

**Production and Characterization of the
Fructosyltransferase (Levansucrase) from *Geobacillus
stearothermophilus*
and its Application for the Synthesis of Novel
Fructooligosaccharides**

by

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ABSTRACT

The production of the fructosyltransferase (levansucrase) by the thermophilic strain *Geobacillus stearothermophilus* was investigated at 55°C over a 32 hr-fermentation time course in a mineral medium supplemented with varying sources of nitrogen, iron and carbon inducers. Both organic nitrogen sources, tryptone and peptone, provided the maximal microbial growth and levansucrase activity. All carbon sources induced the production of levansucrase activity, but sucrose at 1% (w/v) was the most efficient inducer. The supplementation with ferrous and ferric iron sources had no significant effect on the bacterial growth nor on the levansucrase activity. The selected culture medium for the large enzyme recovery was composed of peptone/tryptone mixture (5 g/L, each), FeSO₄ (5 mg/L) and 1% sucrose. Bacterial cells were harvested after 9hr-fermentation period, which corresponded to a specific levansucrase activity of 1.23 U/mL/Abs_{600nm}. The crude levansucrase extract was enriched using selected precipitating agents. The results show that (NH₄)₂SO₄ (50% saturation) and MnCl₂ (50 mM), provided the highest purification factor of 2.44- and 3.16-fold with a recovery yield of 29.3 and 43.9%, respectively. Although PEG-200 and PEG-400 resulted in exceptional purification factors ranging from 231 to 394, the recovery yields were very poor (5.7%). A two-step purification process combining Superdex 75 and MonoQ 5/50 columns resulted in a purification factor of 28.4 and a final yield of 71%. The gel filtration chromatography of the native levansucrase revealed it to be a monomer of 54.4 kDa. The purified levansucrase showed activity over a broad range of temperature (4-67°C) and pH (4-8). However, the transfructosylation and hydrolytic activities were optimal at 47-57°C and at 57°C, respectively, and the transfructosylation activity over the hydrolytic one (2.86 to 3.18 ratio) was favoured within a narrow pH range of 6.0 to 6.5. In addition, the transfructosylation and hydrolytic activities of levansucrase retained 91.7% and 100% of the initial activity after 6 hr-incubation time at 47°C, but were completely inhibited at higher temperatures. The saturation curve of levansucrase activity using sucrose showed typical Michaelis Menten behavior. However, the relatively high K_{mapp} values for transfructosylation (269 mM) and hydrolytic (272 mM) activities indicate the low affinity of the enzyme towards sucrose. Yet, the levansucrase revealed to have higher acceptor specificity towards pyranose- than furanose-containing glycosides.

RÉSUMÉ

La production de la fructosyltransférase (lévansaccharase) par la souche thermophile *Geobacillus stearothermophilus* a été investiguée à 55°C pendant 32 h de fermentation dans un milieu de culture minéral supplémenté avec diverses sources d'azote, de fer et de carbones pour l'induction. Les sources d'azotes organiques, tryptone et peptone, ont conduit à une croissance bactérienne optimale et une production maximale de la lévansaccharase. Toutes les sources de carbones utilisées sont parvenues à induire la production de la lévansaccharase, avec le saccharose (1% m/v) étant l'inducteur le plus efficace. L'addition d'une source ferrique ou ferreuse n'a eu aucun effet significatif ni sur la croissance bactérienne ni sur la production de la lévansaccharase. Le milieu de culture optimal déterminé pour la production maximale de la lévansaccharase est composé d'un mélange de tryptone/peptone (5 g/L, chacun), FeSO₄ (5 mg/L) et 1% saccharose. Les cellules bactériennes ont été récoltées après une période de fermentation de 9 h, correspondant à une activité spécifique de lévansaccharase de 1.23 U/mL/Abs_{600nm}. L'extrait brut de lévansaccharase a été concentré avec des agents de précipitation sélectionnés. Les résultats démontrent que le (NH₄)₂SO₄ (saturation de 50%) et le MnCl₂ (50 mM) fournissaient les facteurs de purification les plus élevés de 2.44 and 3.16 avec un rendement de 29.3 et 43.9%, respectivement. Bien que l'utilisation de PEG-200 et PEG-400 démontraient des facteurs de purification exceptionnels variant entre 231 à 394, les rendements étaient très faibles (5.7%). Un procédé de purification en 2 étapes, combinant les colonnes Superdex75 et MonoQ5/50, a permis de purifier la lévansaccharase 28.4 fois avec un rendement final de 71%. La chromatographie par filtration en gel a révélé que la lévansaccharase est un monomère d'un poids de 54.4 kDa. La lévansaccharase purifiée a démontré une activité à travers un intervalle étendu de température (4-67°C) et de pH (4-8). Par contre, les activités de transfructosylation et d'hydrolyse étaient maximales à 47-57°C et à 57°C, respectivement, avec une préférence pour l'activité de transfructosylation par rapport à l'hydrolyse (ratio de 2.86 à 3.18) à l'intérieur d'un étroit intervalle de pH de 6.0 à 6.5. De plus, les activités de transfructosylation et d'hydrolyse ont conservé 91.7% et 100% de leurs activités initiales après une période d'incubation de 6h à 47°C, mais ont été complètement inhibées à de plus hautes températures. La courbe de saturation de l'activité de la lévansaccharase avec le saccharose illustre un modèle typique de

Michaelis-Menten. Par contre, la valeur élevée de K_{mapp} pour la transfructosylation (269 mM) et l'hydrolyse (272 mM) indique la faible affinité de la lévansaccharase pour le saccharose. Néanmoins, la lévansaccharase a démontré une spécificité plus élevée envers des accepteurs glycosidiques pyranosidique que ceux furanosiques.

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TABLE OF CONTENTS

ABSTRACT	i
RÉSUMÉ	ii
ACKNOWLEDGEMENT	iv
TABLE OF CONTENTS	v
LIST OF FIGURES	viii
LIST OF SCHEMES	x
LIST OF TABLES	xi
LIST OF ABBREVIATIONS.....	xii
INTRODUCTION	xiv
CHAPTER 1: LITERATURE REVIEW.....	1
1.1. Definition, Concept and Health Benefits	1
1.1.1. Probiotics	1
1.1.2. Prebiotics	1
1.1.3. Synbiotics	2
1.1.4. Dietary Soluble Fibers	2
1.2. Oligosaccharides	3
1.2.1. Classification and Applications of Oligosaccharides	3
1.2.2. Non-Digestible Oligosaccharides	3
1.2.3. Prebiotic Activity of Non-Digestible Oligosaccharides	4
1.2.3.1. Prebiotic Activity of Galactooligosaccharides	6
1.2.3.2. Prebiotic Activity of Fructooligosaccharides	6
1.3. Fructooligosaccharides (FOSs)	7
1.3.1. Chemical Structures and Types of Fructooligosaccharides.....	7
1.3.2. Limitations of Current Commercial Fructooligosaccharides	9
1.4. Production of Fructooligosaccharides.....	10
1.4.1. Extraction of Fructooligosaccharides from Natural Food Products	10
1.4.2. Chemical Synthesis of Fructooligosaccharides	11
1.4.3. Hydrolysis of Fructans to Fructooligosaccharides	12
1.4.4. Enzymatic Synthesis of Fructooligosaccharides	12
1.4.4.1. β -fructofuranosidase-Catalyzed Synthesis of Fructooligosaccharides	13
1.4.4.2. Fructooligosaccharide Synthesis by Fructansucrases	14

1.4.5. Levansucrase-Catalyzed Transfructosylation Reaction	14
1.4.5.1. Microbial Sources of Levansucrases	15
1.5. Mechanistic Action of Levansucrase	16
1.5.1. Structure-Function Relationship.....	16
1.5.2. Active Site of Levansucrase	16
1.6. Transfructosylation Product Spectrum	17
1.6.1. Proportionate and Disproportionate Reactions.....	17
1.6.2. Donor and Acceptor Specificities of Levansucrase	17
1.7. Purification of Levansucrase.....	19
1.8. Effect of Reaction Parameters on Levansucrase-Catalyzed Reactions.....	20
1.8.1. Effect of Reaction pH.....	20
1.8.2. Effect of Reaction Temperature.....	20
1.8.3. Effect of Solvents	21
1.8.4. Effect of Substrate Concentration.....	21
1.9. Characterization of Levansucrase Transfructosylation End-Products	22
1.9.1. Thin-Layer Chromatography (TLC) Analysis.....	22
1.9.2. Gas Chromatography	23
1.9.3. High-Performance Liquid Chromatography (HPLC).....	23
1.9.4. High-Pressure Anionic Exchange Chromatograph with Pulsed Amperometric Detection (HPAEC-PAD)	23
1.9.5. Official Method of Detection Assigned by the AOAC	25
CHAPTER 2: MATERIALS AND METHODS	26
2.1. Sources of Chemicals.....	26
2.2. Microbial Strain and Growth Conditions.....	26
2.3. Culture Conditions	26
2.3.1. Effect of Nitrogen Sources	27
2.3.2. Effect of Carbon Sources.....	28
2.3.3. Effect of Iron Sources.....	28
2.4. Fructosyltransferase (Levansucrase) Purification.....	28
2.4.1. Preparation of Crude Fructosyltransferase Extract.....	28
2.4.2. Preparation of Pre-Purified Crude Fructosyltransferase Extract	28
2.4.3. Chromatographic Purification	29
2.4.3.1. Size-Exclusion Chromatography.....	29
2.4.3.2. Anionic-Exchange Chromatography	30

2.4.4. Sodium-Dodecyl-Sulfate-Polyacrylamide-Gel-Electrophoresis (SDS-PAGE)	30
2.4.5. Thin-Layer Chromatography	30
2.4.6. Enzymatic Assay of Fructosyltransferase (Levansucrase) Activity	31
2.4.7. Total Protein Concentration	32
2.5. Characterization of the Catalytic Properties of Fructosyltransferase (Levansucrase)	33
2.5.1. Effect of Temperature	33
2.5.2. Thermal Stability	33
2.5.3. Effect of pH	33
2.5.4. Effect of Substrate Concentration	34
2.6. Characterization of the Product Spectrum and Acceptor Specificity of the Fructosyltransferase (Levansucrase)	34
2.7. Levan Production and Nuclear Magnetic Resonance (NMR) Characterization	35
CHAPTER 3: RESULTS AND DISCUSSION	36
3.1. Optimization of the Production of Fructosyltransferase (FTF) from <i>G. stearothermophilus</i>	36
3.1.1. Effect of Nitrogen Sources	36
3.1.2. Effect of Carbon Sources	42
3.1.3. Effect of Selected Iron Sources	44
3.2. Purification of the Fructosyltransferase (FTF) from <i>G. stearothermophilus</i>	46
3.2.1. Preparation of the Pre-Purified Crude Fructosyltransferase Extract	46
3.2.2. Purification of Fructosyltransferase	48
3.3. Characterization of the Catalytic Properties of Levansucrase from <i>G. stearothermophilus</i>	56
3.3.1. Effect of Reaction Temperature	56
3.3.2. Thermal Stability of Levansucrase	59
3.3.3. Effect of pH	61
3.3.4. Effect of Substrate Concentration and Determination of Kinetic Parameters	64
3.4. Characterization of the Product Spectrum and the Acceptor Substrate Specificity of Levansucrase from <i>G. stearothermophilus</i>	68
3.4.1. Characterization of the Product Spectrum	68
3.4.2. Study of the Acceptor Specificity	70
CONCLUSION	xvii
REFERENCES	xix

LIST OF FIGURES

Figure Number	Page
1. Time course of <i>Geobacillus stearothermophilus</i> growth (□), fructosyltransferase (FTF) production (■) and ratio of FTF activity to Abs _{600nm} (Δ) at 55°C in a mineral culture medium (A) supplemented with peptone (B) yeast (C) and tryptone (D), using 1% sucrose as an inducer.....	37
2. Effect of different carbon sources (w/v) on the growth (■) of <i>Geobacillus stearothermophilus</i> , the fructosyltransferase (FTF) activity (□), and the specific FTF activity (FTF activity/Abs at 600 nm) (Δ).....	43
3. Effect of selected iron sources on the growth (□) of <i>Geobacillus stearothermophilus</i> , the membrane-bound fructosyltransferase (FTF) activity (■) and the extracellular FTF activity (■).....	45
4. Chromatograms* of the fructosyltransferase (FTF) extract from <i>Geobacillus stearothermophilus</i> purified by gel filtration using Superdex 75 (A) then by anionic exchange chromatography using MonoQ 5/50 (B). Elution profile of protein standards (A') and plot of the log of the molecular weights (m.w) of protein standards against retention time** (B').....	50
5. SDS-PAGE of the crude and purified fructosyltransferase (FTF) from <i>Geobacillus stearothermophilus</i> and of fractions collected upon anionic exchange chromatography using MonoQ 5/50.....	52
6. Thin-layer chromatography of the products synthesized by the fructosyltransferase (levansucrase) extract from <i>Geobacillus stearothermophilus</i> obtained after gel permeation chromatography on Superdex 75 (lanes 1 to 2), anionic-exchange chromatography on MonoQ 5/50 (lanes 3 to 8) and after lyophilization of the MonoQ fraction (lane 9).....	54
7. Effect of temperature on the reaction selectivity (A) of the fructosyltransferase (levansucrase) from <i>Geobacillus stearothermophilus</i> : Transfructosylation (■), hydrolysis (□) and transfructosylation to hydrolytic activity ratio (Δ). Arrhenius plot (B) of the transfructosylation (■) and hydrolytic (□) activities of FTF.....	55

8.	Thermal stability of fructosyltransferase (levansucrase) from <i>Geobacillus stearothermophilus</i> . The remaining transfructosylation (■) and hydrolytic (□) activities were determined under optimum reaction conditions after 6h of incubation at different temperatures.....	57
9.	Effect of pH on the reaction selectivity of <i>Geobacillus stearothermophilus</i> fructosyltransferase (levansucrase): Transfructosylation (■), hydrolysis (□) and transfructosylation to hydrolytic activity ratio (Δ).....	60
10.	Michaelis-Menten plot (A) and Line-weaver-Burk plot (B) of transfructosylation (■) and hydrolytic (□) activities of <i>Geobacillus stearothermophilus</i> fructosyltransferase (levansucrase).....	62
11.	¹³ C NMR spectra of the polyfructan synthesized by the levansucrase from <i>Geobacillus stearothermophilus</i>	65
12.	HPAEC-PAD chromatograms of fructooligosaccharides standards (---), 1-kestose (A) and nystose (B), and of transfructosylation products (—) synthesized by <i>G. stearothermophilus</i> fructosyltransferase (levansucrase) using sucrose as the sole substrate. Peaks#1, 2, 3, 4,5 and 6 are identified as bifructose, 1-kestose, blastose, 6-kestose, neo-kestose and nystose, respectively.....	69
13.	HPAEC-PAD chromatogram of the inulin-type fructooligosaccharide standards, 1-kestose (peak#A) and nystose (peak#B) (---), and of the fructooligosaccharides products (—) synthesized by <i>G. stearothermophilus</i> fructosyltransferase (levansucrase) in the presence of the acceptor galactose (A), glucose (B) and lactose (C). Peaks#1 and 1' represent the sucrose analogues galactosyl-fructoside and glucosyl-fructoside, respectively. Peaks# 2' and 3' are identified as bifructose and 1-kestose, respectively.....	72
14.	HPAEC-PAD chromatogram of the inulin-type fructooligosaccharide standards 1-kestose (A) and nystose (B) (---) and of the fructooligosaccharides products (—) synthesized by <i>G. stearothermophilus</i> fructosyltransferase (FTF) in the presence of the acceptor raffinose (A) and maltose (B). Peak#1 represents melibiose, while peak#1' maltose. Peaks#2', 3', 4', 5', 6' and 7' identified as 1-kestose, erlose, blastose, 6-kestose, neo-kestose and nystose, respectively. Peaks#8'-11' are unidentified FOSs of higher degree of polymerization.....	73

LIST OF SCHEMES

Scheme Number	Page
1. Chemical structure of 1-kestose.....	8
2. Chemical structure of 6-kestose.....	8
3. Chemical structure of neo-kestose.....	9
4. Chemical structure of bifurcose.....	89
5. Model of the substrate-binding at the active site and levansucrase-catalyzed proportionate and disproportionation reactions.....	18

LIST OF TABLES

Table Number	Page
1. Commercially available food-grade prebiotics.....	4
2. Concentration of FOS in common food sources.....	11
3. Effect of nitrogen sources on the fructosyltransferase (FTF) activity and on the growth of <i>Geobacillus Stearothermophilus</i>	39
4. Composition of tryptone, peptone and yeast extract from Difco.....	41
5. Purification factor and yield of <i>Geobacillus stearothermophilus</i> fructosyltransferase (FTF) after selected precipitation.....	47
6. Yield and purification factor of <i>Geobacillus stearothermophilus</i> fructosyltransferase (FTF) after chromatographic purification.....	49
7. Catalytic properties of <i>Geobacillus stearothermophilus</i> fructosyltransferase (levansucrase).....	58
8. <i>Geobacillus stearothermophilus</i> fructosyltransferase (levansucrase)-catalyzed transfructosylation reactions in the presence of various acceptors.....	71

LIST OF ABBREVIATIONS

Abs:	Absorbance
A_i :	Pre-exponential constant
AOAC:	Association of Official Analytical Chemists
Arg:	Arginine
Asp:	Aspartic acid
ATCC:	American Type Culture Collection
CV:	Column volume
DEAE-cellulose:	Diethylaminoethyl cellulose
DNA:	Deoxyribonucleic acid
DNS:	3,5-Dinitrosalicylic acid
E_a :	Energy of activation
E_t :	Total enzyme
EC number:	Enzyme classification number
FOS:	Fructooligosaccharide
FPLC:	Fast Protein Liquid Chromatography
Fru:	Fructose
FTF:	Fructosyltransferase
Gal:	Galactose
GH:	Glycosyl hydrolase
Glc:	Glucose
Glu:	Glutamic acid
HPAEC-PAD:	High-pressure-anionic-exchange chromatography with pulse amperometric detection
HPLC:	High pressure liquid chromatography
Inu:	Inulin
IUB-IUPAC:	International Union of Biochemistry - International Union of Pure and Applied Chemistry
K_{cat} :	Turnover number
K_{mapp} :	Apparent Michaelis-Menten constant

M.w: Molecular weight

MALDI-TOF- MS: Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry

NDO: Non-digestible oligosaccharide

-NH₂: Amino-bonded

NMR: Nuclear magnetic resonance

ODU: Optical density unit

PEG: Polyethylene glycol

R: Universal gas constant

RI: Refractive index

Rpm: Rotation per minute

S: Substrate

SA: Specific activity

ScFA: Short-chain fatty acid

SDS-PAGE: Sodium-dodecyl-sulfate-polyacrylamide gel electrophoresis

SHIME: Simulator of Human Intestinal Microbial Ecosystem

Suc: sucrose

T: Temperature

TLC: Thin-layer chromatography

UV: Ultraviolet

V: Reaction rate

V_{maxapp}: Maximum velocity

Xyl: Xylose

INTRODUCTION

Chronic diseases and acute infections are well known health problems associated with aging, putting the elderly at 400 times greater risk of mortality (Garibaldi and Nurse, 1986; Hébuterne, 2003). Most of these diseases have been found to be linked to the intestinal health, which Health Canada has identified as one of its top five health focuses. With the aging of the population, and the increasing costs of health care, functional ingredients and nutraceuticals are becoming of primary importance for preventing chronic diseases and cancers. In this perspective, the potential physiological benefits of non-digestible oligosaccharides (NDOs)- an emerging class of functional ingredients- to support the intestinal health, by selectively stimulating the proliferation of beneficial colonic lactic-acid bacteria at the expense of pathogenic bacteria, are now well established (Gibson and Roberfroid, 1995).

The functional health attributes of NDOs have been shown to be dependent on their chemical structures, in particular, the type of hexose moieties, the extent of polymerization, the glycosidic linkages, and the type of substituents (Sako, 1999). The better understanding of the relationships between the structure of NDOs and their health functions have highlighted the need for efficient biocatalytic approaches to synthesize novel NDO structures (Manning and Gibson, 2004). For instance, levan-type fructooligosaccharides (FOSs) are of increasing interest because of their potential health benefits to selectively support the intestinal health. Indeed, β -(2-6)-FOSs and *neo*FOSs have demonstrated prebiotic activities that surpass those of β -(2-1)-FOSs available for human consumption (Iizuka and Ogyra, 2000; Semjonovs et al., 2004). Because of their low molecular weight, currently available β -(2-1)-FOSs are quickly fermented in the proximal region of the colon and do not reach the distal area, where most of chronic gut disorders originate. Increasing the resistance to gastric digestion and improving colonic persistence of FOSs, by controlling their molecular weight or by modulating their chemical structures are the driving rationale for the development of "*second generation*" FOSs. For instance, a new generation of structurally-well defined FOSs, headed with

different hexoses or pentoses instead of a glucosyl moiety, may conceivably possess high colonic persistence and new health attributes not present in the current FOS generation.

Fructooligosaccharides can be obtained by extraction from natural sources, by chemical synthesis or by enzymatic synthesis. Poor yield of FOS recovery are acquired by the extraction method (Sangheetha et al., 2005), whereas the presence of contaminants, utilization of toxic polluting reagents and low selectivity are the drawbacks associated with the labor-intensive chemical synthesis of FOSs (Palcic, 1999; Warrand and Janssen, 2007). FOSs can also be synthesized from simple sugars by fructosyltransferase-catalyzed transfructosylation reaction or obtained by the controlled hydrolysis of polysaccharides by fructosyl hydrolases. FOSs synthesis by fructosyltransferases offers the advantage of high regio- and stereo-selectivity and high substrate specificity (Plou et al., 2007), which are key requirements for the stereo-specific construction of selected glycosidic bonds (Monchois et al., 1999). Levansucrases (EC 2.4.1.10), which belong to the fructosyltransferase family, have recently gained more interest due to their ability to directly use the free energy of cleavage of non-activated sucrose to transfer the fructosyl group to a variety of acceptors, including mono-, di- and oligosaccharides, resulting in the formation of novel FOS structures. However, the availability of levansucrases, their thermal stability and the understanding of their catalytic mechanism are still limited. In addition, despite considerable interest in identifying novel levansucrase specificities, only few levansucrases have been fully characterized with respect to their acceptor specificity and transfructosylation products. In this context, levansucrase was recovered from a thermophilic microbial source, *Geobacillus stearothermophilus*, and characterized with respect to its catalytic properties and its donor-acceptor specificity to provide more insight into its complex mechanism. The genome sequences of the selected thermophilic strain were reported to carry a novel gene cluster encoding levansucrase and levanases (Li et al., 1997); however, no information is available about the levansucrase from *G. Stearothermophilus*.

The specific objectives of this research were

1. The investigation of the biomass production of *G. stearothermophilus* for the optimal recovery of the fructosyltransferase by varying the nutrient sources in the culture medium.
2. The development of a purification method for the isolation of fructosyltransferase from the crude cell-free extract.
3. The characterization of the catalytic properties of the transfructosylation and hydrolytic activities of the purified fructosyltransferase (levansucrase) and determination of their kinetic parameters.
4. The investigation of the product spectrum and the acceptor specificity of the purified fructosyltransferase (levansucrase) from *G. stearothermophilus*.

CHAPTER 1. LITERATURE REVIEW

1.1. Definition, Concept and Health Benefits

1.1.1. Probiotics

Probiotics, including bifidobacteria, lactobacilli and enterococci, are beneficial microorganisms providing the host with health benefits by restoring the microbial balance in the gut (Fuller, 1989). A stable colonic microflora represents a vital barrier against pathogens (Holzapfel and Schillinger, 2002) because it limits the contact of pathogens to the intestinal epithelium. Moreover, the metabolites derived from probiotics, such as short-chain fatty acids (scFAs), stimulate immunity and inhibit the growth of *Escherichia coli* and *Clostridium perfringens* (Sanders and Gibson, 2006). Gastrointestinal microorganisms may subsist in four defined micro-habitats: the epithelial cell surface, the crypts of the ileum, the caecum (colon), the epithelial mucus layer and the lumen (Freter and de Macias, 1995). However, the growth of bifidobacteria and eubacteria is most desired in the caecum and in the ileum due to the elevated viable count and to the complexity of the microflora (Smith, 1965). In addition to its ability to resist to gastric acids and bile (Lee, 1999; Svensson, 1999), the efficacy of probiotics largely depends on their capacity to permanently adhere, colonize and exert their metabolic activity specifically in the colon (Sip and Grajek, 2010).

1.1.2. Prebiotics

Prebiotics are regarded as a more practical approach for the manipulation of the colonic microflora than probiotics because it targets indigenous bacteria (Macfarlane et al., 2008; Bengmark, 2000). Gibson and Roberfroid (1995) were the first to introduce the concept of prebiotics, which are non-digestible oligosaccharides that are selectively fermented by probiotics, enabling their proliferation and activity specifically in the colon. In fact, the administration of prebiotics has been shown to contribute to the re-establishment of the intestinal microflora balance among unhealthy subjects by selectively nurturing the growth of the endogenous colonic bifidobacteria and lactobacilli, which are the dominant colonic microorganisms among healthy individuals (Gibson et al., 1995; Cummings et al., 2001).

1.1.3. Synbiotics

Synbiotics consist of a combination of both probiotics and prebiotics (Gibson and Roberfroid, 1995) and have been shown to be more effective at increasing the population of colonic bifidobacteria than probiotics administered alone (Bielecka et al., 2002; Pelicano et al., 20052004). The assumption that synbiotics enhance the intestinal persistence of probiotics is derived from an *in vitro* fermentation of synbiotics in a human gut model. The synbiotic preparation consisted of fructooligosaccharides and *L. acidophilus* which were tested on a SHIME (Simulator of Human Intestinal Microbial Ecosystem) reactor. Increased levels of lactobacilli were observed in the ascending colon while bifidobacteria population was improved in the ascending, transverse and descending colons (Gmeiner et al., 2000). However, an *in vitro* comparative study was conducted to investigate the effect of fructooligosaccharides, inulin and synbiotics on the growth of *B. longum*, *B. catanulatum* and *B. animalis*. Results indicated that the growth of these strains was greater with fructooligosaccharides while poor growth resulted from inulin (Bielecka et al., 2002). In fact, inulin was previously reported to have the highest increase in bacterial intestinal population, but the lowest count of lactobacilli and bifidobacteria (Rycroft et al., 2001). Interestingly, the prebiotic preparation were demonstrated to be as effective as the synbiotics (Gibson and Fuller, 2000), suggesting that the administration of fructooligosaccharides alone may induce the persistence of endogenous lactobacilli and bifidobacteria. In comparison to synbiotics, prebiotics can only be effective if the microorganisms able to ferment them are initially present in the host intestinal tract (Van der Westhuizen and Kilian, 2008). Nonetheless, the application of synbiotics becomes relevant when probiotics with diminished survival capacities are used in the preparation (Rastall and Maitin, 2002).

1.1.4. Dietary Soluble Fibers

According to the American Association of Cereal Chemists, dietary fibers are defined as the edible parts of plants or carbohydrate analogues that are non-digestible but that undergo partial or complete fermentation in the colon (Prosky, 2003). Therefore, dietary fibers are comprised of polysaccharides, oligosaccharides, lignin and plant-associated substrates. With the exception of non-digestible oligosaccharides, other forms of soluble dietary fibers do not comply with the criterion of prebiotics because they are partially

digested in the small intestine and most importantly, they are not selectively fermented by bifidobacteria and lactobacilli (Karppinen et al., 2001).

1.2. Oligosaccharides

1.2.1. Classification and Applications of Oligosaccharides

Oligosaccharides are low molecular weight carbohydrates made up of 2 to 10 monosaccharide units linked by *O*-glycosidic linkages (Eggleston and Cote, 2003). The IUB-IUPAC nomenclature classifies oligosaccharides as carbohydrates consisting of 3 to 10 monomers (Mussatto and Mancilha, 2007). Oligosaccharides are differentiated based on their structure, composition, sequence and glycosidic bonds (Bailey, 1963). However, the physicochemical properties of oligosaccharides are dictated by its chemical structure, its degree of polymerization and by the presence of monosaccharides or disaccharides (Roberfroid and Slavin, 2000; Mussatto and Mancilha, 2007). In fact, the viscosity produced by oligosaccharides increases with their molecular weight, resulting in their interesting application as a bulking agent. Additional interesting characteristics of oligosaccharides include their hygroscopic ability for moisture-retention (Crittenden and Playne, 1996) or their non-digestibility for incorporation in foods intended for diabetics (Rivero-Urgell and Santamaria-Orleans, 2001).

1.2.2. Non-Digestible Oligosaccharides

Due to the presence of β -glycosidic bonds between monosaccharide units, some oligosaccharides are further categorized as non-digestible oligosaccharides (NDOs). In fact, α -glucosidase, maltase-isomaltase and sucrase are human digestive enzymes which are specific for the hydrolysis of α -osidic linkages in sugars (Roberfroid, 1996). As a consequence of the β -configuration, the chemical composition of NDOs is comprised of fructose, galactose, glucose and/or xylose units (Cummings et al., 1997). Contrarily to simple sugars, NDOs resist to the degradation by oral microorganisms; therefore preventing the production of cariogenic compounds (Mussatto and Mancilha, 2007). Since NDOs provide relatively mild sweetness and calories, they are considered as safe and suitable sugar substitutes in hypocaloric foods (Crittenden and Playne, 1996). In comparison to soluble fibers, the application of NDOs in foods and beverages is more flexible due to the low dosage needed to provide beneficial effects. Moreover, they offer

high solubility in water, good physical stability, and acceptable texture without leaving an aftertaste (Tomomatsu, 1994). As shown in Table 1, there are currently 12 types of food-grade NDOs commercially produced around the world (Crittenden and Playne, 1996), but the most important NDOs include fructooligosaccharides, galactooligosaccharides, isomaltooligosaccharides, inulooligosaccharides and soybean oligosaccharides (Dhake and Patil, 2007). Dietary sources of prebiotic-oligosaccharides can be extracted from chicory, yeast cell walls, soybeans, Jerusalem artichokes, raw oats, unrefined wheat, garlic, banana and leek (van Loo et al., 1995; Sip and Grajek, 2010).

Table 1: Commercially available food-grade prebiotics (Sako, 1999)

Compound	Molecular structure *
Cyclodextrin	(Glc) _n
Fructooligosaccharides	(Fru) _n -Glc
Galactooligosaccharides	(Gal) _n -Glc
Gentiooligosaccharides	(Glc) _n
Glycosylsucrose	(Glc) _n -Fru
Isomaltooligosaccharides	(Glc) _n
Isomaltulose	(Glc-Fru) _n
Lactulose	Gal-Glc-Fru
Maltooligosaccharides	(Glc) _n
Raffinose	Gal-Glc-Fru
Soybean oligosaccharides	(Gal) _n -Glc-Fru
Xylooligosaccharides	(Xyl) _n

* Gal (Galactose); Glc (Glucose); Fru (Fructose); Xyl (Xylose)

1.2.3. Prebiotic Activity of Non-Digestible Oligosaccharides

Although the non-digestible oligosaccharides listed in Table 1 have been reported to stimulate the growth of bifidobacteria and lactobacilli, they were also consumed by non-probiotic microorganisms. For instance, while FOSs are more selective towards bifidobacteria, gentiooligosaccharides also improves the growth of non-probiotic bacteria (Rastall, 2006). On the other hand, Hopkins et al. (1998) found that galactooligosaccharides and oligofructose provided the highest increase in bifidobacteria,

whereas xylooligosaccharides were poorly consumed. In comparison, Rycroft et al. (2001) obtained the highest increase in bifidobacteria with xylooligosaccharides.

In addition to their diverging selectivity, there is a large variability in the extent to which those non-digestible oligosaccharides are fermented to scFAs, acetate, propionate and butyrate (Rycroft et al., 2001). These biologically active fatty acids are involved in the prevention of colon cancer (Cummings, 1981). In addition to decreasing the intestinal pH, these compounds serve as an energy supply (Delzenne et al., 1995) and provide inhibition against the growth of pathogenic enterobacteria (Roberfroid, 1999). In their undissociated form, volatile scFAs exhibit bacteriostatic and/or bactericidal properties. The release of L-lactate and scFAs further contributes to health by enhancing the bioavailability of essential minerals (Probert and Gibson, 2002) and by improving carbohydrate and lipid metabolism (Gibson et al., 2000; Tuohy et al., 2005). Overall, these effects protect the host against the development of colon cancer and infectious diseases (Probert and Gibson, 2002). Nonetheless, the concentration and releasing rate of scFAs depend on the intestinal microflora and nature of NDOs. For instance, the largest increases in butyrate level and in the population of lactobacilli were obtained with FOSs rather than with xylooligosaccharides and galactooligosaccharides (Rycroft et al., 2001). Therefore, the monomeric composition, molecular weight and type of glycosidic bonds involved are important factors determining the prebiotic activities of NDOs (Sako, 1999; Manning and Gibson, 2004). Rycroft et al. (2001) were one of the only few to carry out a comparative study on prebiotics. Their results indicate that FOSs and lactulose have a much higher rate of propionate production than galactooligosaccharides or soybean oligosaccharides, which produced more lactate. According to Roberfroid (1998) only the inulin-type fructans hold enough supportive experimental evidence to be designated as prebiotics, and these include native inulin, inulin hydrolysates, oligofructosides and synthetic fructooligosaccharides.

1.2.3.1. Prebiotic Activity of Galactooligosaccharides

Human milk is characterized by about 200 types of structurally-complex oligosaccharides (Bode, 2009) that serve as natural prebiotics by interfering with the adhesion of pathogens to the intestinal wall, protecting breast-fed infants against infections and diarrhea (Newburg et al., 2005). Galactooligosaccharides (90%) and long-chain FOSs (10%) have been incorporated in infant milk formula (Boehm et al., 2002; Knol et al., 2003; Schmelzle et al., 2003; Scholtens et al., 2003). As a result, infants fed with this mixture had similar colonic microflora (Moro et al., 2002; Costalos et al., 2008) and scFA profile (Boehm et al., 2004) as those that were breast-fed. Feeding trials involving adult volunteers showed that the consumption of galactooligosaccharides with FOSs increases the population of bifidobacteria (Ito et al., 1990; Gibson et al., 1995) and lactobacilli (McBain & Macfarlane, 2001; Smiricky-Tjardes et al., 2003). However, studies investigating the prebiotic effect of galactooligosaccharides alone are limited to a small population and the results are inconsistent (Macfarlane et al., 2008). Moreover, FOSs and galactooligosaccharides have been reported to be fermented by bacteroides (Duncan et al., 2003) and clostridia (Ohtsuka et al., 1989), respectively. In this perspective, there is a strong need to improve the selectivity of NDOs.

1.2.3.2. Prebiotic Activity of Fructooligosaccharides

In general, FOSs are more selectively fermented by lactobacilli and bifidobacteria than other NDOs (Olano-Martin et al., 2002). This is because these bacteria are equipped with a membrane-bound β -fructofuranosidase enzyme that is capable of hydrolyzing FOSs (Perrin et al., 2001). In fact, several experimental data, both in animal and human trials, have shown that inulin-type FOSs increase the population of these beneficial bacteria in the gut microflora (Videla et al., 2001; Butel et al., 2002; Guigoz et al., 2002; Hoentjen et al., 2005; Osman et al., 2006; Vos et al., 2006). Particularly, FOSs have been shown to enhance the resistance of bifidobacteria against bile, improving their survival and adherence in the colon (Perrin et al., 2000). All these studies have examined the prebiotic activity of the inulin-type FOSs exclusively. Recently, the levan-type FOSs have been demonstrated to be more selective towards beneficial bacteria than other NDOs as well as the commercial FOSs (Marx et al., 2000). Most particularly, neokestose (Kilian et al., 2002) was shown to improve the population of bifidobacteria and lactobacilli to a greater

extent than commercial FOSs (Raftilose), and to inhibit the growth of clostridia. Omori et al. (2010) demonstrated that the β -fructofuranosidase from *B. adolescentis* was able to hydrolyse both the levan-type FOS, neokestose, and the inulin-type FOSs, 1-kestose, but expressed a higher substrate specificity towards neokestose. Similarly, *B. longum*, *breve* and *pseudocatenulatum* are capable of metabolizing $\beta(2\rightarrow6)$ -linked FOSs. Furthermore, the growth of *B. adolescentis* was best supported on this substrate and was associated with the highest acidification (Marx et al., 2000). Kilian et al. (2010) examined the scFA profile produced from these strains and their results indicate that the production of butyric acid was similar with both neokestose and inulin-type FOSs. However, the concentration of lactic, propionic and acetic acids were much higher with neokestose (Kilian et al., 2002). Overall, these findings indicate that the levan-type FOSs may have higher bifidogenic effects than commercial ones and that it may be used as a substrate to specifically target *B. adolescentis*.

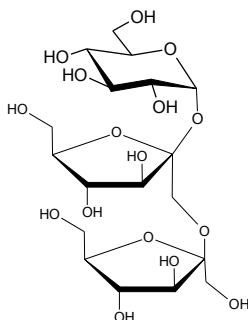
1.3. Fructooligosaccharides (FOSs)

1.3.1. Chemical Structures and Types of Fructooligosaccharides

FOSs are fructan oligomers of 2 to 10 fructosyl residues linked by $\beta(2\rightarrow1)$ or $\beta(2\rightarrow1)$ glycosidic linkages and with a terminal D-glucose group (Roberfroid, 1996). Although the β -glycosidic bonds are a characteristic feature to all FOSs, they are categorized as inulin-type, neoinulin-type, levan-type, neolevan-type and the mixed levan (Vijn and Smeekens, 1999).

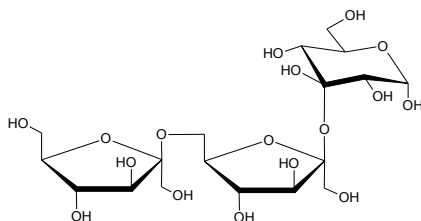
The inulin-type FOSs ($\text{Glc}_{1-2}\text{Fru}_{1-2}\text{Fru}_n$) are composed of 2 to 4 fructosyl units attached by $\beta(2\rightarrow1)$ glycosidic linkages with a D-glucose terminal head (Madlova et al., 2000) positioned at the non-reducing end (Hussein et al., 1998). Commercially available inulin-type FOSs consist of 1-kestose (Glc-Fru_2), nystose (Glc-Fru_3) as well as fructofuranosylnystose (Glc-Fru_4) (Plou et al., 2007). Among those, the trisaccharide 1-kestose (Fig. 1), also known as 1^{Fru}-fructosyl-sucrose (Ferretti et al., 1984), is the smallest and consists of a single D-fructosyl residue attached to a sucrose molecule by a $\beta(2\rightarrow1)$ -glycosidic bond. Similarly, larger inulin-type FOSs are composed of a sucrose moiety which is $\beta(2\rightarrow1)$ -linked to multiple D-fructosyl units also connected by $\beta(2\rightarrow1)$ -glycosidic bonds (Han and Watson, 1992). In the neoinulin-type FOSs, D-fructosyl units

are bound to C₁ and C₆ of the glucopyranosyl portion of sucrose. It comprises the trisaccharide neokestose, which is the smallest inulin neoseries (Plou et al., 2007). Neokestose, or 6^{Glc}-fructosyl-sucrose (Ferretti et al., 1984), is particularly interesting because it has been reported to have greater prebiotic activity than the commercial inulin-type FOSs (Kilian et al., 2002).



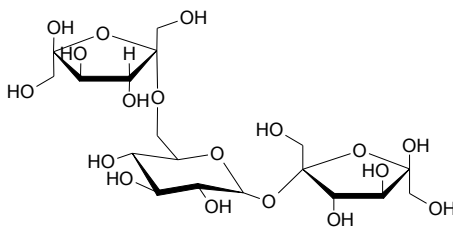
Scheme 1: Chemical structures of 1-kestose.

The levan-type FOSs (Glc₁₋₂Fru₆₋₂Fru_n) consists of a D-glucose unit that is bound to a linear chain of D-fructosyl residues attached by $\beta(2\rightarrow6)$ linkages. The trisaccharide 6-kestose (Fig.2) is the smallest of this category (Han and Watson, 1992) and is also called 6^{Fru}-fructosyl-sucrose because the fructosyl unit is attached to the fructose moiety of sucrose (Ferretti et al., 1984).



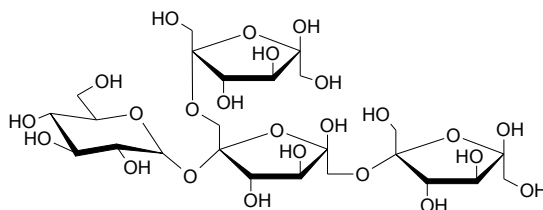
Scheme 2: Chemical structures of 6-kestose.

Neo-levan-type FOSs are made up of a fructosyl molecule that is $\beta(2\rightarrow6)$ -linked to the glucopyranosyl portion from sucrose. If the fructosyl residue are attached to 1-kestose by $\beta(2\rightarrow6)$ bonds, neo-nystose is formed (neo- Glc-Fru₃) whereas neo-kestose (Fig. 3) is produced if the fructosyl unit is attached by a $\beta(2\rightarrow1)$ -glycosidic bond (Plou et al., 2007).



Scheme 3: Chemical structures of neo-kestose.

The mixed levan FOSs contain D-fructosyl residues attached to sucrose by both $\beta(2 \rightarrow 1)$ and $\beta(2 \rightarrow 6)$ glycosidic links. In this subclass of FOSs, the tetrasaccharide bifurcose is the smallest in which the fructosyl moiety of sucrose is $\beta(2 \rightarrow 6)$ -linked to the glucose portion of 1-kestose (Spenger et al., 1995).



Scheme 4: Chemical structures of bifurcose.

1.3.2. Limitations of Current Commercial Fructooligosaccharides

FOSs are notably emerging as an important prebiotic due to their hypocaloric, non-cariogenic and bifidogenic functions (Dhake and Patil, 2007). In fact, the sweetening power of this prebiotic represents only 40 to 60% of that of sucrose (Plou et al., 2007). Yet, the main challenge with currently available prebiotics is the development of non-digestible oligosaccharides intended for targeted functions, and most importantly that are specific towards beneficial bacteria at the species level (Manning and Gibson, 2004). The selectivity of FOSs can be improved by favouring the synthesis of levan-type FOSs. Just recently, neokestose, a $\beta(2 \rightarrow 6)$ -containing FOS, has been demonstrated to exert higher bifidogenic effects than commercial FOSs (Marx et al., 2000; Kilian et al., 2002; Omori et al., 2010).

Up to now, commercial preparations of FOSs are limited to the low molecular-weight inulin-type FOSs: 1-kestose, nystose and fructosyl-nystose (Rastall, 2006). Due to their low molecular weight (Rastall and Maitin, 2002), commercial FOSs are quickly

fermented by the saccharolytic activity of anaerobes in the proximal colon, leading to the formation of scFAs that contribute to the host's welfare at this restricted area. As a result of the depletion of carbohydrates, proteolytic fermentation becomes the main activity of anaerobes in the distal colon, resulting in the generation of phenolic compounds, amines and ammonium, which are toxic metabolites (Manning and Gibson, 2004). Previously, long-chain FOSs of the levan-type have been demonstrated to resist digestive degradation to a greater extent than the short-chain (Nilsson & Björk, 1988). The production of longer-chain FOSs will encourage the prebiotic activity to occur in the distal colon, which is more susceptible to colon cancer (Rastall & Maitin, 2002). Furthermore, FOSs of higher degree of polymerization are less-likely to provoke intestinal discomfort (Fanaro et al., 2005).

The functional properties of current FOSs may be improved by using sucrose analogues (Seibel et al., 2006b) or galactose (Baciu et al., 2005) as acceptors. This may enable the synthesis of FOSs with a terminal galactose residue, which may possess anti-adhesive properties similar to those of galactooligosaccharides. As a summary, the production of FOSs that are structurally well-defined, with higher selectivity and colonic persistence, are the driving rational for the development of the second generation FOSs with functionally-enhanced properties (Rastall & Maitin, 2002).

1.4. Production of Fructooligosaccharides

1.4.1. Extraction of Fructooligosaccharides from Natural Food Products

FOSs may be extracted from flowering plants, which are widely found in temperate to arid climates (Banguela and Hernandez, 2006). Asparagus, garlic, leek, onions, artichoke, Jerusalem artichoke and chicory roots are the edible parts of fructan-containing plant species, which belong to the monocotyledonous and dicotyledonous families *Liliaceae*, *Amaryllidaceae*, *Gramineae* and *Compositae* (van Loo et al., 1995). However, FOSs may also be extracted from frequently consumed foods such as banana, tomato, brown sugar and honey (Flamm et al., 2001) and their FOS content is presented in Table 2.

Table 2: Concentration of FOSs in common foods (Adapted from Sangeetha et al., 2005).

Common sources	Yield of FOS (%)
Barley	0.15
Tomato	0.15
Onion	0.23
Banana	0.30
Brown sugar	0.30
Rye	0.50
Garlic	0.60
Honey	0.75

Although barley, wheat, oat and forage grasses contain up to 70% fructan on a dry weight basis (Fuchs, 1991), the *Gramineae* only contain trace amounts of FOSs (Campbell et al., 1997), because they contain organs which interfere with extraction. On the other hand, due to the presence of bulbs, tubers and tuberous roots, which are storage organs that can be easily processed for purification, plant species within the *Liliaceae*, *Amaryllidaceae*, and *Compositae* families are suited for FOS extraction (Fuchs, 1991). However, as seen in table 2, the yield of FOS obtained from extraction is extremely poor (Sangeetha et al., 2005).

1.4.2. Chemical Synthesis of Fructooligosaccharides

FOSs can be obtained by strategies involving their chemical synthesis. Because of the presence of various functional groups and chiral centers in monosaccharides, sequential selective protection-deprotection steps of these functional groups are needed in order to control the stereochemical and regiochemical specificity of the glycosidic bond formed. The chemical synthesis of FOSs being a laborious multi-step endeavour, it involves toxic pollutant reagents and do not comply with food safety conditions (Palcic, 1999). On the other hand, chemical hydrolysis of a polysaccharide to defined-size oligosaccharides is difficult to control due to the formation of brown contaminants from the conventional heating procedure (Warrand and Janssen, 2007).

1.4.3. Hydrolysis of Fructans to Fructooligosaccharides

FOSs may be produced from the hydrolysis of oligo- or polymeric fructans by levanases (EC 3.2.1.65) or inulinase (EC 3.2.1.7). Exo-levanase is a levan-degrading enzyme that releases levanbiose and fructose by hydrolyzing levan starting from its fructosyl end. Exo-levanases are capable of hydrolyzing inulin, raffinose and sucrose (Menendez et al., 2004) whereas endo-levanases and endo-inulinases have absolute substrate specificity for levan (Miasnikov, 1997) and inulin (Uhm et al., 1999), respectively. Since the hydrolysis of internal β -glycosidic linkages is performed in a random fashion, oligofructans of varying sizes are yielded from these endo-fructanases (Murakami et al., 1990). Up to now, levanase (EC 3.2.1.65), 2,6- β -D-fructan 6-levanbiohydrolase (EC 3.2.1.64) and levan fructotransferase are the three microbial sources of enzymes capable of hydrolyzing levan, resulting in the production of fructose (Baeur and Avigad, 1966), FOSs or levanbiose (Avigad and Zelikson, 1963) and di-D-fructose-2,6':6,2'-dianhydride (Tanaka et al., 1982), respectively.

1.4.4. Enzymatic Synthesis of Fructooligosaccharides

In comparison to the chemical synthesis, enzymatic synthesis offers regiospecificity and stereospecificity to the glycosidic linkages and hence is the actual favored method for the synthesis of commercial oligosaccharides (Plou et al., 2007). FOSs can be synthesized by β -fructofuranosidase (EC 3.2.1.26) or fructosyltransferase (FTF) (EC 2.4.1), also known as fructansucrases (Roberfroid, 2008; Miasnikov, 1997). For instance β -fructofuranosidases from the fungi *Aureobasidium pullulans*, *Aspergillus niger* and *A. oryzae* were used to produce short-chain-FOSs (Fernandez et al., 2004; Shin et al., 2004; Sangeetha et al., 2005). On the other hand, levansucrase (EC 2.4.1.10), which belongs to the fructansucrase enzymes (del Moral et al., 2008), can synthesize the polymer levan as well as short-chain-FOSs (Bekers et al., 2002; Vigants et al., 2003; Ozimek et al., 2006b). Another enzyme capable of synthesizing FOSs is the FTF (EC 2.4.1.162), which is closely similar to levansucrase. However, the only feature that differentiates FTFs from levansucrases is that they mainly catalyze the synthesis of FOSs without producing significant amount of levan (Cheetham et al., 1989; Seibel et al., 2006b).

Although there is ambiguity on whether FOS-producing enzymes should be categorized as β -fructofuranosidase or as fructansucrase (FTF), Plou et al. (2007) argued that the classification should be determined on the basis of the ratio of transfructosylation to hydrolytic activity at low substrate concentration. Fructansucrase (FTF) exerts sufficient transfructosylation activity at low sucrose concentration to synthesize FOSs (Nguyen et al., 2005). According to the Henrissat classification organizing enzymes based on their amino acid sequence, β -fructofuranosidases and fructansucrases belong to the glycoside hydrolase (GH) enzyme family (Coutinho and Henrissat, 1999). Although these enzymes act on common substrates and perform similar glycosylation mechanism (Sanz-Aparicio et al., 1998), bacterial fructansucrases (FTF and levansucrase) belong to family GH68, whereas β -fructofuranosidase and other fructans-hydrolytic enzymes belong to the GH32 family (Coutinho and Henrissat, 1999).

1.4.4.1. β -fructofuranosidase-Catalyzed Synthesis of Fructooligosaccharides

Despite their availability and affordability, the application of β -fructofuranosidases for the synthesis of novel FOS structures is limited by modest yields below 20% (Plou et al., 2007), narrow acceptor specificity (Cote and Tao, 1990) and poor regioselectivity (Ajisaka and Yamamoto, 2002). The ratio of transfructosylation to hydrolytic activity of β -fructofuranosidases relies on the thermodynamic equilibrium of the reaction and on the capacity of the enzyme to bind to the acceptor with high specificity as compared to water (Plou et al., 2007). The synthetic reaction-catalyzed by β -fructofuranosidases may be favored over the hydrolytic one by high substrate concentration, elevated temperatures and the use of organic co-solvents or an acceptor. The yield of products generated from thermodynamically controlled synthesis depends on the initial substrate concentration, pH, temperature, ionic strength and solvent composition (Plou et al., 2007). The hydrolytic activity of fructofuranosidase can be also disfavored by the constant elimination of the transfructosylation end-products by crystallization, selective adsorption to carriers or coupling through another enzymatic reaction. Moreover, the transfructosylation activity can be favored by using high levels of fructosyl acceptor. In fact, the nucleophilic attack of these acceptor substrates by the enzyme is more rapid than with water; thereby inhibiting the hydrolytic reaction (Cobucci-Ponzano et al., 2003). On the other hand, DNA technology can also shift the equilibrium towards the

transfructosylation reaction. Indeed, glycosynthases are enzymes which have been modified by site-directed mutagenesis (Perugino et al., 2004) through the substitution of the catabolic carboxyl nucleophile by non-nucleophilic residues such as glycine, alanine and serine. As a result, the hydrolytic activity of glycosynthases is suppressed but the enzyme still exhibits the transfructosylation activity because the enzyme-glycosyl intermediate complex imitates the covalent fructosyl-enzyme intermediate formed by FTF. Indeed, the mechanism of the transfructosylation of glycosynthases is performed in a similar fashion as natural FTFs. However, the derived transfructosylated products reveal an anomeric configuration that is opposite to the initial substrate. Nonetheless, glycosynthases provide a high yield of oligosaccharides within the range of 95 to 98% (Fairweather et al., 2002).

1.4.4.2. Fructooligosaccharide Synthesis by Fructansucrases

Fructansucrases comprise inulosucrase (EC 2.4.1.9) and levansucrase (EC 2.4.1.10), which are fructosyl-transferring enzymes that use sucrose to produce the fructans inulin (Tungland, 2003) and levan (Gross et al., 1990), respectively. Levan and inulin consist of a terminal D-glucose followed by β -D-fructofuranose units linked by $\beta(2\rightarrow6)$ and $\beta(2\rightarrow1)$ glycosidic bonds, respectively. The enzyme levansucrase is responsible for both the $\beta(2\rightarrow6)$ and $\beta(2\rightarrow1)$ types of linkages found in levan (Hestrin and Avigad, 1958). In addition to the differences in regiospecificity and stereospecificity, inulosucrase and levansucrase are distinguished by the degree of polymerization of the products, which depends on the ratio of transglycosylation to hydrolytic activities (Ozimek et al., 2006a). Previous studies mainly focused on levansucrase than on inulosucrase because of the broader range of acceptors, and therefore to a wider range of products that can be synthesized (Cote and Tao, 1990; Seibel et al., 2005).

1.4.5. Levansucrase-Catalyzed transfructosylation reaction

Levansucrase, or sucrose:2,6- β -D-fructan:6-D-FTF (EC 2.4.1.10), is able to catalyze four types of reactions: exchange, hydrolysis, transfructosylation and polymerization, depending on the acceptor involved. Hydrolysis occurs when levansucrase transfers the fructosyl moiety of sucrose to water (Yun, 1996), whereas the exchange reaction consists of the transfer of the fructose unit of sucrose to a free glucose (Hettwer et al., 1995). Levansucrase can use both sucrose and sucrose analogues because they are

thermodynamically favorable. In fact, the energy released from the hydrolysis of sucrose is sufficient to transfer the fructosyl unit of sucrose to another sucrose or acceptor and to form the high energy glucopyranosyl-fructofuranosyl bond (Chambert et al., 1974; Seibel et al., 2006a). The transfructosylation reaction of levansucrase may result in the synthesis of the polymer levan or to the production of levan-type or inulin-type FOSs (Euzanat et al., 1997; Trujillo et al., 2001). In the presence of other acceptors, such as monosaccharides (Baciu et al., 2005), disaccharides (Park et al., 2003), oligosaccharides (Meng and Futterer, 2008), sucrose analogues (Seibel et al., 2006b), glycerol (Gonzalez-Munoz et al., 1999) or methanol (Kim et al., 2001), levansucrase is able to produce a wide range of hetero-FOSs (Tieking et al., 2005).

1.4.5.1. Microbial Sources of Levansucrases

Levansucrase can be secreted by gram-negative bacteria, including *Pantoea agglomerans* (Cote and Imam, 1989), *Erwinia amylovora* (Gross et al., 1992), *Zymomonas mobilis* (Lyness and Doelle, 1983) *Acetobacter suboxydans* (Loitsyanskaya et al., 1971) *Gluconobacter oxydans* (Elisashvilli, 1980) and *Rahnella aquatilis* (Ohtsuka et al., 1992) and by the gram-positive species such as *Bacillus subtilis* (Tanaka et al., 1978), *B. polymyxa* (Han, 1989), *B. natto* (Takahama et al., 1991), *B. amyloliquefaciens* (Mäntsälä and Puntala, 1982), *Streptococcus mutans* (Sato et al. 1984), and *Actinomyces viscosus* (Pabst et al., 1979). Despite the fact that levansucrases from gram-positive and gram-negative bacteria have similar active site conformation, they differ with respect to their amino sequence (only 20% similarity), their molecular weight, their secretion from the cell-membrane, the degree of polymerization of the product synthesized, the regio-specificity, and the donor or acceptor specificity (van Hijum et al., 2006). For instance, levansucrase from *B. subtilis* and *B. megaterium* were found to catalyze dominantly the synthesis of levan (Chambert et al., 1974; Homann et al., 2007), whereas the levansucrase from *G. diazotrophicus* synthesized mainly the short FOSs (Hernandez et al., 1995). In addition to levan, both *B. megaterium* and *Z. mobilis* levansucrases produced the FOSs, 1-kestose, 6-kestose and neokestose (Beckers et al., 2002; Homann et al., 2007), while *B. megaterium* levansucrase also synthesized nystose and blastose (Homann et al., 2007). Moreover, 1-kestose served as a more effective fructosyl acceptor to the levansucrase of *B. subtilis* as compared to *A. diazotrophicus* levansucrase (Hernandez et al., 1995).

Although some hypotheses have been put forward, it is still poorly understood what structural features could determine the polymerization/oligomerization ratio and the reaction specificity (hydrolysis/transfructosylation) (Ozimek et al., 2006b).

1.5. Mechanistic Action of Levansucrase

1.5.1. Structure-Function Relationship

The structure-function relationship of levansucrase has been examined based on the amino acid sequence alignment, site-directed mutagenesis and structural information (Martinez-Fleites et al., 2005; Ozimek et al., 2006a; Homann et al., 2007). The key functional amino acid residues constituting the catalytic triad of bacterial levansucrase were determined to be Asp95, Glu352 and Asp257. Glu352 is presumed to protonate the osidic bond of sucrose, whereas Asp95 is believed to be the nucleophile that attacks the glucopyranosyl moiety of sucrose by forming an enzyme-fructosyl complex and inverting the glycosidic bond of sucrose into an intermediate state (Chambert and Gonzy-Treboul, 1976). On the other hand, Asp257 is assumed to be involved in the coordination of the hydrogen bonds at positions 3-OH and 4-OH of the fructofuranoside (Kralj et al., 2008). Amino acid substitutions have improved our understanding of the important amino acid residues involved in FOS versus levan synthesis by levansucrase. In fact, various FOSs, including neokestose and blastose were obtained by modifying Arg360 from *B. subtilis* SacB (Chambert and Petit-Glatron, 1991) and Arg370 from *B. megaterium* SacB at subsite -1 (Homann et al. 2007), respectively.

1.5.2. Active Site of Levansucrase

The -1 and +1 acceptor-binding subsites consist of eight amino acid residues participating in the binding of sucrose to the active site of levansucrase (Ozimek et al., 2004; Meng and Fütterer, 2008). Previous work on site-directed mutagenesis on levansucrases strongly indicates that the regions W271, R423 and W340 at subsite -1 are required for substrate recognition and catalysis. On the other hand, the region R423 has been suggested to assist in the interaction of the glucose unit of a bound sucrose located at subsite +1. Substituting the arginine at position R423 with a lysine side chain resulted in a weaker binding to the glucose portion possibly due to the lower hydrogen bonding ability of the lysine in comparison to the longer chain arginine (Ozimek et al., 2006a). It has been previously suggested that R423 is involved in substrate binding, sugar ring recognition and

specificity towards fructopyranosyl unit (Nagem et al., 2004). Ozimek et al. (2006a) observed a substantial drop in enzymatic activity by mutating W340 and interestingly 1-kestose was the sole product detected on TLC. In addition, Yanase et al. (2002) have demonstrated an increase in the polymerization activity of *Z. mobilis* levansucrase mutant by modifying the amino acid residues neighboring the active site W340. Although there seems to be no direct interaction between W340 and the substrate, W340 is assumed to be critical to the shaping of the binding site and to the orientation of sucrose. It has been suggested that removing the tryptophan side chain within the sucrose-binding pocket would result in a larger exposure area of subsite -1, hence favoring polymerization reactions by enhancing the bond between the fructose portion of sucrose and levansucrase (Ozimek et al., 2006a).

1.6. Transfructosylation Product Spectrum

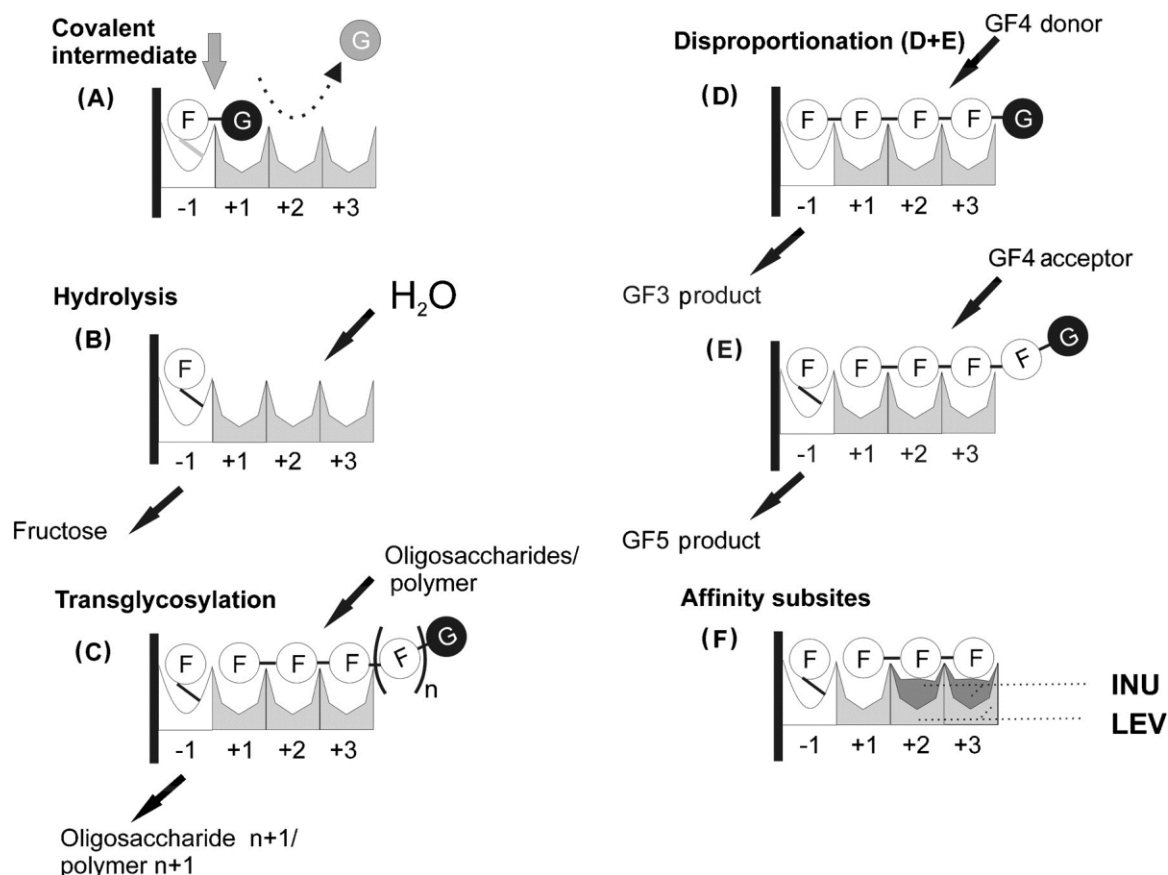
1.6.1. Proportionate and Disproportionate Reactions

According to the 3-D structures and the sequence alignment among GH68 enzyme family, the amino acid residues located at subsite +1 are different among gram-positive and gram-negative bacteria. Nonetheless, these discrepancies do not explain the reaction selectivity towards polymerization, transfructosylation or hydrolysis (Hernandez et al., 1995; van Hijum et al., 2002), and therefore nor the end-products synthesized. In fact, the levansucrase from *B. subtilis* (Chambert et al., 1974), *L. reuteri* (Ozimek et al., 2006a; b) and *B. megaterium* (Homann et al., 2007) synthesize the high-molecular-mass levan, implying that the substrate remained bound to the enzyme during polymerization and that chain elongation proceeded in a processive (proportionate) manner (See Scheme. 5C). In contrast, the short-chain FOSs, kestose and nystose, were synthesized by the levansucrase from *G. diazotrophicus*, *Z. mobilis* and *L. sanfranciscensis* using sucrose as the sole substrate (Doelle et al., 1993; Hernandez et al., 1995; Korakli et al., 2003). The formation of short-chain FOSs by these enzymes must be performed in a non-processive (disproportionate, see Scheme. 5D and 5E) in that the fructan chain is released out of the active site pocket after each transfructosylation reaction (Kralj et al., 2008).

1.6.2. Donor and Acceptor Specificities of Levansucrase

As depicted in Scheme 5A to 5F, subsite +1 of the active site of levansucrase shows affinity for the acceptors glucose (from sucrose or raffinose), fructose (from sucrose),

glycopyranosides (from mannose, galactose, fucose or xylose) and disaccharides (from maltose, lactose or melibiose), whereas subsite -1 has an absolute specificity towards fructose residues (Hernandez et al., 1995; Song et al., 1999; Seibel et al., 2005). For this reason, levansucrase is able to use sucrose, sucrose analogues, raffinose, or stachyose as fructosyl donors (Park et al., 2003; Seibel et al., 2006b; Meng and Fütterer, 2008). The advantages of using sucrose as a substrate include its large availability, purity, low cost (Monchois et al., 1999) and most importantly, its high-energy osidic bond that is almost equivalent to nucleotide-activated sugars. In fact, the Gibbs energy value required for sucrose hydrolysis is $\Delta G^\circ_A = -26.5 \text{ kJ/mol}$ (Goldberg and Tewari, 1989), which is higher than any disaccharides (Tewari and Goldberg, 1991).



Scheme 5: Model of the substrate-binding at the active site and levansucrase-catalyzed proportionate and disproportionation reactions (Ozimek et al., 2006b).
G (Glucose), F (Fructose) and LEV (Levansucrase)

Although levansucrase from *Microbacterium laevaniformis* has a wide acceptor specificity, disaccharides containing a pyranose ring were particularly more effective acceptors than monosaccharides with a furanose ring (Park et al., 2003). It has been previously demonstrated that the chemical structure of acceptors is a strong determinant of the regio-selectivity of enzymes (Robyt, 1995). Park et al. (2003) demonstrated that acceptors comprised of a galactose, maltose, lactose, melibiose or cellobiose residue were more receptive for the levansucrase from *M. laevaniformis* than saccharides containing arabinose, xylose or raffinose within its chemical structure; particularly, maltose, melibiose and cellobiose were shown to be the most suitable acceptors.

1.7. Purification of Levansucrase

Levansucrase can be prepurified using ammonium sulfate (van Hijum et al., 2001), manganese chloride (Vigants et al., 2001) polyethylene-glycol (Oseguera et al., 1996b), protamine sulfate (Euzanat et al., 1997) and acetone (Ben Ammar et al., 2002). However, levansucrase also needs to be purified by gel filtration and/or anionic exchange chromatography. Of the size-exclusion columns that have been previously employed are Sephadex (Tanaka et al., 1979; et al., 1996b), sephacryl S-300 (Cote et al., 1989; Vigants et al., 2003; Goldman et al., 2008) and (TMAE)-Fraktogel (Hettwer et al., 1995). On the other hand, common anionic exchange columns mainly include hydroxyapatite (Gonzy-Treboul et al., 1975) or DEAE-cellulose (Esawy et al., 2008) columns. The purity of levansucrase is usually assessed by SDS-PAGE and the molecular weight is estimated to be around 55 kDa (Sangiliyandi et al., 1999; Abdel-Fattah et al., 2005). The enzyme is believed to exist as a monomer (Tanaka et al., 1978). However, some microbial sources of levansucrase have been shown to have molecular weights of 94 kDa (Yanase et al., 1992; Ohtsuka et al, 1992) and to exist in the dimer form (Goldman et al., 2008). The elevated molecular weights observed in some studies have been proposed to be due to the association of the enzyme to its product, levan (Crittenden and Doelle, 1994; Hettwer et al., 1995; Oseguera et al., 1996b).

1.8. Effect of Reaction Parameters on Levansucrase -Catalyzed Reactions

1.8.1. Effect of Reaction pH

The effect of pH on the reactions catalyzed by levansucrase has been widely studied among various levansucrases from different microbial sources and have been shown to be stable and to exhibit activity within a wide range of pH of 3.0 to 8.0 (Sangiliyandi et al., 1999; Esawy et al., 2008; Rairakhwada et al., 2010). The optimal pH of levansucrase is within near neutral pH, between 5.0 and 7.0 for *Z. mobilis* (Jang et al., 2001; Goldman et al., 2008) and *B. subtilis* (Esawy et al., 2008). However, the levansucrase from lactobacilli species generally catalyze maximal activity at lower pH range of 3.5 to 5.5 (van Hijum et al., 2004; Tieking et al., 2005; Anwar et al., 2010). A few studies have investigated the effect of pH on the different reactions catalyzed by levansucrase (Crittenden and Doelle, 1994; Oseguera et al., 1996b). The results suggest that the effect of pH on the polymerization, transfructosylation and hydrolytic activities of levansucrase is strain-dependent. For instance, the optimum pH for hydrolysis is observed at pH above 6.75 in *Z. mobilis* (Goldman et al., 2008), at pH lower than 3.0 for the levansucrase from *L. panis* (Waldherr et al., 2008), whereas the hydrolytic activity of *B. subtilis* levansucrase remains constant over the wide range of pH (Euzanat et al., 1997). Studies regarding amino acid substitution have pointed the great importance of the steric requirements of the amino acid groups at the active site of levansucrase (Chambert et al., 1991; Yanase et al., 2002; Martinez-Fleites et al., 2005). For this reason, pH is a parameter that is expected to influence the reaction selectivity of levansucrase. However, the mechanisms determining the reactions catalyzed by levansucrase are still unclear (Meng and Futterer, 2003).

1.8.2. Effect of Reaction Temperature

The effect of the variation of temperature on the total activity of levansucrase has also been extensively studied but most authors characterized the total activity rather than separately quantifying the transfructosylation and hydrolytic activities. The optimum temperature for levansucrase is found within the range of 30 to 60°C in the levansucrase from *B. subtilis* (Baciu et al., 2005), *B. megaterium* (Homann et al., 2007), *Z. mobilis* (Sangiliyandi et al., 1999) and *L. reuteri* (Ozimek et al., 2005). Generally, levansucrases

from different microbial sources are stable within a broad range of temperature of 4 to 50°C (Ben Ammar et al., 2002; Esawy et al., 2008; Waldherr et al., 2008; El-Refai et al., 2009). Some have reported that the levan-forming activity was preferred at a lower temperature of 4 to 15°C (Crittenden and Doelle, 1994; Hettwer et al., 1995; Rairakhwada et al., 2009). Interestingly, although the levansucrase from *R. aquatilis* showed maximal polymerization activity at a lower temperature of 17°C, the synthesis of FOS was unaffected by the variations in temperature (Kim et al., 1998). In comparison, FOS-production was demonstrated to be favored under higher temperatures in the levansucrase from *B. subtilis*, *Z. mobilis* and *A. levanicum* (Ebert and Schenk, 1968; Tanaka et al., 1981; Belghith et al., 1996). Therefore, it appears that the effect of temperature on the activity of levansucrases and on their end product spectrum is dependent on their microbiological source.

1.8.3. Effect of Solvents

To improve the enzymatic stability, to change the chemoselectivity, and to alter the thermodynamic equilibrium, levansucrase-catalyzed reactions were investigated in organic solvent media. The organic media enhanced the recovery of levan, which usually requires the use of low molecular weight alcohols to change the dielectric constant and induce their precipitation, and hence preserve the levansucrase activity (Steinberg et al., 2002). When the levansucrase from *B. subtilis* was incubated in an aqueous system with 70% (v/v) acetonitrile, transfructosylation was the sole reaction catalyzed (Chambert and Petit-Glatron, 1989). In contrast, incubating *R. aquatilis* levansucrase in acetonitrile, acetone and dimethyl sulfoxide increased the hydrolytic activity (Kim et al., 1998). These contradictory results support the fact that enzymatic and kinetic activities of levansucrase are source-dependent.

1.8.4. Effect of Substrate Concentration

The hydrolytic activity was the predominant activity expressed by levansucrase at low substrate concentration while polymerization was favored at higher substrate concentration (Morales-Arrieta et al., 2006; Goldman et al., 2008). Moreover, the K_m value of levansucrase is very low for sucrose, ranging from 3 to 76 mM (Ben Ammar et al., 2002; Homann et al., 2007; Waldherr et al., 2008), indicating that levansucrase has a very high affinity for the this fructosyl donor.

1.9. Characterization of Levansucrase Transfructosylation End-Products

Since they contain no functional groups and share structural similarities, the isolation of FOSs is a laborious task. It is, however, possible to isolate them by activated carbon:celite column chromatography (Crittenden and Doelle, 1993; Yun, 1996), but the results are inconsistent (Sims et al., 1991). Although chromatographic separation is a more efficient method (Crittenden and Doelle, 1993; Yun and Song, 1993), it is designed for small samples and the little recovery yield is unsuitable for large scale production (Yun, 1996; Van der Westhuizen and Kilian, 2008).

1.9.1. Thin-Layer Chromatography (TLC) Analysis

FOSs can be characterized by TLC analysis using a developing mobile phase of isopropyl alcohol:ethyl acetate:water (2:1:1) and a detection with phenol sulphuric acid (Park et al., 2001). A more rapid method for the quantification of FOSs uses a modern instrumental TLC, which involves the use of acetonitrile and acetone as solvents, and diol high performance TLC plates; however, derivatization using 4-aminobenzoic acid reagent, glacial acetic acid, water, 5% phosphoric acid and acetone is necessary. Plates are then heated at 115°C for 15 min in order to identify the FOS corresponding to yellowish to brown spots (Vaccari et al., 2001). A more specific detection of fructose-containing carbohydrates, FOSs, consists of using a mixture of 0.1% resorcinol (w/v) and 0.25% thiourea (w/v) in an acetic acid. Reducing sugars and fructose-containing products appear as orange spots and purple spots, respectively (Muro et al., 1999). This represents a very rapid, affordable, specific and sensitive method, which can be relevant for the time-course production of FOS and levan by levansucrase. However, TLC analysis has been mostly replaced by instrumental chromatographic methods for the determination of polysaccharides, monosaccharides, disaccharides and oligosaccharides because of the ease of quantification, rapidity and higher separation efficiency (Folkes and Jordan, 2006).

1.9.2. Gas Chromatography

Gas chromatography followed by mass spectrometry is another technique employed to analyze FOSs. However, FOSs must first undergo methylation with methyl iodide, hydrolysis with 1M H₂SO₄, reduction by sodium tetradeuteroborate (NaBD₄) and alditol acetylation by using acetic anhydride. The methylated FOSs can further be subjected to gas chromatography-mass spectrometry containing a fused silicone column and employing helium as the carrier gas (Hayashi et al., 2000). In the absence of derivatization, liquid chromatography coupled with mass spectrometry should be considered in the characterization of FOSs (Sangeetha et al., 2005).

1.9.3. High-Performance Liquid Chromatography (HPLC)

High performance liquid chromatography (HPLC) is more frequently employed for carbohydrate analysis than gas chromatography due to the major advances in detectors. Elution of FOSs may be more efficient by selecting a polar bonded-phase, such as -NH₂ columns (L'Hocine et al., 2000). Although -NH₂ columns allow the separation of oligosaccharides up to a polymerization degree of 30 to 35 (LaCourse and Johnson, 1993), the formation of glucosamine from the interaction of reducing sugars with the amino groups of the stationary phase is an important drawback (Cataldi et al., 2000). In contrast, K⁺ Aminex HPX-87 K (Le Gorrec et al., 2002), Aminex HPX 87 N columns (Kim et al., 2001; Park et al., 2001) or Aminex HPX 87 C (Trujillo et al., 2001; Crittenden and Playne, 2002) are examples of resin-based columns which elute FOSs in order of decreasing chain length. The use of a resin-based HPLC columns coupled to a refractive index (RI) detectors is very common (Prapulla et al., 2000), but this type of detector has poor selectivity and very low sensitivity (Cataldi et al., 2000).

1.9.4. High-Pressure Anionic Exchange Chromatography with Pulsed Amperometric Detection (HPAEC-PAD)

Separation of carbohydrates is more valuable using HPAEC-PAD than with gas chromatography or HPLC because of its effectiveness for the separation of complex sugars (Cataldi et al., 2000). Moreover, HPAEC-PAD represents a simple, economical and rapid quantifying method requiring small sample volumes and minimal sample preparation (Hogarth et al., 2000). The principle underlying HPAEC-PAD takes advantage of the weakly acidic properties of carbohydrates under highly alkaline medium

(Rendleman, 1973), allowing the separation of closely related mono- and disaccharides (Cataldi et al., 1999). CarboPac PA 100 generally serves as the analytical anion exchange column for the analysis of FOSs by HPAEC-PAD and is packed with macroporous resin consisting of co-polymerized ethylvinyl- and divinylbenzene, making it more suitable for both mono- and oligosaccharide isolation than the divinylbenzene-packed CarboPac PA10 column (Dionex Corp., 1995). However, CarboPac PA200 can also be employed to isolate FOSs by using high concentration of saturated sodium hydroxide and sodium acetate. In addition, this column offers high sensitivity, selectivity, resolution, reproducibility and baseline stability. Moreover, pulsed-amperometric detection is specific to carbohydrates and interferences are eliminated, making HPAEC-PAD more sophisticated than HPLC which uses RI or UV detectors (Folkes and Jordan, 2006). The main disadvantage with this method is the high cost of inulin-type FOS standards and the absence of commercial levan-type FOS standards (Borromei et al., 2009). Another important drawback is the inevitable formation of sodium carbonate from the reaction between carbon dioxide and sodium hydroxide. Sodium carbonate has high affinity to the column, causing a change in retention time (Rocklin et al., 1998). The problem can be averted by cleaning the column with a higher concentration of sodium hydroxide, but the restoration of a stable baseline is time-consuming. In spite of using an inert gas (nitrogen or helium), the effect of sodium carbonate after consecutive injections has a significant impact of retention times and on the column performance (Cataldi et al., 1999). Solutions have been developed to counter this problem and these include the use of carbonate-free sodium hydroxide solutions (Dionex, 1995) and the use of barium or strontium ions to precipitate sodium carbonate (Hammond, 1990). HPAEC-PAD should be used in parallel with matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) to confirm the molecular size of carbohydrates. However, MALDI-MS is reliable with respect to monomer sequencing and molecular mass assignments, but branched and linear FOS are not, making nuclear magnetic resonance (NMR) spectroscopy an indispensable tool for the determination of sugar sequence, branching, glycosidic bonds and anomeric configurations of FOS (Borromei et al., 2009).

1.9.5. Official Method of Detection Assigned by the AOAC

Due to the impurity and poor availability of FOS standards, the official method assigned by the AOAC for the analysis of FOSs, is based on the enzymatic treatment of samples with inulinases. The extraction of inulin and oligofructans is achieved by boiling the samples and hydrolyzing an aliquot of this fraction with amyloglucosidase while the other aliquot remains untreated. An aliquot of the hydrolyzed fraction is further hydrolyzed by the commercial inulinase Fructozyme SP 230. The hydrolysates can be analyzed spectrophotometrically (Steegmans et al., 2004), or by HPLC (Corradini et al., 2004). The inulin concentration is estimated by subtracting the glucose, fructose and sucrose content within the first hydrolysate and untreated sample from the second hydrolysate. Since oligofructans are not extracted by the AOAC-Total Dietary Fiber determination method, FOS may be estimated by subtracting the fructan concentration in the precipitate from the total dietary fiber (Flamm et al., 2001). However the AOAC method is limited since it only quantifies the total amount of FOSs, as opposed to individual FOS. Interestingly, a method has been developed specifically for the separation of FOSs by HPAEC-PAD with CarboPac PA200. The described method proposes a reliable quantification of FOSs with a degree of polymerization greater than 6 and 7, whose commercial standards are unavailable. This method was achieved by constructing standard curves for those compounds using defined quantities of the fiber BeneoTM P95 (Borromei et al., 2010).

CHAPTER 2. MATERIALS AND METHODS

2.1. Sources of Chemicals

In all sets of experiments, deionised water (Millipore) was used. The chemical reagents were of laboratory grade for enzymatic assays, of electrophoresis grade for sodium-dodecyl-sulfate-polyacrylamide-gel-electrophoresis (SDS-PAGE) and of high-pressure-liquid-chromatography (HPLC) grade for structural analysis. Chemical reagents were purchased from Difco (nutrient broth and tryptone), Sigma (sucrose, fructose, glucose, FeSO_4 , FeCl_3 , FeSO_3 , MnCl_2 , 2,5-dinitrosalicylic acid, potassium sodium tartrate, NaOAc , ovalbumin, lysozyme, α -chymotrypsin, 1-kestose and nystose), Fisher (MgSO_4 , $(\text{NH}_4)_2\text{SO}_4$, H_2HPO_4 , CaPO_4 , yeast extract, bacto-peptone, glycerol, ferric ammonium citrate, NaOH , bovine serum albumin, PEG-200, PEG-400), MP Biomedicals (agar, KH_2PO_4), Anachemia (Na_2HPO_4), ACP chemicals (MnSO_4) and Fluka (raffinose and 50% saturated NaOH).

2.2. Microbial Strain and Growth Conditions

Geobacillus stearothermophilus (Donk) was obtained from ATCC and used as a microbial source for the production of FTF. The strain was maintained in a solid agar (15 g/L) media containing peptone (5 g/L) and meat extract (3 g/L) and grown at 55°C in an incubator (Gravity convection incubator, Precision Scientific) for 24 hrs, and then stored at 4°C.

2.3. Culture Conditions

G. stearothermophilus was grown in selected mineral and succinate-salt based media. The mineral salt-based medium (Jarasuriya, 1955) was comprised (in g/L): $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$ (2.67), KH_2PO_4 (1.36), $(\text{NH}_4)_2\text{SO}_4$ (0.5), $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ (0.005), $\text{MnSO}_4 \cdot \text{H}_2\text{O}$ (0.0018), $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$ (0.0025), $\text{CaPO}_4 \cdot 2\text{H}_2\text{O}$ (0.01) and $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (0.2). While Succinate-salt based medium (Caulfield et al., 1979) consisted of (in g/100mL): sodium succinate (10), K_2HPO_4 , (0.7), KH_2PO_4 (0.3), $(\text{NH}_4)_2\text{SO}_4$ (0.2), $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ (0.005), $\text{MnCl}_2 \cdot 6\text{H}_2\text{O}$ (0.005) and $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (0.025). In order to reduce the lag time (Lincoln, 1960), a loopful of *G. stearothermophilus* was inoculated into 100 mL of preculture in a 250 mL Erlenmeyer flask. Moreover, to ensure consistency and prevent variations (Stanbury, 1995), the preculture had a composition identical to the selected culture medium, but it

was deprived of a carbon source. The preculture was incubated at 55°C overnight with continuous orbital agitation of 150 rpm (Lab-Line 3527 Orbit Environ-Shaker) for 15 hours to ensure the transfer of an inoculum at the proper physiological state (metabolically active) (Hockenhull, 1980; Hunt and Stieber, 1986). A defined volume of preculture was withdrawn and transferred into a 1 L baffled Erlenmeyer flask containing 400 mL of culture medium to reach an initial absorbance of 0.7 at 600 nm.

The bacterial growth was assessed spectrophotometrically (DU800 spectrophotometer, Beckman Coulter) by measuring the optical density (OD) of the culture media at 600 nm. The time course for the FTF production was investigated over 32 hr-culture period. Every 2 hrs, 2 mL of culture medium was withdrawn and centrifuged (8,000 rpm for 45 min, Model J2-21 Beckman) at 4°C to isolate the supernatant (extracellular FTF) from the cells (membrane-bound FTF). In order to concentrate and pre-purify the extracellular FTF, an ultrafiltration step (Amicon, Millipore) was carried out using a polyethersulfone membrane (Millipore) with a 10 kDa cut-off. To release the membrane-bound FTF, the recovered bacterial cells were re-suspended in 25 mL of 10 mM potassium phosphate buffer (pH 7.0), and ruptured by ultrasonication at 2 kHz with a cycle of 25/50 seconds (550 Sonic Dismembrator, Fisher). The resulted suspension was homogenized at 4°C for 15 min and then centrifuged (8,000 rpm for 45 min) to liberate the membrane-bound FTF. The activity and the protein content of the crude extracellular and membrane-bound FTF extracts were assessed.

2.3.1 Effect of Nitrogen Sources

To optimize the microbial growth and the FTF production, the mineral salt-based medium was supplemented with different nitrogen sources, including yeast extract (1.0 g/100 mL), tryptone (1.0 g/100 mL), bacto-peptone (0.5 - 2.0 g/100 mL), bacto-peptone/tryptone (0.5 g of each/100 mL) and bacto-peptone/(NH₄)₂SO₄ (0.5 g of each/100 mL). These nitrogen sources supply different levels of free amino acids. The optimal microbial growth and the maximal FTF production were determined and compared using the selected nitrogen sources.

2.3.2. Effect of Carbon Sources

To maximize the FTF induction, the salt-based medium containing bacto-peptone/tryptone was supplemented with selected carbon sources that can act as inducers, including: fructose (10 g/L), glucose (10 g/L), glycerol (10 g/L), raffinose (10 g/L), sucrose (2.5 to 10 g/L), and sucrose/glucose mixture (10 g of each/L) and sucrose/glycerol mixture (10 g of each/L). The optimal microbial growth and the maximal FTF production were ascertained and compared in the presence of the selected carbon sources.

2.3.3. Effect of Iron Sources

In order to trigger the extracellular secretion of FTF, selected sources of ferric and ferrous iron (5 mg/L) were added to the optimal mineral salt medium to which sucrose (10 g/L) and peptone/tryptone mixture (5 g of each/L) were supplemented. The ferric iron sources consisted of FeCl_3 and ferric ammonium citrate, while the ferrous iron sources were $(\text{NH}_4)_2\text{Fe}(\text{SO}_4)_2$, FeSO_4 and FeSO_3 . After 26 hrs of culture, the microbial growth and the activity of the membrane-bound and the extracellular form of FTF were assessed.

2.4. Fructosyltransferase (Levansucrase) Purification

2.4.1 Preparation of Crude Fructosyltransferase Extract

After 8 hrs of culture, the membrane-bound FTF from *G. stearothermophilus* was recovered by ultrasonication of the suspended cells as described before (2.3). The crude FTF extract was dialyzed overnight against potassium phosphate buffer (5 mM, pH 7.0) at 4°C using a regenerated-cellulose-dialysis tubing with a cut-off of 6 to 8 kDa. Then, the dialyzed extract was lyophilized (Labconco freeze-drier, 61×10^{-3} Mbar, -40°C) and stored at -20°C for further purification.

2.4.2. Preparation of Pre-purified Fructosyltransferase Extract

The crude FTF extract was subjected to a pre-purification step through polyethylene glycol (PEG), $(\text{NH}_4)_2\text{SO}_4$ and MnCl_2 precipitation. Low molecular-weight PEGs, including PEG-200 and PEG-400, were added to 25 mL of membrane-bound FTF extract to obtain a final concentration of 40 to 50% (v/v) and 20 to 40% (v/v), respectively. A $(\text{NH}_4)_2\text{SO}_4$ precipitation was carried out at 40, 50 and 80% saturation, whereas 25, 50 and

100 mM concentrations were used for MnCl_2 precipitation. The precipitation step was carried out for 2 hrs at 4°C with continuous gentle stirring. After centrifugation (8,000 rpm for 45 min at 4°C), the FTF activity and protein content were assessed in the precipitate and supernatant fractions. The purification factor and the precipitation yield were then estimated.

2.4.3. Chromatographic Purification

All chromatographic purifications were performed, in a cold chamber at 10°C, on a FPLC system (Fast Protein Liquid Chromatography, Pharmacia), equipped with a LKB Controller LCC 501 Plus (Pharmacia) and UV detector (LKB UV-M-II, Pharmacia). Autochro-2000 1.0 software was used for computerized data handling and analysis (Young Jin, Seoul, Korea). The mobile phases were filtered using nitrocellulose filter membrane (0.22 μm , Millipore) and degassed (Branson 3510). Prior to sample loading, the crude FTF extract was centrifuged at 10,000 $\times g$ for 10 min and the supernatant was then pressure-filtered (Advantec MFS Inc.) through an AcetatePlus membrane (0.4 μm , 13 mm, GE Water and Process Technology). An aliquot of filtrate was withdrawn and assayed for protein content and FTF activity.

2.4.3.1. Size-Exclusion Chromatography

A fresh aliquot of FTF extract (0.5 mg protein/100 μL) was applied to Superdex 75 HR 10/300 gel filtration column (Amersham Pharmacia Biotech.), previously conditioned with 0.01 M potassium phosphate buffer (pH 7.0) containing 0.15 M NaCl. The elution was carried out with 9.6 - 10.4 mL of the same potassium phosphate buffer at a flow rate of 0.4 mL/min. Fractions of 800 μL were collected using a fraction collector (LKB FRAC-100, Pharmacia). In order to determine the purification yield and factor, the fractions, collected from the 15 injections, were gathered in the same tubes, and then assayed for the protein content and FTF activity by the DNS method (Miller, 1959) and the Bradford Microassay method (1976), respectively. The active fractions exhibiting the highest specific activities (elution volume 8.8 - 10.4 mL) were pooled, dialyzed overnight against potassium phosphate buffer (5 mM, pH 7.0) at 4°C and lyophilized.

The size-exclusion column was also used for the estimation of the FTF molecular weight. A protein mixture consisting of bovine serum albumin (2.0 mg/mL, 67 kDa), ovalbumin

(2.5 mg/mL, 43 kDa), lysozyme (5.0 mg/mL, 25 kDa) and α -chymotrypsin (5.0 mg/mL, 14 kDa,) was used as standard. The standard curve was constructed by plotting the log of the molecular weights versus the elution time.

2.4.3.2. Anionic Exchange Chromatography

The lyophilized FTF extract, obtained upon size exclusion chromatography, was re-suspended in potassium phosphate buffer (0.01 M, pH 7.0) and further purified by anionic exchange chromatography using a MonoQ HR 5/50 column (Pharmacia). The pre-purified FTF solution of 0.5 mg protein/100 μ L was applied to the MonoQ column previously equilibrated with potassium phosphate buffer (0.01 M, pH 7.0). To remove the unbound proteins, the column was then washed with 5 mL (5CV) of potassium phosphate buffer (0.01 M, pH 7.0) at a flow rate of 1.0 mL/min. The elution of bound proteins was carried out with two successive linear gradients: 0 to 0.2 M KCl in potassium phosphate buffer (0.01 M, pH 7.0) (total volume 20 mL) and 0.2 to 0.43 M KCl in the same buffer (total volume 32 mL). The FTF activity and protein content of the fractions (1 mL) were assessed. The active fractions with the highest specific activities were pooled, dialyzed overnight against 5 mM potassium phosphate buffer (pH 7.0) at 4°C and lyophilized.

2.4.4. Sodium-Dodecyl-Sulfate-Polyacrylamide-Gel-Electrophoresis (SDS-PAGE)

Aliquots of crude FTF extract and active FTF fractions obtained after gel filtration and anionic exchange chromatography were subjected to the SDS-PAGE electrophoresis analysis (Laemmli, 1970). Sample loading was achieved in a mini protein gel apparatus (Bio-Rad) with a 1.5 mm-thick gel. The acrylamide content in the stacking and resolving gels were 5% and 12%, respectively. The electrophoresis was conducted at a constant current of 120 mV. The gels were then stained for 2 hrs with a staining solution containing 1 g of Coomassie brilliant blue R250 diluted in methanol:water:acetic acid (45:45:10, v/v/v), followed by destaining in methanol:acetic acid:water (1:1:8, v/v/v). The molecular weight of FTF was estimated by comparing its migration distance to those of low molecular weight standard (14.4 - 97.4 kDa, Bio-Rad).

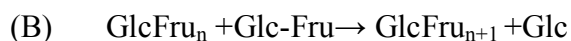
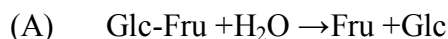
2.4.5. Thin-layer Chromatography

The levan-forming activity of the crude FTF extract and the active FTF fractions, obtained after chromatographic separation, were assessed by thin layer chromatography

(TLC) using sucrose as the sole substrate. The enzymatic reactions were carried out at 10°C for 48 hrs using 0.25 M of sucrose substrate. An aliquot of the reaction was withdrawn and diluted 7-fold with acetonitrile:water (20:80, v/v) prior to being spotted (10 µL) on a TLC silica gel 60F₂₅₄ aluminum sheet (EMD). The TLC plates were immersed in the developing mobile phase composed of butanol:acetic acid:water (5:4:1, v/v/v). For the total sugar detection, the TLC plates were sprayed with 2% (v/v) H₂SO₄ in methanol and heated at 90°C for the development of orange spots. For the detection of fructose-containing carbohydrates, the plates were first sprayed with a resorcinol solution (0.1%, w/v) in acetic acid containing thiourea (0.25%, w/v) and heated at 90°C for the development of purple spots.

2.4.6. Enzymatic Assays of Fructosyltransferase (Levansucrase) Activity

Three enzymatic activities were assayed using sucrose as substrate in a temperature-controlled water incubator (Isothemp 10165, Fisher): total FTF, hydrolytic and transfructosylation activities. The hydrolytic activity (A) and transfructosylation (B) of FTF catalyzes the following reaction:



Where Fru is fructose, Glc-Fru is sucrose, Glc is glucose, GlcFru_n and GlcFru_{n+1} are fructooligosaccharide or levan. One hydrolytic unit of FTF is defined as the amount of the biocatalyst that produces 1 µmol of the fructose per min. One transfructosylation unit of FTF is defined as the amount of the biocatalyst that releases 1 µmol of glucose as a result of transferring fructose, per min. Subtracting the total amount of fructose from that of glucose provides the amount of glucose resulting for transferring fructose. One unit of total FTF activity was defined as the amount of the biocatalyst that liberates 1 µmol of the reducing sugars (glucose and fructose) from sucrose per min at the standard assay conditions.

The total FTF activity was determined colorimetrically using the DNS method (Miller, 1959). 250 µL of enzyme extracts at appropriate dilutions (from 2 to 200) were added to 250 µL of 0.9 M sucrose in the potassium phosphate buffer (0.1 M, pH 7.0). The reaction

mixtures were incubated for 20 min at 37°C. The release of reducing sugar was quantified by adding 750 µL of the DNS solution, consisting of 3,5-dinitrosalicylic acid (1%, w/v) in NaOH solution (1.6%, w/v) and boiled for 5 min. To prevent the 3,5-dinitrosalicylic acid from dissolving oxygen, 250 µL of potassium sodium tartrate solution (50%, w/v) was added as a color stabilizer and the absorbance of the reaction mixtures was measured spectrophotometrically at 540 nm against 0.1 M potassium phosphate buffer as a blank. Two blank assays, without substrate or without enzyme, were conducted in tandem of the trials. All assays were run in triplicate. A standard curve was constructed using glucose at various concentrations from 0.0-4.0 mM.

The hydrolytic and transfructosylation activities were investigated using high-pressure-anionic-exchange chromatography equipped with pulsed amperometric detection (HPAEC-PAD, Dionex) the Chromeleon Software and a CarboPac PA20 column (3 x 150 mm) set at a temperature of 32°C. Isocratic elution was performed with 10 mM NaOH as the mobile phase at a flow rate of 1 mL/min. A typical reaction mixture consisted of 100 µL of enzyme extracts at appropriate dilutions (from 2 to 200) and 100 µL of 0.9 M sucrose in the potassium phosphate buffer (0.1 M, pH 7.0). To stop the reaction, the mixtures were boiled for 5 min. The methanol was added at a ratio of 1:1 (v/v) to precipitate the proteins and levan. Prior injections on HPAEC-PAD, the reaction mixtures were centrifuged (10,000xg, 10 min). The concentration of the products was estimated by constructing standard curves for glucose, sucrose and fructose.

2.4.7. Total Protein Concentration

The concentration of protein was measured using the Standard BioRad Protein Assay (Bradford, 1976) and bovine serum albumin (0.2 to 0.9 mg /mL) as a standard. A volume of 20 µL of the sample and of the standard solution were reacted with 1 mL of the diluted Bradford Dye: H₂O (1:4, v/v). The absorbance of the resulting mixtures was determined at 595 nm using a spectrophotometer. Bio-Rad Protein Microassay was used when the concentration of proteins was very low (1.0 and 10 µg/mL). In this Microassay, 200 µL of the undiluted Bio-Rad dye reagent was added to 800 µL of the enzymatic solution. The slope obtained from the constructed standard curve was used to estimate the protein concentration. The specific activity was obtained by dividing the FTF activity of the

fractions by its protein content. Specific activity was expressed as the μmol of released glucose or fructose per minute of reaction and per mg of protein.

2.5. Characterization of the Catalytic Properties of Fructosyltransferase (Levansucrase)

2.5.1 Effect of Temperature

The effect of temperature on the transfructosylation and hydrolytic activities of FTF was investigated at a wide range of temperature varying from 4 to 67°C. The enzymatic reactions were carried out using a sucrose concentration of 0.25 M and a pH of 7.00 using potassium phosphate buffer (0.1 M).

The energy of activation (E_a) was calculated according to the following Arrhenius equation:

$$V = A_i e^{-E_a/RT}$$

Where, V is the reaction rate at a specific temperature, A_i is a pre-exponential constant, R is the universal gas constant (8.314 J/°K/mol) and T is temperature expressed at the absolute temperature (°K). The optimum temperature was defined as the temperature at which the transfructosylation and hydrolytic activities were maximal.

2.5.2. Thermal Stability

The thermal stability of FTF was examined by incubating the FTF extract for a period of 6 hrs at a selected temperature varying from 4 to 67°C. The remaining transfructosylation and hydrolytic activities were assayed under standard assay conditions (57°C, 0.25 M of sucrose and pH 7).

2.5.3. Effect of pH

The effect of pH was investigated by carrying the enzymatic reactions at a broad range of pH varying from 4.00 to 8.00 using different buffer systems (0.1 M): sodium acetate buffer (pH 4.0-5.5), potassium phosphate buffer (pH 6.0-7.0) and Tris-HCl buffer (pH 7.5-9.0). The transfructosylation and hydrolytic activities were assayed using the standard conditions (57°C, 0.25M sucrose, 40 min).

2.5.4. Effect of Substrate Concentration

The effect of substrate concentration on the transfructosylation and hydrolytic activities of FTF was examined at different sucrose concentration, ranging from 0.25 to 1.0 M using the optimized conditions (57°C, 0.1M potassium phosphate buffer at pH 6.50, 40 min). The measurements were plotted on a saturation curve of the initial reaction rate (V) versus substrate concentration (S) and converted to the Lineweaver-Burk plot shown below in order to compute the apparent Michaelis-Menten constant ($K_{m\ app}$) and the apparent maximum velocity ($V_{max\ app}$) for the transfructosylation and hydrolytic activities of the FTF.

$$v = \frac{v_{max\ app} [S]}{K_{m\ app} + S}$$

Using the estimated molecular weight ($E_t = [E] * m.w$) and the maximum velocity of the FTF, the turnover number k_{cat} was calculated as follow:

$$v_{max\ app} = k_{cat}[E_t]$$

2.6. Characterization of Product Spectrum and Acceptor Specificity of the Fructosyltransferase (Levansucrase)

To study the acceptor specificity of FTF, selected glycosides, including glucose, galactose, lactose, raffinose and maltose, were used as fructosyl acceptors using sucrose to glycoside molar ratio of 0.5: 1 and 1:0.5. The enzymatic reactions were carried out at 15 and 30°C for 32 hrs in potassium phosphate buffer (0.1 M, pH 6.5). At selected reaction time, an aliquot of 20 μ L of the reaction mixture was withdrawn and boiled for 5 min to stop the reaction. The protein and levan polymers were precipitated with methanol (1:1, v/v) and separated by centrifugation. The product spectrum of each reaction was analyzed by HPAEC-PAD using the anionic CarboPac PA200 column. The elution of reaction components was performed using a linear gradient of 0 to 100% 200 mM sodium acetate in 100 mM NaOH for 20 min. 1-Kestose, nystose and FOSs from chicory inulin were used as internal standards to identify the peaks. To estimate the yield, the reaction mixtures were diluted and re-injected until the peak areas of the transfructosylated products were within the concentration range 15.8 to 9 166.6 nM.

2.7. Levan Production and Nuclear Magnetic Resonance (NMR) Characterization

Levan was produced by incubating 200 mL of FTF extract (708 U) at 10°C with a final sucrose concentration of 0.5 M in potassium phosphate buffer (0.06M, pH 6.50). After 7 days of incubation, the levan was recovered by precipitation with ethanol (1:1, v/v) overnight. The levan was recovered in the pellet after centrifugation (8,000xg for 45 min at 4°C). After re-suspension in water, the levan was dialyzed for 3 consecutive days against water at 4°C. The dialyzed levan was lyophilized and the glycosidic linkages were examined by NMR analysis. The sample was dissolved in D₂O and the spectra were obtained at room temperature on a Varian VNMRs-500 operating at 100.5 MHz for ¹³C and 499.9 MHz for ¹H. The ¹³C spectrum is the accumulation of 448 transients with a 45° pulse width, acquisition time of 1.3 s and a recycle delay of 1 s. Lorentzian broadening of 1.0 Hz was applied before Fourier transformation. The ¹H spectrum is the accumulation of 4 transients with a 45° pulse width, acquisition time of 2.0 s and a recycle delay of 1 s. No broadening was applied before Fourier transformation.

CHAPTER 3. RESULTS AND DISCUSSION

3.1. Optimization of Production of Fructosyltransferase from *G. stearothermophilus*

3.1.1. Effect of Nitrogen Sources

Two different minimal media, including succinate-salt (Caulfield et al., 1979) and mineral-based media (Jayasuriya, 1955), were investigated for the production of FTF by *G. stearothermophilus*. As compared to the mineral-based, the bacterial growth was not supported in the succinate-salt based medium (results not shown). Such inhibition of *G. stearothermophilus* growth in the succinate-containing medium could be explained by the formation of sodium carbonate, a highly alkaline salt produced from the oxidation of sodium succinate (Kampen, 2001). The sodium carbonate generated at high levels may have not been buffered by potassium salts present in the succinate-salt culture medium. Using the mineral-based medium, microbial growth could not be sustained, reaching a maximum optical density unit at 600 nm ($OD_{600\text{ nm}}$) of only 0.23 (Fig. 1A). The results show that the lag phase of *G. stearothermophilus* lasted for a period of 10 hrs in the mineral-based medium and the exponential phase was considerably slow, reaching the stationary phase after 24 hrs. In conformity to the low microbial growth, the production of the membrane-bound FTF was poor and started during the exponential growth phase with a maximum production ($0.37\text{ }\mu\text{mol/mL/min}$) after 30 hrs of culture.

The effect of supplementing the selected mineral-salt based medium with different nitrogen sources on the bacterial growth and the FTF production was investigated over a fermentation time-course of 30 to 32 hrs (Fig. 1). Sucrose (1%) was added to all culture media as a FTF inducer and as the sole carbon source. No extracellular FTF was detected in the culture supernatant over the fermentation period, suggesting that *G. stearothermophilus* is only capable of producing the membrane-bound FTF biocatalyst. While most FTFs are extracellular (Arrieta et al., 2004), some studies have shown that the FTFs (levansucrase) from *B. subtilis* (Mäntsälä and Puntala, 1982; Cheetham et al., 1989), *B. amyloliquefaciens* (Mäntsälä and Puntala, 1982) and *Z. mobilis* (Vigants et al., 2001) can be both extracellular and cell-associated enzymes. Similar to *G. stearothermophilus*, some strains of *S. salivarius* are known to produce only the membrane-associated FTF (Chassy et al., 1976; Garszczynski and Edwards, 1973).

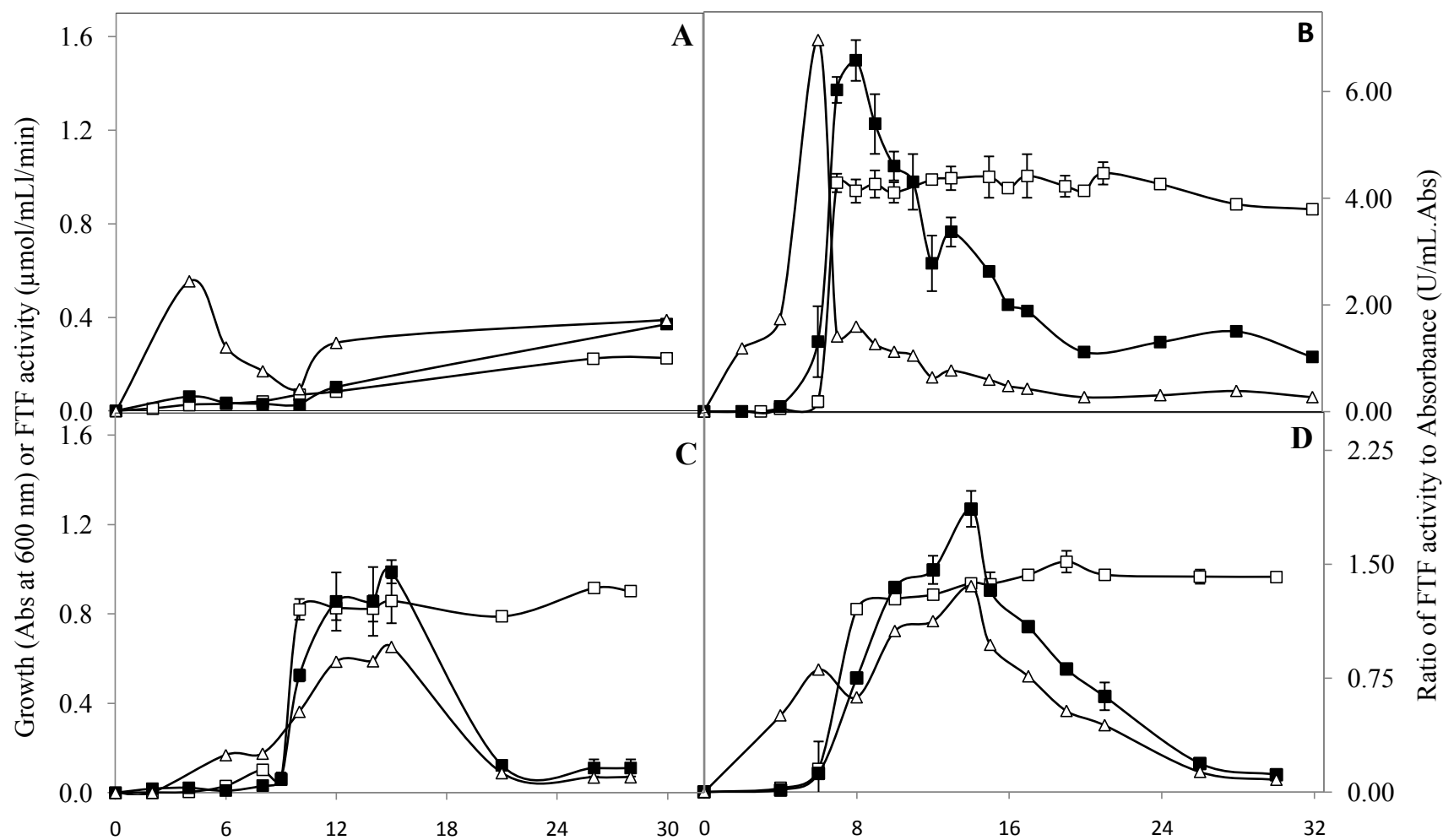


Figure 1. Time course of *Geobacillus stearothermophilus* growth (□), fructosyltransferase (FTF) production (■) and ratio of FTF activity to Abs_{600nm} (Δ) at 55°C in a mineral culture medium (A) supplemented with peptone (B) yeast (C) and tryptone (D), using 1% sucrose as an inducer.

As expected, the supplementation of the mineral-based medium with peptone, yeast and, tryptone as nitrogen sources enhanced both the microbial growth and the FTF production (Figs 1B, C and D). The addition of tryptone and peptone reduced the lag phase of the microbial growth to 6 hrs; however the lag phase remained for 10 hrs when yeast extract was used. Although high level of FTF was produced in the medium comprised of yeast extract and tryptone, the highest FTF activity/ODU_{600 nm} ratio value (U/mL/Abs) was obtained in the presence of peptone (6.97 U/mL/Abs) after only 6 hrs of culture. In comparison, Euzanat et al. (1997) have reported that the use of yeast as a nitrogen source provided 2-fold more FTF production by *B. subtilis* as compared to the use of peptone.

Similarly to the nitrogen-deprived medium, FTF is produced during the exponential growth phase, and the production peaks in the early stationary phase for all the nitrogen-supplied media (Figs 1B, C and D). The fact that the maximum FTF production was achieved in the stationary phase may suggest the occurrence of carbon catabolite repression for *G. stearothermophilus* (Lampen, 1965), a state in which FTF production is suppressed by unmetabolized carbon sources (Rajagopalan and Krishnan, 2008). These results may also indicate that the induction arose once the sugars were depleted. Figures 1C and 1D show that, after 16 hrs of fermentation, FTF production underwent a rapid decline in the yeast and the tryptone -supplemented mineral media. On the other hand, in the peptone-containing mineral medium, the decrease of the FTF production started after 9 hrs with a more severe decline (Fig. 1B). This significant decrease of FTF production can be attributed to the enzymatic degradation by proteases (Le Gorrec et al., 2002). Another explanation could be that sucrose in the medium may have been depleted after 16 hrs of fermentation, resulting in poorer induction of FTF. In addition, it has been shown that low sucrose concentration enhanced sucrase production (Kunst et al., 1974), which may have favored the rapid hydrolysis of the sucrose to the detriment of FTF induction.

The effects of the concentration of selected nitrogen sources on the bacterial growth, the FTF production and the FTF activity/ODU_{600nm} ratio were investigated at selected fermentation times (6 to 10 hrs) and the maximum values are presented in Table 3. The yeast, tryptone or peptone and peptone-tryptone supplemented- mineral media resulted in a comparable microbial growth, with maximum ODU_{600 nm} values varying between 0.82 and 0.93.

Table 3. Effect of nitrogen sources on the fructosyltransferase (FTF) activity and on the growth of *Geobacillus Stearotherophilus*.

Nitrogen Source	Growth (Abs _{600 nm})	FTF Activity ($\mu\text{mol/mL/min}$) ^a	Specific FTF Activity (U/mL/Abs _{600 nm}) ^b
Mineral ^{c, d}	0.23	0.37	1.65
Yeast (10 g/L) ^e	0.82	0.86	1.04
Tryptone (10 g/L) ^e	0.94	1.27	1.36
Peptone			
(5 g/L) ^f	0.85	1.17	1.33
(10 g/L) ^f	0.89	1.17	1.32
(20 g/L) ^f	0.87	1.16	1.31
Peptone (5 /L)			
Tryptone (5 g/L) ^f	0.92	1.23	1.23
(NH ₄) ₂ SO ₄ (5 g/L) ^f	0.50	0.23	0.46

^a Optimum FTF activity was expressed as μmole of reducing sugars per mL of culture per min of reaction.

^b Specific FTF activity was expressed as the optimum FTF activity (U/mL) divided by the Abs_{600 nm} at that corresponding optimal time.

^c The mineral-based media was supplemented with sucrose (10 g/L) and was comprised of (in g/100 mL): KH₂PO₄ (0.136), (NH₄)₂SO₄ (0.05), FeSO₄·7H₂O (0.0005), MnSO₄·H₂O (0.00018), Na₂HPO₄·2H₂O (0.267), Na₂MoO₄·2H₂O (0.00025), CaPO₄·2H₂O (0.001) and MgSO₄·7H₂O (0.02).

^d The optimum specific FTF activity is obtained at 30 hrs of culture.

^e The optimum specific FTF activity is obtained at 14 hrs of culture.

^f The optimum specific FTF activity is obtained at 9 hrs of culture.

Rajagopalan and Krishnan (2008) have demonstrated that the growth of *G. stearothermophilus* may require vitamins and trace elements, which are actually supplied by tryptone, yeast extract and peptone (Table 4). In fact, some microorganisms do not secrete extracellular proteases to degrade the proteins present in the culture medium into amino acids. As an alternative to $(\text{NH}_4)_2\text{SO}_4$, complex organic nitrogen, such as peptone, yeast extract and tryptone are considered as important sources of free amino acids (Kampen, 2001). The results (Table 3) also indicate that the maximum $\text{ODU}_{600\text{nm}}$ (0.85) reached with peptone as the sole nitrogen source is significantly higher than that (0.50) obtained when $(\text{NH}_4)_2\text{SO}_4$ was supplemented to peptone. These results reveal the inhibitory effect of $(\text{NH}_4)_2\text{SO}_4$ on *G. Stearothermophilus* growth. Similarly, a previous study on the effect of culture nutrient composition on the growth of *G. stearothermophilus* has demonstrated the inhibitory effect of the ammonium compounds (Al-Qodah, 2006). In fact, $(\text{NH}_4)_2\text{SO}_4$ can readily be used by microorganisms and their consumption can result in the release of sulfate ions, which may cause a strong pH change to acidity (Kampen, 2001).

The overall results (Table 3) showed that the use of tryptone resulted in the production of the highest level of FTF activity ($1.27 \mu\text{mol/ml/min}$), followed by the peptone (1.16 to $1.23 \mu\text{mol/mL/min}$), and the yeast extract ($0.83 \mu\text{mol/mL/min}$). Interestingly, increasing the peptone content from 5 to 20 g/L did not affect the level of the produced FTF activity (1.16 to $1.17 \mu\text{mol/mL/min}$), nor the specific FTF activity ratio (1.31 to $1.33 \text{ U/mL/Abs}_{600\text{nm}}$). However, the FTF production is about 5-fold lower in the peptone/ $(\text{NH}_4)_2\text{SO}_4$ supplemented medium ($0.23 \mu\text{mol/mL/min}$) as compared to the peptone supplemented medium. Similarly, Dias and Bhat (1962) have demonstrated that FTF production was lower with inorganic nitrogen sources as compared to the organic ones. Although the microbial growth is not significant in the mineral-salt based medium, the specific activity ratio of $1.65 \text{ U/mL/Abs}_{600\text{nm}}$ was within the range obtained for nitrogen supplemented medium (1.04 - $1.36 \text{ U/mL/Abs}_{600\text{nm}}$), with the exception of the peptone/ $(\text{NH}_4)_2\text{SO}_4$ mixture ($0.46 \text{ U/mL.Abs}_{600\text{nm}}$).

For further investigations, the tryptone/peptone mixture was selected as a nitrogen source because the FTF production was stable during the stationary phase of growth in the presence of this source as compared to the tryptone, yeast and peptone.

Table 4. Composition of tryptone, peptone and yeast extract from Difco:

Nitrogen Sources	Yeast Extract	Tryptone	Peptone
Nitrogen content (%)			
Total nitrogen	11.4	13.1	15.4
Amino N/Total N	6.9	0.43	3.5
Elements (µg/g)			
Ca	230	180	18
Mg	800	100	0.6
K	58,013	620	2,542
Na	1,003	26,970	18,440
Cl	0.07	0.35	0.90
SO ₄	0.65	0.22	0.32
PO ₄	3.73	2.25	0.40
Free amino acids (%)			
Alanine	5.7	0.6	1.2
Aspartic acid	2.2	0.1	0.3
Glutamic acid	7.3	1.4	0.7
Histidine	1.1	0.5	0.2
Leucine	4.0	7.5	1.6
Methionine	0.9	1.0	0.3
Proline	1.3	0.2	0.3
Threonine	1.7	0.7	0.3
Tyrosine	0.9	0.5	0.5
Arginine	2.0	3.8	2.8
Cystine	0.2	0.3	---
Glycine	1.6	0.2	0.7
Isoleucine	2.5	1.3	0.6
Lysine	2.7	5.5	2.2
Phenylalanine	2.7	3.0	1.4
Serine	3.9	0.7	0.6
Tryptophan	0.7	0.8	0.3
Valine	3.0	1.7	0.7

--- Below levels of detection

3.1.2. Effect of Carbon Sources

The effects of carbon sources on the microbial growth and on the induction of *G. stearothermophilus* FTF production were examined using the mineral-based medium, containing the tryptone/peptone mixture as the nitrogen source, at different fermentation time. The maximum values for the bacterial growth, the FTF activity and the specific activity are displayed in Figure 2. The results indicate that *G. stearothermophilus* can grow on all investigated carbon sources (concentration of 1% w/v, except when specified), including sucrose, raffinose, glucose, fructose and glycerol. However, the bacterial growth ($ODU_{600\text{ nm}}$) and the extent of FTF production varied depending on the nature and the concentration of the carbon sources. In fact, the highest microbial growth was observed in the presence of the monosaccharides glucose ($ODU_{600\text{ nm}}$ of 1.01) and fructose ($ODU_{600\text{ nm}}$ of 0.99). With the exception of 0.25% sucrose-containing medium (0.61), the maximum $ODU_{600\text{ nm}}$ values varied from 0.81 to 0.99 in all sucrose-containing media. Contrarily to other carbon sources, the use of glycerol and raffinose resulted in a relatively low microbial growth with a maximum $ODU_{600\text{ nm}}$ value of 0.35 and 0.46 after 8 hr-fermentation period, respectively. Similarly, Pabst (1977) reported that when raffinose was used as a carbon source for the production of *A. viscosus* FTF, the bacterial growth was much slower than when glucose, fructose or sucrose were used. The lower biomass can be attributed to the complexity of raffinose, which is less easily metabolized by microorganism than other simple sugars (Kampen, 2001). Moreover, the growth of *G. stearothermophilus* in a mineral-salt based medium supplemented with glycerol as the sole carbon source has previously been demonstrated to be poorer than when the medium was supplied with sucrose or fructose (Welker and Cambell, 1963; Al-Qodah, 2006). Contrarily to other bacterial strains (Shuler and Kargi, 1996), high concentration of carbon source did not result in a decrease in the microbial growth of *G. stearothermophilus*, indicating the absence of the release of toxic wastes (Herbert et al., 1956). Although similar microbial growth was obtained in the mineral medium supplemented with sucrose at a concentration of 1 to 2%, the highest produced FTF activity and specific activity ($U/mL/Abs_{600nm}$) were obtained in the culture medium supplemented with 1% sucrose (Fig. 2).

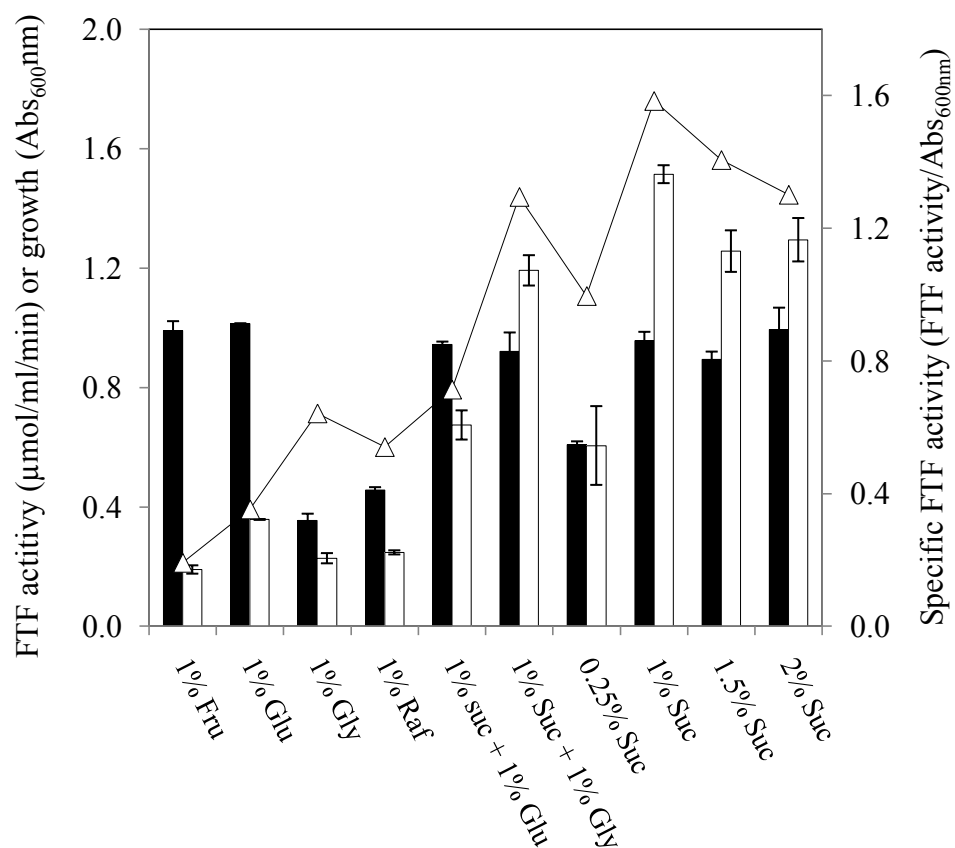


Figure 2. Effect of different carbon sources (w/v) on the growth (■) of *Geobacillus stearothermophilus*, the fructosyltransferase (FTF) activity (□), and the specific FTF activity (FTF activity/Abs at 600 nm) (Δ).

The overall results show that all sucrose-containing media favored the production of the FTF activity and led to higher specific activity values. On the other hand, lower levels of FTF activity and specific activities were obtained when each of fructose, glucose, glycerol and raffinose were used as a carbon source. However, the addition of sucrose to each of glycerol and glucose contributed to a 2-fold increase of the specific activity. These results indicate that the FTF induction is more effective in the presence of 1% sucrose. A constitutive FTF was produced by *G. stearothermophilus*, when glucose, glycerol, fructose and raffinose were used. Similarly, the production of FTF by *A. diazotrophicus* SRT4 was highly induced in the presence of 1% sucrose than 1% glycerol (Hernandez et al., 1995). In contrast, the FTF from *B. Subtilis* NRC 33a, was induced at the same level with 20% sucrose (14.5 U/mL) and 10 % glucose (14.1 U/mL) (Abdel-Fattah et al., 2005). It is important to note that none of the carbon sources tested on *G. stearothermophilus* allowed the production of extracellular FTF. Many evidences support that FTF is a constitutive membrane-bound enzyme (Trujillo et al., 2001; Arrieta et al., 2004).

3.1.3. Effect of Selected Iron Sources

In an attempt to trigger the export of membrane-bound FTF to the extracellular phase, the FeSO_4 present in the mineral-based medium was substituted with selected sources of ferrous iron ($(\text{NH}_4)_2\text{FeSO}_4$ and FeSO_3) and ferric iron (FeCl_3 and $\text{Fe}(\text{NH}_4)_3(\text{C}_6\text{H}_5\text{O}_7)_2$). As shown in Figure 3, none of the ferrous and ferric forms of iron favoured the production of extracellular FTF. Furthermore, the investigated forms of iron appear to have no influence on the growth of *G. stearothermophilus*, resulting in similar $\text{ODU}_{600\text{nm}}$. The results also show that as compared to FeSO_4 used as the control, all forms of iron failed to enhance either membrane-bound or extracellular FTF production with the exception of $\text{Fe}(\text{NH}_4)_3(\text{C}_6\text{H}_5\text{O}_7)_2$. The production of membrane-bound FTF in the presence of $\text{Fe}(\text{NH}_4)_3(\text{C}_6\text{H}_5\text{O}_7)_2$ (0.79 $\mu\text{mol/mL/min}$) was improved as compared to the FeSO_4 (0.65 $\mu\text{mol/mL/min}$). This result may be due to the use of $\text{Fe}(\text{NH}_4)_3(\text{C}_6\text{H}_5\text{O}_7)_2$ as an additional source of carbon.

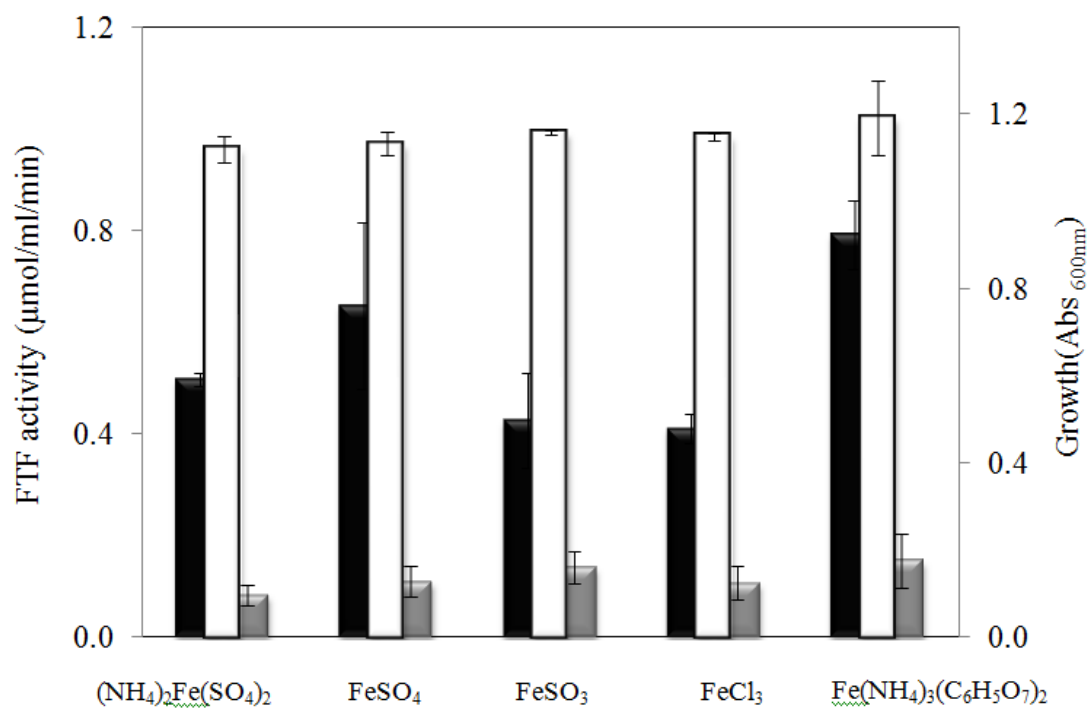


Figure 3. Effect of selected iron sources on the growth (□) of *Geobacillus stearothermophilus*, the membrane-bound fructosyltransferase (FTF) activity (■) and the extracellular FTF activity (▒).

3.2. Purification of Fructosyltransferase from *G. stearothermophilus*.

3.2.1. Preparation of the Pre-purified Fructosyltransferase Extract

In order to prepare the pre-purified FTF extract, the crude FTF extract was subjected to precipitation by polyethylene glycol, ammonium sulfate and manganese chloride at selected saturation levels and concentrations. The purification yields and factors are summarized in Table 5. When the crude FTF extract was brought to 40% saturation with ammonium sulfate, only 6.5% of the FTF activity was recovered with a purification factor of 1.24. However, increasing the saturation of ammonium sulfate to 50% improved the precipitation yield of FTF activity to 29.3% and the purification factor to 2.44-fold. Although increasing the saturation of ammonium sulfate to 80% slightly improved the yield of FTF recovery (37.2%), it led to a decrease in the purification factor to 1.08. The use of the ammonium sulfate at a saturation of 65% (w/v) did not succeed in the precipitation of FTF (levansucrase) from *B. subtilis* (Le Gorrec et al., 2002). While the ammonium sulfate precipitation of FTF from *M. laevaniformans* (Park et al., 2003) and *L. reuteri* (van Hijum et al., 2001) at a saturation of 60 and 65% resulted in a purification degree of 2.6 and 2.3 with an overall yield of 95 and 68%, respectively.

Table 5 also indicates the efficiency of the pre-purification of FTF by manganese chloride precipitation. Upon precipitation with 25, 50 and 100 mM manganese chloride, 77.9, 43.9 and 26.1 % of the initial FTF activity were recovered, respectively. Although the precipitation with the manganese chloride at a concentration of 25 mM resulted in the highest yield, it led to a lower purification factor of 1.58. The highest purification degree of 3.16 was obtained with 50 mM of manganese chloride. The overall results show the higher efficiency of manganese chloride as a precipitant to pre-purify the FTF from *G. stearothermophilus* as compared to the ammonium sulfate. Contrarily to *G. stearothermophilus* FTF, the precipitation of the FTF from *Z. mobilis* by 100 mM manganese chloride resulted in a recovery yield of 96.8% and a 14.5-fold purification factor (Vigants et al., 2001). The same authors have examined the effect of varying protein concentration on the precipitant efficiency of manganese chloride and reported that the yield of FTF recovery (88 to 99%) was similar when the protein concentration was varied from 0.20 to 0.40 mg/mL.

Table 5. Purification factor and yield of *Geobacillus stearothermophilus* fructosyltransferase (FTF) after selected precipitation.

Pre-purification step	Purification factor ^a	Yield (%) ^b
(NH₄)₂SO₄ precipitation		
40% saturation	1.24	6.5
50% saturation	2.44	29.3
80% saturation	1.08	37.2
MnCl₂ precipitation		
25 mM	1.58	77.9
50 mM	3.16	43.9
100 mM	2.31	26.1
Polyethylene glycol-200 precipitation		
40%	262.4	5.7
50%	223.6	5.7
Polyethylene glycol-400 precipitation		
20%	394.4	5.1
30%	267.3	6.1
40%	230.6	5.4

^aThe purification factor is expressed as the ratio of the specific activity (μmol of released reducing sugar per mg protein per min of reaction) of the precipitated FTF and the initial specific activity.

^bThe yield was calculated as the total FTF activity units expressed in the precipitated FTF, divided by the total initial FTF activity units and multiplied by 100.

As opposed to the ammonium sulfate and manganese chloride precipitations, the fractionation by the non-ionic hydrophilic polymer polyethylene glycol (PEG-200 and PEG-400) showed exceptional purification factors ranging from 231 to 394. However, the precipitation yields of FTF (5.1 to 6.1%) upon the PEG fractionation were very poor. Only a few authors have examined the pre-purification of FTF by PEG precipitation (Oseguera et al., 1996a; b; Canedo et al, 1999). Nonetheless, the yield of FTF recovery and purification factor after the PEG precipitation step has only been reported by Oseguera et al (1996b) who have obtained 150% yield and an 8-fold purification factor for the FTF (levansucrase) from *B. circulans*.

3.2.2 Purification of Fructosyltransferase

The FTF from *G. stearothermophilus* was purified using a two-step purification procedure with a purification factor of 28-fold and an overall yield of 71% (Table 6). The purification of FTF by a two-step procedure, involving precipitation followed by chromatographic separation with Sephadex (Oseguera et al., 1996b) and Sephacryl (Vigants et al., 2001; 2003), has been previously described. However, to our knowledge, this is the first report of a two-step chromatographic purification of FTF using Superdex 75 in combination with MonoQ 5/50. The first size-exclusion chromatography on a Superdex 75 column performed the first fractionation of the proteins of the FTF extract into two major fractions with a FTF activity yield of 90% and a purification factor of 6.2. The elution profile shown in Figure 4A indicates that the FTF activity was recovered in the second protein peak, obtained upon 19.8 to 20.8 mL of elution volume. However, proteins but no FTF activity was detected in the first fraction of the size-exclusion elution profile (Fig. 4A). Since the FTF peak was asymmetrical, the fractions were lyophilized and applied to a MonoQ 5/50 column for further purification. This anionic exchange chromatography was the most efficient step with a purification factor of 28.4 and a high FTF activity yield of 71%. The MonoQ 5/50 chromatogram revealed five distinct protein peaks, including two peaks with FTF activity (Fig. 4B). The first active fraction eluted between 0.26 and 0.32 M of KCl represented 20% of the total eluted activity, whereas the second active fraction eluted between 0.35 and 0.43 M KCl showed 24% of the total eluted activity.

Table 6. Yield and purification factor of *Geobacillus stearothermophilus* fructosyltransferase (FTF) after chromatographic purification.

Purification step	Specific activity ^a ($\mu\text{mol}/\text{mg protein}/\text{min}$)	Yield ^b (%)	Purification ^c factor
Crude FTF	5.6	-	-
Size-exclusion chromatography			
Superdex 75 10/300 GL ^d	34.5	90.4	6.2
Anionic-exchange chromatography			
MonoQ 5/50 GL ^e	158.5	71.0	28.4

^a The specific activity is expressed as the total amount of reducing sugar released per mg of protein per minute.

^b The yield was calculated as the FTF units (μmol of released reducing sugar per min of reaction) of the fractions of FTF divided by the crude FTF units, multiplied by 100.

^c The purification factor is the ratio of the specific activity of the fractions of FTF to the specific activity of the crude FTF.

^d Size-exclusion chromatography fractions (9.6 to 10.4 mL) collected with an isocratic elution, using 0.15M NaCl in 10 mM potassium phosphate buffer (pH 7.00) at a flow rate of 0.8 mL/min.

^e Anionic-exchange chromatography fractions (22 to 24 mL) eluted at 0.26 to 0.32 M KCl in potassium phosphate buffer (pH 7.00) with a flow rate of 1.0 mL/min.

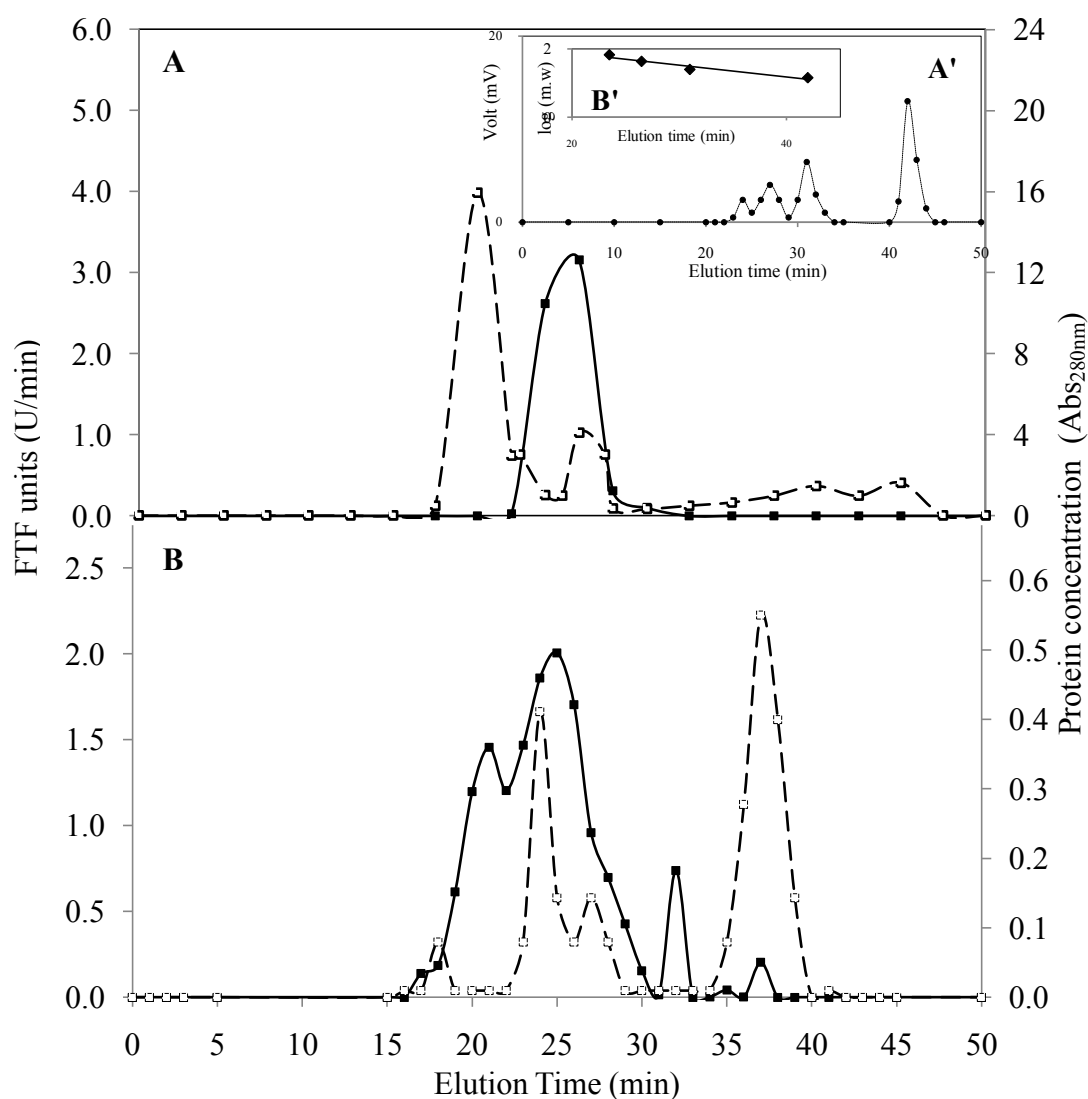


Figure 4. Chromatograms* of the fructosyltransferase (FTF) extract from *Geobacillus stearothermophilus* purified by gel filtration using Superdex 75 (A) then by anionic exchange chromatography using MonoQ 5/50 (B). Elution profile of protein standards (A') and plot (B') of the log of the molecular weights (m.w) of protein standards against retention time**.

* FTF activity (—) and the protein concentration (---) of the collected fractions.

** Selected proteins, including bovine serum albumin, ovalbumin, lysozyme and α -chymotrypsin, were used as standards on Superdex 75

Similar purification degree of 28.6-fold but lower yield of 40.9% were reported for the FTF from *Arthrobacter ureafaciens* upon MonoQ 5/50 purification step (Song et al., 2000). Cheetham et al. (1989) have also successfully purified the FTF from *B. subtilis* by MonoQ 5/50 but the yield and purification factor were unreported.

In order to assess the extent of purification, an SDS-PAGE analysis was carried out on the individual fractions collected upon the anionic exchange chromatography (Fig. 5). The crude FTF extract loaded on lane B of the SDS/PAGE gel showed many peaks over a wide range of molecular weights. The fractions exhibiting the highest FTF specific activity (lanes 19-24) showed two bands on the SDS-PAGE. The protein bands were estimated to have apparent molecular weights of 63.7 and 57.8 kDa by comparing with the mobilities of standard marker proteins. The decrease in the specific activity of fractions 25 to 28 is confirmed by the presence of other contaminating proteins.

From the Superdex 75 size exclusion column, a molecular weight of the native proteins of 54.5 kDa was determined by comparing the elution volume of the FTF proteins with those of the protein standards. Since the FTF applied to the Superdex 75 column was non-denaturated, this indicates that FTF from *G. stearothermophilus* is composed of one monomer. According to Petit-Glatron et al. (1987) and Chambert et al. (1990), membrane-bound FTF exists in two forms (53 and 50 kDa), both are precursors of extracellular FTF (50 kDa). *G. stearothermophilus* may have primarily produced the 53 kDa membrane-bound FTF. On the other hand, it is plausible that both two protein bands revealed on the SDS-PAGE represent the FTF from *G. stearothermophilus*, with one being associated with a low molecular weight levan (Crittenden and Doelle, 1994; Hettwer et al., 1995; Oseguera et al., 1996b). Furthermore, the gene encoding for levansucrase (FTF) (SacB) in *G. stearothermophilus* is found as a gene cluster with the gene encoding for the enzyme levanase (SacC). The latter one is an enzyme responsible for the hydrolysis of levan. It is still unknown whether the induction of SacB and SacC in this gene cluster is separately modulated by sucrose and fructose, respectively, or whether levanase induction is also modulated by sucrose (Li et al., 1997). Taking this into consideration, the contaminating protein in fraction 22 to 24 could also be levanase.

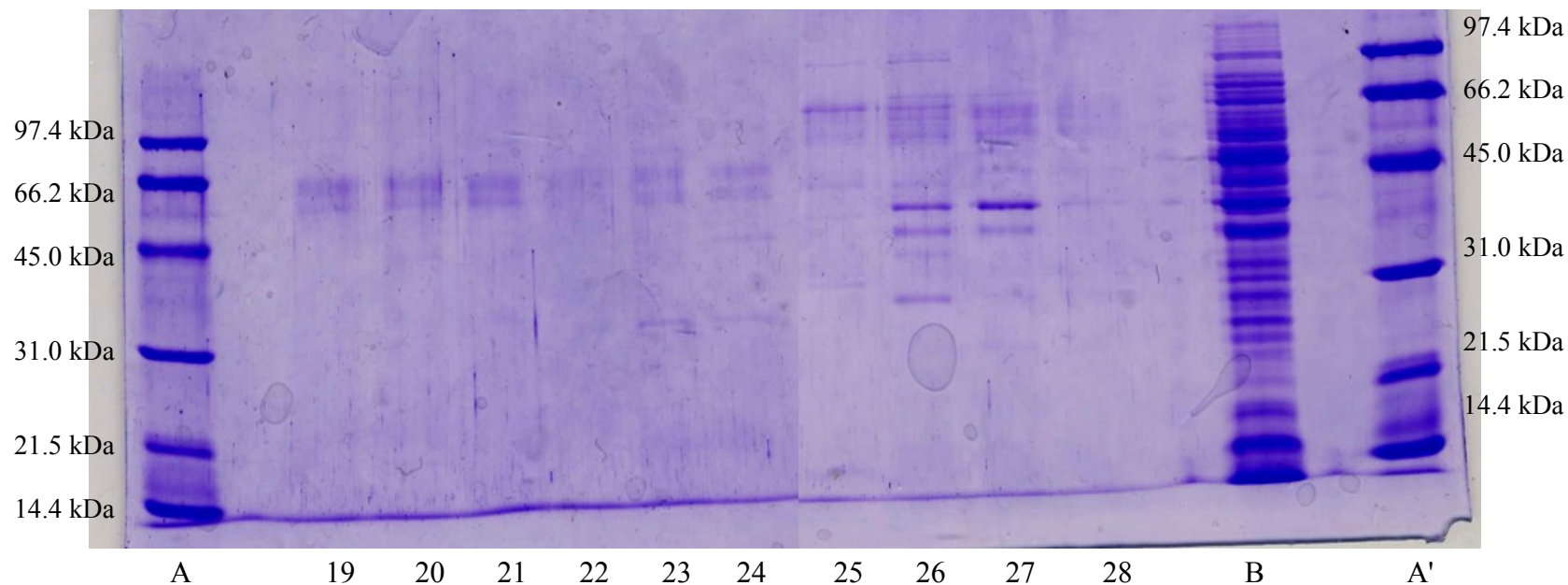


Figure 5. SDS-PAGE of the crude and purified fructosyltransferase (FTF) from *Geobacillus stearothermophilus* and of fractions collected upon anionic exchange chromatography using MonoQ 5/50.

Lane A and A': Low molecular weight standards (phosphorylase b, serum albumin, ovalbumin, carbonic anhydrase, trypsin inhibitor and lysozyme)

Lanes 19 to 28: Fractions 19 to 28 collected by anionic exchange chromatography using a MonoQ 5/50 GL column with a flow rate of 1.0 mL/min and a step gradient of 1.0 M KCl/10 mM potassium phosphate buffer (pH 7.0) for elution.

Lane B: Crude FTF.

The estimated molecular weight of *G. stearothermophilus* FTF falls within the same range as those reported for the FTF (levansucrase) monomers from *B. subtilis* (50 kDa) and *B. amyloliquefaciens* (52 kDa) (Mäntsälä and Puntala, 1982). In contrast, the molecular weight of *Z. mobilis* FTF (levansucrase) was shown to be 94 kDa (Goldman et al., 2008) by size exclusion, while the SDS-PAGE revealed a single protein band of 55 to 56 kDa (Sangiliyandi et al., 1998; 1999). Taking into consideration the molecular weight determined by gel filtration, this suggests that the FTF from *Z. mobilis* exists as a dimer with two subunits of 56 kDa. In fact, the FTF from *R. aqualitis* (Ohtsuka et al, 1992), and *Z. mobilis* (Goldmann et al., 2008) have also been shown to exist in the dimer form.

The active FTF fractions obtained upon the two-step purification procedure were incubated with sucrose (0.25 M) at 10°C for 48 hrs, and their reaction components were analyzed by thin-layer chromatography (Fig. 6). The results indicate that the active size exclusion fractions (lanes 1 and 2) catalyzed the formation of polymer levan and other fructooligosaccharides using sucrose as the sole substrate. On the other hand, lane 3, corresponding to the active MonoQ 5/50 fractions reveals the synthesis of the polymer levan. The NMR spectrum (Fig. 7) of the fructan produced by *G. stearothermophilus* FTF shows six main resonances at 104.1 (C2), 80.2 (C5), 76.2 (C3), 75.13 (C4), 63.2 (C6) and 59.8 (C1) ppm corresponding to the peak position for β -(2-6)-levan. These chemical shifts are very similar to those obtained for the levan synthesized by *B. subtilis* (Shih et al., 2005) and *B. natto* (Han & Clarke. 1990). The detection of β -(2→6)-glycosidic linkages confirms the formation of the polysaccharide levan and the nature of the enzyme produced by *G. stearothermophilus*. Therefore, the *G. stearothermophilus* FTF will be referred to as levansucrase.

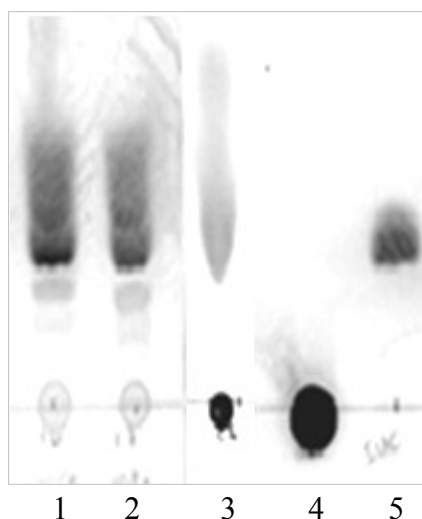


Figure 6. Thin-layer chromatography of the products synthesized by the fructosyltransferase (levansucrase) extract from *Geobacillus stearothermophilus* obtained after gel permeation chromatography on Superdex 75 (lanes 1 to 2), after anionic-exchange chromatography on MonoQ 5/50 (lane 3) and standards (lanes 4 to 5).

- Lanes 1 to 2: Active fractions eluted from gel filtration after 9.6 and 10.4 elution volume were incubated for 48 hrs at 10°C with 0.25M sucrose.
- Lane 3: Active fractions, eluted from MonoQ 5/50 after 22 to 27 elution volumes, were reacted for 48 hrs at 10°C with 0.25 M sucrose.
- Lane 4: Inulin standard.
- Lane 5: Sucrose standard.

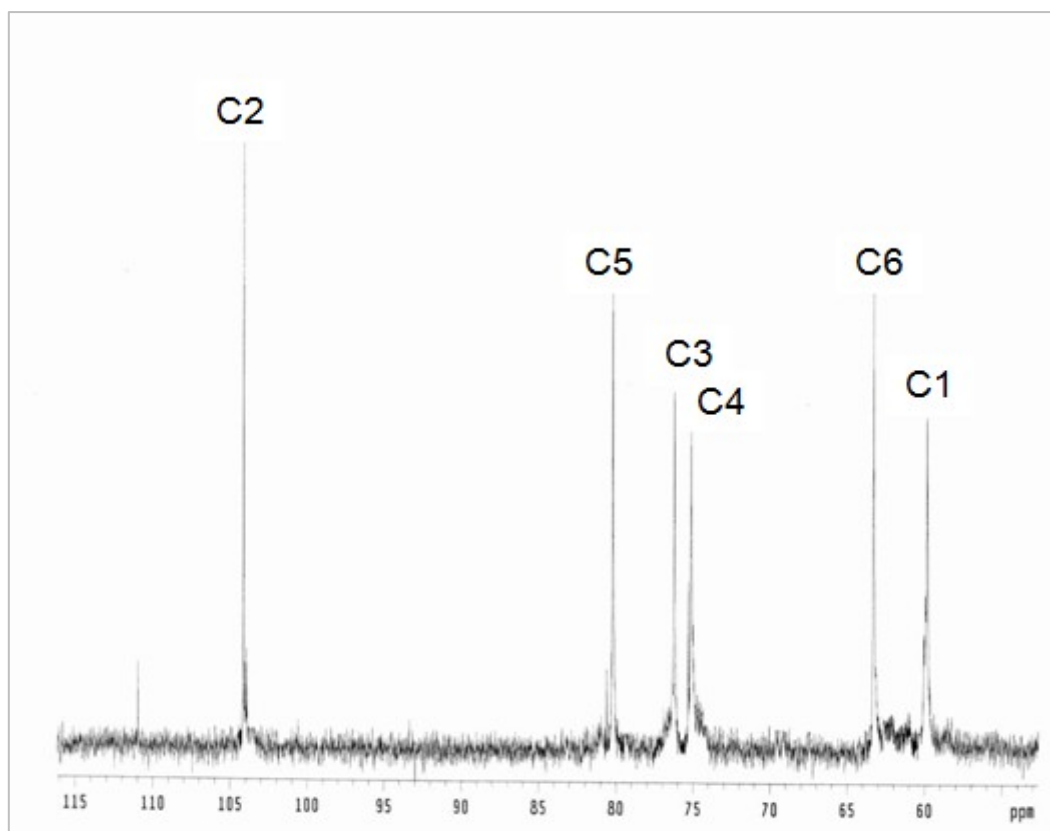


Figure 7. ^{13}C NMR spectrum of the polyfructan synthesized by the levansucrase from *Geobacillus stearothermophilus*.

3.3. Characterization of the Catalytic Properties of Levansucrase from *G. stearothersophilus*.

3.3.1. Effect of Reaction Temperature

The effect of reaction temperature on the transfructosylation and hydrolytic activities of purified levansucrase from *G. stearothersophilus* was investigated under standard reaction conditions. As depicted in Figure 8A, levansucrase is active within a broad range of temperature (4 to 67°C). Both hydrolytic and transfructosylation activities of levansucrase showed similar profiles within the range of 8 to 37°C, in which they increased by a factor of 5. At a higher temperature of 47°C, the hydrolytic activity of *G. stearothersophilus* levansucrase reached its maximum value and remained constant at 57°C; while the maximum transfructosylation activity value was obtained at 57°C. At a higher temperature of 67°C, the transfructosylation and the hydrolytic activities of levansucrase decreased significantly, with a loss of up to 56 and 49% of its maximum value, respectively. The optimum temperature of *G. stearothersophilus* levansucrase of 57°C falls within the range of 30 to 60°C, as reported for the hydrolytic and transfructosylation activities of levansucrases from *B. subtilis* (Baciu et al., 2005; Esawy et al., 2008), *B. megaterium* (Homann et al., 2007), *Z. mobilis* (Sangiliyandi et al., 1999) and *L. reuteri* (Ozimek et al., 2005). Contrary to *G. stearothersophilus* levansucrase which was active up to 67°C, levansucrases from *B. amyloliquefaciens* (Rairakhwada et al., 2010) and *L. panis* (Waldherr et al., 2008) showed no activity at 60°C. The overall results reveal that *G. stearothersophilus* levansucrase is relatively more stable than FTFs from other microbial sources. This relative high thermal stability may be explained by the fact that *G. stearothersophilus* is a thermophilic bacterium, and that thermophiles tend to secrete enzymes that are more resistant to higher temperatures (Sonnleitner, 1983).

The results (Fig. 8A) also show that, for every 10°C increase in temperature, there is a 5.85-fold increase in the transfructosylation activity up to 57°C. In comparison, there is a 4.37-fold increase in the hydrolytic activity for every rise of 10°C, until 57°C. From the Arrhenius plots of the ascending part (Fig 8B), the energy of activation (E_a) for the hydrolytic and transfructosylation activities was estimated to be 35.6 and 33.16 kJ/mol, respectively (Table 7). The E_a being close for both hydrolytic and transfructosylation activities of levansucrase suggests that their limiting catalytic step is similar.

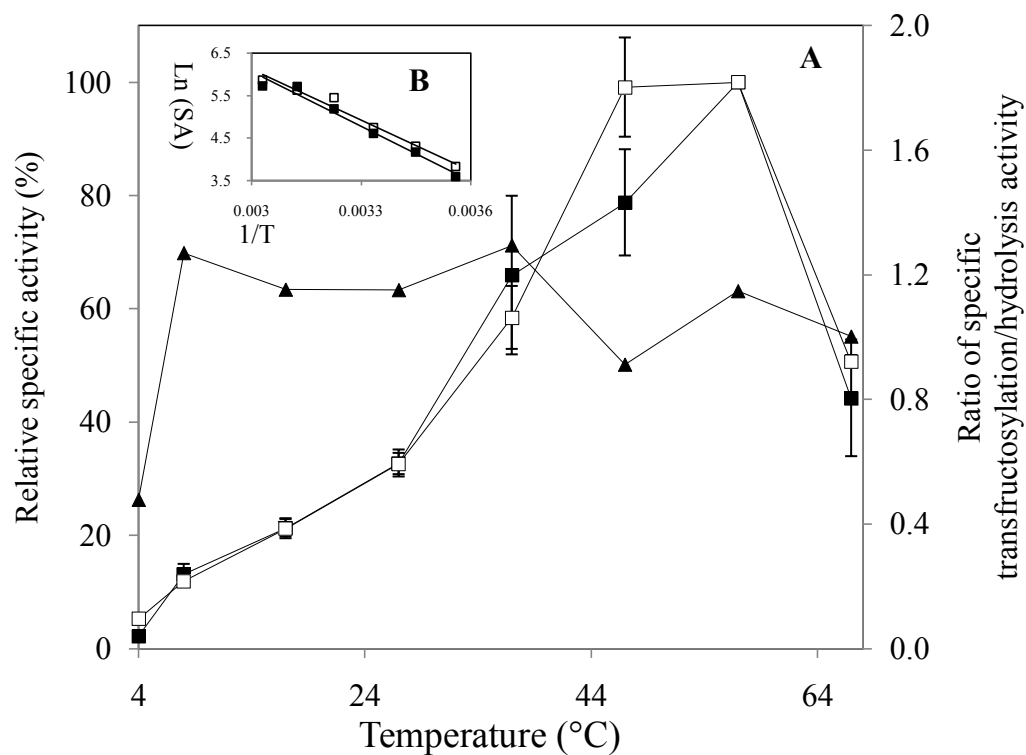


Figure 8. Effect of temperature on the reaction selectivity (A) of the fructosyltransferase (levansucrase) from *Geobacillus stearothermophilus*: Transfructosylation (■), hydrolysis (□) and ratio of transfructosylation to hydrolytic activity (Δ). Arrhenius plot (B) of the transfructosylation (■) and hydrolytic (□) activities of FTF.

Table 7. Catalytic properties of *Geobacillus stearothermophilus* fructosyltransferase (levansucrase).

Catalytic properties	Specific hydrolytic ^a activity	Specific transfructosylation ^b activity
Optimum pH ^c	6.75	6.75
Optimum temperature ^d	47-57°C	57°C
Thermostability	6h at 47°C	6h at 47°C
K _{mapp} ^e	272	269
V _{maxapp} ^f	27.70	58.48
k _{cat} ^g	25.12	53.02
k _{cat} /K _{mapp} ^h	92.52	197.10
E _a ⁱ	35.60	33.16

^a The specific hydrolytic activity was calculated from the total amount of fructose units that is transferred from sucrose to water and it is expressed as the μmol of released fructose per mg of protein per min of reaction.

^b The specific transfructosylation activity represents the total amount of fructose units that is transferred from sucrose to an acceptor and it is expressed as the μmol of transferred fructose (total glucose released minus total fructos released) per mg of protein and per min of reaction.

^c The optimum pH is the pH at which the specific activity is maximal.

^d The optimum temperature is the temperature at the specific activity is maximal.

^e K_{mapp}, Michaelis-Menten constant, is expressed in mM.

^f V_{maxapp}, maximum reaction rate is expressed in μmol per mg proteins per min of reaction.

^g k_{cat}, the turnover number, is expressed as s⁻¹.

^h k_{cat}/K_{mapp}, catalytic efficiency, is expressed as M⁻¹ s⁻¹..

ⁱ E_a the energy of activation, is expressed in kJ/mol.

In addition, the E_a values indicate that both activities have the same sensitivity to the variations of temperatures. This is the first report on the determination of the activation energy of the hydrolytic and transfructosylation activities separately. In fact, the activation energy of the total activity of levansucrase (FTF) from *B. circulans* (El-Refai et al., 2009) and *B. subtilis* (Euzanat et al., 1997; Baciú et al., 2005; Esawy et al., 2008) has been reported to vary from 6.62 to 53.9 kJ/mol.

The results (Fig. 8A) also indicate that the ratio of transfructosylation to hydrolytic activities (ranging from 0.91 to 1.29) was not affected by the reaction temperature changes, with the exception at 4°C at which the ratio was 0.48. The transfructosylation activity of FTF was reported to be predominant at lower temperatures for levansucrases from *Pseudomonas syringae* pv. (18°C) (Hettwer et al., 1995), *Z. mobilis* (15°C) (Crittenden and Doelle, 1994) and *B. amyloliquefaciens* (4°C) (Rairakhwada et al., 2009).

3.3.2. Thermal Stability of Levansucrase

The thermal stability of the purified levansucrase extract from *G. stearothermophilus* was investigated by incubating it for a period of 6 hrs at various temperatures ranging from 4 to 67°C. The remaining enzymatic activities of levansucrase were assessed under standard conditions and are presented in Figure 9. Both the hydrolytic and transfructosylation activities of the purified levansucrase remained stable for 6 hrs at temperatures ranging from 4 to 47°C. However, a complete loss of these activities was obtained at a higher temperature of 57°C. In contrast, the transfructosylation activity of the levansucrase from *B. circulans* was more resistant to temperature than its hydrolytic activity, with a half-life 2-fold higher as reported by Oseguera et al. (1996b). At the elevated temperatures of 57°C and 67°C, levansucrase undergoes an irreversible denaturation, which may be due to the permanent temperature-dependent unfolding (Ozimek et al., 2005) and the higher rate of covalent inactivation processes, such as deamination (Kvittingen, 1994).

In fact, unlike other β -propellers-containing enzymes, levansucrase is devoid of a molecular velcro, resulting in a loosely folded enzyme conformation (Meng and Fütterer, 2003). The thermal instability of levansucrases was also attributed to its dissociation from the polymer levan at high temperatures (Crittenden and Doelle, 1994).

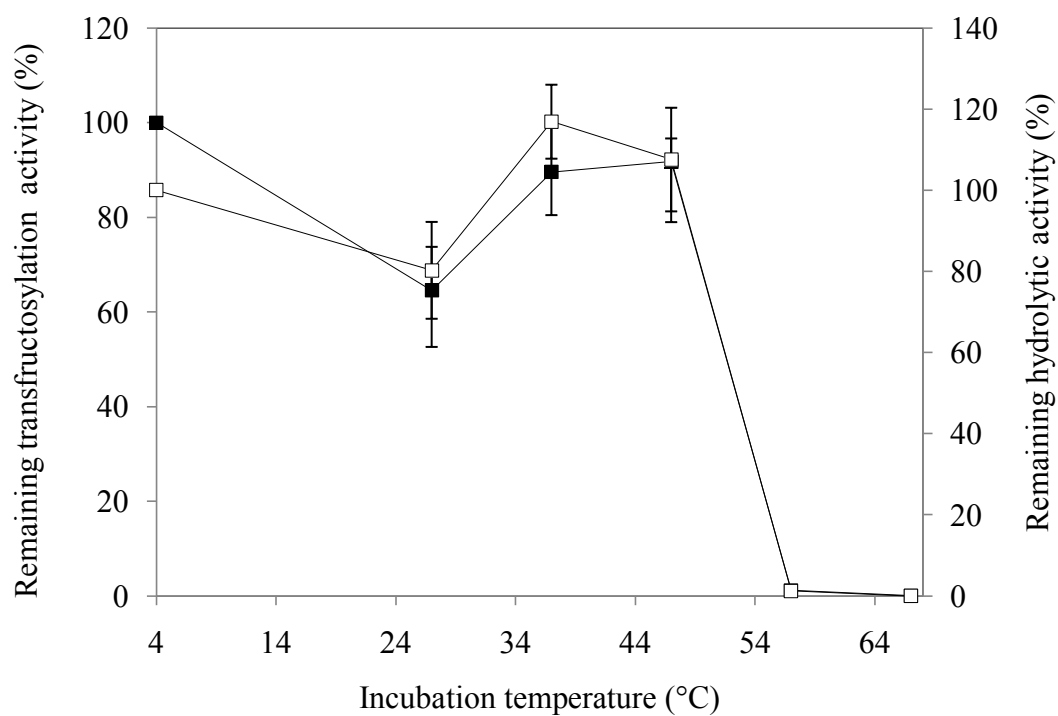


Figure 9. Thermal stability of fructosyltransferase (levansucrase) from *Geobacillus stearothermophilus*. The remaining transfructosylation (■) and hydrolytic (□) activities were determined under optimum reaction conditions after 6h of incubation at different temperatures.

Indeed, it has been reported that levansucrase molecules tend to aggregate together (Petit-Glatron et al., 1980; L'Hocine et al., 2000) or with carbohydrates (Crittenden and Doelle, 1994; Vigants et al., 2003); such aggregations at medium range temperature contribute to the thermal stability of levansucrases. The thermal stability of the levansucrase from *G. stearothermophilus* is comparable to that of *Z. mobilis* levansucrase (Sangiliyandi et al., 1999), which retained 70% of the initial total activity after 6 hrs incubation at 40°C and was totally inhibited at 50 and 60°C, after 100 and 30 min, respectively. However, the transfructosylation activities of levansucrases from *B. circulans* (El-Refai et al., 2009) and *B. cereus* (Fawkia et al., 2009) showed lower thermal stability. In fact, they were completely inactivated after only 60 min of thermal treatment at 50 and 60°C, respectively, whereas the levansucrase from *G. stearothermophilus* still showed 91.7% of the transfructosylation activity after 6 hrs incubation at 47°C. Furthermore, only 41% of the transfructosylation activity was reported for *Z. mobilis* levansucrase after 15 min incubation at 45°C (Jang et al., 2001). Likewise, the levansucrase from *B. natto* was reported to retain 50% of its initial activity after 30 min incubation at 40°C, while a complete loss of its activity was obtained after 60 min at 45°C (Ben Ammar et al., 2002).

3.3.3. Effect of pH

The effect of pH on the transfructosylation and hydrolytic activities of purified levansucrase from *G. stearothermophilus* was investigated within a broad range of pH. The results (Fig. 10) indicate that *G. stearothermophilus* levansucrase displayed a hydrolytic activity over a wide pH range of 4.00 to 8.00, but remained highly active in the neutral range. A maximal hydrolytic activity was observed at pH 6.75 and more than 50% of this activity was retained at a narrow pH range of 6.7 to 7.2. On the other hand, the transfructosylation activity of *G. stearothermophilus* levansucrase exhibited a different profile, showing maximum activity at pH 6.75 and retaining more than 50% of this activity over a broader pH range of 5.9 to 7.25. Little hydrolytic activity remained at pH 8.00, while the transfructosylation activity was completely abolished. The effect of pH on the reaction selectivity of levansucrase was higher within a very narrow pH range of 6.00 to 6.50 with a transfructosylation to hydrolytic ratio of 2.86 to 3.18. However, a rapid decline of the transfructosylation to hydrolytic ratio to 0.09 and 1.00 was obtained at a pH value of 5.0 and 6.75, respectively.

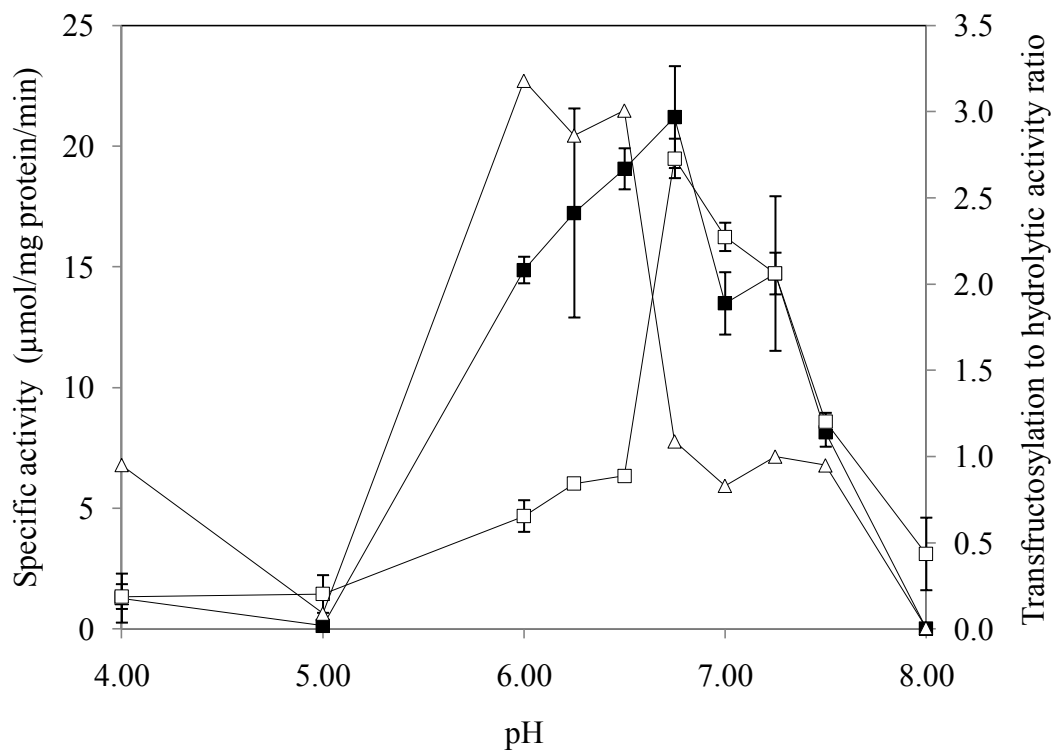


Figure 10. Effect of pH on the reaction selectivity of *Geobacillus stearothermophilus* fructosyltransferase (levansucrase): Transfructosylation (■), hydrolysis (□) and transfructosylation to hydrolytic activity ratio (Δ).

Based on the pH profile, pH was the most significant parameter that influenced the reaction selectivity of the levansucrase from *G. stearothermophilus* as compared to temperature. The pH activity profile of *G. stearothermophilus* levansucrase showed common features than those of levansucrases from other bacterial sources, which were active within a wide pH range of 3.5 to 8.0 (Hernandez et al., 1995; Euzanat et al., 1997; Sangiliyandi et al., 1999; Jang et al., 2001; Esawy et al., 2008; Rairakhwada et al., 2010). Indeed, the levansucrase from *B. megaterium* has been reported to display a maximal activity at pH 6.6 with lower activity at pH values below 5.6 and above 7.6. The pH optimum for the total *G. stearothermophilus* levansucrase activity is within the range of 5.0 to 7.0 as reported for the levansucrases from *Z. mobilis* (Jang et al., 2001; Goldman et al., 2008) and *B. subtilis* (Euzanat et al., 1997; Esawy et al., 2008). However, lower pH optimum values in the pH range of 3.5 to 5.5 were obtained for levansucrases from lactobacilli species (van Hijum et al., 2004; Tieking et al., 2005; Waldherr et al., 2008).

Only a few studies have investigated the effect of pH on the polymerization, transfructosylation and hydrolytic activities (Crittenden and Doelle, 1994; Oseguera et al., 1996b; Euzanat et al., 1997; Goldman et al., 2008; Waldherr et al., 2008). Indeed, levansucrase from *Z. mobilis* exhibited maximum hydrolytic, polymerization and transfructosylation activities at pH greater than 6.75, lower than 6.0 and higher than 7.0, respectively. Goldman et al. (2008) have confirmed, using transmission electron microscopy, that the change in reaction selectivity of *Z. mobilis* levansucrase was initiated by the modification of the enzyme quaternary structure rather than by the variation in pH. These authors suggested that the micro-fibril structure, as opposed to the dimeric form, favoured the attachment of the polymer levan to the surface of the enzyme, therefore promoting chain elongation. In contrast, Euzanat et al. (1997) have demonstrated that the levansucrase from *B. subtilis* maintains a constant hydrolytic rate regardless of the variations in pH. In addition, several catalytic studies have demonstrated that the steric requirements of the catalytic amino acid groups have a greater impact on the levan-forming activity of levansucrase than on its hydrolytic activity (Yanase et al., 2002; Martinez-Fleites et al., 2005), mainly by slowing the rate of formation of the fructosyl-enzyme intermediate (Li et al., 2008). In fact, the acidic residues, Asp86 and Glu342,

were found to play an important role in the catalysis of glycosyl hydrolase enzymes (Davies and Henrissat, 1995) and particularly in the substrate binding.

3.3.4. Effect of Substrate Concentration and Determination of Kinetic Parameters.

In spite of the complexity, due to the fact that FTF catalyzed simultaneously several reactions (hydrolysis, transfructosylation, and polymerization), it was still considered worthwhile to determine and analyze the kinetic parameters. The effect of sucrose concentration on the reaction rate of the purified levansucrase extract from *G. stearothermophilus* was investigated using a substrate concentration of 0.125 to 1.0 M under standard reaction conditions. The results (Fig. 11) reveal that the kinetics of levansucrase followed the Michaelis-Menten model, as indicated by the linearity of the corresponding Lineweaver-Burk plots. Figure 11 also shows that the rate of hydrolysis and transfructosylation of the levansucrase from *G. stearothermophilus* were not significantly inhibited at high substrate concentration (1.0 M). In addition, the transfructosylation to hydrolysis ratio of *G. stearothermophilus* levansucrase remains constant over the investigated sucrose concentration. The maximum reaction rate ($V_{\max\text{app}}$) for transfructosylation (58.48 μmol of transferred fructose/mg protein/min) was 2-fold higher than the hydrolytic reaction (27.7 μmol of released free fructose/mg protein/min). On the other hand, the K_{mapp} Michaelis-Menten values (272 – 269 mM sucrose) were very similar for both reactions, whereas the k_{cat} turnover number for transfructosylation (53.02 s^{-1}) was 2-fold higher than that of hydrolysis (25.12 s^{-1}). As a result, the catalytic efficiency ($k_{\text{cat}}/K_{\text{mapp}}$) for the transfructosylation activity (197.10 $\text{M}^{-1}\cdot\text{s}^{-1}$) was much higher than for the hydrolytic activity (92.52 $\text{M}^{-1}\cdot\text{s}^{-1}$) (Table 7).

These results reveal a very important characteristic of *G. stearothermophilus* levansucrase with regards to the independence of its transfructosylation to hydrolysis ratio to the variations of the substrate concentration, making it different from most of the previously described levansucrases from other microbial sources (Park et al., 2003; Martinez-Fleites et al., 2005; Homann et al., 2007). Indeed, the hydrolytic activity of levansucrases from other microbial sources was predominant at low sucrose concentration (Chambert and Petit-Glatron., 1991; Crittenden and Doelle, 1994; Morales-Arrieta et al., 2006; Goldman et al., 2008).

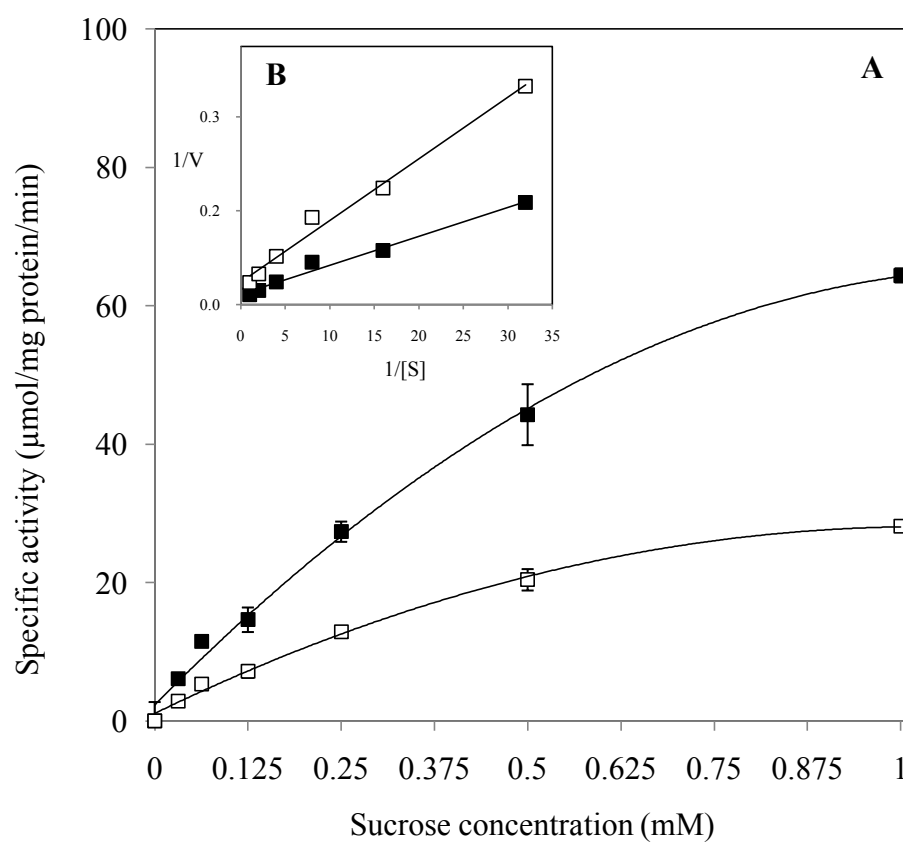


Figure 11. Michaelis-Menten plot (A) and Line-weaver-Burk plot (B) of transfructosylation (■) and hydrolytic (□) activities of *Geobacillus stearothermophilus* fructosyltransferase (levansucrase).

In contrast to other levansucrases, for which K_m values range between 3 and 76 mM (Ben Ammar et al., 2002; van Hijum et al., 2004; Kang et al., 2005; Homann et al., 2007; Goldman et al., 2008; Waldherr et al., 2008; Anwar et al., 2010), the levansucrase from *G. stearothermophilus* displayed a very high apparent K_m value (272 mM and 269 mM). This high K_{mapp} value is an indication that the levansucrase from *G. stearothermophilus* has a low affinity towards the substrate sucrose. Similarly, Yanase et al. (1992) have also reported a relatively high K_m value for the levansucrase from *Z. mobilis* (122 mM), which was attributed to the absence of enzyme saturation at elevated sucrose concentration (Goldman et al., 2008). A comparable K_m value (0.2 M) has been reported for a FTF from *B. subtilis* NCIMB 11871, and has been classified as EC 2.4.1.162 (Rathbone et al., 1986; Cheetham et al., 1989; Rathbone et al., 1990). The ability of this FTF to synthesize sucrose analogues was subsequently investigated by Seibel et al. (2005; 2006b) and Beine et al. (2008; 2009). In fact, this FTF has shown similar feature to the levansucrase enzyme (Jones et al., 1992), with regards to its ability to catalyze hydrolysis and transfructosylation in the presence of a wide variety of acceptors. However, in contrast to levansucrases, this FTF was unable to produce a significant amount of levan but was capable of producing FOSs containing a maximum of 7 units (Cheetham et al., 1989).

Although the levansucrase from *G. stearothermophilus* had a very low affinity for the substrate sucrose, the higher turnover number for transfructosylation ($197.10 \text{ M}^{-1}\cdot\text{s}^{-1}$), as compared to hydrolysis ($92.52 \text{ M}^{-1}\cdot\text{s}^{-1}$), demonstrates that levansucrase favors the transfructosylation reaction. Goldman et al. (2008) reported that the levansucrase from *Z. mobilis* has a greater catalytic efficiency for the transfructosylation reaction ($10527 \text{ M}^{-1}\cdot\text{s}^{-1}$) than the hydrolytic reaction ($4611 \text{ M}^{-1}\cdot\text{s}^{-1}$). In contrast, the levansucrase from *L. reuteri* favoured the hydrolytic reaction ($10400 \text{ M}^{-1}\cdot\text{s}^{-1}$) over the transfructosylation one ($2430 \text{ M}^{-1}\cdot\text{s}^{-1}$) (van Hijum et al., 2004).

As previously mentioned, the rate of fructose liberation (hydrolysis) reaches a definite saturation whereas transfructosylation undergoes a minor inhibition at a sucrose concentration of 1.0M. This may be explained by a competition between two acceptors, a sucrose molecule and a growing levan chain, for the binding at subsite -1 and +1 at the active site of levansucrase. At high substrate concentration, sucrose is the preferred

acceptor and two possible reactions occur: a FOS is synthesized or levan is elongated. In such condition, the rate of glucose release (oligomerization) remains unaffected although polymerization occurs at a slower rate (Goldman et al., 2008). To support this explanation, other studies have previously demonstrated that the addition of levan to the reaction mixture inhibits hydrolysis and favors the transfructosylation reaction (Chambert et al., 1974; Tanaka et al., 1979; Oseguera et al., 1996b). Interestingly, Tanaka et al. (1979) found that levan served as an activator of transfructosylation reaction rather than an acceptor because the molecular weight of levan did not increase. Moreover, the rate of transfructosylation still reaches saturation (Oseguera et al., 1996b). This can be attributed to the fact that the concentration of free glucose increases as a result of transfructosylation. Glucose has been reported to inhibit the hydrolysis, transfructosylation and levan-forming activities of the levansucrase from *Z. mobilis*, while fructose did not significantly affect the velocities of those reactions (Crittenden and Doelle, 1994).

3.4. Characterization of the Product Spectrum and the Acceptor Substrate Specificity of Levansucrase from *Geobacillus stearothermophilus*.

3.4.1. Characterization of the Product Spectrum

The product spectrum of levansucrase-catalyzed reaction was investigated using sucrose as the sole substrate. The reaction conditions used for the determination of the product spectrum favour the transfructosylation (75.04%) over the hydrolysis (24.96%) reaction. The HPAEC chromatogram (Fig. 12) shows that, besides the polyfructan, the *G. stearothermophilus* levansucrase synthesized six different FOSs. The characterization of the FOSs was based on the comparison of their elution times with those of the injected standards (Peaks#A, B) and those reported in the literature (Ozimek et al., 2006b; Homann et al., 2007). The results show that *G. stearothermophilus* levansucrase produces significant amount of 1-kestose (Peak#2, *O*- β -D-fructofuranosyl-(2 $\rightarrow$ 1)-*O*- β -D-fructofuranosyl-(2 $\rightarrow$ 1)- α -D-glucopyranoside) and nystose (Peak#6, *O*- β -D-fructofuranosyl-(2 $\rightarrow$ 1)-*O*- β -D-fructofuranosyl-(2 $\rightarrow$ 1)-*O*- β -D-fructofuranosyl-(2 $\rightarrow$ 1)- α -D-glucopyranoside with a retention time of 7.28 and 8.78 min, respectively. By comparing the retention times and the order reported in the literature (Homann et al., 2007), the transfructosylation products eluted at 7.50, 7.85 and 8.00 min were characterized as blastose (Peak#3, *O*- β -D-fructofuranosyl-(2 $\rightarrow$ 6)-*O*- α - β -D-glucopyranose), 6-kestose (Peak#4, *O*- β -D-fructofuranosyl-(2 $\rightarrow$ 6)- β -fructofuranosyl-(2 $\rightarrow$ 1)- α -D-glucopyranoside) and neokestose (Peak#5 *O*- β -D-fructofuranosyl-(2 $\rightarrow$ 6)- α -D-glucopyranosyl-(1 $\rightarrow$ 2)- β -D-fructofuranoside), respectively. The blastose was reported to be the results of the hydrolysis of neokestose at the β (2 $\rightarrow$ 1) linkage (Homann et al., 2007). In addition to these five identified FOS products, unknown product at significant amount was eluted at 6.2 min and may be characterized as bifructose (Peak#1, *O*- β -D-fructofuranosyl-*O*- β -D-fructofuranoside) (Ozimek et al., 2006b). The transfructosylation product spectrum of *G. stearothermophilus* levansucrase-catalyzed reaction is very similar to that of *B. megaterium* levansucrase (Homann et al., 2007). Contrarily to *B. megaterium*, the levansucrase from *G. stearothermophilus* did not accumulate a large amount of blastose from sucrose. Homann et al. (2007) have reported that the accumulation of blastose may be attributed to the presence of β (2 $\rightarrow$ 6) linkage between glucose and fructose, making it an unfavorable fructosyl donor substrate.

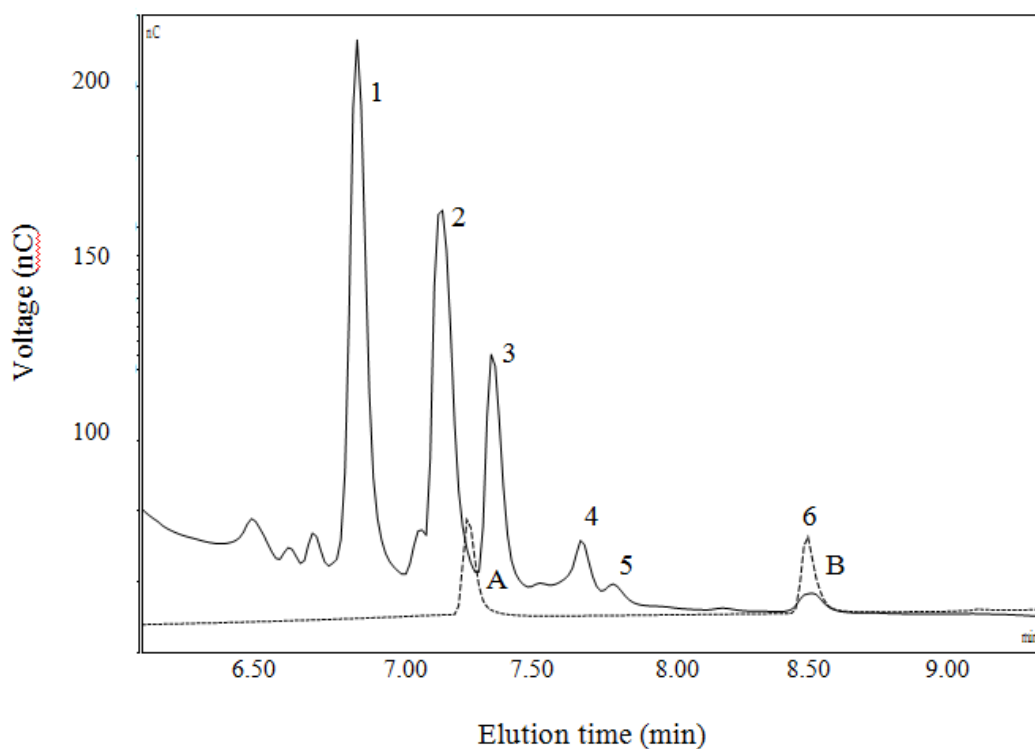


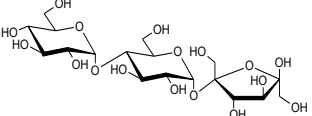
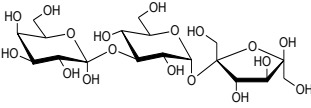
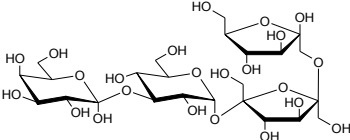
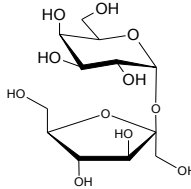
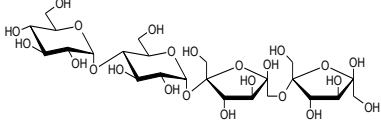
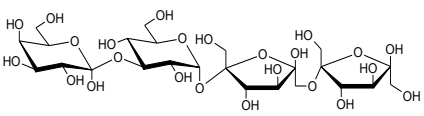
Figure 12: HPAEC-PAD chromatograms of fructooligosaccharides standards (---), 1-kestose (A) and nystose (B), and of transfructosylation products (—) synthesized by *G. stearothermophilus* fructosyltransferase (levansucrase) using sucrose as the sole substrate. Peaks#1, 2, 3, 4, 5 and 6 are identified as bifructose, 1-kestose, blastose, 6-kestose, neo-kestose and nystose, respectively.

3.4.2. Study of the Acceptor Specificity.

The specificity of *G. stearothermophilus* levansucrase towards different acceptors, including glucose, galactose, maltose, lactose and raffinose, were investigated at 15 and 30°C, using sucrose as the fructosyl donor. A fructosyl donor to acceptor molar ratio of 0.5:1.0 and 1.0:0.5 were used. For each acceptor, the optimal reactions conditions for fructosyl transfer were determined by estimating the concentration of the transfructosylation products using HPAEC-PAD. Overall, the transfructosylation was more favorable at 30°C than at 15°C regardless of the acceptor used. With the exception of maltose, all the acceptors produced more FOSs at a fructosyl donor to acceptor molar ratio of 1.0:0.5. The optimum reaction time was determined to be 8 hrs; beyond this time, the FOSs were either hydrolyzed or used as acceptors for chain elongation. The inulin-type FOS standards, 1-kestose (Peak#A) and nystose (Peak#B), were used as external standards (Fig. 13). The FOS products were identified by comparing their retention times with those of injected standards as well as with those reported in the literature.

The results (Table 8) show that galactose (0.50 μ M/hr) was a good monosaccharide acceptor as compared to glucose (0.20 μ M/hr) to form *O*- β -D-fructofuranosyl-(2 $\rightarrow$ 1)-*O*- α -D-galactopyranoside (Gal-Fru, Peak#1) and β -D-fructofuranosyl-(2 $\rightarrow$ 1)- α -D-glucopyranoside (Glc-Fru, Peak#1'), respectively. Figures 13A and 13B show that sucrose analogues, Gal-Fru and Glc-Fru, were the main products synthesized by levansucrase in the presence of galactose and glucose. In contrast to the galactose, levansucrase was able to produce a significant amount of bifructose in the presence of glucose (Fig. 13B, Peak#2'). The product profiles also indicate that 1-kestose (Peak#3') was synthesized in detectable amount. The presence of 1-kestose in the reaction mixtures can be attributed to the fact that levansucrase was unable to use it efficiently as a fructosyl donor (Crittenden and Doelle, 1994). In fact, fructosyl units from oligosaccharides can only be transferred by levansucrase when the β -fructofuranosyl moiety is attached to the anomeric carbon of an aldose ring such as in raffinose (Hestrin et al., 1956). *B. subtilis* levansucrase was capable of using 1-kestose as a fructosyl-acceptor with a greater efficiency as compared to the levansucrase from *A. diazotrophicus* (Hernandez et al., 1995). In contrast, Ozimek et al. (2006b) have demonstrated that inulin-type FOSs are poorer fructosyl acceptors for the levansucrase from *L. reuteri*, as compared to the levan-type FOSs.

Table 8. *Geobacillus stearothermophilus* fructosyltransferase (levansucrase)-catalyzed transfructosylation reactions in the presence of various acceptors.

Acceptor	Maltose ^d	Lactose ^e	Raffinose ^e	Glucose ^e	Galactose ^e
Productivity of Acceptor-Fru ($\mu\text{M/h}$) ^a	7.52 	1.52 	0.01 	0.20	0.50 
Productivity of Acceptor-(Fru) ₂ ($\mu\text{M/h}$) ^b	0.72 	0.11 	-	-	-
Yield ^c from sucrose (%)	98	37	50	50	50

^a The productivity of acceptor-Fru represents the rate of synthesis of the compound at the optimum incubation time of 8 hrs.

^b The productivity of acceptor-(Fru)₂ represents the rate of synthesis of the compound at the optimum incubation time of 8 hrs.

^c The product yield from sucrose represents the conversion yield of sucrose into fructosylated products, by assuming that sucrose is the sole fructosyl donor. The product yield from sucrose is expressed in percentage and it is obtained by dividing the consumed sucrose by the initial sucrose concentration.

^d The molar ratio of fructosyl donor:acceptor is 0.5:1.0.

^e The molar ratio of fructosyl donor:acceptor is 1.0:0.5.

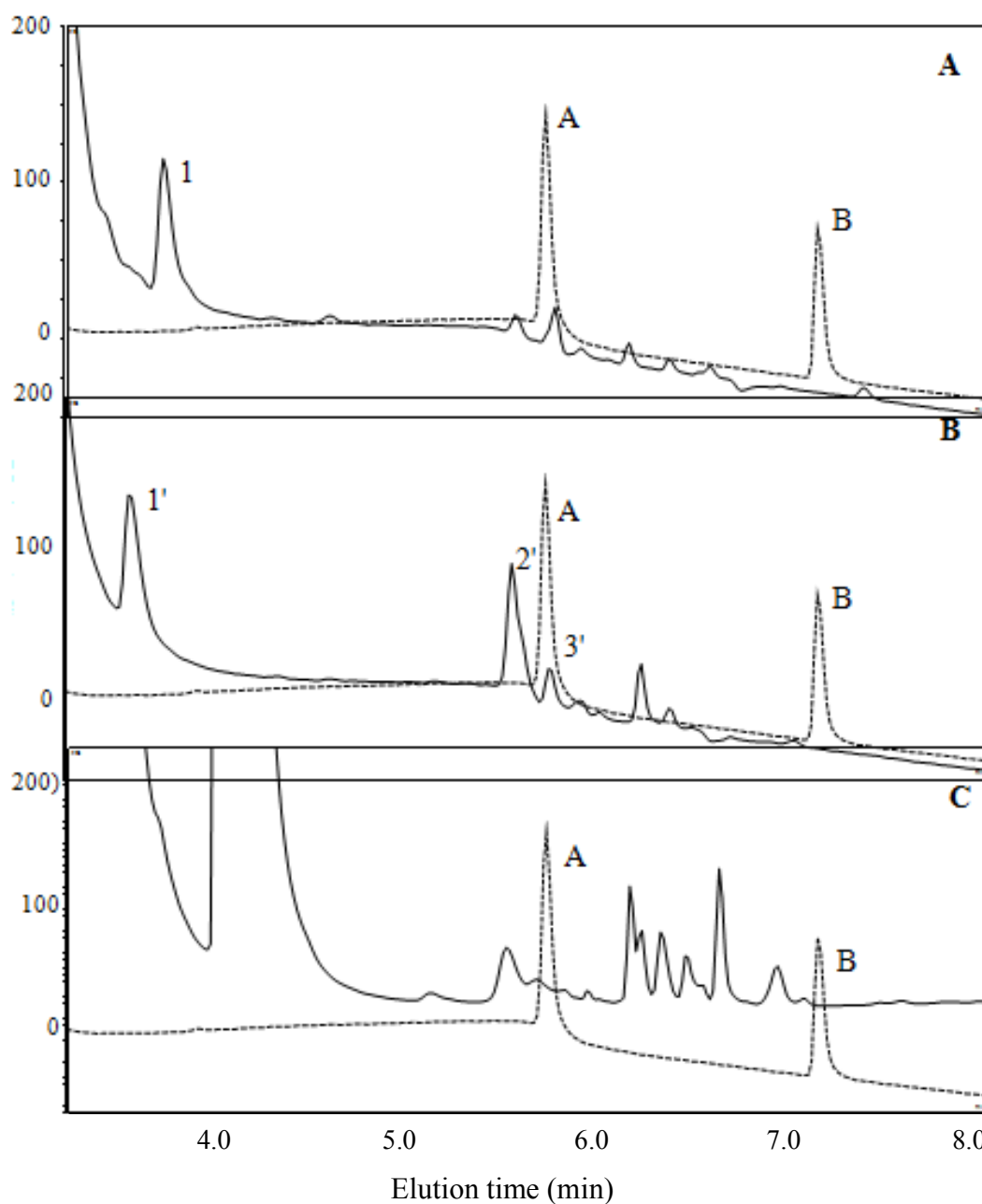


Figure 13: HPAEC-PAD chromatogram of the inulin-type fructooligosaccharide standards, 1-kestose (peak#A) and nyctose (peak#B) (---), and of the fructooligosaccharides products (—) synthesized by *G. stearothermophilus* fructosyltransferase (levansucrase) in the presence of the acceptor galactose (A), glucose (B) and lactose (C). Peaks#1 and 1' represent the sucrose analogues galactosyl-fructoside and glucosyl-fructoside, respectively. Peaks# 2' and 3' are identified as bifructose and 1-kestose, respectively.

Table 8 also indicates that a yield of 50% conversion of sucrose to transfructosylation products were obtained with the levansucrase from *G. stearothermophilus* using both galactose and glucose as acceptors; these results suggest that in addition to sucrose analogues, levan was also produced. The limit production of sucrose analogues may be attributed to the rapid quasi-equilibrium of the reaction (Seibel et al., 2006b) as sucrose analogues is thermodynamically favorable and can also be used as a donor. In fact, the energy released from the hydrolysis of the $\beta(2\rightarrow1)$ glycosidic bond in sucrose analogues (Gal-Fru) is sufficient to drive the non-Leloir-type of transfructosylation reaction (Seibel et al., 2006a; van Hijum et al., 2006). Beine et al. (2008) further provided evidence that the transfructosylation reaction catalyzed by FTF from *B. subtilis* 11871 using Gal-Fru follows the same transfructosylation step as with sucrose and results in the production of predominant polysaccharide of Gal-(Fru)_n-Fru (where $n > 12$). Indeed, the same authors have speculated that the fructosyl residue of the sucrose analogue, Gal-Fru, could bind to the subsite +2 of the active site in the same manner as raffinose.

The results (Table 8) also show that the yield of conversion of sucrose into transfructosylation products was of 37 and 50% in the presence of lactose and raffinose, respectively. Melibiose (Peak#1, *O*- α -galactopyranosyl-(1 $\rightarrow$ 6)- α -D-glucopyranoside) with a retention time of 4.25 min was identified as the main FOS product in the presence of raffinose (Fig. 14A). Using lactose (Fig.13C) or raffinose (Fig. 14A) as fructosyl acceptors, the levansucrase from *G. stearothermophilus* synthesized other various unidentified FOSs, with undetectable amount of 1-kestose. Tieking et al. (2005) have identified the main products of the levansucrase from *L. sanfranciscensis* using raffinose as the acceptor, as β -fructosylraffinose (Gal-Glc-Fru₂) and melibiose (Gal-Glc). The same authors have reported that when raffinose was used as the fructosyl donor, heterooligosaccharides of up to only five units were synthesized by the levansucrase from *L. sanfranciscensis*. Since the FOS synthesized by *G. stearothermophilus* levansucrase with lactose and raffinose did not comprise the inulin-type FOS, 1-kestose and nystose, it may be assumed that the unidentified FOS are the levan-type FOS linked by $\beta(2\rightarrow6)$ -glycosidic linkages.

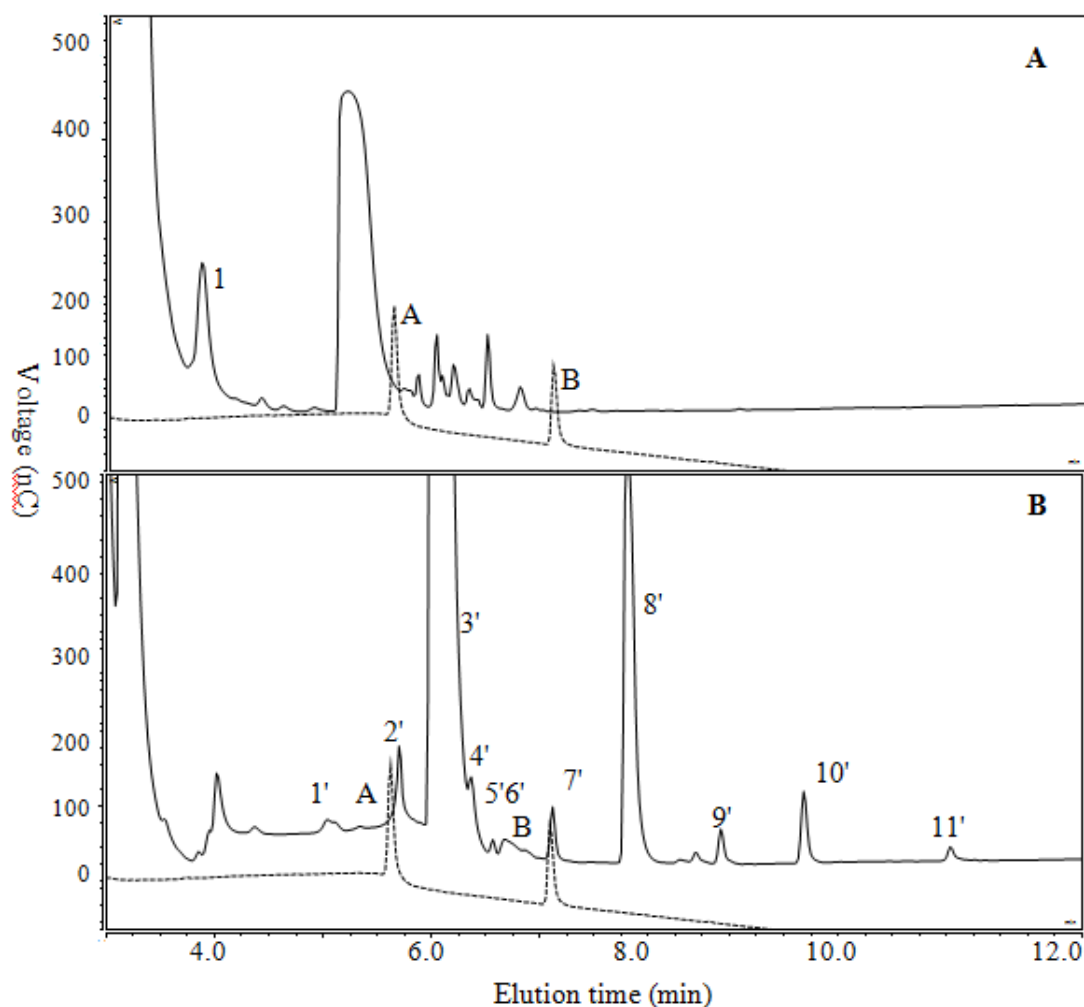


Figure 14: HPAEC-PAD chromatogram of the inulin-type fructooligosaccharide standards 1-kestose (A) and nystose (B) (---) and of the fructooligosaccharides products (—) synthesized by *G. stearothermophilus* fructosyltransferase (FTF) in the presence of the acceptor raffinose (A) and maltose (B). Peak#1 represents melibiose, while peak#1' maltose. Peaks#2', 3', 4', 5', 6' and 7' identified as 1-kestose, erlose, blastose, 6-kestose, neo-kestose and nystose, respectively. Peaks#8'-11' are unidentified FOSs of higher degree of polymerization.

The overall results suggest that glycosides composed of a pyranoside ring (galactose, maltose, lactose) were preferably used as fructosyl acceptors by *G. stearothermophilus* levansucrase as compared to those containing furanose rings, such as raffinose. Maltose (7.52 $\mu\text{M/hr}$) was shown to be the most suitable acceptor of fructosyl units as demonstrated in the chromatogram (Fig.14B, Peak#1'). Maltose (1.0 M) has been almost completely consumed by *G. stearothermophilus* levansucrase and a higher amount of the compound erlose (6.15 min. Peak#3', *O*- α -D-glucopyranosyl-(1 $\rightarrow$ 4)-*O*- α -D-glucopyranosyl- β -(1 $\rightarrow$ 2)-D-fructofuranoside) was produced. Series of other FOSs were produced, including 1-kestose (Peak#2'), blastose (Peak#4'), 6-kestose (Peak#5'), neokestose (Peak#6') and nystose (Peak# 7'), as well as oligofructosides of higher degree of polymerization (Peak# 8'-11'). Previous studies investigating the product spectrum of levansucrase using maltose as acceptor have also reported the formation of erlose as the predominant product (Kang et al., 2005; Tieking et al., 2005; Seibel et al., 2006b).

In general, the results demonstrated the broad specificity of the levansucrase from *G. stearothermophilus*, with maltose as the preferred fructosyl acceptor. It is important to mention that, contrarily to the accumulation of 1-kestose, the compound 6-kestose was not detected with the investigated acceptors. Similar results have been reported and attributed the absence of 6-kestose to its elongation to the β (2 $\rightarrow$ 6)-linked polyfructan levan (Ozimek et al., 2006b). Moreover, the levansucrase was shown to be able of synthesizing both inulin- and levan-type FOSs containing, β (2 $\rightarrow$ 1) and β (2 $\rightarrow$ 6) glycosidic bonds, respectively. This finding is in agreement with other levansucrases from different microbial sources (Korakli et al., 2003; Euzenat et al., 2005). Nevertheless, the elucidation of the structural components responsible for the determination of the degree of polymerization and type of glycosidic bonds formed is yet to be resolved. However, a mechanism of non-processive or processive transfructosylation reaction has been suggested by Ozimek et al. (2006b) with the aim of explaining the production of FOSs versus levan, respectively.

CONCLUSION

The experimental results provided in this study have determined the optimal conditions for the expression of FTF (levansucrase) in *G. stearothermophilus*. The formation of levan was confirmed by TLC and NMR analyses, further testifying the production of levansucrase (FTF) by the thermophilic strain. This work constitutes the first study on the catalytic properties and the acceptor specificity of levansucrase from *G. stearothermophilus*.

The hydrolytic and transfructosylation activities of the levansucrase from *G. stearothermophilus* were shown to be significantly more resistant to high temperatures than levansucrases from other microbial sources. The catalytic features of this levansucrase are similar to those of other levansucrase with respect to its hydrolytic activity representing 50% that of the total activity, its optimal pH, and its optimal temperature. The reaction temperature did not affect the ratio of transfructosylation to hydrolytic activities. However, the highest transfructosylation activity (75%) over the hydrolytic one (25%) was obtained within a narrow pH range of 6.0 to 6.50. In contrast to the majority of levansucrases, the levansucrase from *G. stearothermophilus* demonstrated a very low affinity towards the substrate sucrose. However, the levansucrase from this strain had a high catalytic efficiency for the transfructosylation reaction. Furthermore, the use of acceptors enhanced the diversity of the transfructosylation end-products. In fact, using sucrose as the sole substrate, levansucrase was able of synthesizing six FOSs, bifructose, 1-kestose, blastose, 6-kestose, neo-kestose and nystose, whereas additional FOSs were formed using galactose, lactose, raffinose and particularly maltose as fructosyl acceptors. Indeed, the use of maltose as a fructosyl acceptor resulted in a 98% yield of transfructosylation products. The overall results revealed the wide acceptor specificity of *G. stearothermophilus* levansucrase, enabling the synthesis of FOSs-headed with a terminal fructosyl- or galactosyl-residue, for which health attributes are expected to be superior to currently available inulin-FOSs.

In sum, the production and the catalytic characterization of levansucrase from the thermophilic *G. stearothermophilus* establishes exciting grounds for prospective studies

aiming at the synthesis of structurally-prominent FOSs. It would be considered worthwhile to explore the synthesis of FOSs by the levansucrase from *G. stearothermophilus* and assess the prebiotic activities of those emerging “second generation” FOSs.

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