_LIC_R 5'-GTGGTGGTGATGGTGATGGCCttagcttttaatcagctctccag-3’

Using designed primer set, the PCR processes were carried out with Phusion High-Fidelity DNA Polymerase (Thermo Scientific, USA). The PCR product of the successfully amplified GS40 gene was purified using the GeneJET Gel Extraction Kit (Thermo Scientific, USA) before the cloning reaction. EZNA Plasmid DNA Mini Kit I (Omega Bio-Tek, USA) was used for plasmid extraction. Colony PCR processes were performed with Dream Taq DNA Polymerase (Thermo Scientific, USA)

The purification step of the GS40 enzyme, which was determined to have activity, was started, and proteins containing His-tag were purified using the HisPur Ni-NTA Purification Kit (Thermo Scientific, USA) for the purification of the enzyme.

Human ELISA kits (SunRed Biotech, China) were used to quantify the levels of IL-4, IL-10, IL-12 and TNF-α cytokines.

2.1.3 Chemicals and Reagents

Sucrose, maltose, raffinose, melibiose and cellobiose were obtained from Sigma-Aldrich (Steinheim, Germany). Chemicals used for the other analysis were all purchased from Sigma-Aldrich (Steinheim, Germany) and were analytically grade.

2.2 Method

2.2.1 Whole Genome Sequencing of *L. mesenteroides* YTU-40

Whole genome sequencing of *L. mesenteroides* YTU-40 was performed using Illumina HiSeq 4000 platform (Whole genome sequence analysis was performed at Quadram Institute Bioscience, Norwich, UK). For this at first genomic DNA was isolated from strain YTU-40 using a Promega Maxwell Cultured Cells DNA Extraction Kit (AS1620) and DNA concentration was quantified with a Qubit reagent (Promega) prior to the DNA sequencing. Following the quantification, a DNA library was assembled using a modified protocol based on Illumina's Nextera, specifically designed as Low Input Transposase Enabled libraries. Sequencing yielded 947,409 paired-end reads of 150 bp length on the Illumina HiSeq 4000 platform. The reads underwent quality trimming using bbduk (v.38.06) (available at sourceforge.net/projects/bbmap), with a minimum quality threshold set at 3. For long-read sequencing, a DNA library was created following the manufacturer's guidelines using the native barcoding genomic DNA expansion 1–12 kit (EXP-NBD104, EXP-NBD114, and SQK-LSK109, Nanopore, UK) and sequenced via a MiniION platform, provided by Quadram Institute Bioscience.

To obtain a high-quality closed genome, Prokka system was used and the formed contigs were then searched for the presence of a glucansucrase gene using BLAST comparison with other glycoside hydrolase 70 (GH70) enzymes. In the next step, the possible glucansucrase enzyme was identified by performing multiple amino acid sequence alignments with the Clustal Omega Tool (https://www.ebi.ac.uk/Tools/msa/clustalo). ExPASy Compute pI/ Mw (http://web.expasy.org/compute_pi/) was used to determine the theoretical Mw (molecular weight) of the potential glucansucrase. A putative glucansucrase gene was identified in the genome of *L. mesenteroides* YTU-40 and this gene was submitted to GenBank with the PP922942 accession number.

2.2.2 Genomic DNA Extraction

After the gene sequence responsible for glucansucrase production was determined, the genomic DNA of *L. mesenteroiedes* YTU-40 was extracted to use as a template in PCR reactions. For this purpose, bacteria were grown in MRS medium at 30°C, the night before. 1 mL of the culture was centrifuged (6000 rpm, 10 min) and DNA extraction was carried out by following the procedure recommended by the manufacturer. The extracted DNA was stored at -20°C throughout the study.

2.2.3 Amplification of GS40 Gene with PCR

Using primer set, the PCR reaction was carried out as given in Table 2.1.

Table 2.1 PCR mix prepared for amplification of the GS40 gene

H_20	30,5 µL
GC Buffer	10µL
10 mM dNTPs	4 µL
GS40_LIC_F (20 µM)	1,25µL
GS40_LIC_R (20 µM)	1,25 µL
DMSO	1,5 µL
Template DNA	1 µL
Phusion DNA Polymerase	0,5 µL

The relevant gene region was amplified by performing a PCR reaction according to the conditions given in Table 2.2.

Table 2.2 Optimized PCR reaction conditions for amplification of the GS40 gene

Denaturation	98°C for 30 sec	
Denaturation Annealing Extension	98°C for 10 sec 54°C for 1 min 72°C for 1.5 min	25 cycle
Final Extension	72°C for 10 min	1 cycle

2.2.4 Agarose Gel Electrophoresis

Agarose gel electrophoresis was carried out to control the size of the product obtained from the PCR reaction. 10 µL of PCR product was loaded onto the gel prepared using 1% agarose and Tris Borate EDTA (TBE, 0.5X) buffer, after being colored with loading dye. Electrophoresis was performed using TBE buffer at 100

V for 45 min. After this period, the agarose gel was kept in a solution containing ethidium bromide for 30 minutes for staining and then rinsed in distilled water. Observation was performed under UV light (Gel Doc XR+Imager, Bio-Rad). GeneRuler 1 kb DNA Ladder (Thermo Scientific, USA) was used as a DNA sizer. The PCR product of the successfully amplified GS40 gene was purified before the cloning reaction.

2.2.5 Construction of pLATE31-GS40 Expression Cassette

In order to calculate the amount of PCR product for LIC reaction, the DNA concentration in the purified PCR product was determined with Biospec-Nano (Shimadzu, Japan). The LIC cloning reaction using 6 mL PCR product, considering the 4428 bp length of the GS40 gene and the DNA concentration, is given in Table 2.3.

Table 2.3 LIC cloning reaction composition

LIC Buffer (5X)	2 μL
PCR product	6 μL
Water	1 μL
T4 DNA Polymerase	1μL

The reaction mixture was vortexed, centrifuged briefly, and incubated at 25 °C for 5 min. After the incubation period, the reaction was terminated by adding 0.6 μL of 0.5M EDTA, and then the annealing reaction was started by adding 1 μL of pLATE31 to the mixture. This reaction was vortexed and centrifuged briefly, then incubated at 25 °C for at least 5 min and used as vector in the transformation process within 2 h.

2.2.6 Preparation of Electrocompetent *E. coli* Cells and Transformation in *E. coli* TOP10

Cloning vector *E. coli* TOP10 and expression vector *E. coli* BL21 cells were used as electrocompetent in the transformation. For this purpose, cells were grown in LB medium at 37°C and 200 rpm and incubated until they reached an optical density of 0.5-0.6 at OD600 nm. Cultures that reached the desired density were centrifuged (4500 rpm, 10 min), and the cell pellet was washed twice by centrifugation with cooled 10% sterile glycerol solution. All steps were performed on ice. After the washing process, the cell pellet was suspended in 500 µL of cooled 10% glycerol solution and aliquoted into 50 µL. While the competent cells were kept on ice for use, after the electroporation process was completed, the excess cells were stored at -20°C for use in subsequent studies.

In the next step, the pLATE31-GS40 was transferred to electrocompetent cells using an electroporator (Eppendorf, Germany) to perform the transformation. For electroporation, 0.5-1 µL of annealing mix mixture was added to 50 µL of electrocompetent cells, which were kept on ice, and mixed carefully and incubated on ice for 1 minute. Additionally, while distilled water was used as a negative control, the control pLATE31-Cm plasmid was used as a positive control. The transformation process was carried out by placing the mixture containing competent cells and vector into electroporation cuvettes. Immediately after electroporation, 450 µL of SOC medium was added to the cuvettes, and the mixture was incubated at 37°C and 200 rpm for 2 hours. After this period, the cells were incubated in LB agar medium (100 µg/mL ampicillin) for a night at 37°C. As a result of incubation, colonies were selected, and colony PCR process was applied.

2.2.7 Colony PCR and Transformation in *E. coli* BL21

After electroporation, control PCR was carried out to check the pLATE 31-GS40 vector on selected colonies. For this purpose, colonies were taken into 10 µL distilled water, and the PCR reaction given in Table 2.4 was carried out using the primer set **LIC Forward Sequencing primer** 5'-TAATACGACTCACTATAGGG-3' and **LIC Reverse Sequencing primer** 5'-GAGCGGATAACAATTTCACACAGG-3'.

Table 2.4 Composition for colony PCR mixture

Buffer (10X)	2 µL
dNTPs (10 mM)	1,6 µL
LIC F (10 µM)	1 µL
LIC R (10 µM)	1 µL
H_2O	13,8 µL
DNA	0,5 µL
Taq DNA Polimeraz	0,1 µL

Colony PCR processes were performed with the PCR conditions given in Table 2.5. After the colony PCR process, agarose gel electrophoresis and imaging were performed as stated in section 2.2.4. One of the colonies determined to have the relevant size was selected and transferred into 10 mL LB broth (100 µg/mL ampicillin), incubated at 200 rpm at 37°C, and glycerol stock was prepared and stored at -80°C.

Table 2.5 Conditions used for colony PCR reaction

Denaturation	95°C for 3 min	1 cycle
Denaturation Annealing Extension	94°C for 30 sec 50°C for 30 sec 72°C for 4 min	25 cycle
Final extension	72°C for 5 min	1 cycle

For the second stage of transformation, plasmid extraction was performed in accordance with the kit procedure. The extracted plasmid was electroporated into *E. coli* BL21. Preparation of *E. coli* BL21 cells and electroporation of the target plasmid were carried out as described before. After incubation of cells and selection of colonies, the size of the product was reconfirmed by performing colony PCR.

2.2.8 Expression and Extraction of Glucansucrase in Recombinant Bacterial Cells

The GS40 gene was cloned into pLATE31 vector and expressed in *E. coli* BL21. For enzyme expression, colonies were grown in LB broth (with 100 µg/mL ampicillin) at 200 rpm at 37°C for a night, and then cells were passaged into LB broth medium under the same conditions and incubated until they reached an optical density of 0.5-0.6 at OD600 nm. To initiate protein expression in pLATE31, 0.1 M IPTG was added to the cells that reached the desired optical density and continued for 24 hours at 200 rpm at 25°C. After this period, the cells were centrifuged (4000 rpm, 15 min, 4°C), and the cell pellet was kept at -20°C for 1 night before extraction. For extraction, the cell pellet was suspended in 20 mM NaAc (pH 5.5) at a ratio of 1:5 and performed with an ultrasonic water bath for 1 min of sonication at 35 kHz and 1 min of incubation on ice, for a total of 10 cycles. Following extraction, the crude enzyme solution was centrifuged (4500 rpm, 15 min) to eliminate cellular debris. Subsequently, the procedures for verifying enzyme activity and purifying the active enzyme were undertaken.

2.2.9 Enzyme Activity and Purification of Glucansucrase

The enzyme activity of GS40 was observed by the dinitrosalicylic acid method. For this purpose, 500 uL of buffer containing 100 mM sucrose solution, 1 mM $CaCl_2$ and 50 mM NaAC (pH 5.5) was prepared, and 20 uL of enzyme was added to the reaction medium. The reaction was continued at 37°C for 15 minutes, and at the end of the period, 100 µL of the reaction medium was placed in a glass tube, 900 µL of distilled water, and 3 mL of DNS solution were added and kept at 95°C for 5 minutes. At the end of the period, the absorbance of the solutions cooled to room temperature was measured at 540 nm. Additionally, a calibration curve was drawn to determine the amount of fructose formed in the reaction medium. After its activity was determined, the GS40 enzyme was purified using a purification kit, which targets 6xHis tagged proteins in the plasmid. The kit protocol was followed with some slight modifications, and the purified protein was stored at −20°C containing 10% pure glycerol during the study.

2.2.10 Determination of Protein Amounts of Purified Enzymes by Bradford Method

The Bradford method was used to determine the amount of protein in the purified enzyme. The analysis was carried out in a 96-well plate, and 5 µL of enzyme solution and 250 µL of Bradford Reagent were added to the wells and incubated for 10 minutes at 25°C. At the end of the period, absorbance was measured at 595 nm using a spectrophotometer (Epoch 2 BioTek, USA). Additionally, solutions containing different concentrations (0-2000 µg mL^{-1}) of bovine serum albumin (BSA) were prepared, and a calibration curve was drawn.

2.2.11 SDS-PAGE

The presence and size of GS40 were detected using the SDS-PAGE method. After determining the protein concentration by the Bradford method, the sample volume was calculated as 10 µg μL^{-1} protein. Lithium dodecyl sulfate (LDS) buffer and reducing agent were added to the sample and denatured by heat treatment (70°C, 10 min). Electrophoresis was carried out using 1X uPAGE MES SDS Running Buffer (Invitrogen, USA) for 35 min at 200 V. Following the electrophoresis process, the gel was washed 3 times with distilled water and stained with Simply Blue Safestain (Invitrogen). After staining, the gel was rinsed in distilled water for at least one hour, and observing was performed under UV light using a UV transilluminator (Gel Doc XR+Imager, Bio-Rad). SeeBlue Plus ladder (Invitrogen, USA) was used as a reference to check the sizes of the proteins.

2.2.12 Effect of Temperature and pH on GS40 Enzyme

To determine the optimum operating temperature and optimum pH value of the GS40 enzyme, the reaction medium was prepared as section 2.2.9. The effect of temperature on enzyme activity was determined by carrying out the reactions between 20-60 ºC and the optimum operating temperature was determined. To determine the optimum pH, the pH of the reaction medium was adjusted to the range of pH 3-9.

2.2.13 *In vitro* Glucan Production

For glucan production with purified GS40 enzymes, 100 mm sucrose, 1 mM $CaCl_2$ and 50 mM NaAC (pH 5.5) reaction conditions were prepared and continued at 30 °C for approximately 1 day. At the end of 24 hours, the presence of sucrose in the medium was observed by thin layer chromatography (TLC) described in section 2.2.17. When the sucrose in the reaction was finished, ethanol at twice the total reaction voume was added to precipitate the glucan in the medium. Glucan was dialyzed using a 12-14 kDa dialysis membrane. After the purification process was completed, the polysaccharide was dried in a lyophilizer.

2.2.14 Determination of Monosaccharide Composition of the Glucan by HPLC

The monomer composition of the pure glucan was determined by HPLC (Shimadzu, Japan). For analysis, 800 µL polysaccharide solution (20 mg/mL) was taken and heated with 72% perchloric acid at 95°C for 2 h to release the monosaccharide units. Then, 500 µL of 5 M KOH was added to this solution to neutralize, and the sample was centrifuged (13.500 rpm, 5 min) to precipitate the salt. It was then filtered through a 0.22 µm pore size filter and analyzed by HPLC. HPLC conditions and details of the analysis are given elsewhere [22].

2.2.15 Structural Analysis of the Glucan by NMR Spectroscopy

^{1}H and ^{13}C NMR spectroscopy analyses were applied to determine the bond composition of glucan produced by GS40 enzyme. For analysis, 20 mg of glucan sample was dissolved in 500 µl of D_2O, and one-dimensional ^{1}H and ^{13}C NMR analysis was taken with AVANCE III 400 MHz NMR (Bruker BioSpin International AG) at 300 K. The results were analyzed with MestReC and the bond structure was revealed.

2.2.16 Production of Gluco-oligosaccharides with Different Acceptor Sugar

In order to test the ability of the GS40 enzyme to add the glucose released by the breakdown of sucrose to different acceptor, reactions were prepared with maltose, glucose, lactose, raffinose, cellobiose, mannose, melibiose, fructose and cellobiose. For this purpose, sucrose (0.4 mg mL^{-1}) and acceptor sugar (0.1 mg mL^{-1}) were added to the 1 mM $CaCl_2$, 20 mM NaAC (pH 5.4) reaction medium, and the acceptor reactions were continued at 30°C [46]. The formation of gluco-oligosaccharides was monitored by TLC.

2.2.17 Thin Layer Chromatography (TLC) Analysis of Different Gluco-oligosaccharides

The formation of gluco-ligosaccharides and depletion of sucrose in the acceptor reactions were monitored by TLC using Silica gel 60 F254 (20x20 cm - Merck, Darmstadt, Germany) plates. For TLC analysis, reaction products were diluted 50-fold with ethanol, loaded onto TLC plates, and run for 90 min using 80% acetonyltrile. At the end of the period, the plate was washed with methanol/sulfuric acid/N-1-naphthyl(ethylenediamine) dihydrochloride, and sugar fragments were observed with heat application.

2.2.17.1 Removal of Sugars by Application of Immobilized Yeast from Gluco-oligosaccharides Reaction

In acceptor reactions, simple sugars (fructose, sucrose, etc.) in the medium were removed from the medium using *Saccharomyces cerevisiae* (commercial baker's yeast) immobilized in sodium alginate, and cells in bead form were obtained, as described previously (Hu, Winter, Chen, & Ganzle, 2017). 10% immobilized yeast cells were added to the acceptor reactions, and the mixture was incubated at 30°C until the other sugars in the reaction were consumed (at least 24 hours).

2.2.18 Structural Characterization of Different Oligosaccharides with NMR Analysis

The structural characterisation of the maltose, cellobiose and cellobiose derivative gluco-oligosaccharides was applied by ^{1}H NMR analysis as described in part 2.2.15.

2.2.19 Determination of the Molecular Masses of the Oligosaccharides

For the determination of the molecular masses of maltose, cellobiose, raffinose and cellobiose derivative gluco-oligosaccharides with respect to their DPs, LC/MS/Q-TOF analysis was performed with an Agilent 6530 LC/MS/Q-TOF instrument and details of the analysis were given elsewhere [41].

2.2.20 Determination of the Prebiotic Potential of Glucan and Different Oligosaccharides

The *in vitro* fecal fermentation was applied with the ethical approval of Necmettin Erbakan University, Health Sciences Scientific Ethical Committee with the approval number of 2022/259 within the scope of the project numbered FBI-2022-5143, supported by Yıldız Technical University Scientific Research Projects Coordination Unit. In this context, glucan and gluco-oligosaccharides produced by GS40 (produced using maltose, melibiose, raffinose, and cellobiose as acceptors) were subjected to *in vitro* fermentation with colon microorganisms. Before this process, the samples were treated as described before [53] with upper digestive system enzymes to show that they could reach the colon without being digested in the upper digestive system of the human body, which is a prerequisite for qualifying as prebiotic.

2.2.20.1 Fermentation of Specific Glucans and Different Oligosaccharides by Colon Microorganisms

In order to monitor short-chain fatty acids (SCFA), which are formed as a result of the fermentation of glucans and different oligosaccharides by colon microorganisms, *in vitro* fecal fermentation was carried out as previously described [53, 54]. Fecal samples for analysis were taken from 3 healthy, omnivorous female donors who had not used antibiotics for the last three months and whose body mass index was within the normal range. After the fecal samples were collected, they were quickly transferred to the anaerobic cabinet with a gas composition of 90%

N_2, 5% CO_2 and 5% H_2, and analysis was started within 2 hours. For analysis, 50 mg of glucan or oligosaccharide samples and 4 mL of sterile carbonate-phosphate buffer were added to each Balch tube (to be analyzed at time intervals of 0, 6, 12, 24 and 48 hours). The feces were homogenized and filtered using the same buffer. The filtrates were mixed, and 1 mL was added to each tube. The tubes were closed airtight to prevent anaerobic conditions and were left for incubation (37°C, 150 rpm).

2.2.20.2 Determination of Microbial Metabolites (Short-Chain Fatty Acids) Formed During Fermentation

The identification of short-chain fatty acids produced by the microbial fermentation of glucans and different oligosaccharides in the large intestine was performed using Gas Chromatography (GC) according to the method described by Tuncil et al. [54]. At the end of each time interval, the relevant tubes were opened, and 1 mL of sample was taken, and an internal standard was added and aliquoted. Collected samples were stored at -20°C until analysis. For analysis, the samples were at room temperature and centrifuged, and the supernatant was analyzed for the quantitative and qualitative determination of microbial short-chain fatty acid production.

2.2.21 Determination of Immune Modulation Properties of Glucan and Different Oligosaccharides

Caco-2 cells, a human colorectal adenocarcinoma cell line, were used to determine the immune regulatory properties of the glucans and oligosaccharides to be produced on pro-inflammatory TNF-α, IL-12 and anti-inflammatory IL-10, IL-4 cytokines. For this purpose, the Caco-2 cell line (ATCC, USA) was obtained from the cell culture stock of YTU Food Engineering Department. Caco-2 cell line was developed in DMEM (Gibco, USA) medium containing 10% FBS (fetal bovine serum) (Gibco, USA) and 1% Penicillin-Streptomycin (Gibco, USA), at 37ºC and in an incubator containing 5% CO_2. The cells were passaged as stated in Horn et al., (2013), and when they were thought to have reached sufficient maturity (at the end of 2-3 passages), the induction process with glucan and different oligosaccharides was started. For this purpose, freeze-dried samples were dissolved in DMEM medium with a total concentration of 1 mg mL^{-1}. Caco-2 cells were passaged and counted, and they were seeded in 6-well cell culture dishes at 400,000 cells per well and incubated in 3 mL DMEM for 24 hours. After the incubation period, 100 µg mL^{-1} of sample mixtures were applied to the Caco-2 cells and the cells were incubated for 24 hours. DMEM was added to the blank sample. After the 24 hours incubation period, the media in the 6-well plates was collected and stored at -20ºC until the ELISA experiment was performed to determine the amounts of IL-4, IL-10, IL-12 and TNF-α cytokines. Following the manufacturer's methodology, the amounts of IL-4, IL-10, IL12 and TNF-α induced by the glucan to be produced were determined in the microplate reader (Epoch2 BioTek, USA) spectrophotometer at 450 nm.

2.2.22 Statistical Analysis

Immunomodulatory effect and SCFA production analysis were performed in triplicate. Results were represented as means± standard deviation. SCFA production data were subjected to one-way analysis of variance (ANOVA) using SPSS (Version 22, SPSS Inc, Chicago, IL, USA). Tukey's multiple comparison test was used to compare differences at the $p<0.05$ significance level. GraphPad Prism® (Version 10, GraphPad Software Inc., La Jolla, CA 92037 USA) statistical package program was used for graphical drawings.

3
RESULTS AND DISCUSSION

3.1 Glucansucrase 40 Nucleotide and Protein Sequence Analysis

BLASTn analysis of *L. mesenteroides* YTU-40 whose whole genome was sequenced, showed the presence of a 4428 bp glucansucrase gene in this genome that is highly similar to other glucansucrase enzymes belonging to the GH70 family. GS40 nucleotide sequence demonstrated a similarity of 98.12% and 94.81% with the GSs from *L. mesenteroides* subsp. *suionicum* strain DSM 20241 (CP015247.1) and *L. mesenteroides* strain KNU-2 (CP089782.1), respectively. In terms of protein similarity, GS40 with a 1475 amino acid length showed 95.57% protein sequence similarity to *L. mesenteroides* DPC 7261, according to BLASTp similarity test. Comparison of GS40 with biochemically characterised GTFA E81 from *Lactobacillus reuteri* E81 (accession no. MH051191, 1767 aa) based on the Clustal Omega tool alignment, revealed a significant level of alterations in the amino acid sequence of GS40, especially in the catalytic region of the GS, suggesting the potential novelty of the GS40.

3.2. Cloning of the GS40 Gene from *L. mesenteroides* YTU-40

3.2.1 Amplification of GS40 Gene with PCR

After the gene region responsible for glucansucrase production was identified, amplification of this region was carried out using primers whose 5' end contained vector-specific sequences and whose 3' end was designed to provide sufficient overlap with the gene of interest. Figure 3.1 shows the agarose gel image showing the successful acquisition of the 4428 bp long GS40 gene, which was amplified as a result of the PCR reaction performed using GS40_LIC_F and GS40_LIC_R primers.

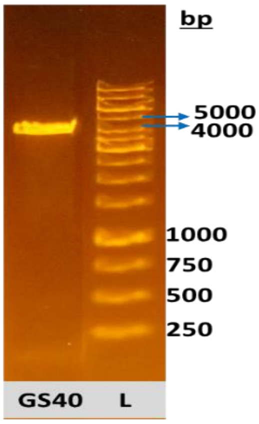

Figure 3.1 Amplification of the GS40 gene

3.2.2 Construction of pLATE31-GS40 and Transformation in *E. coli* TOP10 and *E. coli* BL21

Before setting up the LIC reaction, the PCR product of GS40 amplification was purified, and the amount to be used in the reaction was calculated. The LIC reaction product was then used directly for bacterial cell transformation. In the first step of transformation, electrocompetent *E. coli* TOP10 cells were used. After electroporation, cells were incubated on LB agar containing ampicillin (100 µg/mL) for 1 night at 37 °C. Figure 3.2 shows colonies growing on LB agar at the end of the incubation period.

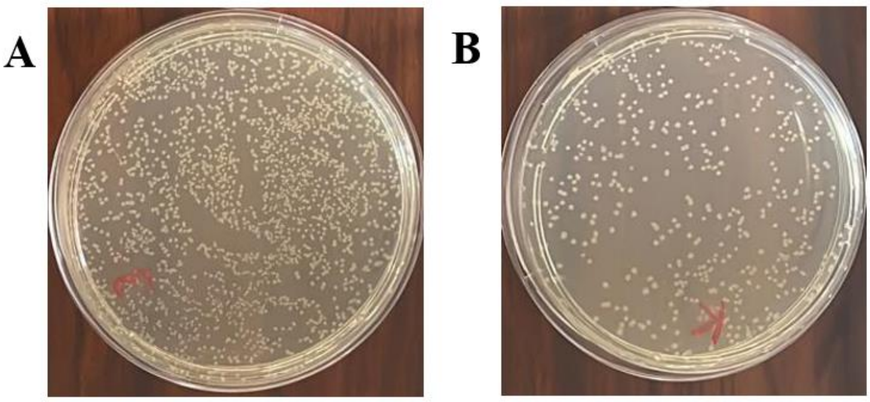

Figure 3.2 *E. coli* TOP10 cells with pLATE31-GS40 plasmid (A) and pLATE31-Cm plasmid as a positive control (B) grown on LB agar containing 100 µg/mL ampicillin as a result of electroporation.

The presence of pLATE31-GS40 plasmid in the selected colonies was checked by colony PCR using LIC_F and LIC_R primers. The agarose gel image of the products obtained as a result of colony PCR is given in Figure 3.3.

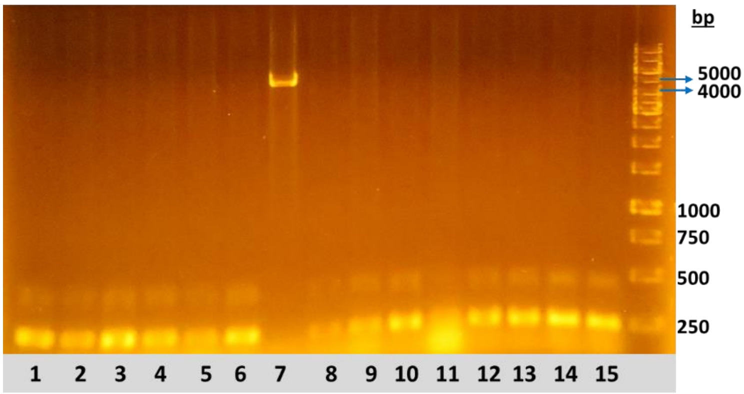

Figure 3.3 Testing of pLATE31-GS40 positive colonies from *E. coli* TOP10 colonies by colony PCR.

Obtaining a product with a length of 4633 bp (GS40 gene is 4428 bp, the relevant region of the pLATE31 vector is 205 bp long) with the LIC_F and LIC_R primer set is an indication that the cloning was performed correctly.

One of the *E. coli* TOP10 cells confirmed as a result of colony PCR was selected and developed in LB Broth (100 µg/mL ampicillin) and plasmid extraction was performed. After extraction, the plasmid was transformed into an *E. coli* BL21 cell. Developing colonies were confirmed by colony PCR as mentioned in 2.2.7 (Figure 3.4).

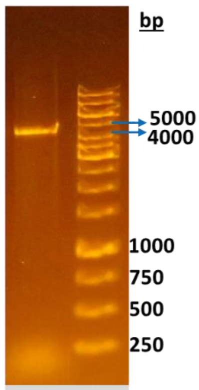

Figure 3.4 Testing of pLATE31-GS40 positive colonies from *E. coli* BL21 colonies by colony PCR.

3.3 Expression of Recombinant GS40

The pLATE31-GS40 expression cassette created for the expression of GS40 was transformed into *E. coli* BL21 and grown for protein production. Protein expression in cells was achieved by IPTG induction and extracted by sonication. SDS-PAGE was performed to check the activity and purified enzyme protein expression.

The theoretical molecular weight of the potential glucansucrase was determined to be approximately 162 kDa by ExPASy Compute pI/ Mw (http://web.expasy.org/compute_pi/). Electrophoresis against the 196 kDa SeeBlue Plus ladder of the GS40 enzyme, given in Figure 3.5, confirmed the size. Glucansucrase enzymes have very large molecular weights, 110-300 kDa [55]. SDS-PAGE analysis of glucansucrase produced from *Lactobacillus kunkeei* AP-37 by İspirli et al. [35] revealed the presence of an enzyme with a size of 300 kDa.

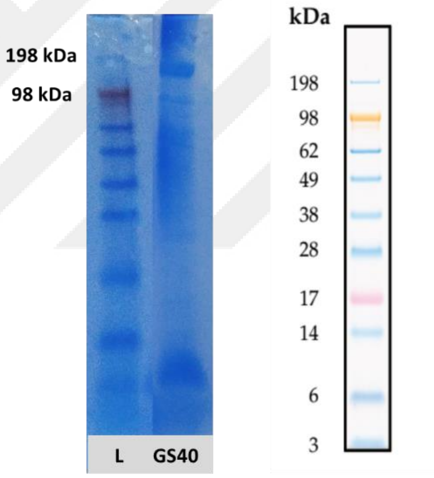

Figure 3.5 Control of protein expressions by SDS-PAGE

3.4 Effect of Temperature and pH on GS40 Enzyme

The optimum working temperature of the GS40 enzyme was measured between 10-60 °C and the optimum working pH was between 3-9 by determining the amount of fructose released into the environment. As can be seen from Figure 3.6A, the optimum operating temperature of GS40 is 30 °C, and its activity decreases rapidly when the optimum temperature is exceeded. In general, the result is similar to the optimum operating temperature previously determined for recombinant glucansucrases. Similar results were obtained when the enzyme activity and temperature relationship of glucansucrase *L. mesenteroides* TDS2-19 were examined [56]. On the contrary, glucansucrace from *Lactobacillus reuteri* 180 showed its highest enzyme activity at 50 °C [43]. The optimum working pH of glucansucrases is between 5.0-6.0 [29]. Figure 3.6B shows that glucansucrase has the highest enzyme activity at pH 6. It has been reported that the optimum working pH of glucansucrases obtained from *Leuconostoc* strains is 5.5 [57].

3.5 In Vitro Glucan Production and Characterization

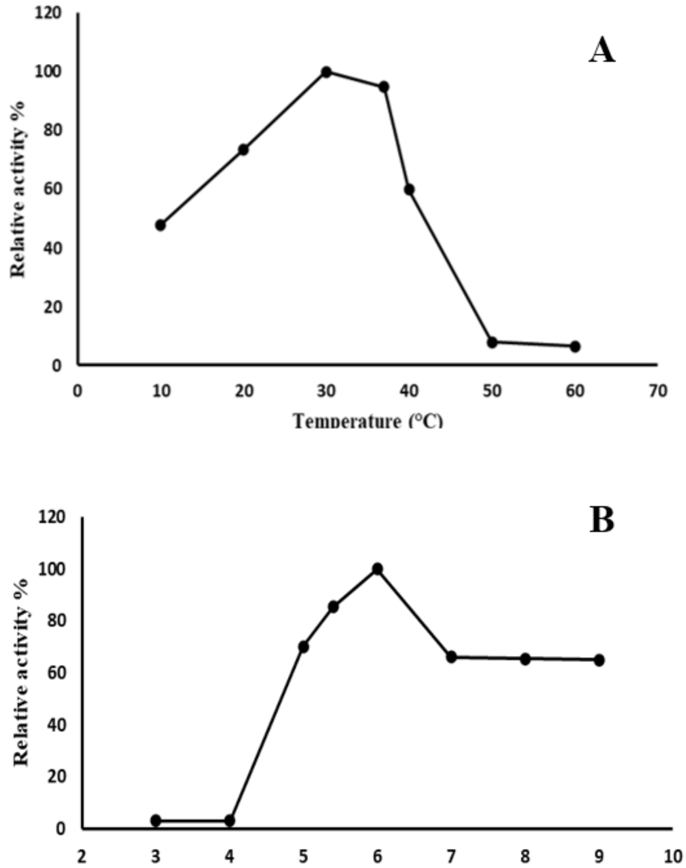

Figure 3.6 Effect of temperature (A) and pH (B) on GS40 enzyme

The monosaccharide unit of the glucan produced under *in vitro* conditions using the GS40 enzyme was revealed by HPLC. According to the chromatogram given in Figure 3.7, it was revealed that the glucan consisted only of glucose units.

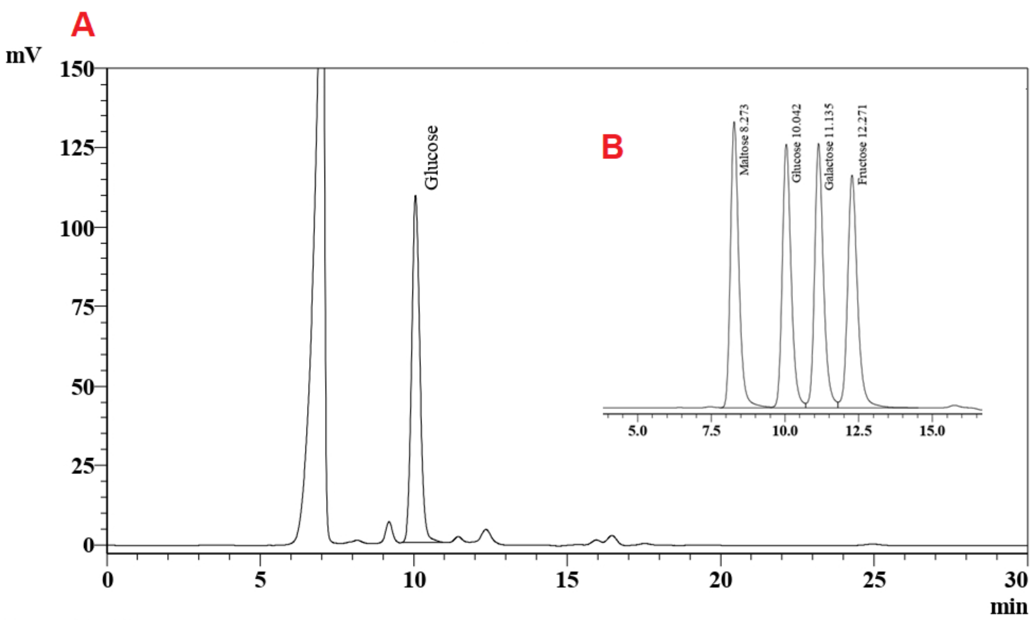

Figure 3.7 HPLC chromatograms showing the monosaccharide content of the polysaccharide produced by the GS40 enzyme (A) and the standard sugars used in calibration (B).

Structural analysis of α-glucan produced by GS40 enzyme was performed by ^{1}H and ^{13}C NMR. Figure 3.8A and B shows the ^{1}H and ^{13}C NMR spectra of the glucan polymer, respectively. Figure 3.8A, ^{1}H spectra demonstrated the specific spectral resonances at 3.3 ppm and 5.3 ppm. In the ^{1}H spectrum, the characteristic anomeric signals appeared at 4.8 and 5.13 ppm and represent the presence of (1→6)-linked α-D-glucose units and (1→3)-linked α-D-glucose units in the structure, respectively [22, 58]. Another important observation for the ^{1}H spectra was the fact that H-4 signal which (appeared to be at ~3.40–3.45 ppm) was observed to be at a high intensity which arose from the terminal glucosyl residues, revealing the high level of branching linkages (~ 20%) [35, 59, 60]. In addition, no peak was observed at around ~ 4.20 ppm in the ^{1}H spectra of glucan 40, which was previously allocated to H-5 signal of [(→6)-α-D-Glcp-(1→3))-] residue, indicating that the branching α-(1→3) glucosyl residues were all terminal [60]. The ^{1}H spectra of glucan 40 revealed that GS40 was responsible for the production of a highly branched dextran containing ~ 20% α-(1→3) glucosyl residues. The peaks observed in the region between 3.2 and 4.0 ppm in this spectrum originate from other protons in the glucan structure [22].

^{13}C NMR spectra of glucan 40 revealed six signals at 98.9 ppm, 73.9 ppm, 72.1 ppm, 70.8 ppm, 70.01 ppm and 65.6 ppm that corresponded to C-1, C-3, C-2, C-5, C-4 and C-6, respectively (Figure 3.8B) [18, 35, 58]. Overall, these findings revealed that the expressed GS40 was an active novel glucansucrase responsible for the production of branched dextran with ~ 20% α-(1→3) glucosyl residues. *L. mesenteroides* strains are well-known for being classical dextran producers containing low levels of (1→3)-linked α-D-glucopyranosyl units as branching points (~5%) [58] although recent studies also revealed the production of branched dextrans produced by other *Leuconostoc spp*. [18, 58]. Nevertheless, the capability of obtaining a pure GS40 producing a branched dextran from sucrose can be important to produce this novel dextran at high levels without any impurities that can be further used for distinct technological purposes, including gluco-oligosaccharide production.

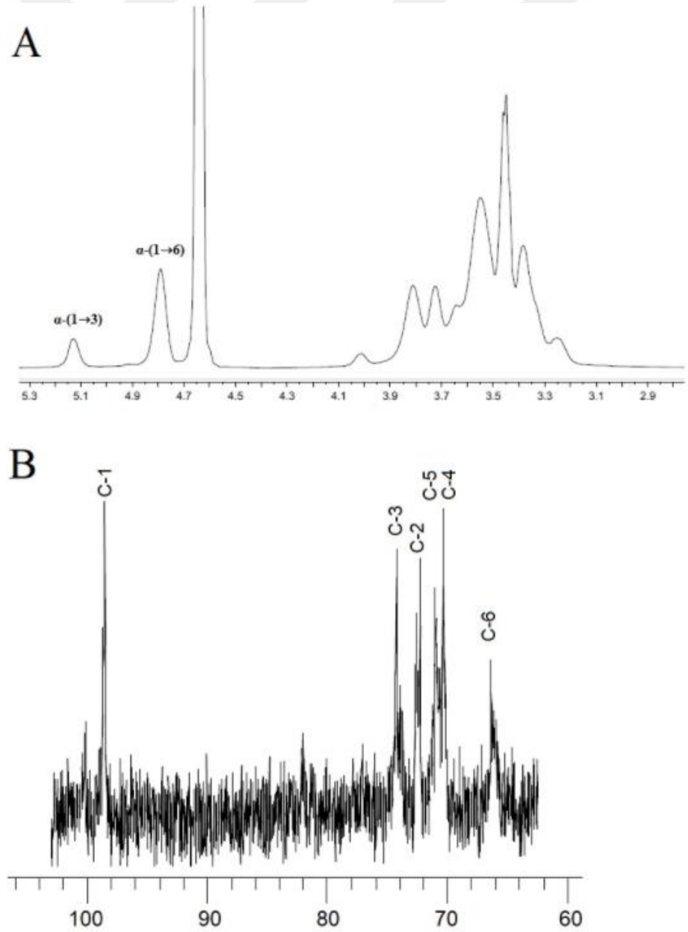

Figure 3.8 The ^{1}H (A) and ^{13}C (B) NMR spectra of α-glucan produced by GS40

3.6 Production and Characterization of Oligosaccharides with Different Sugar Acceptors with GS40 Enzyme

The ability of the GS40 enzyme to transfer glucose to other acceptor sugars in the reaction after sucrose is broken down was tested using glucose, lactose, raffinose, cellobiose, mannose, melibiose, and fructose. The progress of the reactions was observed by Thin Layer Chromatography (TLC) and the products formed as a result of the reactions were analyzed by Nuclear Magnetic Resonance (NMR).

3.6.1 TLC Identification of Different Oligosaccharides

Figure 3.9 shows the reactions with 8 different sugars used and TLC monitoring of the products after 48 hours of incubation at 30°C. TLC results revealed that among the different sugars used as acceptor sugars, only maltose, melibiose, raffinose, and cellobiose were effectively used as acceptors by GS40.

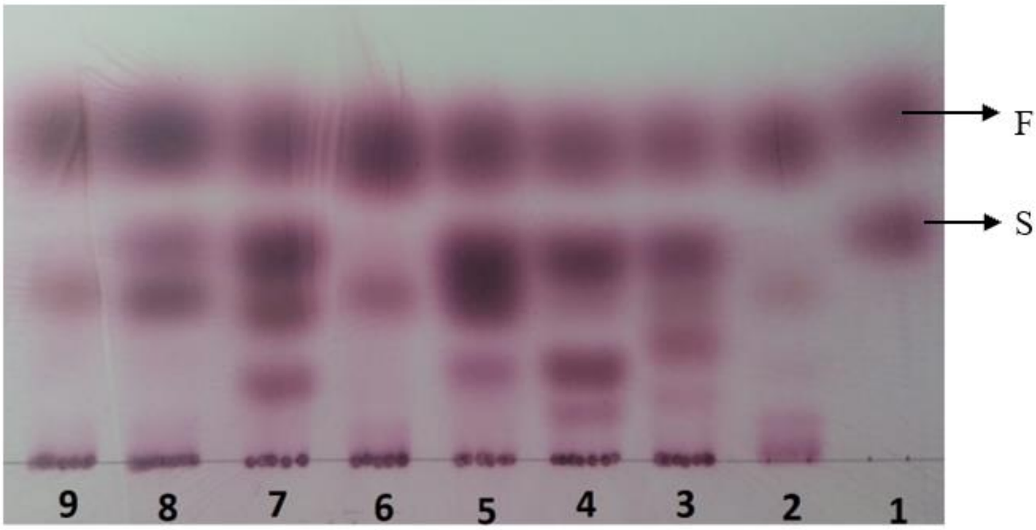

Figure 3.9 Monitoring the reactions by TLC after 48 hours of incubation at 30°C, (2: maltose, 3: melibiose, 4: raffinose, 5: cellobiose, 6: glucose, 7: lactose, 8: fructose, 9: mannose, F: fructose, S: sucrose)

The reactions of the sugars that were found to be effective acceptors were continued at 30 °C for 60 hours, and the complete consumption of sucrose in the reaction was monitored by TLC at the end of the period. Figure 3.10 shows the TLC observation of these reactions at the end of the incubation.

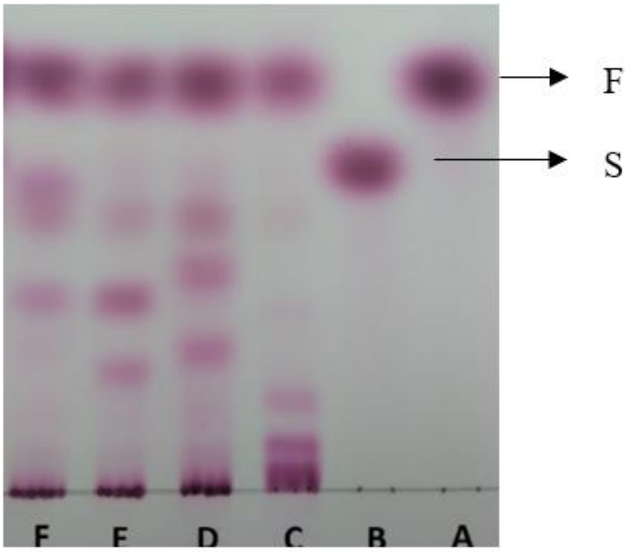

Figure 3.10 Monitoring the reactions by TLC after 60 hours of incubation at 30°C (A: fructose (F), B: sucrose (S), C: maltose, D: melibiose, E: raffinose and F: cellobiose)

After the completion of acceptor reactions, immobilized yeast was applied to remove the remaining simple sugars in the medium. Figure 3.11 shows the TLC showing that simple sugars were depleted in the medium after yeast application in the production of cellobiose and maltose-derived oligosaccharides.

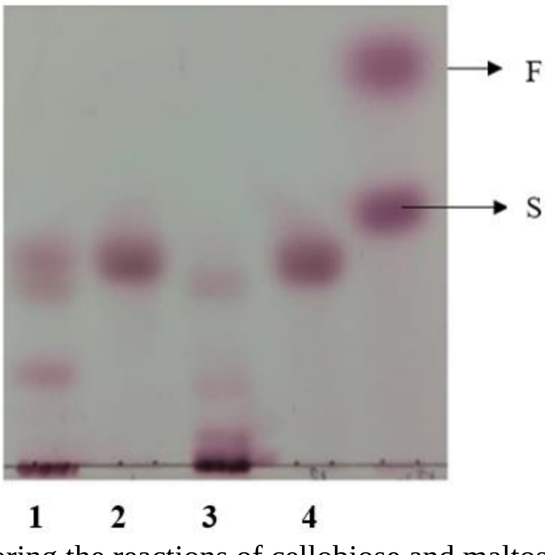

Figure 3.11 Monitoring the reactions of cellobiose and maltose derived oligosaccharides by TLC after immobilized yeast application (F: fructose, S: sucrose, 1: cellobiose-derived oligosaccharides, 2: cellobiose standard, 3: maltose- derived oligosaccharides, 4: maltose standard)

3.6.2 Structural Characterization of the Oligosaccharides by NMR Analysis

After showing by TLC analysis that GS40 could effectively use maltose, melibiose, raffinose, and cellobiose as acceptor sugars, structural analysis of glyco-oligosaccharides produced with these acceptor sugars was carried out by NMR. Figures 3.12, 3.13, and 3.14 show the results of NMR structural analysis of glyco-oligosaccharides produced using maltose, melibiose, and cellobiose as acceptors, respectively (^{1}H NMR spectrum of raffinose-derived oligosaccharides is not given).

As can be seen in Figure 3.12, the anomeric region revealed specific peaks demonstrating the modification of maltose following the acceptor reaction with GS40. Importantly, the ^{1}H spectrum of GS40 maltose reaction product was aligned with the spectra of GTFA-ΔN E81 [45], where specific signals for α-Glc→3 (δ ~ 5.13) and α-Glc→6 (δ ~ 4.69) units were observed, revealing the addition of the α-linked glucose to maltose at either the 3-O position or the 6-O position by GS40.

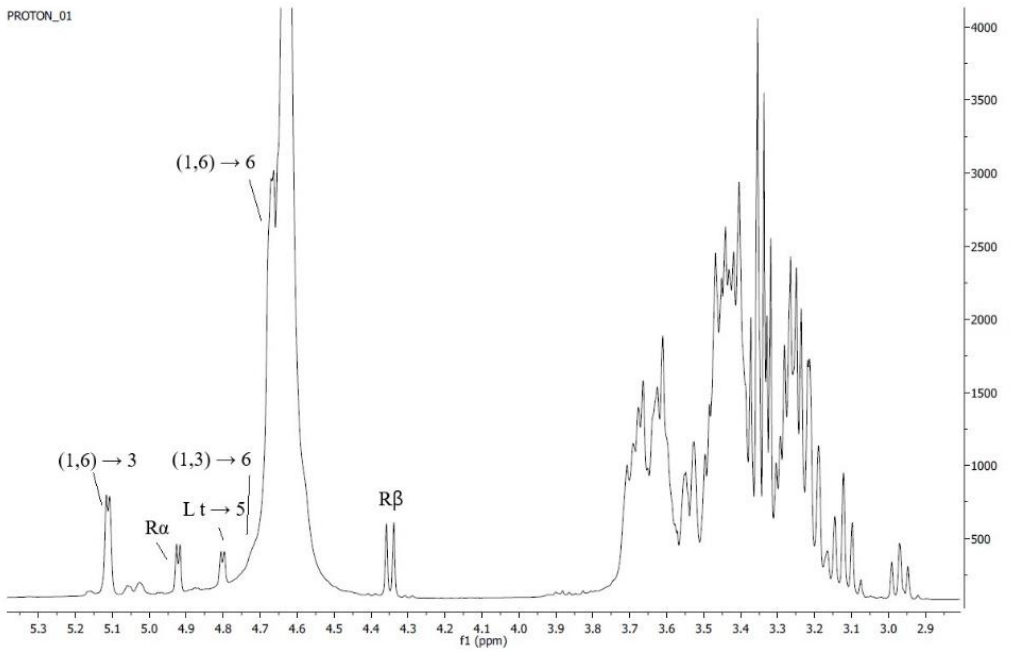

Figure 3.12 ^{1}H NMR spectrum of oligosaccharides produced by GS40 with maltose as the acceptor

Additionally, specific signals at δ ~ 4.93 and δ ~ 4.35 were observed representing the oligosaccharide reducing end Rα and Rβ, respectively [45]. Similar to the maltose acceptor reaction of GTFA-ΔN E81, ^{1}H NMR profile revealed the formation of Leucrose (α-D-Glcp-(1→5)-D-Frup) (L: δ~4.8) during the acceptor reaction of GS40. In terms of structural features of the maltose-derived glucosoligosaccharides, a similar profile was observed for the acceptor reaction of GTFA-ΔN E81 [45], whereas for the acceptor reaction of GTF180-ΔN, α-Glc→2 units were observed [61], suggesting the potential role of the distinct glucansucrases and their conformations.

The structure of the oligosaccharides produced using melibiose with the acceptor reactions of GS40 was elucidated by the ^{1}H NMR spectrum given in Figure 3.13. In this spectrum, there are signals indicating the addition of glucose units at positions 1,6 (δ~4.72) and 1,3 (δ~5.09) to the acceptor melibiose, and this indicates that state (1,6)Glc and (1,3)Glc units are incorporated into the structure.

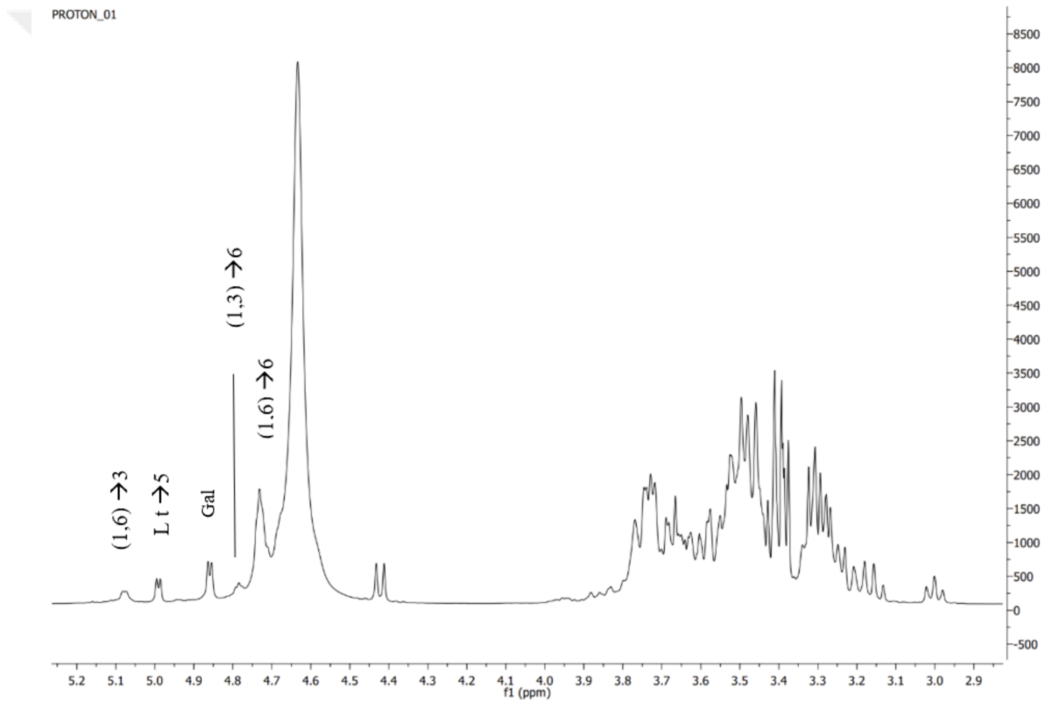

Figure 3.13 ^{1}H NMR spectrum of oligosaccharides produced by GS40 with melibiose as the acceptor

The chemical shift around δ~4.85 indicates the presence of Galactose (Gal) units. The formation of Leucrose (α-D-Glcp-(1→5)-D-Frup) (L:δ~5.01) was also observed. This structure is similar to the oligosaccharide produced from mellibiose using glucansucrase E81[44]. Figure 3.14 demonstrates the ^{1}H spectrum of cellobiose derivative gluco-oligosaccharides, where modification of cellobiose can be seen with the formation of specific peaks.

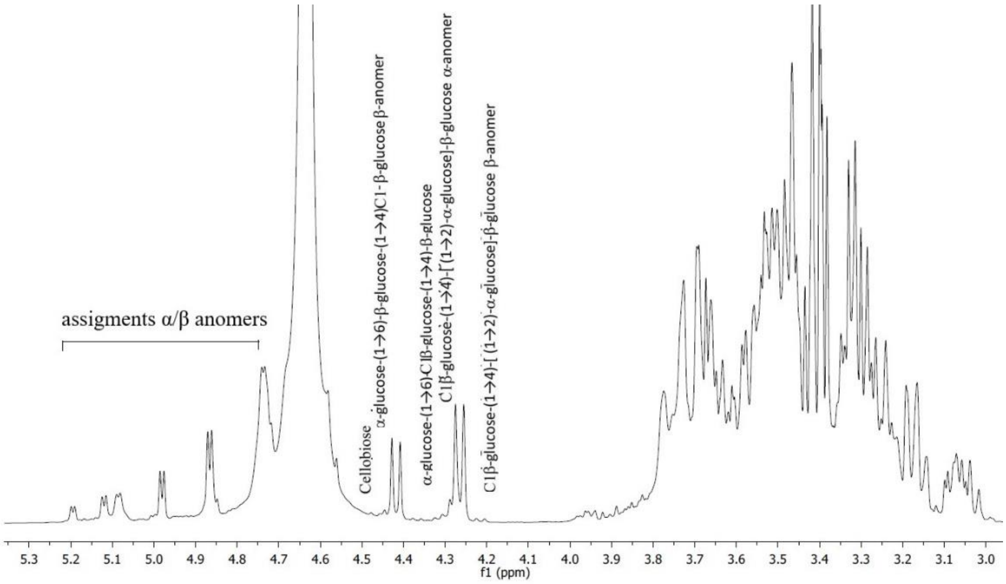

Figure 3.14 ^{1}H NMR spectrum of oligosaccharides produced by GS40 with cellobiose as the acceptor

Importantly, the spectrum of cellobiose derivative gluco-oligosaccharides produced by GS40 was comparable with the previous studies in which two alternansucrases and one dextransucrase were used for the production of the cellobiose-derived oligosaccharides [46, 62, 63]. The ^{1}H spectra of the GS40 acceptor product with cellobiose were aligned with the spectra of GTF E81, where 12 doublets with a chemical shift from 4.40 ppm to 5.45 ppm were reported previously and these doublets were observed between 4.20 ppm and 5.25 ppm for GS40 acceptor products. In accordance with previous reports, this data revealed the α- and β-anomers of the glucose reducing end of the formed oligosaccharides and the addition of the α-linked glucose to cellobiose at either the 2-O position or the 6-O position (Figure 3.14). This data suggested the formation of Glc units linked →6 and linked →2 during the elongation of the oligosaccharide chain length of cellobiose-derived oligosaccharides produced by GS40. Previously, the formation of gluco-oligosaccharides containing Glc units linked →6 and linked →2 was also reported for other glucansucrases using cellobiose as the acceptor sugar [46, 62, 63] suggesting the potential stereochemistry effect of cellobiose as an acceptor molecule. However, more studies are required to identify the function of cellobiose as an acceptor for the formation of glucooligosaccharides containing Glc units linked →6 and linked →2. The chemical characterisation of the maltose-derived oligosaccharides produced by GS40 acceptor reaction was also applied using ^{1}H NMR analysis.

3.6.3 Structural Characterization of Oligosaccharides in Terms of Degree of Polymerization

For the determination of the final length of the maltose, cellobiose, raffinose, and melibiose derivative gluco-oligosaccharides with respect to their DPs, LC/MS/Q-TOF analysis was performed. Figures 3.15, 3.16, 3.17, and 3.18 demonstrate the total ion chromatogram of the oligosaccharides recorded at [M]− scan for maltose, melibiose, raffinose, and cellobiose and derivative gluco-oligosaccharides, respectively (Maltose was used as a representative in naming the lengths of oligosaccharides in the spectra).

As can be seen in Figure 3.15, using maltose as the acceptor sugar, gluco-oligosaccharides up to DP 9 oligosaccharides were formed as the final peak of M]− ions was recorded at m/z of 1475.2, corresponding to the analogue of maltononaose.

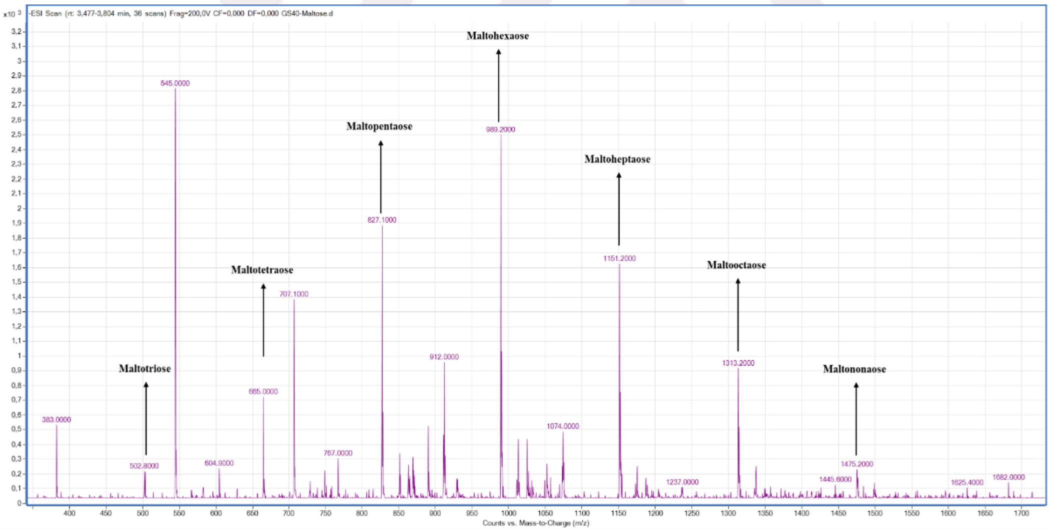

Figure 3.15 Gluco-oligosaccharides produced by GS40's acceptor reaction with maltose

This was in accordance with previous observations where the acceptor reaction of GTFA-ΔN E81 with maltose resulted in the formation of gluco-oligosaccharides at DP 9 length [45]. Other studies also reported the formation of similar length [64, 65] or shorter gluco-oligosaccharides as DP ≤ 6 [33, 43] by the action of distinct glucansucrases suggesting the enzyme specific role of the glucansucrases in the formation of novel gluco-oligosaccharides.

Another important acceptor sugar tested in this study was melibiose, and as can be seen in Figure 3.16, melibiose-derived oligosaccharides up to DP 11 length were produced by GS40. The acceptor reaction of GTFA-ΔN E81 with melibiose led to the production of only DP3 and 4 oligosaccharides [44].

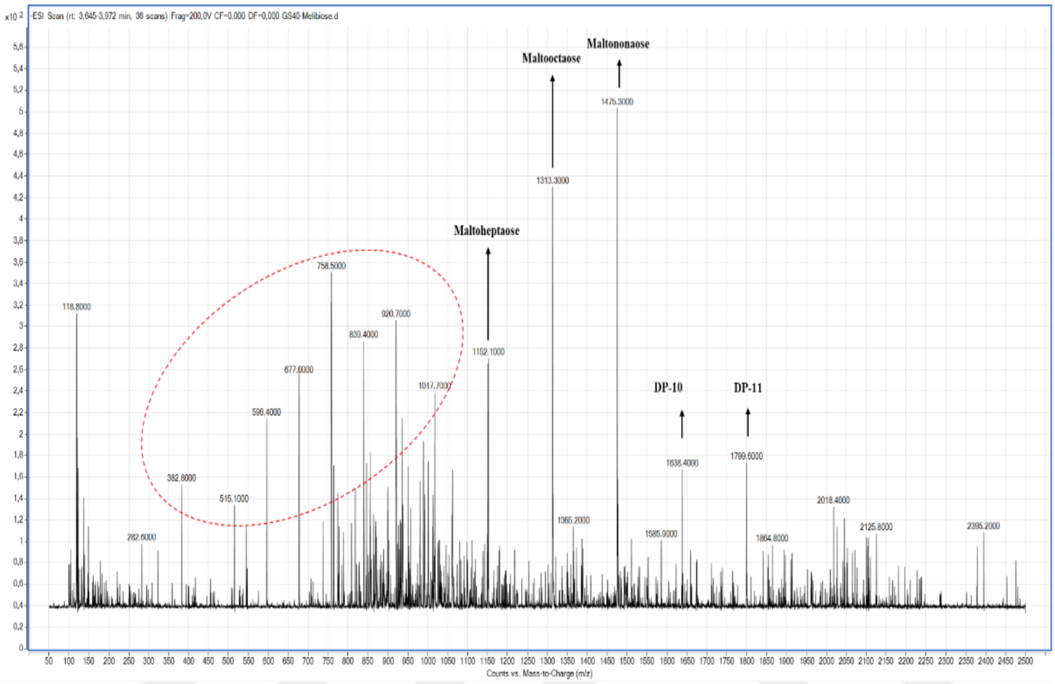

Figure 3.16 Gluco-oligosaccharides produced by GS40's acceptor reaction with melibiose

Another important acceptor sugar used to produce gluco-oligosaccharides by GS40 was raffinose. Figure 3.17 shows the LC-MS profile of the gluco-oligosaccharides produced by GS40 from raffinose. Importantly, gluco-oligosaccharides up to DP 11 length were produced by GS40. Only DP4 oligosaccharides could be produced from *L. mesenteroides* URE 13 as a result of the raffinose acceptor reaction of the glucansucrase URE 13-300 enzyme. In fact, it has been observed that as the donor and acceptor ratio changes, the degree of polymerization does not change; only the oligosaccharide yield changes [50]. The presence of oligosaccharides with a maximum length of DP7 was demonstrated in raffinose-derived acceptor reactions of GTF E81 [46].

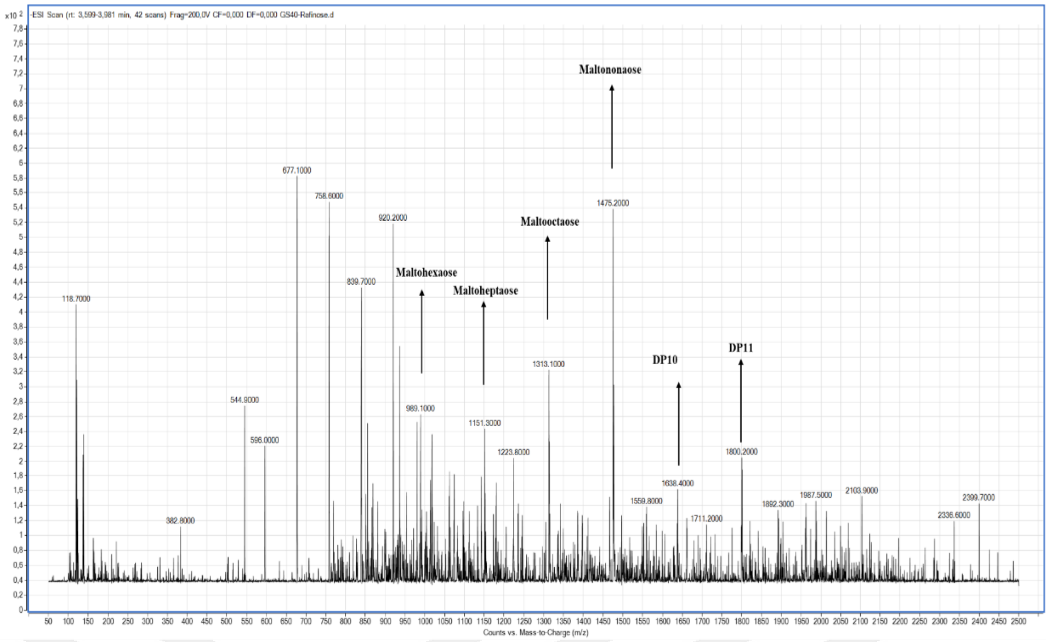

Figure 3.17 Gluco-oligosaccharides produced by GS40's acceptor reaction with raffinose

Finally, cellobiose was used as an acceptor sugar for the production of gluco-oligosaccharides with GS40. As can be seen in Figure 3.18, gluco-oligosaccharides up to DP 12 length were produced with GS40. Previously, the formation of cellobiose-derived oligosaccharides up to DP 7 was observed by the activity of distinct glucansucrases, and the production of longer oligosaccharides can be important to alter the specific bioactive characteristics of these oligosaccharides. These findings were important as longer gluco-oligosaccharides were efficiently produced with the action of GS40 using acceptor sugars such as maltose, melibiose, raffinose and cellobiose.

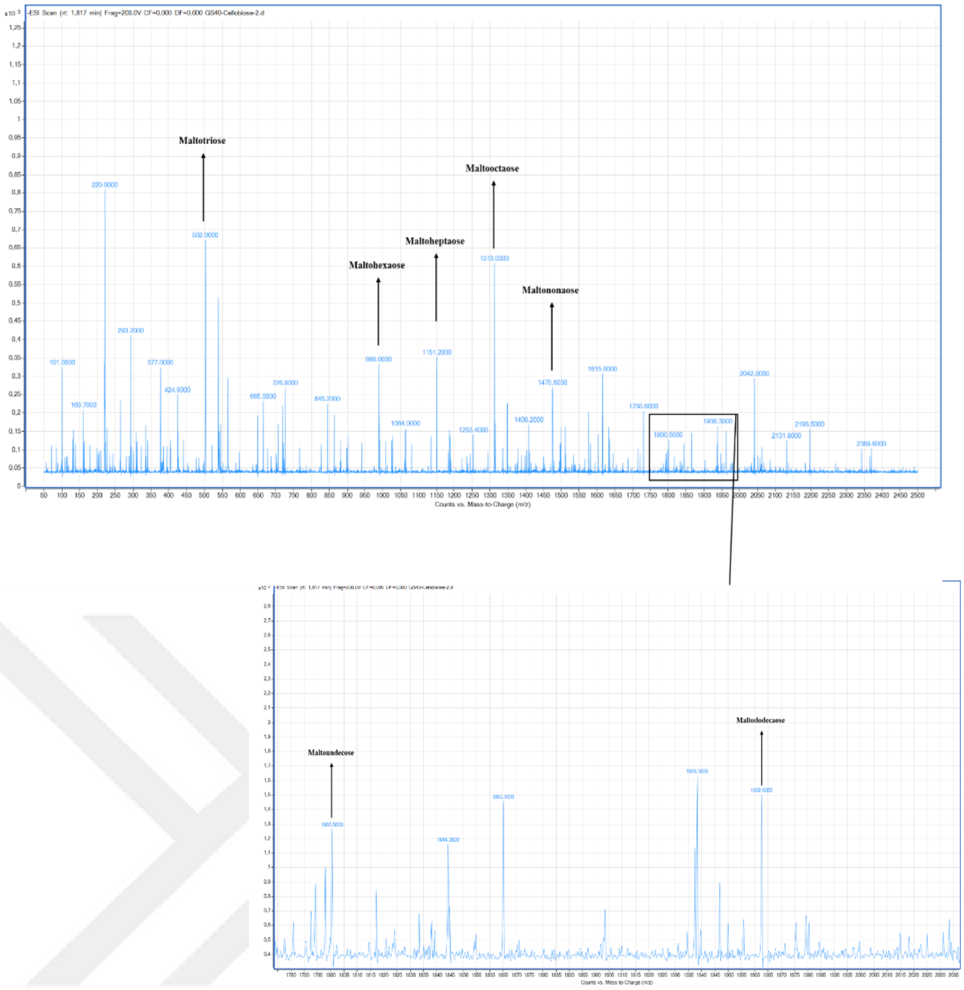

Figure 3.18 Gluco-oligosaccharides produced by GS40's acceptor reaction with cellobiose

3.7 Monitoring the Production of Short-Chain Fatty Acids

Glucan and different oligosaccharides produced using the GS40 enzyme were fermented *in vitro* by the colon microbiota. The formation of SCFA was examined by taking samples at certain time intervals for 48 hours. Figures 3.19, 3.20, 3.21 and 3.22 show the amounts of acetate, propionate, butyrate, and total SCFA (determined by summing the molar concentrations of acetate, propionate, and butyrate) released as a result of fermentation, respectively, at 0, 6, 12, 24 and 48 hours of fermentation. Short-chain fatty acids are produced as a result of the digestion of carbohydrates by the colon microbiota. These carbohydrates are dietary fiber-like structures that are not digested by the host in the intestine.

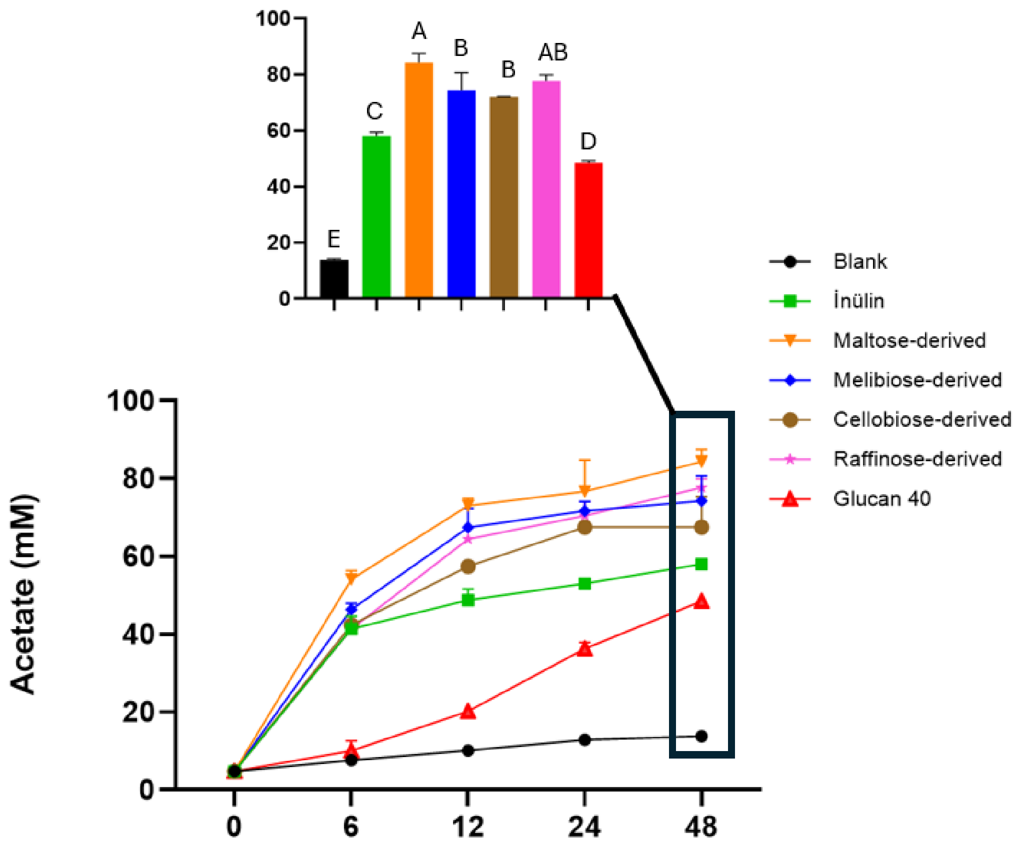

Figure 3.19 Acetate generated throughout the *in vitro* fermentation

Acetate, propionate and butyrate constitute the majority of short-chain fatty acids in the colon, and these 3 fatty acids can represent the total short-chain fatty acids. A very small part of the total SCFA consists of branched chain SCFAs under the influence of amino acids (valine, leucine and isoleucine) in the environment [66].

While the production of short-chain fatty acids can be considered evidence of the prebiotic effect, SCFAs formed as a result of indigestible carbohydrate fermentation have various benefits for host health. These structures improve gastrointestinal health and play a role in preventing obesity and cardiovascular diseases [67]. Because they contribute to the growth of probiotic species, they are described as non-digestible prebiotics, such as oligosaccharides and microbial polysaccharides. These structures enable the development of the immune system by promoting the production of SCFAs such as acetate, lactate, propionate, and butyrate. [36]. The prebiotic effects and host benefits of oligosaccharides synthesized by classical methods [40] were investigated. However, studies on the mentioned effects of products produced with recombinant enzymes are limited. The results obtained here are expected to help explain how these structures affect microbial fermentation.

In general, one of the most widely used prebiotics is inulin, and inulin was used as positive control and the blank did not contain any carbon substrate. Overall, all samples tested produced similar amounts of total SCFA and showed similar levels of activity to inulin. An important point here is the change in SCFA amounts caused by glucan 40 depending on time. Fermentation of the majority of total SCFA production occurs in the second half, revealing that GS40 (glucan) is fermented by the colonic microbiota at a slower rate compared to the others. This may indicate that glucan 40 exhibits ideal prebiotic properties. Slowly digested carbohydrates encourage short-chain fatty acid production throughout the colon during the later hours of fermentation. Additionally, this may prevent colon-related disorders because late fermentation encourages the growth of microorganisms in the part of the colon associated with these diseases [68, 69].

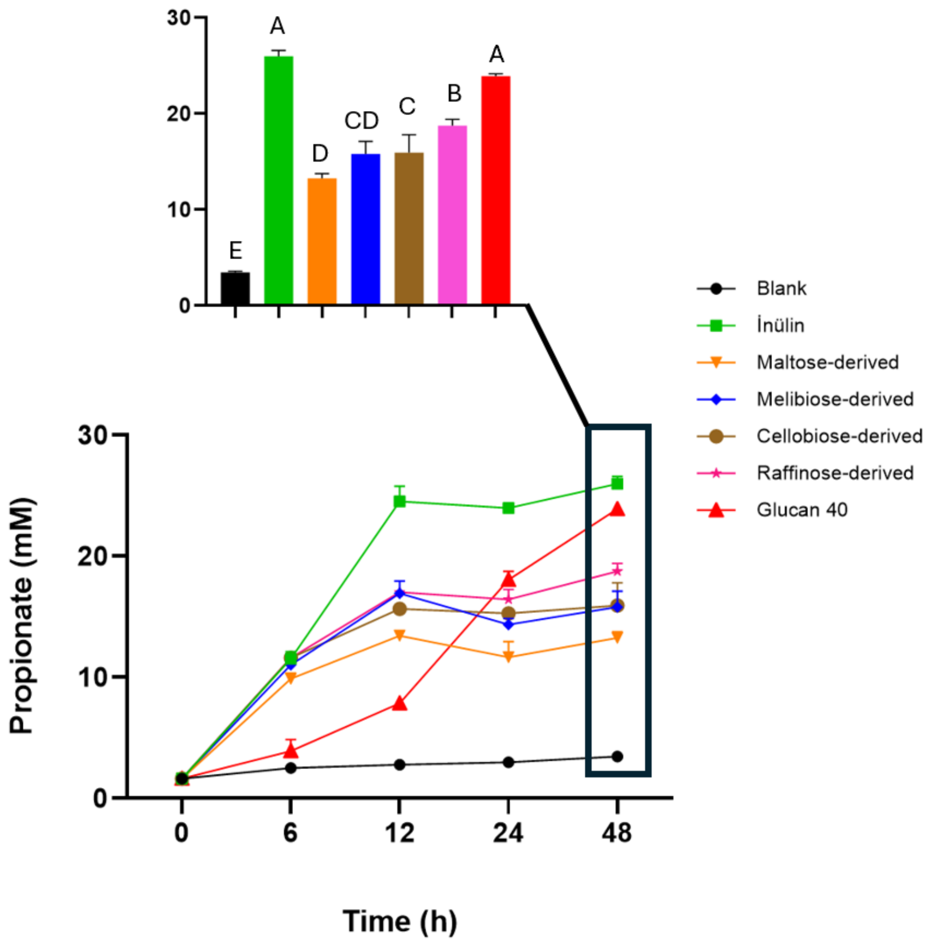

Figure 3.20 Propionate generated throughout the *in vitro* fermentation

Another interesting finding was that GS40 (glucan) resulted in a generation of butyrate as high as that observed in inulin. Beyond being an energy source for intestinal epithelial cells, butyrate has effects on the intestinal tract and functions in maintaining immune homeostasis. Butyrate deficiency can cause various inflammatory disorders. Butyrate is one of the most important microbial metabolites produced in the colon due to its anti-carcinogenic, anti-inflammatory, and anti-obesity effects [70, 71]. When the acetate (Figure 3.19), propionate (Figure 3.20), and butyrate (Figure 3.21) levels resulting from 48-hour fermentation of oligosaccharides were compared with inulin fermentation, all gluco-oligosaccharides increased the acetate level far higher than inulin.

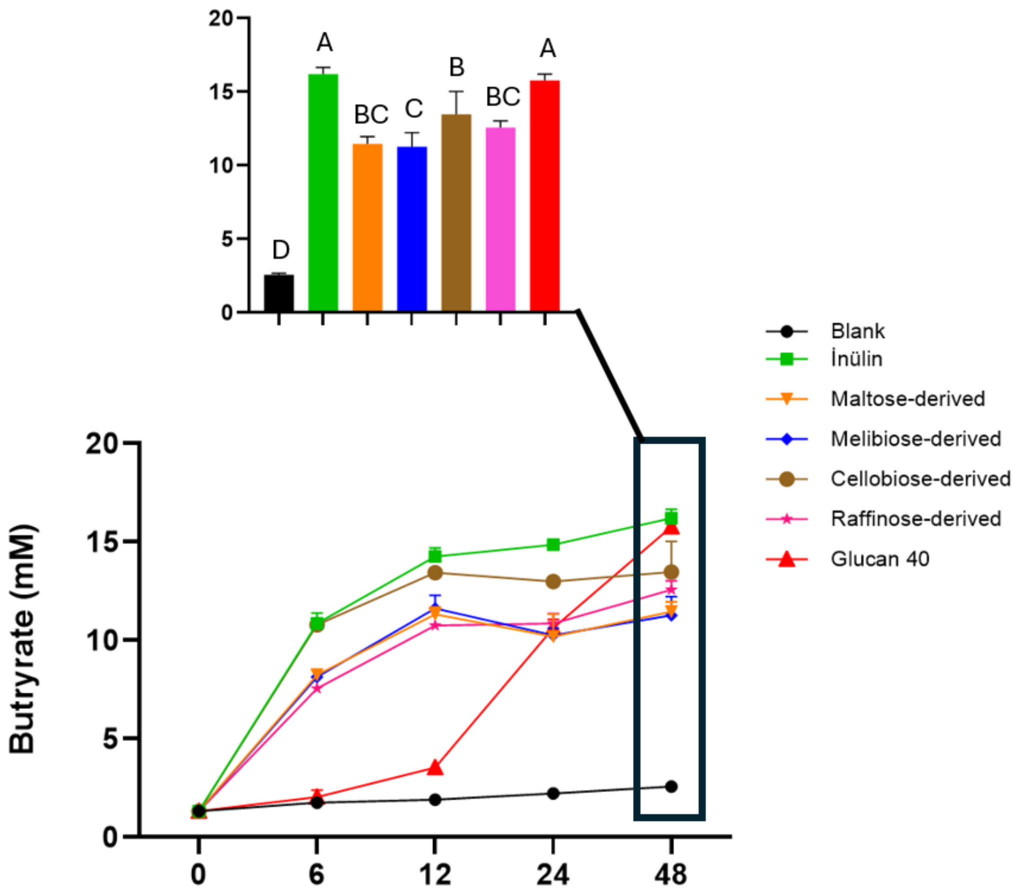

Figure 3.21 Butryrate generated throughout the *in vitro* fermentation

In terms of gluco-oligosaccharides, maltose-based oligosaccharides resulted in higher levels of acetate production and, more importantly, all glyco-oligosaccharides increased the acetate level to a much higher level than inulin. This was not seen for propionate production, but glyco-oligosaccharides also produced significant amounts of propionate. For butyrate production, cellobiose-based oligosaccharides resulted in similar levels of butyrate formation, indicating that its activity is similar to that of inulin. It can be concluded here that oligosaccharides produced by different acceptors are fermented differently by the intestinal microbiota, which changes SCFA production.

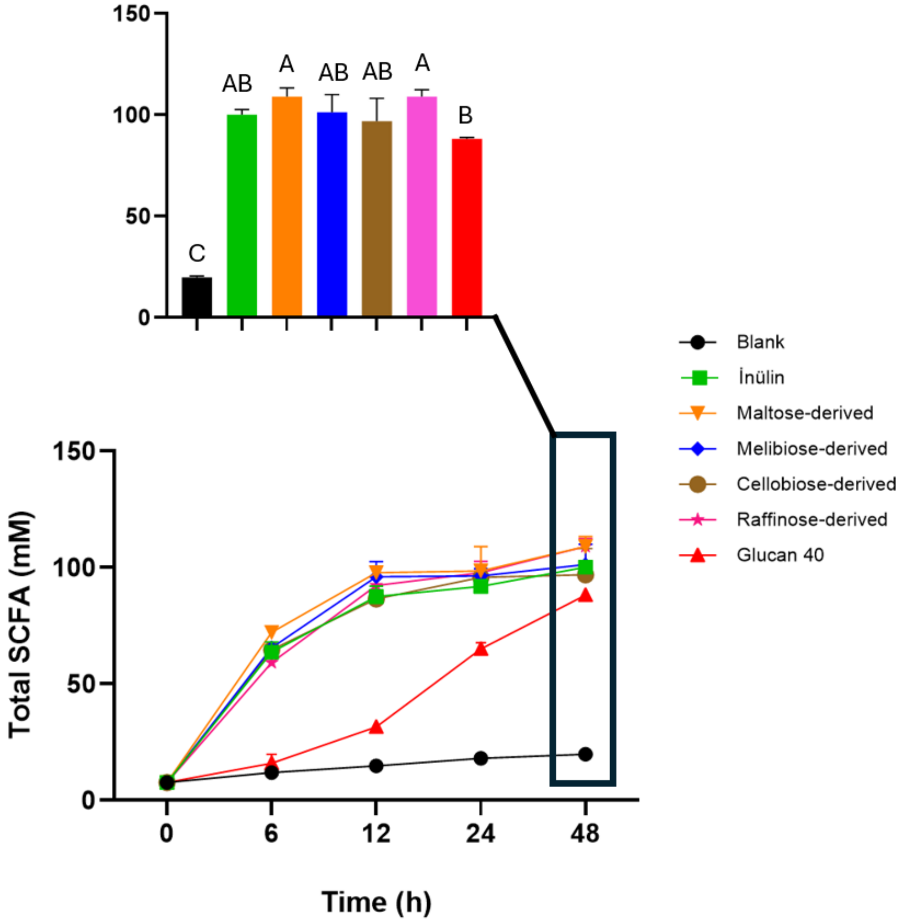

Figure 3.22 Total SCFA generated throughout the *in vitro* fermentation

3.8 Immune-modulatory Roles of Glucan and Different Oligosaccharides

The immunomodulatory effects of glucan and different oligosaccharides were determined by measuring TNF-α, IL-12, IL-10 and IL-4 cytokine levels in Caco-2 cells line. Immunomodulatory agents have a stimulating effect through the induction of molecules that regulate the immune system, such as cytokines, and their mechanisms are still being investigated. In particular, the regulation of anti-inflammatory (IL-4, IL-10) and pro-inflammatory (TNF-α, IL-12) cytokine levels through these structures is an important indicator of immunomodulation [4]. In this sense, the immune regulation abilities of different glucan structures and oligosaccharides, whose prebiotic properties have been widely investigated, have also been demonstrated [11, 41, 72]. However, further studies are needed to elucidate the immunomodulatory mechanisms of different glucans and oligosaccharides synthesized with glucansucrase enzymes *in vitro*. The induction amounts of glucans and oligosaccharides for IL-4, IL-10, IL-12, and TNF-α tested in our study are shown in Figures 3.23, 3.24, 3.25, and 3.26, respectively.

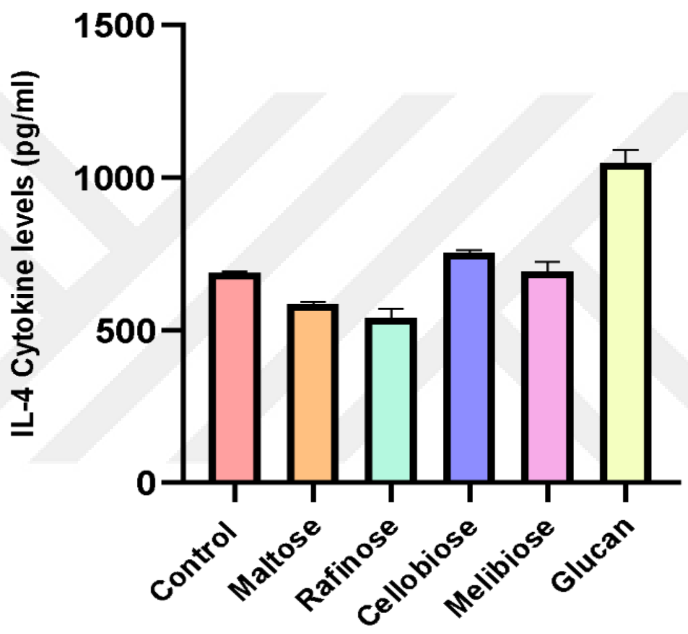

Figure 3.23 IL-4 cytokine induction in Caco-2 cell line

Figure 3.23 demonstrates the IL-4 cytokine response in Caco-2 cells to glucan 40 and different gluco-oligosaccharides, which revealed that a strong induction for IL-4 production was observed for glucan 40 whereas no induction and a relatively low level of induction were detected for maltose and cellobiose derivative gluco-oligosaccharides, respectively. No induction was observed in maltose- and raffinose-derived oligosaccharides. Previously, malto-oligosaccharides containing ($\alpha 1 \rightarrow 4$) and ($\alpha 1 \rightarrow 6$) linkages strongly induced the IL-4 cytokine induction [45] but no induction for maltose derivative gluco-oligosaccharides was observed in this study, suggesting the potential role of linkage type between glucose units in affecting the IL-4 induction. Strong induction of important anti-inflammatory cytokine IL-4, which could modulate several biological processes by glucan 40 can be important to reveal its bioactive role.

When anti-inflammatory IL-10 induction (Figure 3.24) was examined, only cellobiose derivative gluco-oligosaccharides induced the production of anti-inflammatory cytokine IL-10 as an important cytokine that can be stimulated by probiotic bacteria during probiotic action [73]. This result might also reveal the structure related stimulation potential of gluco-oligosaccharides as well as glucans for distinct cytokines. According to previous findings, malto-oligosaccharides produced from maltoheptaose by a glucanotrasferase specifically stimulated IL-10 levels among the tested oligosaccharides [74].

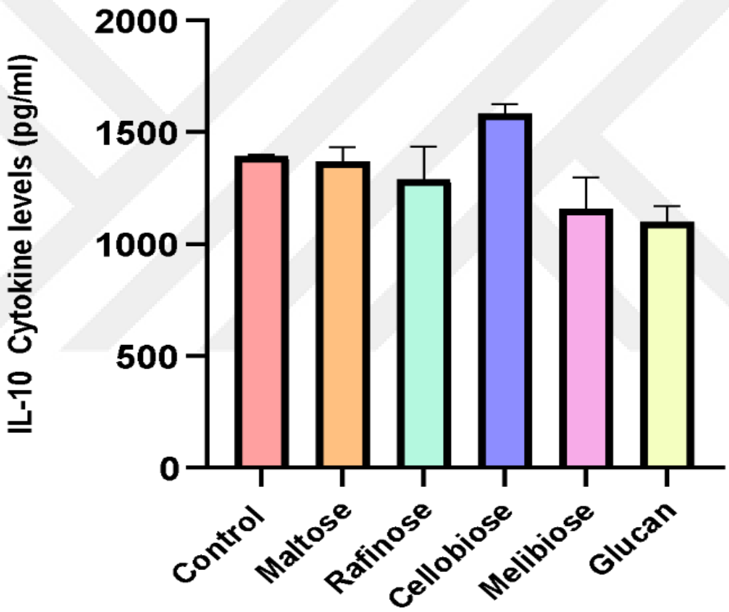

Figure 3.24 IL-10 cytokine induction in Caco-2 cell line

While IL-12 cytokine induction (Figure 3.25) was not observed with maltose-derived oligosaccharides, other oligosaccharides were observed to increase IL-12 levels and cellobiose and melibiose-derived oligosaccharides revealed a strong induction. The case for the pro-inflammatory cytokine IL-12 as glucan 40 strongly induced its production. Previously, the role of exopolysaccharides for immune modulation was comprehensively discussed [75, 76]. However, there is a lack of information on the α-glucans and these findings can be important to unveil the immune-modulatory roles of α-glucans.

Both glucan 40 and cellobiose derivative gluco-oligosaccharide application resulted in the bipolar induction of the production of pro-inflammatory cytokine IL-12 and anti-inflammatory cytokine IL-4, and this was previously suggested to be a positive factor for the preservation against food allergies as well as infectious diseases tested for weaned piglets [77].

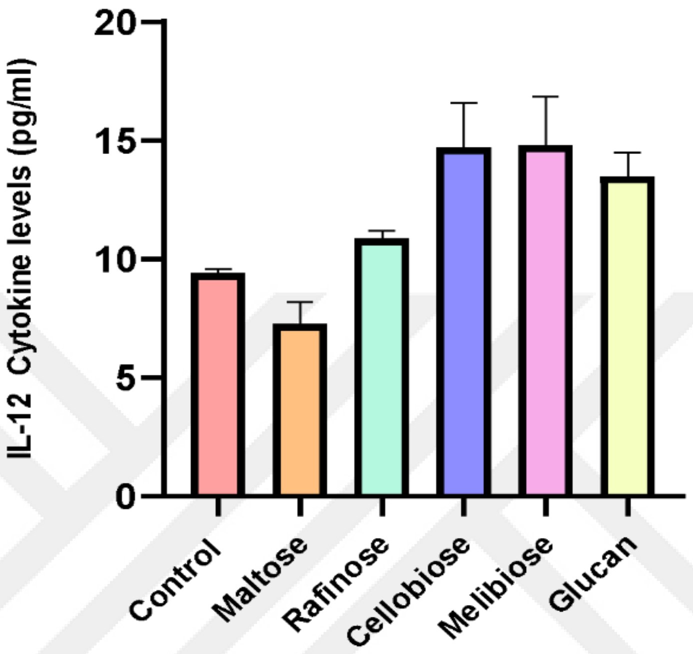

Figure 3. 25 IL-12 cytokine induction in Caco-2 cell line

Finally, maltose, raffinose and melibiose derivative gluco-oligosaccharides stimulated the production of pro inflammatory TNF-α (Figure 3.26) which was indicated to be an important stimulant for non-specific immune responses which can be triggered by Lactobacilli exopolysaccharides [78]. The potential of maltose, raffinose and mellibiose-derived oligosaccharides to increase TNF-α (tumor necrosis factor) induction is important. Because TNF-α is a cytokine that takes part in cell differentiation, the defense system against pathogenic microorganisms, and the formation of immune cell responses, and is essential for the regular functioning of the immune system [79, 80]. In contrast to our data, previously malto-oligosaccharides were reported to be ineffective for the TNF-α induction [74] which again suggests the role of distinct molecular structures for cytokine inductions. Previous studies have shown that the chemical composition of oligosaccharide and glucan structures affects cytokine induction [72], and this effect may be due to the different interactions of monomers in the structure with receptors on the surface of immune cells [11].

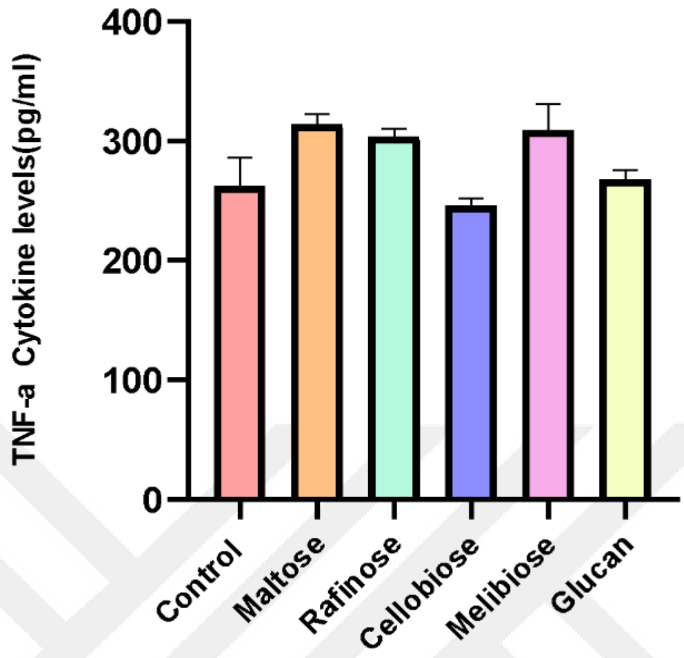

Figure 3.26 TNF-a cytokine induction in Caco-2 cell line

In previous analysis, it was revealed that the glucan produced by the GS40 enzyme consists only of glucose units and contains α(1,6) and α(1,3) in the structure. In a study examining the effect of monosaccharide composition on the immunomodulatory properties of exopolysaccharides, it was reported that increasing galactose content increased the anti-inflammatory effects [72]. Overall, these findings suggest the importance of the final structural features of the gluco-oligosaccharides as well as α-glucans for the induction of specific immune responses in terms of anti-inflammatory and pro-inflammatory cytokines, and more studies are required to unveil the role of glucansucrase-based oligosaccharides for bioactive functional properties. No study has been found in the literature comparing the immunomodulatory effects of oligosaccharides synthesized with different acceptor sugars using glucansucrase enzymes. In this sense, the study carried out is important in understanding the effect of glucan and gluco-oligosaccharides on anti-inflammatory and pro-inflammatory cytokines.

4
CONCLUSION

In this study, the gene region responsible for glucansucrase production encoded in *L. mesenteroides* YTU-40 was revealed, and the method followed for the detection, cloning, and expression of this gene was described. BLASTn analysis of *L. mesenteroides YTU*-40 showed the presence of a 4428 bp glucansucrase gene in this genome, which is highly similar to other glucansucrase enzymes belonging to the GH70 family. Ligation-independent cloning technology and the IPTG-inducible vector pLATE31 were used to clone the GS40 gene from *L. mesenteroides*. The corresponding plasmid cassette was transformed into *E. coli* BL21, and GS40 was heterologously expressed.

In subsequent studies, the ability of this enzyme to produce glucans *in vitro* and its ability to form acceptor reactions with different sugars were tested. The chemical structure of glucan 40 was elucidated by NMR and HPLC. The results revealed that the glucan produced by this enzyme contains only glucose units and is responsible for the production of a branched dextran with ~20% α-(1,3) glucosyl residues. In addition, reactions were carried out for the production of oligosaccharides using different acceptor sugars, and GS40 was shown to be active in acceptor reactions with maltose, melibiose, raffinose, and cellobiose, and new glyco-oligosaccharide structures were produced.

The chemical structures and polymerization degrees of maltose, melibiose, celibiose, and raffinose-derived oligosaccharides were elucidated by NMR and LC/MS/Q-TOF analyses, respectively. GS40 added α-(1,2) Glu or α-(1,6) Glu units to cellobiose during elongation of the oligosaccharides and gluco-oligosaccharides up to DP 12 length were formed. For maltose acceptor reactions, α-(1,3) Glu or α-(1,6) Glu units were added by GS40 and up to DP 9 gluco-oligosaccharides were produced. In melibiose-derived oligosaccharides, it was observed that α-(1,3) Glu or α-(1,6) Glu units were included in the structure and up to DP 11 length were produced by GS40. As a result of the reactions carried out with raffinose, oligosaccharides up to DP 11 could be produced by the GS40 enzyme.

The final stage involved revealing the bioactive properties of these created structures, and the immunomodulatory and prebiotic properties of both branched dextran and different oligosaccharides were tested. The effects of glucan 40 and different oligosaccharides on the colon microbiota were investigated using the *in vitro* fecal fermentation method for 48 hours, and the changes in SCFA (acetate, propionate, and butyrate) were monitored. While the production of short-chain fatty acids indicates the existence of a prebiotic effect, SCFAs formed as a result of fermentation have various benefits for host health.

Overall, all samples tested produced similar amounts of total SCFA and showed similar levels of activity to inulin, one of the most commonly used prebiotics. It is important here that glucan 40 shows low SCFA production in the first half of the fermentation and high SCFA production in the second half. This indicates that the branched dextran structure is digested slowly and may ferment in the further parts of the colon. This may also prevent colon-related disorders, as late fermentation encourages the growth of microorganisms in the part of the colon associated with these diseases. When the SCFA production of oligosaccharides synthesized as a result of receptor reactions is examined, it can be concluded that these products are fermented differently by the intestinal microbiota. One of the most important results here is that all glyco-oligosaccharides increase the acetate level to a much higher level than inulin. This was not observed in the formation of butyrate and propionate, but still resulted in the production of high amounts of SCFA. These results may be useful in understanding the effects of polysaccharide and oligosaccharide structures produced by recombinant enzymes.

The immunomodulator effect was determined by measuring TNF-α, IL-12, IL-10 and IL-4 cytokine levels in Caco-2 cell line. The results showed that these constructs could generate specific immune responses on Caco-2 cells, with each product resulting in different levels of induction of IL-12, IL-10, IL-4, and TNF-α cytokine responses. These different responses support the idea that immunomodulatory effects will change as chemical structures change. For example, anti-inflammatory IL-10 induction was induced only by cellobiose-derived glyco-oligosaccharides. This induction is important because IL-10 is an important cytokine that can be stimulated by probiotic bacteria during probiotic action. Gluco-oligosaccharides showed little or no induction of IL-4. In contrast, glucan 40 had a strong induction of the important anti-inflammatory cytokine IL-4, which can modulate various biological processes. While IL-12 cytokine induction was not observed with maltose-derived oligosaccharides, other oligosaccharides were observed to increase IL-12 levels, and cellobiose and melibose-derived oligosaccharides were observed to produce a strong induction. Glucan 40 strongly triggered the production of the proinflammatory cytokine IL-12. These findings may be important for uncovering the immune regulatory roles of α-glucans and to reveal the bioactive role of branched dextran. Finally, TNF-α (tumor necrosis factor) is an important cytokine involved in cell differentiation, cell defense, and the formation of immune responses, and has been observed to increase the induction of maltose, raffinose and mellibiose-derived oligosaccharides.

In conclusion, in this study, the existence of recombinant GS40 was demonstrated and its ability to produce glucans and different oligosaccharides *in vitro* was proven. The structures of the products have been elucidated, and it has been shown that they have prebiotic and immunomodulatory effects. In this context, identifying new glucansucrases and testing these enzymes for the production of different glyco-oligosaccharides with bioactive properties as well as α-glucans are important in many respects. First of all, these reactions make it possible to obtain products in a shorter time and at higher yields than complex, costly traditional production methods. *In vitro* enzymatic syntheses can be carried out sustainably with inexpensive substrates. These features of the resulting products show that they have the potential to be used in nutraceutical and pharmaceutical applications, especially in the functional food industry.

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A
GENE SEQUENCE OF GS40

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GAGAGCTGATTAAAAGCTAA

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Conference Papers

1. Dere, S., Ispırlı, H., Korkmaz Edıs, K. Sagdıc, O and Dertli, E. Characterization of a glucansucrase from *Leuconostoc mesenteroides* AP-40 and production of different oligosaccharides with acceptor reactions, International Food Innovation and Sustainability Congress (IFIS 2024), 2024.