Characterization of Dextransucrase and Branching Sucrase of *Apilactobacillus kunkeei* DSM 12361

by

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Abstract

Glycoside hydrolase family 70 (GH 70) glucansucrases have various applications in both food and non-food industries. This study reports the characterization of two GH70 enzymes, dextransucrase and branching sucrase, of *Apilactobacillus kunkeei* DSM 12361 on a range of acceptor substrates. GtfZ of *A. kunkeei* DSM 12361 possesses two catalytic domains, CD1 and CD2, which are interconnected by a glucan binding domain (GBD). For this study, dextransucrase CD1-GBD is a combination of the first catalytic domain CD1 with GBD, while branching sucrase GBD-CD2 is composed of GBD combined with the second catalytic domain CD2. In addition to sucrose as the sole substrate, CD1-GBD was active when dextran was available as an acceptor substrate. It was also found that CD1-GBD was active on reuteran and modified potato amylose. GBD-CD2 was also active on various substrates such as dextran, reuteran, and modified amylose, which proves the ability of GBD-CD2 to introduce branches on polymer chains with different linkage types. The activity of CD1-GBD and GBD-CD2 showed a preference for α-(1→6) glycosidic linkage in the acceptor substrate. The ability of CD1-GBD and GBD-CD2 to act on starch-derived molecules, such as potato amylose, is crucial in synthesizing polymers at a low cost. The activity on a wide range of substrates increases the options for producing polymers with different properties.

Preface

This thesis is an original work by Clement Niyirora.

The cloning part for the chimeric enzymes was performed by Dr. Julia Bechtner, who provided the constructs GBD-GtfZ, SBD-GtfZ, GtfZ-A, and GtfZ-B for further analysis. Yuqi Shao provided the clone for the branching enzyme GlgB, and I conducted protein expression and further analysis related to my research. The strains carrying gtfa used in this study were obtained from the lab 2-50 strain collection and were previously cloned by Dr. Xiao Yan Chen. All laboratory work and data analysis presented in this thesis were completed under the supervision and guidance of Dr. Michael Gänzle.

I would like to dedicate this thesis to my family, particularly my mother, Valérie Mukundwangendo, whose love, guidance, and unrelenting belief in my abilities have been the foundation of my success.

Acknowledgement

I would like to extend my sincere appreciation to all the people who have contributed to the successful completion of this thesis.

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I would like to express my appreciation, especially to my mentor, Dr. Gautam Gaur, for helping me get started with my project and being there to answer any questions. I am grateful to lab 2-50 members for their constant support and encouragement. Their stimulating discussions and constructive feedback have been a driving force in shaping my research and sharpening my analytical skills. I am also thankful for their friendship and camaraderie, which have made my time in the lab truly enjoyable.

I would also like to express my gratitude to the faculty and staff of the department for providing a challenging and stimulating academic environment. Their passion for teaching and research has inspired me to pursue excellence in my own work.

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Table of Contents

List of Tables

List of Figures

List of Abbreviations

^{1}H NMR	Proton Nuclear Magnetic Resonance
ANOVA	Analysis of Variance
BLAST	Basic Local Alignment Search Tool
CD	Catalytic Domain
DNA	Deoxyribonucleic acid
EFSA	European Food Safety Authority
GBD	Glucan Binding Domain
GH	Glycoside Hydrolases
IPTG	Isopropyl β-D-1-thiogalactopyranoside
LAB	Lactic Acid Bacteria
mMRS	Modified de Man, Rogosa & Sharpe
Ni-NTA	Nickel-Nitrilotriacetic Acid
PCR	Polymerase Chain Reaction
QPS	Qualified Presumption of Safety
SDS-PAGE	Sodium dodecyl sulfate–Polyacrylamide Gel Electrophoresis

1 Introduction

Historically, fermentation was performed for preservation purposes and for improving organoleptic properties of food (Hutkins, 2018). Specifically, lactic acid fermentation has been used for thousands of years (Siedler et al., 2019) and lactic acid bacteria (LAB) are important in this fermentation for enzymatic conversion of the original food to a final product with preferred properties such as improved texture, flavor, and nutritional value (Baruah & Goyal, 2022; Marco et al., 2017). All these functions of LAB are not only linked to the production of lactic acid but also to their ability to form other compounds including but not limited to exopolysaccharides (EPS) (Torino et al., 2015).

LAB produce EPS and form biofilms to adapt to adverse environmental conditions, however studies have proven their potential for application in the food industry (Baruah & Goyal, 2022; Han et al., 2016). Interestingly, EPS from EPS-forming microbes that are used in food fermentations, strains of the genera *Limosilactobacillus*, *Lactobacillus*, *Lactococcus*, *Latilactobacillus*, *Lactiplantibacillus*, *Lentilactobacillus*, *Lacticaseibacillus*, *Fructilactobacillus*, *Leuconostoc*, *Pediococcus* and *Streptococcus mainly streptococcus thermophilus*, are not included as additives on the food label because these LAB have been granted a Qualified Presumption of Safety (QPS) by the European Food Safety Authority (EFSA 2021) (Koutsoumanis et al., 2022). As a result, EPS producing cultures have been widely used in various fermented foods. For example, in yogurt EPS contribute to the thickness and smoothness enhancement (Han et al., 2016), and in sourdough production, EPS improves rheology, shelf life and the volume of the bread (Lynch et al., 2018; Xu et al., 2020).

EPS produced by LAB are structurally classified as homopolysaccharide (HoPS) and heteropolysaccharides (HePS). The main difference between these EPS is the composition of the monosaccharides. HoPS are composed of multiple monosaccharide units of a single type. However, HePS have more than one type of monosaccharide unit linked together (Mozzi et al., 2006). Most HoPS from LAB are synthesized extracellularly from sucrose as a donor molecule by glycansucrases of bacteria such as *Lactobacillus*, *Leuconostoc, Streptococcus*, *Weissella, Liquorilactobacillus*, *Limosilactobacillus* and *Apilactobacillus* using (Moulis et al., 2006; Torino et al., 2015). HoPS are divided into two categories, glucans and fructans. Glucans are made of glucose molecules linked together by α-linkages, whereas fructans are fructose linked with β-linkages (Monsan et al., 2001). Depending on whether glucan or fructan is being synthesized, glycansucrases are termed glucansucrases or fructansucrases respectively (Cantarel et al., 2009). The linkage type available in the polysaccharide chain can also be used to classify as glucosidic or fructosidic. A typical example of HoPS composed of glucosidic linkages is dextran with predominantly α-(1→6) glucosidic linkage. Inulin is an example of HoPS with fructosidic linkages where it has β-(2→1) fructosidic linkages (Van Kranenburg et al., 1999).

In addition to EPS production, LAB, especially species of the genus *Limosilactobacillus* colonize the gastrointestinal tract of humans and other vertebrates (Duar et al., 2017; Frese et al., 2011). *Limosilactobacillus reuteri* is one species of this genus and its strains have been isolated from humans, ruminants, pigs, mice, chicken and turkey. The ability of *Lm. reuteri* to colonize intestinal tract have attracted exceptional attention due to its positive contribution to gut health (Oh et al., 2010; Walter et al., 2011). *Lm. reuteri* adheres to the mucin and intestinal epithelia and studies have proven that some stains are capable of attaching to gut epithelial cells (Li et al., 2008). This attachment and persistence in the intestinal tract of the host is assisted by a range of

factors including but not limited to biofilm formation (Frese et al., 2013) and the capability of *Lm. reuteri* TMW1.106 biofilm formation and cell aggregation are dependent on the production of the reuteransucrases GtfA *in vivo* (Walter et al., 2008). The ability for LAB to colonize has been linked to production of glucansucrases. In water kefir, *Lentilactobacillus hilgardii* synthesizes dextran to form kefir granules (Hu et al., 2017). *Streptococcus mutans* glucansucrase has been reported to form dextran-based biofilms to colonize the oral cavity of humans (Fernandes et al., 2017).

1.1 LAB Glucansucrases, Branching Sucrases and Glucanotranferases

LAB glucansucrases have been characterized because of their applications in synthesis of important polysaccharides used in food and non-food industries (Baruah & Goyal, 2022; Bounaix et al., 2009; Patel et al., 2012). These enzymes are named after their products α-glucan, and their activity can be assisted by branching sucrases. Branching sucrases are responsible for introducing branches to a linear α-(1→6) linked glucan mainly dextran which results in highly branched polymers (**Figure 1.1**) (Vuillemin et al., 2016). Branching sucrases and glucansucrases belong to glucoside hydrolase 70 family (GH 70) and are known to use sucrose as a substrate to synthesize complex carbohydrate structures (Brison et al., 2012; Moulis et al., 2006; Robyt, 1995). Glucanotransferases are also classified as GH 70, but they belong to their own subfamily. They share similarities with glucansucrases of the same family however they are inactive on sucrose. These enzymes are known to use starch as substrates to synthesize α-glucan (Bai et al., 2015).

As of December 2022, a total of 972 GH 70 enzymes were recorded on the CAZY database (http://www.cazy.org/GH70.html) however only fifty-nine have been characterized (**Table 1**). This number is still small; however, there are other enzymes of this family that are not reported

on the CAZY database even though they are available in literature (**Table 1**). The most studied glucansucrase in the GH70 family is dextransucrases because of their applications in industry, but still there are others such as reuteransucrases, mutansucrases, and alternansucrases. Dextransucrases synthesize dextran with mostly α-(1→6) linkages in their main chain but can also contain various branching points (Robyt, 1995). Reuteransucrases have been reported to produce reuterans that are water soluble glucan with mainly α-(1→4) linkages with a significant amount of α-(1→6) and α-(1→4,6) glycosidic linkages (Kralj et al., 2005; Kralj, van Geel-Schutten, van der Maarel, et al., 2004). Mutansucrases are responsible for the formation of water insoluble mutans with predominantly α-(1→3) linkages (Z. Chen et al., 2021). Alternansucrases synthesize alternan that contains alternating α-(1-6) and α-(1→3) linkages (Bounaix et al., 2009).

Table 1 Characterized GH70 enzymes from CAZy database and publications. They are sorted by specificity and then by genus and species.

Enzyme	Organism	GenBank	Specificity	Reference
GTF-0	*Lm. reuteri*	AAY86923.1	Reuternasucrase	(Kralj et al., 2005)
GTF-A	*Lm. reuteri*	AAU08015.1	Reuternasucrase	(Kralj et al., 2002)
ASR	*Ln. mesenteroides*	CAB65910.2	Alternansucrase	(Arguello-Morales et al., 2000)
ASR	*Ln. citreum*	AIM52834.1	Alternansucrase	(Wangpaiboon et al. NP)
GFT-ML1	*Lm. reuteri*	AAU08004.1	Mutansucrase	(Kralj, van Geel-Schutten, Dondorff, et al., 2004)
GTF-I	*S. criceti*	BAF62338.1	Mutansucrase	(Shinozaki-Kuwahara et al., 2008)
GTF-I	*S. downei*	AAC63063.1	Mutansucrase	(Ferretti et al., 1987)
GTF-SI	*S. mutans*	BAA26114.1	Mutansucrase	(Bowen & Koo, 2011)
GTF-B	*S. mutans*	AAA88588.1	Mutansucrase	(Shiroza et al., 1987)
GTF-F	*S. orisuis*	BAF62337.1	Mutansucrase	(Shinozaki-Kuwahara et al., 2008)
GFT-L	*S. salivarius*	AAC41412.1	Mutansucrase	(Simpson et al., 1995)
GTF-J	*S. salivarius*	AAA26896.1	Mutansucrase	(Simpson et al., 1995)
GTF-I	*S. sobrinus*	BAA02976.1	Mutansucrase	(Sato et al., 2009)
DSR	*Lb. animalis*	CCK33644.1	Dextransucrase	(Rühmkorf et al., 2013)
GTF-Kg3	*Lb. fermentum*	AAU08008.1	Dextransucrase	(Kralj, van Geel-Schutten, van der Maarel, et al., 2004)
GTF-33	*Lb. parabuchneri*	AAU08006.1	Dextransucrase	(Kralj, van Geel-Schutten, van der Maarel, et al., 2004)
GTF-Kg15	*Lb. sakei*	AAU08011.1	Dextransucrase	(Kralj, van Geel-Schutten, van

Enzyme	Organism	GenBank	Specificity	Reference
				der Maarel, et al., 2004)
GTF-180	*Lm. reuteri*	AAU08001.1	Dextransucrase	(Kralj, van Geel-Schutten, van der Maarel, et al., 2004)
DSR106.1	*Lm. reuteri*	ABP88725	Dextransucrase	(Rühmkorf et al., 2013)
DSR-B	*Ln. citreum*	AAB95453.1	Dextransucrase	(Monchois et al., 1998)
DSR-A	*Ln. citreum*	AAB40875.1	Dextransucrase	(Monchois et al., 1996)
DSR-X	*Ln. mesenteroides*	AAQ98615.2	Dextransucrase	(Yalin et al., 2008)
DSR-T	*Ln. mesenteroides*	BAA90527.1	Dextransucrase	(Funane et al., 2000)
DSR-S	*Ln. mesenteroides*	AAD10952.1	Dextransucrase	(Monchois et al., 1997)
DSR-P	*Ln. mesenteroides*	AAS79426.1	Dextransucrase	(Olvera et al., 2007)
DSR-D	*Ln. mesenteroides*	AAG61158.1	Dextransucrase	(Neubauer et al., 2003)
DEX-YG	*Ln. mesenteroides*	ABC75033.1	Dextransucrase	(Zhang et al., 2008)
GTF-S	*S. downei*	AAA26898.1	Dextransucrase	(Gilmore et al., 1990)
GTF-G	*S. gordonii*	AAC43483.1	Dextransucrase	(Vickerman et al., 1997)
GFT-D	*S. mutans*	AAA26895.1	Dextransucrase	(Shimamura et al., 1994)
GTF-R	*S. oralis*	BAA95201.1	Dextransucrase	(Fujiwara et al., 2000)
GTF-M	*S. salivarius*	AAC41413.1	Dextransucrase	(Simpson et al., 1995)
GTF-K	*S. salivarius*	CAA77898.1	Dextransucrase	(Simpson et al., 1995)
GTF-P	*S. sanguinis*	BAF43788.1	Dextransucrase	(Kralj, van Geel-Schutten, van der Maarel, et al., 2004)
GTF-U	*S. sobrinus*	BAC07265.1	Dextransucrase	(Hayakawa et al., 1993)
GTF-I	*S. sobrinus*	BAA14241.1	Dextransucrase	(Hoshino et al., 2012)
GTF-Tl	*S. sobrinus*	AAX76986.1	Dextransucrase	(Hanada et al., 1993)
DSR-WC	*W. cibaria*	ACK38203.1	Dextransucrase	(Kang et al., 2009)
DSR-C39-2	*W. confusa*	CCF30682.1	Dextransucrase	(Amari et al., 2013)
DSR-E	*Ln. citreum*	CAD22883.1	Dextransucrase + α-1-2 Branching sucrase	(Fabre et al., 2005)
GtfZ	*Ap. kunkeei*	KRK22577.1	Dextransucrase + α-1-3 Branching sucrase	(Meng, Gangoiti, Wang, et al., 2018)
BRS-A	*Ln. citreum*	CDX66896.1	α-1,2 branching sucrase	(Passerini et al., 2015)
BRS-B	*Ln. citreum*	CDX65123.1	α-1,3 branching sucrase	(Vuillemin et al., 2016)
BRS-C	*Ln. fallax*	WP_010006776.1	α-1,3 branching sucrase	(Vuillemin et al., 2016)
BRS-D	*Ap. kunkeei*	WP_051592287.1	α-1,2 branching sucrase	(Vuillemin et al., 2016)
GBD-CD2	*Ln. citreum*	CAD22883.1	α-1,2 branching sucrase	(Brison et al., 2010)

1.1.1 Glucansucrases and Branching Sucrases Catalytic Mechanism

Enzymes belonging to the GH70 family are known to mainly catalyze transglucosylation reactions using a mechanism referred to as double displacement. The first reaction is the formation of a β-D-glucosyl-enzyme intermediate from sucrose. This intermediate compound is a

result of aspartate nucleophile which attacks the anomeric carbon of the glucosyl unit. This nucleophilic attack is assisted by the acid-base catalyst glutamic acid that donates a proton to fructosyl moiety, resulting in a release of fructose and formation of β-D-glycosyl-enzyme and followed by the stabilization of covalent intermediate by a third aspartate. The second step involves the obstruction of the intermediate by either a water molecule or the hydroxyl group of the acceptor substrate and this results in hydrolysis or transglucosylation, respectively. Other than hydrolysis, the transfer of a glycosyl group can be on sucrose itself, on fructose which results in formation of sucrose isomers, or on an introduced hydroxylated acceptor substrate. The formation of the polymer is due to multiple transfer of glycosyl groups to the non-reducing end of the α-glucan chain (**Figure 1.1**) (MacGregor et al., 1996; Mooser et al., 1991; Moulis et al., 2006).

For branching sucrases, transferase activity is present when dextran is available, otherwise only hydrolytic activity will be predominant. If case fructose is available in excess, branching sucrases can transfer to fructose to form leucrose (Moulis et al., 2006). Various glucansucrases have shown the ability to form polymers. For example, ASR from *Ln. citreum* NRRL B-1355 and DSR-S from *Ln. citreum* NRRL B-1355 synthesize polymer by transferring glycosyl unit to glucose or sucrose resulting in oligosaccharides production. Multi-chain elongation is the first part of oligosaccharide production which is replaced by single chain elongation and results in the production of polymer with high molar mass. The characteristics of the products depend on the ratio of transglucosylation and hydrolysis. In addition, some studies show that the structure and specificity of the product generated before in the beginning steps can limit chain elongation (Molina et al., 2019; Moulis et al., 2006).

Glucansucrases have been tested on wide range of acceptors from disaccharides to polysaccharides (McCabe & Smith, 1978) and they are reported to recognize even polyphenols (Meulenbeld et al., 1999), alkyl-glycosides (Richard et al., 2003) or terpenoids (Gerwig et al., 2017) depending on the source and the type of the enzyme. In contrast, compared to glucansucrases, branching sucrases have not been studied on many acceptor molecules. Reported branching sucrases such as α-(1→2) and α-(1→3) are active on fructose but they do not recognize maltose even though maltose is preferred by many glucansucrases. Branching sucrases prefer glycosyl chain with α-(1→6) with a minimum four glycosyl units to introduce branches (Meng, Gangoiti, Wang, et al., 2018; Vuillemin et al., 2016).

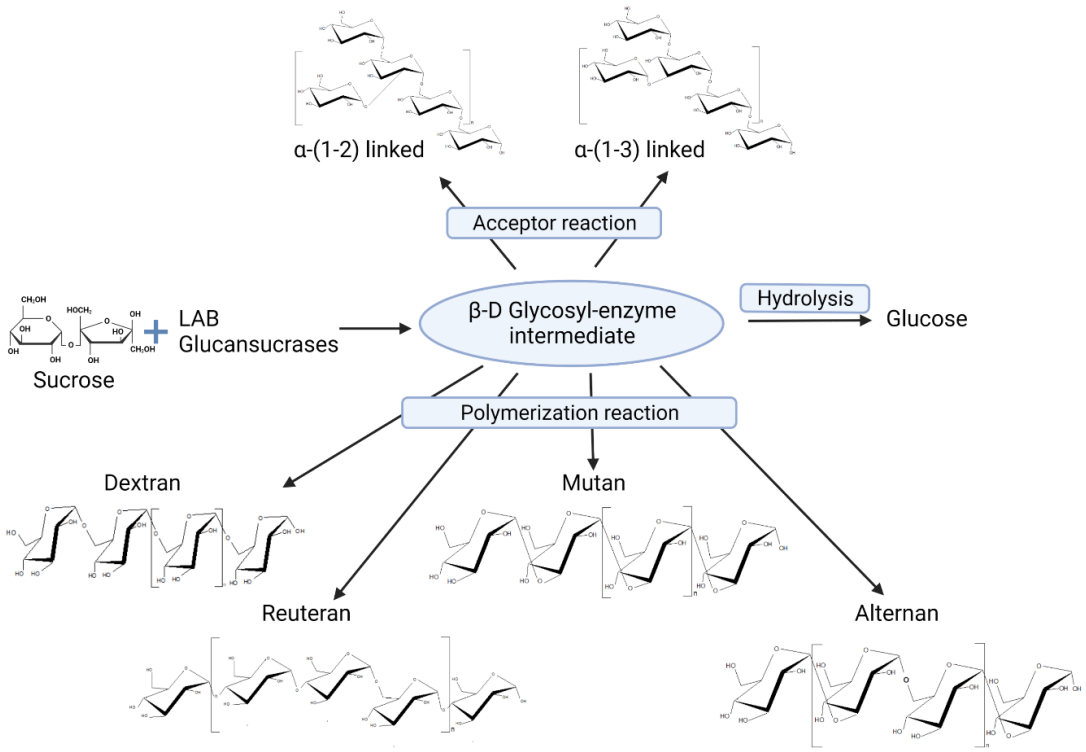

Figure 1.1 Reactions catalyzed by LAB glucansucrases and branching sucrases (Z. Chen et al., 2021)**.**

1.1.2 Dextransucrases and Products

Dextransucrases are extracellular enzymes produced by LAB genera such as *Leuconostoc*, *Limosilactobacillus*, *Streptococcus*, *Pediococcus*, and *Weissella* (Monsan et al., 2001). Dextransucrases attracted a strong attention in both food and non-food industries because of their ability to synthetize mostly used polysaccharide such as dextran, using sucrose as substrate (Baruah et al., 2017). In the presence of other sugar molecules in the reaction, dextransucrases can transfer glycosyl groups from sucrose to the non-reducing end of these molecules and resulting in formation of acceptor products (Richard et al., 2003). Dextran is predominantly composed of the main linear chain of α-(1→6) and with different branching points such as α-(1→2), (1→3), (1→4) glycosidic linkage depending on the source of dextransucrase used in synthesis. This change in percentages of different linkages contributes to physicochemical properties of dextran (Patel et al., 2012). Dextransucrases from *Leuconostoc mesenteroides* NRRL B-512F produce dextran with 95% α-(1-6) and 5% of α-(1→3) glycosidic linkages branches whereas dextran from *Leuconostoc mesenteroides* NRRL B1299 contains 63% α-(1→6) in backbone chain and 27% of α-(1→2) and 8% of α-(1→3) branches (Dols et al., 1998).

1.1.3 Branching sucrase

Recently branching sucrases were discovered by genome analysis of highly branched dextran producing bacteria. These bacterial species include *Leuconostoc citreum* NRRL B-1299 and *Leuconostoc citreum* NRRL B-742. The comparison of sequence similarities of a new gene with different glucansucrases such as dextransucrase, mutansucrases and alternansucrases have proven a discovery branching sucrase which is specialized introducing α-(1→2) or α-(1→3) branches on dextran (Passerini et al., 2015; Vuillemin et al., 2016). From this discovery, numerous studies were conducted to identify other branching sucrases in *Leuconostoc fallax*

KCTC3537 and *A. kunkeeii* EFB6 (Vuillemin et al., 2016). A glucansucrase produced by *Leuconostoc mesenteroides* BD3749 was suggested to be an intermediate between glucansucrases and branching sucrases because of their products with α-(1→6)/ α-(1→4) and α-(1→3) linkages, in addition to their sequence similarities (Yan et al., 2018).

The interest of studying branching sucrases has led to the characterization of genes that contain two different catalytic domains, one with glucansucrase activity and the other one with branching sucrase activity. In *Ln. citreum* NRRL B-1299, DSR-E contains catalytic domain 1, CD1 which is responsible for dextransucrase activity and the second catalytic domain CD2 that possesses α-(1→2) branching activity (Fabre et al., 2005). Most recently, *gtfZ* glucansucrase was characterized from *A. kunkeeii* DSM 12361 and contain two catalytic domains one with dextransucrase and the second one was reported to have a branching sucrase activity by introducing α-(1→3) glucosidic branches on dextran (Meng, Gangoiti, Wang, et al., 2018). These previously described enzymes were proposed to belong in GH70 family in a subfamily referred to as branching sucrase enzymes (Brison et al., 2012; Fabre et al., 2005).

1.2 LAB Glucanotransferases

GH70 glucansucrases synthesize EPS from sucrose, however in recent studies there are several GH70 enzymes identified among LAB which use starches and starch-delivered oligosaccharides as substrates instead of sucrose to synthesize α-glucan (Bai et al., 2015). LAB glucanotransferases include 4,6- α-glucanotransferase and 4,3- α-glucanotransferase (**Figure 1.2**). There is a structural similarity among LAB glucanotransferases and GH70 glucansucrases, but their function is similar to GH13 α-amylases because of their ability to act on starches. They were reported to be evolutionary intermediate of GH13 and GH70 enzymes (Gangoiti et al., 2018; Meng et al., 2016). A comparison of glucansucrases with 4,6- α-glucanotransferases show

that they share 45% to 50% of their sequence identity but they belong in different GH70 subfamilies (Kralj et al., 2011; Leemhuis et al., 2013).

The first reported LAB glucanotransferase was 4,6-α-glucanotransferases from *Lm. reuteri* 121. This enzyme is able to act on starch-delivered oligosaccharide and synthesize α-glucans which contain a higher degree of α-(1→6) linkages (Bai et al., 2017). When donor substrates, such as starch and maltooligosaccharides are available, 4,6- α-glucanotransferase cleaves α-(1-4) linkages from the non-reducing end of the donor and transfer the glycosyl unit to the non-reducing end of the receptor and forming a new chain of α-(1→6) linkages (Kralj et al., 2011). The reported hydrolytic activity of 4,6- α-glucanotransferase enzymes is low.

There are other characterized 4,6-α-glucanotransferases enzymes which have the ability to produce isomalto/maltooligosaccharides. These enzymes are GtfML4, GtfX, GtfW and GTFB-E81 characterized from *Lm. reuteri* ML1, *Ligilactobacillus aviarius* subsp. *aviarius* DSM 200655, *Lm. reuteri* DSM 20016 and *Lm. reuteri* E81, respectively (İspirli et al., 2019; Leemhuis et al., 2013; Meng, Gangoiti, de Kok, et al., 2018). Interestingly the 4,6-α-glucanotranferase from *Lm. reuteri* NCC 2613 synthesizes a different product from other characterized 4,6-α-glucanotransferases described above. Instead of producing isomalto/malto polysaccharides with a linear chain, *Lm. reuteri* NCC 2613 4,6-α-glucanotransferases synthesize reuteran like polymer with branches when amylose is used as a substrate (Gangoiti, Van Leeuwen, et al., 2017).

Recently, the genomic analysis of *Limosilactobacillus fermentum* NCC 2970 has led to the identification of 4,3- α-glucanotransferases with high similarity to *Lm. reuteri* 121 4,6- α-glucanotransferases; however, the enzyme revealed uniqueness in its product specificity (Gangoiti, van Leeuwen, et al., 2017). This enzyme uses amylose to produce α-glucan composed

with maltoologosaccharides units connected by α-(1→3) linkages. The products from 4,3-α-glucanotransferases consist of alternating α-(1-3) and α-(1→4) linkages with α-(1→3,4) branches (Gangoiti, van Leeuwen, et al., 2017).

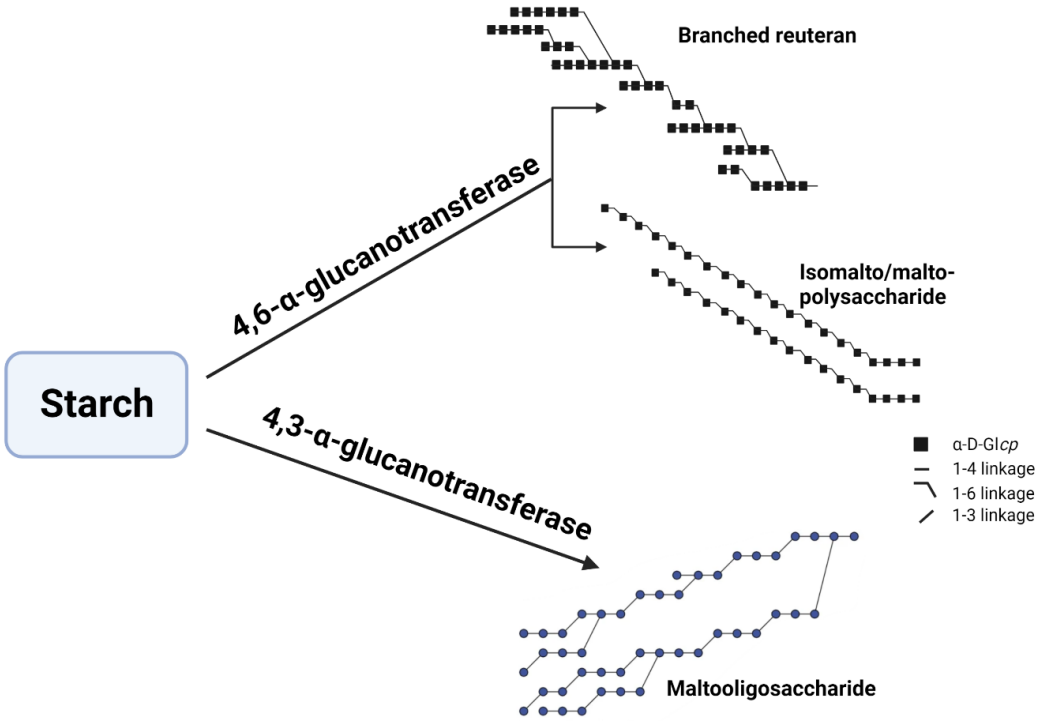

Figure 1.2 Reactions catalyzed by LAB glucanotransferases (Z. Chen et al., 2021).

1.3 Applications: Hydrocolloids, oligosaccharide synthesis, dietary fibre, pharmaceutical applications.

Glucansucrases contribute to the synthesis of important products with various applications in various fields including but not limited to food and pharmaceutical industry. The most widely applied α-glucan is dextran (Z. Chen et al., 2021). In the food industry, dextran with more than

95% α-(1→6) linear linkages contributes to water solubility of these polysaccharides, which results in their use as viscosifier, stabilizer, and hydrocolloids in food industry (Goulas et al., 2004; Purama & Goyal, 2005). During sourdough fermentation, EPS including dextran has been associated with increasing loaf volume and softness of the bread (Baruah et al., 2017; Katina et al., 2009) and in jam and ice cream, dextran is used as a thickener (McCurdy et al., 1994). Dextran have been reported to enhance the texture of gluten free products (Katina et al., 2009); however, it is important to use *Weissella* that does not produce acetic acid with fructose as electron acceptor (Galle et al., 2010). In multiple food products, dextran has been reported to enhance moisture retention, improve the texture and flavor, and prevent crystallization of sugar (Naessens et al., 2005; Purama & Goyal, 2005, 2008).

Glucansucrases can synthesize functional oligosaccharides when an appropriate acceptor molecule is available (Gangoiti et al., 2020). These oligosaccharides include α-1,2 branched glucooligosaccharides (Dols et al., 1997; Hasselwander et al., 2017) and isomaltooligosaccharides (Djouzi et al., 1995). Glucooligosaccharides produced by *Ln. citreum* NRRL B-1299 possess a linear chain of α-(1→6) with α-(1→2) branches. An in vivo study by Hasselwander et al. show that the presence of α-(1→2) branches increases the resistance to digestion (Hasselwander et al., 2017). Isomaltooligosaccharides are partially digestible oligosaccharides with α-(1→6) and α-(1→4) linkages (Hu et al., 2013).

In addition to functional oligosaccharides, different polysaccharides produced by glucansucrases have been reported to have dietary fibre properties. The products from 4,6-α-glucanotransferase contain a high percentage of α-(1→6) when debranched starches are used as substrates. When potato amylose is available as acceptor, 4,6-α-glucanotransferase synthesize isomalto/malto polysaccharides which contain up to 92% of α-(1→6) linkages and these polysaccharides are

considered as soluble dietary fibers as they show resistance to digestion in gastrointestinal tract thus reach colon (Leemhuis et al., 2014). Dextransucrase from *Weissella cibaria* RBA12 produced dextran with 97% α-(1→6) linkages in their main chain and 3% α-(1→3) branches. In the Baruah et al. (2017) study, dextran showed high resistance to human digestive enzymes but fermented in the colon and stimulated growth of beneficial microbes (Baruah et al., 2017). The presence of the α-(1→6) linkages in the dextran backbone is responsible for this digestion resistance in the gastrointestinal tract of human (Kothari et al., 2015).

1.4 Knowledge Gap

LAB glucansucrases of GH70 family especially dextransucrases have been widely studied. According to (http://www.cazy.org/GH70.html) more than 75% of GH70 enzymes characterized are dextransucrases whereas branching sucrases are underrepresented in this family. In this family, another group of enzymes, glucanotransferases attracted interest because of their ability to use starches and starch-delivered molecules as substrates, thus a large range of substrates have been tested on these enzymes to understand their activity (Z. Chen et al., 2021). In contrast, branching sucrases of GH70 have been studied on a limited number of substrates.

Currently, only six branching sucrases have been characterized and among them, four have a detailed characterization such as DsrE-CD2, Brs-A, Brs-B and GtfZ-CD2 (Brison et al., 2012; Meng, Gangoiti, Wang, et al., 2018a; Passerini et al., 2015; Vuillemin et al., 2016). In addition to the relatively low number of characterized branching sucrases, all the studies have only tested enzymes on sucrose and dextran. There is no reported study demonstrating the activity of branching sucrases on other polysaccharides except dextran. Therefore, there is no available knowledge on how branching sucrase might act in presence of other polysaccharides including amylose and reuteran.

1.5 Hypotheses and Objectives

Hypotheses

- The transferase activity of *A. kunkeei* 12361 dextransucrase and branching sucrase is dependent on the linkage type present in the acceptor.
- *A. kunkeei* 12361 dextransucrase and branching sucrase is higher in the presence of α-(1→6) over α-(1→4) linkages in the acceptor molecule.
- *A. kunkeei* 12361 dextransucrases and branching sucrase are active on potato amylose.

Objectives

- Evaluate the activity of *A. kunkeei* 12361 dextransucrase and branching sucrase on different substrates and understand the influence of linkages available in the acceptor molecule on the enzyme activity.

2 Materials and methods

2.1 Bacterial growth condition and DNA isolation.

A. kunkeei DSM 12361 was cultured anaerobically for 48 h at 30 °C in mMRS media containing 10 g/L, 5 g/L glucose and 5 g/L fructose. *A. kunkeei* DSM 12361 DNA was extracted with DNeasy Blood & Tissue Kit (Qiagen, Toronto, ON, Canada). *E. coli* strain DH5α carrying pET28a(+) plasmid was cultured aerobically for 16 h at 37 °C in Luria broth supplemented with 50 mg/L kanamycin. pET28a(+) bacterial plasmid DNA was extracted with QIAprep Spin Miniprep Kit (Qiagen, Toronto, ON, Canada). *E. coli* BL21 (DE3) containing plasmids pET28a(+) carrying reuteransucrase gtfA, and mutants ΔN gtfA (S1135N:A1137S mutation), pET28a+ carrying ΔN gtfA (V1024P:V1027I:S1135N:A1137S mutation) (X. Y. Chen et al., 2016) were cultured aerobically for 16 h at 37 °C in Luria broth supplemented with 50 mg/L kanamycin. E. coli BL21 (DE3) with pET28b(+) plasmids carrying branching enzyme *glgB* was cultured aerobically for 16 h at 37 °C Luria broth supplemented with 50 mg/L kanamycin.

2.2 Cloning and transformation

The glucansucrase *gtfZ* gene was identified in *A. kunkeei* DSM 12361 by nucleotide blast from *A. kunkeei* (KRK22577.1) (Meng et al. 2018). Fragments of *gtfZ* such as CD1-GBD (4083bp) and GBD-CD2 (4020bp) (Figure 1) were amplified using the primers pairs shown in **Table 1** and ligated fragments were constructed in pET28a(+) plasmid at XhoI and BamHI restriction sites. PCR amplicons were digested with restriction endonucleases XhoI and BamHI (Thermo Scientific, Ottawa, Canada) and ligated with T4 ligase (Thermo Scientific) according to the manufacturers' protocols, generating the plasmids pET28a(+)-CD-GBD and pET28a(+)-GBD-

CD2. pET28a(+)- CD1-GBD and pET28a(+)-GBD-CD2 were transformed in *E.coli* BL21 star (DE3) (Invitrogen, Toronto, ON, Canada) as described (Meng et al. 2018).

Cloning of chimeric glucansucrases and glucanotransferases was performed by Dr. Julia Bechtner and constructs GBD-GtfZ, SBD-GtfZ, GtfZ-A and GtfZ-B were provided for protein expression. Briefly, GBD-GtfZ resulted from a replacement of glucan-binding domain of the *gtfZ* branching sucrase by the glucan-binding domains of the reuteransucrase of *Lm. reuteri* TMW 1.656. The replacement of glucan-binding domains of the *gtfZ* branching sucrase part were replaced by the glucan-binding domains of the 4,6-α- glucanotransferase of *Lm. reuteri* DSM 20016 resulted in SBD-GtfZ. For GtfZ-A, the catalytic GH70 domain of *gtfZ* branching sucrase was replaced by GH70 catalytic domain of reuteransucrase of *Lm. reuteri* TMW 1.656. GtfZ-B represents the enzyme that results from replacing the catalytic GH70 domain of *gtfZ* branching sucrase with the GH70 catalytic domain of *Lm. reuteri* DSM 20016 4,6-α-glucanotransferase (**Figure 2.2**).

Table 2.1 Primers used in cloning of GtfZ of *A. kunkeei* DSM12361. The lower-case sequences represent the 5' extension of primers to introduce the restriction site in pET28a+ as indicated.

Primer	Sequence (5' – 3')	Restriction site
CD1-GBD forward primer	ctgtctcgagATTTGTTTCACTTTCACCAA	XhoI
CD1-GBD reverse primer	ccgggatccatgAACAACACATACTATTAT	BamhI
GBD-CD2 forward primer	cctgtctcgagATCATCAAAACTATTTCTATAAG	XhoI
GBD-CD2 reverse Primer	ccgggatccatgAATGTTGAATATGGTTTA	BamhI

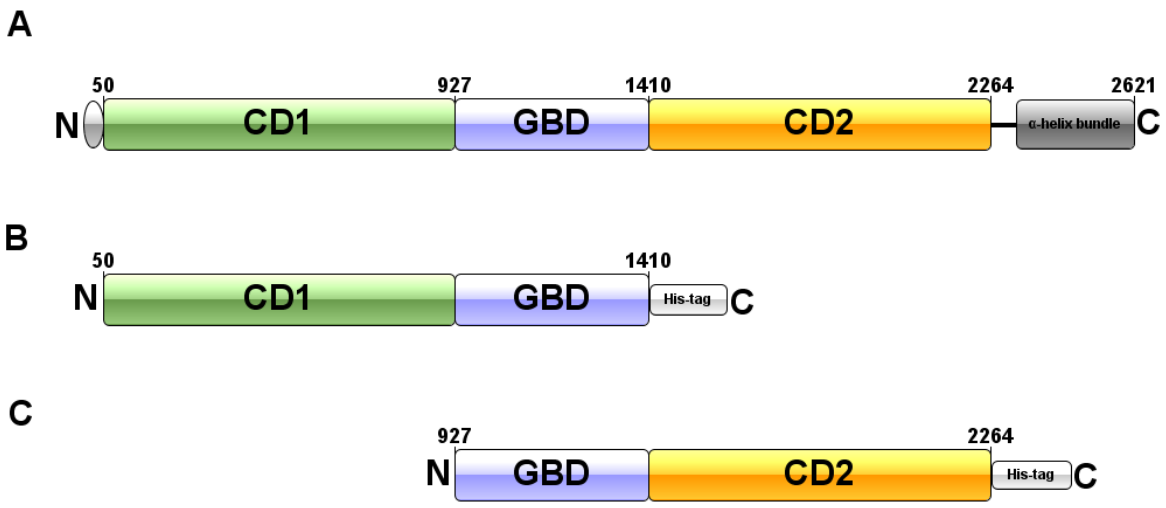

Figure 2.1 Linear representation of the domains in *GtfZ* of *A. kunkeei* DSM 12361 (A). CD1 and CD2 are connected by glucan-binding domain (GBD). An α-helical bundle is present on the C-terminus of *GtfZ* and 50 amino acid residues on N-terminus with unknown function**.** The constructs for the his-tagged dextransucrase (CD1-GBD) (B) and branching sucrase (GBD-CD2) (C) are represented in the figure.

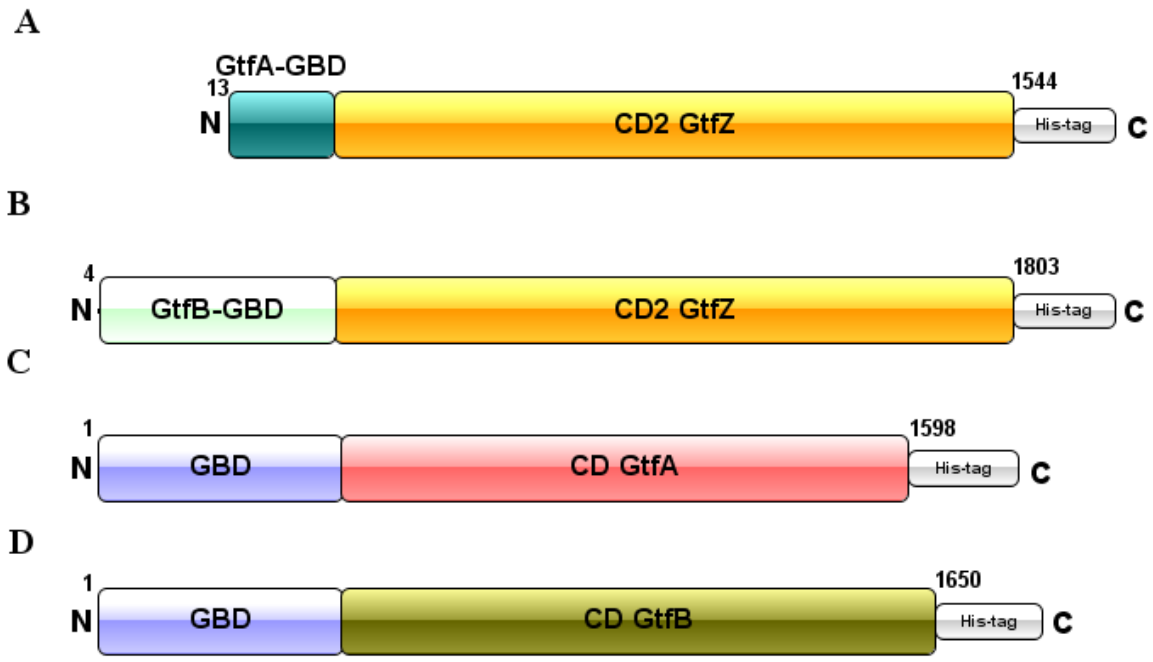

Figure 2.2 Linear representation of domain swapping. Glucan-binding domain of the GtfZ branching sucrase part were replaced by the glucan-binding domains of the reuteransucrase GtfA of *Lm. reuteri* TMW 1.656 (**GtfA-GBD**) (**A**). Glucan-binding domains of the GtfZ branching sucrase part were replaced by the glucan-binding domains of the 4,6-α-glucanotransferase GtfB (**GtfB-GBD**) of *Lm. reuteri* DSM 20016 (**B**). Catalytic GH70 domain of GtfZ branching sucrase was replaced by GH70 catalytic domain of reuteransucrase of *Lm. reuteri* TMW 1.656 (**C**), Catalytic GH70 domain of GtfZ branching sucrase was replaced by GH70 catalytic domain of *Lm. reuteri* DSM 20016 4,6-alpha-glucanotransferase (**D**). Every domain swapping construct has his-tag on the C-terminus.

2.3 Protein extraction and purification

Luria broth media supplemented with 50 mg/L kanamycin and 250 mM sorbitol was inoculated with overnight cultures of transformed constructs. The cultures were incubated in a shaking incubator at 37 °C until OD600 nm reached 0.4 - 0.6. Protein expression was induced by 0.2 mM isopyl-B-D-1-thiogalactobapyrnoside and incubated in a shaking incubator at 200 rpm for 12 h at 20 °C. Cells were harvested by centrifugation (5000 rpm, 10 min, 4°C) disrupted by bead beating. Cell debris was removed by centrifugation to obtain the crude protein extracts. Protein extracts were purified by Ni-NTA anion-exchange chromatography using HisPur Ni-NTA Spin Columns (Thermo Scientific, Ottawa, Canada). Protein extract was prepared by mixing with the equilibrium buffer of 10 mM imidazole until two resin-bed volumes and loaded to the equilibrated columns with two resin-bed volume of equilibrium buffer of 10mM imidazole. Protein extracts were washed with 25 mM imidazole and purified protein was eluted with elution buffer of 250 mM imidazole. The SDS page was conducted (Bio-Rad, Mississauga, ON, Canada) to assess the purity of the protein. The same methods were used for chimeric glucansucrases and glucanotransferases. Reuteransucrase GtfA, and mutants ΔN gtfA (S1135N:A1137S mutation), pET28a+ carry ΔN gtfA (V1024P:V1027I:S1135N:A1137S mutation) were purified as described by (X. Y. Chen et al., 2016). Protein concentration was determined using protein assay reagent (Bio-Rad) and serum albumin was used as standard. Branching enzyme *glgB* was extracted and purified as described by (Shao et al., 2023).

2.4 Enzyme activity assays

Modified amylose was produced from a reaction of potato amylose with 200 nM *glgB* in 50 mM phosphate buffer (pH 7.4) at 37 °C overnight as by Shao et al. (2023) Reuteran was produced by incubating 200 nM reuteransucrase GtfA with 50 mM sucrose and 20 g/L dextran in 50 mM

acetate buffer (pH 4.84) supplemented with 1 mM $CaCl_2$. The reaction was conducted at 37 °C overnight. Reuteran from wild type GtfA was produced by incubating dextran and sucrose with the enzyme. For reuteran from mutant GtfA was produced by incubating the mutants GtfA with sucrose.

The total and hydrolytic enzymatic activities were measured by the release of fructose and glucose respectively (Meng, Gangoiti, Wang, et al., 2018) **.** The transferase activity was calculated by subtracting the hydrolytic activity from total activity. One unit of enzyme activity was defined as the release of 1 μmol of glucose or fructose per min.

Enzyme activity assays for dextransucrase and branching sucrase were conducted at 30 °C in 50 mM acetate buffer (pH 5.42) supplemented with 1 mM $CaCl_2$. Reactions were conducted with 200 nM enzymes, 50 mM sucrose as a donor substrate and 20 g/L acceptor substrate including dextran, reuteran, reuteranNS, reuteranPINS, modified amylose and amylose **(Table 3).** The reaction conditions for reuteransucrase were similar to dextransucrase and branching sucrase except the buffer pH and incubation temperature were 4.84 and 37 °C, respectively. Samples were collected every 3 min for 15 min and the enzyme was deactivated by heating at 95 °C for 10 min. The concentration of fructose and glucose was measured by the release of NADPH using Glucose and Fructose Assay Kit (Sigma-Aldrich, Oakville, ON, Canada).

Table 2.2 Substrates used in enzymatic reactions and their corresponding linkage types.

Acceptor substrates	Linkage %		
	α-(1→4)	α-(1→6)	α-(1→4,6)
Dextran	-	>95 [a)]	
Reuteran	11	89[b)]	
ReuteranNS	39	61[c)]	
ReuteranPINS	51	49[c)]	
Modified amylose	96		4[d)]
Amylose	100	-[a)]	

[a)] Based on specifications of SigmaAldrich

[b)] Based on NMR analysis.

[c)] (X. Y. Chen et al., 2016)

[d)] (Shao et al., 2023)

2.5 Polysaccharide purification

Polysaccharide purification was achieved by ethanol precipitation and dialysis. Briefly, polysaccharides were precipitated by adding two volumes of 70 % chilled ethanol, followed by centrifugation (5000 rpm, 15 min, 4 °C). The precipitate was redissolved in demineralized water, and two volumes of cold ethanol were added. After centrifugation, the polysaccharide materials were dissolved in demineralized water and purified by dialysis using membrane tubing with molecular weight cut-off 3500 (Spectra/Por 3 membrane tubing; Spectrum Laboratory Inc., Rancho Dominguez, CA, USA) for 28 hours to remove monosaccharides, disaccharides, and oligosaccharides. The final polysaccharides were freeze dried, the amount was determined by measuring the wight of the dry matter and stored at -20 °C for further analysis.

2.6 Product Analysis by NMR

Purified polysaccharides from reactions of GtfA with sucrose and modified amylose, GtfA with sucrose and dextran, and GtfA with only sucrose. were exchanged in 99.99 % D_2O then samples were sent for NMR analysis (NMR Facility, University of Alberta). One dimensional ^1H NMR spectra were generated in D_2O at a probe temperature of 27 °C, and chemical shifts were recorded in parts per million with reference to external acetone at δ 2.225. Integration of the surface area was used to determine the ratio of different glycosidic linkages.

2.7 Statistical Analysis

Differences in enzyme activities with respect to the substrates (n = 3) were analyzed using one-way analysis of variance (ANOVA) followed by Bonferroni test in SAS version 9.4 (SAS Institute Inc., Cary, NC, USA).

3 Results

3.1 Cloning and protein purification of dextransucrase, branching sucrase, chimeric glucansucrases and glucanotransferases

Truncated dextransucrase CD1-GBD (amino acid residue 50 - 1410) and branching sucrase GBD-CD2 (amino acid residue 927 – 2264) were cloned from *A. kunkeei* DSM 12361 (**Figure 2.1**). CD1-GBD and GDB-CD2 were successfully expressed in *E. coli* BL21 DE3 star. After purification with Ni-NTA anion-exchange chromatography, SDS-PAGE analysis of CD1-GBD and GBD-CD2 resulted in bands that matched the expected molecular masses of 151 kDa and 148 kDa (**Figure 3.1**). Protein expression of chimeric glucansucrases and glucanotransferases was successful; however, after multiple attempts to purify the protein, no pure band was obtained, therefore no further analysis was performed.

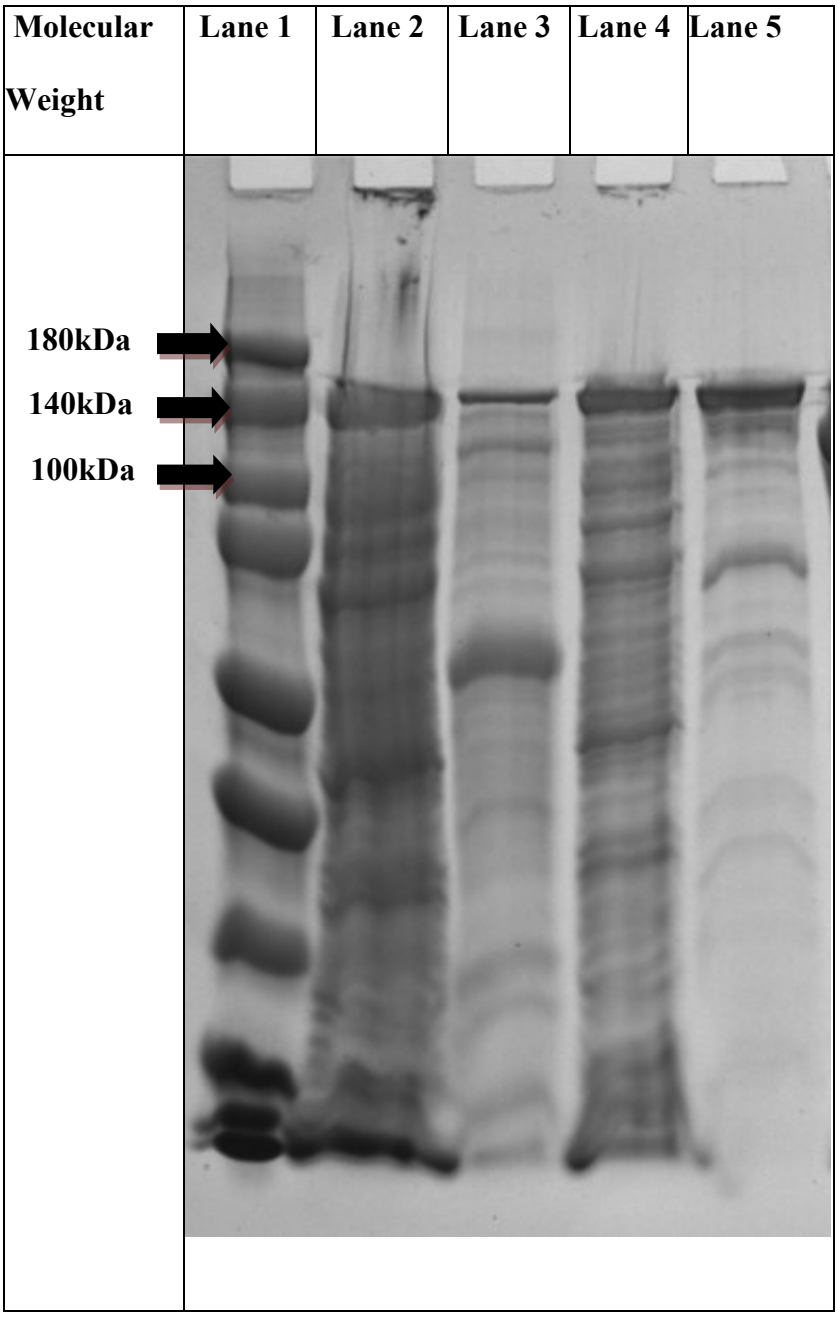

Figure 3.1 SDS-PAGE of CD1-GBD and GDB-CD2. Lane 1 is the protein ladder, Lane 2 represents CD1-GBD flow through, Lane 3 represents eluted CD1-GBD. Lane 4 is the flow through of GDB-CD2 and Lane 5 is the eluted protein GDB-CD2.

3.2 Activity of dextransucrase, branching sucrase and reuteransucrase with different acceptor polymers

Dextransucrase has high transferase activity in presence of dextran and reuteran but not in presence of amylose (**Figure 3.2**). The branching sucrase has high transferase activity with dextran and reuteran but not with amylose (**Figure 3.3)**. Reuteransucrase has high transferase activity in presence of dextran, reuteran and amylose (**Figure 3.4)**. For both branching sucrase and dextransucrase, the transferase activity was higher when dextran was used as an acceptor molecule compared to when sucrose was used a sole substrate and when reuteran, modified amylose and amylose were used as acceptor substrates (**Figure 3.2 & 3.3).** Reuteransucrase is active on α-(1→4) and α-(1→6) linked substrates while the dextransucrase and branching sucrase are specific for α-(1→6) linkages. Dextransucrase activity is higher with reuteran than with modified amylose (**Figure 3.2**). Branching sucrase transferase activity is higher with modified amylose compared to when reuteran is available as acceptor molecule (**Figure 3.3**).

A decrease in branching sucrase and dextransucrase activity from reuteran with high percentage of α-(1→6) to the ones with less percentage (**Figure 3.5 & 3.6**). Dextransucrase is more active on reuteran with high percentage α-(1→6) and slightly goes down as the percentage decrease. The decrease is observed for branching sucrase, however the as for dextransucrase. For reuteransucrase, the transferase activity was high to all substrates used **(Figure 3.7**).

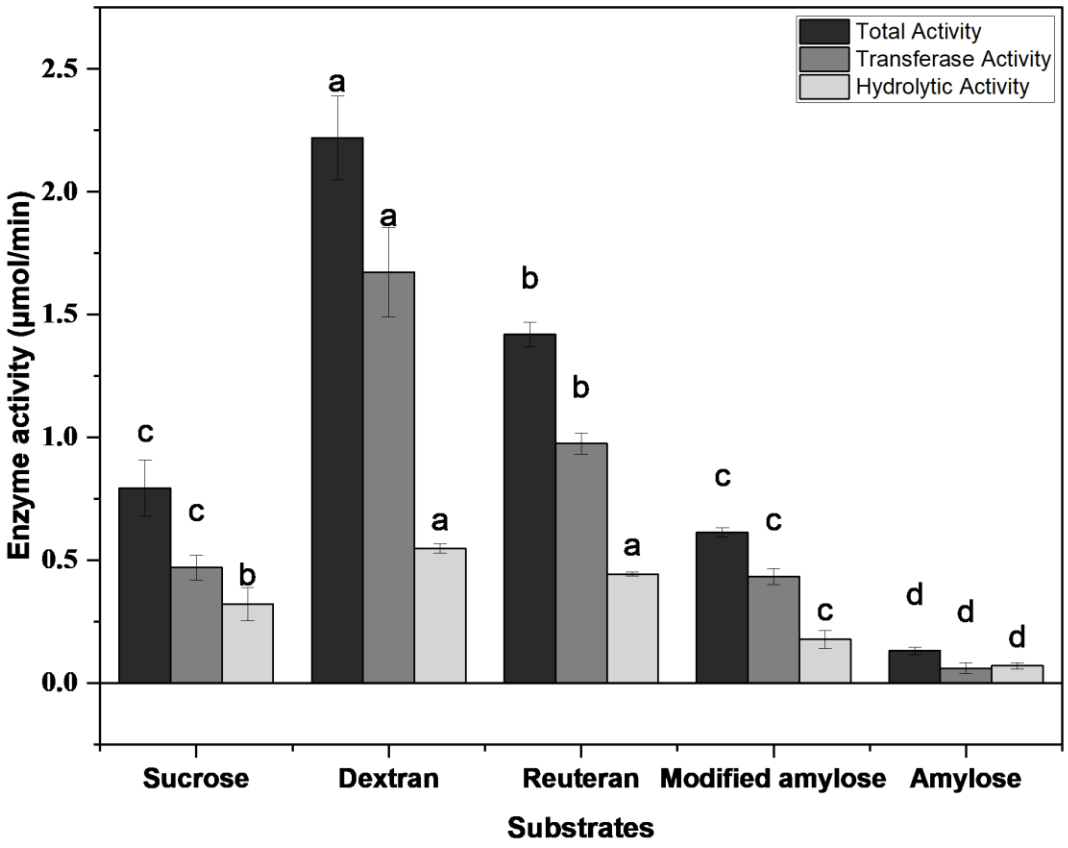

Figure 3.2 Dextransucrase activity (**CD1-GBD**) in the presence of sucrose as sole substrate, and when dextran, reuteran, modified amylose, and amylose are available as acceptor molecules of glycosyl group from sucrose.

a, b, c Different letters for enzyme activity indicate a significant difference among the mean values ($p < 0.05$).

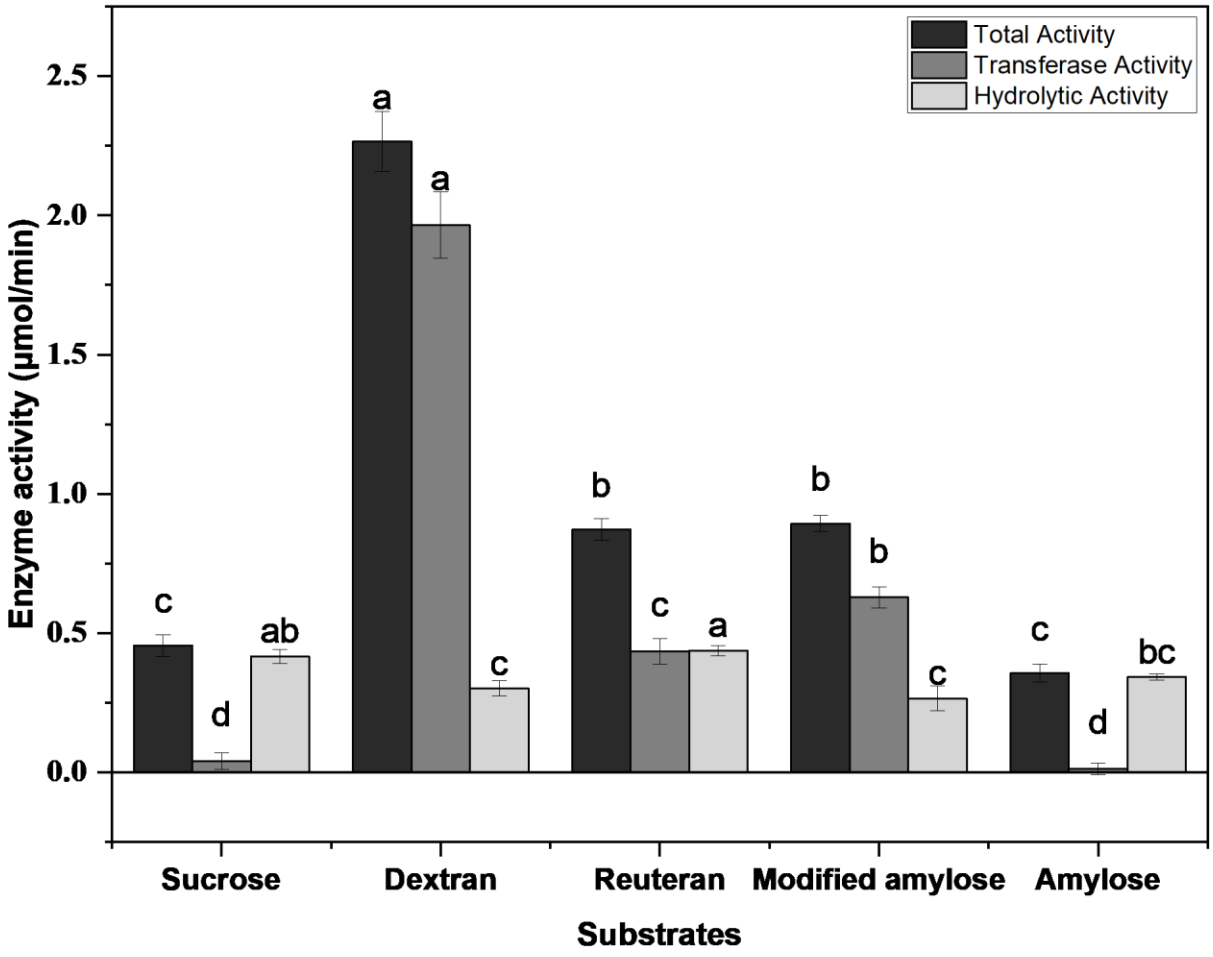

Figure 3.3 Branching sucrase activity (**GBD-CD2**) in the presence of sucrose as sole substrate, and when dextran, reuteran, modified amylose, and amylose are available as acceptor molecules of glycosyl group from sucrose.

a, b, c Different letters for enzyme activity indicate a significant difference among the mean values ($p < 0.05$).

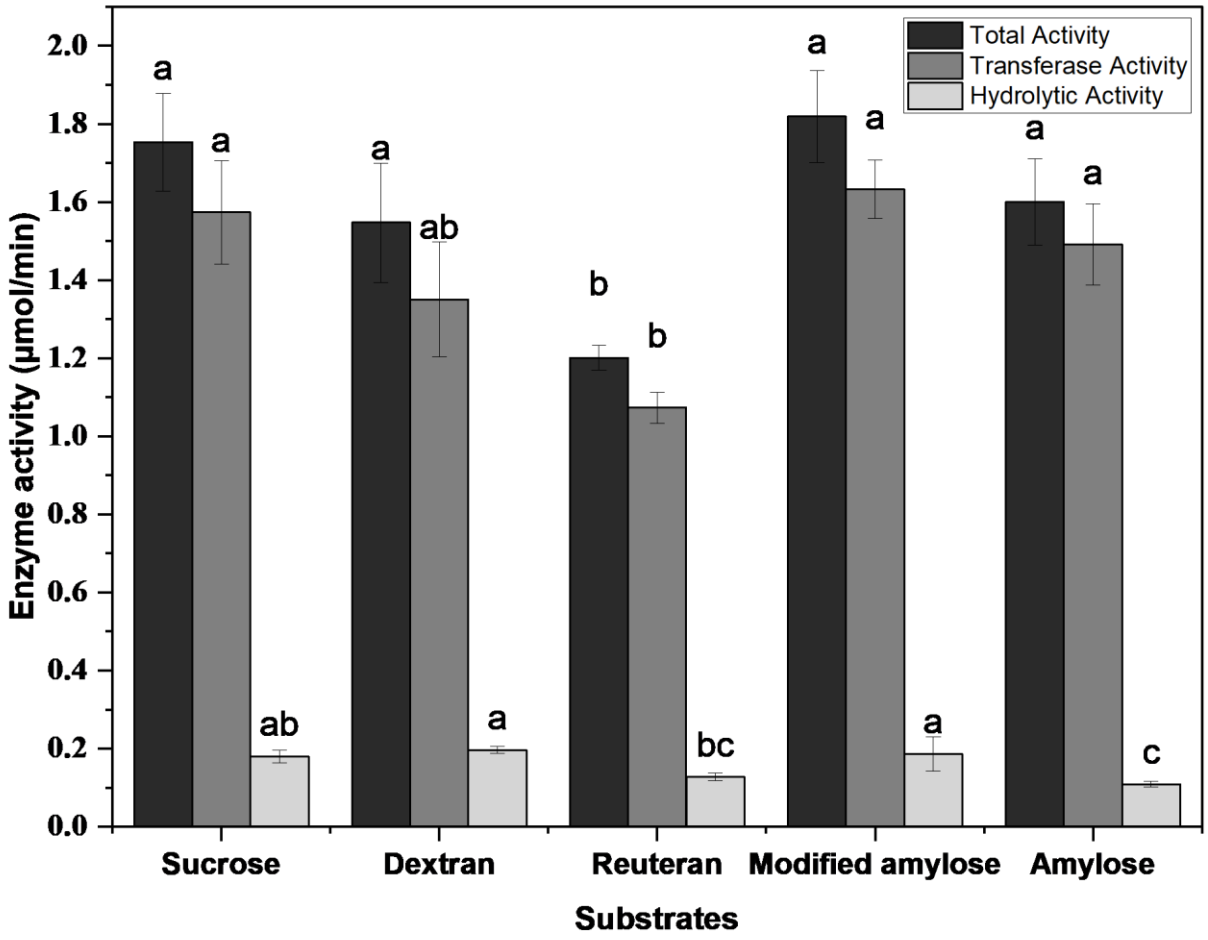

Figure 3.4 Reuteransucrase activity (**GtfA**) in the presence of sucrose as sole substrate, and when dextran, reuteran, modified amylose, and amylose are available as acceptor molecules of glycosyl group from sucrose.

a, b, c Different letters for enzyme activity indicate a significant difference among the mean values ($p < 0.05$).

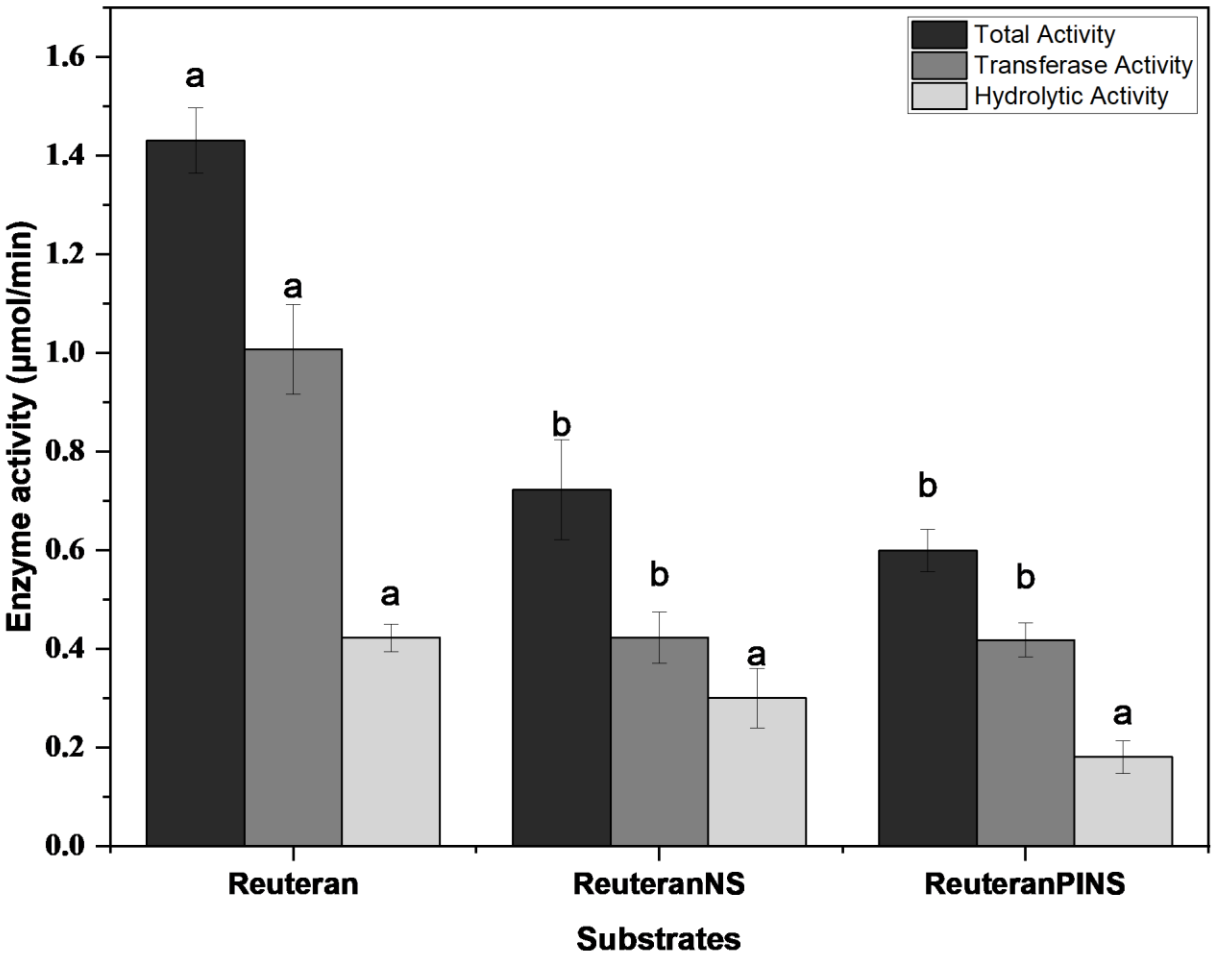

Figure 3.5 Dextransucrase activity (**CD1-GBD**) when reuteran (88 % α-(1→6)), reuteranNS (61 % α-(1→6)) and reuteranPINS (49 % α-(1→6)) are available as acceptor molecules for glucosyl group from sucrose.

a, b, c Different letters for enzyme activity indicate a significant difference among the mean values ($p < 0.05$).

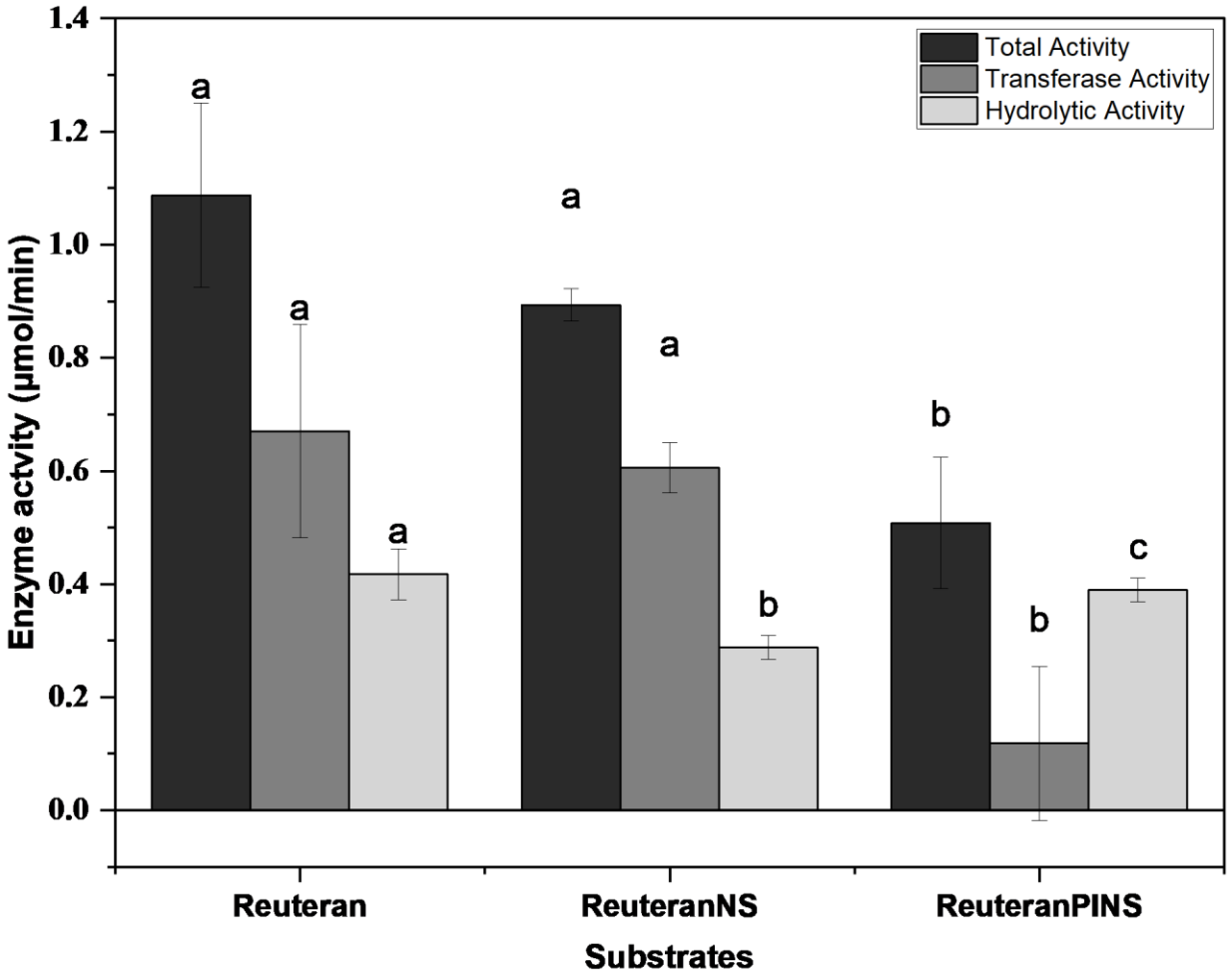

Figure 3.6 Branching sucrase activity (**GBD-CD2**) when reuteran (88 % α-(1→6)), reuteranNS (61 % α-(1→6)) and reuteranPINS (49 % α-(1→6)) are available as acceptor molecules for glucosyl group from sucrose.

a, b, c Different letters for enzyme activity indicate a significant difference among the mean values ($p < 0.05$).

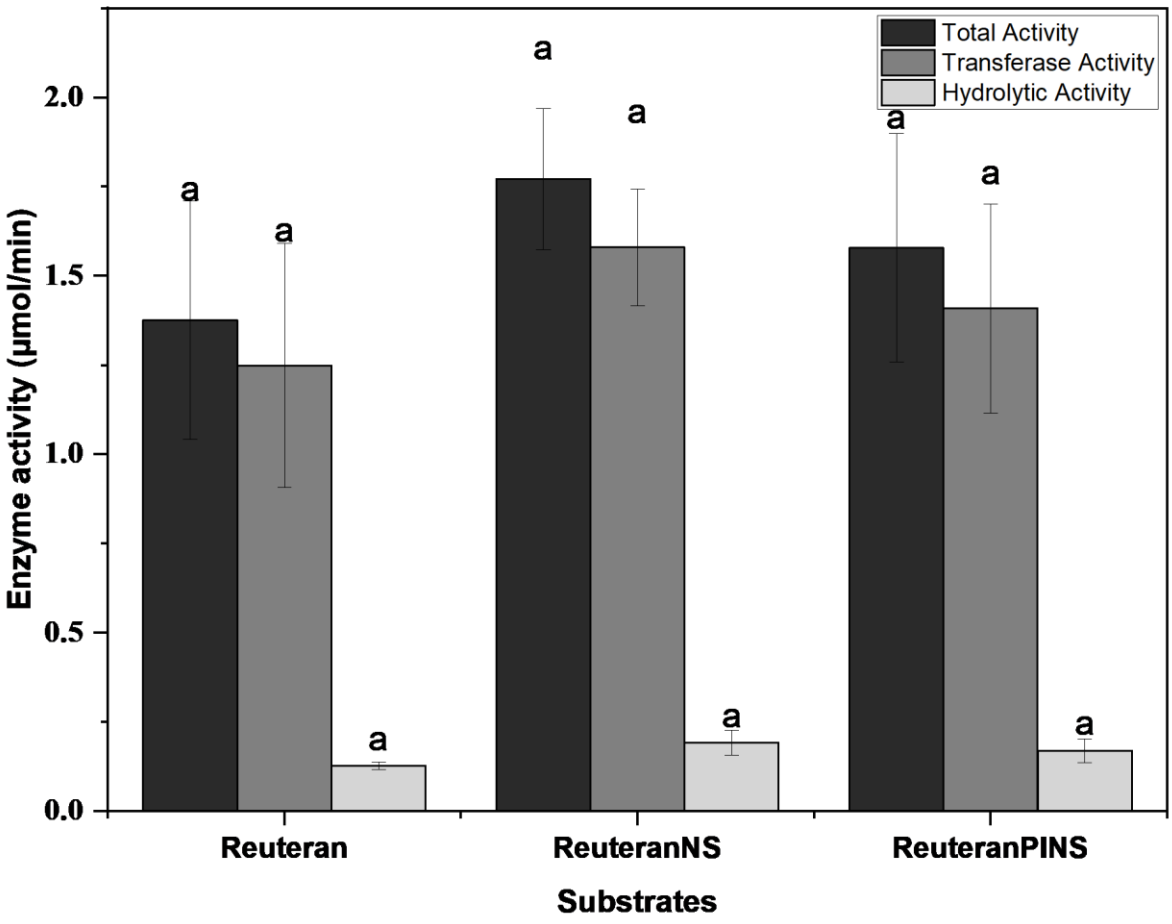

Figure 3.7 Reuteransucrase activity (**GtfA**) when reuteran (88 % α-(1→6)), reuteranNS (61 % α-(1→6)) and reuteranPINS (49 % α-(1→6)) are available as acceptor molecules for glucosyl group from sucrose.

a, b, c Different letters for enzyme activity indicate a significant difference among the mean values ($p < 0.05$).

3.3 Polysaccharides ^{1}H NMR Analysis

Polysaccharide isolated from incubation of GtfA with sucrose and modified amylose, has a high percentage of α-(1→6) compared to α-(1→4). The α-(1→6) signal is displayed at 4.956 ppm and α-(1→4) is at 5.297 ppm (**Figure 3.8**). Reuteran synthesized from sucrose has 84 % of α-(1→6) with δ 4.955 and 16 % of α-(1→4) with δ 5. 297 (**Figure 3.9**). When dextran and sucrose were as substrates for GtfA, the polysaccharide produced had 89 % of α-(1→6) (**Figure 3.10**) which is slightly high compared to when *gtfA* reacted with only sucrose (**Figure 3.9**). The signals α-(1→6) and α-(1→4) were displayed at 4.953 ppm and 5.297 ppm, respectively (**Figure 3.10**).

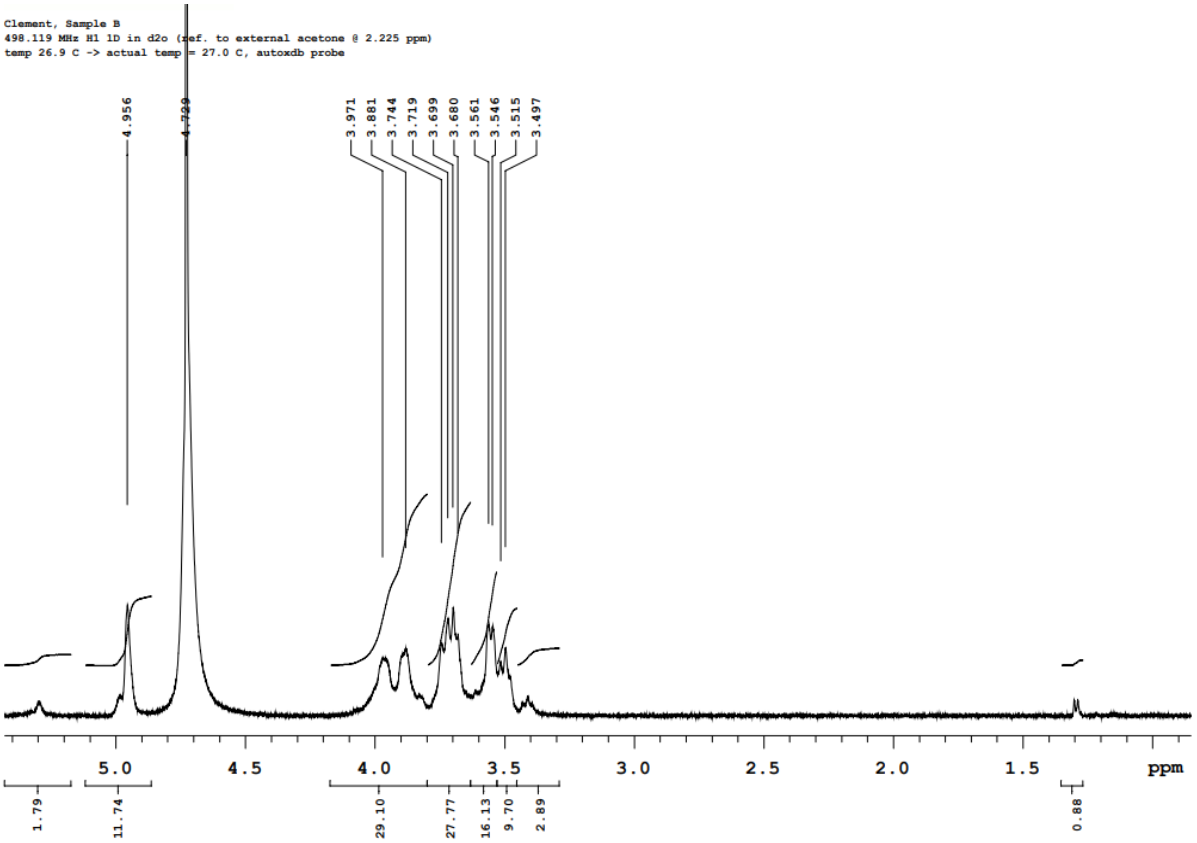

Figure 3.8 ^{1}H NMR analysis of isolated polysaccharide produced by incubation of GtfA with sucrose and modified amylose. The α-(1→6) signal is displayed at 4.956 ppm and α-(1→4) is at 5.297.

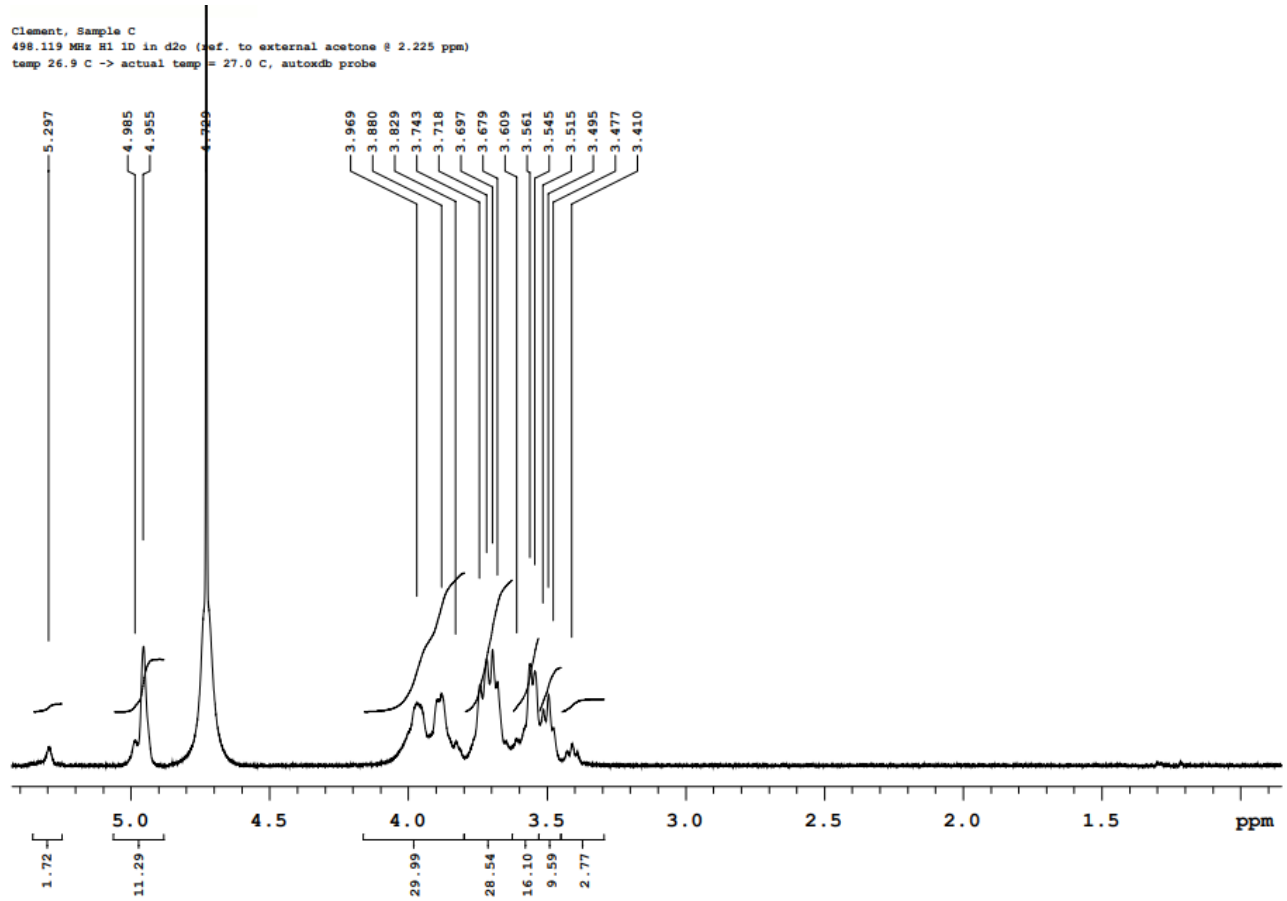

Figure 3.9 ^{1}H NMR analysis of isolated polysaccharide produced by incubation of GtfA with only sucrose as the substrate, the α-(1→6) signal is displayed at 4.955 ppm and α-(1→4) with 5. 297 ppm.

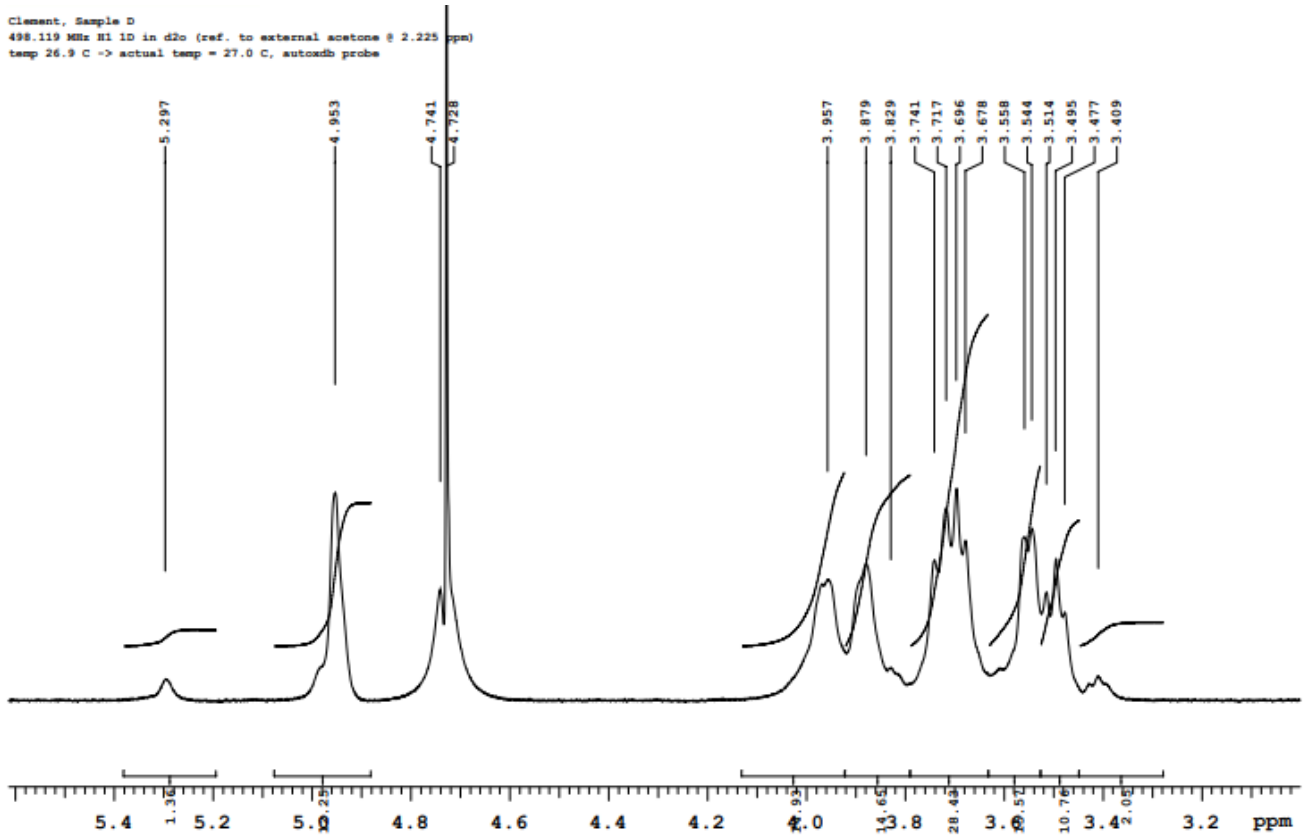

Figure 3.10 ^{1}H NMR analysis of isolated polysaccharide produced by incubation of GtfA with sucrose and dextran. The signals α-(1→6) and α-(1→4) were displayed at 4.953 ppm and 5.297 ppm, respectively.

4 Discussion

Although many GH70 glucansucrases have been studied in general, with a majority being dextransucrases, only a few branching sucrases have been characterized. In addition, dextransucrases and reuteransucrases have only been characterized using sucrose as the glucosyl donor and diverse oligosaccharides as acceptors. Different polysaccharides have not been evaluated as acceptor molecules. Likewise, the characterization of branching sucrases to date has only used dextran as an acceptor, and the preference of these enzymes in terms of the linkage type or molecular size of the acceptor molecules remains unknown. To investigate the preferences of dextransucrase and branching sucrase on linkage type and their activity on amylose, enzymatic assays were conducted on a range of substrates with different ratios of α-(1→6) and α-(1→4) glycosidic linkages. Recently, glucansucrase GtfZ harboring two catalytic domains, CD1 and CD2 was characterized from *A. kunkeeii* DSM 12361 and these catalytic domains are interconnected by a glucan-binding domain GBD (Meng, Gangoiti, Wang, et al., 2018). The ability of GBD to bind dextran was previously reported by Brison et al (Brison et al., 2016), CD1 has dextransucrase specificity while CD2 has branching sucrase specificity (Meng, Gangoiti, Wang, et al., 2018). In addition to GtfZ, DsrE of *Leuconostoc citreum* NRRL 1299 with both dextransucrase and branching sucrase has been characterized. Catalytic domains in GtfZ and DsrE are connected by glucan-binding domain (Fabre et al., 2005; Meng, Gangoiti, Wang, et al., 2018). This domain arrangement suggests that the dextran intermediate product is synthesized by dextransucrase and carried by a glucan-binding domain toward branching sucrase where the branches are introduced (Brison et al., 2016).

In the recent study by (Meng, Gangoiti, Wang, et al., 2018) a dextransucrase construct was created with a combination of CD1 and part of glucan-binding domain, and the branching

sucrase was made of a remaining part of a glucan-binding domain combined with CD2. Contrary, in the current study, the constructs for dextransucrase and branching sucrase was a combination of the glucan binding domain in a full length with catalytic domain responsible for dextransucrase specificity (CD1-GBD) and catalytic domain with branching sucrase activity combined with a glucan binding domain (GBD-CD2) **(Figure 3.3**). A combination of catalytic domains and glucan binding domain resulted in proteins that were difficult to express under the conditions used in Meng et al. probably because of the high molecular weight of 151 kDa and 148 kDa for CD1-GBD and GBD-CD2, respectively. According to Meng et al. (2018) protein was expressed using the incubation time of 20 h and resulted in protein in inclusion body (Meng, Gangoiti, Wang, et al., 2018). In contrast, lowering the expression time to 12 h and supplementing the medium with 250 mM sorbitol, resulted in a successful expression of CD1-GBD (**Figure 3.1**).

All reported dextransucrases (**Table 1**) have glucan binding domain on their C-terminal with full repeats. In contrast, the reported branching sucrase have full repeats (Brison et al., 2012; Passerini et al., 2015; Vuillemin et al., 2016) or partial repeats (Meng, Gangoiti, Wang, et al., 2018) of glucan binding domains that are used in binding glucan. However, different small molecules including disaccharides such as maltose and isomaltose are good acceptors for dextransucrase, different from leucrose which is not an acceptor for dextransucrase (Dols et al., 1997; Robyt & Eklund, 1982). Similarly, in one study, branching sucrase GtfZ-CD2 was tested, and it was active on isomaltotriose as acceptor substrate (Meng, Gangoiti, Wang, et al., 2018). The ability of dextransucrase and branching sucrase to transfer glycosyl units to these small molecules shows that the acceptor molecules do not necessarily need to bind to the glucan binding domain of the enzyme, as long as a good substrate is available.

Dextransucrase CD1-GBD had low activity when sucrose was used as a sole substrate compared to when dextran was available in the reaction (**Figure 3.2**), which suggests that the glucosyl unit from sucrose hydrolysis is being transferred to the dextran to make a high molecular weight polysaccharide. This finding was not expected because dextransucrases were previously shown to be active with only sucrose as sole substrate (Bounaix et al., 2010; Naessens et al., 2005; Rühmkorf et al., 2013). When there is an adequate amount of glucose donor molecule (sucrose), polymerization continues and dextran is formed unless an acceptor molecule causes the release of dextran from the active site (Robyt & Eklund, 1982). Contrary, in this study, it was unusual to notice that the activity of dextransucrase was favored by the presence of dextran in the reaction. The observed unexpected results of dextransucrase activity, where the activity was low with only sucrose compared to when dextran was available might be explained by the kinetics of enzymatic reaction. Briefly, the reaction starts hydrolysis of sucrose which yields glucose and glucose is not a good acceptor. However, as the reaction continues, there is a synthesis of isomaltose which is a suitable acceptor compared to glucose. (Hu et al., 2017). When isomaltose is available as an acceptor substrate, the growing polymer can be extended and contribute to the formation of a high molecular weight polymer. Therefore, conducting a reaction for only 15 min is the main reason for the low dextransucrase activity on sucrose as there might be not enough glucose to glycosylates substrate and growing polymer.

Branching sucrase GBD-CD2 has shown low transferase activity when only sucrose was used as a substrate, mainly hydrolytic activity was dominant (**Figure 3.3**). The GBD-CD2 activity was low because branching sucrase requires the presence of acceptor molecules in order to synthesize polysaccharides and a study by Meng et al has reported that branching sucrase prefer dextran as acceptor, specifically (Meng, Gangoiti, Wang, et al., 2018). The branching sucrase preference of

dextran was also observed in our study because compared to other acceptor molecules used in the study, GBD-CD2 activity was high when dextran was available in the reaction. This confirms ability of branching sucrase to transfer glycosyl unit to dextran to form a highly branched dextran (Meng, Gangoiti, Wang, et al., 2018). Depending on the concentration of sucrose in the reaction, sometimes the accumulation of glucose from sucrose hydrolysis can result in a transfer to fructose thus increasing the transferase activity with only sucrose as sole substrate; however, leucrose does not act as an acceptor substrate. Therefore, there will be no synthesis of polysaccharides from leucrose (Vuillemin et al., 2016). Passerini et al reported that with sucrose as sole substates and the branching sucrase BRS-A as catalyse, glucose, leucrose and other oligosaccharides were produced (Passerini et al., 2015). The DsrE branching sucrose catalytic domain did not produce any polymer from sucrose, only sucrose hydrolysis or a formation of leucrose from a transfer of glycosyl residue to the previously produced fructose. Also, DsrE branching sucrase did not show any activity on maltose which is different from other glucansucrases, however the activity was observed when dextran and glucooligosaccharides were used as acceptor substrates (Fabre et al., 2005). This is similar to the recently characterized branching sucrases, as they are not able to synthesize polymers from sucrose alone as a sole substrate (Meng, Gangoiti, Wang, et al., 2018; Vuillemin et al., 2016).

Reuteransucrase transferase activity was not different when sucrose was used as a sole substrate, and when dextran, modified amylose and amylose were used as acceptor molecules (**Figure 3.4**). The reason might be that GtfA is only using sucrose available in the reaction instead of dextran and the activity being observed might be from the synthesis of polysaccharides with similar characteristics as reuteran produced from GtfA and sucrose. The ^{1}H NMR data show that the polysaccharides from a reaction with dextran and sucrose and GtfA from only sucrose (**Figure**

3.9) were similar; however, for GtfA with dextran and sucrose in the reaction the percentage of α-(1→6) was 89 % (**Figure 3.10**), a sign that the polysaccharide being synthesized is reuteran even though dextran was used in the reaction as an acceptor substrate. The explanation for this might be that reuteransucrase is already bound to the substrate and starts to synthesize polymer and leaves no space for the dextran to bind. It also shows how sucrose is a good substrate for reuteransucrase (Kralj et al., 2005). In the presence of only sucrose, reuteransucrase GtfO of *Limosilactobacillus* did not convert all sucrose into polymer as 50 % of 100 mM of sucrose were converted into glucose and there was a production of leucrose and isomaltose (Kralj et al., 2005). However, for GtfA a high amount of reuteran is produced when the same concentration of sucrose is used in the reaction (Kralj et al., 2005; Kralj, van Geel-Schutten, van der Maarel, et al., 2004).

The presence of reuteran with 89 % α-(1→6) and 11 % α-(1→4) (produced from a reaction of GtfA with dextran and sucrose) as acceptor substrate for CD1-GBD resulted in high transferase activity (**Figure 3.2**) which is from the transfer of a glycosyl unit to reuteran. The presence of a good acceptor substate reduce the synthesis of dextran polymer and can even inhibit the formation of dextran as the concentration increases (Seibel & Buchholz, 2010). Even though the transferase activity was observed when GBD-CD2 reacted with reuteran, there is a decrease in activity compared to when dextran was used as acceptor substrate for GBD-CD2. The main possibility could be enzyme preference because of different linkages available in acceptor molecules. As there was a decrease in α-(1→6), the branching sucrase activity decreased. A slight decrease was also observed when GtfA reacted with reuteran and sucrose. The decrease in activity of CD1-GBD and GBD-CD2 was also observed when the different reuterans from mutant GtfA were used in assays **(Figure 3.5 & 3.6**). Compared to when reuteran with 89 % α-

(1→6) CD1-GBD showed a decrease activity, when reuteranNS (61 % α-(1→6)) was used and slightly continue to decrease when reuteranPINS (49 % α-(1→6)) were used as an acceptor substrate (**Figure 3.5**). A similar trend was observed when the GBD-CD1 was tested on reuteranNS and reuteranPINS (**Figure 3.6**). This suggests that both GD-CD1 and GBD-CD2 prefer the presence of α-(1→6) in the acceptor molecule because there is a decrease in their transferase activity as the percentage of this linkage decreased.

The enzymes CD1-GBD and GBD-CD2 have shown a very low activity in the presence of amylose with only α-(1→4); however, when modified amylose with 4 % of α-(1→4,6) were used, the transferase activity increased (**Figure 3.2 & 3.3**). Introduction of α-(1→4,6) in potato amylose made the product preferable to both CD1-GBD and GBD-CD1. The previously studied branching sucrase has shown a high transferase activity on dextran rich in α-(1→6) and small molecule isomaltose (Meng, Gangoiti, Wang, et al., 2018); however, it was not tested on amylose or modified amylose. The results from our study show that CD1-GBD and GBD-CD2 are not able to transfer glucosyl group to amylose but were able to transfer a glucosyl group to modified amylose due to the presence of α-(1→4,6) in the product. Reuteransucrase gtfA transferase activity was still high for both amylose and modified amylose (**Figure 3.4**) and the polysaccharide ^{1}H NMR showed that that the product being synthesized is reuteran (**Figure 3.10**). This proves that reuteransucrase uses only sucrose even though modified amylose is available in reaction. There is no evidence of transfer of glycosyl group to the modified amylose, the observed activity might be from a transfer of glycosyl unit to growing polymer to form reuteran. Reuteransucrase has been tested on small acceptor molecules, such as maltose and isomaltose, as acceptors and reuteransucrase prefers maltose over isomaltose for the acceptor reaction (Kralj et al., 2005). The preference of maltose (with α-(1→4)) over isomaltose might

predict that reuteransucrase can be active on amylose as well because it has a high percentage of α-(1→4); however, it seems that the size of the molecule might play a role in being recognized or rejected by reuteransucrase as acceptor.

Reported dextransucrases generally include glucan binding domain (**Table 1**) and studies suggested that the glucan binding domain is necessary for the activity (Brison et al., 2016; Moulis et al., 2006). Data in this study showed that dextran binding increases activity of dextransucrases and branching sucrase (**Figure 3.3**). It was suggested that manipulating acceptor specificity by swapping glucan binding domains from different proteins. Domain swapping did not result in a successful purification, which might be because of not having his-tag well attached to the protein fragment of interest. It is suggested to sequence the construct before proceeding with protein expression and purification to confirm the presence of his-tag. In addition, changing the his-tag to a different terminal might make it accessible and result in successful purification. The intention to perform domain swapping was to assess the responsibility of glucan binding domain and catalytic domain on branching sucrase activity. If the activity stays the same on a good acceptor such as dextran when the branching sucrase glucan binding domain was replaced by either reuteransucrase glucan binding domain, or 4,6-α-glucanotransferase glucan binding domain **(Figure 2.2 A & B)** would confirm that the catalytic domain is the one responsible for the activity. The responsibility of branching sucrose glucan-binding domain would be evaluated by replacing branching sucrase catalytic domain by reuteransucrase catalytic domain and 4,6-α-glucanotransferase catalytic domain (**Figure 2.2 C & D**). If the reuteransucrase with the glucan binding domain from the branching sucrase is active on dextran and reuteran, or if 4,6-α-glucanotransferase with the glucan binding domain from branching sucrase is active on starch, it

would show that the branching sucrase glucan-binding domain binds glucans with different linkage proportions and then the catalytic domain would be responsible for the specificity.

5 Application

The ability of dextransucrases, branching sucrases and reuteransucrase to act on different substrates is important in their application in food and non-food industry. Low molecular weight dextrans are used in clinical application (Patel et al., 2012) while high molecular weight soluble dextrans are used in food as versifier, solubilizer and hydrocolloids (Goulas et al., 2004). The transfer of glycosyl unit to dextran by CD1-GBD and introduction of branches on dextran by GBD-CD2 is beneficial because different degree of branching and chain length offer a wide range of applications depending on the polymer properties. Similarly, producing various reuterans with different proportions of linkage types can result in diversifying their applications. Reuteran with 61 % of α-(1→6) has been reported to improve bread volume and texture (Chen et al., 2016) whereas highly branched reuterans have shown the ability to form biofilm in vivo (Walter et al., 2008). In addition, depending on properties of the polysaccharides produced by these enzymes, some have been reported to have dietary fibre properties. For example, *Weissella cibaria* RBA12 produce dextran with 97 % α-(1→6) linkages in their main chain and 3 % α-(1→3) branches (Baruah et al., 2017).

Even though sucrose is a disaccharide composed of glucose and fructose, only glucosyl units are involved in the synthesis dextran and reuteran which results in a loss of 50 % total sucrose molecule. In this study, the transferase activity of dextransucrase and branching sucrase was observed when modified amylose were used as acceptor substrate. The ability of CD1-GBD and GBD-CD2 to use modified amylose would be cheaper than using sucrose in the reaction as there

would be no loss because every component of starch delivered molecules would contribute to polysaccharide synthesis. The modified potato amylose used in this study has 4 % of α-(1→4,6) linkages and it was preferred by CD1-GBD and GBD-CD2 compared to when amylose with 100 % α-(1→4) were used as acceptor substrate. However, there are other enzymes such as 4,6-α-glucanotransferases that can produce starch derived products with a high percentage of α-(1→6) such as isomalto/maltooligosaccharides (İspirli et al., 2019; Leemhuis et al., 2013; Meng, Gangoiti, de Kok, et al., 2018). Therefore, this study suggests that the starch derived products with high percentage of α-(1→6) would be preferred by branching sucrase and dextransucrases as these enzymes showed a high preference for α-(1→6) linkages in the acceptor molecule. In addition, starch derived products from glucanotransferases which contain up to 92 % of α-(1→6) linkages have been reported to have resistance to digestion in gastrointestinal tract and are considered as soluble dietary fibers (Leemhuis et al., 2014). Therefore, the ability of branching sucrase to act on these products would introduce new branching points and may result in lowering the carbohydrate digestibility.

The findings from this study prove that the transferase activity of *A. kunkeei* 12361 dextransucrase and branching sucrase is dependent on the linkage type present in the acceptor. It is also confirmed that *A. kunkeei* 12361 dextransucrase and branching sucrase activity is higher in the presence of α-(1→6) compared to α-(1→4) linkages in the acceptor molecule. However, the third hypothesis was not proven to be true because the *A. kunkeei* 12361 dextransucrases and branching sucrase are not active on potato amylose. The activity was observed after the modification of potato amylose by introducing α-(1→4,6) branching points.

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