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*In vitro* synthesis and structural analysis of selected acceptor products of *Weissella confusa* VTT E-90392 dextransucrase

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<p>Non-digestible oligosaccharides possess important physicochemical and physiological properties. They have gained great attention because of their potential prebiotic properties. The oligosaccharides have wide food industrial applications as dietary fibers, sweeteners, humectants, and possible weight controlling agents.</p> <p>In this thesis, cloned <i>Weissella confusa</i> VTT E-90392 dextranucrase was used to catalyze the synthesis of oligosaccharides (acceptor products) by acceptor reaction, when sucrose was the glucosyl unit donor. Maltose acceptor reaction was selected to analyze the effects of the concentrations of sucrose and maltose, as well as the dosages of dextranucrase on maltose acceptor products. Other acceptor reactions were also evaluated, with primary lactose and cellobiose acceptor products being purified and partially characterized by MS/MS.</p> <p>Concentrations of sucrose and maltose, as well as their interactions were the most important factors regarding isomalto-oligosaccharides production. The production of overall oligosaccharides could be increased by increasing sucrose and maltose concentrations. Lactose, cellobiose, other disaccharides and trisaccharides could be acceptors for <i>W. consufa</i> dextranucrase. In combination with the structures from previous publications, 2-<math>\alpha</math>-D-glucopyranosyl-cellobiose and 2-<math>\alpha</math>-D-glucopyranosyl-lactose were probably the primary products for cellobiose and lactose, although one trisaccharide synthesized by lactose acceptor reaction remains unknown. In order to verify the characterization, NMR spectroscopic analysis is needed for further study. In conclusion, <i>W. consufa</i> dextranucrase is capable of catalyzing oligosaccharides synthesis, and acceptor reactions would be promising methods in producing prebiotic oligosaccharides.</p>			
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## **PREFACE**

The thesis was conducted at the Department of Food and Environmental Sciences, Food Chemistry Division.

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Helsinki, April 2014

Yaxi Hou

## LIST OF ABBREVIATIONS

DP	degree of polymerization
EPS	exopolysaccharides
ESI	electrospray ionization
GRAS	generally recognized as safe
HPAEC-PAD	high-performance anion exchange chromatography with pulse amperometric detection
IMO	isomalto-oligosaccharide
LAB	lactic acid bacteria
MS	mass spectrometry
MS/MS	tandem mass spectrometry
MS <sup>2</sup>	second MS/MS circle
MS <sup>3</sup>	third MS/MS circle
<i>m/z</i>	mass to charge ratio
NMR	nuclear magnetic resonance

## TABLE OF CONTENTS

ABSTRACT

PREFACE

LIST OF ABBREVIATIONS

1. INTRODUCTION.....	7
2. LITERATURE REVIEW .....	9
2.1 <i>Weissella confusa</i> and dextransucrase .....	9
2.1.1 <i>Weissella confusa</i> .....	9
2.1.2 Dextransucrase.....	12
2.2 Dextransucrase acceptor reaction .....	15
2.2.1 Categories of acceptors .....	15
2.2.2 Mechanism of acceptor reaction .....	17
2.2.3 Factors affecting acceptor reaction .....	20
2.2.4 Characterization and structural analysis of acceptor products.....	20
2.3 Functional properties of acceptor products in food applications.....	22
2.4 Key challenges and further developments .....	22
2.5 Safety evaluation .....	23
2.5.1 Safety evaluation of <i>Weissella confusa</i> and dextransucrase.....	23
2.5.2 Safety evaluation of products synthesized by dextransucrase .....	25
3. EXPERIMENTAL RESEARCH .....	27
3.1 Aims of the study.....	27
3.2 Materials .....	27
3.3 Methods .....	28
3.3.1 Dextransucrase activity assay .....	28
3.3.2 Protein determination.....	29
3.3.3 Experimental design and acceptor reactions.....	29
3.3.4 Dextran content estimation.....	32
3.3.5 Analysis of monosaccharides, disaccharides and oligosaccharides .....	33
3.3.6 Purification of acceptor products .....	33
3.3.7 Characterization of primary acceptor products using MS/MS .....	34
3.4 Results.....	34
3.4.1 Dextransucrase activity and protein content estimation.....	34
3.4.2 Preliminary design results of maltose acceptor reaction .....	34
3.4.3 Optimization design results of maltose acceptor reaction.....	37
3.4.4 Other acceptor reactions.....	43
3.4.5 Primary acceptor products characterization using MS/MS .....	45
3.5 Discussion .....	52
3.5.1 Factors affecting maltose acceptor reaction .....	52

3.5.2	Other acceptor reactions and primary acceptor products characterization.....	55
4.	CONCLUSION.....	58
	REFERENCES .....	60
	APPENDIX .....	66

## 1. INTRODUCTION

*Weissella* species are Gram-positive, non-spore-forming, non-motile and heterofermentative organisms with irregular, short or rod-shaped morphology (Collins et al. 1993). Up to now, around 19 *Weissella* species have been found and isolated from various food sources, such as fresh and fermented vegetables (Patel et al. 2013; Dellaglio and Torriani 1986), meat and meat products (Tsakalidou et al.1997; Koort et al. 2006), sourdough (Amari 2013), fermented rice grains (Tohno et al. 2013), carrot juice and row milk (Hammes and Vogel 1995). Genus *Weissella* are capable of synthesizing dextrans by dextransucrases. Dextrans are  $\alpha$ -glucans with  $\alpha$ -D-(1 $\rightarrow$ 6) glycosidic linkages as their major structural features, while  $\alpha$ -(1 $\rightarrow$ 2),  $\alpha$ -(1 $\rightarrow$ 3) and  $\alpha$ -(1 $\rightarrow$ 4) linkages are synthesized as branch linkages (Amari 2013). Dextran produced by *Weissella confusa* contains more linear structures with even fewer  $\alpha$ -(1 $\rightarrow$ 3)-linked branches (2.7%) (Maina et al. 2008). This endows *Weissella confusa* a diversity of food applications, especially in bakery industry. For example, dextran produced during sourdough bread fermentation can improve the shelf-life, volume, moisture retention and nutritional values of the final products (Kati et al. 2009; Galle et al. 2012)

In the presence of sucrose, dextransucrases catalyze the transfer of glucosyl moiety from sucrose to growing dextran, while free fructose is released. Dextransucrases (EC 2.4.1.5) are extracellular enzymes. They have an average molecular weight of 170 kDa and are classified in glycoside hydrolase family 70 (GH70) (<http://www.cazy.org>). Dextransucrases are capable of synthesizing oligosaccharides by transferring glucosyl units from sucrose (donor) to other compounds (acceptors), such as maltose and maltotriose, which is known as the acceptor reaction (Monsan et al. 2010). Apart from synthesizing dextrans and oligosaccharides, dextransucrases can hydrolyze sucrose by directly transferring glucosyl units to H<sub>2</sub>O.

Dextransucrases have gained increasing popularity for their abilities to produce novel oligosaccharides, which are potential non-digestible prebiotics (Goffin et al. 2011). Non-digestible oligosaccharides have a long history of use, especially in Asia (Goffin et al. 2011). These ingredients pass through the digestive tract and selectively stimulate the growth of colonic beneficial bacteria, mainly the *Bifidobacteria* species (Kolida et al. 2002; Mussatto and Mancilha 2007). In addition, they have the potential to decrease the risk of infections and diarrhea (Mussatto and Mancilha 2007). Prebiotic oligosaccharides have a

wide range of applications in food industries and are becoming more and more popular due to their postulated health benefits.

Generally speaking, acceptors are divided into two types based on their efficiencies: strong and weak acceptors. Among all the acceptors, maltose has been most intensively studied. It is the most effective acceptor, and during its acceptor reaction a series of isomaltoligosaccharides is produced (Dols et al. 1997). The structures and biological activities of the oligosaccharides are dominantly dependent on dextransucrase specificity. A lot of other compounds have also been proved to be acceptors of dextransucrase, such as lactose, sorbitol and flavonoids. Nevertheless, except for maltose acceptor reaction, other acceptor reactions for *Weissella confusa* dextransucrase remain unclear.

The aim of the study was to deeper explore *Weissella confusa* dextransucrase acceptor reactions and to evaluate its ability to synthesize oligosaccharides. VTT provided cloned *Weissella confusa* VTT E-90392 dextransucrase. In this thesis, dextransucrase activity and protein content of the dextransucrase extract were firstly measured. Since maltose acceptor reaction has been intensively explored, it was used to study in detail the effects of concentrations of sucrose (donor) and maltose (acceptor), and dosages of dextransucrase on produced oligosaccharides. Other ten acceptors: disaccharides (cellobiose, lactose, isomaltose, laminaribiose, mannobiose, melibiose, and nigerose), and trisaccharides (arabinoxylbiose, isopanose, and maltotriose) were tested for their potentialities of being acceptors. Analysis of mono-, di- and oligo-saccharides was done by HPAEC-PAD. Cellobiose and lactose were selected as substrates to synthesize potential prebiotic oligosaccharides, and their acceptor products were separated by gel filtration using a P2 column. Additionally, MS/MS was used to analyze the possible structures of produced oligosaccharides.



## 2. LITERATURE REVIEW

### 2.1 *Weissella confusa* and dextransucrase

#### 2.1.1 *Weissella confusa*

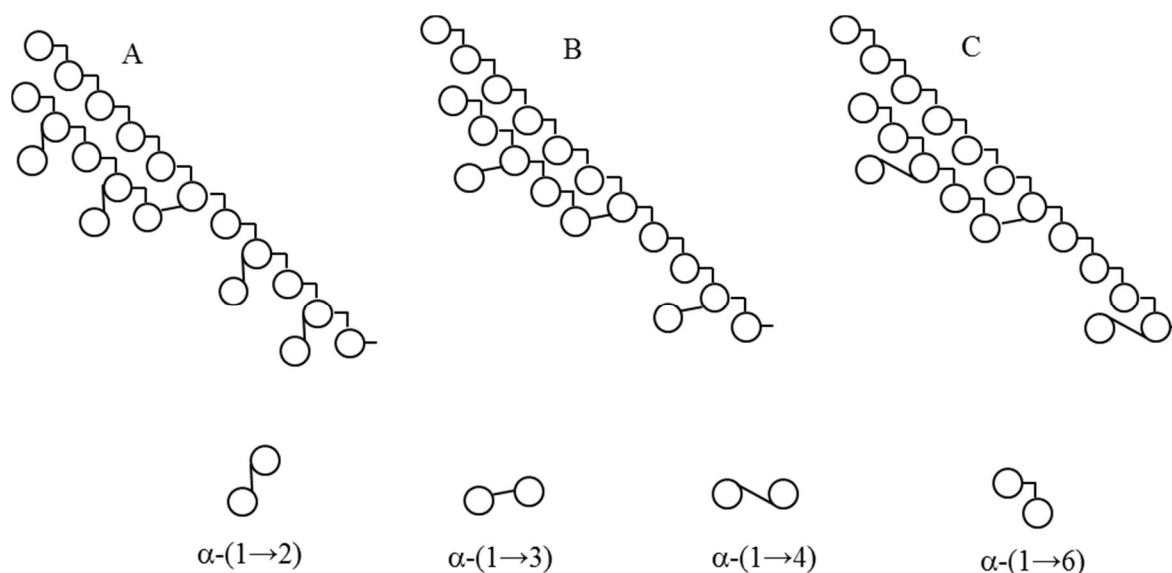
*Weissella* species are Gram-positive, non-spore-forming, non-motile and heterofermentative organisms with irregular, short or rod-shaped morphology (Collins et al. 1993). Up to now, around 19 *Weissella* species have been found (Table 1). They have been isolated from various food sources, such as fresh and fermented vegetables (Patel et al. 2013; Dellaglio and Torriani 1986), meat and meat products (Tsakalidou et al. 1997; Koort et al. 2006), sourdoughs (Amari 2013), fermented rice grains (Tohno et al. 2013) carrot juice and raw milk (Hammes and Vogel 1995). Apart from food sources, *Weissella* species are also present in natural habitats, such as soils and desert plants (Magnusson et al. 2002), as well as in mammals (Björkroth et al. 2002 and Kumar et al. 2011).

Genus *Weissella* belong to lactic acid bacteria (LAB) and produce exopolysaccharides (EPS), mainly dextrans. Apart from *Weissella* species, other dextran producing LAB are mainly from *Streptococcus*, *Leuconostoc* and *Lactobacillus*. Among them *Leuconostoc* species are regarded as the main dextran producers (Sidebotham 1974). Although *Weissella* species (formerly affiliated to the genus *Leuconostoc* and *Lactobacilli*) were isolated in 1957, at that time they belonged to genus *Lactobacillus*. Only until 1993, Collins et al. proposed the genus *Weissella* during the reclassification of some *Leuconostoc*-like and *Lactobacillus*-like species for the first time. Then six strains, which had been isolated before 1993, were reclassified as *Weissella* species. They are *Weissella confusa* (formerly named *Lactobacillus confusus*), *Weissella halotolerans* (formerly named *Lactobacillus halotolerans*), *Weissella kandleri* (formerly named *Lactobacillus kandleri*), *Weissella minor* (formerly named *Lactobacillus minor*), *Weissella paramesenteroides* (formerly named *Leuconostoc paramesenteroides*) and *Weissella viridescens* (formerly named *Lactobacillus viridescens*).

**Table 1.** Isolated *Weissella* strains from various sources

Strains	Sources	First reported publication
<i>Weissella beninensis</i>	Submerged cassava fermentations	Padonou et al. 2010
<i>Weissella ceti</i>	Beaked whales	Vela et al. 2011
<i>Weissella cibaria</i>	Malaysian foods and clinical samples	Björkroth et al. 2002
<i>Weissella confusa</i>	Sugarcane, carrot juice, fermented foods, saliva, sewage and clinical samples	Holzappel and Kandler 1969
<i>Weissella diestrammenae</i>	Gut of a camel cricket	Oh et al. 2013
<i>Weissella fabalis</i>	Spontaneous fermented cocoa bean	Snauwaert et al. 2013
<i>Weissella fabria</i>	Ghanaian cocoa fermentation	De Bruyne et al. 2010
<i>Weissella ghanensis</i>	Ghanaian cocoa fermentation	De Bruyne et al. 2008
<i>Weissella halotolerans</i>	Fermented meat	Kandler et al. 1983
<i>Weissella hellenica</i>	Fermented Greek sausage	Collins et al. 1993
<i>Weissella kandleri</i>	Desert spring	Holzappel and van Wyk 1982
<i>Weissella kimchii</i>	Kimchi and fermented Chinese cabbage	Choi et al. 2002
<i>Weissella koreensis</i>	Kimchi	Lee et al. 2002
<i>Weissella minor</i>	Sludge of milking machines	Kandler et al. 1983
<i>Weissella oryzae</i>	Fermented rice grains	Tohno et al. 2013
<i>Weissella paramesenteroides</i>	Cucumber	Garvie 1967
<i>Weissella soli</i>	Soil	Magnusson et al. 2002
<i>Weissella thailandensis</i>	Thailand fermented fish	Tanasupawat et al. 2000
<i>Weissella viridescens</i>	Discolored cured meat products and pasteurized milk	Niven and Evans 1957

The ability to produce dextrans is one of the key factors in classifying genus *Weissella* (Collins et al. 1993). Strictly speaking, dextrans are  $\alpha$ -glucans with  $\alpha$ -D-(1 $\rightarrow$ 6) glucosidic linkages as the backbones, while  $\alpha$ -(1 $\rightarrow$ 2),  $\alpha$ -(1 $\rightarrow$ 3) and  $\alpha$ -(1 $\rightarrow$ 4) are branched linkages (Amari 2013). Figure 1 presents the schematic structures of the dextrans. The  $\alpha$ -(1 $\rightarrow$ 6) glucosidic linkages may account for 50% to 97% of the total glucosidic linkages (Jeans et al. 1954). There usually presents more than one branch in their structures. As shown in Figure 1, structure A stands for dextran with  $\alpha$ -(1 $\rightarrow$ 2) and  $\alpha$ -(1 $\rightarrow$ 3) linkages as branches, structure B presents dextran with  $\alpha$ -(1 $\rightarrow$ 3) linkages as branches, while structure C is dextran with  $\alpha$ -(1 $\rightarrow$ 3) and  $\alpha$ -(1 $\rightarrow$ 4) linkages as branches. In addition, there are two other types of  $\alpha$ -glucans: alternans and mutans. Alternans contain  $\alpha$ -(1 $\rightarrow$ 6) and  $\alpha$ -(1 $\rightarrow$ 3) linkages and the linkages also present in the branches, whereas mutans have  $\alpha$ -(1 $\rightarrow$ 3) linkages in the main chains and  $\alpha$ -(1 $\rightarrow$ 6)-linked branches (Morales et al. 2001).



**Figure 1.** Schematic representation of dextrans (adapted from Maina 2012). A, dextran with  $\alpha$ -(1→2) and  $\alpha$ -(1→3) linkages as branches; B, dextran with  $\alpha$ -(1→3) linkages as branches; C, dextran with  $\alpha$ -(1→3) and  $\alpha$ -(1→4) linkages as branches

The use of dextran dates back to 1947, when a 6% solution of dextran was approved for clinical use as a blood extender in Sweden (de Belder 1990). Nowadays dextran is generally recognized as safe (GRAS) when it is used as a component of food-packaging material (European Commission [EC] CS/NF/DOS/7/ADD 3 FINAL 2000). But in 1977, dextran was deleted from the GRAS ingredients food list (Federal Register No 223 1977). Not until 1993 was dextran considered safe again in clinical nutrition products on condition that a dextran fraction had to contain at least 95% of  $\alpha$ -(1→6) glucosidic linkages in the main chain and less than 5% of branches (Advisory Committee on Novel Foods and Processes [ACNFP] 1993). Since 2000, dextran has become commercialized novel food ingredient and been used in bakery products (European Commission [EC] CS/NF/DOS/7/ADD 3 FINAL 2000). The dextran is produced by *Leuconostoc mesenteroides* and contains 4.6%  $\alpha$ -(1→3) glucosidic branched linkages. It is able to improve rheological and physico-chemical properties of bakery products.

Dextran produced by *Weissella confusa* contains more linear structures with even fewer  $\alpha$ -(1→3)-linked branches (2.7%) (Maina et al. 2008). This endows *Weissella confusa* a diversity of food applications. For example, dextran produced during wheat sourdough fermentation improves loaf volume, moisture retention, crumb softness and shelf life (Katina et al. 2009). Dextrans have a variety of structural forms and the differences in their structures contribute to their unique physical characteristics, such as solubility and rheological properties (van Hijum et al. 2006). Dextran is synthesized by transferring

glucosyl moieties from sucrose to dextran growing chain under the function of dextransucrase, while fructose is released. Dextransucrase also catalyzes the synthesis of oligosaccharides by transferring glucosyl units from sucrose to other compounds, such as maltose and maltotriose (Monsan et al. 2010). The produced polymers and oligosaccharides by *Weissella confusa* dextransucrase have gained growing interest in clinical, cosmetics, food and feed industries (Naessens et al. 2005, Patel and Goyal 2010). Their structures and biological activities are dominantly dependent on dextransucrase specificity, thus the study of dextransucrase has gained lots of attention and popularity from scholars.

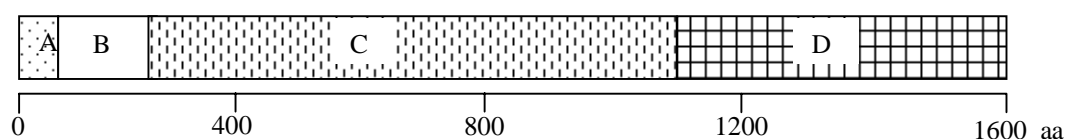
### 2.1.2 Dextransucrase

Dextransucrases (EC 2.4.1.5), belonging to glucansucrase, are extracellular enzymes. They have an average molecular weight of 170 kDa and are classified in the glycoside hydrolase family 70 (GH70) (<http://www.cazy.org>). The other members of glucansucrases are mutansucrases (EC 2.4.1.5) and alternansucrases (EC 2.4.1.140), which are in charge of synthesizing another two types of  $\alpha$ -glucans: mutans and alternans. Purified dextransucrases have the optimal pH ranging 5.0~5.5 and reaction temperature around 30~35 °C (Shukla and Goyal 2011). Calcium in low level activates the enzymes, however acts as an inhibitor in high concentration (Miller and Robyt 1986). The optimal ionic strength for dextransucrases is 10~20 mM (Shukla and Goyal 2011).

#### Structures

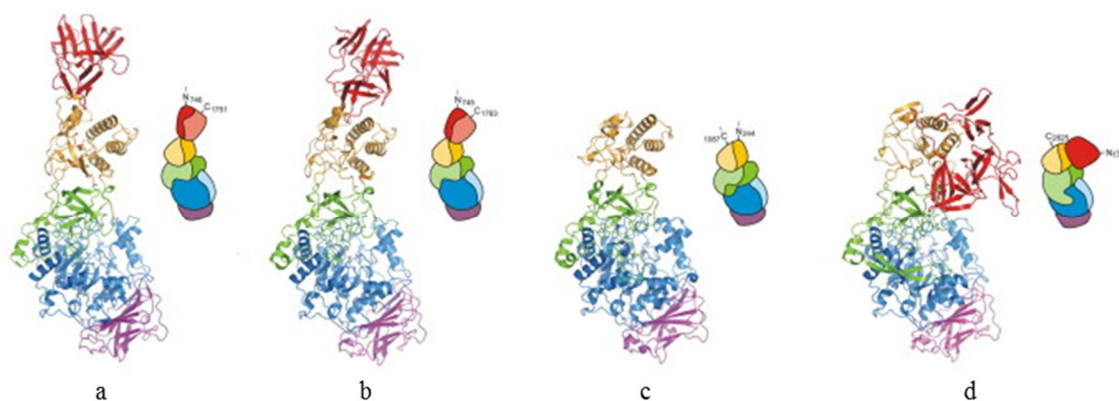
So far more than 40 glucansucrase-coding genes have been isolated and sequenced (Bounaix et al. 2010). Not until 2012 was the complete gene sequence encoding *Weissella confusa* dextransucrase identified, which revealed common structural features of GH70 family (Amari et al. 2013). There are four distinct structural domains in their amino acid sequences: signal peptide, variable region, N-terminal and C-terminal domain, shown in Figure 2 (Monchois et al. 1999). The encoding genes start with a signal peptide (A) with around 30 amino acids, followed by a variable region (B) with around 120 amino acids (Monchois et al. 1999). The variability of the amino acids in the variable region explains the product specificity of GH70 family. N-terminal domain (C) is the catalytic domain, which binds sucrose and cleaves sucrose, containing around 1000 amino acids; whereas the

C-terminal domain (D) is composed of around 500 amino acids and provides the enzyme with glucan binding functionality (Monchois et al. 1999; Leemhuis et al. 2013).



**Figure 2.** Schematic structure of glucansucrase-coding genes (adapted from Monchois et al. 1999). A, signal peptide; B, variable region; C, N-terminal catalytic domain; D, C-terminal domain.

However, only four GH70 glucansucrases' three-dimensional structures are available, and they are from *Lactobacillus reuteri* 180, *Lactobacillus reuteri* 121, *Streptotoccus Mutans* and *Leuconostoc Mesenteroides* (Leemhuis et al. 2013). The cartooned three-dimensional structures of glucansucrases are presented in Figure 3. Previously, it was assumed that the domains on the glucansucrases were arranged one after another as described in Figure 2 (Monchois et al. 1999). The availability of glucansucrase 3D structures reveals that the arrangement of the domains is different from previous assumption. There are three domains (I, II and III) in the catalytic core, and two extra domains called IV and V (Figure 3). For example, in *Lactobacillus reuteri* 180 glucansucrase (Figure 3a), domain I, II, IV and V are from two discontinuous polypeptide chains and domain III is the joint where the two discontinuous polypeptide chains meet (Leemhuis et al. 2013).



**Figure 3.** Three-dimensional structures of glucansucrases and schematic domain arrangements of *Lactobacillus reuteri* 180 (A), *Lactobacillus reuteri* 121 (B), *Streptotoccus mutans* (C) and *Leuconostoc Mesenteroides* (D), colored by domain (I: blue, II: green, III: magenta, IV: yellow, V: red) (adapted from Leemhuis et al. 2013).

### Catalytic mechanism

Biosynthesis of dextran consists of three steps: initiation, elongation and termination (Tsuchiya et al. 1953). The energy, released from the cleavage of the glycosidic bond of

sucrose (27.6 kJ/mol), is sufficient to maintain the reaction, thus no extra energy is required for the elongation step (van Hijum et al. 2006). Dextranase can also hydrolyze sucrose directly by transferring glucosyl units onto water molecules, and free glucose is released. Glucose and sucrose act as initiator primers of dextran polymerization. After the released glucose accumulates to a sufficient amount, the synthesis of dextran is about to happen (Moulis et al. 2006). No detectable oligosaccharide is available at the beginning of the reaction (Monchois et al. 1999). Also, the synthesis reaction can be accelerated by adding exogenous dextran (Monchois et al. 1999).

The mechanism during the elongation step of synthesizing dextran is still under debate: whether the glucosyl moieties from sucrose are transferred to the non-reducing or the reducing ends of growing dextran chains. Nowadays it is widely accepted that dextran chains are elongated by adding glucosyl units ( $C_1$ -OH) to the non-reducing ends of the dextran chains ( $C_6$ -OH), referring as one active site insertion mechanism. Dextranase contains one nucleophilic residue and one protonated residue. The nucleophilic residue attacks the  $C_1$ -OH of the glucosyl moiety of the sucrose, forming a covalent glucosyl-enzyme complex; whereas the protonated residue acts as a proton donor, stimulating the release of fructose (Monchois et al. 1999). The following glucosyl units are continuously added to the non-reducing ends of growing dextran chains (Monchois et al. 1999). Studies carried out by Moulis et al. (2006) and van Hijum et al. (2006) support this mechanism by providing the biochemical and kinetic characterization on dextranase. Lately, the study on three-dimensional structures of dextranases, as well as on its complexes with sucrose, provides further information supporting the one active site insertion mechanism (Vujičić-Žagar et al. 2010). Besides, some other dextranases, such as amylosucrase from *Neisseria polysaccharea*, have been proved to use the same mechanism in transferring glucosyl moieties (Jensen et al. 2004).

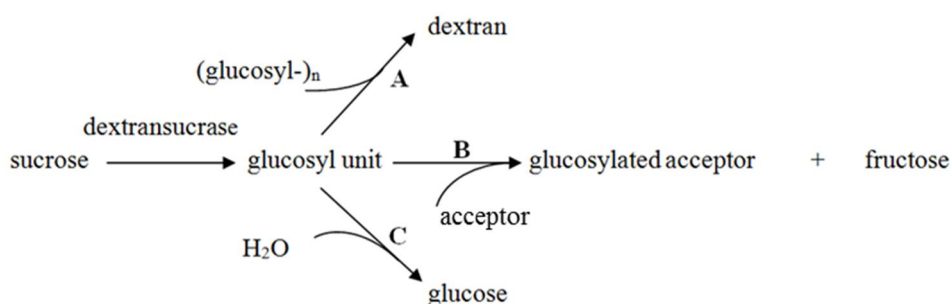
There are also studies claiming that dextran is synthesized by transferring glucosyl units to the reducing-ends of growing dextran chains (Robyt et al. 1974). By labeling sucrose with  $^{14}C$ , Robyt et al. (1974) proposed a two-site insertion mechanism. It reveals that there were two nucleophilic sites involving in the synthesis reaction: two sucrose molecules were attacked by two nucleophilic residues, forming two covalent glucosyl-enzyme complexes; and one  $C_6$ -OH of glucosyl unit was attacked to  $C_1$  position of the other one, which allowed the elongation of dextran from the reducing ends.

## Production of dextran

Dextran cannot be produced by dextransucrase without the presence of sucrose. Sucrose is believed to be the only natural substrate in synthesizing dextran (Monchois et al. 1999). Although *p*-nitrophenyl- $\alpha$ -D-glucopyranoside is also a substrate for dextransucrase, the production efficiency is much lower than that of sucrose (Binder and Robyt 1983). Also, the presence of sucrose may stimulate the release of dextransucrase in some dextransucrase-producing strains (Robyt et al. 2008).

### 2.2 Dextransucrase acceptor reaction

In the presence of sucrose, dextransucrase is capable of synthesizing dextran by transferring the glucosyl units to the growing chains. However, when other compounds, such as maltose and isomaltose, are introduced to the reaction mixture, the enzyme starts to synthesize oligosaccharides by transferring the glucosyl units from sucrose (donor) to these compounds (acceptors). This is known as the acceptor reaction, which was firstly described by Koepsell et al. (1953). Glucose can be regarded as a side product during the reactions. Dextransucrase catalyzed-reactions are briefly summarized in Figure 4.



**Figure 4.** Reactions catalyzed by dextransucrase (adapted from Monchois et al. 1998). A, dextran synthesis by successive transferring glucosyl units; B, oligosaccharides synthesis by transferring glucosyl units to acceptor; C, sucrose hydrolysis by transferring glucosyl units to  $\text{H}_2\text{O}$ .

#### 2.2.1 Categories of acceptors

Generally speaking, acceptors are divided into two categories based on their efficiencies: strong acceptors and weak acceptors. Among all the acceptors, maltose has been most intensively studied, followed by isomaltose and maltotriose. Maltose is the strongest acceptor and when studying other acceptors, maltose is often used as a reference sugar. During maltose acceptor reaction a series of isomalto-oligosaccharides is produced (Dols et al. 1997). This is because the maltose primary acceptor reaction product, panose, can

further be served as an acceptor to give more acceptor products, finally yielding a series of homologous acceptor products (Fu and Robyt 1991). Similarly, isomaltose and maltotriose are also good dextransucrase acceptors (Robyt and Eklund 1983, Seo et al. 2007). But in the case of poor acceptors, fructose only gives a single and low amount of acceptor-product, leucrose (Daum and Buchholz 2002). Therefore, the efficiency of acceptor reaction depends largely on the particular acceptor. In addition, the acceptors are enzyme-dependent. For example, fructose is a more efficient acceptor for alternansucrase, which continuously transfers glucosyl units to leucrose, and the acceptor reaction produces unusual glucosyl-fructose oligosaccharides (Côté et al. 2008). These strong and poor acceptors belong to three different categories: conventional saccharide acceptors, modified saccharide acceptors and non-saccharide acceptors.

#### Conventional saccharide acceptors

In the presence of dextransucrase, a large variety of di-, tri- and higher oligosaccharides are produced by transferring glucosyl units from sucrose to the acceptors. The acceptor products are often associated with postulated health beneficial properties. For example, maltose acceptor products are associated with boosting the growth of beneficial bacteria, and could be used as prebiotics (Sanz et al. 2005). The conventional saccharide acceptors consist of monosaccharides, disaccharides and some oligosaccharides. The monosaccharides include: glucose (Robyt and Eklund 1983), fructose (Daum and Buchholz 2002), galactose (Robyt and Eklund 1983), mannose (Robyt and Eklund 1983), and xylose (Robyt and Eklund 1983). The disaccharides are cellobiose (Robyt and Eklund 1983; Kim et al. 2010), isomaltose (Robyt and Eklund 1983), lactose (Robyt and Eklund 1983; Seo et al. 2007), lactulose (Díez-Municio et al. 2012), maltose (Robyt and Eklund 1983; Rabelo et al. 2006), melibiose (Robyt and Eklund 1983), nigerose (Robyt and Eklund 1983), and turanose (Robyt and Eklund 1983). Besides, tri- and oligosaccharides may also act as acceptors for dextransucrase, such as maltotriose (Fu and Robyt 1990), maltodextrin (Fu and Robyt 1991) and raffinose (Robyt and Eklund 1983).

#### Modified saccharide and non-saccharide acceptors

The potential applications of the synthesized oligosaccharides intrigue the desire for synthesizing new oligosaccharides derivatives. Some modified saccharides, such as fructose dianhydride, alditols, sugar acids and alkyl saccharides, are able to serve as



acceptors (Demuth et al. 2002). Some other compounds, which are not even saccharides, are also dextransucrase acceptors, such as flavonoids (Bertrand et al. 2006). Woo HJ et al. (2012) used dextransucrase to increase the water solubility and browning resistance of ampelopsin (flavonoids) by glucosylation.

### 2.2.2 Mechanism of acceptor reaction

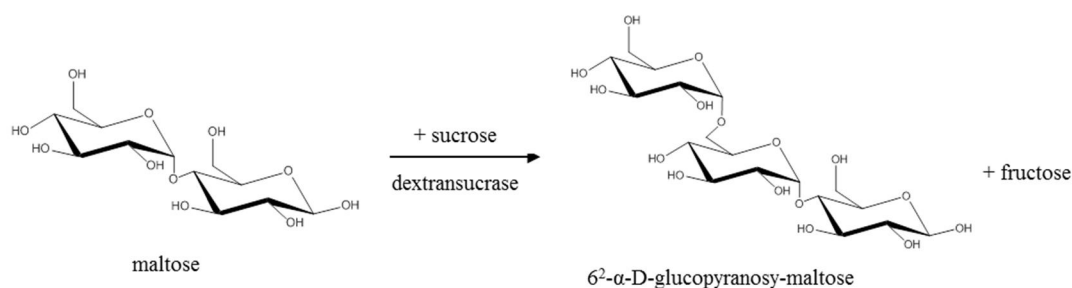
During dextran synthesis, the glucosyl unit is transferred to the C<sub>6</sub>-OH of the growing dextran chain to form a  $\alpha$ -(1→6) glycosidic linkage, which is also the case for many acceptors. Moreover, dextransucrases are capable of connecting glucosyl moieties to acceptors via (1→1), (1→2) (1→3), (1→4), and (1→5) linkages, which depends on the structures of the acceptors (Demuth et al. 2002). For example, the primary acceptor product of maltose, panose, is synthesized by transferring the glucosyl unit to maltose via  $\alpha$ -(1→6) linkage, whereas  $\alpha$ -D-glucopyranosyl-cellobiose, which is cellobiose acceptor product, is produced through the formation of  $\alpha$ -(1→2) glycosidic linkage (Ruiz-Matute et al. 2011).

Acceptor interferes with the covalent glucosyl-enzyme complex, and competes the enzyme-active site with dextran (Naessens et al. 2005). The dextran synthesis is terminated and acceptor reaction is initialized when the acceptor replaces the growing dextran chain from the enzyme-active site (Naessens et al. 2005).

Maltose is the strongest acceptor for dextransucrase. Because of its high availability and high efficiency in the synthesis of oligosaccharides, maltose acceptor reaction and its products have been most intensively studied. Panose (6<sup>2</sup>- $\alpha$ -D-Glc-maltose) is the primary acceptor product, the glucosyl unit from sucrose attacking non-reducing end of C<sub>6</sub>-OH of maltose and facilitating the formation  $\alpha$ -(1→6) glycosidic linkage, shown in Figure 5 (Naessens et al. 2005). When panose accumulates to a certain amount, it also acts as acceptor to form isomaltosyl- $\alpha$ -(1→6)-maltose (Rabelo et al. 2006). Similarly, isomaltosyl-maltose also acts as acceptor, finally forming a series of 6<sup>2</sup>-isomaltodextrinosyl maltoses, which are also called isomalto-oligosaccharides (IMOs) (Naessens et al. 2005).

IMOs show prebiotic effects on *Bifidobacterium spp.* (Goffin et al. 2011). IMOs naturally exist in various foods and are popular functional oligosaccharides in Asia. Traditionally,

commercial available IMO are produced by  $\alpha$ -amylase,  $\alpha$ -glucosidase and pullulanase (Goffin et al. 2011). Currently IMOs produced by dextransucrase have received growing attention. For example, Cho et al. (2014) explored an improved process of producing IMOs in kimchi by adding a dextransucrase producer, sucrose and maltose.



**Figure 5.** Dextranucrase acceptor reaction for panose production (Naessens et al. 2005)

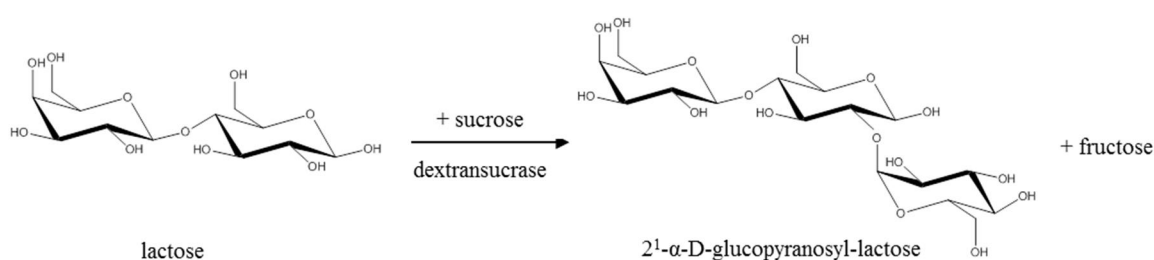
Other maltose-like compounds are also suitable as acceptors. Isomaltose has almost the same effectiveness as maltose, and the primary isomaltose product is 6<sup>2</sup>- $\alpha$ -D-Glc-isomaltose (Roby and Eklund 1983). Maltotriose is also a good acceptor, although it is 40% as effectiveness as maltose (Fu and Robyt 1990). The mechanism of synthesizing maltotriose acceptor products by dextransucrase is quite similar as maltose. 6<sup>2</sup>- $\alpha$ -D-Glc-maltotriose is the primary maltotriose product, the glucosyl unit from sucrose being transferred to the non-reducing residue (Fu and Robyt 1990). The primary product can serve as an acceptor, leading to the accumulation of a series of oligosaccharides. In addition, the glucosyl unit could also be transferred to the reducing residue of maltotriose, forming (1 $\rightarrow$ 2) linkage. But this product is not capable of acting as an acceptor. And its production is low (Fu and Robyt 1990).

Lactose and cellobiose are also acceptors for dextransucrase. Compared to maltose, their relative efficiencies are low, which are 10% as effectiveness as maltose (Roby and Eklund 1983, Fu and Robyt 1990). In lactose acceptor reaction, glucosyl- $\alpha$ -(1 $\rightarrow$ 2)-lactose is the major product, which is also an excellent potential prebiotic ingredient (Sanz et al. 2006). Further structure analysis reveals that the glucosyl unit from sucrose attacks C<sub>2</sub>-OH of the glucosyl moiety, and forms 2<sup>1</sup>- $\alpha$ -D-Glc-lactose (see Figure 6) (Díez-Municio et al. 2012). This is also the possible mechanism to synthesize cellobiose acceptor product: 2<sup>1</sup>- $\alpha$ -D-Glc-cellobiose (see the first reaction in Figure 7) (Ruiz-Matute et al. 2011). Another cellobiose acceptor of DP 3 has also been identified, which is 6<sup>2</sup>- $\alpha$ -D-Glc-cellobiose (see the second reaction in Figure 7) (Ruiz-Matute et al. 2011). The reaction could be continued by

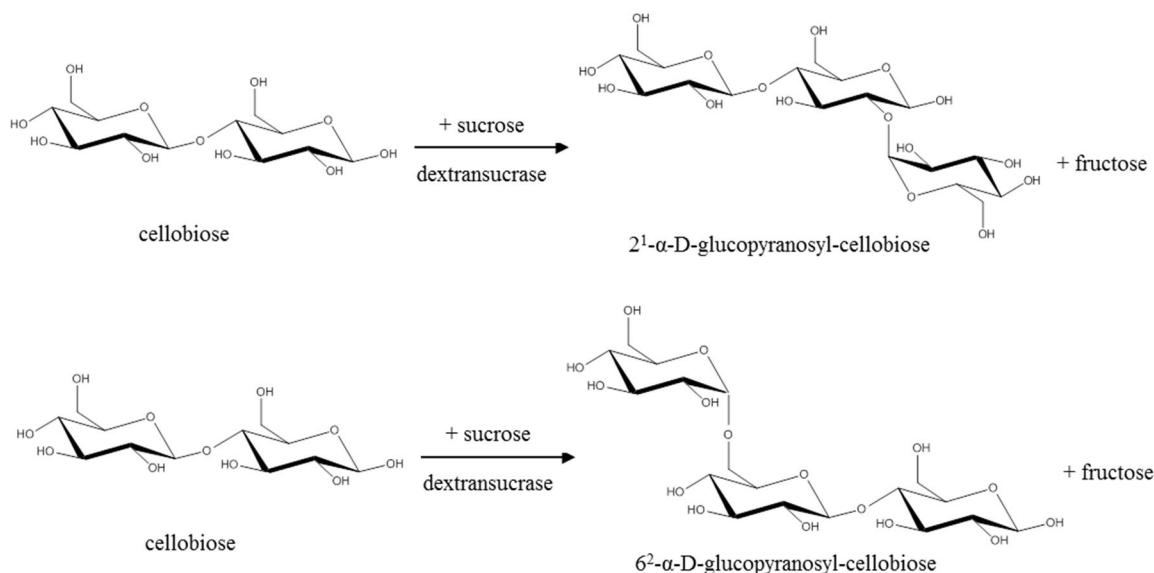
forming more  $\alpha$ -(1 $\rightarrow$ 6) glucosidic linkages, finally synthesizing a series of oligosaccharides (Ruiz-Matute et al. 2011). The acceptor products for maltose, isomaltose, maltotriose, lactose and cellobiose are summarized in the following table.

**Table 2.** Acceptor and its primary product synthesized by dextransucrase acceptor reaction

Acceptor	Primary product(s)
Maltose	6 <sup>2</sup> - $\alpha$ -D-Glc-maltose
Isomaltose	6 <sup>2</sup> - $\alpha$ -D-Glc-isomaltose
Maltotriose	6 <sup>2</sup> - $\alpha$ -D-Glc-maltotriose
Lactose	2 <sup>1</sup> - $\alpha$ -D-Glc-lactose
Cellobiose	6 <sup>2</sup> - $\alpha$ -D-Glc-cellobiose and 2 <sup>1</sup> - $\alpha$ -D-Glc-D-cellobiose



**Figure 6.** Dextransucrase acceptor reaction for 2<sup>1</sup>- $\alpha$ -D-glucopyranosyl-lactose production (Díez-Municio et al. 2012)



**Figure 7.** Dextransucrase acceptor reaction for 2<sup>1</sup>- $\alpha$ -D-glucopyranosyl-cellobiose and 6<sup>2</sup>- $\alpha$ -D-glucopyranosyl-cellobiose production (Ruiz-Matute et al. 2011)

However, acceptor products for lactose and cellobiose remain inconclusive. For example, according to Kang et al. (2013), glucosyl-, isomaltosyl- and isomaltotriosyl-cellobiose have been produced during cellobiose acceptor reaction. Except for 2<sup>1</sup>- $\alpha$ -D-Glc-D-cellobiose, the structures of other products are not the same as summarized in Table 2,

even though the origins of dextransucrases are the same. Also, more lactose acceptor products have been identified in earlier publications, such as isomaltosyl-lactose; however the structure of one DP3 product remains unclear (Kang et al. 2013).

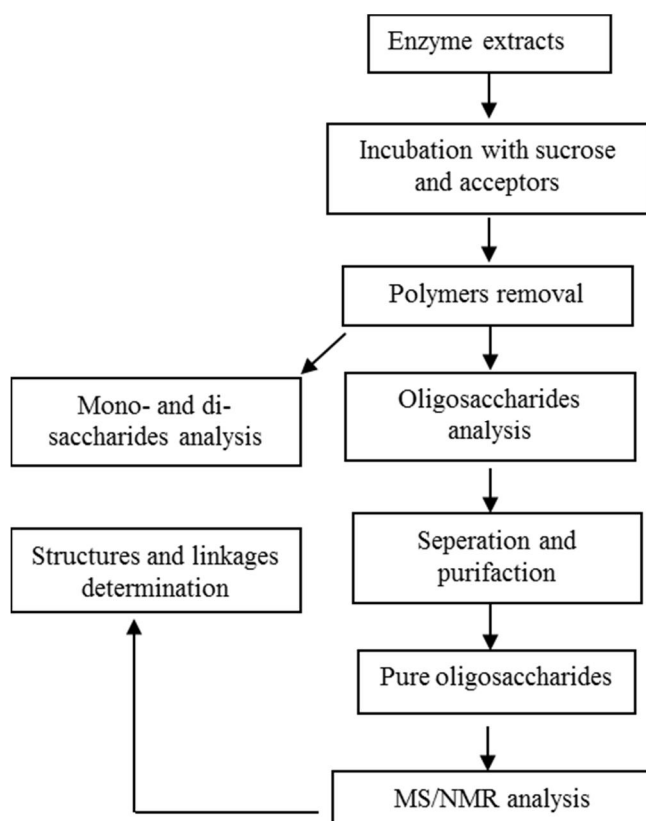
### **2.2.3 Factors affecting acceptor reaction**

The factors affecting dextransucrase acceptor reactions have been investigated at different experimental conditions, among which maltose acceptor reaction has been intensively studied. Early in 1957, Bailey et al. discovered that by increasing the dosage of dextransucrase, more fructose was released. This was the same for other acceptor reactions, such as isomaltose, glucose, galactose, lactose, and cellobiose acceptor reactions (Bailey et al. 1957). In 1983, Robyt and Eklund proved that in the presence of maltose, the yield of dextran decreased, indicating maltose acted as an inhibitor in synthesizing dextran. This study also showed that acceptors with higher efficiencies performed better inhibition behaviors in dextran synthesis (Robyt and Eklund, 1983). In addition, increasing the initial concentrations of maltose and sucrose led to the improvement of panose production (Heincke et al. 1999; Rabelo et al. 2006). Rabelo et al. (2006) reported that in order to get higher panose production and lower dextran formation, lower sucrose to maltose ratio was preferred. There are also publications focusing on overall oligosaccharides production (Lee et al. 1997; Iliev et al. 2007). However, there are many factors determining the oligosaccharides production during an acceptor reaction, such as the concentrations of donor and acceptor, the origin, activity and dosage of dextransucrase, as well as the structure of acceptor. It is difficult to come to a conclusion that which are the most influential effects regarding oligosaccharides production (Kim et al. 2001; Rabelo et al. 2006; Vassileva et al. 2008).

### **2.2.4 Characterization and structural analysis of acceptor products**

The types of glucosidic linkages and degrees of polymerization of synthesized oligosaccharides are important in studying their prebiotic properties, which determine their applications in health and food industries (Goffin et al. 2011). Therefore, it is essential to acquire the information related to characterization and structural analysis of acceptor products. Figure 8 describes the general experimental approaches in analyzing the oligosaccharides synthesized by dextransucrase. The analysis includes oligosaccharides

isolation and purification, size and glycosidic linkages determination, as well as branches lengths determination (Leemhuis et al. 2013).



**Figure 8.** Experimental approaches in analyzing oligosaccharides (adapted from Leemhuis et al. 2013)

Oligosaccharides have to be isolated and purified before the structural analysis starts. Ethanol precipitation is able to remove polysaccharides from the incubation mixture (Maina et al. 2008). The purification of oligosaccharides of various DP is achieved by applying size-exclusion chromatography (SEC), which is most widely used. Besides, high performance anion exchange chromatography (HPAEC) technique has been well developed in separating oligosaccharides since 1980s (Baenziger and Natowicz 1981). Some emerging techniques, such as electrophoretic separation with a novel fluorescent tag, are also able to separate oligosaccharides (Kazarian et al. 2010). Molar masses of oligosaccharides can be determined by gas chromatography with mass spectrometry (GC-MS) when the oligosaccharides are methylated. LC-MS (liquid chromatography) is also a preferable technique in molecular weight determination and structural analysis of oligosaccharides. MS ( $MS^2$ ) spectra can provide oligosaccharides structural information. In Maina et al. (2012), it is possible to use ESI-MS (Electrospray ionization) to determine the linkage types of dextran branches. NMR spectroscopic analysis provides exact structural

information of oligosaccharides, including types of monosaccharides, types and positions of linkages, as well as anomeric configurations (Leemhuis et al. 2013).

### **2.3 Functional properties of acceptor products in food applications**

Functional oligosaccharides are gaining popularity in food industries because of their beneficial effects in boosting the growth of *Bifidobacteria* in human intestines and increasing bowel functions and metabolism (Goffin et al. 2011). For example, the primary lactose acceptor product is resistant to the digestive enzymes in humans and animals; however served as selective growth substrate for beneficial bacteria living in large intestines (Díez-Municio et al. 2012). Maltose and cellobiose acceptor products also stimulate the growth of *Bifidobacteria* in human intestines (Goffin et al. 2011; Ruiz-Mature et al. 2011). Moreover, the oligosaccharides have technical properties, can be used as an anti-fading agent in food pigments, and antifungal agents in baking products (Rabelo et al. 2005; Ruiz-Mature et al. 2011; Gänzle 2012). Their caloric contents are low and have taste sweet, thus could replace sucrose as sweeteners (Oku and Nakamura 2002). Also, they are non-fermentable by oral microorganisms, and can be used as anti-cariogenic substances (Day and Kim 2009). In summary, the method of synthesizing oligosaccharides by dextransucrase acceptor reaction provides a novel method in producing functional oligosaccharides with defined structures, which endows dextransucrase based acceptor reaction a great industry value. However, at present no health claims according to the European nutrition and health claims regulation (EC1924/2006) are accepted.

### **2.4 Key challenges and further developments**

The developing technology allows us to have deeper investigations and further insights in understanding dextransucrase and its related reactions, as well as products characterization. But there are still challenges. For example, the structures of complete dextransucrases remain unclear (Leemhuis et al. 2013). The limited understanding in the completed structures and the functional regions of dextransucrases set barriers in synthesizing new prebiotic products. Also, the limited recognition in the mechanism of dextransucrase action makes it difficult to predict the structures of synthesized oligosaccharides. Moreover, dextransucrase acceptor reactions are strains and acceptors dependent, indicating the final acceptor products are determined by the enzyme origins and activities, as well as the structures of the acceptors. The complexities of the effects and their interactions make

dextranucrase acceptor reactions even more puzzling. Nevertheless, considering the rapid development in the study of dextranucrase in last two decades, it is foreseeable to have dextranucrase acceptor reactions widely spread in food and health industries, or even wider areas.

## **2.5 Safety evaluation**

According to the Regulation (EC) No 258/97, novel foods (NF) are foods and food ingredients that were not used for human consumption to a significant degree within the European Community before 15 May 1997. Generally speaking, there are six categories of novel foods: Class 1, pure chemicals or simple mixtures from non-GM sources; Class 2, complex NF from non-GM sources; Class 3, GM plants and their products; Class 4, GM animals and their products; Class 5, GM microorganisms and their products; and Class 6, foods produced using a novel process (European Council and Parliament Regulation [EC] No 258/97 1997). Dextranucrase produced by *Weissella confusa* has not been used before during food processing in EU and non-EU countries, and should be subject to safety evaluation.

Dextranucrase can synthesize non-digestible oligosaccharides. The oligosaccharides are water-soluble, low-calorie, and have prebiotic properties (Goffin et al. 2011). These properties increase the popularity of dextranucrase in food industrial applications. Dextranucrase can be applied into food industry directly or indirectly by inoculation of *Weissella confusa*. Purified dextranucrase can be used as a processing aid to synthesize prebiotic oligosaccharides directly, which is related to NF Class 1. Also, if *Weissella confusa* is inoculated to sourdough during bakery process, the products are classified to NF Class 6. However, when dextranucrase is produced in a large scale, clone technique should be included. The application of dextranucrase is associated with genetically modified (GM) product. Therefore, the safety of *Weissella confusa*, dextranucrase and its end products should all be assessed and evaluated.

### **2.5.1 Safety evaluation of *Weissella confusa* and dextranucrase**

#### *Weissella confusa*

*Weissella confusa* was firstly identified in 1969 and gradually it was identified naturally existing in many foods, such as fermented meats, sugarcanes, sourdoughs, acidic-carbohydrate foods, and milk (Flaherty et al. 2003). It belongs to lactic acid bacteria (LAB) and LAB are generally regarded as safe (GRAS). Due to its properties in synthesizing dextrans and oligosaccharides, the application of *Weissella confusa* into sourdough preparation has been studied extensively (Maina et al. 2008; Katina et al, 2009; Amari et al. 2013; Shukla et al. 2014). It has received widespread popularity in biotechnology, especially in sourdough fermentation process. Therefore food derived *Weissella confusa* could be regarded as of no safety concerns under this purpose of use.

### Dextranucrase

Dextranucrase (EC 2.4.1.5), produced by *Weissella confusa*, is capable of synthesizing dextran and acceptor products. Partially purified *Weissella confusa* dextranucrase has a molar mass of 178 kDa (Shukla et al. 2014). Temperature, pH and ionic strength affect the activity of dextranucrase (Shukla and Goyal 2011). The nature of the enzyme is protein, thus considered to be of no safety concerns when applied to food products. However, as more complex enzymes have been produced with the help of advanced techniques and efficient production methods, the European Food Safety Authority (EFSA) started to conduct safety evaluation of food enzymes in 2009 (European Council and Parliament Regulation [EC] No 1331/2008). Information related to identification, manufacturing process, assessment of dietary exposure and toxicological data (toxicological and allergenic studies) should be provided in order to assess the safety of *Weissella confusa* dextranucrase. Detailed requirements are available at the EFSA journal (EFSA 1305/2009).

*Weissella confusa* dextranucrase could be acquired by either extracting from the microorganism directly or using genetic engineering. Even though dextranucrase is purified, it is inevitable to contain varying traces of other naturally occurring constituents, such as residues from the microorganism. Since lactic acid bacteria are GRAS, food derived *Weissella confusa* could be regarded as of no safety concerns. However, dextranucrase can also be produced by the GM technique. For example, in this thesis, the gene encoding *Weissella confusa* dextranucrase was transferred into food-graded *Lactococcus lactis* (unpublished). This technique allows better production of *Weissella confusa* dextranucrase. Genetically modified organism, in this case *Lactococcus lactis*, as



well as its product, dextransucrase, should be subject to safety evaluation (European Council and Parliament Regulation [EC] No 258/97 1997).

Dextransucrase is able to catalyze several reactions in foods which are considered to have beneficial effects in food production: dextrans and oligosaccharides synthesis. Dextrans produced by *Leuconostoc mesenteroides* dextransucrase are already commercial available. Oligosaccharides synthesized during dextransucrase acceptor reactions have also gained attention among scholars for their potential prebiotic properties. The non-digestible ingredients pass through the digestive tract and selectively stimulate the growth of beneficial bacteria in colon (Kolida et al. 2002; Mussatto and Mancilha 2007). Apart from that, they have the potential to decrease the risk of infections and diarrhea (Mussatto and Mancilha 2007). Prebiotic oligosaccharides have large applications in food industries and are becoming more and more popular due to their health benefits.

Dextransucrase is present in finished food product either in functional form or in non-functional form, depending on its activity. However, it should be noted that if the food product is suitable for dextransucrase to perform its activity, dextransucrase is regarded as a food ingredient and has to be appeared on the label; if its activity is reverted or denatured, or it is removed from the end-product, it would be considered as a processing aid and there is no need for labeling (European Council and Parliament Regulation [EC] No 1331/2008).

### **2.5.2 Safety evaluation of acceptor products synthesized by dextransucrase**

Among all the acceptor reactions, maltose acceptor reaction has been most intensively studied. Therefore maltose acceptor products – isomalto-oligosaccharides (IMOs) are chosen as the end products for dextransucrase in assessing their safety. Under this circumstance, produced IMOs are categorized to novel foods Class 1 if genetic technique is not involved.

According to the Regulation (EC) No 258/97 of the European Parliament and of the Council concerning novel foods and novel food ingredients, if novel foods have similar composition, nutritional value, metabolism, intended use and level of undesirable substances as the existing products, the novel foods are substantially equivalent to the existing products.

Isomalto-oligosaccharides synthesized by *Weissella confusa* dextransucrase could be regarded as the substantial equivalences to IMOs produced by BioNetura Inc. (2008). IMOs are naturally present in foods such as miso, sake, soy sauce and honey (Goffin et al. 2011). In Asia, IMOs are already commercial available functional foods, especially in Japan (Goffin et al. 2011). Canada has already approved IMOs as novel foods in 2012. In July 2013, European Committee has finalized the application of isomalto-oligosaccharide as a novel food ingredient by BioNetura Inc., allowing IMOs being applied to foods (Food Standards Agency (UK) 2013). Nowadays, isomalto-oligosaccharides could be used in beverages, cereal products, sugar confectioneries and nutritionally complete and fortified foods; with the corresponding maximum use-levels being explicitly required (Food Standards Agency (UK) 2013).

The differences between dextransucrase synthesized IMOs and the substantial equivalence exist in the monosaccharides contents and the ratios of  $\alpha$ -(1 $\rightarrow$ 4) to  $\alpha$ -(1 $\rightarrow$ 6) glucosidic linkages. IMOs synthesized by dextransucrase are high in fructose. Also, the oligosaccharides might have  $\alpha$ -(1 $\rightarrow$ 3) glucosidic linkages because *Weissella confusa* dextransucrase can synthesize dextran with  $\alpha$ -(1 $\rightarrow$ 3) linked branches (Shukla et al. 2014). These differences might affect digestibility of the IMOs. In conclusion, IMOs synthesized by dextransucrase should not give rise to any safety concerns.

### 3. EXPERIMENTAL RESEARCH

#### 3.1 Aims of the study

The aim of this master thesis was to explore *Weissella confusa* dextransucrase acceptor reactions and to evaluate its potential to synthesize oligosaccharides. Maltose acceptor reaction was chosen to analyze the effects of concentrations of donor (sucrose) and acceptor (maltose), as well as dosages of dextransucrase on maltose acceptor products. Other acceptors, some disaccharides and trisaccharides, were also evaluated for their potentialities of being acceptors. Primary cellobiose and lactose acceptor products were selected for more detailed studies. They were purified by gel filtration and MS/MS was used to evaluate their potential structures, which also provided further information to understand *W. confusa* dextransucrase acceptor reaction mechanism.

#### 3.2 Materials

Glucose, fructose, lactose, maltose monohydrate and cellobiose were acquired from Merck (Darmstadt, Germany). Sucrose was from AnalaR (England). Isomaltose and panose were from TCI Europe N.V. (Zwiindrecht, Belgium). Maltotriose was purchased from Sigma Chemical Co.(St. Louis, MO, USA). Nigerose and melibiose were from Sigma-Aldrich (Steinheim, Germany). Laminaribiose and mannobiose were obtained from Megazyme (Wicklow, Ireland). Isopanose was purchased from Carbosynth Limited (Berkshire, UK). Laboratory prepared arabinoxylobiose was produced as described by Rantanen et al. (2007). The structures of used oligosaccharides are summarized in Appendix Table 1.

The gene coding dextransucrase from *Weissella confusa* VTT E-90392 was transferred to *Lactococcus lactis* (unpublished). Dextransucrase extract was prepared and partially purified by Technical Research Centre of Finland (VTT).

Dextransucrase activity assay reagent A: 2.5 g of sodium carbonate anhydrous ( $\text{Na}_2\text{CO}_3$ ) (Merck, Darmstadt, Germany), 2.5 g of sodium potassium tartrate tetrahydrate ( $\text{KNaC}_4\text{H}_4\text{O}_6 \cdot 4\text{H}_2\text{O}$ ) (Merck, Darmstadt, Germany), 2.0 g of sodium bicarbonate ( $\text{NaHCO}_3$ ) (Merck, Darmstadt, Germany) and 20 g of sodium sulfate anhydrous ( $\text{Na}_2\text{SO}_4$ ) (VWR International BVBA, Belgium) dissolved into 100 ml water. Reagent B: 4.5 g of copper sulphate ( $\text{CuSO}_4$ ) (Merck, Darmstadt, Germany) dissolved into 30 ml water with one drop of concentrated sulphuric acid ( $\text{H}_2\text{SO}_4$ ) (J.T.Baker, Holland). Reagent C: 0.3 g of Sodium

arsenate dibasic heptahydrate ( $\text{Na}_2\text{HAsO}_4 \cdot 7\text{H}_2\text{O}$ ) (Sigma-Aldrich, Steinheim, Germany) dissolved into 2.5 ml water, and added to a 45 ml water solution containing 2.5 g of ammonium heptamolybdate tetrahydrate ( $(\text{NH}_4)_6\text{Mo}_7\text{O}_{24} \cdot 4\text{H}_2\text{O}$ ) (Merck, Darmstadt, Germany) and 2.1 ml of  $\text{H}_2\text{SO}_4$ . Reagent D: Freshly prepared by mixing reagent A and reagent B with the ratio of 25/1.

Protein determination reagent A (alkaline copper tartrate solution) and B (dilute Folin reagent) were purchased from Bio-Rad Laboratories, Inc. (USA). Standard bovine serum albumin was from Sigma-Aldrich (Steinheim, Germany).

Sodium citrate dihydrate and calcium chloride ( $\text{CaCl}_2$ ) were acquired from Merck (Darmstadt, Germany). Citric acid monohydrate was from Sigma Chemical Co. (St. Louis, MO, USA). Milli-Q water was prepared using Millipore system (Bedford, MA, USA).

### **3.3 Methods**

#### **3.3.1 Dextranucrase activity assay**

The released fructose was measured by Somogyi-Nelson method to evaluate dextranucrase activity (Nelson 1944; Somogyi 1952).

Dextranucrase activity was measured in 1 ml 20 mM sodium citrate buffer (pH 5.4) containing 2 mM  $\text{Ca}^{2+}$  reaction mixture, in which there were 5% (w/v) of sucrose and 20  $\mu\text{l}$  of enzyme extract, which was diluted to 1/8 of its original concentration. The mixture was incubated at 30 °C for 15 min and after that 100  $\mu\text{l}$  of the reaction mixture was aliquoted to 100  $\mu\text{l}$  of reagent D. Four fructose standard solutions were prepared at the concentrations of 125  $\mu\text{g/ml}$ , 175  $\mu\text{g/ml}$ , 225  $\mu\text{g/ml}$  and 275  $\mu\text{g/ml}$  respectively. 100  $\mu\text{l}$  of reagent D was added to each standard solution. All solutions were boiled in a water bath for 20 min before cooling down to the room temperature. 100  $\mu\text{l}$  of reagent C was added to the solution, followed by 700  $\mu\text{l}$  water. The color formed was measured at 500 nm with a Perkin Elmer Lambda 25 (Shelton, USA) UV/Vis spectrophotometer against blank. Each experiment was performed twice and the samples were prepared in triplicate.

One unit (U) of dextranucrase activity is defined as the amount of enzyme that catalyzes the release of 1  $\mu\text{mol}$  of fructose per min. Its activity was calculated based on the following

equation (1), where  $A_{500}$  is the absorbance at 500 nm against blank,  $C$  is 1 optical density (OD) equivalent fructose concentration (mg/ml), calculated from the standard plot,  $V$  is the volume of the reaction mixture (ml),  $k$  is the dilution fraction, 180 is the molecular weight of fructose,  $t$  stands for the reaction time (min), and  $v$  is initial enzyme extract volume used in this assay. In this test,  $k$  equals 20.

$$1 \text{ U} = \frac{A_{500} \times C \times V \times k}{180 \times t \times v} \quad (1)$$

### 3.3.2 Protein determination

The protein content of dextransucrase extract was estimated by Lowry et al. (1951).

The bovine serum albumin standard stock (3mg/ml) was diluted into four concentrations: 0.2 mg/ml, 0.6 mg/ml, 1.0 mg/ml and 1.5 mg/ml solution. Dextransucrase extract was diluted to 1/20 of its original concentration. 125  $\mu$ l of protein determination reagent A was added to 25  $\mu$ l of diluted enzyme extract and the standards, followed by 1 ml of reagent B. The final volume was 1.15 ml. The color formed was measured at 750 nm on the spectrophotometer against blank. Each experiment was performed twice and in each experiment the samples were prepared in triplicate.

The protein content of dextransucrases extract was calculated based on the following equation (2), where  $A_{750}$  is the absorbance at 750 nm against blank,  $C$  is 1 optical density (OD) equivalent protein concentration (mg/ml) and  $k$  is the dilution factor. In this test,  $k$  equals 20.

$$\text{Protein content} = A_{750} \times C \times k \quad (2)$$

### 3.3.3 Experimental designs and acceptor reactions

#### Preliminary design

Preliminary design was intended to acquire concentration regions of maltose and sucrose, during which synthesis of oligosaccharides was preferred and synthesis of dextrans was suppressed. For isomalto-oligosaccharides synthesis, maltose acceptor reactions were

prepared in 1 ml scale. Sucrose and maltose concentrations, as well as dextransucrase dosages are indicated in Table 3. The mixtures were incubated at 30 °C for 24h in 20 mM sodium citrate buffer (pH 5.4) containing 2 mM CaCl<sub>2</sub>. Finally the reactions were terminated in a boiling water bath for 10 min.

**Table 3.** Preliminary design for maltose acceptor reaction

Coding	Sucrose conc. (mol/l)	Maltose conc. (mol/l)	Dextransucrase conc. (U/g sucrose)
A 1	1	0.5	1
A 10	1	0.5	10
B 1	0.5	0.5	1
B 10	0.5	0.5	10
C 1	0.5	1	1
C 10	0.5	1	10
D 1	0.3	0.15	1
D 10	0.3	0.15	10
E 1	0.15	0.15	1
E 10	0.15	0.15	10
F 1	0.15	0.3	1
F 10	0.15	0.3	10

### Optimization design

Three independent factors: sucrose (0.15-1 mol/l), maltose (0.15-1 mol/l) and dextransucrase dosages (1-10 U/g sucrose) were selected in this design, in order to predict their effects on the production of DP3, DP4, DP5, DP6 and overall products, as well as consumed percentage of maltose, within the experimental regions. The optimization design with 18 runs was called central composite design, in which four replicates were made at the center point to estimate the reproducibility. Table 4 displays the sucrose and maltose concentrations, and dextransucrase dosages of the central composite design. For each response, a quadratic model was used, as indicated in the following equation (3), where  $y$  is the response,  $x_1$ ,  $x_2$  and  $x_3$  are the three independent factors,  $\beta_0$  is the constant term,  $\beta_1$ ,  $\beta_2$  and  $\beta_3$  are the model coefficients, and  $\varepsilon$  is the residual response variation not explained by the model (Umetri 1998). Regression analysis and contour plot were finished by Modde 6.0 (Umetrics AB, Umeå, Sweden).

$$y = \beta_0 + \beta_1x_1 + \beta_2x_2 + \beta_3x_3 + \beta_{12}x_1x_2 + \beta_{13}x_1x_3 + \beta_{23}x_2x_3 + \varepsilon \quad (3)$$

The evaluation of the optimization model was determined by two parameters,  $R^2$  and  $Q^2$ .  $R^2$  is called the goodness of fit, with its value between 0 and 1, which measures how well the regression model can be made to fit the raw data.  $R^2$  equaling to 1 indicates a perfect model is built, while  $R^2$  being 0 means the model is a failure.  $Q^2$  is called the goodness of prediction, measuring how well the model predicts.  $Q^2$  has the upper limit 1 and lower limit minus infinity. A larger  $Q^2$  indicates the model has good predictive ability thus the prediction errors should be small (Umetri 1998). In a good model, the values of  $R^2$  and  $Q^2$  are both high and not separated by more than 0.2-0.3 (Umetri 1998). As the general rule, accurate predictions require  $Q^2$  being no less than 0.5 (Umetri 1998).

The reaction mixtures were prepared the same way as the preliminary design.

**Table 4.** Central composite design for maltose acceptor reaction

Run	Sucrose conc. (mol/l)	Maltose conc. (mol/l)	Dextranucrase conc. (U/g sucrose)
1	0.15	0.15	1
2	0.15	1	1
3	1	0.15	1
4	1	1	1
5	0.15	0.15	10
6	0.15	1	10
7	1	0.15	10
8	1	1	10
9	0.575	0.15	5.5
10	0.575	1	5.5
11	0.15	0.575	5.5
12	1	0.575	5.5
13	0.575	0.575	1
14	0.575	0.575	10
15	0.575	0.575	5.5
16	0.575	0.575	5.5
17	0.575	0.575	5.5
18	0.575	0.575	5.5

### Other acceptor reactions

Other disaccharides and trisaccharides: lactose, cellobiose, isomaltose, isopanose, maltotriose, melibiose, nigerose, laminaribiose and mannobiose, and arabinoxylobiose were selected as acceptors. The concentrations of donor and acceptors, and the dextransucrase dosages are presented in Table 5 and 6. Table 5 displays the 5 experiments carried out for lactose acceptor reactions. Also based on the results in the study, acceptor products seemed to be most abundant when donor to acceptor ratio was around 4. The ratio determined their concentrations in Table 6. The reaction mixtures were prepared the same as the preliminary design but in 200  $\mu$ l scales.

**Table 5.** Lactose acceptor reactions

Coding	Sucrose conc. (mol/l)	Lactose conc. (mol/l)	Dextransucrase conc. (U/g sucrose)
L 1	1	0.15	1
L 2	1	0.15	10
L 3	0.575	0.15	5.5
L 4	0.15	0.15	1
L 5	0.15	0.15	10

**Table 6.** Acceptor reactions with various acceptors

Donor	Donor conc. (mol/l)	Acceptor	Acceptor conc. (mol/l)	Dextransucrase conc. (U/g sucrose)
Sucrose	0.08	Arabinoxylobiose	0.02	10
Sucrose	0.1	Isomaltose	0.025	10
Sucrose	0.2	Isopanose	0.05	10
Sucrose	0.2	Cellobiose	0.05	10
Sucrose	0.2	Mannobiose	0.05	10
Sucrose	0.2	Maltotriose	0.05	10
Sucrose	0.2	Melibiose	0.05	10
Sucrose	0.2	Nigerose	0.05	10
Sucrose	0.2	Laminaribiose	0.05	10

### **3.3.4 Dextran content estimation**

Dextran content was estimated using phenol sulfuric acid assay, which was modified based on Dubois et al. (1956). Dextran was precipitated and washed twice by 50% ethanol to remove sucrose, fructose and oligosaccharides. The pellet was re-suspended with H<sub>2</sub>O. 500



$\mu\text{l}$  of dextran sample, blank (water) or standards (glucose) were added into glass tubes followed by 500  $\mu\text{l}$  5% phenol solution. After vortexing, 2.5 ml of concentrated sulphuric acid was added firmly to the middle of the solution, and mixed. The mixtures were allowed to incubate at room temperature for 30 min. The color formed was measured at 480 nm with a Perkin Elmer Lambda 25 (Shelton, USA) UV/Vis spectrophotometer against blank.

### **3.3.5 Analysis of monosaccharides, disaccharides and oligosaccharides**

Samples were centrifuged at 13000 rpm for 10 min in Heraeus Biofuge Pico Microlitre centrifuge (Kendro, Germany) and filtered through 10-kDa Amicon Ultra 0.5 ml filters (Millipore, Billerica, MA, USA). Monosaccharides, disaccharides and oligosaccharides were analyzed by HPAEC-PAD equipment, which had a CarboPac PA-100 column (250  $\times$  4 mm, i.d, Dionex, USA), a Decade gold electrode (Antec Leyden, The Netherlands), a Waters 717 autosampler and two Waters 515 pumps. The analytical method of oligosaccharides was improved based on Rantanen et al (2007). Two eluents: A (75 mM NaOH) and B (1M NaOAc in 75mM NaOH) were used. Eluent A started the gradient at 8 min, reached to 10% at 35 min, and lasted for 5 min. The flow rate was 1 ml/min and the injection volume was 10  $\mu\text{l}$ . Glucose, fructose, sucrose and maltose were used as the external quantitative standards. Quantification of maltose products (DP3-6) was carried out using panose as the external standard.

### **3.3.6 Purification of acceptor products**

Cellobiose and lactose acceptor products were selected for further purification. Cellobiose products were synthesized under the condition described in Table 6, while lactose products under the condition in Table 5 (coding L2). Both were in 50 ml scales. The purification of acceptor products was done using gel filtration method described by Shukla et al. (2014). After the reaction was terminated, two volumes of ethanol (Altia Etax A, Finland) were added to reaction mixture to precipitate polymeric dextran. The mixture was centrifuged at 13000 rpm for 20 min using superspeed centrifuge (Sorvall RC5C, 28 for SLA-1500, Thermo Scientific). The supernatant was collected and concentrated to approximately 2 ml using rotatory vacuum evaporator (Heidolph Laborota 4001 Digital). The concentrated solution was diluted with water to 30 ml, followed by filtration with 0.45  $\mu\text{m}$  membrane filter (Acrodisc 13, Pall Corporation, Ann Arbor, USA). 5 ml of filtrated solution was further purified by Biogel P2 column (5  $\times$  95 cm; Biorad, Hercules, USA), and the

acceptor products were separated according to the degrees of polymerization. Water was used as the eluent. The flow rate was set at 0.7 ml/min and the void volume was around 850ml. Two ml of fraction was collected per tube and the total collection volume was 400 ml. Fractions with most purity were selected for further characterization.

### **3.3.7 Characterization of primary acceptor products using MS/MS**

The molar masses and structures of the isolated oligosaccharides were determined by ESI-MS in negative mode. The MS equipment (Bruker Daltonik GmbH, Bremen, Germany) was coupled with Bruker Esquire quadrupole ion trap (QIP). 10  $\mu$ l of oligosaccharide fractions mixed with 189  $\mu$ l of MeOH/water/formic acid solution (50:49:1) and 1  $\mu$ l of  $\text{NH}_4\text{Cl}$  (10 mg/ml). The samples were injected directly at a flow rate of 5 $\mu$ l/min. The capillary and end-plate voltages were 3200 V and -500 V, respectively. The temperature of drying gas ( $\text{N}_2$ ) was 325  $^\circ\text{C}$  at a flow rate of 4.00 l/min. Nebulizer pressure was 15.00 psi. The scan range was 100.00 – 800.00  $m/z$ . The MS fragmentation amplitudes were between 0.45 V and 0.65 V. The MS fragmentation amplitudes for  $\text{MS}^2$  and  $\text{MS}^3$  were both 0.65 V. MS spectra were processed by DataAnalysis for LC/MSD Trap Version 3.2 (Bruker Daltonik GmbH, Bremen, Germany).

## **3.4 Results**

### **3.4.1 Dextransucrase activity and protein content estimation**

Dextransucrase activity was 28.3 U/ml. Protein content of dextransucrase extract was 24.2 mg/ml. Thus, the specific activity of dextransucrase was 1.2 U/mg.

### **3.4.2 Preliminary design results of maltose acceptor reaction**

Twelve experiments of maltose acceptor reactions were done. The concentrations of glucose, fructose, maltose, sucrose, overall acceptor products and dextran were determined after the reactions were terminated (see Table 7). The twelve experiments could be evenly divided into two sets. Under each set, there were three pairs of experiments. The chromatograms of pair A, B, and C are in Figure 9, while those of pair D, E, and F are presented in Figure 10. A1 indicates the dosage of dextransucrase was 1 U/g sucrose, and A10 means 10 U/g sucrose. Also, acceptor products of DP3-6 accounted for the overall

products because the production of oligosaccharides with  $DP > 6$  was too little to be quantified.

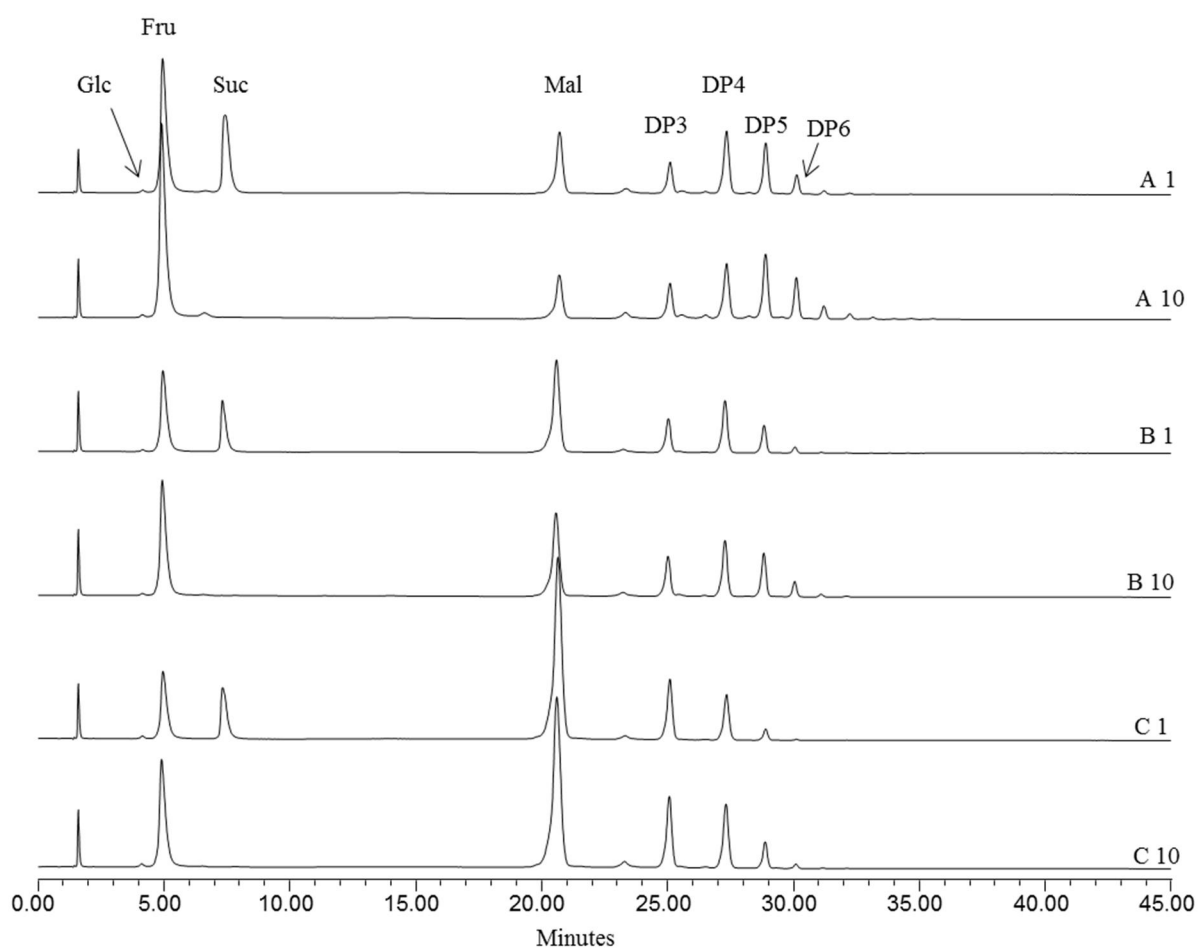
When sucrose and maltose were at their highest concentrations (sucrose 1M and maltose 0.5M) and catalyzed by dextransucrase (10 U/g sucrose), the maximum yield of acceptor products was as high as 253.3 mg/ml. Minimum production of 33.8 mg/ml was acquired when sucrose and maltose were at their lowest concentrations (sucrose and maltose both 0.15M), and catalyzed by dextransucrase (1 U/g sucrose) (Table 7). The production of dextran was between 0.2 mg/ml ~ 0.5 mg/ml, much lower than total acceptor products. Under the selected experimental regions, dextrans synthesis was largely suppressed. Maltose was not totally consumed. Although free glucose and fructose were released during the reaction, the released glucose could be neglected compared to fructose. More than 97% of glucosyl units from sucrose were transferred to maltose. Also, when the dosage of dextransucrase was 10 U/ g sucrose, sucrose was totally consumed.

By comparing chromatograms of A1 and A10 in Figure 9, it is clear that the dosages of dextransucrase had an effect on the utilization of maltose and sucrose, but did not affect much on the profiles of isomalto-oligosaccharides. The same conclusion could be drawn by comparing the chromatograms in other pairs. Moreover, the interactions between maltose and sucrose affected the isomalto-oligosaccharides profiles. In chromatograms of A1, B1 and C1 of Figure 9, when the ratio of sucrose to maltose dropped from 2:1 to 1:2, the production of products with lower DP (DP3 and DP4) seemed to increase, while those higher DP products (DP5 and DP6) decreased significantly. This is also the same for sample D1, E1 and F1, by comparing their chromatograms in Figure 10. In addition, the samples in Figure 9 were four times more diluted than those in Figure 10, explaining the phenomenon that some samples in Figure 10 had more observable oligosaccharides.

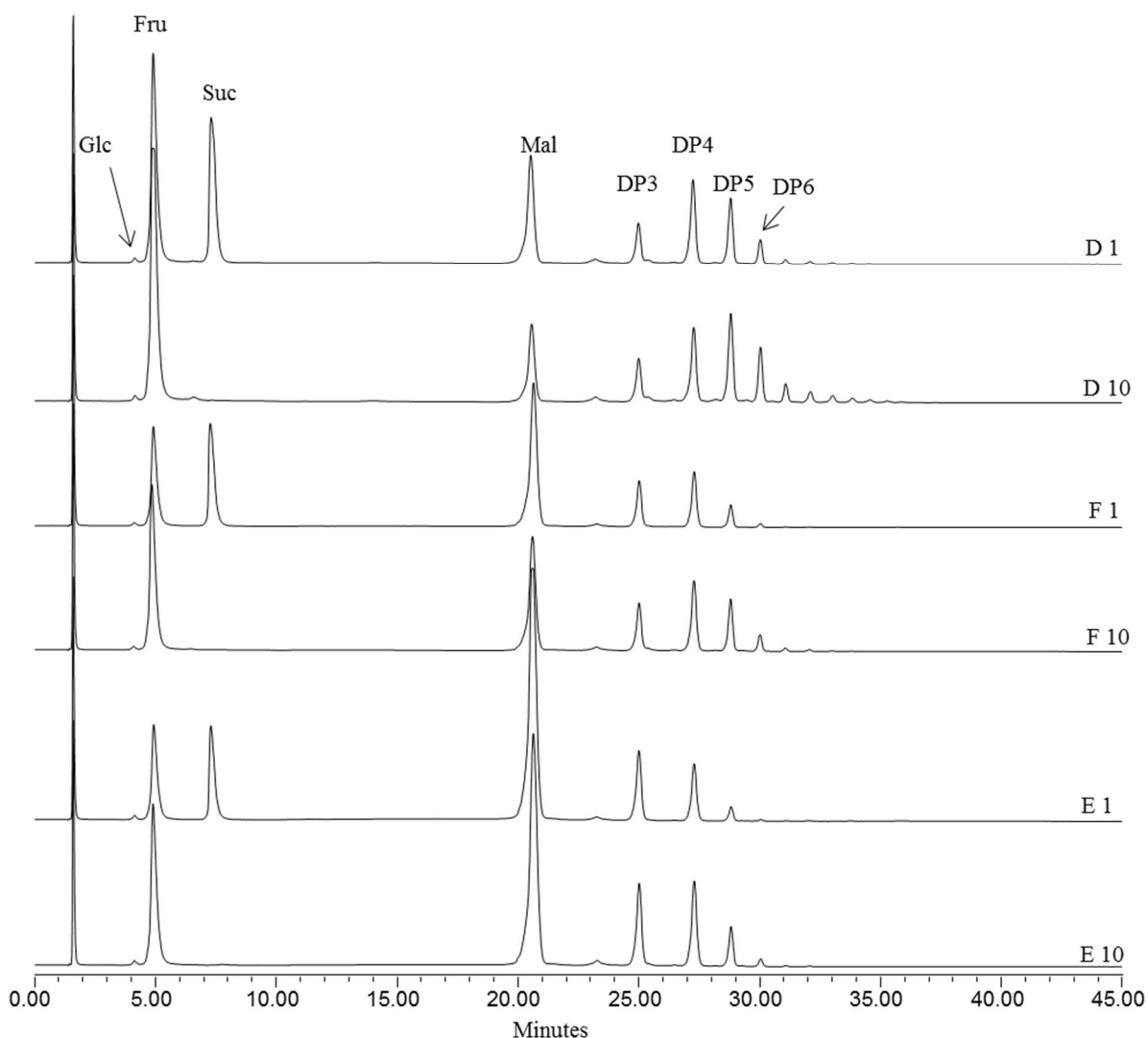
**Table 7.** Concentrations of saccharides after incubation

Before incubation <sup>a</sup>				After incubation <sup>b</sup>						
Coding	Sucrose conc. (mg/ml)	Maltose conc. (mg/ml)	Dextranase conc. (U/g sucrose)	Glucose conc. (mg/ml)	Fructose conc. (mg/ml)	Sucrose conc. (mg/ml)	Maltose conc. (mg/ml)	Overall acceptor products (mg/ml)	Dextran content (mg/ml)	
A 1	342 (1 M)	171 (0.5 M)	1	1.0	97.5	74.3	67.1	184.3	0.27	
A 10	342	171	10	1.2	146.6	0.0	48.0	253.3	0.35	
B 1	171 (0.5 M)	171 (0.5 M)	1	0.8	59.0	46.5	105.7	122.4	0.29	
B 10	171	171	10	0.8	85.1	0.0	94.9	165.1	0.24	
C 1	171 (0.5 M)	342 (1 M)	1	1.1	48.4	47.9	216.8	124.3	0.15	
C 10	171	342	10	1.2	78.9	0.0	197.9	180.7	0.28	
D 1	97 (0.3 M)	49 (0.15 M)	1	0.4	41.3	39.0	32.4	64.2	0.50	
D 10	97	49	10	0.5	49.8	0.0	23.0	87.5	0.53	
E 1	49 (0.15 M)	49 (0.15 M)	1	0.3	19.0	25.2	43.3	33.8	0.34	
E 10	49	49	10	0.3	32.6	0.0	34.6	51.8	0.29	
F 1	49 (0.15 M)	97 (0.3 M)	1	0.4	18.5	23.0	77.8	41.7	0.20	
F 10	49	97	10	0.4	31.6	0.0	72.1	62.5	0.29	

<sup>a</sup>One sample was prepared under each experimental condition. <sup>b</sup>The concentrations were analyzed twice and their average values are presented in the table.



**Figure 9.** HPAEC-PAD profiles of maltose acceptor products in the presence of : A 1, sucrose (1 mol/l) and maltose (0.5 mol/l) catalyzed by dextransucrase (1 U/g sucrose); A 10, sucrose (1 mol/l) and maltose (0.5 mol/l) catalyzed by dextransucrase (10 U/g sucrose); B 1, sucrose (0.5 mol/l) and maltose (0.5 mol/l) catalyzed by dextransucrase (1 U/g sucrose); B 10, sucrose (0.5 mol/l) and maltose (0.5 mol/l) catalyzed by dextransucrase (10 U/g sucrose); C 1, sucrose (0.5 mol/l) and maltose (1 mol/l) catalyzed by dextransucrase (1 U/g sucrose); C 10, sucrose (0.5 mol/l) and maltose (1 mol/l) catalyzed by dextransucrase (10 U/g sucrose). Labeled peaks are as follows: glucose (Glc), fructose (Fru), sucrose (Suc), maltose (Mal), and acceptor products with the degree of polymerization (DP) 3, 4, 5 and 6. (The samples in Figure 9 are four times more diluted than samples in Figure 10)



**Figure 10.** HPAEC-PAD profiles of maltose acceptor products in the presence of : D 1, sucrose (0.3 mol/l) and maltose (0.15 mol/l) catalyzed by dextransucrase (1 U/g sucrose); D 10, sucrose (0.3 mol/l) and maltose (0.15 mol/l) catalyzed by dextransucrase (10 U/g sucrose); E 1, sucrose (0.15 mol/l) and maltose (0.15 mol/l) catalyzed by dextransucrase (1 U/g sucrose); E 10, sucrose (0.15 mol/l) and maltose (0.15 mol/l) catalyzed by dextransucrase (10 U/g sucrose); F 1, sucrose (0.15 mol/l) and maltose (0.3 mol/l) catalyzed by dextransucrase (1 U/g sucrose); C 10, sucrose (0.15 mol/l) and maltose (0.3 mol/l) catalyzed by dextransucrase (10 U/g sucrose). Labeled peaks are as follows: glucose (Glc), fructose (Fru), sucrose (Suc), maltose (Mal), and acceptor products with the degree of polymerization (DP) 3, 4, 5 and 6.

### 3.4.3 Optimization design results of maltose acceptor reaction

The preliminary showed that, in the selected experimental regions, oligosaccharides synthesis reaction had much higher priority over dextran synthesis. Therefore an optimization design was followed to analyze in more detail on how sucrose, maltose and dextransucrase affected the isomalto-oligosaccharides.

Eighteen experiments were performed in the optimization design. Three independent variables and six responses were selected. Maltose acceptor products with different degrees of

polymerization (DP3-6), overall products, and consumed percentage of maltose were calculated at each experimental point. The production of overall acceptor products was the sum of acceptor products DP3-6.

Table 8 lists the concentrations of fructose, sucrose, maltose, DP3-6 and overall DP products in the optimization design. The coefficients of the model, goodness of fit ( $R^2$ ) and predictive power ( $Q^2$ ) for all responses were relatively high:  $R^2$  value varying between 0.8 and 1, while  $Q^2$  varying between 0.5 and 0.9 (Table 9). The model represented the data well. Also, the reproducibility was high. Model coefficient plots were used instead of the regression equations. The model coefficient plots in Figure 11 display the effects of linear (mal, suc and enz), quadratic (mal\*mal, suc\*suc and enz\*enz) and interactions (mal\*suc, mal\*enz, and suc\*enz) on the six responses (DP3-6) and consumed percentage of maltose.

Acceptor products (DP3-6) were obtained under nearly all conditions. However, their contents varied. The maximal yields of acceptor products (DP3-6) were 0.25 mol/l, 0.22 mol/l, 0.12 mol/l and 0.05 mol/l, respectively. The highest yield of overall acceptor products (0.59 mol/l) was achieved when maltose and sucrose concentrations were at 1 mol/l, catalyzed by dextransucrase (10 U/g sucrose). The lowest production of the overall acceptor products (0.05 mol/l) was obtained when maltose and sucrose were at their lowest concentrations (both were 0.15 mol/l), catalyzed by dextransucrase (1 U/g sucrose). Based on the modeling results, high maltose and sucrose concentrations and their interactions were the main factors resulting in the desired increase in acceptor products DP3, DP4 and overall products (Figure 11A, B and E). High concentration of sucrose had positive effects on the production of acceptor products DP5 and DP6 (Figure 11C and D). The effects of maltose and sucrose on consumed percentage of maltose were different: the utilization of maltose was improved by high sucrose concentration, however decreased by increasing maltose concentration (Figure 11F). Other modelled linear (enz) and quadratic effects (mal\*mal, suc\*suc and enz\*enz), as well as their interactions (mal\*enz, and suc\*enz), seemed had little influences on the six responses.

**Table 8.** Results of central composite design for maltose acceptor reaction

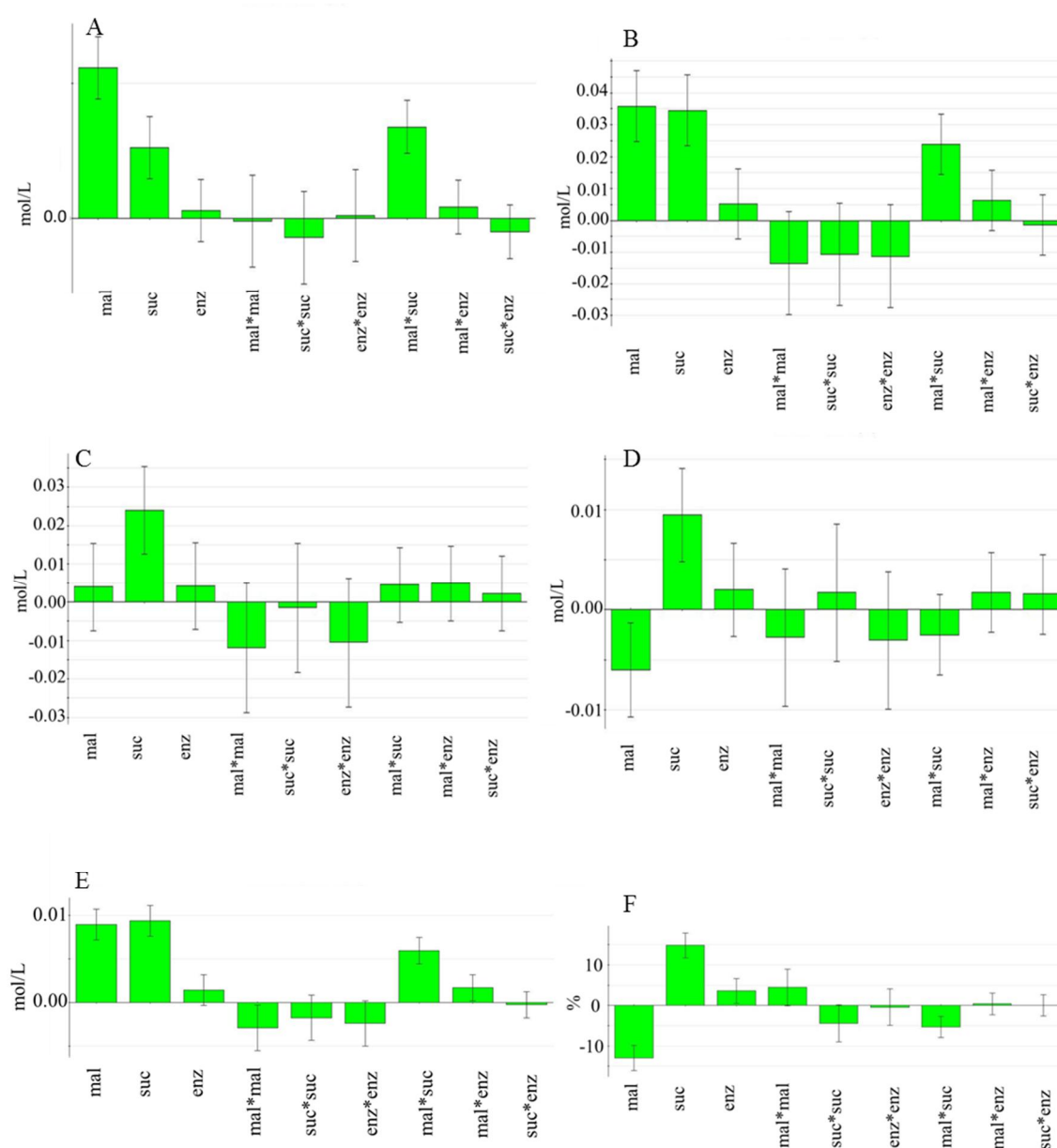
Before incubation <sup>a</sup>				After incubation <sup>b</sup>							
Run	Sucrose conc. (mol/l)	Maltose conc. (mol/l)	Dextranucrase conc. (U/g sucrose)	Fructose (mol/l)	Sucrose (mol/l)	Maltose (mol/l)	DP3 (mol/l)	DP4 (mol/l)	DP5 (mol/l)	DP6 (mol/l)	Overall DP (mol/l)
1	0.15	0.15	1	0.09	0.04	0.09	0.02	0.02	0.01	0.00	0.05
2	0.15	1	1	0.03	0.14	0.79	0.04	0.00	0.00	0.00	0.04
3	1	0.15	1	0.51	0.38	0.02	0.00	0.03	0.05	0.03	0.12
4	1	1	1	0.61	0.36	0.62	0.25	0.17	0.04	0.00	0.46
5	0.15	0.15	10	0.16	0.00	0.09	0.03	0.04	0.02	0.00	0.09
6	0.15	1	10	0.13	0.00	0.71	0.10	0.03	0.00	0.00	0.13
7	1	0.15	10	1.06	0.00	0.01	0.00	0.02	0.03	0.03	0.08
8	1	1	10	1.05	0.00	0.50	0.25	0.22	0.10	0.02	0.59
9	0.575	0.15	5.5	0.50	0.00	0.03	0.01	0.03	0.04	0.03	0.11
10	0.575	1	5.5	0.55	0.00	0.59	0.19	0.16	0.05	0.00	0.39
11	0.15	0.575	5.5	0.15	0.00	0.44	0.08	0.05	0.01	0.00	0.13
12	1	0.575	5.5	1.03	0.00	0.20	0.09	0.16	0.12	0.05	0.42
13	0.575	0.575	1	0.42	0.17	0.34	0.10	0.13	0.05	0.01	0.28
14	0.575	0.575	10	0.39	0.00	0.23	0.09	0.10	0.06	0.01	0.25
15	0.575	0.575	5.5	0.48	0.00	0.29	0.10	0.13	0.07	0.02	0.31
16	0.575	0.575	5.5	0.54	0.00	0.31	0.10	0.13	0.07	0.02	0.32
17	0.575	0.575	5.5	0.50	0.00	0.29	0.10	0.13	0.07	0.02	0.31
18	0.575	0.575	5.5	0.56	0.00	0.29	0.10	0.14	0.07	0.02	0.32

<sup>a</sup>One sample was prepared under each experimental condition, and Run 15-18 are the four replicates. <sup>b</sup>The concentrations were analyzed twice and their average values are presented in the table.



**Table 9.** Coefficients of the quadratic model for the six responses

Responses	Goodness of fit ( $R^2$ ) and predictive power ( $Q^2$ )
DP3	$R^2 = 1$ , $Q^2 = 0.8$
DP4	$R^2 = 1$ , $Q^2 = 0.7$
DP5	$R^2 = 0.8$ , $Q^2 = 0.5$
DP6	$R^2 = 0.8$ , $Q^2 = 0.5$
Overall DP	$R^2 = 1$ , $Q^2 = 0.9$
Consumed percentage of maltose	$R^2 = 1$ , $Q^2 = 0.8$



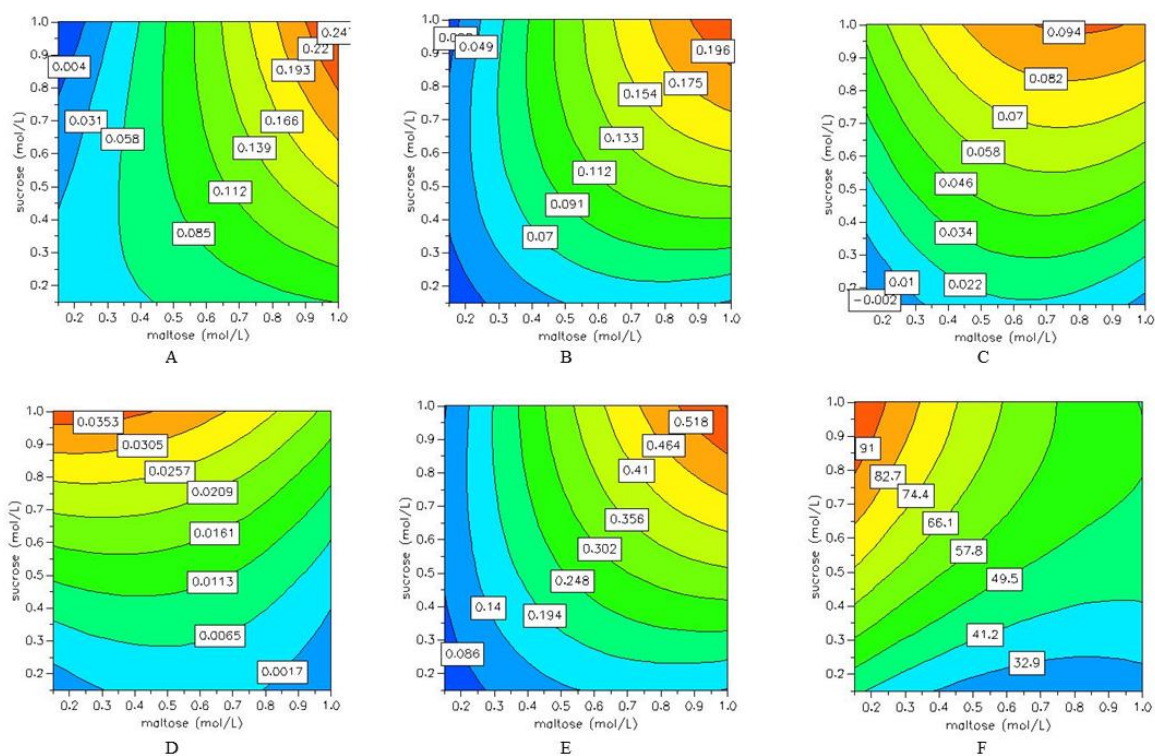
**Figure 11.** Regression coefficient plots of the optimization design for DP3 (A), DP4 (B), DP5 (C), DP6 (D) products, overall DP products I and of consumed percentage of maltose (F). Mal: maltose linear effect; suc: sucrose linear effect; enz: dextransucrase linear effect; mal\*mal: maltose quadratic effect; suc\*suc: sucrose quadratic effect; mal\*suc: maltose and sucrose interaction effect; mal\*enz: maltose and dextransucrase interaction effect; suc\*enz: sucrose and dextransucrase interaction effect.

The model contour plots (Figure 10-15 in Appendix) present the predictive results of the six responses: acceptor product DP3, DP4, DP5, DP6, overall acceptor products and the percentage of consumed maltose. Each figure consists of a triple-contour plot, which displays the predicted responses from sucrose (0.0 ~ 1.0 mol/l) and maltose (0.0 ~ 1.0 mol/l) under the function of three dosages of dextransucrase (1, 5.5 and 10 U/g sucrose). Since the six responses were independent of dextransucrase dosage used in the optimization design, one contour plot from the six figures, in which the dosage of dextransucrase was 10 U/g sucrose, was extracted to make up of Figure 12 (A-F). The products DP3 and DP4 were the predominant oligosaccharides, thus the effects on them were the same for the overall products.

Production of acceptor products DP3-6 and overall products, as well as the percentage of consumed maltose, were highly dependent on sucrose concentrations. All six responses were improved simply by increasing the concentration of sucrose. Therefore in order to maximize the production and maltose utilization, sucrose should be kept highest within the experimental regions.

Maltose had different effects on the responses. Production of acceptor products DP3, DP4 and overall products, as well as the percentage of consumed maltose, were highly dependent on maltose concentrations. Maltose had little effects on the production of DP5 product, but had negative effects on that of DP6 product. Figure 12F indicates that high maltose utilization was reached by decreasing maltose concentration, whereas highest production of acceptor products DP3 and DP4, and overall products were obtained when maltose concentration was at its maximum.

In summary, maximal yields of DP3 (0.25 mol/l), DP4 (0.22 mol/l) and overall acceptor products (0.59 mol/l) were attained when maltose and sucrose were both 1 mol/l (see Figure 12 A, B and E). Higher concentrations of sucrose and moderate concentrations of maltose were preferred to achieve higher production of DP5 and DP6 products (Figure 12 C and D). As far as oligosaccharides with higher DP are concerned, lowering the concentration of maltose seemed to increase their production. Thus in order to produce more isomalto-oligosaccharides with higher DP and to increase the maltose utilization, high sucrose but low maltose reaction condition was preferred.



**Figure 12.** Predictive responses (A-F) with dextran sucrose (10 U/g sucrose), expressed in terms of sucrose (0.0 ~ 1.0 mol/l) and maltose (0.0 ~ 1.0 mol/l). A, acceptor product DP3 (mol/l); B, acceptor product DP4 (mol/l); C, acceptor product DP 5 (mol/l); D, acceptor product DP6 (mol/l); E, overall acceptor products (mol/l); F, consumed percentage of maltose (%)

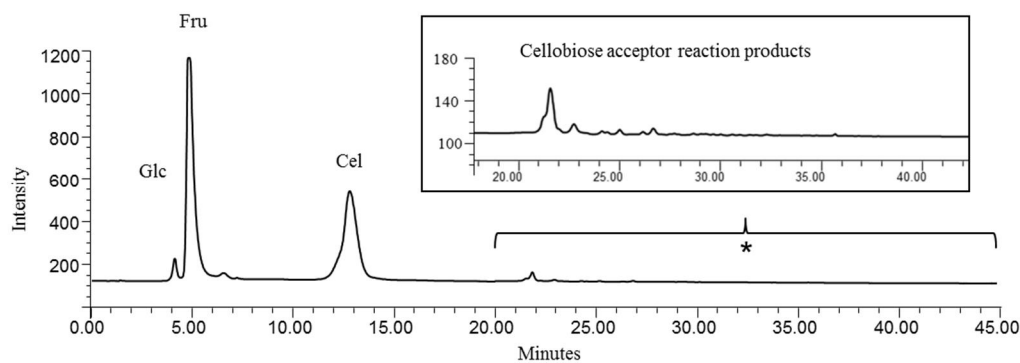
### 3.4.4 Other acceptor reactions

After maltose acceptor reaction, the possibilities of other acceptors to produce oligosaccharides were evaluated. The used conditions had some differences, due to solubility limitations of some studied oligosaccharides, like lactose, or limited availability of the less common oligosaccharides, like isopanose. But judging from their corresponding chromatograms, the effectiveness of the acceptors were as follows in a decreasing order: isomaltose, maltotriose, nigerose, lactose and cellobiose. Other tested acceptors, arabinoxylobiose, isopanose, mannoibiose, melibiose and laminaribiose had relatively low effectiveness. A large amount of fructose was released in all reactions, thus it is difficult to interpret from the chromatograms whether sucrose was totally consumed or not. However, in maltose acceptor reaction, when the dosage of dextran sucrose was 10 U/g sucrose, sucrose was totally consumed. Therefore it is reasonable to assume that in other acceptor reactions all the sucrose was utilized, either to dextran or oligosaccharides, depending on the effectiveness of the tested acceptors.

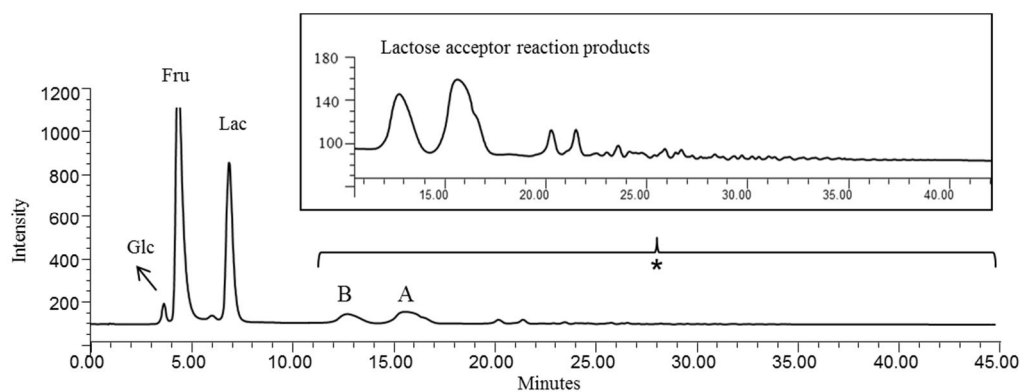
The chromatograms of cellobiose and lactose acceptor reactions are given in Figure 13 and Figure 14, whereas others are presented in Appendix Figure 2-9. The acceptor products of isomaltose and maltotriose have been earlier thoroughly studied. Nigerose, a product of the caramelization of glucose, is not as commercially available as cellobiose or lactose, thus not suitable to produce potential prebiotics. Therefore, cellobiose and lactose acceptor reactions were selected for further studies.

During cellobiose and lactose acceptor reactions, a large amount of dextrans were observed and much fewer oligosaccharides were produced compared to maltose acceptor reaction. It is clear that sucrose was totally consumed judging from Figure 13 and Figure 14. In addition, if some sucrose remained unconsumed, its retention time was unfortunately the same as that of lactose, thus could not be identified in the chromatogram. Although acceptors were not all converted into acceptor products, a series of oligosaccharides were produced by both reactions. Cellobiose acceptor products began to be eluted at around 21 min and the reaction seemed to produce one major acceptor product (Figure 13). This is quite different for lactose acceptor reaction. Lactose acceptor products began to be eluted at 12 min and two predominant oligosaccharides (B and A in Figure 14) seemed to be produced, being eluted at 13 min and 16 min, respectively. Lactose has solubility advantage over cellobiose, therefore a series of lactose acceptor reactions were also carried out. Their corresponding chromatograms are in Appendix Figure 1. The concentration of lactose was the same in the five experiments, while the concentrations of sucrose varied. Maltose optimization design showed the enzyme dosage was not a significant influential factor regarding producing oligosaccharides. Therefore the differences revealed from the chromatograms were mainly from sucrose. Clearly higher sucrose to lactose ratios increased the production of oligosaccharides with higher DP, which is the same as maltose acceptor reaction.

The main cellobiose and lactose acceptor products were separated by gel filtration using a P2 column. After separation, the purity was confirmed by HPAEC-PAD. The three fractions with the most purity were analyzed by MS/MS to gain structural information.



**Figure 13.** HPAEC-PAD profile of cellobiose acceptor products in the presence of sucrose (0.2 mol/l) and cellobiose (0.05 mol/l) catalyzed by dextransucrase (10 U/g sucrose). Labeled peaks are as follows: glucose (Glc), fructose (Fru) and cellobiose (Cel). \*The inset shows a zoom area of the eluted cellobiose acceptor products



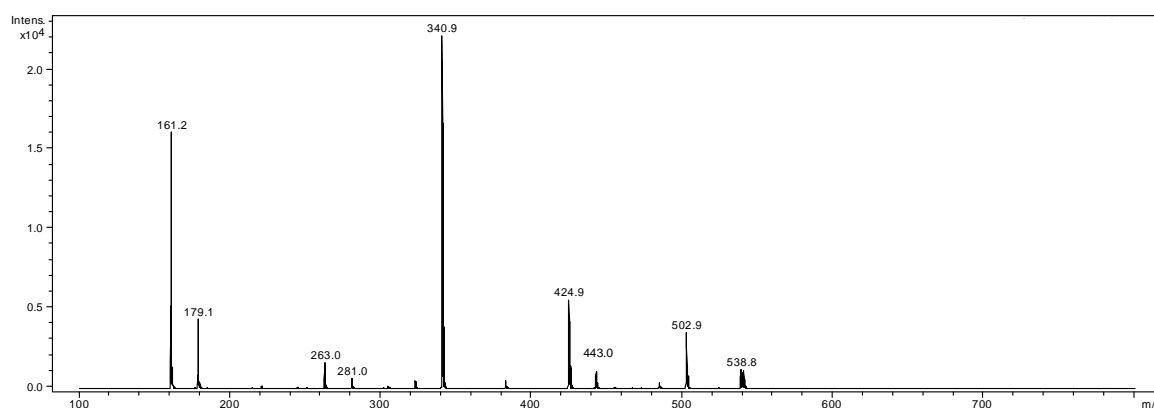
**Figure 14.** HPAEC-PAD profile of lactose acceptor products in the presence of sucrose (1 mol/l) and lactose (0.15 mol/l) catalyzed by dextransucrase (10 U/g sucrose). Labeled peaks are as follows: glucose (Glc), fructose (Fru) and lactose (Lac). \*The inset shows a zoom area of the eluted lactose acceptor products

### 3.4.5 Primary acceptor products characterization using MS/MS

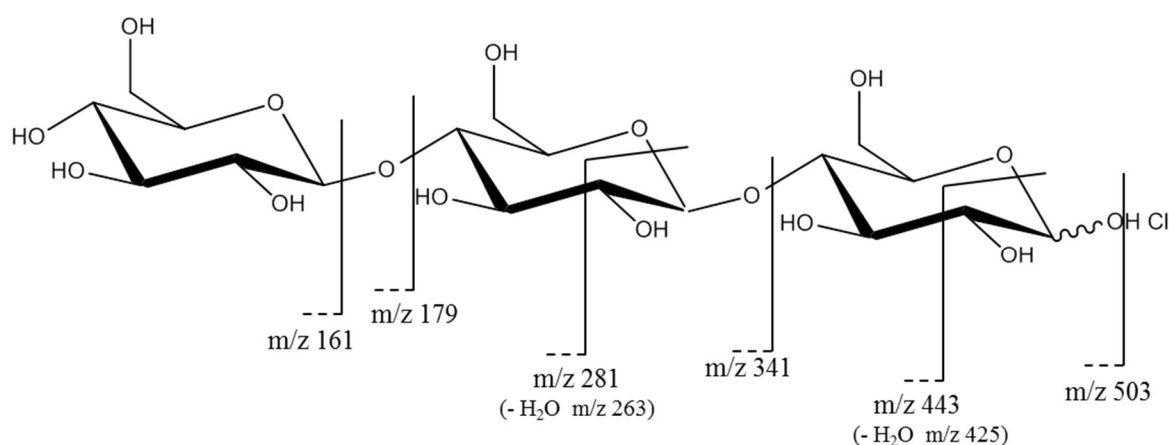
#### Model trisaccharide

Cellotriose [ $\beta$ -D-Glc-(1 $\rightarrow$ 4)-  $\beta$ -D-Glc-(1 $\rightarrow$ 4)-D-Glc] was selected as a model trisaccharide to analyze the linkages in produced oligosaccharides. The negative mode MS<sup>2</sup> spectrum of chloride adduct ion [M+Cl]<sup>-</sup> of cellotriose is in Figure 15. The molecular mass of chloride adduct ion was 539. The  $m/z$  503 was the deprotonated ion [M-H]<sup>-</sup> when chloride ion was lost as HCl. There were two pathways of forming fragments: glycosidic cleavage and cross-ring cleavage. The structure of cellotriose and the formation of fragments are presented in Figure 16.

The fragmentation started from the reducing end under negative MS mode. The fragment ion  $m/z$  341 with the loss of 162 Da from  $m/z$  503 was the most abundant ion in this spectrum, followed by  $m/z$  161. Fragment ion  $m/z$  341 was formed due to glucosyl bond cleavage by losing a glucosyl unit from  $[M-H]^-$ , the same for  $m/z$  179. The  $m/z$  161 also formed during glycosidic cleavage. The cross-ring cleavage formed fragment ions  $m/z$  263 and  $m/z$  425. Fragment ions at  $m/z$  443 (loss of 60 Da) and  $m/z$  425 (loss of 78 Da) indicate a (1 $\rightarrow$ 4) glucosidic linkage at the reducing end of cellotriose. Additionally, fragment ions at  $m/z$  281 (loss of 60 Da from  $m/z$  341) and  $m/z$  263 (loss of 78 Da from  $m/z$  341) imply the other (1 $\rightarrow$ 4) glucosidic linkage in cellotriose. The configuration of the linkage type was determined by relative peak intensities. The ratios, ( $m/z$  263) : ( $m/z$  281), and ( $m/z$  425) : ( $m/z$  443), being larger than 1, indicate that cellotriose is linked by  $\beta$ -glucosyl linkage (Guan and Cole 2008). The diagnostic fragment ions and neutral losses are summarized in Table 10.



**Figure 15.** MS<sup>2</sup> spectrum of chloride adduct ion  $[M+Cl]^-$  of cellotriose ( $m/z$  539)



**Figure 16.** Structure of chloride adduct ion  $[M+Cl]^-$  of cellotriose ( $m/z$  539) and fragments formation during MS/MS

**Table 10.** Structure diagnostic neutral losses and fragments peaks (in parentheses) of chloride adducts of glucopyranosyl glucoses as well as other hexose: hexose disaccharides. Mass differences resulting from neutral losses are calculated from deprotonated saccharides. (Adapted from Guan and Cole 2008)

Link	Presence	Absence	161:179 ratio				
1-6	- 162 (179)	- 120 (221)	- 90 (251)	- 60 (281)	- 78 (263)	< 1	
1-4	- 180 (161)	- 162 (179)		- 78 (263)	- 60 (281)	- 90 (251)	> 1
1-3	- 180 (161)	- 162 (179)	- 120 (221)				> 1
1-2		- 162 (179)	- 120 (221)	- 78 (263)		- 60 (281)	< 1
1-1	- 162 (179)						< 1

### Cellobiose acceptor products

MS/MS was carried out to the purified cellobiose product under the negative mode. The MS and MS<sup>2</sup> spectra of primary cellobiose acceptor product are presented in Figure 17 and Figure 18, respectively. The product was a trisaccharide, proved by the presence of  $m/z$  539 (chloride adduct ion  $[M+Cl]^-$ ). The acceptor product fragmented easily and several fragments were already acquired at MS stage. Chloride adduct ion  $[M+Cl]^-$  of cellobiose acceptor product was further fragmented by MS/MS. MS<sup>2</sup> spectrum revealed that  $m/z$  425 was the most abundant fragment, followed by fragments  $m/z$  503,  $m/z$  341,  $m/z$  179, and  $m/z$  263 with their abundance in a decreasing order (see Figure 18). Fragment  $m/z$  503 was the deprotonated ion  $[M-H]^-$  when chloride ion was lost as HCl.

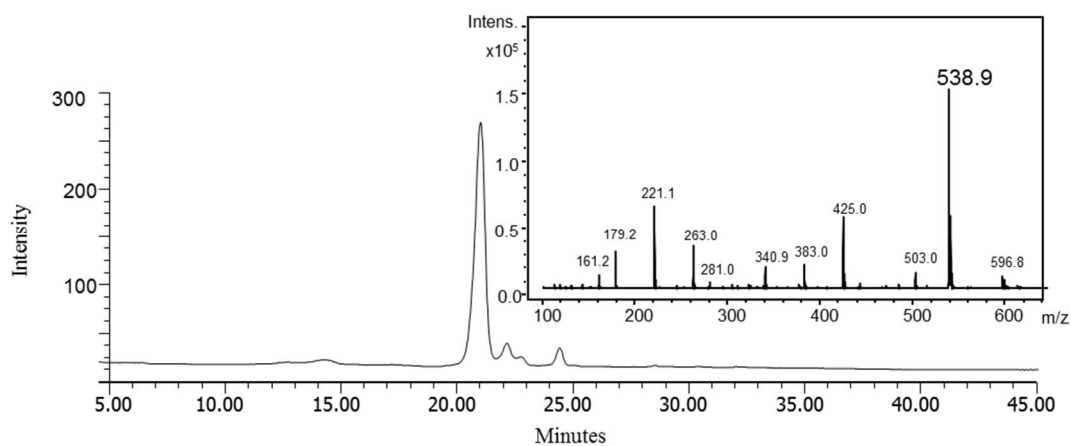
The MS<sup>2</sup> spectrum of the trisaccharide was different from cellotriose. Judging from the neutral losses, it is clear that the two glycosidic linkages in the trisaccharide were different. The major neutral losses for the trisaccharide were 162 Da and 78 Da, corresponding to the abundant fragment ions  $m/z$  341 and  $m/z$  425. In combination with the possible mechanism of dextranase, an assumption is made: the glucosyl unit was linked to cellobiose by (1→2) glycosidic linkage in the synthesized trisaccharide, namely 2<sup>1</sup>- $\alpha$ -D-Glc-cellobiose. Based on this assumption, the possible structure of the glucosylated cellobiose and fragmentation patterns are given in Figure 19.

In 2<sup>1</sup>-  $\alpha$ -D-Glc-cellobiose, the middle glucosyl unit, with the exposure of its reducing end, started to fragment under negative MS mode. This leads to the loss of 60 Da ( $m/z$  443), followed by the loss of one H<sub>2</sub>O molecular (18 Da) to a more stable structure, forming the most abundant fragment ion  $m/z$  425. Also, another cross-ring fragmentation might happen, forming ion  $m/z$  263. This cross-ring fragmentation was the same in cellotriose. A neutral glucosyl unit (the loss of 162 Da) was removed directly from the deprotonated ion [M-H]<sup>-</sup>, forming fragment ion  $m/z$  341. This could happen on both terminal (end) glucosyl units. Besides, one glucosyl unit could be separated from chloride 2-glucopyranosyl-cellobiose and charged by breaking down (1→2) or (1→4) glucosidic linkage, forming fragment ion  $m/z$  179.

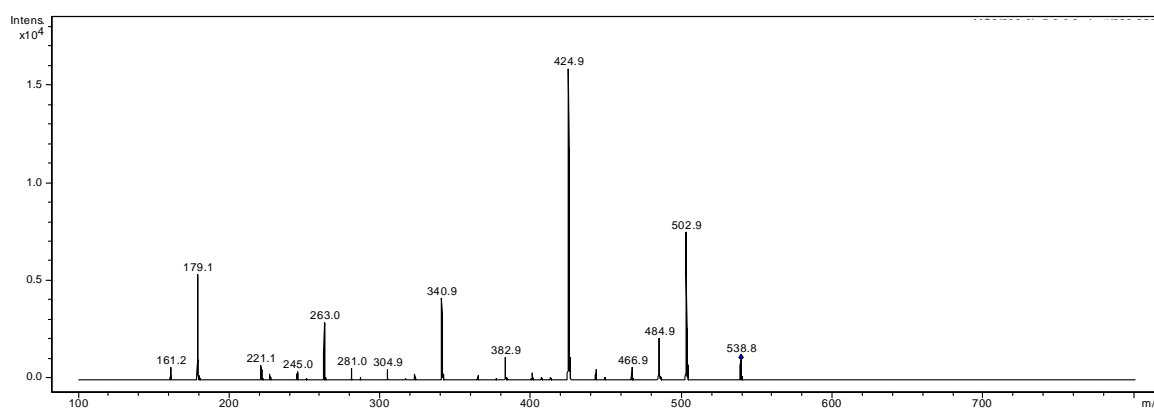
However, the speculated structure of the synthesized cellobiose product could not explain the presence of  $m/z$  383 and  $m/z$  221 in its MS<sup>2</sup> spectrum, corresponding to the neutral loss of 120 Da. It is impossible to have a neutral loss of 120 Da in such a branched structure. The presence of  $m/z$  383 and  $m/z$  221 was relatively low, so it is possible that the isolated trisaccharide was contaminated with some impurities, and synthesized 2-glucopyranosyl-cellobiose was not the only trisaccharide present in the sample. HPAEC-PAD chromatogram indeed shows some minor peaks in addition to the main one (Figure 17).

The other possibility of the trisaccharide product is 6<sup>2</sup>-  $\alpha$ -D-Glc-cellobiose (Ruiz-Matute et al. 2010). In this trisaccharide, the glucosyl unit from sucrose was transferred to the non-reducing glucosyl residue of cellobiose, forming a linear structure. The structure better explains the presence of fragment ions  $m/z$  383 and  $m/z$  221. However, it is impossible to have the neutral loss of 78 Da, which is corresponding to the most abundant fragment ion  $m/z$  425. In order to accurately determine the structures of the synthesized glucopyranosyl-cellobioses, NMR spectroscopic analysis is needed.

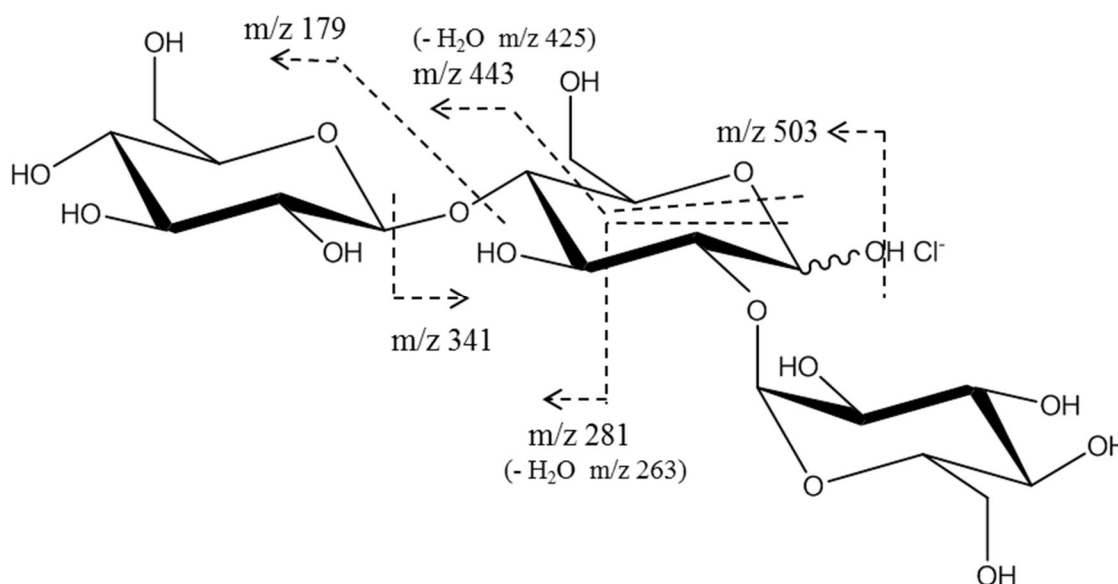




**Figure 17.** HPAEC-PAD chromatogram of isolated cellobiose acceptor product (retention time 21 min). Mass spectrum ( $[M+Cl]^-$ ) of this fraction is shown as an inset. Note, analysis at different times explains the slight difference in retention time of Figure 13



**Figure 18.** MS<sup>2</sup> spectrum of chloride adduct ion  $[M+Cl]^-$  of glucopyranosyl-cellobiose ( $m/z$  539) synthesized by cellobiose acceptor reaction

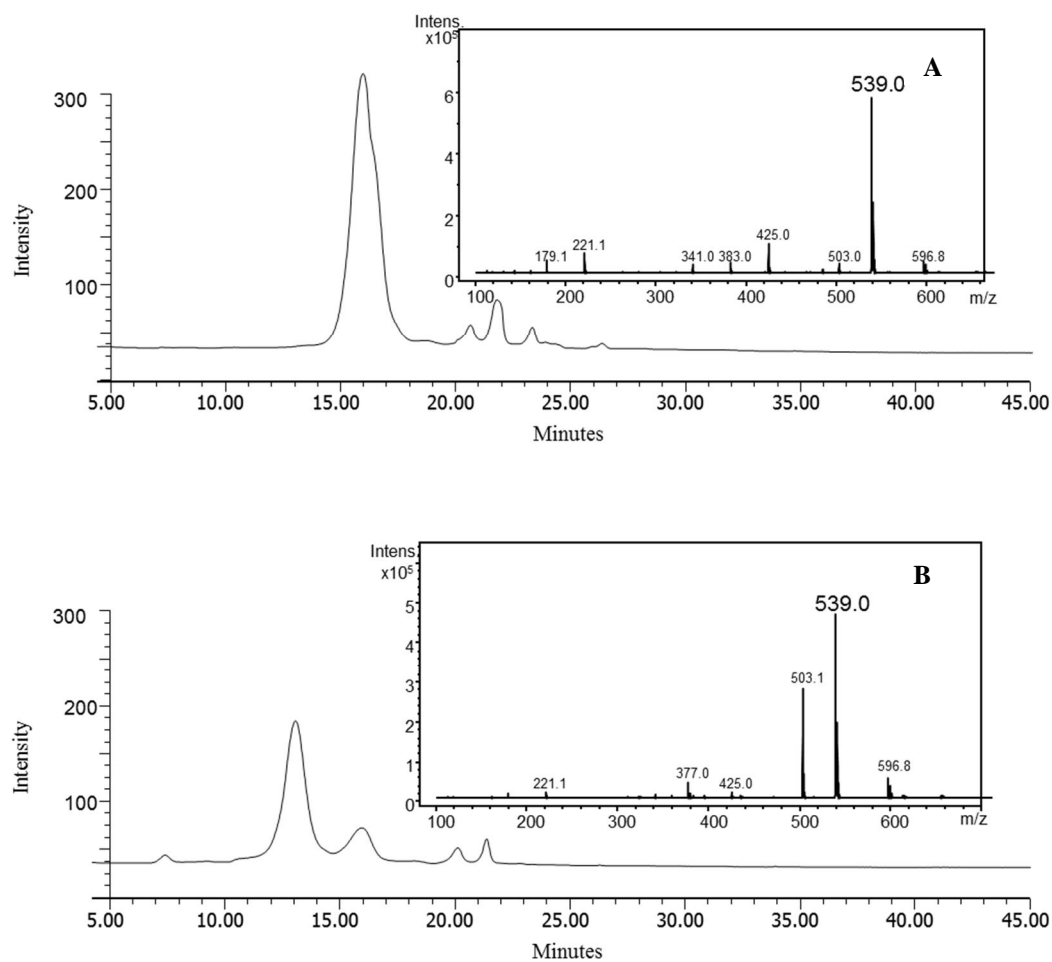


**Figure 19.** Possible fragmentation patterns of chloride 2'- $\alpha$ -D-Glc-cellobiose

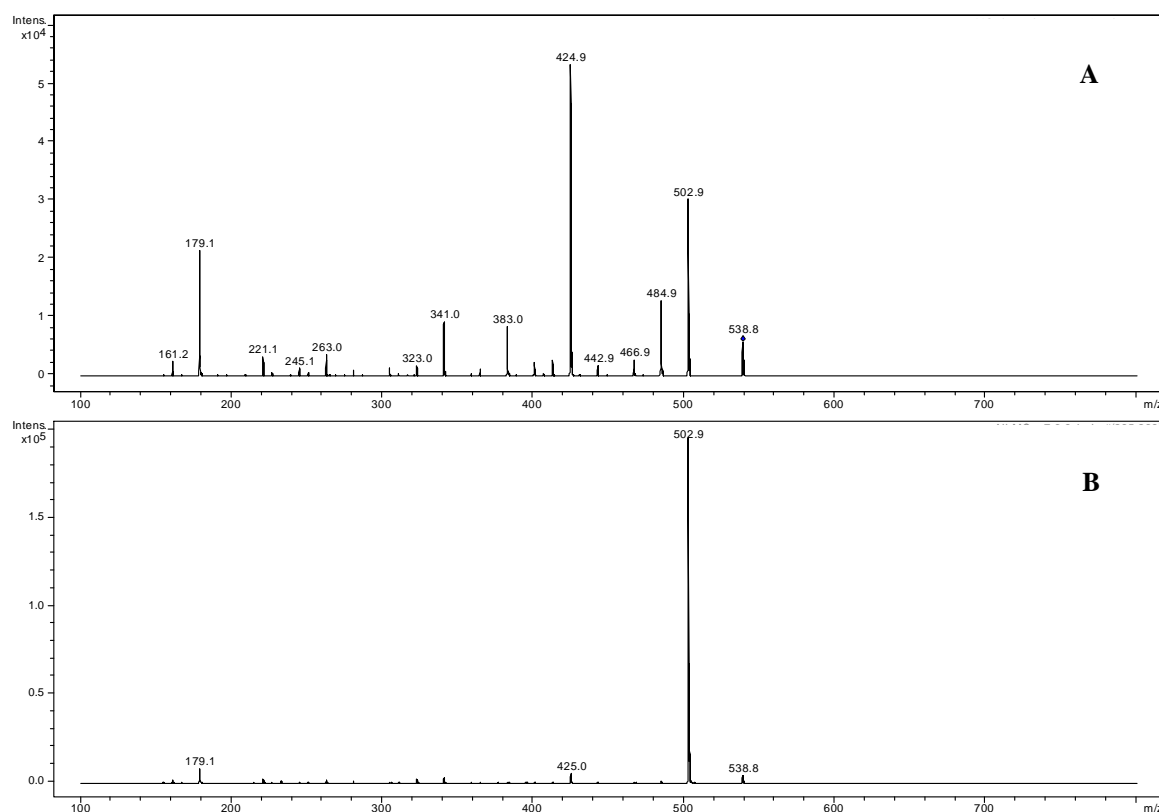
### Lactose acceptor products

Two major products (A and B) were isolated from lactose acceptor products mixture. MS/MS analysis was carried out to primary lactose acceptor products under the negative mode. Their MS and MS<sup>2</sup> spectra are presented in Figure 20 (A and B) and Figure 21 (A and B), respectively. Lactose acceptor products fragmented less compared to cellobiose product. The two major products were also confirmed to be trisaccharides. The  $m/z$  539 was chloride adduct ion  $[M+Cl]^-$  and  $m/z$  503 was the deprotonated ion  $[M-H]^-$  when chloride ion was lost as HCl. The process from  $[M+Cl]^-$  to  $[M-H]^-$  of lactose product B happened easily, thus accumulating a significant amount of  $[M-H]^-$  (Figure 20B).

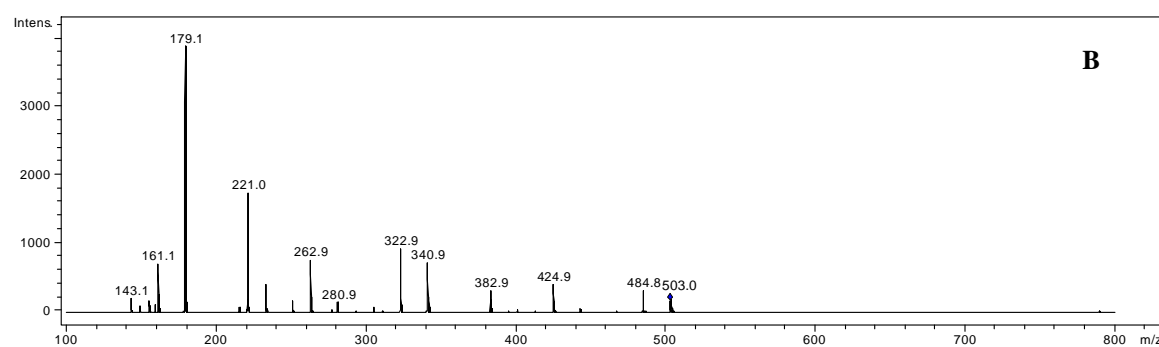
At MS<sup>2</sup> stage (Figure 21), lots of fragmented ions were acquired for lactose product A. The fragment ion  $m/z$  503 was the most abundant ion, followed by  $m/z$  425,  $m/z$  323,  $m/z$  538 and  $m/z$  179. However, lactose product B could not be further fragmented at this stage, only resulting in the accumulation of  $[M-H]^-$ . Therefore, one more MS stage was added to  $[M-H]^-$  of lactose acceptor product B, and its MS<sup>3</sup> spectrum is shown in Figure 22. Lactose acceptor product A and B had different profiles of fragment ions, thus had different glycosidic linkages. Lactose lactose acceptor product A and the cellobiose product had similar MS spectra, implying their similar structures. The possible structure for lactose product A (2<sup>1</sup>- $\alpha$ -D-Glc-lactose) and fragmentation patterns are the same as cellobiose product (see Appendix Figure 16). MS<sup>3</sup> spectrum of lactose product B is different from those of cellotriose and cellobiose acceptor product (see Figure 22), so it is reasonable to assume that the structure of lactose product B differed from the structures mentioned above. Also, the intensity of fragment ions (lower than 4000) is too low to be taken into consideration. The separation efficiency of the trisaccharide was not satisfactory (Figure 20B). Therefore there was no enough information to identify the structure of the lactose product B in the study.



**Figure 20.** HPAEC-PAD chromatograms of isolated cellobiose acceptor product A (retention time 16 min) and B (retention time 13 min). Mass spectra ( $[M+Cl]^-$ ) of the two fractions are shown as insets. Note, analysis at different times explains the slight difference in retention time of Figure 14



**Figure 21.** MS<sup>2</sup> spectra of chloride adduct ion [M+Cl]<sup>-</sup> of glucopyranosyl-lactose A (*m/z* 539) and B (*m/z* 539) synthesized by lactose acceptor reaction



**Figure 22.** MS<sup>3</sup> spectrum of deprotonated ion [M-H]<sup>-</sup> of glucopyranosyl-lactose B (*m/z* 503) synthesized by lactose acceptor reaction

## 3.5 Discussion

### 3.5.1 Factors affecting maltose acceptor reaction

During maltose acceptor reaction, synthesis of dextran and oligosaccharides both happened. But the production of oligosaccharides gained significant priorities over dextrans. The results are in agreement with Rabelo et al. (2007). Dextrans synthesis is inhibited in the presence of acceptors, and the degree of inhibition is affected by the efficiencies of the acceptors (Rodrigues et al. 2005). If the efficiency of the acceptor is high, most of sucrose

would be consumed to produce acceptor products, and dextrans synthesis reaction is inhibited to a large extent, and vice-versa. For example, when the initial maltose concentration is sufficiently high, dextrans synthesis is almost completely eliminated (Heincke et al. 1999). This explains that little dextrans were produced in the preliminary and optimization design of maltose acceptor reaction. It also explains the phenomenon observed in this study that a large amount of dextrans were precipitated in cellobiose and lactose acceptor reactions although little sucrose remained unconsumed.

A few earlier publications studied the effects of sucrose to maltose ratios on the production of isomalto-oligosaccharides (Lee et al. 1997; Iliev et al. 2007). It has been proved that the ratios of sucrose to maltose affect the oligosaccharide profiles in maltose acceptor reaction. This partially reveals that the interactions between sucrose and maltose influence the produced oligosaccharides. As indicated in the preliminary design, the chain length of isomalto-oligosaccharides decreased when sucrose to maltose ratio decreased from 2:1 to 1:2. Lee et al. (1997) also proved that synthesis of longer oligosaccharides was preferred by increasing sucrose to maltose ratio. Also, Iliev et al (2007) discovered that when sucrose to maltose ratios were increased from 2 to 7, the quantity of synthesized oligosaccharides with a higher DP also increased.

Some studies have evaluated factors affecting maltose acceptor reactions, and most of them are concentrating on the production of panose and total oligosaccharides. Also the dextransucrases applied are mainly from *Leuconostoc mesenteroides*. This study is the first time to use *Weissella confusa* dextransucrase acceptor reaction, investigating the effects of sucrose and maltose on the production of acceptor products DP3, DP4, DP5 and DP6 separately by an optimization design. The optimization design results revealed that the most significant variables influencing the formation of acceptor products DP3 (panose) were maltose and sucrose concentrations, as well as their interactions. Its production was increased by increasing both sucrose and maltose concentrations. The behavior of panose formation is the same in Rabelo et al. (2005). Also, Rodrigues et al.' (2006) studied the effects of linear (mal and suc), quadratic (mal\*mal and suc\*suc) and interactions (mal\*suc, mal\*enz) on panose, and have proved that maltose and sucrose concentrations are the most significant variables in terms of panose production. Fernandes and Rodrigues (2006) optimized panose productivity using the maltose to sucrose ratio as a parameter. The results have revealed that the productivity of panose varies as maltose to sucrose ratios change. As far as the production of total oligosaccharides is concerned, the effects on their

production are the same as panose (Rodrigues et al. 2006). In order to attain maximal isomalto-oligosaccharides production, the highest concentrations of sucrose and maltose are required. A study of fitted surface response for isomalto-oligosaccharides formation has also reached to the same conclusion (Rabelo et al. 2007). Other factors, such as quadratic effect of sucrose (suc\*suc) and maltose (mal\*mal), are not important (Rabelo et al. 2007).

The factors affecting the production of products DP4, DP5 and DP6 were also evaluated in the optimization design of maltose acceptor reaction. The effects on product DP4 were the same as panose. However, the regression coefficient plots (Figure 11) show that maltose had no influences on product DP5, and it had negative effects on product DP6. The amounts of oligosaccharides with DP >6 were too low to be quantified, so there was not enough experimental data to prove the effects of maltose on longer oligosaccharides. However, Iliev et al. (2007) proved that when the concentration of sucrose was seven times higher than maltose, produced oligosaccharide with DP7 accounted for 7% of the total oligosaccharides production. Combined with the results in this study, it is reasonable to assume that higher sucrose and lower maltose conditions favor the production of oligosaccharides with higher DP. There are limited studies focusing on the produced oligosaccharides separately, thus difficult to relate the results herein to earlier publications.

Temperature and dosage of dextransucrase affect maltose acceptor reaction more or less. The temperature has an effect on the activity of dextransucrase, thus affects the reaction. The dosage of dextransucrase applied in the experiment impacts the chances of the substrate contacting with enzyme's active site, which leads to the changes in the reaction rate. Pereira et al. (1998) and Seo et al. (2007) proved that temperature affected acceptor reaction. However, most of the acceptor reactions are done under the optimal working temperature of dextransucrase, which is about 30~35 °C (Shukla and Goyal 2011). The dosages of dextransucrase have also been assayed as one of the variables in optimizing panose production at levels of 250, 375, and 500 U/l, but the results did not reveal a significant influence (Rodrigues et al. 2006).

In addition, a few studies estimated the productivity of oligosaccharides in maltose acceptor reaction. The isomalto-oligosaccharides productivity of purified *Leuconostoc mesenteroides* dextransucrase in Heincke et al. (1999) was 35 mmol/L·h, whereas Kubik et al. (2004) obtained 7.26 mmol/L·h from an immobilized mixture of dextransucrase and

dextranase. Rabelo et al. (2009) obtained a productivity of 42.95 mmol/L·h. The synthesis conditions, as well as dextransucrases, used in the studies are different. But this partially clarifies the fact that enzymes from various origins have different activities.

### 3.5.2 Other acceptor reactions and primary acceptor products characterization

Among other evaluated acceptors, the structures of maltotriose and isomaltose products have been clearly identified (Robyt and Eklund 1983; Fu and Robyt 1990). Cellobiose and lactose acceptor products were selected to the following characterization. Cellobiose can be easily obtained by enzymatic or acidic hydrolysis of cellulose. Cellulose is the important structural component of green plant cell walls and is widely distributed in nature. Lactose is commonly present in dairy products and not tolerant by a specific group of people. Modification of lactose with glucosyl units by dextransucrase is capable of changing the digestibility of lactose, and it may endow the new compounds with health benefits (Díez-Municio et al. 2012). Therefore, the selected two disaccharides are potential substrates in synthesizing new prebiotic ingredients.

As mentioned earlier in the results, a large amount of dextrans were obtained in cellobiose and lactose acceptor reactions. The presence of dextrans is not of a food safety concern. Dextran produced by *W. confusa* dextransucrase has more than 97% of  $\alpha$ -(1→6) glucosidic linkages in the backbones and less than 3% of  $\alpha$ -(1→3) glucosidic branches (Maina et al. 2008). The  $\alpha$ -(1→6) linkages in the backbones of dextran increase its water solubility (Jeanes et al. 1954). Also, dextransucrase used in this study has been applied in sourdough fermentation to produce dextran, and proved to have some technological properties, such as improved shelf-life and volume (Katina et al. 2009). In addition, according to the opinion of the Scientific Committee, the dextran containing more than 95% of  $\alpha$ -(1→6) glucosidic linkages does not constitute a safety concern when added at a level of maximum 5% in bakery products (European Commission [EC] CS/NF/DOS/7/ADD 3 FINAL 2000).

The produced oligosaccharides are regarded as the only food safety concern of applying *W. confusa* dextransucrase to produce potential prebiotics by cellobiose and lactose acceptor reactions. Moreover, the prebiotic properties of oligosaccharides are structurally related (Sanz et al. 2005). Therefore, it is necessary to characterize cellobiose and lactose acceptor products.

### Cellobiose acceptor products

The speculated cellobiose acceptor product,  $2^1$ - $\alpha$ -D-Glc-cellobiose, is supported by earlier published results (Robyt 1995; Morales et al. 2001; Ruiz-Matute et al. 2010; Kang et al. 2013). In addition, another trisaccharide has also been produced during the cellobiose acceptor reaction, which is  $6^2$ - $\alpha$ -D-Glc-cellobiose. The two synthesized trisaccharides are present together in a few works (Morales et al. 2001; Ruiz-Matute et al. 2010; Kang et al. 2013).

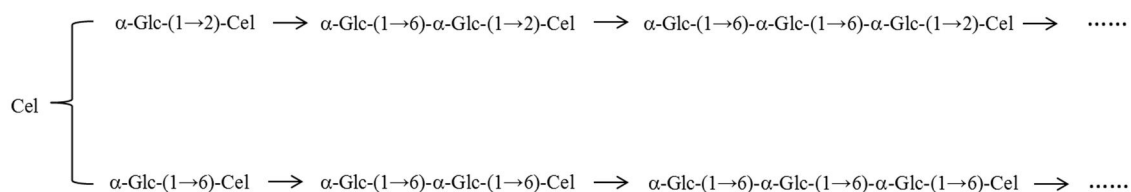
Actually during maltose acceptor reaction, panose is the only trisaccharide synthesized at the beginning (Robyt and Eklund 1983, Fu and Robyt 1990, Dols et al. 1997). However, as reaction continues, two oligosaccharides with DP 4 are formed:  $6^2$ - $\alpha$ -D-Glc-panose and  $2^1$ - $\alpha$ -D-Glc-panose (Dols et al. 1997). The synthesis of  $6^2$ - $\alpha$ -D-Glc-panose is preferred and its production is much higher than  $2^1$ - $\alpha$ -D-Glc-panose. Also, this DP4 product is an acceptor to give the third product, etc. to give a homologous series (Dols et al. 1997). The other DP4 product,  $2^1$ - $\alpha$ -D-Glc-panose, in which glucosyl unit is added to the reducing end of panose, only serves as a very poor acceptor to give a small amount of next homologous series (Dols et al. 1997). This mechanism is the same for maltotriose to maltooctose (DP3-8) acceptor reactions. The glucosyl unit from sucrose can be transferred either to non-reducing end or the reducing end residues, resulting in the formation of  $\alpha$ -(1 $\rightarrow$ 6) or  $\alpha$ -(1 $\rightarrow$ 2) linkage (Fu and Robyt 1990). In summary, during dextransucrase acceptor reactions, two predominant types of glucosidic bonds are formed:  $\alpha$ -(1 $\rightarrow$ 6) and  $\alpha$ -(1 $\rightarrow$ 2).

According to the earlier publications on cellobiose acceptor reaction, a series of cellobiose products have been identified. Among them are  $2^1$ - $\alpha$ -D-glucopyranosyl-cellobiose,  $2^1$ - $\alpha$ -D-isomaltosyl-cellobiose and  $2^1$ - $\alpha$ -D-isomaltotriosyl-cellobiose (Kang et al. 2003). Another series of oligosaccharides are also cellobiose acceptor products:  $6^2$ - $\alpha$ -D-glucopyranosyl-cellobiose,  $6^2$ - $\alpha$ -D-isomaltosyl-cellobiose and  $6^2$ - $\alpha$ -D-isomaltotriosyl-cellobiose (Ruiz-Matute et al. 2010).

Therefore, it is possible that at the beginning of cellobiose acceptor reaction two trisaccharides are formed. But their effectiveness is weak and their amounts are too low to go through further analysis. A possible mechanism in synthesizing cellobiose acceptor



products has been proposed herein. Two trisaccharides are firstly produced: 2<sup>1</sup>- $\alpha$ -D-Glc-cellobiose and 6<sup>2</sup>- $\alpha$ -D-Glc-cellobiose. Then another glucosyl unit could continuously be transferred to the trisaccharides via  $\alpha$ -(1 $\rightarrow$ 6) glycosidic linkage. Finally a series of isomalto-cellobiose oligosaccharides are produced. This process can be exemplified in Figure 24. However, in order to prove the accuracy of this assumption, more data should be provided, which should not only include the structural information of acceptor products, but also the quantitative information of each acceptor product.



**Figure 24.** Proposed cellobiose acceptor products synthesized by dextransucrase. Cel, cellobiose; Glc glucose.

In addition, the structural differences in the acceptor products also depend on the origin of dextransucrase. For example, maltose acceptor products synthesized by *L mesenteroides* NRRL B-512F dextransucrase are all  $\alpha$ -(1 $\rightarrow$ 6) linked (Robyt and Walseth 1978, Paul et al. 1986). But when *L mesenteroides* NRRL B-1299 dextransucrase is used, the produced oligosaccharides also contain  $\alpha$ -(1 $\rightarrow$ 2), linking to the reducing residues (Dols et al. 1997, Monsan and Paul 1995). Most importantly,  $\alpha$ -(1 $\rightarrow$ 2) linkages are resistant to digestive enzymes, and are beneficial to the bacteria in large intestines (Remaud-Simeon et al. 2000). Hence the oligosaccharides with  $\alpha$ -(1 $\rightarrow$ 2) linkages can be used as prebiotics and should be without food safety concerns.

### Lactose acceptor product

The proposed structure of lactose product A, 2<sup>1</sup>- $\alpha$ -D-Glc-lactose, has also been reported in previous publications (Robyt 1995; Díez-Municio et al. 2012; Kang et al. 2013; Moreno et al. 2014). But only in Díez-Municio et al. (2012), its structure has been clearly identified by NMR spectroscopic characterization. Also, 2<sup>1</sup>- $\alpha$ -D-Glc-lactose is capable of serving as another acceptor to produce 2<sup>1</sup>- $\alpha$ -isomaltosyl-lactose (Kang et al. 2013). Two publications reported one unknown lactose product (DP3) (Díez-Municio et al. 2012; Kang et al. 2003). This study also found an unknown trisaccharide. Thus NMR spectroscopy is needed for fully structural analysis.

#### 4. CONCLUSION

Acceptor reactions are alternative methods to produce prebiotic oligosaccharides. In the thesis, acceptor reactions catalyzed by the *Weissella confusa* VTT E-90392 dextranucrase were investigated. The study focused on the factors affecting maltose acceptor products and structural analysis of cellobiose and lactose acceptor products. A preliminary and optimization design (central composite model) were used for maltose acceptor reaction to evaluate the influential behaviors of sucrose (donor) and maltose (acceptor) concentrations, as well as dextranucrase dosages on the production of oligosaccharides (DP3-6) and on the utilization of maltose. Some common disaccharides and trisaccharides were evaluated for their potential as acceptors to produce novel prebiotic oligosaccharides. Primary cellobiose and lactose acceptor products were isolated and preliminarily characterized. In this study, synthesis and characterization of cellobiose and lactose acceptor products of *W. confusa* dextranucrase are studied for the first time.

The preliminary design for maltose acceptor reaction provided experimental regions for the optimization design, during which the oligosaccharides synthesis was preferred and dextran synthesis was suppressed. The effectiveness of maltose was high for *Weissella confusa* dextranucrase, and a large amount of isomalto-oligosaccharides was produced. The preliminary design revealed that the ratios of sucrose to maltose affected the oligosaccharides profiles. For example, when sucrose to maltose ratio was increased from 1:2 to 2:1, the quantity of synthesized oligosaccharides with a higher DP also increased. The optimization design deeper studied the effects of the concentrations of sucrose and maltose, and the dosages of dextranucrase. The results clearly revealed that sucrose, maltose and their interactions had positive effects on the production of products DP3, DP4 and overall oligosaccharides. The effects of maltose started to change in producing product DP5 and maltose negatively affected the production of product DP6. This is the first study to analyze the effects on acceptor products (DP3-6) separately. Moreover, higher sucrose and lower maltose favored the production of oligosaccharides with higher DP. High utilization of maltose was reached simply by reducing maltose concentration.

In the study, one cellobiose acceptor product (DP3) and two lactose acceptor products (DP3) were separated by gel filtration. MS/MS in the negative mode was used to explore the possible structures of these trisaccharides. Judging from their MS<sup>2</sup> spectra and the results from earlier publications, 2<sup>1</sup>- $\alpha$ -D-Glc-cellobiose was supposed to be the isolated

cellobiose acceptor product, and 2<sup>1</sup>- $\alpha$ -D-Glc-lactose in the case for lactose. The other trisaccharide synthesized during lactose acceptor reaction remains unknown. It is quite possible that during cellobiose acceptor reaction, two trisaccharides are firstly produced: 2<sup>1</sup>- $\alpha$ -D-Glc-cellobiose and 6<sup>2</sup>- $\alpha$ -D-Glc-cellobiose. Then glucosyl units are continuously transferred to the trisaccharides via  $\alpha$ -(1 $\rightarrow$ 6) glucosydic linkage, finally producing a series of longer isomalto-cellobiose oligosaccharides. But the synthesized glucosyl-cellobiose products are weak acceptors, therefore only a few longer oligosaccharides could be observed from the chromatogram. In addition, all the tested disaccharides and trisaccharides were able to serve as acceptors for *W. confusa* dextranucrase, although clear differences were found in their efficiencies.

Unfortunately, until now there are no commercially available acceptor products for cellobiose and lactose. Such commercially available products could help us confirm the structures of trisaccharides synthesized in this study. In the near future, NMR spectroscopic analysis needs to be included for the accurate characterization of the synthesized trisaccharides. Also, *Weissella confusa* VTT E-90392 dextranucrase is not a feasible enzyme to produce potential prebiotic oligosaccharides using cellobiose and lactose, since the yields of their acceptor products were very low.

As presented in the thesis, the optimization model of maltose acceptor reaction reflects the variables affecting the production of isomalto-oligosaccharides. The concentrations of sucrose (donor) and maltose (acceptor) are the two most important parameters involved, which provides reference information for industrial scale production of isomalto-oligosaccharides. In addition, since the prebiotic properties of non-digestible oligosaccharides are structure-function related, characterization of prebiotic oligosaccharides is essential to the fully understanding of their functional behaviors. Also, it is recommended to explore more effective acceptors, and the future of *Weissella confusa* VTT E-90392 dextranucrase in synthesizing prebiotic oligosaccharides is promising.

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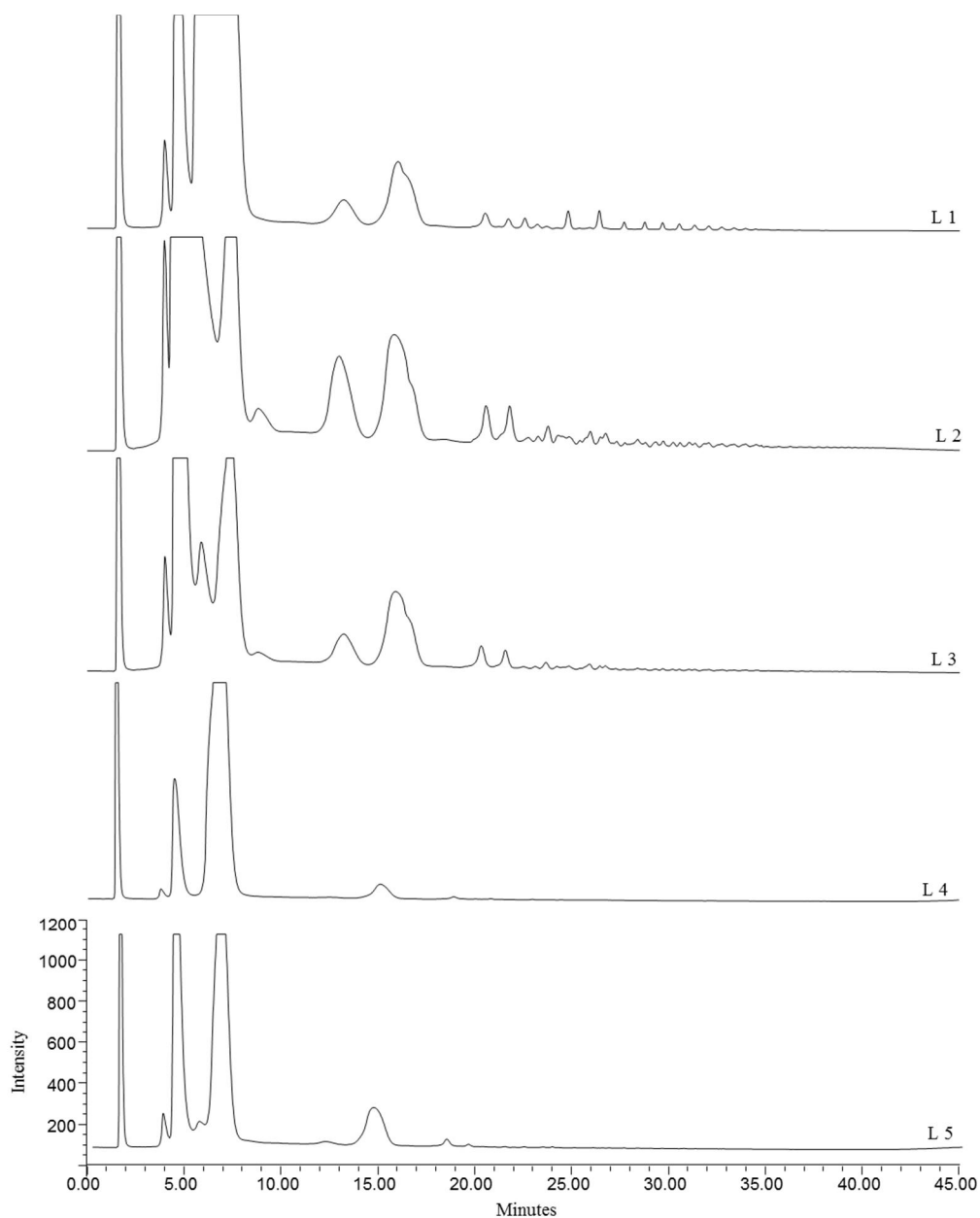


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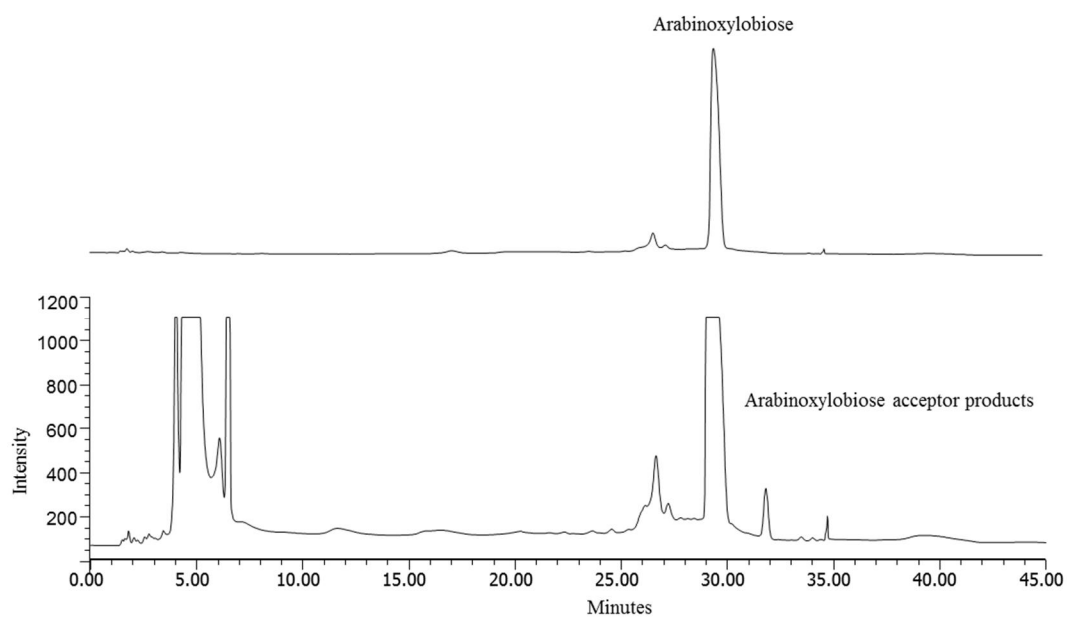
## APPENDIX

**Table 1.** Structures of oligosaccharides used

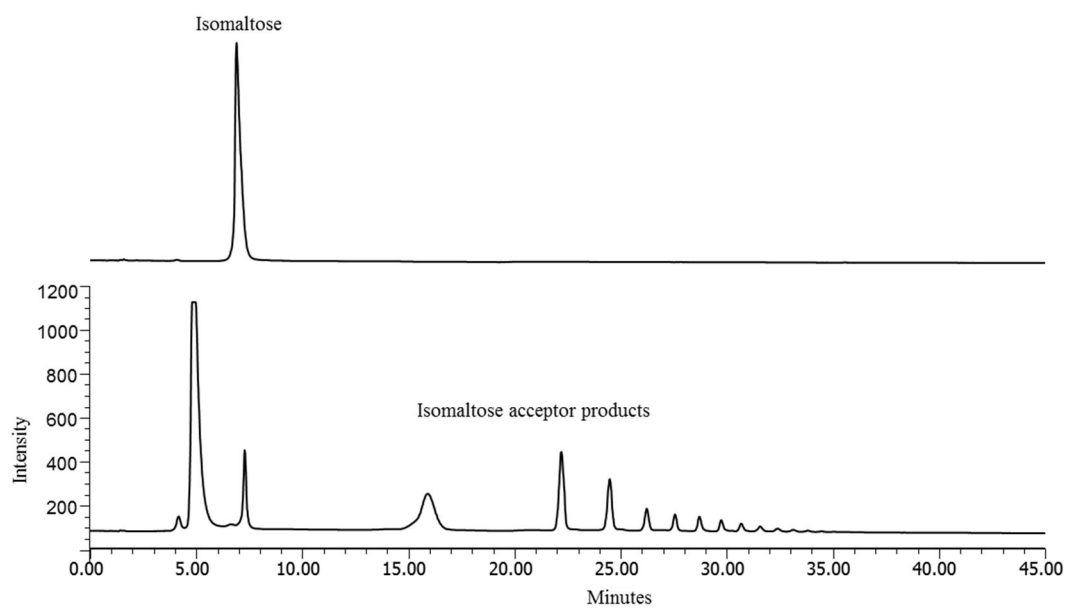
Acceptor	Structure
<b>Disaccharides</b>	
Maltose	$\alpha$ -D-Glc-(1→4)-D-Glc
Cellobiose	$\beta$ -D-Glc-(1→4)-D-Glc
Lactose	$\beta$ -D-Gal-(1→4)-D-Glc
Isomaltose	$\alpha$ -D-Glc-(1→6)-D-Glc
Laminaribiose	$\beta$ -D-Glc-(1→3)-D-Glc
Mannobiose	$\beta$ -D-Man-(1→4)-D-Man
Melibiose	$\alpha$ -D-Gal-(1→6)-D-Glc
Nigerose	$\alpha$ -D-Glc-(1→3)-D-Glc
<b>Trisaccharides</b>	
Arabinoxyllobiose	$\alpha$ -D-Ara-(1→3)- $\beta$ -D-Xyl-(1→4)-D-Xyl
Isopanose	$\alpha$ -D-Glc-(1→4)- $\alpha$ -D-Glc-(1→6)-D-Glc
Maltotriose	$\alpha$ -D-Glc-(1→4)- $\alpha$ -D-Glc-(1→4)-D-Glc
Panose	$\alpha$ -D-Glc-(1→6)- $\alpha$ -D-Glc-(1→4)-D-Glc



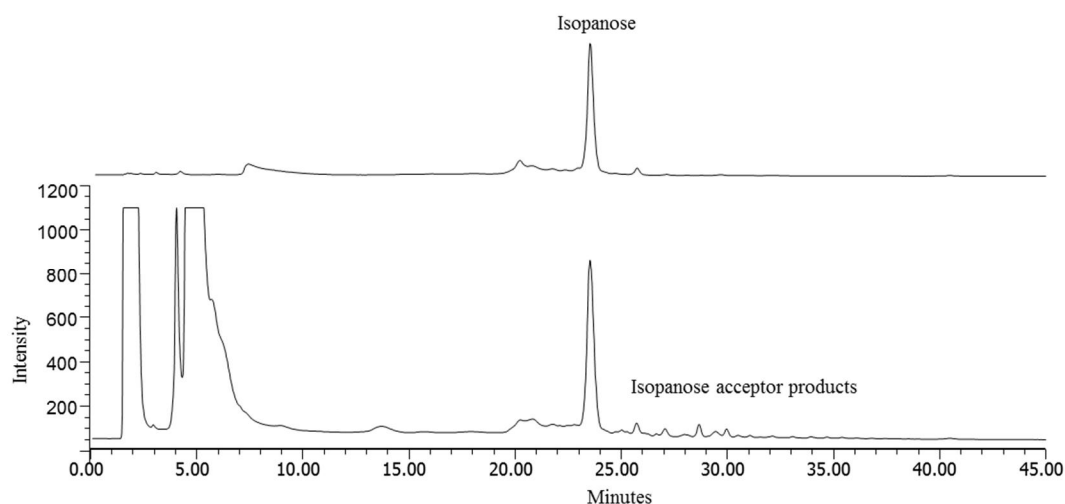
**Figure 1.** HPAEC-PAD profiles of lactose acceptor products in the presence of: L1, sucrose (1 mol/l) and lactose (0.15 mol/l) catalyzed by dextransucrase (1 U/g sucrose); L2, sucrose (1 mol/l) and lactose (0.15 mol/l) catalyzed by dextransucrase (10 U/g sucrose); L3, sucrose (0.575 mol/l) and lactose (0.15 mol/l) catalyzed by dextransucrase (5.5 U/g sucrose); L4, sucrose (0.15 mol/l) and lactose (0.15 mol/l) catalyzed by dextransucrase (1 U/g sucrose); L5, sucrose (0.15 mol/l) and lactose (0.15 mol/l) catalyzed by dextransucrase (10 U/g sucrose).



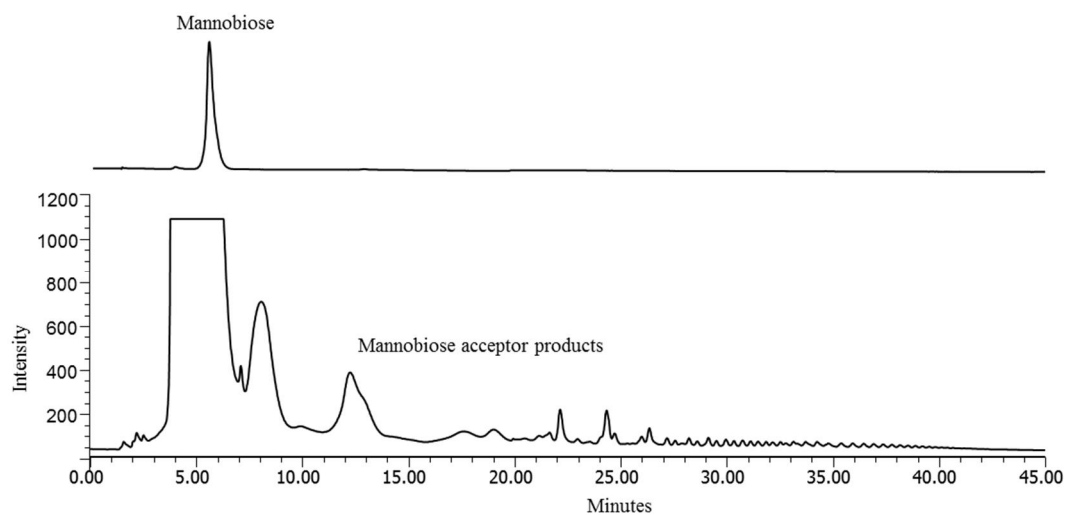
**Figure 2.** HPAEC-PAD profile of arabinoxylobiose acceptor products in the presence of sucrose (0.08 mol/l) and isomaltose (0.02 mol/l) catalyzed by dextransucrase (10 U/g sucrose).



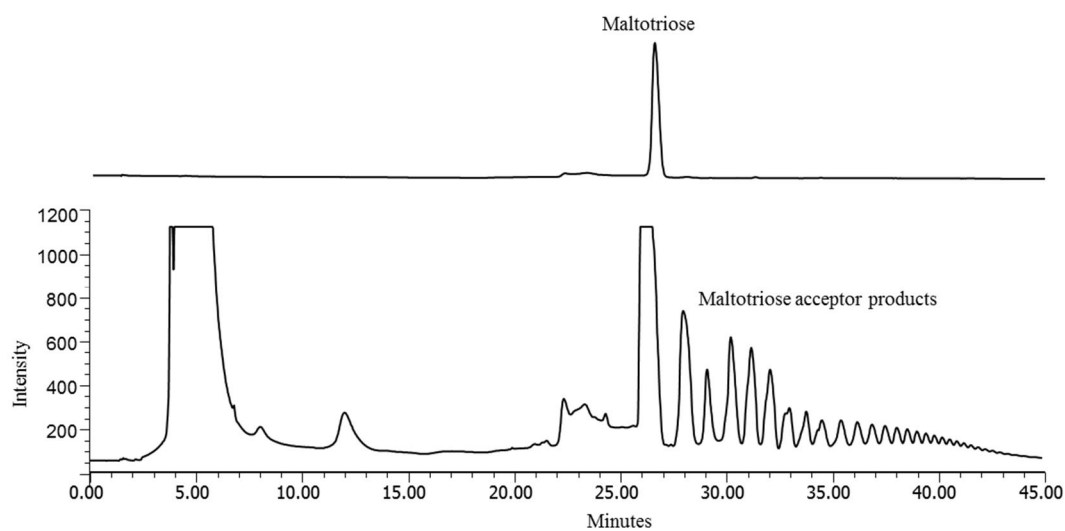
**Figure 3.** HPAEC-PAD profile of isomaltose acceptor products in the presence of sucrose (0.1 mol/l) and isomaltose (0.025 mol/l) catalyzed by dextransucrase (10 U/g sucrose).



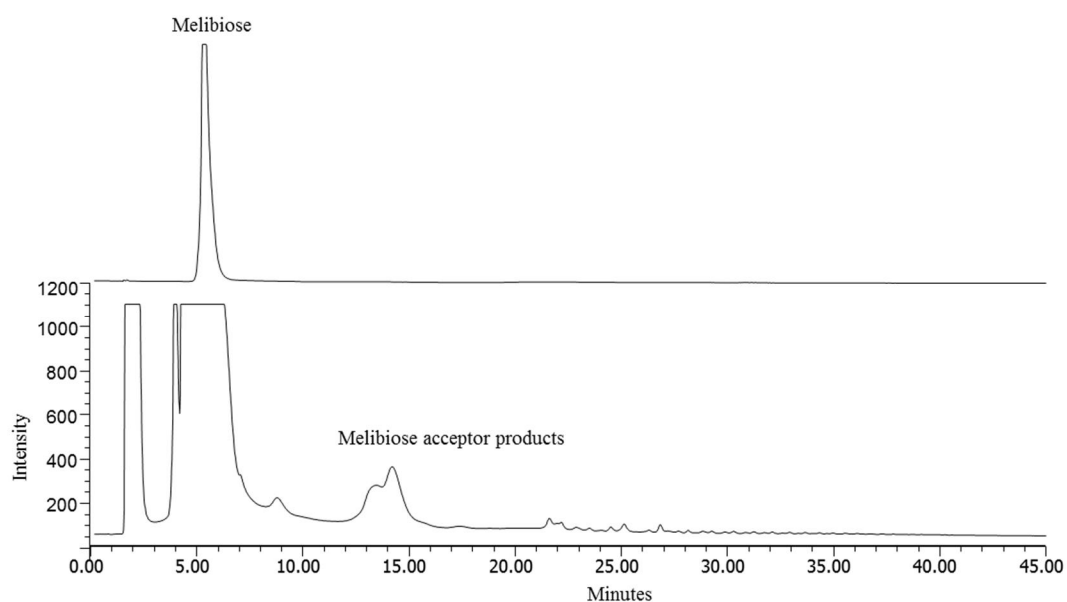
**Figure 4.** HPAEC-PAD profile of isopanose acceptor products in the presence of sucrose (0.2 mol/l) and isomaltose (0.05 mol/l) catalyzed by dextransucrase (10 U/g sucrose).



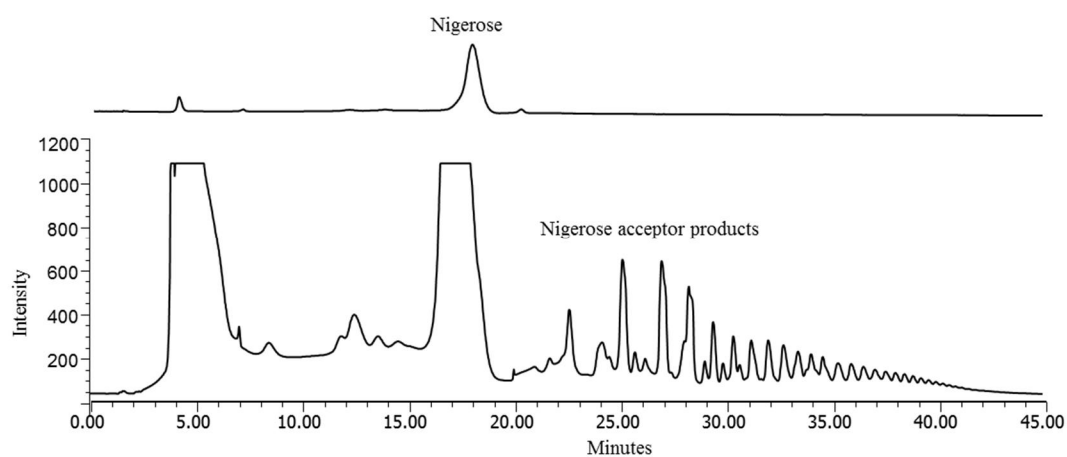
**Figure 5.** HPAEC-PAD profile of mannobiose acceptor products in the presence of sucrose (0.2 mol/l) and 1,4- $\beta$ -D-mannobiose (0.05 mol/l) catalyzed by dextransucrase (10 U/g sucrose).



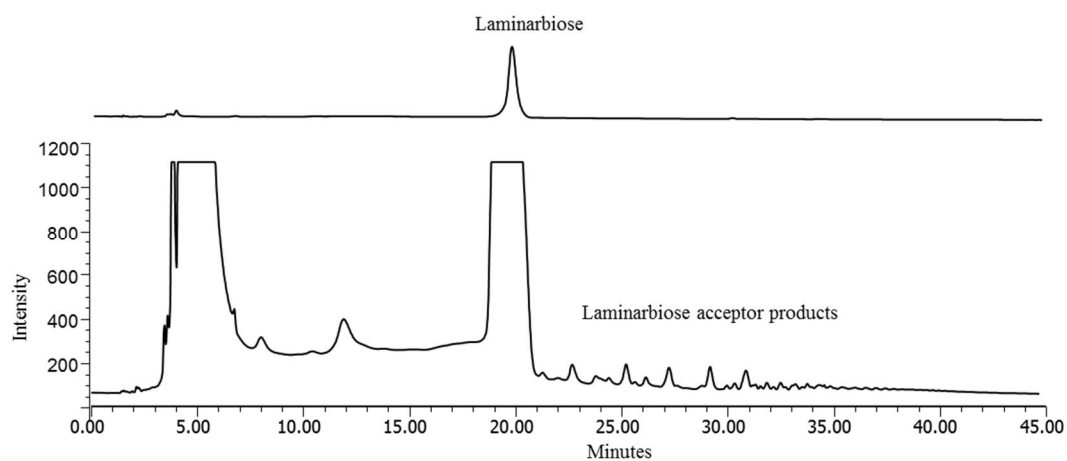
**Figure 6.** HPAEC-PAD profile of maltotriose acceptor products in the presence of sucrose (0.2 mol/l) and maltotriose (0.05 mol/l) catalyzed by dextransucrase (10 U/g sucrose).



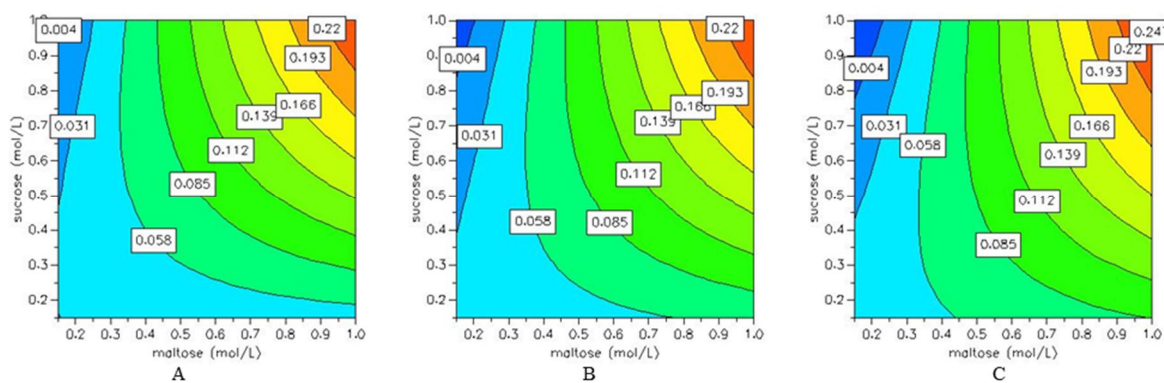
**Figure 7.** HPAEC-PAD profile of melibiose acceptor products in the presence of sucrose (0.2 mol/l) and isomaltose (0.05 mol/l) catalyzed by dextransucrase (10 U/g sucrose).



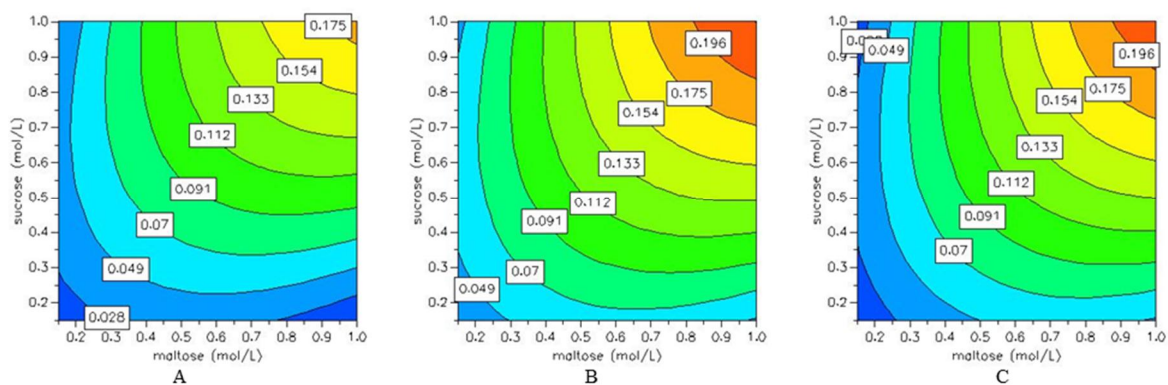
**Figure 8.** HPAEC-PAD profile of nigerose acceptor products in the presence of sucrose (0.2 mol/l) and nigerose (0.05 mol/l) catalyzed by dextransucrase (10 U/g sucrose)



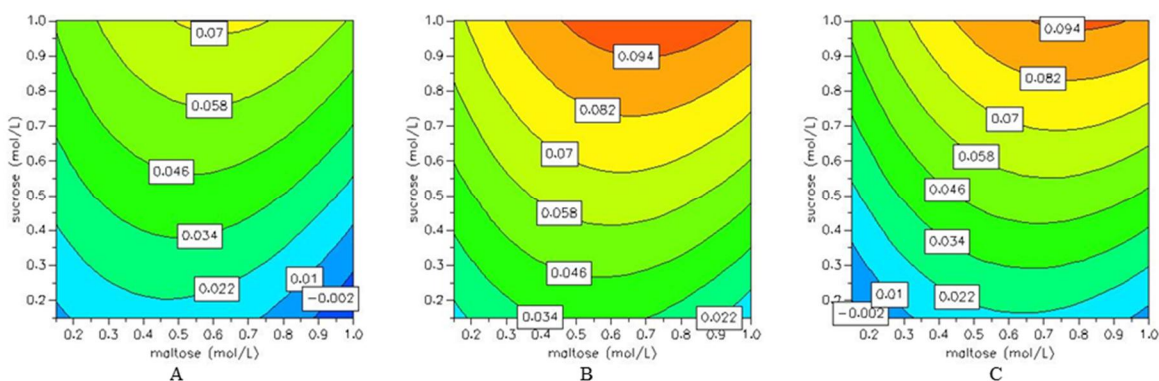
**Figure 9.** HPAEC-PAD profile of laminaribiose acceptor products in the presence of sucrose (0.2 mol/l) and laminaribiose (0.05 mol/l) catalyzed by dextransucrase (10 U/g sucrose)



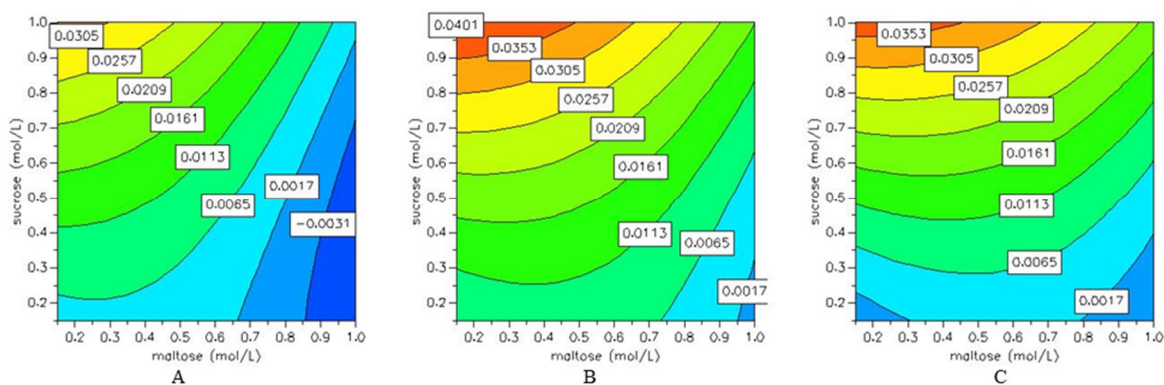
**Figure 10.** Predictive effects of sucrose (0.0 ~ 1.0 mol/l) and maltose (0.0 ~ 1.0 mol/l) on DP3 product (mol/l) under the function of different dextransucrase dosages (U/g sucrose). A, 1 U/g sucrose; B 5.5 U/g sucrose; C, 10 U/g sucrose.



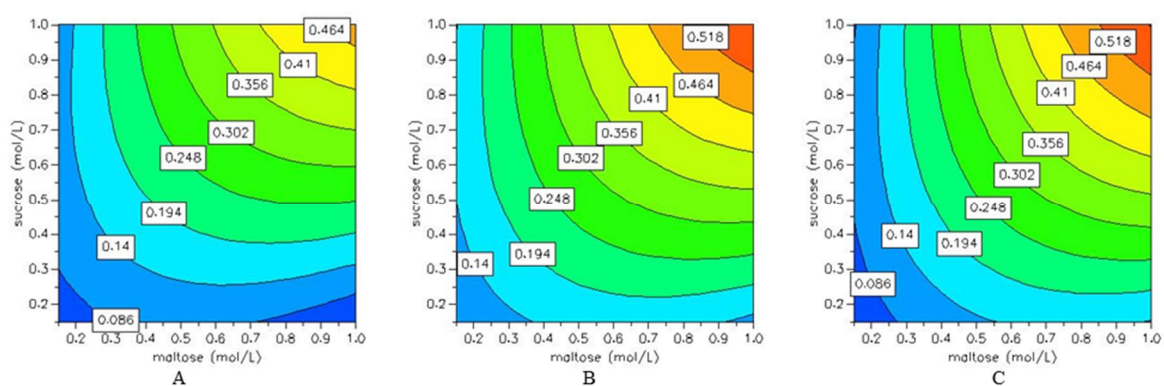
**Figure 11.** Predictive effects of sucrose (0.0 ~ 1.0 mol/l) and maltose (0.0 ~ 1.0 mol/l) on DP4 product (mol/l) under the function of different dextransucrase dosages (U/g sucrose). A, 1 U/g sucrose; B 5.5 U/g sucrose; C, 10 U/g sucrose.



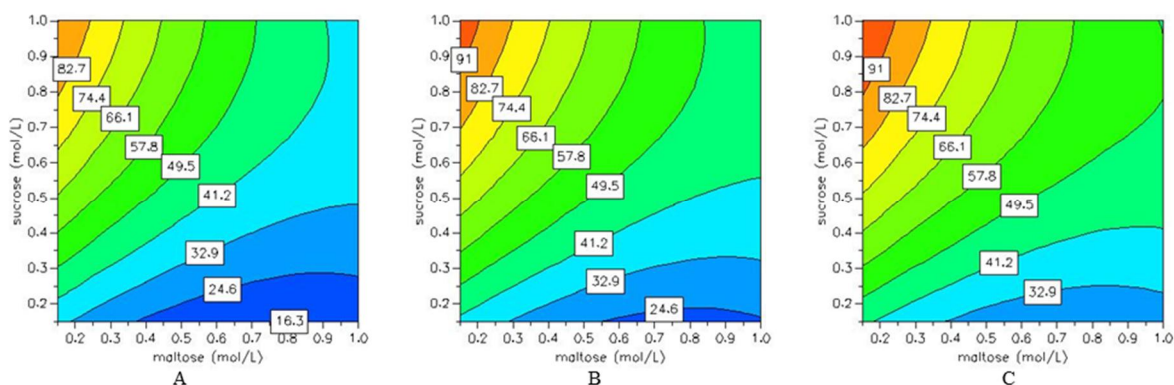
**Figure 12.** Predictive effects of sucrose (0.0 ~ 1.0 mol/l) and maltose (0.0 ~ 1.0 mol/l) on DP5 product (mol/l) under the function of different dextransucrase dosages (U/g sucrose). A, 1 U/g sucrose; B 5.5 U/g sucrose; C, 10 U/g sucrose.



**Figure 13.** Predictive effects of sucrose (0.0 ~ 1.0 mol/l) and maltose (0.0 ~ 1.0 mol/l) on DP6 product (mol/l) under the function of different dextranucrase dosages (U/g sucrose). A, 1 U/g sucrose; B 5.5 U/g sucrose; C, 10 U/g sucrose.

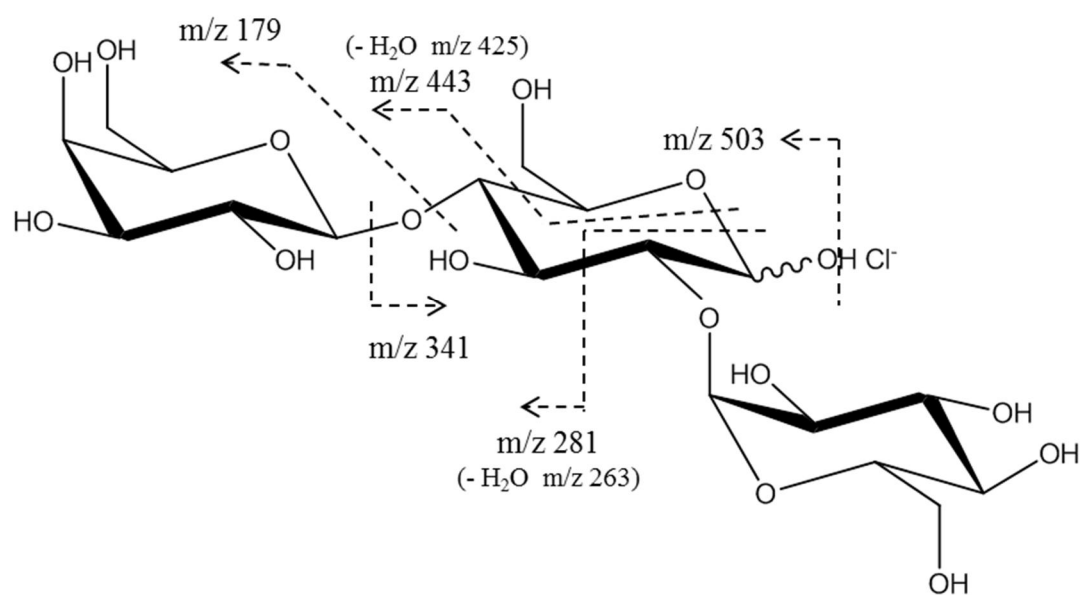


**Figure 14.** Predictive effects of sucrose (0.0 ~ 1.0 mol/l) and maltose (0.0 ~ 1.0 mol/l) on overall DP products (mol/l) under the function of different dextranucrase dosages (U/g sucrose). A, 1 U/g sucrose; B 5.5 U/g sucrose; C, 10 U/g sucrose.



**Figure 15.** Predictive effects of sucrose (0.0 ~ 1.0 mol/l) and maltose (0.0 ~ 1.0 mol/l) on consumed percentage of maltose under the function of different dextranucrase dosages (U/g sucrose). A, 1 U/g sucrose; B 5.5 U/g sucrose; C, 10 U/g sucrose.





**Figure 16.** Possible fragmentation patterns of chloride 2<sup>1</sup>-α-D-Glc-lactose