CHARACTERIZING GENOME-MINED MICROBIAL LEVANASES FOR THE SYNTHESIS OF PREBIOTIC β-(2,6)-FRUCTOOLIGOSACCARIDES AND OLIGOLEVANS

By

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SYNTHESIS OF LEVAN-TYPE FRUCTOOLIGOSACCHARIDES AND OLIGOLEVANS BY NEWLY DISCOVERED BIOCATALYSTS

ABSTRACT

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The recent emergence of prebiotics in the food and pharmaceutical industries is attributed with compounding awareness of their wide array of systemic and targeted health benefits, including, but not limited to, the promotion of gastrointestinal health and prevention of increasingly prevalent chronic diseases such as diabetes and obesity. Inulin- and levan-type fructooligosaccharides (FOSs) are two prominent classes of non-digestible oligosaccharides which have been investigated for their ability to meet prebiotic classification, though there are significantly fewer reports in literature regarding the latter due to limited accessibility to natural sources of levan and inefficient methods of generating prebiotic FOSs from such. Recently, there has been a shift in interest from the commercially available inulin-type prebiotics, which solely contain β-(2,1)-glycosidic linkages, to levanoligosaccharides, which are characterized by β-(2,6)-linked fructosyl residues in the fructan main chain. This differentiation in structure has been attributed with higher prebiotic capacity as it is correlated with increased colonic persistence and selective fermentation by beneficial bacteria in the intestinal tract, which are two crucial criteria for prebiotics. The focus of the present work was to develop an industrially feasible enzymatic approach for the synthesis of novel levan-type FOS compounds to be administered as second-generation prebiotics with enhanced functionality. Endo-levanases (E.C.3.2.1.65) are glycosyl-hydrolytic enzymes (GH32) which characteristically catalyze the hydrolysis of levan β-(2,6)-linkages, yielding fructooligosaccharides (FOSs) and oligolevans promising higher prebiotic potential than commercial β-(2,1)-/inulin-type FOSs. Due to their scarcity, data mining of microbial genomes was performed using reference levanase levanase genes in a representative protein database. This entailed a mass phylogenetic screening conducted using BLASTP against UniProtKB to elucidate all microbial species containing genetic material bearing strong homology to previously annotated levanase-encoding genes. Subsequently, a new collection of 1902 genetic sequences amenable to cloning was obtained, from which 123 representative candidates were screened according to their individual specific activities on low- and high-molecular weight levan and inulin, as well as differences in behaviour owing to substrate specificity and thermal stability. This ultimately revealed 10 viable, highly evolved and diverse bacterial candidate levanases. In particular, those from *Belliella baltica* (LEV4-A10), *Dyadobacter fermentans* (LEV4-D3),

Capnocytophaga ochracea (LEV4-D4), *Vibrio natriegens* (LEV4-H2) and *Arthrobacter aurescens* (LEV5-A7) exhibited the highest levels of endo-hydrolytic activity on levan and demonstrated preferential hydrolysis of such over inulin. These biocatalysts were further investigated in terms of their kinetic and optimal reaction parameters, as well as thermal stability. In addition, the use of different enzyme units was explored in order to define the optimal parameters for the synthesis of β-(2,6)-FOSs and oligolevans. Results demonstrated that the biocatalytic activity of the selected levanases remained relatively stable across varying reaction conditions, but generally performed optimally at 37°C and pH 6.4. Notably, *B. baltica* and *C. ochracea* levanases exhibited the highest thermal stability, retaining 62.2% and 64.9% of their initial activity, respectively, after 6 hours at 50°C. Employed simultaneously with levansucrase, these levanases also demonstrated the highest bioconversion of levan into oligolevans and β-(2,6)-FOSs varying in degree of polymerization. The maximal yield of FOS products was observed in a one-step bi-enzymatic system involving *G. oxydans* levansucrase and *B. baltica* levanase, which bears promising applications in the food and pharmaceutical industries in the form of functional ingredients and nutraceuticals.

RÉSUMÉ

L'émergence récente des prébiotiques dans les industries alimentaire et pharmaceutique est associée à une prise de conscience croissante de leur large gamme de bénéfices systémiques et ciblés, y compris, mais sans s'y limiter, la promotion de la santé gastro-intestinale et la prévention de maladies chroniques telles que le diabète et l'obésité. Les fructo-oligosaccharides de type inuline et lévane (FOS) sont deux classes importantes d'oligosaccharides non digestibles qui ont été étudiés pour leur capacité à satisfaire la classification prébiotique, bien qu'il y ait beaucoup moins de rapports dans la littérature concernant ce dernier en raison de l'accessibilité limitée aux sources naturelles. Levan et des méthodes inefficaces de générer des FOS prébiotiques à partir de tels. Récemment, il y a eu un changement d'intérêt des prébiotiques de type inuline disponibles dans le commerce, qui contiennent uniquement des liaisons β-(2,1)-glycosidiques, aux levanoligosaccharides, qui sont caractérisés par des résidus fructosyle β-(2,6)-liés dans le chaîne principale de fructan. Cette différenciation dans la structure a été attribuée à une capacité prébiotique plus élevée car elle est corrélée avec une augmentation de la persistance colique et une fermentation sélective par des bactéries bénéfiques dans le tractus intestinal, qui sont deux critères cruciaux pour les prébiotiques. L'objectif du présent travail était de développer une approche enzymatique industriellement réalisable pour la synthèse de nouveaux composés FOS de type levan à administrer en tant que prébiotiques de deuxième génération avec une fonctionnalité améliorée. Les endo-levanases (E.C. 3.2.1.65) sont des enzymes glycosyl-hydrolytiques (GH32) qui catalysent de manière caractéristique l'hydrolyse des liaisons β-(2,6) du levane, donnant des fructooligosaccharides (FOS) et des oligolevans promettant un potentiel prébiotique plus élevé que les β-(2,1)- / FOS de type inuline. En raison de leur rareté, l'exploration de données de génomes microbiens a été effectuée en utilisant des gènes de levanase levanase de référence dans une base de données de protéines représentatives. Ceci a impliqué un criblage phylogénétique de masse effectué en utilisant BLASTP contre UniProtKB pour élucider toutes les espèces microbiennes contenant du matériel génétique portant une forte homologie avec des gènes codant pour la lévanase précédemment annotés. Par la suite, une nouvelle collection de 1902 séquences génétiques pouvant être clonées a été obtenue, parmi lesquelles 123 candidats représentatifs ont été sélectionnés en fonction de leurs activités spécifiques sur le levan et l'inuline de bas et haut poids moléculaire ainsi que des différences de comportement dues à la spécificité du substrat et la

stabilité thermique. Ceci a finalement révélé 10 levanases bactériennes viables, hautement évoluées et diverses. En particulier, ceux de *Belliella baltica* (LEV4-A10), de *Dyadobacter fermentans* (LEV4-D3), de *Capnocytophaga ochracea* (LEV4-D4), de *Vibrio natriegens* (LEV4-H2) et d'*Arthrobacter aurescens* (LEV5-A7) présentaient les plus hauts taux d'endotoxicité activité hydrolytique sur le levan et hydrolyse préférentielle démontrée d'une telle inuline. Ces biocatalyseurs ont été étudiés plus en détail en termes de paramètres de réaction cinétiques et optimaux, ainsi que de stabilité thermique. De plus, l'utilisation de différentes unités enzymatiques a été explorée afin de définir les paramètres optimaux pour la synthèse des β-(2,6)-FOS et des oligolevanes. Les résultats ont démontré que l'activité biocatalytique des levanases choisies restait relativement stable dans diverses conditions de réaction, mais généralement de façon optimale à 37 ° C et pH 6,4. Notamment, *B. baltica* et *C. ochracea* levanases présentaient la stabilité thermique la plus élevée, conservant 62,2% et 64,9% de leur activité initiale, respectivement, après 6 heures à 50 ° C. Employées simultanément avec la levansucrase, ces levanases ont également démontré la plus haute bioconversion du lévane en oligolevanes et en β-(2,6) -FOS variant en degré de polymérisation. Le rendement maximal des produits FOS a été observé dans un système bi-enzymatique en une étape impliquant *G. oxydans* levansucrase et *B. baltica* levanase, qui présente des applications prometteuses dans l'industrie alimentaire et pharmaceutique sous la forme d'ingrédients fonctionnels et de nutraceutiques.

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The thesis must be more than a collection of manuscripts. All components must be integrated into a cohesive unit with a logical progression from one chapter to the next. In order to ensure that the thesis has continuity, connecting texts that provide logical bridges between the different papers are mandatory.

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When previously published copyright material is presented in a thesis, the candidate must obtain, if necessary, signed waivers from the co-authors and publishers and submit these to the Thesis Office with the final deposition.

PREFACE AND CONTRIBUTION OF AUTHORS

This thesis includes five chapters. Chapter I provides a general introduction to the concept of prebiotics, with emphasis on the current trend towards the development of novel fructooligosaccharides (FOSs), and outlines the specific research objectives of the present study.

Chapter II consists of a literature review of topics and studies relevant to prebiotic classification, current methods of producing such (with focus on enzymatic approaches), and the shift in scientific efforts from developing inulin-type prebiotic compounds to the functionally superior levan-type. In addition, the chapter provides a detailed discussion of the enzymes employed for prebiotic synthesis (i.e., levansucrases and levanases) in terms of reaction selectivity, product spectrum, and substrate specificity. In accordance with this, a novel bi-enzymatic approach for the synthesis of controlled-size prebiotic FOS (developed in our laboratory) is explained. The chapter concludes with an examination of the analytical techniques for quantitative and qualitative FOS analysis.

Chapter III and VI are presented in the form of manuscripts, which will be submitted for publication. The corresponding connecting statement provides the framework for the progression of the present work. Specifically, Chapter III describes our efforts in discovering new candidate levanases and subsequent screening of such on the basis of levan hydrolysis, substrate specificity and end-product profile. In Chapter IV, the top candidate levanases (determined in Chapter III) are further characterized according to their catalytic and reaction parameters. This is followed by the end-product investigation of one-step and step-wise bi-enzymatic systems employing the top two levanases and two newly discovered levansucrases bearing high levan-producing capacity. The final chapter provides an overall summary of the experimental outcomes of the present work with foresight into future research extensions.

The present author was responsible for the experimental work and the preparation of the first draft of the manuscripts for publication and dissertation;

Dr. Salwa Karboune, the M.Sc. student's supervisor, guided the entire research framework and reviewed the aforementioned manuscripts prior to their submission;

Dr. Veronique Berardinis, Jean-Louis Petit and Andrea Hill, co-authors of Chapter III, were integral to the discovery and screening of the levanases investigated in the present work. Dr.

Berardinis and Jean-Louis are credited with the bioinformatics-based query for new levanase candidates and genetic analyses of these post-purification.

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NOMENCLATURE/LIST OF ABBREVIATIONS

BLAST: Basic local alignment search tool

DNS: 3,5-Dinitrosalicyclic acid

DP: Degree of polymerization

EC number: Enzyme Classification number

FOS: Fructooligosaccharide

Fru: Fructose

GF_4: 1^F -fructofuranosylnystose

Glc: Glucose

HMW: High-molecular weight

HPAEC-PAD: High-performance anionic exchange chromatography with pulsed amperometric detection

k_{catapp}: Apparent turnover number

LMW: Low-molecular weight

MW: Molecular weight

n: Hill coefficient/Hill constant (indicating the degree of cooperativity)

PIPES: piperazine-N,N′-bis(2-ethanesulfonic acid)

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

SE-HPLC: Size-exlcusion high-performance liquid chromatography

Suc: Sucrose

CHAPTER I:

GENERAL INTRODUCTION

The human alimentary tract is host to a highly diverse and evolved microbial community that is integral to overall health (including metabolism, physiology, nutrition and immune function) (Guinane and Cotter, 2013; Marshall, 2008). Recently, an increasing body of literature has been directing attention to this hidden metabolic 'organ' as an emerging culprit of chronic metabolic syndromes (i.e., type 2 diabetes mellitus and obesity). In particular, disruption of this microbiota has been implicated in the development of chronic gastrointestinal conditions, which currently rank among Health Canada's top five health concerns requiring intervention (Guinane and Cotter, 2013; Health Canada, 2017). It has been reported that annually, more than 20 million Canadians suffer from gastrointestinal disorders, with cases of colorectal cancer, Crohn's disease, and irritable bowel syndrome becoming increasingly prevalent (Canadian Digestive Health Foundation, 2017). Taking this into consideration along with the increasing costs of health care, functional ingredients and/or nutraceuticals targeting the prevention of the aforementioned diseases are becoming increasingly necessary. This highlights the demand for investigation into an emerging class of prebiotic carbohydrates- non-digestible oligosaccharides (NDOs), specifically of the fructooligosaccharide type, which not only confer numerous systemic health benefits, but also promote intestinal health by selectively stimulating the proliferation of beneficial colonic bacteria at the expense of pathogenic species (Marshall, 2008).

The functionality of NDOs, specifically their health benefits, are dependent on their chemical structures (i.e., the type(s) of hexose moieties present, the extent of polymerization, the glycosidic linkages and the type(s) of substituents (Sako, 1999). Recently, insight has been gained into the relationship between the structure of NDOs and their health functions, which has contributed to the development of efficient biocatalytic approaches for the synthesis of novel NDO compounds possible (Manning and Gibson, 2004). Previously, levan-type fructooligosaccharides (FOSs) were seldom investigated, in part owing to limited availability; however, they are receiving increased interest due to their ability to modulate colonic microflora such that the growth of beneficial populations is favored, thereby stimulating intestinal health. This has been supported by reports of higher prebiotic activity from β-(2,6)-FOSs and neo-FOSs as compared with their β-(2,1)-counterparts (Iizuka and Ogyra, 2000; Semjonovs *et al*., 2004). The only commercially available prebiotic preparations, β-(2,1)-/inulin- type FOSs, are hampered by numerous deficits, a key one being their reputably low molecular weight, and, inevitably, the rapid rate of fermentation during their transit through the digestive system, which consequently prevents them from reaching the

distal colon (where the vast proportion of chronic gut disorders originate) (Slavin, 2013). Increasing resistance to gastric digestion and improving the colonic persistence of FOSs by controlling their molecular size and by producing ones with novel chemical structures are the pillars for the development of "second generation" FOSs with enhanced prebiotic activity.

In the context of commercial applications, FOSs can be obtained by extraction from natural sources, by chemical synthesis, or by enzymatic synthesis, with the latter being the most favorable method when efficiency and quantity of yield are weighed. Generally, it is only possible to extract low quantities of FOSs from natural sources (Sangheetha *et al*., 2005), and the presence of contaminants, utilization of toxic reagents, and low selectivity associated with chemical synthesis of these compounds makes it laborious and unsustainable (Palcic, 1999; Warrand and Janssen, 2007). Enzymatic approaches are a more feasible alternative, as they involve the bioconversion of widely abundant starting materials (i.e., simple sugars) to prebiotic FOSs by the transfructosylation action of fructosyltransferases, or alternatively, by the controlled hydrolytic activity of fructosyl hydrolases on polysaccharides. In this context, the production of FOSs by fructosyltranferases provides the advantage of high regio- and stereo-selectivity and high substrate specificity (Plou *et al*., 2007), which are integral to the stereo-specific construction of the selected glycosidic linkage type (Monchois *et al.*, 1999). Classified in this family of enzymes are levansucrases (EC 2.4.1.10), which directly employ 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 synthesis of novel FOS structures. Additionally, levanases (belonging to the class of fructanhydrolases), characteristically hydrolyze β-(2,6)-linked fructans consisting of more than 3 fructose units to fructose or various levanoligosaccharides (Lim *et al.*, 1998; Mardo *et al.*, 2017). They can further be categorized according to two types: 1) exo-levanase (EC 3.2.1.64), and 2) endo-levanase (EC 3.2.1.65). Fructose is essentially the only product of levan hydrolysis by exo-levanases, while endo-levanases degrade levan into various fructooligosaccharides and oligolevans as the final hydrolysis products (Miasnikov, 1997). Endo-levanases have a 5-bladed β-propeller fold, and they hydrolyze glycosidic substrates by employing a proton donor (i.e., glutamate), and a nucleophile (predominantly aspartate) in their mechanism of catalysis (Mardo *et al*., 2017). Typically, in addition to levan, these enzymes are also capable of hydrolyzing sucrose and inulin (Miasnikov, 1997). The increased awareness of fructooligosaccharide-producing levan hydrolases can be mainly attributed to the discovery of the superior prebiotic properties these

compounds exhibit. Detailed study of this class of enzymes is an intriguing challenge as the majority of levans characterized to date are highly branched and heterogeneous in terms of molecular weight, and the structural elements responsible for substrate binding and specificity have not yet been elucidated.

It has previously been demonstrated by Tian and Karboune (2012) that levansucrase from *Bacillus amyloliquefaciens* acts synergistically with (commercially available) endo-inulinase in the production of FOSs. Investigated in one-step and two-step bi-enzymatic systems, the levansucrase catalyzed the synthesis of levan and various oligolevans from sucrose as the starting material, while the endo-inulinase (introduced simultaneously with levansucrase in the one-step system, or following levansucrase in the two-step system) hydrolyzed these products to FOSs and oligolevans varying in degree of polymerization. The present work examined the end-products from the pairing of newly discovered levansucrases and levanases under the same premises.

Data-mining of bacterial and archaeal genomes for the discovery of novel levanases and the subsequent bioinformatics-based phylogenetic analyses of these candidates advanced the current scope of knowledge regarding levanases, and served as the foundation of the present work. Specifically, the research objectives were to:

1. Screen candidate levanases obtained by genome-mining according to level of activity on low- and high-molecular weight levan, as well as substrate specificity (determined by the extent of hydrolysis of levan versus inulin)
2. Characterize the top 10 candidate levanases with respect to their individual end-product profiles, kinetics parameters (i.e., v_{max}, k_m, k_{cat}), optimal reaction conditions (i.e., pH and temperature) and thermal stabilities for the determination of the ideal bio-catalysts for FOS production
3. Develop bi-enzymatic systems comprising high levan-producing levansucrases and endo-hydrolytically active levanases (as an extension to the previous study performed in our laboratory) for the production of β-(2,6)-FOSs with enhanced prebiotic potential.

CHAPTER II:
LITERATURE REVIEW

2.1 Introduction

The human alimentary tract harbors a diverse and complex microbial community along its entire length which has recently become subject to extensive characterization owing to its significant influences on physiology, metabolism, nutrition status, and immune function (Marshall 2008). In particular, disruption of this microbiota has been implicated in the development of chronic gastrointestinal conditions, which currently rank among Health Canada's top five health concerns requiring intervention (Guinane and Cotter 2013); Health Canada, 2017). It has been reported that annually, more than 20 million Canadians suffer from gastrointestinal disorders, with cases of colorectal cancer, Crohn's disease, and irritable bowel syndrome becoming increasingly prevalent (Canadian Digestive Health Foundation, 2017). Coupled with the increasing costs of health care, functional ingredients and nutraceuticals targeting prevention of the aforementioned diseases are becoming increasingly necessary. Among these are an emerging class of prebiotic carbohydrates- non-digestible oligosaccharides, which not only confer numerous systemic health benefits, but also promote intestinal health by selectively stimulating the proliferation of beneficial colonic bacteria at the expense of pathogenic species (Marshall, 2008).

2.2 The Gut Microbiome

The human alimentary tract is host to a diverse microbial community that is a crucial determinant of overall health (including metabolism, physiology, nutrition and immune function), and hence frequently referred to as a hidden metabolic 'organ' (Guinane and Cotter, 2013; Marshall, 2008). An increasing body of literature is directing attention to an emerging culprit of chronic metabolic syndromes (i.e., type 2 diabetes mellitus and obesity): the human gut microbiota. The encompassed microorganisms and thereby their bacterial genomes (microbiomes) are becoming more widely regarded as integral pathogenic factors in various diseases ranging from gastrointestinal conditions (e.g., inflammatory bowel syndrome) to obesity (Vrieze *et al.*, 2010). Compounding evidence suggests that the stimulated growth of beneficial bacteria can reduce the risk of disease through pathogen inhibition and the production of benign and favorable metabolites (Kolida and Gibson, 2007). Until recently, insight into the composition and functionality of the human gut microbiota has been limited, owing to the lack of techniques and technology for exploration of such. Despite the fact that a major proportion of the dominant (anaerobic) microorganisms in the gastrointestinal tract still remains impossible to culture, the development of

16S ribosomal RNA gene-based approaches has allowed for the ongoing identification and classification of bacteria in this environment (Vrieze *et al.*, 2010). The large intestine is one of the most diversely colonized and metabolically active organs in the human body, with microbial populations comprising 10^{11}-10^{12} cfu/g of contents. This environment promotes bacterial growth due to its slow transit time, readily available nutrients, and favorable pH (Slavin, 2013). Specific regions of the gut are characterized by different population numbers and species distributions of these microorganisms, of which bacteria are the most demographically dominant and diverse (Marshall, 2008). In fact, the microbiome exceeds the human genome by more than 100 times, and comprises a community of at least 10^{14} bacteria which can be classified into 500-1000 different species according to culture-based data (Vrieze *et al*., 2010; Kolida and Gibson, 2007). Under these premises, Vrieze *et al.* (2010) has referred to the human intestinal microbiota as an 'exteriorized organ that contributes to overall metabolism and plays a role in converting food into nutrients and energy.' On a quantitative basis, 10-20 genera occur prevalently in the colon, including *Bacteroides*, *Lactobacillus*, *Clostridium*, *Fusobacterium*, *Bifidobacterium*, *Eubacterium*, *Peptococcus*, *Peptostreptococcus*, *Escherichia* and *Veillonella* (Kolida and Gibson, 2007). In adults, 3 bacterial divisions, Firmicutes (Gram-positive), Bacteroidetes (Gram-negative) and Actinobacteria (Gram-positive) dominate the gut microbiota. Firmicutes is the broadest bacterial phylum and contains over 200 genera, including *Lactobacillus*, *Mycoplasma*, *Bacillus* and *Clostridium*. Bacteroidetes, comprising approximately 20 genera, and Actinobacteria are also prevalently found in the human intestinal tract; however, the latter are frequently neglected by RNA gene sequencing, and thus can only be detected by fluorescent *in situ* hybridization (FISH) (Vrieze *et al.*. 2010).

Past the mouth, microbial colonization is markedly determined, partially by luminal pH, and by the progressively slower transit of food materials towards the colon. As compared with the movement of digesta through the stomach and small intestine, typical colonic transit time is far less rapid, allowing for the establishment of a complex and relatively stable bacterial community in the large intestine. In addition, the near-neutral pH and the relatively low absorptive state of the colon encourages extensive microbial colonization and proliferation (Marshall, 2008). The human large intestine comprises the caecum, ascending colon, transverse colon, descending colon, sigmoid colon, and rectum. Resident microflora allow the colon to conduct complex hydrolytic-digestive functions, which involves the breakdown of dietary components, principally complex

carbohydrates as well as select proteins that are not hydrolyzed nor absorbed in the upper digestive tract. As various dietary residues pass from the proximal colon to the transverse and distal bowel, carbohydrate availability is diminished (Marshall, 2008). The gut microbiome co-evolves with its host, and therefore alterations to this population in terms of species demographics can result in substantial consequences, either beneficial or detrimental (Guinane and Cotter, 2013; Roberfroid, 2014). The intestinal microbiota of healthy individuals confers numerous health benefits pertaining to defense mechanisms against pathogens, nutrition status, host metabolism and immune modulation (Guinane and Cotter, 2013; He and Shi, 2017). Culture-based analysis has historically demonstrated similarities in the bacterial species present in the guts of healthy adults; however, advancements in technologies which facilitate culture-independent examination of the gut microbiota have led to findings that there is significant inter-individual microbial diversity, with minimal phylogenetic overlap between hosts (Guinane and Cotter, 2013). Fetuses are sterile *in utero*; however, during birth and rapidly thereafter, bacteria from the mother and the surrounding environment colonize the infant's gut. Post-inoculation, the microbiota changes rapidly in composition, presumably due to dietary influences. In the first year of life the infant intestinal tract transitions from sterility to extremely dense colonization with a mixture of microorganisms that is generally parallel to that present in the adult intestines, and at the age of 4 years, the gut microbiota in the host individual is considered to be fully mature. As reported by Vrieze *et al.* (2010), the final composition of the human gut microbiota can be attributed to host genotype, colonization history, the physiology of the host, as well as an array of environmental factors. Several studies have also demonstrated that the genetic makeup of the individual is an integral variable contributing to the ultimate composition of the core gut microbiota. The total bacterial count in the gastric contents of an adult is typically below 10^3/g, while numbers in the small intestine range from approximately 10^4/ml at the site of the jejunum to 10^7/ml at the terminal ileum (Kolida and Gibson, 2007). The significantly lower microbial content of the stomach can be attributed to its low pH, as well as the rapid transit times in this region of the gastrointestinal tract. Further, bile/pancreatic secretions into the small intestine maintains relatively low microbial populations. In contrast, the colon is inarguably the most densely colonized portion of the gastrointestinal tract, with numbers typically reaching 10^{12}/g of contents. This environment is favorable for bacterial growth due to a significantly slower transit time, readily accessible nutrients and higher pH (Kolida and Gibson, 2007).

According to recent advancements in technologies for the quantification of the human gut microbiome, while the general composition of the human intestinal microbiota is similar among most healthy individuals, with more than 90% of the cells belonging to the Firmicutes or Bacteroidetes phyla, the species composition is highly personalized (Sonnenburg et al., 2010). Generally, bacteria whose metabolism is almost exclusively saccaharolytic (i.e., minimally proteolytic) can be considered potentially beneficial, as attributed with lactobacilli and bifidobacteria (Slavin, 2013). In synergism with the gut immune system, colonic and mucosal microflora significantly contribute to the establishment of the barrier that prevents pathogenic bacteria from invading the gastrointestinal tract. Numerous factors determine the diversity of and relative proportions of different species encompassed by the gut microflora, including changes in the host's physiological conditions (e.g., age, stress, health status), dietary composition, and environmental circumstances (e.g., antibiotic therapy) (He and Shi, 2017; Slavin, 2013). Recent recognition of the health-promoting properties of select gut bacteria has encouraged dietary-based modulation of the human intestinal microflora towards a more beneficial composition and metabolism, which can be accomplished with fermentable fibres (prebiotics). In this context, stimulation in the growth and/or activity of beneficial indigenous bacteria (i.e., lactobacilli and bifidobacteria) is expected to be observed, in addition to improvement in gut barrier function and host immunity, reduction in subpopulations of potentially pathogenic bacteria (e.g., clostridia), and enhanced short-chain fatty acid (SCFA) production (Gibson and Roberfroid, 2007; Slavin, 2013).

2.2.1 Colonic Microflora and Fermentation

Through the activities of the resident microflora, the status of the human gut is integral to host nutrition and well-being. This 'organ' is among the most active in the body, owing to the highly profuse and diverse colonic microflora (Kolida and Gibson, 2007). Bacteria of the human gut feed primarily on complex carbohydrates (i.e., food fibre, resistant starch, oligosaccharides, etc.) that are resistant to degradation by gastric acid and digestive enzymes of the host (Mardo et al., 2017). The 2 main types of fermentation carried out in the colon are saccharolytic and proteolytic. However, saccharolytic fermentation is more advantageous to the host due to the range of metabolic end-products generated (Kolida and Gibson, 2007). Numerous complex plant polysaccharides in the human diet are resistant to host-mediated degradation, either due to

insolubility or lack of human-encoded hydrolytic enzymes (Sonnenburg *et al*., 2010). These carbohydrates are not absorbed in the upper intestinal tract and serve as a major source of carbon and energy for the distal gut microbial community (Sonnenburg *et al.*, 2010). A crucial mechanism of action for dietary fibre and prebiotics is fermentation in the colon and alterations in gut microflora (Slavin, 2013). These products include SCFAs (predominantly butyrate acetate and propionate) and other metabolites of microbial origin confer an array of beneficial functions for the host, including the production of vitamins, modulation of the immune system, enhanced digestion and absorption, inhibition of harmful species, as well as the removal of carcinogens and other toxins (Kolida and Gibson, 2007). In the colon, carbohydrates are fermented by resident microflora to short chain fatty acids (SCFAs, predominantly acetate, propionate, and butyrate), as well as other types of metabolites including the electron sink products (lactate, pyruvate, ethanol, and succinate) and gases (H_2, CO_2, CH_4 and H_2S). SCFAs are rapidly absorbed by the colonic mucosa and supply the energy requirements of the host (Marshall, 2008).

The large intestine can harbor pathogens either belonging to the resident flora, or exist as transient members. Attachment and subsequent overgrowth of these undesirable microorganisms generally results in acute diarrheal infections, or can be manifested in the form of chronic intestinal diseases, including inflammatory bowel diseases (ulcerative colitis and Crohn's disease) and colon cancer. To varying extents, microflora composition and activities, and thereby the composition of the human diet since it provides the major source for their growth, have been implicated in the incidence of these disorders. In response to this, the concept of probiotics was generated to influence the gut microbiota in a beneficial manner. While the definition is continuously evolving, the consensus designates probiotics as 'non-pathogenic, live microbial, mono- or mixed-culture preparations, which, when applied to humans or animals in sufficient doses, beneficially affect the host by improving the intestinal microbial balance and its properties.' The presence of prebiotic carbohydrates can substantially enhance their rate of survival, especially if these compounds have been evidenced in the selection for useful species of *Bifidobacterium* and *Lactobacillus*. Mixtures of probiotics and prebiotics, referred to as synbiotics, have the capacity to improve therapeutic potential in the gastrointestinal tract (Marshall, 2008).

Rapid accumulation of new data on the gut microbiome and its plethora of functions is fueling the discovery of prebiotics for other potentially beneficial commensal bacterial populations such

as *Faecalibacterium* and *Bacteroides* which are abundantly present in the intestinal tract of healthy adults (Mardo *et al*., 2017). The bacteroidetes bacteria occurring in the human gut are classified under the order *Bacteroidales*, which has 3 dominant genera: 1) *Bacteroides*; 2) *Parabacteroides*; and 3) *Prevotella*. Individual strains and species of *Bacteroidales* have been found to be highly abundant in the gut, reaching populations of 10^9-10^{10} CFU/g of feces. The main reasoning for the *Bacteroides* genus to be of interest is its repertoire of polysaccharide-degrading enzymes which act to consume nutrients available in the colon. For instance, bacteria belonging to this genus are equipped to degrade resistant starch, pectin, galactomannan, glucomannan, arabinogalactan, alignate, laminarin, xylan, β-glucan, rhamnogalactan and cellulose (Mardo *et al*., 2017). One abundant species of *Bacteroides* in the gut is *B. thetaiotamicron*, which is considered to be either a commensal or a symbiont, and is gaining increased awareness in the scientific community owing to its ability to grow on levan, as reported by Sonnenburg et al. In this context, the author proved that the endo-levanase (BT1760; BT_1760) was indispensable to the microorganism, for which no homologue is found in the fructan utilization loci of other tested species of *Bacteroides* (i.e., *B. caccae*, *B. ovatus*, *B. fragilis*, *B. vulgatus* and *B. uniformis*) *(*Mardo *et al.*, 2017).

2.3 Probiotics and Prebiotics in Human Health and Nutrition

2.3.1 *Probiotics*

Due to the growing body of evidence establishing the crucial role of the gut microbiota in human gastrointestinal health and disease, extensive research efforts have been undertaken to modulate this community of bacteria as a therapeutic strategy to mitigate chronic disease. Approaches include the administration of prebiotics, supplementation with probiotics and the reconstitution of select bacterial populations via fecal transplantation (Guinane and Cotter, 2013). The modulation of activities directed towards improving gut microbial function and composition, and the associated health benefits of such has historically been primarily driven by the concept of probiotics. Bifidobacteria and lactobacilli are the most common ingredients of live microbial feed preparations as they are purported to demonstrate broad anti-pathogenic capabilities and are responsible for ‘colonization resistance’ in the colon (Kolida and Gibson, 2007). A stable colonic microflora represents a vital barrier against pathogens because it defends the intestinal epithelium against harmful invasion. In addition, the metabolites derived from probiotics, such as short-chain fatty acids (SCFAs), stimulate immunity and inhibit the growth of *Escherichia coli* and

Clostridium perfringens (Gibson et al., 2017). Gastrointestinal microorganisms may subsist in four defined micro-habitats: the epithelial surface, the crypts of the ileum, the cecum (colon), as well as the epithelial mucus layer and the lumen. However, it should be noted that the growth of bifidobacteria and eubacteria is most desirable in the cecum and in the ileum owing to elevated viable count and the complexity of the microflora. The efficacy of probiotics is dependent not only on their ability to resist gastric acids and bile, but also their capacity to permanently adhere to, colonize, and perform their metabolic activities in the colon (Rastall and Gibson, 2015). The application of oral probiotic cultures for the restoration of gut microbiota has contributed to the treatment of intestinal disorders such as ulcerative colitis and obesity; however, oral probiotic doses do not provide the necessary microbial population to alter the bacterial demographics of the colon (Guinane and Cotter, 2013). It is suspected that the bacteria present in probiotic supplements exert their influence through indirect pathways, such as via the production of anti-microbial compounds or by modulation of the immune system (Guinane and Cotter, 2013).

2.3.2 *Synbiotics*

Synbiotics consist of a combination of both probiotics and prebiotics (Rastall and Gibson, 2015) and have exhibited more efficacy in increasing the population of colonic bifidobacteria than probiotics administered alone. 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 an increase in the bifidobacteria population was observed in the ascending, transverse and descending colons (Gmeiner *et al*., 2000). Further, 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 fructooligosaccharides triggered high growth of these strains, while poor growth resulted from inulin (Bielecka *et al*., 2002). Previously, it was reported that inulin promotes the highest increase in the overall bacterial intestinal population, but the lowest count of lactobacilli and bifidobacteria (Rycroft *et al.*, 2001). Interestingly, the prebiotic preparation was 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. Compared 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).

2.3.3 *Prebiotics*

Prebiotics are gaining increased awareness owing to their ability to promote the proliferation of beneficial commensals, and hence the potential to improve gastrointestinal health (Guinane and Cotter, 2013). They are regarded as a more practical approach for manipulating the microflora of the human colon than probiotics because they directly target indigenous bacteria present in the digestive tract. The prebiotic approach advocates the targeting of selected indigenous bacteria though nonviable food ingredients. Gibson and Roberfroid were the first to introduce the prebiotic concept, defined as a 'selectively fermented ingredient that allows specific changes, both in the composition and/or activity in the gastrointestinal tract that confers benefits to host well-being and health' specifically at the site of the colon (Rastall and Gibson, 2015). Prebiotics are therefore non-digestible carbohydrates that are administered as a more tailored approach to modulating microbial balance in the intestinal tract (Kolida and Gibson, 2007). Since the introduction of prebiotics, numerous food components, particularly oligosaccharides and polysaccharides (including dietary fibre), have been attributed with prebiotic activity without regard to the necessary criteria (Roberfroid, 2007). All prebiotics are dietary fibres by definition; however, not all fibre is prebiotic (Slavin, 2013). Certain poly- and oligosaccharides (e.g., galactooligosaccharides, inulin, and other β-(2,1)-linked (inulin-type) fructooligosaccharides) are well-established and widely available prebiotics with specific functions in promoting the growth of the approved probiotic gut bacteria-lactobacilli and bifidobacteria (Mardo *et al.*, 2017; Rastall and Gibson, 2015). These are compounds which fulfill the recently reviewed criteria for prebiotic classification: 1) resistance to host digestion and absorption, as well as adsorption processes; 2) fermentation by the microflora colonizing the gastrointestinal system; and 3) selective stimulation of the growth and/or activity of 1 or a limited number of favorable bacteria within the gastrointestinal tract (Kolida and Gibson, 2007). Among intestinal bacteria stimulated by prebiotics are *Bifidobacteria* and *Lactobacilli* species are considered to be the most important. In this context, beneficial effects to the host include protection from enteric infection, activation of intestinal function, cholesterol-lowering

activity (Delzenne & Kok, 2001), enhanced calcium absorption and bone mineralization (Franck, 2006), as well as a reduction of serum triacylglycerol and phospholipid concentrations (Roberfroid, 2000), which in turn promotes cardiovascular health. Prebiotics are capable of stimulating significant and specific shifts in the populations of bacterial groups in the gut ecosystem and direct carbon flux from carbohydrate substrates to metabolic end-products such as organic acids. Under these premises, accumulation of organic acids is purported to improve local and systemic health. For instance, acetate is employed for the generation of ATP in muscle tissue, propionate is implicated in the regulation of liver cholesterol synthesis, and butyrate is a crucial source of energy for colonocyte function (Rastall and Gibson, 2015). It can thus be deduced from these effects that saccharolytic fermentation by microbial populations in the gastrointestinal tract is beneficial for health. In obese women, the administration of prebiotics has been evidenced to increase levels of bifidobacteria by HitChip and qPCR analyses (Rastall and Gibson, 2015).

Studies have demonstrated that the administration of prebiotics contribute to the re-establishment of a balanced intestinal microflora among unhealthy subjects by selectively nurturing the proliferation of endogenous colonic *Bifidobacteria* and *Lactobacilli* (which are the dominant colonic microorganisms among healthy individuals). Lactobacilli and bifidobacteria are the predominant target genera for prebiotics. Changes in bifidobacteria are more frequently observed compared to lactobacilli, possibly owing to the higher proportion of resident bifidobacteria to lactobacilli in the human colon and the fact that they exhibit a preference for oligosaccharides (Slavin, 2013). Only select carbohydrate compounds can be considered prebiotics, which must possess the following characteristics: 1) resistance to gastric acidity, to hydrolysis by mammalian enzymes and to gastrointestinal absorption; 2) fermentation by intestinal microflora; and 3) selective stimulation of the growth and/or activity of specific intestinal bacteria which have established roles in health and well-being (Roberfroid, 2007). Resistance to the factors required for the first criterion does not necessarily denote that the prebiotic is indigestible; however, a sufficient amount of the compound should remain un-metabolized as it reaches the large intestine, where it can serve as a fermentation substrate (Rastall and Gibson, 2015; Roberfroid, 2007; Slavin, 2013). While each of the criteria a food component must possess in order to be classified as a prebiotic is equally important, the third is generally the most challenging to fulfill (Roberfroid, 2007). Merely reporting fermentation in pure cultures of single microbial strains or an increase in a limited number of bacterial genera in complex mixtures of bacteria (e.g.,

fecal slurries), whether in vitro or in vivo is not sufficient for claiming the effect of a prebiotic, as these results do not take population interactions into consideration (Roberfroid, 2007; Slavin, 2013). Anaerobic sampling of the host's feces followed by reliable and quantitative microbiological analysis of a diversity of bacterial genera (e.g., total anaerobes/aerobes, bacteroides, bifidobacteria, clostridia, enterobacteria, eubacteria and lactobacilli) are necessary in demonstrating the selective stimulation of growth and/or activity of intestinal bacteria that contribute to health and well-being (Roberfroid, 2007).

The consumption of foods high in prebiotics can be traced to prehistoric times. For instance, analysis of well-preserved coprolites suggests that dietary intake of inulin was ~135 g/day for the typical adult male hunter-forager (Rastall and Gibson, 2015; Slavin, 2013). Prebiotics occur naturally in foods such as leeks, asparagus, chicory, Jerusalem artichoke, garlic, onions, wheat, oats, and soybeans (Slavin, 2013). The consumption of these dietary fibres in typical US and European diets is estimated to be several grams per day. Calorically, non-digestible oligosaccharides are valued between 1 and 2 kcal/g (Slavin, 2013).

2.3.4 *Purported Health Benefits of Prebiotics*

To date, the health outcome data for prebiotic consumption is significantly more limited and controversial than for dietary fibre. Despite this, it has been suggested that consumption of prebiotics may:

- Reduce the prevalence and duration of infectious and antibiotic-associated diarrhea;
- Reduce the inflammation and symptoms observed with inflammatory bowel disease;
- Exert protective effects against colon cancer;
- Enhance the bioavailability and uptake of minerals (e.g., calcium, magnesium, and possibly iron);
- Reduce certain risk factors for cardiovascular disease; and
- Promote satiety and weight loss (thereby preventing obesity) (Slavin, 2013).

2.3.5 Established and Candidate Prebiotics

2.3.5.1 *Non-Digestible Oligosaccharides*

The IUB-IUPAC nomenclature classifies oligosaccharides as carbohydrates consisting of 3 to 10 monomers (Mussatto and Mancilha, 2007). These compounds are differentiated based on

their structure, composition, sequence and orientation of glycosidic bonds (Bailey, 1963). However, the physicochemical properties of oligosaccharides are dictated by their chemical structure, degree of polymerization and by the presence of monosaccharides or disaccharides (Roberfroid and Slavin, 2000; Mussatto and Mancilha, 2007). For instance, the viscosity produced by oligosaccharides increases with their molecular weight, resulting in their promising application as bulking agents. 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). Due to the presence of β-glycosidic bonds between monosaccharide units in select oligosaccharides, they are further defined as non-digestible oligosaccharides (NDOs). As a consequence of the β-configuration, the chemical composition of NDOs comprises fructose, galactose, glucose and/or xylose units (Cummings *et al*., 1997).

In contrast to simple carbohydrates, NDOs resist degradation by oral microorganisms, and are hence considered to be non-cariogenic (Mussatto and Mancilha, 2007). Since NDOs provide relatively mild sweetness and are relatively hypocaloric, they are also regarded safe and suitable sugar substitutes (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 necessary to provide beneficial effects. In addition, non-digestible oligosaccharides are considered to possess an array of useful characteristics such as the ability to promote the growth of beneficial bacteria in the intestinal tract, enhancing the immune system, as well as other physiological functions (Lim *et al.*,1998). Of the 12 types of NDOs currently classified, the most important ones that exhibit these effects are fructooligosaccharides, galactooligosacchardies, isomaltulose, lactulose, inulooligosaccharides and levanoligosaccharides. These compounds are all selectively hydrolyzed by *Bifidobacterium* spp. (Lim *et al.*, 1998). The concentration and release rate of SCFAs as a consequence of metabolism by these bacteria are dependent on the nature of NDOs. For instance, the most significant increases in butyrate production 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). 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.

2.3.5.2 *Fructooligosaccharides and Their Classification*

Fructooligosaccharides (FOSs) are fructans with degree of polymerization ≤10, and considered with inulin, constitute 2 of the most important ingredients employed in the formulation of functional foods, particularly those claiming prebiotic properties (Kolida and Gibson, 2007). These are not digested during transit through the gastrointestinal tract, but rather are fermented to SCFAs and lactic acid in the colon, which are subsequently absorbed by the host, resulting in an increase in available energy and a reduction in intestinal pH (Kilian *et al.*, 2002). Historically, commercial FOSs have either been obtained by enzymatic synthesis from sucrose, or by partial enzymatic hydrolysis of chicory inulin by endo-inulinases. Oligofructose differs from inulin in that it is highly soluble and possesses technological advantages reminiscent of sugar and glucose syrups (e.g., increased viscosity), resulting in improved mouthfeel properties. For these reasons, despite its moderate sweetness, oligofructose is employed as a sugar replacement (Franck, 2000). Other industrially relevant properties of this class of oligosaccharides include a lower freezing temperature imparted to foods, limited browning during heat processing, as well as a high moisture-retaining capacity that prevents excessive drying (Crittenden and Playne, 1996). FOSs which are derived from the plant storage polysaccharide inulin are already widely recognized as prebiotics (Mardo *et al*., 2014). The prebiotic effect of inulin-type FOSs, which contain predominantly β-(2,1)-linkages, have been extensively explored. In contrast, there are limited reports on levan-type FOSs, whose fructosyl residues are β-(2,6)-linked, are significantly less common in nature, and are not commercially produced. Despite this, several authors who have investigated the potential benefits of levan-type FOSs have reported that they are selectively fermented by bifidobacteria and demonstrate even more potent prebiotic effects than their β-(2,1)-linked counterparts (Mardo *et al*., 2014). In general, FOSs are more selectively fermented by lactobacilli and bifidobacteria than other NDOs (Olano-Martin *et al*., 2002), owing to the fact that these bacteria are equipped with a membrane-bound β-fructofuranosidase enzyme capable of hydrolyzing FOSs (Perrin *et al.*, 2001). Experimental data generated from both animal and human trials have confirmed that inulin-type FOSs increase the population of these beneficial bacteria in the intestinal tract (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). 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*, *B. breve* and *B. pseudocatenulatum* are capable of metabolizing β-(2,6)-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). A study conducted by (Kilian *et al*., 2002) reported the prebiotic effect of neo-kestose (a trisaccharide comprising fructose units linked to the glucosyl residue of sucrose) from static batch cultures inoculated with feces. It was shown that this oligosaccharide, produced from sucrose by the fungus *Xanthophyllomyces dendrorhous*, selectively increases *bifidobacteria* and *lactobacilli* communities in cultures containing potentially detrimental coliforms, clostridia and bacteroides, an effect, which, according to the authors, was more pronounced than that demonstrated by commercial preparations of prebiotic FOSs (Mardo, *et al*., 2014). The proposed beneficial properties of bifidobacteria stem from the fact that they produce vitamins (primarily of the B type), are effective stimulators of the immune response and contributes to the restoration of intestinal flora post-antibiotic therapy (Kilian *et al.*, 2002). Simultaneously, they inhibit the growth of detrimental bacteria such as *Escherichia coli* and *Clostridium perfringens* either through the secretion of an inhibitory substance or due to a decrease in intestinal pH following their metabolic processes, which creates an unfavorable environment for these bacteria to thrive in (Kilian *et al.*, 2002).

FOSs are an emerging class of NDOs. Chemically, they are fructan oligomers of 3 to 10 fructosyl residues attached by β-(2,1)- or β-(2,6)-glycosidic linkages and contain a terminal D-glucose group (Roberfroid, 1996). In general, FOSs belong to one of four major classifications: inulin-, levan-, mixed levan-, and neoseries type (Monsan, 2009). Inulin-type FOSs ($G_{1\text{-}2}F_{1\text{-}2}F_n$) (Figure 2.1) are composed of β-(2,1)-linked D-fructofuranosyl units with a D-glucose terminal head (Westhuizen, 2008). Commercially available inulin-type FOSs consist of 1-kestose (Glc-Fru2), nystose (Glc-Fru3) as well as fructofuranosylnystose (Glc-Fru4) (Plou *et al.*, 2007).

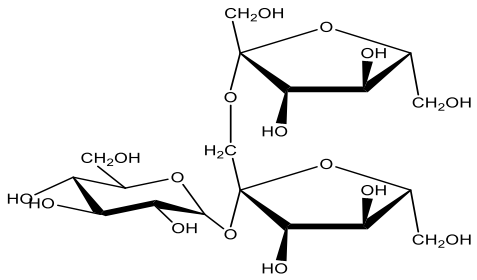


Figure 2.1: Inulin-type FOS, 1-ketose

Levan-type FOSs ($G_{1\text{-}2}F_{6\text{-}2}F_n$) are composed of β-(2,6)-linked D-fructofuranosyl units with a β-(2,1)-link to sucrose. The trisaccharide 6-kestose (Figure 2.2) is the smallest compound in this category (Westhuizen, 2008).

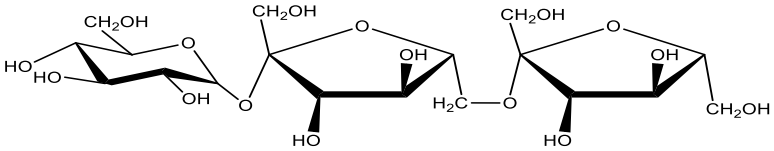


Figure 2.2: Levan trisaccharide, 6-ketose

Mixed levan-type FOSs contain both β-(2,1)- and β-(2,6)-linked D-fructofuranosyl units. In this subclass of FOSs, the tetrasaccharide bifurcose (Figure 2.3) is the smallest in which the fructosyl moiety of sucrose is β-(2,6)-linked to the glucose moiety of 1-kestose (Monsan and Ouarne, 2009).

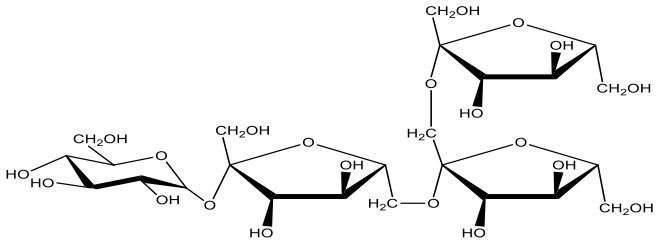


Figure 2.3: Mixed levan tetrasaccharide, bifurose

FOSs belonging to the inulin and levan neoseries classification contain fructose units bonded to the C1 and C6 carbons of glucose in sucrose. In the inulin neoseries (Figure 2.4), bonded to the C1 and C6 carbons are β-(2,1)-linked D-fructanfuranosyl units. The levan neoseries consists of β-(2,6)-linked D-fructanfuranosyl units attached to either side of a glucose unit from sucrose (Monsan and Ouarne, 2009; Westhuizen, 2008).

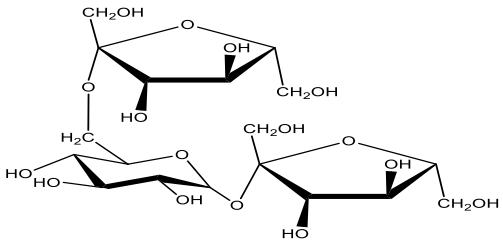


Figure 2.4: Neo-inulin-type FOS, trisaccharide neoketose

2.3.5.3 *Inulin*

Inulin is an umbrella term that encompasses all β-(2,1)-linked linear fructans. The terms oligofructose and/or inulin can be used, respectively, when it is necessary to identify oligomers versus polymers (Roberfroid, 2007). The linear chain of inulin is either an α-D- glucopyranosyl-[β-D-fructofuranosyl]$_{n-1}$-β-D-fructofuranoside ($G_{py}F_n$) or a β-D-fructopyranosyl-[β-D-fructofuranosyl]$_{n-1}$- β-D- fructofuranoside ($F_{py}F_n$) (Roberfroid, 2007). The fructosyl-glucose linkage is always β-(2↔1) as in sucrose, but the fructosyl-fructose linkages are β-(2→1) (Roberfroid, 2007). Chicory inulin is a heterogeneous mixture of oligo- and polymers in which the degree of polymerization (DP) varies from 2 to ~60 units with a DP_{av} = 12 (Roberfroid, 2007). Approximately 10% of the fructan chains in native chicory inulin have a DP ranging between 2 (F_2) and 5 (GF_4). The partial enzymatic hydrolysis of inulin using an endoinulinase (EC 3.2.1.7) produces oligofructose, which is a mixture of both $G_{py}F_n$ and $F_{py}F_n$ molecules, in which the DP varies from 2 to 7 with a DP_{av} = 4 (Roberfroid, 2007). Alternatively, oligofructose can be obtained via enzymatic synthesis (transfructosylation) employing the fungal enzyme β-fructosidase (EC 3.2.1.7) from *Aspergillus niger* (Roberfroid, 2007). The DP varies from 2 to 4 with DP_{av} = 3.6 among these synthetic compounds, and all oligomers are of the $G_{py}F_n$ type (Roberfroid, 2007).

2.4 Limitations of Current Commercial Prebiotic Preparations

FOSs are emerging as an important class of prebiotics primarily owing to their hypocaloric, non-cariogenic and bifidogenic functions (Dhake and Patil, 2007). As previously reported, the sweetening power of these fructans represents 40 to 60% of that of sucrose (Plou *et al*., 2007), thus making them suitable as sugar alternatives and/or enhancers of conventional sweeteners. However, commercially available prebiotics are hampered by one major obstacle: the development of non-digestible oligosaccharides equipped with targeted functions, and most importantly that are

specific towards beneficial bacteria at the species level (Manning and Gibson, 2004). The production of levan-type FOSs is becoming an increasingly attractive method of improving currently available prebiotics. Recently, neokestose, a β-(2,6)-linked FOS, has been demonstrated to exert higher bifidogenic effects than commercial FOSs (Marx *et al*., 2000; Kilian *et al*., 2002; Omori *et al*., 2010). However, to date, commercial prebiotic preparations 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 rapidly fermented by the saccharolytic activity of anaerobic bacteria in the proximal colon, leading to the formation of SCFAs that contribute to the host's welfare in this restricted length of the intestinal tract. 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 and 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 and 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.*, 2006) 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. In summary, the production of FOSs which are structurally well-defined, with higher selectivity and colonic persistence, are the driving rationale for the development of second generation FOSs with functionally-enhanced properties (Rastall and Maitin, 2002).

2.5 Levan

Levan is a homopolysaccharide comprising D-fructofuranosyl residues joined by β-(2,6)-linkages and exhibits differing degrees of β-(2,1)-branching depending on its origin (Byun, Lee, and Mah, 2014). Levan-type fructans are primarily synthesized by bacterial enzymes, but also occur in select plants. Examples include timothy grass (*Phleum pratense*) and orchard grass (*Dactylis glomerata*), linear β-(2→6)-linked fructans which are referred to as phleins or plant levans (Mardo *et al*., 2017). Mixed levans (graminans), characterized by the presence of both β-

(2→1) and β-(2→6) linkages, can be found in many *Poales* species (e.g., ryegrass), but also in agave and in common cereals such as wheat, rye and barley (Mardo *et al*., 2017). Bacterial levan is not currently widely produced, and only by few companies such as Montana Polysaccharides Corporation (USA). It serves as a functional ingredient in foods, beverages, medicine and nanotechnology. Typically, microbial levan is extracellularly generated from sucrose-based substrates by levansucrase, which catalyzes levan biosynthesis by transferring fructose (cleaved from sucrose or other fructose donor) to an acceptor molecule (either sucrose or oligo-/polymeric levan) (Byun, Lee, and Mah, 2014). Recently, levan has become the subject of increasing attention owing to its numerous health benefits, including the reduction of total body fat and cholesterol (Yamamoto *et al*., 1999), stimulatory effects on immunity (Xu *et al.*, 2006), and cancer-prevention properties (Yoo *et al*., 2004). Additionally, it has been reported that levan possesses prebiotic properties, as it has been evidenced to stimulate the growth of latic acid bacteria in animal models (Jang *et al.*, 2003). The physiological functions and applications of this fructan have been investigated and reviewed by various authors recently, including Mellet and Fernandez (2010), who reported levan to be low-calorie and non-cariogenic and hence suitable as an alternative sweetener. They also established the integral role of levan in human gastrointestinal health, attributed to its ability to stimulate the growth of favourable microflora at the expense of pathogenic/detrimental strains (Byun, Lee, and Mah, 2014). In their investigation of the potential anti-bacterial properties of levan, Byun, Lee, and Mah observed that low-molecular weight levan demonstrated the strongest *in vitro* inhibitory effect, as compared with high-molecular weight levan and difructose dianhydride. Although these authors were not able to elucidate the underlying mechanism by which levan compounds inhibit the growth of spoilage and pathogenic bacteria, they postulated that these effects may be the result of 2 different modes of action: 1) induction of osmotic stress and/or reduction in water activity, or 2) competitive interference with bacterial absorption of essential nutrients (Byun, Lee, and Mah, 2014).

As previously mentioned, β-linked fructans are generally considered to be resistant to gastric acid and human digestive enzymes, allowing them to reach the site of the distal colon intact and to serve as a selective food for resident microbiota. However, several publications have described moderate hydrolysis of β-linked fructans by gastric acid (Mardo *et al.*, 2017). Generally, the pH in the stomach of healthy adults ranges from 1.5-3.5, whereas this is level of acidity is lower in children under the age of 2 and in elderly populations. Mardo et al. assayed the resistance of levans

to hydrolysis by 0.01 M hydrochloric acid (pH 2.0) at 37°C (physiological temperature). In this context, inulin extracted from dahlia was studied as a β-(2,1)-linked reference fructan. The authors monitored the production of reducing sugars from fructans for 24 hours, and demonstrated that all studied fructans were acid-stable. In fact, after 2 hours of incubation in 0.01 M hydrochloric acid, less than 2% of reducing sugars was released from the polymers, and this value remained low up to 7 hours of incubation, after which time hydrolysis products could be visualized by thin layer chromatography. It was noted the dahlia inulin experienced the highest level of degradation, while levan from timothy grass exhibited the highest level of resistance, although the reason remains unclear. Interestingly, this assay revealed that levan synthesized by levansucrase Lsc3 using raffinose as a starting material withstood acid hydrolysis better than when levan was synthesized from sucrose (Mardo *et al.*, 2017).

Conclusions from previous studies suggest that presence of levan in the colon can exert protective and health-promoting effects on colonocytes. For instance, a recent *in vitro* assay performed by Bondarenko *et al*. demonstrated that Lsc3-produced levan had no harmful effect on metabolic activity and integrity of the Caco-2 cells (Mardo *et al.*, 2017). Concordant to these results, levan from a halophilic bacterium *Halomonas* sp. did not affect proliferation of osteoblasts and murine macrophages *in vitro* and protected a marine crustacean *Artemia salina* from chemical-induced toxicity (Mardo *et al.*, 2017). In addition to the protection of colonocytes, levan and levan-type FOS are potentially prebiotic. Numerous strains of probiotic bacteria, including but not limited to bifidobacteria and lactobacilli, can grow on either levan or levan-type FOS (Mardo *et al.*, 2017). Levan utilization by a gut commensal *Bacteroides thetaiotaomicron* was first reported by Sonnenburg *et al* (2010). Adamberg *et al*. (2015) showed that *B*. *thetaiotaomicron* ferments Lsc3-produced levan to SCFA (mostly acetic, D-lactic, propionic and succinic acids were detected). If growth of fecal consortia on Lsc3-produced levan was addressed by the same group, acetic, lactic, butyric, propionic, succinic acids and carbon dioxide were detected as the main excreted metabolites. Association between the growth of levan-degrading (e.g. *Bacteroides*) and butyric acid-producing (e.g. *Faecalibacterium*) bacteria was detected in the fecal consortia suggesting feeding of butyrate-producing bacteria on levan-derived metabolites. Butyrate production in the gut is important–it is the main energy source for colonocytes, inhibits proliferation of colon cancer cells and induces their apoptosis. Cross- feeding hypothesis by Adamberg *et al*. agrees with data by Rakoff-Nahoum *et al*. who showed that when grown on levan,

B. *thetaiotaomicron* releases levan breakdown products (FOS and fructose) into the medium to be consumed by other gut symbionts. In a recent study conducted by (Mardo *et al.*, 2017), it was demonstrated that *Bacteroides thetaiotamicron*, an abundant commensal of the human intestinal tract, is capable of degrading various types of levan, specifically those originating from microbial and plant sources. The authors observed that *B. thetaiotamicron*, with a cell surface bound endo-levanase, BT1760, grew on levan produced by *Zymomonas mobilis* and produced FOSs in the process. In addition, this bacterium exhibited activity on levans synthesized by levansucrases such as Lsc3 of *Pseudomonas syringae* pv. tomato, its mutant Asp300Asn, as well as those from *Z. mobilis*, *Erwinia herbicola*, *Halomonas smyrnensis*. Notably, *B. thetaiotamicron* also effectively hydrolyzed levan isolated from timothy grass, which led this report to become the first one describing the use of a plant levan as a suitable substrate for an endo-fructanase of a human gut bacterium (Mardo *et al.*, 2017). When the end-products of BT1760 were examined in greater detail by these authors, it was shown that this endo-levanase degraded levans to FOSs with degree of polymerization ranging from 2-13. Further, it was observed that low molecular weight (<60 kDa) levans (i.e., those from timothy grass and synthesized from sucrose by Lsc4Asp300Asn) were degraded much more rapidly than that produced from Lsc3 from raffinose. BT1760 performed optimally at physiological temperature (37°C) and under moderately acidic conditions (pH 5-6) which are typical of the gut lumen. Mardo *et al.* (2017) concluded that levans of both bacterial and plant origin can potentially serve as prebiotic fiber for *B. thetaiotamicron* and contribute to the synthesis of SCFAs by gut microbiota. Apart from the protection of colonocytes, levan and levan-type FOSs are potentially prebiotic as multiple strains of probiotic bacteria have exhibited growth from metabolizing these compounds, including bifidobacteria and lactobacilli (Mardo *et al.*, 2017). In addition, an association has been made between the growth of levan-degrading (e.g., *Bacteroides*) and butyric acid-producing (e.g., *Faecalibacterium*) bacteria in the human fecal consortia, suggesting the feeding of butyrate-producing bacteria on levan-derived metabolites. This is significant in the context of the human gut as butyrate is the main source of energy for colonocytes and has been implicated in preventing the proliferation of colon cancer cells, as well as inducing their apoptosis (Mardo *et al.*, 2017).

Since several gut *Bacteroides* species (e.g., *B. caccae*, *B. vulgatus*) can grow on levan-breakdown products, their proliferation should also be promoted by these substrates. Levan can also stimulate the growth of other gut bacteria, including *B. xylanisolvens* and *Butyrivibrio*

fibrisolvens, which Mardo et al. predicted to be levan-degraders. Recently, one strain of *B. xylanisolvens* isolated from the human gut has been classified as safe and confirmed not to possess any virulence potential. While *Bacteroides* species do not produce butyrate as metabolites, *Butyrivibrio* species do. These bacteria can be considered potential probiotics as they selectively ferment levan (Mardo *et al.*, 2017).

Levan is permitted as a functional food additive in Japan and South Korea whereas it is currently not commercially produced and applied in Europe (Mardo *et al.*, 2017). Levan-type FOS are not commercially manufactured, but rather produced at small scale for research using either acid-aided or enzymatic hydrolysis of bacterial levan (Mardo *et al*., 2017). Because there is an existing enzymatic process for the production of inulin-type FOSs, one involving the hydrolysis of levan to produce levan-type FOSs and oligolevans is feasible and necessary for the investigation of β-(2,6)-type prebiotics. In this context, there are numerous reports describing the synthesis of levans from sucrose by levansucrases available from a wide range of microbial sources. However, reports on endolevanases, which hydrolyze levan into FOSs and oligolevans, are scarce, owing to their limited availability. This demands exploration into the potential sources of levanases with high endo-hydrolytic activity. The focus of the present work will be on the production of levan-type FOSs and olioglevans by the application of a levansucrase-levanase bi-catalytic system. End-products obtained from the concerted actions of these enzymes are expected to demonstrate higher prebiotic potential than currently available prebiotic FOSs of the inulin type.

2.6 *Other Emerging Prebiotic Candidates*

Preliminary and promising data pertaining to the prebiotic potential of glucooligosaccharides, isomaltooligosaccharides, lactosucrose, polydextrose, soybean oligosaccharides, and xylooligosaccharides currently exist. However, the evidence for prebiotic status remains insufficient, preventing them from presently being classified as prebiotics (Roberfroid, 2007). Nevertheless, polydextrose consumption was observed to result in a dose-dependent decrease in bacteroides, as well as an increase in lactobacilli and bifidobacteria (Slavin, 2013). The prebiotic potential of several other compounds has also been investigated. However, evidence pointing toward any prebiotic effect is too sparse to justify a detailed review. These compounds include germinated barley foodstuffs, oligodextrans, gluconic acid, gentiooligosaccharides, pectic oligosaccharides, mannan oligosaccharides, lactose, glutamine, and hemicellulose-rich substrate,

resistant starch and its derivatives, oligosaccharides from melibiose, lactoferrin-derived peptide, and N-acetylchi- tooligosaccharides (Roberfroid, 2007). Consumption of wheat dextrin has been shown to increase lactobacilli and bifidobacteria, while reducing *Clostridium perfringens* (Slavin, 2013).

2.7 Methods of Synthesizing Prebiotics

2.7.1 *Extraction of Fructooligosaccharides from Natural Products*

FOSs may be extracted from flowering plants, which are prevalent 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, Amaryllidacea, 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). Although barley, wheat, oat and forage grasses contain up to 70% fructan on a dry weight basis (Fuchs, 1991), only trace quantities of FOSs can be obtained from these sources (Campbell *et al*., 1997) as they possess organs which interfere with extraction. In contrast, plant storage organs (i.e., bulbs, tubers and tuberous roots) in species within the Liliaceae, Amaryllidacea, and Compositae families are highly accessible for FOS extraction (Fuchs, 1991). In general, however, the yield of FOSs obtained from extraction methods is considered to be exceptionally low, and thus impractical for industrial applications (Sangeetha *et al*., 2005).

2.7.2 *Chemical Synthesis of FOSs*

Another method by which FOSs can be obtained is chemical synthesis. However, due to the presence of various functional groups and chiral centers in monosaccharides, sequential selective protection-deprotection steps are necessary to control the stereochemical and regiochemical specificity of the glycosidic bond formed. The chemical synthesis of FOSs is genrally a laborious multi-step endeavor, as it involves toxic reagents and does not comply with food safety conditions (Palcic, 1999). In addition, chemical hydrolysis of a polysaccharide to defined-size oligosaccharides is difficult to manipulate due to the formation of brown contaminants from the conventional heating procedure (Warrand and Janssen, 2007).

2.7.3 *Enzymatic Synthesis of FOSs*

In comparison to chemical synthesis, enzymatic approaches to the production of FOSs confer regiospecificity and stereospecificity to glycosidic linkages present in the end-products, and hence are more promising for the synthesis of commercial oligosaccharides with enhanced prebiotic potential (Plou *et al*., 2007). FOSs can be synthesized by β-fructofuranosidases (EC 3.2.1.26) or fructosyltransferases (EC 2.4.1), also referred to as fructansucrases (Roberfroid, 2008; Miasnikov, 1997). For instance, β- fructofuranosidases from the fungi *Aureobasidium pullulans*, *Aspergillus niger* and *A. oryzae* have previously been reported to produce short-chain-FOSs (Fernandez *et al*., 2004; Shin *et al*., 2004; Sangeetha *et al*., 2005). Additionally, levansucrase (EC 2.4.1.10), a fructansucrase (del Moral *et al*., 2008), can synthesize polymeric levan as well as short-chain FOSs (Bekers *et al*., 2002; Vigants *et al.*, 2003). Numerous authors have investigated the enzymatic production of FOSs from levansucrase, including Mardo *et al.* (2017), who recently reported FOSs with degree of polymerization (DP) of 3–8 by reacting a highly active levansucrase Lsc3 of *Pseudomonas syringae* pv. tomato with sucrose under conditions favoring FOS production. It was further observed that the FOS mixture synthesized by the levansucrase Lsc3 was heterogeneous, as it not only contained β-(2,6)-linked FOS, but also β-(2,1)-linked oligosaccharides (e.g., 1-kestose) (Mardo *et al*., 2017).

2.7.3.1 *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 as well as the capacity of the enzyme to bind to the acceptor with high specificity as compared to water (Plou *et al*., 2007). The synthesis 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 consistent elimination of the transfructosylation end-products through crystallization, selective adsorption to

carriers or coupling through another enzymatic reaction. Further, transfructosylation activity can be favored by employing high levels of a fructosyl acceptor (Cobucci-Ponzano *et al.*, 2003).

2.7.3.2 *FOS Synthesis by Fructansucrases*

Fructansucrases comprise inulosucrase (EC 2.4.1.9) and levansucrase (EC 2.4.1.10), and are fructosyl-transferring enzymes that employ sucrose for the synthesis of inulin (Tungland, 2003) and levan (Gross *et al.*, 1990), respectively. Levansucrases are responsible for both the β-(2,6)- and β-(2,1)-linkages present in the main fructan chain and branches of 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 their end-products, which is dependent on the proportion of transglycosylation to hydrolytic activity (Ozimek *et al*., 2006). Previous studies have mainly focused on levansucrases rather than on inulosucrases due to the broader range of acceptors, and hence wider range of expected end-products (Cote and Tao, 1990; Seibel *et al.*, 2005).

2.7.3.3 *Levansucrase-Catalyzed Synthesis of Prebiotic FOSs*

Levansucrases (EC 2.4.1.10) are extracellular bacterial enzymes belonging to the GH68 family of glycoside hydrolases. The employ the free energy of cleavage of non-activated sucrose to transfer the fructosyl group to a variety of acceptors, including monosaccharides (for which an exchange reaction occurs), oligosaccharides (producing FOSs), or a growing fructan chain (resulting in polymer synthesis) (Strube *et al*., 2011). Of these 4 possible reaction mechanisms (hydrolysis, exchange, transfructosylation and polymerization) (Li *et al.*, 2015); the transfructosylation and polymerization activity of levansucrases are most relevant in the context of prebiotic production. These bio-catalyzed reactions involve the cleavage of sucrose (or other fructose donor) and subsequent synthesis of β-(2,6)-linked oligo- and polyfructans, including high molecular weight levan. These enzymes are present in numerous plant-related bacteria such as *Pseudomonas syringae*, *Gluconobacter diazotrophicus*, *Zymomonas mobilis* and *Erwinia amylovora*, as well as in *Bacillus subtilis*, *B. megaterium* and several species of lactic acid bacteria including *Lactobacillus sanfranciscensis*, *L. Reuteri* and *Leuconostoc mesenteroides* (Mardo *et al*., 2014). Extensive investigation of levansucrases has unveiled the wide acceptor specificity of these biocatalysts, as they have exhibited the ability to transfer fructosyl residues from not only

sucrose, but also a variety of other disaccharides (other than lactose) to yield a range of hetero-oligosaccharides (Li *et al*., 2015). Certain monosaccharides can also serve as effective acceptors in levansucrase-catalyzed transfructosylation reactions. This is exemplified by *Bacillus subtilis* levansucrase, which displays even higher transfer efficiency toward D-galactose, D-xylose and D-fucose than sucrose and other disaccharides (Seibel *et al.*, 2006). Notably, *B. licheniformis* levansucrase exhibits high activity towards an unconventional pentose, L-arabinose (Lu *et al*., 2014), a low-calorie sweetener with promising beneficial properties (Krog-Mikkelsen *et al*., 2011).Typically, levansucrases from Gram-negative bacteria (e.g., *Gluconobacter diazotrophicus* and *Zymomonas mobilis*) produce primarily FOSs and low amounts of levan, while levansucrases from Gram- positive bacteria (e.g., *Bacillus subtilus*, *Bacillus amyloliquefaciens*, and *Microbacterium levaniformans*) synthesize predominantly high-molecular weight levan with a mass of up to 10^4 kDa (Tian, Inthanavong, and Karboune, 2011). The transfructosylation activity of levansucrase can be inhibited by high substrate concentration (i.e., sucrose) (Oseguera *et al*., 1996).

2.7.3.4 *Levanase-Catalyzed Synthesis of Prebiotic FOSs*

Levan-degrading enzymes, referred to as levanases (2,6-β-D-fructanohydrolase, EC 3.2.1.65), hydrolyze β-(2,6)-linked fructans consisting of more than 3 fructose units to fructose or various levanoligosaccharides (Lim *et al.*, 1998; Mardo *et al*., 2017). They are categorized in glycoside hydrolase (GH) family 32 with invertases (EC 3.2.1.26), endo-inulinases (EC 3.2.1.7), exo-inulinases (EC 3.2.1.80), and several other enzymes which exhibit homologous sequence motifs and topology (Mardo *et al.*, 2017). They can further be classified according to two types: 1) exo-levanase (EC 3.2.1.64), and 2) endo-levanase (EC 3.2.1.65). Exo-type levanases have been identified in *Pseudomonas* species (Avigad and Zelikson, 1963), *S*. *salivarius* KTA- 19 (Takahashi *et al*., 1983), *Streptomyces* species No. 7-3 (Murakami *et al*., 1990), *Streptomyces exfoliates* F3-2 (Yokota *et al*., 1993), and *Streptomyces* sp. K52 (Kang *et al*., 1998). Endo-type levanases have been identified in *Arthrobacter* species (Avigad and Bauer, 1966), *Bacillus* species (Miasnikov, 1997), and *G*. *diazotrophicus* SRT4 (Menedez *et al*., 2004). Unidentified-type levanases have been identified in *Actinomyces viscosus* strains ATCC15987 (Miller and Somers, 1978), and ATCC 19246 (Igarashi *et al.*, 1987), as well as *Rhodotorula* species (Chaudhary *et al*., 1996). In addition, levanases commonly occur in yeasts and filamentous fungi (Dahech *et al*., 2013). Fructose is

essentially the only product of levan hydrolysis by exo-levanases, while endo-levanases degrade levan into various fructooligosaccharides as the final hydrolysis products (Miasnikov, 1997). Endo-levanases have a 5-bladed β-propeller fold, and they hydrolyze glycosidic substrates by employing a proton donor (i.e., glutamate), and a nucleophile (predominantly aspartate) in their mechanism of catalysis (Mardo *et al.*, 2017).Typically, in addition to levan, these enzymes are also capable of hydrolyzing sucrose and inulin (Miasnikov, 1997). The increased awareness of fructooligosaccharide-producing levan hydrolases can be mainly attributed to the discovery of prebiotic properties these compounds possess. The detailed study of this enzyme is an intriguing challenge as the majority of levans are highly branched and heterogeneous in terms of molecular weight, and the structural elements responsible for substrate binding and specificity have not yet been elucidated.

While several studies have been performed on microbial levanases, specifically focused on the production of levanoligosaccharides, the end-products generated by these enzymes were generally observed to be heterogeneous, with various degrees of polymerization. Levan-derived oligosaccharides produced by levanases fall into 2 main groups: 1) DFA IV (di-D-fructose (2,6’):(2’,6)-dianhydrous); and 2) linear fructooligosaccharides (Lim et al. 1998). Exo-levanases hydrolyze inulin, raffinose and sucrose (Menedez *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 produced from these endo-fructanases (Murakami *et al.*, 1990). Exo-levanase from *Streptococcus salivarius* KTA-19 demonstrated an optimum pH and temperature range of 6.5 and 40-50 °C, respectively. The enzyme did not hydrolyze inulin, raffinose, sucrose, or melezitose, but exhibited sole specificity for levan. Fructose was the only by-product of the enzymatic reaction on levan (Takahashi *et al.*, 1983). Exo-levanase from *Streptomyces* species No. 7-3 hydrolyzed levan and released mainly levanbiose (80%), some levantriose (17%) and fructose (3%) (Murakami *et al*., 1990). *G*. *diazotrophicus* SRT4 exo-levanase could release fructose from sucrose, raffinose, inulin and levan, but not melezitose (Menedez *et al*., 2004). On the other hand, endo-inulinase from *Bacillus* species was not able to hydrolyze sucrose, levanbiose, and difructose anhydride IV DFA IV), but hydrolyzed levan and phlein (Miasnikov, 1997).

The mechanism of action of levanases and inulinases is analogous to that of other glycosyl hydrolases or glycosidases. The hydrolysis of the glycosidic bond occurs with the generation of two possible stereotypes: inversion or retention of the anomeric configuration. The reaction pathway of levanases has not been elucidated in detail; however, the catalytic residues involved in the hydrolysis of levan were potentially identified for a levanase from *Microbacterium laevaniformans*. Through multiple sequence alignments with other fructosylhydrolases and through the analysis of conserved residues, the catalytically active residues were identified as Asp86 and Glu2707/Cys271/Pro272 (Song *et al.*, 2002). Generally, levanase molecular weights range from 38-135 kDa (Murakami *et al*., 1992). All levanases have a low isoelectric point, ranging from 4.1 (Lim *et al*., 1998) to 4.8 (Kang *et al.,* 1998), and their optimum pH varies from 5.0 (Menedez *et al*., 2004) to 7.0 (Lim et al., 1998). Their optimum reaction temperatures were reported to be in the range of 30 °C (Menedez *et al*., 2004) to 60 °C (Yokota *et al*., 1993)**.**

Although levansucrase and levanase share a similar overall structure, they are catalytically different. There are appreciable variations even among levansucrases of different bacteria, as certain levansucrases, including the ones from *B*. *subtilis* (Beine *et al*., 2008), *L*. *reuteri* 121 (Ozimek, Kralj, van der Maarel, and Dijkhuizen, 2006), and *B*. *megaterium* (Homann, Biedendieck, Götze, Jahn, and Seibel, 2007), mainly produce long-chain levan, whereas others, such as the ones from *G*. *diazotrophicus* (Batista *et al*., 1999), *Zymomonas mobilis* (Doelle, Kirk, Crittenden, Toh, and Doelle, 1993), and *Lactobacillus sanfranciscensis* (Korakli, Pavlovic, Gänzle, and Vogel, 2003) synthesize primarily short-chain FOS. This difference in product spectrum is determined by whether the enzyme is able to catalyze the reaction with a processive or disproportionate (nonprocessive) mechanism. In a processive mechanism, the product of a catalytic step remains bound to the enzyme and is ready for the next catalytic step. Conversely, a disproportionate mechanism requires that the "intermediate" products dissociate from the enzyme after every step of catalysis. The difference in mechanism seems to be dictated by structural determinants (Kralj *et al*., 2008; Ozimek *et al.*, 2006) that may be located outside the core active site structure (Anwar *et al*., 2012); however, respective structural determinants remain poorly characterized.

Similarly, levanases from different bacterial sources exhibit differentiable levels and modes of activity. For example, *Streptomyces* sp. No. 7-3 (Murakami *et al*., 1990) and *Streptomyces exofoliaticus* F3-2 (Yokota, Kondo, Nakagawa, Kojima, and Tomita, 1993) produce levanases that

hydrolyzed levan to levanbiose as the main product by an exo- acting mechanism. In contrast, the levanase produced from *Bacillus* sp. No. 71 predominantly hydrolyzed levan to levanheptaose, but also produced other oligosaccharides with degrees of polymerization exceeding 7 in the early stage of the reaction, which suggests that the mode of action of this levanase is an endo-type (Murakami *et al.*, 1992). Miasnikov (1997) has previously screened for microbial sources of levan-degrading enzymes, and found that these types of microorganisms are abundant in soil. It was observed that the majority of the enzymes isolated from these sources hydrolyzed levan predominantly or solely to fructose. However, the *L7* levanase in *Bacillus subtilis* hydrolyzes levan to a mixture of polysaccharides with fructose being a minor end-product, and hence was cloned for further investigation. (Miasnikov, 1997) observed that the specificity of this levanase appeared to be limited to endo-type hydrolysis of levan, and that it had no activity towards sucrose even when a 100-fold higher enzyme concentration was used than the author's levanase assays. The end-products of levan hydrolysis by L7 levanase were a mixture of fructose and a series of fructooligosaccharides ranging in degree of polymerization, with the largest one being a 12-mer and levantriose being the fructooligosaccharide obtained in highest yield (estimated at approximately 24%). Low concentrations of heavy-metal ions completely inhibited the activity of this levanase, while calcium and magnesium ions, as well as chelating agents such as EDTA appeared to have no appreciable effects on activtiy. However, sucrose, at low concentrations, demonstrated a weak inhibitory effect.

2.7.4 Bi-Enzymatic Production of Prebiotic FOSs

To date, there has been limited investigation of the combined use of different biocatalysts for the production of oligosaccharides. High concentration/yield of FOSs (i.e., up to 90%) was obtained from the combined use of fructosyl-transferase from *A. pullulans* KFCC 10524 and glucose oxidase (EC 1.1.3.4) from *A. niger* (Yun and Song, 1993). The maximum conversion yield from sucrose to FOSs ranged from 55 to 60%, potentially owing to the inhibitory effect of unreacted sucrose and glucose on fructosyl-transferase activity (Jung *et al*., 1989). To release glucose inhibition, glucose isomerase and glucose oxidase were introduced into the bi-enzymatic system. Glucose isomerase, which converted glucose to fructose, was not very useful in releasing fructosyl-transferase inhibition, whereas glucose oxidase was very efficient in suppressing the glucose inhibition by complete removal of glucose and its conversion to gluconic acid (Yun and

Song, 1993). A later study on FOSs production using β-fructofuranosidase (from *A. japonicas* CCRC 93007) and commercial glucose oxidase (Gluzyme, Novo Nordisk) achieved more than 90% (w/w) yield on a dry weight basis (Sheu *et al*., 2001).

Most recently, Tian and Karboune (2013) investigated the combined use of levansucrase and endo-inulinase in one-step and two-step bi-enzymatic systems. At selected reaction times, aliquots of the reaction mixtures were withdrawn, and methanol was added at a ratio of 1:1 (v/v) followed by boiling for 5 min to inactivate the enzymes. Analysis of end-products was performed using HPAEC and high performance size exclusion chromatography (HPSEC). In the two-step system, the levansucrase-catalyzed transfructosylation reaction of sucrose was first conducted for 12 hours prior to the addition of endo-inulinase, ultimately resulting in an enzyme ratio of 1:1 (Tian and Karboune, 2012). The conversion of sucrose by *B. amyloliquefaciens* levansucrase during the first step of the bi-enzymatic system reached a yield of 50%. Subsequent addition of endo-inulinase resulted in an increase of the transfructosylation products (scFOSs and oligolevans) and in a rapid depletion of sucrose. This increase in the conversion rate of sucrose can be attributed to lower inhibition of levansucrase by high-MW levan, which is hydrolyzed by endo-inulinase, and/or a shift of the reaction equilibrium of levansucrase-catalyzed reaction towards the hydrolysis reaction. The results also revealed that the maximum concentration of the transfructosylation products obtained in the sequential bi-enzymatic system was low compared to that produced in the one-step bi-enzymatic system. These results may be attributed to the high proportion of high molecular weight levans (>10 000 kDa) formed by levansucrase in the first stage, which are more difficult for endo-inulinase to hydrolyze owing to steric hindrance. When both enzymes were engaged in product formation in the one-step bi-enzymatic system, levans with MW higher than 10 000 kDa were not produced. The constant concentration of short chain FOSs over the reaction time course indicates that these intermediates of levan hydrolysis do not serve as acceptors by levansucrase. However, the decrease in the concentration of oligolevan reveals their use as fructosyl acceptors (Tian, Hill, and Karboune, 2013).

2.8 Discovery of Biocatalysts by High-Throughput Sequence-Based Screening

Sequence-based screening is an increasingly attractive method for the identification of new levanases due to the availability of rapidly expanding biological databases. To prospect for genes encoding levanase in microbial communities, homologous sequences in a selected database (e.g.,

UniProtKB) can be identified employing Basic Local Search Tool (BLAST) analysis with a collection of representative sequences, for instance the levanase gene YveB from *B. subtilis*. Resulting homologues can be filtered according to a set of specific and stringent criteria, such as an expect value lower than e-10 and a sequence identity with the query higher than 50%. For the purpose of the present work, candidate levanase genes to be selected for further investigation must possess the following criteria: 1) exhibit enzyme function of interest (i.e., higher FOS production over the production of fructose; 2) substrate specificity; and 3) high quality sequence, taking into consideration gene length and homology. A study conducted by Porras-Dominguez *et al*. (2014) identified sequences exhibiting high identity with the endolevanase sequence from *B. subtilis* strain 168 through a BLASTP analysis. Among the high identity sequences, they discovered a potential endolevanase from *B. licheniformis* and expressed it in *Escherichia coli*. The identified enzyme subsequently demonstrated high efficiency in producing levan-type FOSs. Following related sequence identification, multiple sequence alignments can be performed on the selected sequences to determine the degree of conservation of structurally and/or functionally important amino acids during evolution. Based on the sequence evolution analysis, residues that are important in enzymatic catalytic activity and specificity can be predicted since evolutionarily conserved residues tend to be essential in providing the enzyme's catalytic activity, whereas the amino acids that form specific interactions with substrates are likely to confer selectivity to the enzyme.

2.8.1 *Heterologous Expression of Levanases*

The majority of levanases which have been purified and characterized in more detail have been obtained from yeasts and filamentous fungi, and only a few bacterial sources of this enzyme have been elucidated (Wanker, Klingsbichel, and Schwab, 1995). The levanase from *Bacillus subtilis* is capable of hydrolyzing levan, inulin and sucrose. Kunst et al. were the first to describe this levanase, and classified it as a levanase owing to its activity on various substrates. However, the Avigad and Bauer classification for this enzyme is a non-specific β-fructofuranosidase to be distinguished from true levanases since it does not possess sole specificity for β-(2,6)-linkages (Wanker, Klingsbichel, and Schwab, 1995). Levanase expression in *B. subtilis* is stringently regulated and detectable enzyme quantities are only found with regulatory mutants (*sacL* mutants). The gene encoding this levanase has been cloned in *Escherichia coli*, sequenced, and characterized in detail. It has also been over-expressed in *E. coli*, the result of which was an enzyme of

approximately 75 kDa present primarily in the intracellular space, despite the presence of a secretion signal (Wanker, Klingsbichel, and Schwab, 1995).

To produce high levels of a protein, its gene is typically cloned downstream of a well-characterized, regulated promoter. Bacterial expression systems are attractive for the high-level production of heterologous proteins due to their rapid rate of proliferation using cost-effective substrates as well as the availability of a wide range of cloning vectors and mutant host strains. *E. coli* is the most commonly employed species in this context due to its well established status as a universal expression host (Terpe, 2006). There has been limited research into the heterologous expression of levanases. The majority of these studies have examined the cloning of reference levanase genes in various hosts for the purpose of screening the microbial genome to identify the levanase gene of interest and its amino acid sequence. The levanase gene (*YveB*) of *B. subtilis* was expressed in *E. coli* XL-1 Blue employing a pCR (+) vector. The identified levanase enzyme produced oligosaccharides spanning a degree of polymerization lower than 50 as the main products with an endo-acting mechanism (Pereira, Petit-Glatron, and Chambert, 2001). The levanase gene from *G. diazotrophocus* was cloned in *E. coli* XL1-Blue after being ligated to pET3d. The resulting recombinant gene was expressed in *E. coli* BL21(DE3) (Menedez *et al*., 2002). Miasnikov (1997) previously cloned a levanase gene from *B. subtilis* that was isolated from the L7 gene library by phenotypic selection. Two plasmids containing this levanase gene were independently isolated, and appeared identical by restriction analysis. Homology searches using the BLASTP program revealed that the L7 levanase exhibits amino acid sequence similarity with multiple fructofuranosidases from bacteria, fungi and higher plants. The author further noted that the highest level of similarity was found between the L7 levanase sequence and other levanase sequences found in *B. subtilis*, *B. polymyxa*, and *Bacteroides fragilis* exo-levanases. A more thorough analysis of the homology search results revealed that the C-terminal domain of L7 levanase (residues 390-700) is similar to essentially all of the hydrolases acting on β-fructofuranoside linkages, including invertases, sucrose-6-phosphate hydrolases, inulinases, etc. However, it was revealed that the N-terminal half of L7 levanase beared no similarity to the majority of fructofuranosidases, although two sequence fragments in this region were found to be homologous to the C-terminal (residues 558-685) sequence of *B. subtilis* levanase. The author speculated that this presumptive domain may be responsible for levan-binding (Miasnikov, 1997).

2.9 Purification of Recombinant Enzymes Using Polyhistidine Affinity Tags

Immobilized metal-affinity chromatography (IMAC) is a widely employed method for the expedited purification of recombinant proteins containing a short affinity tag comprising polyhistidine residues. This technique relies on the interactions between a transition metal ion (e.g., Co^{2+}, Ni^{2+}, Cu^{2+}, Zn^{2+}) immobilized on a matrix and specific amino acid side chains fused with the peptide of interest. Histidine is the amino acid that exhibits the strongest interaction with immobilized metal ion matrices, attributed to the fact that electron donor groups on the histidine imidazole ring readily form coordination bonds with the immobilized transition metal (Bornhorst and Falke, 2000). Peptides which contain consecutive sequences of histidine residues are efficiently retained on IMAC column matrices, which can be easily eluted by either modifying the pH of the column buffer or with the addition of free imidazole following washing of the matrix material (Bornhorst and Falke, 2000). IMAC is a robust method that can be employed to achieve 100-fold enrichments in polyhistidine affinity-tagged proteins in a single purification step, with up to 95% purity. Polyhistidine tag-based purification has been performed successfully using a number of expression systems, including *Escherichia coli*, *Saccharomyces cerevisiae*, mammalian cells, and baculovirus-infected insect cells (Bornhorst and Falke, 2000). Non-specific binding of untagged proteins can occur during purification using polyhistidine affinity tags. While the presence of histidine is relatively low (constituting 2% of all protein residues), select cellular proteins contain 2 or more adjacent histidine residues, which confers affinity for the IMAC matrix, and may cause these extraneous proteins to co-elute with the one of interest, resulting in substantial contamination of the final product (Bornhorst and Falke 2000). In *E. coli* systems, this is generally not a concern. In addition, the polyhistidine affinity tag purification system possesses several crucial advantages, including the fact that it allows for the affinity tag to be easily fused with the protein of interest. IMAC purification of highly expressed proteins, as in the case of *E. coli* systems, also provides purities of up to 95% with 90% recovery of the tagged protein in a single purification step (Bornhorst and Falke, 2000). Furthermore, the relatively minute size and charge of the polyhistidine affinity tag practically guarantees that it rarely affects protein function. Perhaps one of the most pertinent benefits of purifying proteins using polyhistidine affinity tags is that elution of the protein of interest can occur under mild conditions from the IMAC resin, allowing its biological activity to be preserved.

2.10 Methods of Identification and Quantification FOSs and Oligolevans

2.10.1 *High Performance Liquid Chromatography*

Currently, high-performance liquid chromatography (HPLC) is universally employed to conduct most types of carbohydrate analyses. Coupled with a refractive index detector, this method can quantitate mono-, di- and oligosaccharides, as well as polysaccharides with or without hydrolysis. Additionally, HPLC can yield qualitative (carbohydrate identification) results (BeMiller, 2010), which is integral to examining the FOS product spectrum catalyzed by levansucrase/levanase in the present work.

2.10.2 *High Performance Anion Exchange Chromatography*

Alternatively, carbohydrates can be separated based on their affinities for the oppositely charged stationary phase and the competition between ionic groups in the mobile phase in high-performance anion-exchange chromatography (HPAEC). Separation of carbohydrates by HPAEC coupled with pulsed amperometric detection (PAD) is preferred over gas chromatography and HPLC because of its high efficacy in resolving complex mixtures of carbohydrates (Cataldi *et al*., 2000). In addition, HPAEC-PAD represents a simple, economical and rapid quantifying method requiring minute sample volumes and minimal sample preparation (Hogarth *et al*., 2000). Further, PAD 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 principle underlying HPAEC-PAD takes advantage of the weakly acidic properties of carbohydrates under highly alkaline conditions (Rendleman, 1973), allowing the separation of closely related mono- and disaccharides (Cataldi *et al*., 1999). HPAEC can be used for analysis not only of monosaccharides but also oligosaccharides varying in degree of polymerization, including complex mixtures of oligosaccharides (Brummer and Cui, 2005). In HPAEC, two eluents are generally required, one containing only sodium hydroxide and another with sodium hydroxide and sodium acetate. Previous studies have compared the use of acetate, carbonate, nitrate and sulfate for the efficiency of carbohydrate elution, and acetate was recommended because its affinity for the anion-exchange resin is similar to that of hydroxide and thus maximizes resolution of the carbohydrates. Therefore, as an acetate gradient is run, the acetate anion exchanges for the carbohydrate anion and displaces the carbohydrates, causing them to be eluted (Rocklin and Pohl, 1983). It should be understood that the concentration of sodium hydroxide is the most important

factor in determining the range of carbohydrates that can be resolved in HPAEC. 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).

CONNECTING STATEMENT I

A comprehensive literature review focusing on enzymatic approaches for prebiotic levan-type FOS synthesis, including the mechanistic properties of levanase and quantitative methods of FOS analysis was provided in Chapter II. Chapter III details the discovery of novel levanases obtained by genome-mining, and discusses the screening parameters from which the top biocatalytic candidates were selected. In addition, their thermal stability and product spectra are compared since these are crucial considerations for industrial viability.

The results of this chapter were presented in the poster sessions at the Institute of Food Technologists Annual Meeting 2016, held in Chicago, Illinois.

CHAPTER III.

DISCOVERY AND SCREENING OF GENOME-MINED MICROBIAL LEVANASE CANDIDATES

3.1 Abstract

The focus of the present work was to develop an industrially feasible enzymatic approach for the synthesis of novel levan-type FOS compounds to be administered as second-generation prebiotics with enhanced functionality. A mass phylogenetic screening of bacterial and archaeal species was conducted employing BLASTP against UniProtKB to elucidate all candidates whose genomic profile contains a region bearing strong homology to previously annotated genetic sequences encoding endo-levanase (EC 3.2.1.65), a glycosyl hydrolase (GH32), which specifically hydrolyzes levan to short-chain FOSs and oligolevans. A new collection of 1902 gene sequences amenable to cloning was obtained, from which 140 representative candidates were screened according to their individual specific activities on low- and high-molecular weight levan, as well as differences in behaviour owing to substrate specificity and thermal stability. This ultimately revealed 10 viable, highly evolved and diverse bacterial candidate levanases. In particular, those from *Belliella baltica* (LEV4-A10), *Dyadobacter fermentans* (LEV4-D3), *Capnocytophaga ochracea* (LEV4-D4), *Vibrio natriegens* (LEV4-H2) and *Arthrobacter aurescens* (LEV5-A7) exhibited the highest levels of endo-hydrolytic activity on levan and demonstrated preferential hydrolysis of such over inulin.

3.2 Introduction

There has been increased scientific intent in the elucidation of sustainable sources of biocatalysts involved in the hydrolysis of fructans (i.e., inulin and levan) to non-digestible oligosaccharides, driven by important applications of these prebiotic compounds in the food and pharmaceutical industries. The consumption of prebiotics is attributed with a plethora of physiological benefits, the most widely recognized being the maintenance and promotion of gastrointestinal health, owing to selective fermentation by *Bifidobacterium* (predominantly occurring in the intestinal tract), resulting in their stimulated growth at the expense of harmful bacteria (Lim *et al.*, 1998; Rastall and Gibson, 2015; Slavin, 2013). Levan, which serves as a promising material for the production of fructooligosaccharides (FOSs), comprises a β-(2,6)-fructan main chain and β-(2,1)-linked side chains. Owing to its structure, oligosaccharides derived from this polyfructan can be applied as low-calorie, non-cariogenic sweeteners. Levan has several plant sources (e.g., ryegrass, timothy grass), and can also be obtained from a wide selection of bacteria during their assimilation of sucrose by the action of levansucrase (EC 2.4.1.10) (Lim *et al.*, 1998; Mardo *et al*., 2017). Bacterial levans can in turn be converted to levanoligosaccharides by the action of endo-levanases (EC 3.2.1.65), which characteristically hydrolyze the β-(2,6)-linked main chain to oligosaccharides of varying size and fructose as an inevitable reaction product (Lammens, 2009; Lim *et al.*, 1998). The aforementioned enzymes belong to glycosyl hydrolase family 32 (GH32), in which all members have previously been established to share a similar mechanism of action involving overall retention of the configuration of the anomeric carbon atom of the substrate (Pouyez *et al.*, 2012). Hydrolysis of glycosyl linkages by levanases occurs via a general acid catalysis mechanism requiring 2 catalytic residues: 1) a proton donor; and 2) a nucleophile (Pouyez *et al.*, 2012). Primary protein structures of multiple exo-levanases have been deduced from their corresponding genetic sequences, while no sequence information has been accessible for endo-levanases until recently (Miasnikov, 1997). Miasnikov (1997) previously investigated a then novel endo-levanase isolated from the soil bacterium *Bacillus* sp. L7. Upon characterization, this enzyme was found to exhibit no exo-β-fructofuranosidase activity, and was hence cloned and sequenced. Homology searches performed in this study concluded that the C-terminal domain of this levanase is homologous to several known β-fructofuranosidases and that the N-terminal region appears to be a levan-binding domain. In addition to levan, levanases are typically also capable of hydrolyzing inulin and sucrose (Miasnikov, 1997) to varying extents,

although preferential activity is demonstrated on the former. To date, few levanases have been discovered and characterized relative to other GH32 enzymes, and the majority within the existing collection have been isolated from yeasts and filamentous fungi, as other microbial kingdoms (i.e., bacteria and archaea) have only been explored to minimal extents (Wanker, Huber, and Schwab, 1995).

The vast proportion of levanases possess an exo-hydrolytic mechanism of biocatalysis, which involves the hydrolysis of levan from the fructose (reducing) end, consequently resulting in its liberation as the sole end-product (Wanker, Huber, and Schwab, 1995). Constrastingly, endo-levanases (EC 3.2.1.65) generate a wider array of end-products from levan (and potentially other fructans), particularly fructooligosaccharides varying in degree of polymerization (Miasnikov, 1997), and therefore, digestibility. As expected, levanases obtained from different microbial sources demonstrate varying levels and types of catalytic behavior. For instance, *Streptomyces* sp. No. 7-3 (Hiroshimi *et al*., 1990) and *Streptomyces exofoliaticus* F3-2 express levanases which hydrolyze levan to levanbiose as the predominant end-product. On the other hand, the levanase from *Bacillus* sp. No. 71 primarily converts levan to levanheptaose, as well as other oligosaccharides with DP higher than 7 in early reaction stages, thus suggesting that this enzyme possesses endo-hydrolytic machinery, which is rare in its class (Kametani and Umezawa, 1966).

Given that biocatalysts are integral to the bioconversion of levan and other fructans to prebiotic fructooligosaccharides, and that studies of levanases are hampered by limited availability, inadequate reaction selectivity and low thermal stability, it is necessary to explore the genetic diversity of microbial kingdoms in order to elucidate a new, broader collection of these enzymes. High-throughput sequence-based homology screening is an increasingly attractive method for the identification of new levanases due to the availability of rapidly expanding genome and protein databases. Few authors have achieved success from this bioinformatics approach for the discovery of endo-levanases. For instance, a study conducted by Porras-Dominguez *et al.* (2014) reported sequences exhibiting high identity with the endo-levanase-encoding gene from *Bacillus subtilis* strain 168 upon BLAST analysis. Among the results was a potential endo-levanase from *B. licheniformis*, which the authors subsequently expressed in *Escherichia coli* and confirmed to produce levan-type FOSs. While their efforts in synthesizing prebiotic FOSs from a then novel levanase were successful, this line of investigation has seen minimal advancements

since then, which makes our discovery of new candidate levanases especially pertinent, given the limited availability and overall incomplete characterization of levanases.

In order to produce sufficient quantities of a biocatalyst, it is typically necessary to first clone its genetic information downstream of a well-characterized, regulated promoter. Bacterial expression systems are ideal for high-level production of heterologous proteins owing to their rapid rate of proliferation using cost-effective substrates, as well as the wide availability of cloning vectors. Among mutant host strains, *E. coli* is the most universally employed due to the ubiquitous successes of recombinant gene expression by this microorganism (Terpe, 2006). While there has been limited research into the heterologous expression of levanases, several authors have cloned genes encoding this enzyme in other hosts for the purpose of analyzing their amino acid sequences and in the screening of microbial genomes. The levanase gene (*YveB*) isolated from *B. subtilis* has previously been over-expressed in *E. coli* XL-1 Blue, from which the authors subsequently identified an enzyme that produced oligosaccharides spanning a degree of polymerization (DP) lower than 50 as the predominant end-products. Considering these examples, it is necessary to investigate all candidate microbial levanase sources in order to determine the ideal biocatalysts for producing prebiotic FOSs, which should not only demonstrate high levels of specific endo-hydrolytic activity towards levan, but also possess other industrially desirable attributes such as thermal stability.

The present work focuses on the exploration of genomic diversity within the bacteria and archaea kingdoms for the discovery of new candidate levanases exhibiting predominant endo-hydrolytic activity on levan and high thermal stability. To prospect for candidate sources of this biocatalyst, Basic Local Alignment Search Tool (BLASTP) was employed against UniProtKB in order to identify all genetic sequences bearing high homology to those encoding levanases previously reported in literature. From these homologues, selections for further investigation were made according to the following criteria: 1) enzyme function of interest (i.e., higher FOS production compared to the release of fructose); 2) the predicted selectivity of the enzyme; and 3) sequence quality (including gene length and homology). In our ongoing work, we investigated the catalytic behavior of 123 bacterial levanase candidates that were over-expressed in *E. coli* BL21(DE3). They were representative of 1902 newly discovered homologues and possessed genetic sequences of high quality that were amenable to cloning. This resulted in the selection of

10 promising new levanases capable of synthesizing FOSs varying in size. These candidates will be discussed in terms of genetic diversity, specific activity and thermal stability.

3.3 Materials and Methods

3.3.1 Chemicals and Materials

Growth media preparations, lysogeny broth (LB) constituents (tryptone, NaCl, and yeast extract) broad spectrum antibiotics (ampicillin and carbenicillin), and the protein expression inducer, IPTG, were purchased from Thermo Fisher Scientific (Mississauga, ON, Canada). Mono- and disaccharide standards (i.e., D-(-)-fructose, D-(+)-glucose and sucrose) were obtained from Sigma-Aldrich (Oakville, ON, Canada). Fructooligosaccharide standards (i.e., 1-kestose, nystose and 1-fructofuranosylnystose were purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan). Other chemicals and reagents, including inulin, 3,5-dinitrosalicylic acid, potassium sodium (+)-tartrate, K_2HPO_4, KH_2PO_4, NaOH and NaOAc, imidazole, PIPES, bovine serum albumin, and Bradford Reagent were also purchased from Sigma-Aldrich (Oakville, ON, Canada). Purification of levanases was performed using GE Healthcare Life Sciences HisTrap™ FF 1 mL columns (Mississauga, ON, Canada).

3.3.2 Acquiring New Levanase Candidates

Candidate microbial sources of levanase were obtained by high-throughput sequence-based screening with a reference set of levanase genes compiled from previous studies found in literature. In this context, BLASTP was employed against UniProtKB with minimum 80% identity and e <0.03. The query returned 1902 genes which were phylogenetically clustered, resulting in the selection and subsequent investigation of 123 representative candidates. These were obtained from the library of microbial genomes at CEA-Genoscope (Evry, France), where they were over-expressed in competent *Eschericia coli* BL21(DE3) with the utilization of pLysS vectors.

3.3.3 Levanase Production, Recovery and Purification

E. coli BL21(DE3)-pLysS transformed by the insertion of levanase-encoding genes were aerobically pre-cultured in LB media (40% (w/v) tryptone, 20% (w/v) yeast extract, 40% (w/v) NaCl) with the addition of ampicillin (0.1 mg/mL final concentration) overnight at 37°C and 250 rpm with continuous agitation (New Brunswick Scientific *Excella E24 Incubator Shaker Series*)

until an optical density (OD) of 1.2-1.4 at 600 nm was reached. Inoculation of commercial TB media (47.6 g/L) with carbenicillin (0.1 mg/mL final concentration) performed using a 50-times dilution of the aforementioned LB mixture followed under the same conditions for 20-24 hours hours (to achieve a final OD of 1.2-1.4) prior to the addition of IPTG (1 mM final concentration) to induce protein expression. Incubation proceeded at 20°C for 20 hours before cell mass was harvested by centrifugation at 4°C (7003 x g for 20 minutes). Cells frozen at -80°C were re-suspended in pH 7.2 sonication buffer (10% (w/v) glycerol, 30 mM PIPES, 30 mM NaCl) prior to being treated with lysozyme (4 mg/g cell mass) and DNase (2000 U, 4 μL/g cell mass), followed by a 1-hour incubation period at 18°C and 50 rpm in an orbital shaker. This cell suspension was ultrasonicated and centrifuged at 4°C (8817 x g for 1 hour) to obtain crude enzyme extract, which was subsequently dialyzed against 5 mM potassium phosphate buffer (pH 6.0) with a membrane cut-off of 5-6 kDa at 4°C, and lyophilized at -40°C. Purification was performed by immobilized metal anion chromatography (IMAC) on HisTrap™ FF 1 mL columns using an imidazole gradient ranging from 5 mM-1 M in pH 6.4 wash buffer (30 mM NaCl, 10 % (v/v) glycerol, 30 mM PIPES) as the eluent.

3.3.4 Electrophoretic Analysis of Purified Levanases

The efficacy of crude levanase purification by IMAC was verified by performing SDS-PAGE on each collection fraction (sonication buffer, wash buffer, 5 mM imidazole, 10 mM imidazole, 100 mM imidazole, 200 mM imidazole, 1 M imidazole). 15% polyacrylamide gels were handcasted according to Bio-Rad (Saint-Laurent, QC, Canada) specifications. SDS-PAGE was conducted at 120 V until full sample migration was achieved. Generally, pure enzyme fractions eluted when the imidazole gradient reached concentrations of 100 mM-200 mM.

3.3.5 Levanase Activity Assays

The glycosyl-hydrolytic activity of each levanase was assayed by measuring the release of reducing sugars by the 3,5-dinitrosalicylic acid (DNS) method using low- and high-molecular weight levan (5.5-6.25 kDa and 2.8^3 kDa, obtained from *Bacillus amyloliquefaciens* levansucrase) and inulin as substrates. Two blank assays neither containing substrate nor enzyme, as well as an additional blank assay solely containing substrate were performed in tandem for each trial. 250 μL levanase (6-25 μg protein) diluted in 50 mM potassium phosphate buffer (pH 6.0) was reacted

with 250 μL substrate (1% w/v) for 20 minutes at 37°C. This was followed by the addition of 750 μL DNS reagent (1% (w/v) DNS, 1.6% (w/v) NaOH), at which point the reaction mixture was boiled for 5 minutes for complete enzyme inactivation. 250 μL potassium sodium tartrate (50% w/v) was subsequently added to stabilize the colorimetric reaction. All assays were conducted in triplicate and absorbance readings were measured spectrophotometrically at 540 nm against the aforementioned blanks. One unit of levanase activity was defined as the release of 1 μmol of fructose per minute. Following purification, the micro-assay procedure derived from the Bradford method for total protein quantification was performed with bovine serum albumin standards (1-20 μg/mL) and results were correlated with those for total enzyme activity to determine specific levanase activity in μmol/mg*min.

3.3.6 Characterization of Levanase End-Product Profiles by HPAEC-PAD

The end-product profiles of levanase-catalyzed hydrolysis of levan and inulin were quantified by high performance anion exchange chromatography coupled with pulsed amperometric detection (HPAEC-PAD) on the Dionex ICS-3000. This system was equipped with Chromeleon™ 7.2 Chromatogrpahy Data System Software and a CarboPac® PA200 column (3 x 150 mm). A linear gradient of 0-200 mM NaOAc in 100 mM NaOH was applied over a duration of 20 minutes at a rate of 0.4 ml per minute to elute carbohydrate compounds of interest. Identification and quantification of specific carbohydrates were performed using glucose, fructose, sucrose and fructooligosaccharide (i.e., 1-kestose, nystose, 1-fructofuranosylnystose) standards (1-100 μM).

3.3.7 Thermal Stability of Levanases

Crude levanases were heat-treated at 50°C in a stagnant waterbath for specific durations ranging from 0-60 minutes. Enzymatic activity assays as previously described followed in triplicate at 37°C for 20 min using low-molecular weight levan (1% w/v) as the substrate. Decay trends were monitored spectrophotometrically at 540 nm until more than 50% of the initial levanase activity was lost.

3.4 Results and Discussion

3.4.1 Data-Mining Microbial Genomes for the Discovery of New Levanases

The discovery of new levanases commenced with a genome-mining approach that screened microbial communities based on a collection of annotated levanase genes compiled from literature. Genetic sequences encoding exo-levanases from various bacterial sources, as shown in Figure 3.1, were employed in the data-mining of archived bacterial and archaeal genomes to prospect for strong homologues. As shown in Table 3.1, the majority of currently known levanases function in an exo-hydrolytic manner, releasing fructose as the sole end-product. The end-product profiles of these biocatalysts are generally highly heterogeneous (ranging from fructose to short-chain FOSs), which highlights the need to investigate those that possess endo-hydrolytic mechanisms and to develop an approach for the synthesis of controlled-size FOSs. The optimal reaction conditions under which these levanases exhibit maximal activity and conformational stability are major determinants of their catalytic efficiency and their usefulness in the production of levan-derived end-products, with minor exceptions. For instance, the levanase from *S. exfoliates* F3-2 demonstrates adequate levels of activity at pH 3.5; however, generally, the optimal reaction parameters tend to group around pH 6.0 and 40°C.

Our query for new microbial levanases returned 1902 genetic sequences amenable to cloning. Phylogenetic clustering of these results revealed a remarkable degree of diversity in the origin of these sources, underlining the ubiquity of levanases. 123 representative candidates were selected for further investigation, and as depicted in Figure 3.1, the majority of these are highly divergent from the strains containing levanase-encoding genes referenced in literature. Although the scope of genome-mining in our investigation was extended across the bacteria and archaea kingdoms, it was interesting to note that homologues bearing at least 80% identity to levanase-encoding genes only appeared in bacterial species. This can be attributed to the fact that in archaea, there is a general absence of many ‘classical’ pathways for central carbohydrate metabolism (Brasen *et al.*, 2014). Rather, these microorganisms are unique for the use of modified variants which require unconventional enzymes. Additionally, it was observed that in certain cases, microbial levanase genes occur co-localized with that of levansucrase (E.C. 2.4.1.10), the enzyme responsible for synthesizing levan by transfructosylation and polymerization of disaccharides (predominantly sucrose). For instance, in *Bacillus subtilis*, the gene encoding levanase was found co-localized with that for levanase. This has also been previously observed by (Menéndez *et al.*, 2002), who reported

the genes encoding levansucrase (*lsdA*) and levanase (*lsdB*) in *G. diazotrophicus* SRT4 to form a two-gene operon. In addition, in the Gram-negative bacterium *Zymomonas mobilis*, transcription of the genes coding for the extracellular sucrolytic enzymes levansucrase (*levU*) and invertase (*invB*) is regulated by the same promoter. The branching pattern of the levanase phylogenetic tree (Figure 3.1) that these members of the GH32 are widely prevalent in lower-order organisms (i.e., there appears to be no correlation between the taxonomy of a bacterium and the presence of a levanase-encoding sequence in its genome). This implies that, among bacteria, the gene for levanase is ubiquitously conserved, which can be rationalized given that levan is a major storage carbohydrate from which these microorganisms derive the energy required for survival (Vijin and Smeekens, 1999). The evolution of GH32 genes among bacteria has not been investigated; however, Parrent *et al.* (2009) were the first to detect 9 lineages of such in various fungi. They reported 0-12 GH32 genes in the fungal genomes they examined, and revealed a strong correlation between nutritional mode and the abundance of this gene, as deduced from ancestral state reconstruction. Data mining of complete fungal genomes allowed Parrent *et al.* (2009) to observe a definite signature of both ecological strategy and species phylogeny on GH32 gene number. Phylogenetic reconstructions indicated that expansions of GH32 genes coincided with switches to a plant pathogenic habitat, and conversely, loss of all GH32 genes was observed on branches leading to nearly all animal pathogens. This trend can be applied to the diverse phylogenetic origins of levanase-encoding genes among bacterial species and explains the absence of GH32 genes in archaea, which are of higher order than bacteria, and as previously discussed, possess more complex metabolic machinery. Despite the highly evolved and diversified nature of bacteria, the data presented in Figure 3.1 speaks to the prevalence of levanase-encoding genes among these communities, suggesting they are ubiquitously employed for the metabolism of polysaccharides (i.e., levan).

In our work, data mining of microbial genomes also revealed significant distinctions in levanase-encoding genes that can occur in a single strain of bacteria. For instance, multiple levanases differing in catalytic behavior can be obtained from the host *Arthrobacter aurescens* TC1, as with LEV4-B7 and LEV5-A7 (Figure 3.1). These sequence homologues were observed to only bear 26% identity to one another, which can account for their remarkable differences in substrate specificity and overall catalytic behavior. As indicated in Table 3.2, LEV5-A7 is capable of hydrolyzing levan (both low- and high-molecular weight) much more efficiently than LEV4-

B7, and it is also unique in its family as it possesses a susbstantially higher affinity for high-molecular weight levan. Notably, all levanase-encoding genes from *A. aurescens* TC1 appear to result in the expression of enzymes that preferentially hydrolyze high-molecular weight levan over its low-molecular weight counterpart, which is a common trend observed among bacterial levanases. This behavior can perhaps be attributed to the prevalence of high-molecular weight levan as the predominant storage carbohydrate in the cellular makeup of this bacterial species.

3.4.2 Screening of Select Candidate Levanases

In the screening of 123 bacterial candidate levanases, widely different levels of hydrolytic activity on low- and high-molecular levans were observed. In general, low-molecular weight levan was more readily hydrolyzed, with several notable exceptions, as in the case of all candidate levanases from *A. aurescens* TC1 and *Bacillus amyloliquefaciens* (Table 3.2). In general, the lower affinity of most bacterial levanases for high-molecular weight levan can be explained by steric hindrance caused by its bulkiness in the enzyme's active site, reducing the accessibility to glycosyl linkages. It was further noted from the genome-mining process that there appears to be a correlation between bacterial taxonomy and substrate specificity. Specifically, Gram-negative bacteria appear to preferentially hydrolyze high-molecular weight levan, as exemplified by the levanases from *A. aurescens* (which demonstrated a specific activity of 121.67 μmol/mg*min on low-molecular weight levan and 329.83 μmol/mg*min on high-molecular weight levan), *Asticcacaulis benevestitus, Azotobacter vinelandii,* and *Bacteroides* spp. among others. Most notably, the levanase from Gram-negative *Subdoligranulum variabile* exhibited hydrolytic activity on high-molecular weight levan that exceeded its activity on low-molecular weight levan by almost 100-fold. With regards to Gram-positive bacterial sources of levanase, trends were less apparent, as certain species exhibited higher levels of activity on low-molecular weight levan (e.g., *Actinomyces naeslundii*, and *Bifidobacterium* spp. among others), while others demonstrated higher affinity for high-molecular weight levan (e.g., *Beijerinckia indica* subsp. *indica, Bacillus*

Table 3.1 Properties of microbial levanases employed as genomic references for the discovery of new candidates

Microbial Levanase Source	MW (kDa)	Optimal Conditions		Stability Range		Products from Levan	Action /Effect on β-(2,6)-Linkages	References
		pH	T (°C)	pH	T (°C)			
Streptomyces sp. No. 7-3	57	6.5	40	5.5-8.5	≤ 40	F (3%), F2 (80%), F3 (17%)	Exo-type /No	(Murakami *et al.*, 1990)
Arthrobacter sp. 51A	60	5.8	65	7.0-9.0	≤ 45	F, F2	Endo-type /Yes	(Murakami, Fukui, Nakano, and Kitahara, 1994)
Pseudomonas sp. No. 43	36	7.0	40	6.0-8.0	≤ 50	F2	Exo-type /No	(Kang *et al.*, 1999)
Streptomyces exofoliatus F3-2	54	5.5	60	3.5-8.0	≤ 50	F, F2	Exo-type /Yes	(Saito, Kondo, Kojima, Yokota, and Tomita, 2000)
Microbacterium laevaniformans ATCC 15953	68	6.0	30	5.5-7.0	≤ 45	F, F2, F3	Exo-type /Yes	(Song *et al.*, 2002)
Streptomyces sp. 366L	80	7.0	40	6.0-10.0	≤ 45	F7	Exo-type /No	(Lim *et al.*, 1998)
Rhodotorula sp.	39	6.0	40	5.5-6.5	≤ 40	F (21%), F2 (79%5)	Exo-type /Yes	(Chaudhary *et al.*, 1996)
Bacillus subtilis	ND	ND	ND	ND	ND	≤ F50	Endo-type /ND	(Pereira *et al.*, 2001)
Bacillus sp. L7	86	5.5	50	5.4-6.6	≤ 60	F, F2-F12	Endo-type /ND	(Miasnikov, 1997)

ND: no data; F: followed by degree of polymerization of FOS with glucose head moiety

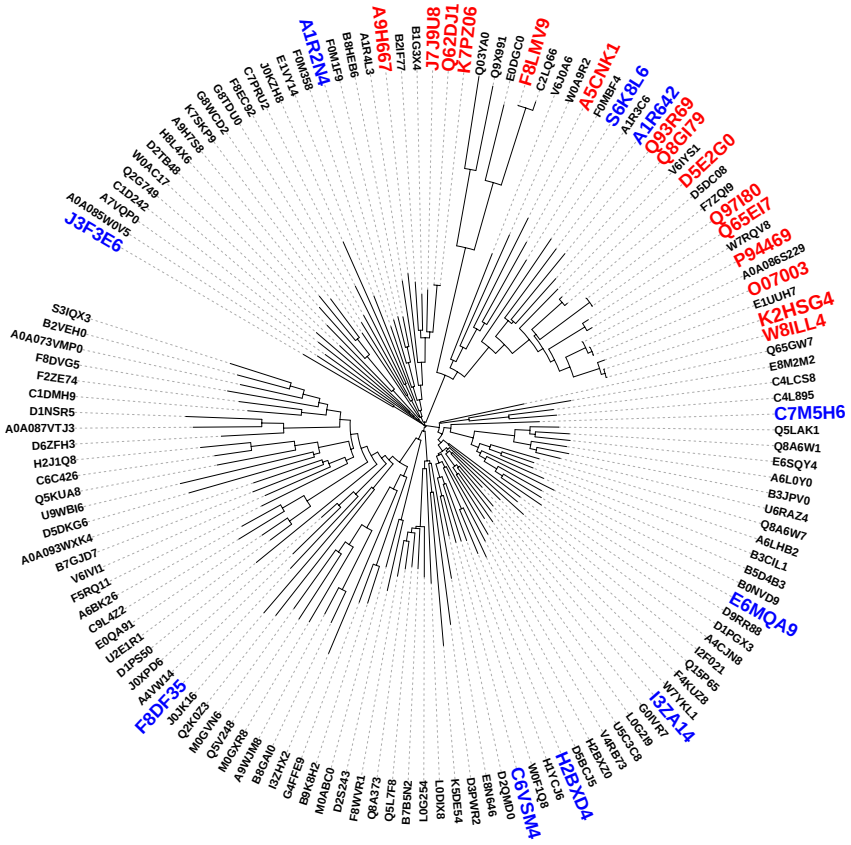


Figure 3.1 Phylogenetic origins of reference levanases reported in literature and the top 10 newly discovered candidate levanases

Bacterial sources of reference levanase genes: *Guconoacetobacter diazotrophicus* (A9H667); *G. diazotrophicus* (Q8G179); *G. diazotrophicus* (Q9RBJ1); *Microbacterium laevaniformans* (Q93R69); *Bacillus subtilis* 168 (O07003); *B. licheniformis* (Q65EI7)

Bacterial sources of genes encoding selected candidate levanases: LEV4-E4: *Prevotella salivae* (E6MQA9); LEV4-F7: *Gillisia limnaea* (H2BXD4); LEV4-E11: *Streptococcus parasanguinis* (F8DF35); LEV5-C5: *Actinomyces naeslundii* (J3F3E6); LEV5-A7: *Arthrobacter aurescens* TC1 (A1R642); LEV4-B7: *A. aurescens* TC1 (A1R2N4); LEV4-D4: *Capnocytophaga ochracea* (C7M5H6); LEV4-D3: *Dyadobacter fermentans* (C6VSM4); LEV4-A10: *Belliella Baltica* (I3ZA14); LEV4-H2: *Vibrio natriegens* (S6K8L6)

spp. and *A. aurescens*). A significant proportion of candidate levanases shown in Table 3.2 interestingly appear to have similar levels of activity on low- and high-molecular weight levan, as with *Streptococcus parasanguinis*, *Anaerolinea thermophile*, *Centipeda periodontii* and *Belliella baltica* to name a few. It can therefore be concluded from a general assessment of the data presented in Table 3.2 that although there appears to be an association between Gram-negative bacterial sources of levanase and the enzyme's substrate specificity for high-molecular weight levan, catalytic behavior is regulated more so by genetic determinants. Further data mining is necessary to justify the cause of unquantifiably low levels of levanase activity in certain candidates and to confirm whether weak homology to levanase-encoding reference genes is the sole reason.

10 candidate levanases were selected from the collection summarized in Table 3.2 for further characterization (i.e., in terms of thermal stability and end-product profile) based on their ability to hydrolyze low- and high-molecular weight levan, with more emphasis being placed on the former since it is typically hydrolyzed more readily than its high-molecular weight counterpart. These are shown in Figure 3.4, with levanases originating from: 1) *Belliella baltica* (LEV4-A10); 2) *Arthrobacter aurescens* (LEV4-B7); 3) *Dyadobacter fermentans* (LEV4-D3); 4) *Capnocytophaga ochracea* (LEV4-D4); 5) *Prevotella salivae* (LEV4-E4); 6) *Streptococcus parasanguinis* (LEV4-E11); 7) *Gillisia limnaea* (LEV4-F7); 8) *Vibrio natriegens* (LEV4-H2); 9) *Arthrobacter aurescens* (LEV5-A7); and 10) *Actinomyces naeslundii* (LEV5-C5). Among these selections, LEV4-A10, LEV4-D3 and LEV4-D4 levanases demonstrated the highest levels of activity on low-molecular weight levan, while LEV5-A7 showed both high activity on low-molecular weight levan and a substantially higher activity on high-molecular weight levan. It can be hypothesized where there is preferential hydrolysis of high-molecular weight levan, the levanase may possess several ligand-binding sites that facilitate the accessibility of the enzyme to glycosidic linkages joining the fructosyl residues in the polymer chain.

Table 3.2 Specific activities of the top 10 candidate levanases on various substrates

Bacterial Levanase Source	Gram +/-	LMW Levan	HMW Levan
		Specific Activity (μmol/mg*min)	Specific Activity (μmol/mg*min)
Actinomyces naeslundii (strain ATCC 12104 / DSM 43013 / JCM 8349 / NCTC 10301 / Howell 279)	+	44.00	<0.03
Anaerolinea thermophila (strain DSM 14523 / JCM 11388 / NBRC 100420 / UNI-1)	-	23.76	24.47
Anoxybacillus flavithermus (strain DSM 21510 / WK1)	+	2.62	7.59
Arthrobacter arilaitensis (strain DSM 16368 / CIP 108037 / JCM 13566 / Re117)	+	18.48	14.50
Arthrobacter aurescens (strain TC1)	+	<0.03	12.31
Arthrobacter aurescens (strain TC1)	+	<0.03	7.08
Arthrobacter aurescens (strain TC1)	+	121.67	329.83
Arthrobacter aurescens (strain TC1)	+	43.81	39.73
Arthrobacter chlorophenolicus (strain A6 / ATCC 700700 / DSM 12829 / JCM 12360)	+	2.36	19.54
Arthrobacter phenanthrenivorans (strain DSM 18606 / JCM 16027 / LMG 23796 / Sphe3)	+	<0.03	6.30
Arthrobacter phenanthrenivorans (strain DSM 18606 / JCM 16027 / LMG 23796 / Sphe3)	+	0.15	3.47
Arthrobacter phenanthrenivorans (strain DSM 18606 / JCM 16027 / LMG 23796 / Sphe3)	+	2.24	7.15
Asticcacaulis benevestitus DSM 16100 = ATCC BAA-896	-	41.97	56.20
Azotobacter vinelandii (strain DJ / ATCC BAA-1303)	-	<0.03	5.52
Bacillus amyloliquefaciens	+	<0.03	0.26
Bacillus amyloliquefaciens (strain ATCC 23350 / DSM 7 / BCRC 11601 / NBRC 15535 / NRRL B-14393)	+	18.70	29.22
Bacillus clausii	+	<0.03	0.85
Bacillus licheniformis (strain DSM 13 / ATCC 14580)	+	49.73	54.51
Bacillus licheniformis S 16	+	<0.03	4.37
Bacillus megaterium (strain DSM 319)	+	<0.03	0.10
Bacillus megaterium (strain DSM 319)	+	<0.03	0.61
Bacillus subtilis subsp. *subtilis*	+	<0.03	4.41
Bacteroides coprocola DSM 17136	-	<0.03	2.14
Bacteroides fragilis (strain ATCC 25285 / NCTC 9343)	-	<0.03	0.68
Bacteroides fragilis (strain ATCC 25285 / NCTC 9343)	-	<0.03	1.42
Bacteroides helcogenes (strain ATCC 35417 / DSM 20613 / JCM 6297 / P 36-108)	-	<0.03	1.04
Bacteroides intestinalis DSM 17393	-	<0.03	0.90
Bacteroides massiliensis B84634 = Timone 84634 = DSM 17679 = JCM 13223	-	<0.03	1.13
Bacteroides plebeius (strain DSM 17135 / JCM 12973 / M2)	-	5.53	9.19

Bacteroides stercoris ATCC 43183	-	<0.03	0.96
Bacteroides thetaiotaomicron (strain ATCC 29148 / DSM 2079 / NCTC 10582 / E50 / VPI-5482)	-	<0.03	<0.03
Bacteroides thetaiotaomicron (strain ATCC 29148 / DSM 2079 / NCTC 10582 / E50 / VPI-5482)	-	<0.03	1.54
Bacteroides thetaiotaomicron (strain ATCC 29148 / DSM 2079 / NCTC 10582 / E50 / VPI-5482)	-	<0.03	7.85
Bacteroides vulgatus (strain ATCC 8482 / DSM 1447 / NCTC 11154)	-	<0.03	0.51
Beijerinckia indica subsp. *indica* (strain ATCC 9039 / DSM 1715 / NCIB 8712)	-	57.78	76.23
Belliella baltica (strain DSM 15883 / CIP 108006 / LMG 21964 / BA134)	-	109.16	93.25
Bifidobacterium breve JCP7499	+	<0.03	0.23
Bifidobacterium dentium ATCC 27679	+	10.03	2.33
Bifidobacterium gallicum DSM 20093 = LMG 11596	+	35.94	<0.03
Bifidobacterium indicum LMG 11587 = DSM 20214	+	<0.03	7.46
Blautia hansenii DSM 20583	+	<0.03	1.87
Burkholderia graminis C4D1M	-	19.87	40.40
Capnocytophaga ochracea (strain ATCC 27872 / DSM 7271 / JCM 12966 / VPI 2845)	-	137.79	102.79
Cedecea davisae DSM 4568	-	<0.03	1.28
Centipeda periodontii DSM 2778	-	16.30	17.17
Chitinophaga pinensis (strain ATCC 43595 / DSM 2588 / NCIB 11800 / UQM 2034)	-	<0.03	0.35
Chloroflexus aggregans (strain MD-66 / DSM 9485)	-	<0.03	<0.03
Chloroflexus aurantiacus (strain ATCC 29366 / DSM 635 / J-10-fl)	-	<0.03	0.57
Clostridium acetobutylicum DSM 1731	+	<0.03	0.22
Clostridium leptum DSM 753	+	<0.03	<0.03
Corynebacterium matruchotii ATCC 14266	+	10.00	12.83
Cyclobacterium marinum (strain ATCC 25205 / DSM 745)	-	<0.03	0.30
Cytophaga fermentans DSM 9555 = JCM 21142	-	<0.03	9.93
Deinococcus deserti (strain VCD115 / DSM 17065 / LMG 22923)	+	<0.03	<0.03
Dickeya dadantii (strain Ech703)	-	<0.03	<0.03
Dorea longicatena DSM 13814	+	<0.03	0.92
Dyadobacter fermentans (strain ATCC 700827 / DSM 18053 / NS114)	-	98.51	93.00
Dysgonomonas mossii DSM 22836	-	<0.03	1.15
Echinicola vietnamensis (strain DSM 17526 / LMG 23754 / KMM 6221)	-	<0.03	<0.03
Echinicola vietnamensis (strain DSM 17526 / LMG 23754 / KMM 6221)	-	1.65	3.14
Emticicia oligotrophica (strain DSM 17448 / GPTSA100-15)	-	27.04	20.07
Erwinia pyrifoliae (strain DSM 12163 / CIP 106111 / Ep16/96)	-	<0.03	0.31

Erwinia tasmaniensis (strain DSM 17950 / Et1/99)	-	<0.03	1.09
Escherichia coli 5-172-05_S1_C1	-	<0.03	<0.03
Frateuria aurantia (strain ATCC 33424 / DSM 6220 / NBRC 3245 / NCIMB 13370)	-	<0.03	5.32
Geobacillus kaustophilus (strain HTA426)	+	<0.03	<0.03
Gillisia limnaea DSM 15749	-	48.91	30.24
Gillisia limnaea DSM 15749	-	49.30	29.27
Gluconacetobacter diazotrophicus (strain ATCC 49037 / DSM 5601 / PAl5)	-	<0.03	5.63
Gluconobacter oxydans H24	-	<0.03	0.09
Haliscomenobacter hydrossis (strain ATCC 27775 / DSM 1100 / LMG 10767 / O)	-	1.24	3.22
Haloarcula marismortui (strain ATCC 43049 / DSM 3752 / JCM 8966 / VKM B-1809)	-	<0.03	15.90
Haloferax gibbonsii ATCC 33959	+	<0.03	0.48
Haloferax gibbonsii ATCC 33959	+	<0.03	0.54
Haloterrigena turkmenica (strain ATCC 51198 / DSM 5511 / NCIMB 13204 / VKM B-1734)	-	<0.03	0.96
Klebsiella oxytoca (strain ATCC 8724 / DSM 4798 / JCM 20051 / NBRC 3318 / NRRL B-199 / KCTC 1686)	-	2.52	1.01
Lactobacillus mali KCTC 3596 = DSM 20444	+	47.24	51.39
Leuconostoc mesenteroides subsp. *mesenteroides* (strain ATCC 8293 / NCDO 523)	+	<0.03	<0.03
Mucilaginibacter paludis DSM 18603	-	<0.03	<0.03
Natrialba taiwanensis DSM 12281	-	<0.03	7.54
Niabella soli DSM 19437	-	47.24	<0.03
Niastella koreensis (strain DSM 17620 / KACC 11465 / GR20-10)	-	<0.03	0.70
Novosphingobium aromaticivorans (strain DSM 12444 / F199)	-	7.99	4.13
Parabacteroides distasonis (strain ATCC 8503 / DSM 20701 / NCTC 11152)	-	<0.03	0.66
Parabacteroides johnsonii DSM 18315	-	<0.03	0.22
Prevotella copri DSM 18205	-	5.94	7.09
Prevotella melaninogenica (strain ATCC 25845 / DSM 7089 / JCM 6325 / VPI 2381 / B282)	-	<0.03	0.03
Prevotella salivae DSM 15606	-	73.19	50.20
Pseudoalteromonas atlantica (strain T6c / ATCC BAA-1087)	-	7.95	4.49
Pseudomonas syringae pv. oryzae str. 1_6	-	<0.03	0.10
Rahnella aquatilis (strain ATCC 33071 / DSM 4594 / JCM 1683 / NBRC 105701 / NCIMB 13365 / CIP 78.65)	-	<0.03	0.54
Rhizobium etli (strain CFN 42 / ATCC 51251)	-	<0.03	0.44
Rhizobium leguminosarum bv. viciae WSM1455	-	1.54	3.21
Rhodonellum psychrophilum GCM71 = DSM 17998	-	<0.03	1.56
Rhodopirellula baltica SH28	-	<0.03	2.91
Robiginitalea biformata (strain ATCC BAA-864 / HTCC2501 / KCTC 12146)	-	<0.03	0.86

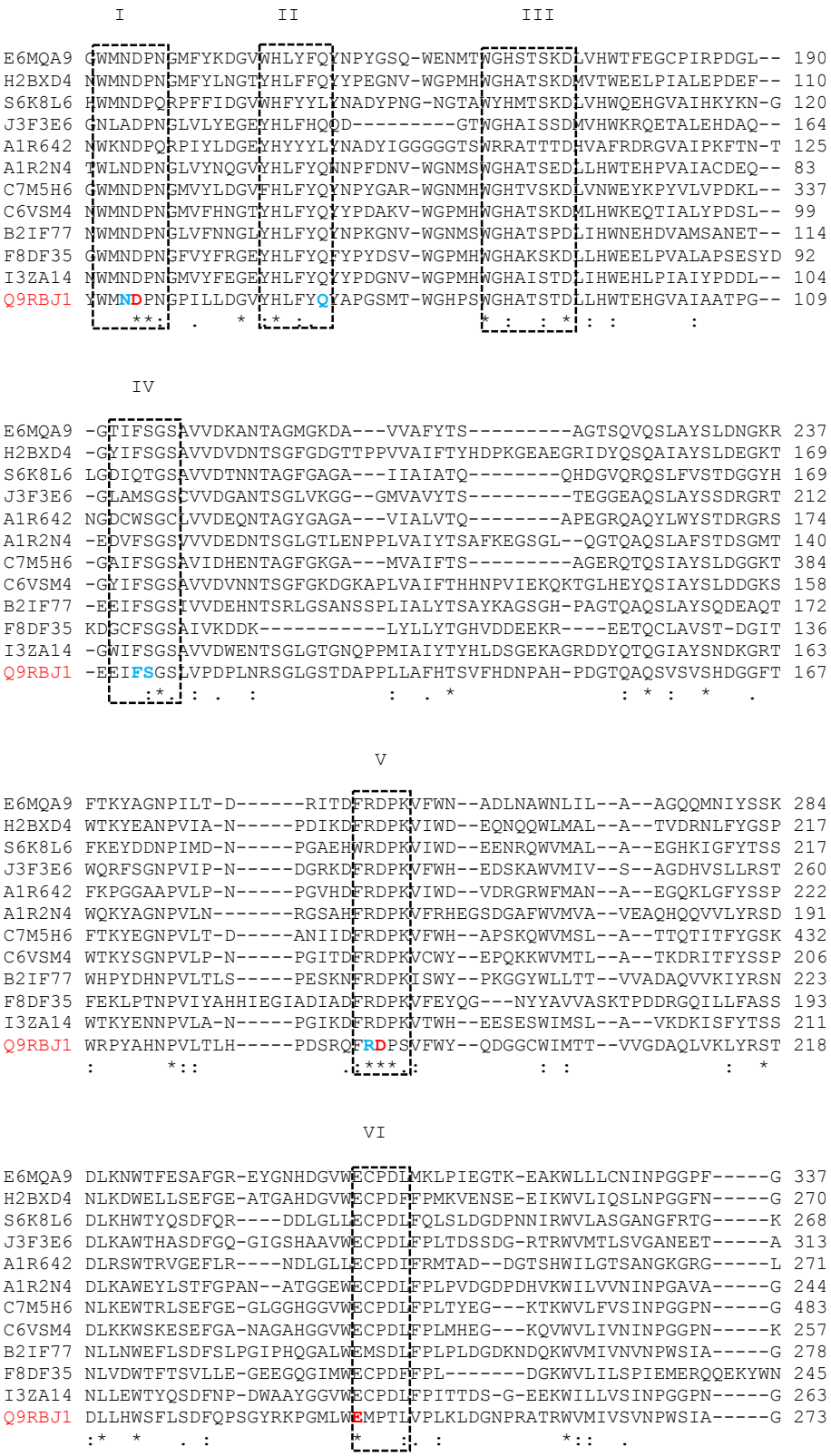
Runella slithyformis (strain ATCC 29530 / DSM 19594 / LMG 11500 / NCIMB 11436 / LSU 4)	-	11.21	6.12
Segniliparus rotundus (strain ATCC BAA-972 / CDC 1076 / CIP 108378 / DSM 44985 / JCM 13578)	-	<0.03	10.32
Singulisphaera acidiphila (strain ATCC BAA-1392 / DSM 18658 / VKM B-2454 / MOB10)	-	<0.03	7.54
Sphingomonas sanxanigenens DSM 19645 = NX02	-	<0.03	4.94
Sphingomonas sanxanigenens DSM 19645 = NX02	-	8.86	<0.03
Spirosoma linguale (strain ATCC 33905 / DSM 74 / LMG 10896)	-	13.68	24.52
Sporolactobacillus laevolacticus DSM 442	+	<0.03	<0.03
Sporolactobacillus laevolacticus DSM 442	+	<0.03	1.10
Sporolactobacillus laevolacticus DSM 442	+	<0.03	1.83
Stackebrandtia nassauensis (strain DSM 44728 / NRRL B-16338 / NBRC 102104 / LLR-40K-21)	+	2.71	6.01
Streptococcus mutans	+	60.43	104.55
Streptococcus parasanguinis (strain ATCC 15912 / DSM 6778 / CIP 104372 / LMG 14537)	+	68.32	73.08
Streptococcus pneumoniae 2082239	+	<0.03	<0.03
Streptococcus pyogenes GA41039	+	<0.03	0.54
Streptococcus salivarius SK126	+	<0.03	<0.03
Streptococcus suis (strain 05ZYH33)	+	<0.03	<0.03
Streptomyces viridochromogenes Tue57	+	<0.03	5.00
Subdoligranulum variabile DSM 15176	-	<0.03	95.43
Terriglobus roseus (strain DSM 18391 / NRRL B-41598 / KBS 63)	-	<0.03	4.37
Thermotoga maritima (strain ATCC 43589 / MSB8 / DSM 3109 / JCM 10099)	-	<0.03	0.41
Thermotoga neapolitana (strain ATCC 49049 / DSM 4359 / NS-E)	-	<0.03	<0.03
Tolumonas auensis (strain DSM 9187 / TA4)	-	19.92	14.45
Tolumonas auensis (strain DSM 9187 / TA4)	-	2.43	3.53
Vibrio natriegens NBRC 15636 = ATCC 14048 = DSM 759	-	66.67	65.68
Vibrio sinaloensis DSM 21326	-	<0.03	<0.03
Zunongwangia profunda (strain DSM 18752 / CCTCC AB 206139 / SM-A87)	-	26.29	14.69
Zymomonas mobilis subsp. *mobilis* (strain ATCC 10988 / DSM 424 / LMG 404 / NCIMB 8938 / NRRL B-806 / ZM1)	-	<0.03	0.66

Specific activity: reducing sugars quantified in μmol, released per mg of enzyme, per minute
LMW: low-molecular weight; HMW: high-molecular weight

3.4.3 Structure of Levanase Genes and the Role of Conserved Sequence Motifs

While the mechanistic and structural characteristics of microbial levanases remain poorly defined, numerous authors have investigated this with inulinases, which are also members of GH32 (Pouyez *et al.*, 2012). GH32 enzyme crystal structure consists of a biomodular arrangement: a N-terminal 5-fold β-propeller catalytic domain with 4 β-sheets and C-terminal β- sandwich domain organized in 2 β-sheets with 5 β-strands, which seems to maintain structural stability and may have a role in protein oligomerization (van Wyk *et al.*, 2013). Interestingly, it was noted that endo-inulinase contains a glutamate nucleophile residue, while in exo-inulinases, this is exchanged with an aspartate. Pouyez *et al.* (2012) speculated that this change is attributed with endo-acting glycosyl hydrolases. Indeed, it is evident in the genetic sequences of the selected levanases (Figure 3.3) that there are numerous glutamate residues (E) among the conserved sequence motifs.

It has been established that GH32 members possess catalytic amino acid residues that are conserved in the form of an aspartic acid (D) residue conducting the nucleophilic attack while a glutamic acid (E) residue acts as the general catalytic acid/base (Lammens *et al*., 2009). These 2 bases are generally located within conserved regions known as the WMN*D*PNG and *E*C motifs (catalytic residues italicized). As indicated by the multiple sequence alignments in Figure 3.2, this was true for the gene sequences encoding the levanases explored in our work. Another aspartic acid residue within a conserved R*D*P motif has been identified as the transition state stabilizer and forms a constituent of the catalytic triad with the other 2 residues. We observed this conserved sequence motif in the fifth catalytic site determined by multiple sequence alignment in our exploration of the top 10 candidate levanases. Wanker, Klingsbichel, and Schwab (1995) previously investigated the substrate specificity of *B. subtilis* levanase which was over-expressed in *E. coli*, and reported that the enzyme was able to hydrolyze levan, inulin, sucrose, and to a lesser extent, raffinose. Indeed, our investigation of the top candidate levanases shown in Table 3.3 reveal that they are all capable of hydrolyzing inulin (albeit to different degrees), which may


I
II
III
IV
V
VI
E6MQA9 GWMNDPNGMFYKDGVWHLYFQYNPYGSQ-WENMTWGHSTSKDLVHWTFEGCPIRPDGL-- 190
H2BXD4 NWMNDPNGMFYLNGTYHLFFQYYPEGNV-WGPMHWGHATSKDMVTWEELPIALEPDEF-- 110
S6K8L6 HWMNDPQRPFFIDGVWHFYYLYNADYPNG-NGTAWYHMTSKDLVHWQEHGVAIHKYKN-G 120
J3F3E6 GNLADPNGLVLYEGEYHLFHQQD---------GTWGHAISSDMVHWKRQETALEHDAQ-- 164
A1R642 NWKNDPQRPIYLDGEYHYYYLYNADYIGGGGGTSWRRATTTDHVAFRDRGVAIPKFTN-T 125
A1R2N4 TWLNDPNGLVYNQGVYHLFYQYNPFDNV-WGNMSWGHATSEDLLHWTEHPVAIACDEQ-- 83
C7M5H6 GWMNDPNGMVYLDGVFHLFYQYNPYGAR-WGNMHWGHTVSKDLVNWEYKPYVLVPDKL-- 337
C6VSM4 NWMNDPNGMVFHNGTYHLFYQYYPDAKV-WGPMHWGHATSKDMLHWKEQTIALYPDSL-- 99
B2IF77 NWMNDPNGLVFNNGLYHLFYQYNPKGNV-WGNMSWGHATSPDLIHWNEHDVAMSANET-- 114
F8DF35 GWMNDPNGFVYFRGEYHLFYQFYPYDSV-WGPMHWGHAKSKDLLHWEELPVALAPSESYD 92
I3ZA14 NWMNDPNGMVYFEGEYHLFYQYYPDGNV-WGPMHWGHAISTDLIHWEHLPIAIYPDDL-- 104
Q9RBJ1 YWMNDPNGPILLDGVYHLFYQYAPGSMT-WGHPSWGHATSTDLLHWTEHGVAIAATPG-- 109
E6MQA9 -GTIFSGSAVVDKANTAGMGKDA---VVAFYTS---------AGTSQVQSLAYSLDNGKR 237
H2BXD4 -GYIFSGSAVVDVDNTSGFGDGTTPPVVAIFTYHDPKGEAEGRIDYQSQAIAYSLDEGKT 169
S6K8L6 LGDIQTGSAVVDTNNTAGFGAGA---IIAIATQ--------QHDGVQRQSLFVSTDGGYH 169
J3F3E6 -GLAMSGSCVVDGANTSGLVKGG--GMVAVYTS---------TEGGEAQSLAYSSDRGRT 212
A1R642 NGDCWSGCLVVDEQNTAGYGAGA---VIALVTQ--------APEGRQAQYLWYSTDRGRS 174
A1R2N4 -EDVFSGSYVVDEDNTSGLGTLENPPLVAIYTSAFKEGSGL--QGTQAQSLAFSTDSGMT 140
C7M5H6 -GAIFSGSAVIDHENTAGFGKGA---MVAIFTS---------AGERQTQSIAYSLDGGKT 384
C6VSM4 -GYIFSGSAVVDVNNTSGFGKDGKAPLVAIFTHHNPVIEKQKTGLHEYQSIAYSLDDGKS 158
B2IF77 -EEIFSGSIVVDEHNTSRLGSANSSPLIALYTSAYKAGSGH-PAGTQAQSLAYSQDEAQT 172
F8DF35 KDGCFSGSAIVKDDK-----------LYLLYTGHVDDEEKR----EETQCLAVST-DGIT 136
I3ZA14 -GWIFSGSAVVDWENTSGLGTGNQPPMIAIYTYHLDSGEKAGRDDYQTQGIAYSNDKGRT 163
Q9RBJ1 -EEIFSGSLVPDPLNRSGLGSTDAPPLLAFHTSVFHDNPAH-PDGTQAQSVSVSHDGGFT 167
E6MQA9 FTKYAGNPILT-D------RITDFRDPKVFWN--ADLNAWNLIL--A--AGQQMNIYSSK 284
H2BXD4 WTKYEANPVIA-N-----PDIKDFRDPKVIWD--EQNQQWLMAL--A--TVDRNLFYGSP 217
S6K8L6 FKEYDDNPIMD-N-----PGAEHWRDPKVIWD--EENRQWVMAL--A--EGHKIGFYTSS 217
J3F3E6 WQRFSGNPVIP-N-----DGRKDFRDPKVFWH--EDSKAWVMIV--S--AGDHVSLLRST 260
A1R642 FKPGGAAPVLP-N-----PGVHDFRDPKVIWD--VDRGRWFMAN--A--EGQKLGFYSSP 222
A1R2N4 WQKYAGNPVLN-------RGSAHFRDPKVFRHEGSDGAFWVMVA--VEAQHQQVVLYRSD 191
C7M5H6 FTKYEGNPVLT-D-----ANIIDFRDPKVFWH--APSKQWVMSL--A--TTQTITFYGSK 432
C6VSM4 WTKYSGNPVLP-N-----PGITDFRDPKVCWY--EPQKKWVMTL--A--TKDRITFYSSP 206
B2IF77 WHPYDHNPVLTLS-----PESKNFRDPKISWY--PKGGYWLLTT--VVADAQVVKIYRSN 223
F8DF35 FEKLPTNPVIYAHHIEGIADIADFRDPKVFEYQG---NYYAVVASKTPDDRGQILLFASS 193
I3ZA14 WTKYENNPVLA-N-----PGIKDFRDPKVTWH--EESESWIMSL--A--VKDKISFYTSS 211
Q9RBJ1 WRPYAHNPVLTLH-----PDSRQFRDPSVFWY--QDGGCWIMTT--VVGDAQLVKLYRST 218
E6MQA9 DLKNWTFESAFGR-EYGNHDGVWECPDLMKLPIEGTK-EAKWLLLCNINPGGPF-----G 337
H2BXD4 NLKDWELLSEFGE-ATGAHDGVWECPDFFPMKVENSE-EIKWVLIQSLNPGGFN-----G 270
S6K8L6 DLKHWTYQSDFQR----DDLGLLECPDLFQLSLDGDPNNIRWVLASGANGFRTG-----K 268
J3F3E6 DLKAWTHASDFGQ-GIGSHAAVWECPDLFPLTDSSDG-RTRWVMTLSVGANEET-----A 313
A1R642 DLRSWTRVGEFLR----NDLGLLECPDIFRMTAD--DGTSHWILGTSANGKGRG-----L 271
A1R2N4 DLKAWEYLSTFGPAN--ATGGEWECPDLFPLPVDGDPDHVKWILVVNINPGAVA-----G 244
C7M5H6 NLKEWTRLSEFGE-GLGGHGGVWECPDLFPLTYEG---KTKWVLFVSINPGGPN-----G 483
C6VSM4 DLKKWSKESEFGA-NAGAHGGVWECPDIFPLMHEG---KQVWVLIVNINPGGPN-----K 257
B2IF77 NLLNWEFLSDFSLPGIPHQGALWEMSDLFPLPLDGDKNDQKWVMIVNVNPWSIA-----G 278
F8DF35 NLVDWTFTSVLLE-GEEGQGIMWECPDFFPL-------DGKWVLILSPIEMERQQEKYWN 245
I3ZA14 NLLEWTYQSDFNP-DWAAYGGVWECPDLFPITTDS-G-EEKWILLVSINPGGPN-----G 263
Q9RBJ1 DLLHWSFLSDFQPSGYRKPGMLWEMPTLVPLKLDGNPRATRWVMIVSVNPWSIA-----G 273


VII
VIII

Figure 3.2 Conserved sequence motifs in the top 10 candidate levanases as compared to the exo-levanase from *Gluconobacter diazotrophicus* (Q9RBJ1)

∗: Conserved sequence motif

Bacterial sources of reference levanase genes: *Guconoacetobacter diazotrophicus* (A9H667); *G. diazotrophicus* (Q8G179); *G. diazotrophicus* (Q9RBJ1); *Microbacterium laevaniformans* (Q93R69); *Bacillus subtilis* 168 (O07003); *B. licheniformis* (Q65EI7)

Bacterial sources of genes encoding selected candidate levanases: LEV4-E4: *Prevotella salivae* (E6MQA9); LEV4-F7: *Gillisia limnaea* (H2BXD4); LEV4-E11: *Streptococcus parasanguinis* (F8DF35); LEV5-C5: *Actinomyces naeslundii* (J3F3E6); LEV5-A7: *Arthrobacter aurescens* TC1 (A1R642); LEV4-B7: *A. aurescens* TC1 (A1R2N4); LEV4-D4: *Capnocytophaga ochracea* (C7M5H6); LEV4-D3: *Dyadobacter fermentans* (C6VSM4); LEV4-A10: *Belliella Baltica* (I3ZA14

	S6K8L6	Q65EI7	O07003	A1R642	Q93R69	Q8GI79	F8DF35	J3F3E6	E6MQA9	C6VSM4	C7M5H6	H2BXD4	I3ZA14	A9H667	Q9RBJ1	A1R2N4
S6K8L6	100	40.84	41.15	36.59	35.95	38.02	24.11	27.47	32.09	32.92	33.8	29.41	32.64	31.06	31.06	34.69
Q65EI7	40.84	100	65.37	45.36	47.83	51.11	26.12	29.88	30.85	32.65	33.95	32.34	35.2	28.69	28.69	32.9
O07003	41.15	65.37	100	44.4	46.26	51.51	27.68	28.95	32.42	31.77	35.11	32.07	34.78	28.63	28.63	34.41
A1R642	36.59	45.36	44.4	100	48.73	52.87	20.85	28.03	28.42	29.07	32.72	27	29.92	29.44	29.44	32.54
Q93R69	35.95	47.83	46.26	48.73	100	54.9	22.67	30.17	27.16	29.61	32.01	27.04	29.9	29.68	29.68	30.47
Q8GI79	38.02	51.11	51.51	52.87	54.9	100	23.94	29.88	29.6	31.2	34.15	31.72	33.19	28.69	28.69	34.2
F8DF35	24.11	26.12	27.68	20.85	22.67	23.94	100	27.93	26.87	29.45	30.96	33.33	31.97	28.2	28.2	34.17
J3F3E6	27.47	29.88	28.95	28.03	30.17	29.88	27.93	100	30.19	35.6	32.83	30.14	37.18	35.21	35.21	37.88
E6MQA9	32.09	30.85	32.42	28.42	27.16	29.6	26.87	30.19	100	43.01	43.47	38	39.53	33.4	33.4	39.64
C6VSM4	32.92	32.65	31.77	29.07	29.61	31.2	29.45	35.6	43.01	100	46.41	47.23	45.29	35.73	35.73	41.98
C7M5H6	33.8	33.95	35.11	32.72	32.01	34.15	30.96	32.83	43.47	46.41	100	44.51	49.17	33.73	33.73	41.88
H2BXD4	29.41	32.34	32.07	27	27.04	31.72	33.33	30.14	38	47.23	44.51	100	49.8	30.31	30.31	36.49
I3ZA14	32.64	35.2	34.78	29.92	29.9	33.19	31.97	37.18	39.53	45.29	49.17	49.8	100	36.11	36.11	39.09
A9H667	31.06	28.69	28.63	29.44	29.68	28.69	28.2	35.21	33.4	35.73	33.73	30.31	36.11	100	100	43.88
Q9RBJ1	31.06	28.69	28.63	29.44	29.68	28.69	28.2	35.21	33.4	35.73	33.73	30.31	36.11	100	100	43.88
A1R2N4	34.69	32.9	34.41	32.54	30.47	34.2	34.17	37.88	39.64	41.98	41.88	36.49	39.09	43.88	43.88	100

Figure 3.3 Degree of homology between the top 10 candidate levanases (black) and reference levanases (red) reported in literature
Bacterial sources of reference levanases: *Guconoacetobacter diazotrophicus* (A9H667); *G. diazotrophicus* (Q8G179); *G. diazotrophicus* (Q9RBJ1); *Microbacterium laevaniformans* (Q93R69); *Bacillus subtilis* 168 (O07003); *B. licheniformis* (Q65EI7)
Bacterial sources of candidate levanases: LEV4-E4: *Prevotella salivae* (E6MQA9); LEV4-F7: *Gillisia limnaea* (H2BXD4); LEV4-E11: *Streptococcus parasanguinis* (F8DF35); LEV5-C5: *Actinomyces naeslundii* (J3F3E6); LEV5-A7: *Arthrobacter aurescens* TC1 (A1R642); LEV4-B7: *A. aurescens* TC1 (A1R2N4); LEV4-D4: *Capnocytophaga ochracea* (C7M5H6); LEV4-D3: *Dyadobacter fermentans* (C6VSM4); LEV4-A10: *Belliella Baltica* (I3ZA14)

allude to the different types of substrates that are available in the environments of the bacteria from which they were sourced. The wide availability of 3D structures of plant GH32 members boosted structure-function studies (Van Den Ende *et al.*, 2009), which showed that enzymes preferentially employing sucrose as the donor substrate show a functional Asp/Arg or Asp/Lys couple in a hypervariable loop. Generally, the absence of this couple denotes the use of fructose as the preferred donor substrate. These authors further established that all hydrolases contain an intact hypervariable loop within the WMNDPNG motif and an intact WGW motif. Notably, only select amino acids in the vicinity of the active site appear to directly control substrate specificity. However, Van den Ende *et al.* (2009) were not able to elucidate which amino acids are ultimately responsible for the formation of β-(2,1)- versus β-(2,6)-linkages in fructans; this remains a question to be answered. Of interest in our investigation are the conserved motifs (denoted in blue) present in catalytic sites 1, 2, 4 and 5 (Figure 3.3) which remain uncharacterized. These conserved fragments are associated with larger levanases, specifically that from *C. ochracea*, which is 82.8 kDa (more than 20 kDa larger than previously characterized levanases found in literature and others in our study).

Tsujimoto *et al.* (2003) cloned the gene (*inu*A) encoding an exo-inulinase from *Geobacillus stearothermophilus* that was 56.7 kDa and exhibited high sequence similarity to not only *Pseudomonas mucidolens* exo-inulinase, but also *Bacillus subtilis* levanase and *Paenibacillus polyxma* fructosyltransferase among other enzymes, which justified the authors' conclusion that this enzyme is a member of GH32. As supported by other studies, analysis of multiple alignments of the *inu*A gene indicated that the N-terminal region of GH32 is highly conserved. Specific to this investigation, the inuA product sequence contains 3 conserved regions: 1) 21-WMN*D*ANGLVY-30; 2) 149-DFR*D*PKVFWH-158; and 3) 202-W*E*CP-205 (putative catalytic residues italicized). These same conserved motifs and conservation of the N-terminal region are evident in the genetic sequences of the selected levanases (Figure 3.3) and justify their further characterization.

3.4.4 Catalytic Properties of Top Levanase Candidates

Comparison of the top candidate levanases (Table 3.3) and those referenced in literature revealed a high degree of differentiability in terms of non-conserved sequence motifs (Figure 3.2). This mirrored results obtained from phylogenetic clustering, which showed broad distances in the familial origins of microbial levanases, and consequently, their similarities to one another.

Notably, the reference levanase-encoding genes (Figure 3.3) employed in microbial genome-mining for new homologues did not share more than 65.37% identity with the candidates selected for further characterization, which rationalizes the differences in biocatalytic activity observed among them (i.e., in terms of the mechanism for hydrolysis (exo- vs. endo-) and end-product yield), as well as their differences in molecular size. Of the levanases selected for reference, those from *B. subtilis* 168 (O07003) and *B. licheniformis* ATCC 14580 (Q65EI7) were most genetically similar according to Figure 3.3. It would be interesting to determine whether this relatively high level of genetic correspondence has any effect on their catalytic behaviour, especially in terms of the type(s) of end-products generated. Levanases among those selected as the top 10 biocatalysts in the new collection displayed much lower levels of similarity to one another and to those serving as reference.

While the majority of the levanases selected as the top candidates demonstrated preferential hydrolysis of levan over inulin (Figure 3.4), there were several exceptions, specifically those from *A. naeslundii* (LEV5-C5) and *P. salivae* (LEV4-E4). LEV5-C5 lacks the ability to hydrolyze high-molecular weight levan, while LEV4-E4 appears to display no differentiable substrate preferences where levans of low and high molecular weight and inulin are concerned. Contrastingly, the catalytic behavior of *A. aurescens* (LEV5-A7), *S. parasanguinis* (LEV4-E11) and *C. ochracea* (LEV4-D4) levanases was revealed to be highly substrate specific, as they only hydrolyzed inulin to minor extents and there was a distinct preference for low-molecular weight levan by LEV4-D4, while LEV4-E11 appeared to have equal affinities for low- and high-molecular weight levan, and LEV5-A7 hydrolyzed high-molecular levan much more readily than its low-molecular weight counterpart. With a few exceptions, the ranges of specific levanase activity on low- versus high-molecular weight levan were highly smilar. Among the top candidate levanases (Table 3.3), hydrolysis of low-molecular weight levan occurred at rates of 43.81-137.79 μmol/mg*min, with LEV4-D4 demonstrating the highest level of activity in this context. With regards to high-molecular weight levan, hydrolysis occurred at rates of 0-329.83 μmol/mg*min, with LEV5-A7 being most efficient in doing so. It should be noted that the upper limit in this range (329.83 μmol/mg*min) is not reflective of the general behavior of the selected candidates, which generally hydrolyze low-molecular weight levan more rapidly.

The strongest homology, 49.8%, occurs between *B. baltica* and *G. limnaea* levanases, which exhibit similar substrate specificities towards levan versus inulin (Figure 3.4). The genes encoding

C. ochracea and *B. baltica* (both Gram-negative) levanases also share a high degree of homology, and these biocatalysts not only exhibit similar behavior in terms of substrate specificity and end-product profile, but are also among the most thermally stable candidate levanases. Notably, crude extracts of *B. baltica* and *C. ochracea* levanases retained 77.97% and 84.62% of their maximal glycosyl hydrolase activity after a 1 hour heat treatment at 50°C (Table 3). In general, trends observed in the thermal stability of the selected levanases surpassed previous findings of other authors. After a 1-hour heat treatment at 50°C, only *A. naeslundii* (LEV5-C5), *D. fermentans* (LEV4-D3) and *G. limnaea* (LEV4-F7) retained less than 50% of their maximal catalytic activity, suggesting that the recombinant levanases discovered in our work are more heat durable than levanases reported in literature (Table 3.1). There appears to be no association between the taxonomy (i.e., Gram-stain, origin) of a bacterial levanase source and the thermal stability of the enzyme, thus suggesting that this property is solely determined by the microorganisms's genetic makeup and perhaps its dwelling habitat.

Table 3.3 Properties of the top 10 candidate bacterial levanases obtained by genome-mining

Enzyme Identifier	Bacterial Source	Gram +/-	Molecular Weight (kDa)	Residual Activity (%) After 1 hr at 50°C	Specific Activity on Substrate (μmol/mg*min)		
					LMW Levan	HMW Levan	Inulin
LEV4-A10	*Belliella baltica*	-	57.0	77.97	109.16	93.25	54.82
LEV4-B7	*Arthrobacter aurescens*	-	54.3	50.73	43.81	39.74	34.52
LEV4-D3	*Dyadobacter fermentans*	-	56.7	8.98	98.51	93.00	46.29
LEV4-D4	*Capnocytophaga ochracea*	-	82.8	84.62	137.79	102.79	42.56
LEV4-E4	*Prevotella salivae*	-	63.4	78.81	73.19	50.20	65.06
LEV4-E11	*Streptococcus parasanguinis*	-	55.8	76.58	68.32	73.08	20.44
LEV4-F7	*Gillisia limnaea*	+	61.7	10.08	48.91	120.11	34.78
LEV4-H2	*Vibrio natriegens*	-	60.4	73.29	66.67	65.68	30.14
LEV5-A7	*Arthrobacter aurescens*	+	57.6	65.02	121.67	329.83	37.69
LEV5-C5	*Actinomyces naeslundii*	-	61.1	46.99	43.99	0.00	50.21

Specific activity: release of reducing sugars per unit enzyme per minute (μmol/mg*min); residual activity: proportion of initial (maximal) glycosyl-hydrolytic activity retained post-heat treatment (1 hour at 50°C)

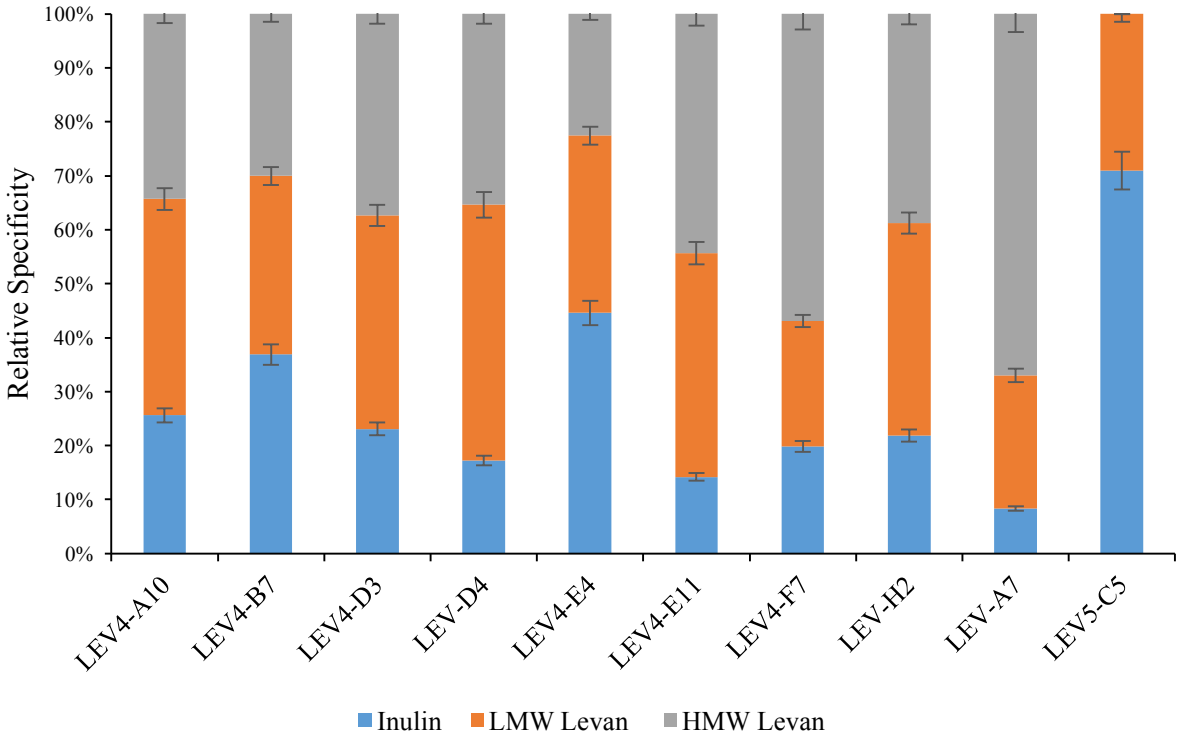


Figure 3.4 Relative substrate specificities of the top 10 candidate levanases
Bacterial levanase source: *Belliella baltica* (LEV4-A10); *Arthrobacter aurescens* (LEV4-B7); *Dyadobacter fermentans* (LEV4-D3); *Capnocytophaga ochracea* (LEV4-D4); *Prevotella salivae* (LEV4-E4); *Streptococcus parasanguinis* (LEV4-E11); *Gillisia limnaea* (LEV4-F7); *Vibrio natriegens* (LEV4-H2); *Arthrobacter aurescens* (LEV5-A7); and *Actinomyces naeslundii* (LEV5-C5)

3.4.5 End-Product Spectra of Top Candidate Levanases

As previously established, the majority of microbial levanases function exo-hydrolytically (Kang *et al.*, 1999; Lim *et al.*, 1998; Murakami *et al.*, 1990; Song *et al.*, 2002), with the exception of a select few (Table 3.1), such as the one present in *Bacillus* sp. No. 71, which produces oligosaccharides with DP > 7 (Murakami *et al.*, 1992). HPAEC-PAD analysis of the end-products generated by the candidate levanases investigated in our work not only supports the discovery of new members of the GH32 family, but more importantly, biocatalysts which convert levan to FOSs of varying sizes by endo-hydrolysis. Figure 5 shows that levanases sourced from *B. baltica* (LEV4-A10), *D. fermentans* (LEV4-D3), *C. ochracea* (LEV4-D4), *V. natriegens* (LEV4-H2), and *A. aurescens* (LEV5-A7) produce significantly higher levels of FOSs as compared to the remaining levanases studied in our work. It was interesting to note that in all cases, levantriose and levanbiose were the major FOS end-products generated, indicating that the selected candidate levanases preferentially synthesize short-chain FOSs. These findings are reflective of those observed by Song *et al.* (2002) for *Microbacterium laevaniformans* ATCC 15953. Notably, the activity of these levanases on inulin appeared to primarily result in the release of fructose and sucrose/blastose. LEV4-D4 generated the most diverse range of end-products, including FOSs of DP 2-4 with low-molecular weight levan as the substrate. In general, the same types of end-products were observed with high-molecular weight levan as for its low-molecular weight counterpart, with one exception: LEV5-C5, which evidently has no ability to hydrolyze high-molecular weight levan. The differences in end-products obtained from each candidate levanase in terms of quantity and degree of polymerization can be attributed to the differences in genetic makeup of the selected biocatalysts. In our ongoing work, the end-product spectra of the top candidate levanases will be further investigated by size-exclusion high-performance liquid chromatography in order to characterize any FOS end-products of DP>3, which have previously been obtained from *Streptomyces* sp. 366L (Lim *et al.*, 1998) and *Bacillus* sp. L7 (Miasnikov, 1997).

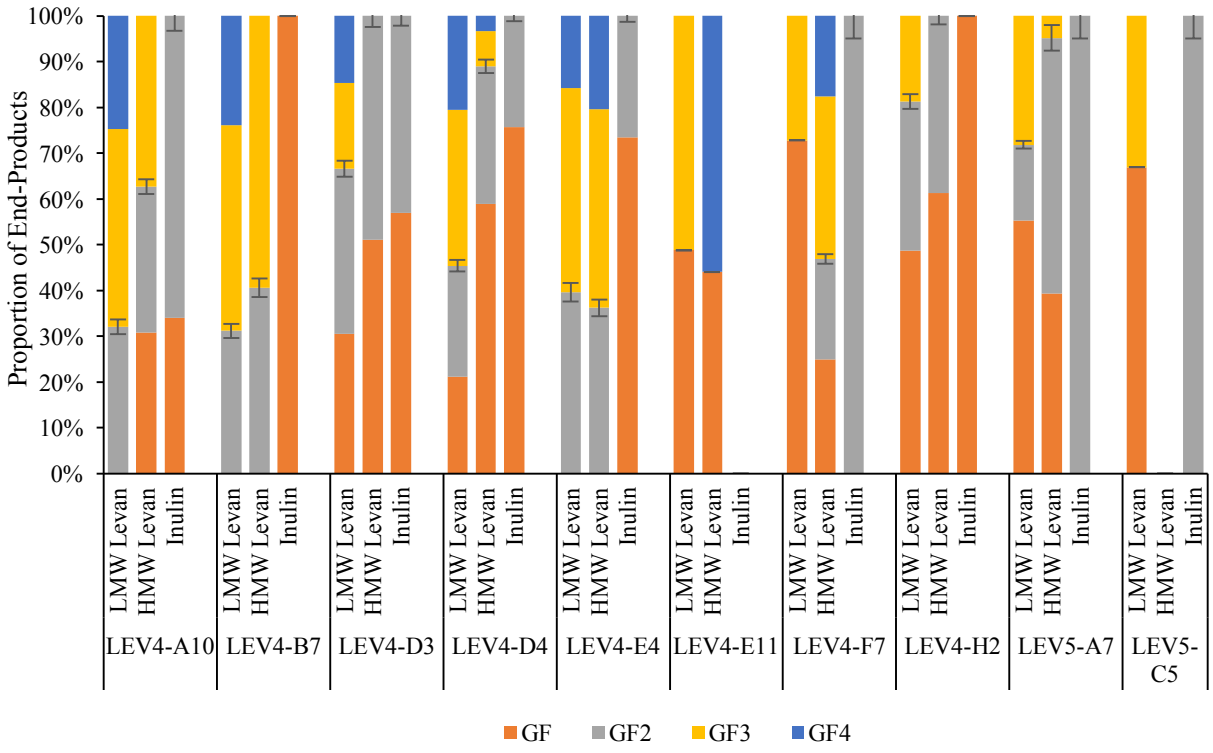


Figure 3.5 End-product spectra of the top 10 candidate levanases, with emphasis on the FOSs generated

Bacterial levanase source: *Belliella baltica* (LEV4-A10); *Arthrobacter aurescens* (LEV4-B7); *Dyadobacter fermentans* (LEV4-D3); *Capnocytophaga ochracea* (LEV4-D4); *Prevotella salivae* (LEV4-E4); *Streptococcus parasanguinis* (LEV4-E11); *Gillisia limnaea* (LEV4-F7); *Vibrio natriegens* (LEV4-H2); *Arthrobacter aurescens* (LEV5-A7); and *Actinomyces naeslundii* (LEV5-C5)

3.5 Conclusion

Data-mining of microbial genomes for sequences homologous to exo- and endo-levanases produced 1902 hits amenable to cloning. Among 140 representative candidates expressed in *E. coli* BL21(DE3) and screened for levanase activity, substantial diversity was observed not solely in terms of phylogenetic origin, but also the degree to which low- and high-molecular weight levans were hydrolyzed. This resulted in the selection of 10 viable biocatalysts found in various bacterial species which exhibited preferential hydrolysis of levan, while also demonstrating varying levels of glycosyl hydrolase activity on inulin. The end-product spectra of these levanases were investigated by HPAEC-PAD, which revealed differing levels of endo- versus exo-activity, and provided an indication of the types of FOS end-products which could be obtained from this new collection of microbial levanases. Generally, levanbiose and levantriose were the major FOSs obtained from the reaction of these enzymes with low-molecular weight levan. This is promising as the majority of microbial levanases investigated to date function in an exo-hydrolytic manner, releasing fructose as the sole reaction product. The top candidate levanases, selected according to their specific hydrolytic activity on levan with an end-product profile reflecting the mechanistic action of an endo-glycosyl hydrolase, were subjected to thermal stability assays, which revealed that several candidates, specifically *B. baltica* and *C. ochracea* levanases, far surpassed the heat durability of levanases previously characterized in literature. They, along with other viable levanases discovered in our work, will be further investigated in terms of enzyme kinetics, optimal reaction parameters, and will undergo more thorough profiling of their FOS product yields when different quantities of enzyme are employed.

CONNECTING STATEMENT II

The screening of 123 new, genome-mined levanases on the basis of levan hydrolysis, substrate specificity and end-product spectrum led to the elucidation of 10 top candidates in Chapter III. Chapter IV investigates these biocatalysts further, specifically with regards to their individual catalytic and reaction parameters, thermal stability and end-product profiles when different enzyme units are employed. The present work concludes with the design and trialling one-step and step-wise bi-enzymatic systems employing the top 2 candidate levanases alongside high levan-producing levansucrases for the synthesis of prebiotic levan-type FOS.

The results of this chapter were presented in the poster sessions at BioTrans 2017, the 13th International Symposium on Biocatalysis and Biotransformations, held in Budapest, Hungary.

CHAPTER IV.

CHARACTERIZATION OF TOP LEVANASE CANDIDATES AND THEIR APPLICATION IN A BI-ENZYMATIC SYSTEM FOR THE SYNTEHSIS OF LEVAN-TYPE FOSs

4.1 Abstract

Endo-levanases (E.C.3.2.1.65) are glycosyl-hydrolytic enzymes, which characteristically catalyze the hydrolysis of levan β-(2,6)-linkages, yielding fructooligosaccharides (FOSs) and oligolevans promising higher prebiotic potential than commercial β-(2,1)-/inulin-type FOSs. Due to their scarcity, genome-mining for novel endo-levanases was performed with reference genes in a representative protein database, revealing 140 bacterial candidates upon phylogenetic screening. 5 viable levanases were selected for further investigation from this new collection as they demonstrated the highest levels of hydrolysis of levan and product spectra which reflected the higher yields of FOS products compared to the levanases they were screened against. *Belliella baltica*, *Dyadobacter fermentans*, *Capnocytophaga ochracea*, *Vibrio natriegens*, and *Arthrobacter aurescens* levanases produced the highest yields of β-(2,6)-FOSs on low-MW levan as the substrate. These levanases were relatively stable across varying reaction conditions, but generally performed optimally at 37°C and pH 6.4. *B. baltica* and *C. ochracea* levanases exhibited the highest thermal stability, retaining 62.2% and 64.9% of their initial activity, respectively, after 6 hours at 50°C. Employed simultaneously with levansucrase, these levanases also demonstrated the highest bioconversion of levan into oligolevans and β-(2,6)-FOSs varying in degree of polymerization. The maximal yield of FOS products was observed in a one-step bi-enzymatic system involving *G. oxydans* levansucrase and *B. baltica* levanase, which has promising applications in the food and pharmaceutical industries for the production of functional ingredients and nutraceuticals.

4.2 Introduction

Awareness of prebiotics is becoming increasingly widespread among consumers, owing to their integral roles in promoting gastrointestinal and overall health. Fructooligosaccharides (FOSs) constitute an emerging class of non-digestible oligosaccharides which fulfill the criteria for prebiotic classification (Roberfroid, 2007). This is evidenced by their resistance to degradation during transit through the upper intestinal tract (host to the majority of human digestive enzymes), and by the manner in which they serve as selective energy sources for beneficial microbial populations in the human colon (Slavin, 2013; Bouhnik *et al*., 2004). Further health-promoting activities of fructans have been reported, including, but not limited to, their anti-tumor and anti-obesity effects in animal models (He and Shi, 2017). In addition to the health benefits conferred by short-chain FOSs, they are also suitable for industrial applications in the form of low-calorie and non-cariogenic sweeteners due to their sweet taste. Contrastingly, longer chain fructans generally possess a neutral taste and tend to form emulsions which simulate fat in texture (Caputi *et al*., 2013).

The increasing recognition of the health benefits of FOSs and a more comprehensive awareness of their structure-attribute relationships underline the need to develop efficient biocatalytic approaches for the synthesis of novel and controlled-size FOS products. While β-(2,6)-FOSs and neoFOSs exhibit prebiotic effects surpassing those of β-(2,1)-FOSs (the only commercially available type), reports of their biocatalytic production are few, attributable to limited exploration in this context (Bello *et al*., 2001). However, evidence to support the higher prebiotic capacity of β-(2,6)-FOSs (Kilian *et al*., 2002) and their anti-adhesion activity against pathogens (Shibata *et al.*, 2009) justify the quest for new sources and methods for the synthesis of tailor-made fructans with more useful physical and biological properties (Rastall and Gibson 2015; Tian, Khodadadi, and Karboune, 2014). Enzymatic strategies for the production of FOSs are generally based on the action of fructofuranosidases (EC 3.2.1) and fructosyltransferases (EC 2.4.1) (Lombard, 2014). Despite the wide availability of fructofuranosidases, however, their application in this context is limited by narrow acceptor specificity, as well as low to modest yields (Yun, 1996). Levansucrase (EC 2.4.1.10), a fructosyltransferase, is gaining increased interest owing to its ability to directly employ the free energy of cleavage of non-activated sucrose to transfer the fructosyl group to a variety of acceptors including monosaccharides (exchange), oligosaccharides (FOS synthesis), and growing fructan chains (polymer synthesis) (Strube *et al*.,

2011). Alternate sources which can be exploited for the production of FOSs include bacterial levan that can be hydrolyzed to controlled-size FOSs by the activity of levanase (EC 3.2.1.65). Murakami *et al.* (1990) previously classified these enzymes into 6 categories: 1) producing only fructose (exo-levanases); 2) producing primarily levanbiose (levanbiohydrolases); 3) producing mainly levanbiose and levantriose; 4) producing di-D-fructofuranose-(2,6'):(2',6)-dianhydride; 5) producing FOSs with degrees of polymerization (DP) in the range of 5-9; and 6) producing a series of FOSs. The majority of levanases function as fructose- or levanbiose-producing exo-hydrolases, exemplified by those occurring in *Bacillus subtilis*, *Actinomyces viscosus*, *Bacteroides fragilis* and *B. stearothermophilus* (Menéndez *et al*., 2002; Miasnikov, 1997; Pereira *et al.*, 2001). Generally, these enzymes are not only capable of hydrolyzing levan, but also inulin, raffinose and sucrose, although with different substrate specificities. In nature, numerous bacterial species synthesize levan from exogenous sucrose by the action of extracellular levansucrases. Interestingly, a major proportion of these microorganisms also secrete levanases to degrade levan under circumstantial conditions (Menéndez *et al.*, 2002). This alludes to the ability to levansucrases and levanases to work in sync for the production of short-chain FOSs and oligolevans. A bi-enzymatic system employing levansucrase and levanase for the production of FOSs was previously developed in our laboratory, specific for the bioconversion of sucrose (a cost-effective and widely available starting material) to FOSs with prebiotic potential (Tian, Karboune, and Hill, 2012). Tian, Khodadadi and Karboune (2014) were the first to optimize the production of controlled-size FOS products from *Bacillus amyloliquefaciens* and a commercial inulinase (also capable of hydrolyzing levan). It was determined that a one-step system, in which *B. amyloliquefaciens* levansucrase and the inulinase were simultaneously reacted with sucrose, produced the most desirable results in terms of FOS yield and end-product profile. The unavailability of levanases at the time the study was performed may have hampered the types and quantity of FOSs synthesized (since inulinases are not specific for levan), demanding further insight into the cooperativity between true levansucrase-levanase pairs, as investigated in the present work. A major focus in our investigation was on the detailed characterization of top candidate levanases (obtained by employing Basic Local Alignment Search Tool (BLASTP) was employed against UniProtKB) in terms of their kinetic and reaction (pH, temperature) parameters, with emphasis on the FOS end-products generated. Due to the high specific activities (towards levan) and endo-hydrolytic behavior of *B. baltica* and *C. ochracea* levanases, these biocatalysts were selected for investigation with newly discovered levansucrases

from *Gluconobacter oxydans* and *Vibrio natriegens*, which are both characterized by high levels of levan production. The aforementioned levansucrases and levanases were paired in a simultaneous and step-wise manner, allowing for detailed analysis of the reaction kinetics of FOS production, as well as the concerted action of these enzymes. The present work details advancements in the exploration of endo-levanases which has revealed enzymes that demonstrate stability across a wide range of reaction parameters (i.e., pH and temperature), and are promising for the production of levan-type FOSs bi-catalytically with levansucrase.

4.3 Materials and Methods

4.3.1 Chemicals and Materials

Growth media preparations, lysogeny broth (LB) constituents (tryptone, NaCl, and yeast extract) broad spectrum antibiotics (ampicillin and carbenicillin), and the protein expression inducer, IPTG, were purchased from Thermo Fisher Scientific (Mississauga, ON, Canada). Mono- and disaccharide standards (i.e., D-(-)-fructose, D-(+)-glucose and sucrose) were obtained from Sigma-Aldrich (Oakville, ON, Canada). Fructooligosaccharide standards (i.e., 1-kestose, nystose and 1-fructofuranosylnystose were purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan). Other chemicals and reagents, including inulin, 3,5-dinitrosalicylic acid, potassium sodium (+)-tartrate, K_2HPO_4, KH_2PO_4, NaOH and NaOAc, imidazole, PIPES, bovine serum albumin, and Bradford Reagent were also purchased from Sigma-Aldrich (Oakville, ON, Canada). Purification of levanases was performed using GE Healthcare Life Sciences HisTrap™ FF 1 mL columns (Mississauga, ON, Canada).

4.3.2 Levanase Production, Recovery and Purification

E. coli transformed by the insertion of levanase-encoding genes (from *Belliella baltica* (LEV4-A10), *Dyadoacter fermentans* (LEV4-D3), *Capnocytophaga ochracea* (LEV4-D4), *Vibrio natriegens* (LEV4-H2) and *Arthrobacter aurescens* (LEV5-A7)) were aerobically pre-cultured overnight in LB media with the addition of ampicillin (0.1 mg/mL final concentration) at 37°C and 250 rpm (New Brunswick Scientific *Excella E24 Incubator Shaker Series*). Inoculation of TB media (47.6 g/L) with carbenicillin (0.1 mg/mL final concentration) followed under the same conditions for 24 hours prior to the addition of IPTG (1 mM final concentration) to induce protein expression. Incubation proceeded at 18°C for 20 hours before cell mass was obtained by

centrifugation at 4°C (7003 x g for 20 minutes). Cells frozen at -80°C were re-suspended in sonication buffer (glycerol, PIPES, NaCl) prior to being treated with lysozyme (4 mg/mL) and DNase (2000 U; 4 μL/mL). This cell suspension was ultrasonicated and centrifuged (8817 x g rpm for 1 hour) to obtain crude enzyme extract, which was subsequently dialyzed against 5 mM potassium phosphate buffer (pH 6.0) with a membrane cut-off of 5-6 kDa at 4°C, and lyophilized at -40°C. Purification was performed by immobilized metal anion chromatography (IMAC) on HisTrap™ FF 1 mL columns using an imidazole gradient ranging from 5 mM-1 M in pH 6.4 wash buffer (30 mM NaCl, 10 % (v/v) glycerol, 30 mM PIPES) as the eluent.

4.3.3 Electrophoretic Analysis of Purified Levanases

The efficacy of crude levanase purification by IMAC was verified by performing SDS-PAGE on each fraction collected along an imidazole gradient (sonication buffer, wash buffer, 5 mM imidazole, 10 mM imidazole, 100 mM imidazole, 200 mM imidazole, 1 M imidazole). 15% polyacrylamide gels were handcasted according to Bio-Rad (Saint-Laurent, QC, Canada) specifications. SDS-PAGE was conducted at 120 V until full sample migration was achieved.

4.3.4 Levanase Activity Assays

The glycosyl-hydrolytic activity of each levanase was assayed by quantitating the release of reducing sugars by the 3,5-dinitrosalicylic acid (DNS) method using low- and high-molecular weight levan (5.5-6.25 and 2.8[3] kDa, respectively, obtained from *Bacillus amyloliquefaciens* levansucrase), as well as inulin as substrates. 250 μL levanase (6-25 μg protein) diluted in 50 mM potassium phosphate buffer (pH 6.0) was reacted with 250 μL substrate (1% w/v) for 20 minutes at 37°C. This was followed by the addition of 750 μL DNS reagent (DNS, NaOH) before boiling for 5 minutes to inactivate enzyme activity. 250 μL potassium sodium tartrate (50% w/v) were subsequently added to stabilize the colorimetric reaction. Total levanase activity was defined as the release of 1 μmol of fructose per minute. Specific levanase activity following purification was determined by the Bradford method with the use of bovine albumin standards (1-30 μg/mL) were used.

4.3.5 Determination of Kinetic Parameters

To determine the kinetic parameters (i.e., v_{max}, K_m, k_{cat}) and catalytic efficiency of each candidate levanase, glycosyl hydrolytic activity was investigated at select low- and high-MW levan concentrations, ranging from 0.1-1.0% (w/v). The response of each levanase to incrementally increasing substrate concentration was plotted on a saturation curve and kinetic parameters were computed using SigmaPlot® 13 software based on the Hill model, as indicated below:

$$v = \frac{v_{max\,app}[S]^n}{(K_{m\,app})n + [S]^n}$$

Using the estimated molecular weight (E_t = [E] * MW) and the maximum velocity (v_{max}), the turnover number k_{cat}, representing catalytic efficiency, was calculated for each levanase as follows:

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

4.3.6 Effects of pH and Temperature

The effect of pH on levanase activity was investigated at pH 3-9 by using various 50 mM buffers as follows: sodium citrate (pH 3.0-3.5), sodium acetate (pH 4.0-5.5), potassium phosphate (pH 6.0-7.0) and Tris-HCl (pH 7.5-9.0). The optimal reaction temperature for each levanase was determined by performing activity assays over a wide temperature range (20-60°C) in 50 mM pH 6.0 potassium phosphate buffer. These assays were carried out using 1.0% (w/v) low-molecular weight levan as the substrate.

4.3.7 Thermal Stability

Candidate levanases were incubated at 50°C after initial glycosyl-hydrolytic activites were measured at 37°C. Every 15 min for 120 min, an aliquot was taken and the residual hydrolytic activity of each levanses according to the reducing sugar assay previously described.

4.3.8 Investigation of Levanase-Catalyzed End-Products by HPAEC-PAD and SE-HPLC

The product spectra of levanase-catalyzed reactions on levan (low- and high-molecular weight) and inulin were quantified using high performance anion exchange chromatography coupled with pulsed amperometric detection (HPAEC-PAD) on the Dionex ICS-3000 equipped with a CarboPac® PA200 column (3 x 150 mm) and Chromeleon™ 7.2 software. A linear gradient of 0-200 mM NaOAc in 100 mM NaOH was applied over a duration of 20 minutes to elute carbohydrate compounds of interest. Prior to chromatographic investigation, reaction mixtures

consisting of diluted levanase solution and substrate were boiled to inactivate enzyme activity, and any un-hydrolyzed substrate polymers (i.e., levan/inulin) were precipitated with the addition of methanol (1:1 v/v) followed by centrifugation (8817 x g for 5 minutes). Identification and quantification of specific carbohydrates were performed using glucose, fructose, sucrose and fructooligosaccharide (i.e., 1-kestose, nystose, 1-fructofuranosylnystose) as standards (1-100 μM). Size-exlusion high performance liquid chromatography (SE-HPLC) on the Waters® 1525 system equipped with TSKgel PWXL-5000 and PWXL-3000 columns and Breeze™2 software was employed for the identification and quantitation of products obtained from levansucrase-levanase bi-enzymatic systems examined in our research. In this context, 0.1 M NaCl served as the eluent and dextran standards of 1-670 kDa (0.5-1.5 mg/ml) were used in addition to fructose and sucrose.

4.4 Results and Discussion

4.4.1 *Kinetic Parameters of Top Candidate Levanases*

Kinetic parameters of levanases from *B. baltica* (LEV4-A10), *D. fermentans* (LEV4-D3), *C. ochracea* (LEV4-D4), *V. natriegens* (LEV4-H2) and *A. aurescens* (LEV5-A7) were determined using low- and high-molecular weight levan as substrates. We observed that the kinetics of the levanases investigated in our work (Table 4.1) follow the Hill model rather than the Michaelis-Menten model. This was owing to the fact that each levanase demonstrated exponential increases in hydrolytic activity in response to linearly increasing substrate concentration preceding the saturation stage. A Hill coefficient (nH) less than 1 reveals negative cooperativity among the levanase's binding sites for the hydrolysis of levan, while a value higher than 1 indicates synergistic cooperativity. As summarized in Table 4.1, the Hill coefficients indicate that, in general, there was positive cooperativity between the active site and other ligand-binding sites on the enzyme surface. In fact, this type of behavior is common to a number of polysaccharide hydrolases, which possess a single catalytic domain linked at either the N- or C-terminus to one or more polysaccharide (ligand) binding sites (Miasnikov, 1997). It is interesting to note that *A. aurescens* levanase (LEV5-A7) was the only candidate to demonstrate more rapid hydrolysis of high-molecular weight levan than low-molecular weight levan, as reflected in the corresponding v_{max} values (of 122.52 μmol/mg*min and 325.53 μmol/mg*min, respectively). This is supported by its Hill coefficient for high-molecular weight levan, which is the only one showing positive cooperativity among the top 5 candidates. It can be hypothesized that, contrary to other levanases

which were investigated alongside it, *A. aurescens* levanase has preference for high-molecular weight levan due to exposure of the host microorganism to this substrate in its natural habitat. As indicated in Table 4.1, the maximum rate of levan hydrolysis (v_{max}), defined as the quantity of fructose released in μmol per mg enzyme per minute, is highly differentiable among the levanases studied. LEV4-D4, the levanase from *C. ochracea*, exhibited the highest hydrolytic activity on low-molecular weight levan (140.00 μmol/mg*min) and affinity for this substrate (k_m = 1.91μM) with a lower rate of biocatalysis on high-molecular weight levan, a trend observed for the majority of the top candidate levanases in Table 4.1. Notably, LEV5-A7 demonstrated the highest specific activity considering all other candidates in Table 1, specifically towards high-molecular weight levan (325.52 μmol/mg*min), and its k_m of 1.13 μM further supports its high affinity for high-molecular weight levan. Comparatively, LEV4-H2 had the lowest activity among the top candidate levanases investigated, releasing fructose at 75.41 and 71.03 μmol/mg*min, respectively, and according to Figure 2, also exhibited significantly higher exo-hydrolytic activity rather than the desired endo-type. In addition, it is evident from Table 2.1 that it has substantially lower affinity for low- and high-molecular weight levan than the other top candidate levanases, with k_m values of 3.70 and 4.01 μM , respectively. Wanker, Klingsbichel, and Schwab (1995) previously characterized the enzyme kinetics of *B. subtilis* levanase for which they found maximum velocity of levan hydrolysis to be 0.96 nmol/s, with an associated K_m of 1.2 μM and k_{cat} of 370 s^{-1}. As indicated in Table 4.1, the candidate levanases selected in our investigation are much more efficient in hydrolyzing levan than this characterized levanase. In other studies, Dahech *et al.* (2013) reported the maximum specific activity of the levanase they isolated from a Tunisian thermal source to be 226.66 μmol/mg*min, which is more consistent with the activity levels exhibited by our top candidate levanases.

Table 4.1 Kinetic parameters of the top 5 levanase candidates following the Hill model

Enzyme Identifier	Levanase Source	Substrate	v_{max} ($\mu_{mol/mg*min}$)	K_m (μM)	k_{cat} (s^{-1})	Catalytic Efficiency (k_{cat}/K_m)	Hill Coefficient
LEV4-A10	*B. baltica*	LMW Levan	112.22 ± 14.20	3.68	106.61	28.97	1.34
		HMW Levan	103.25 ± 18.31	3.75	98.09	26.16	1.00
LEV4-D3	*D. fermentans*	LMW Levan	106.98 ± 11.56	3.79	101.10	26.68	0.98
		HMW Levan	104.98 ± 13.03	3.79	99.21	26.18	0.98
LEV4-D4	*C. ochracea*	LMW Levan	140.00 ± 12.46	1.91	193.20	101.15	2.09
		HMW Levan	114.52 ± 9.98	3.63	158.04	43.54	0.82
LEV4-H2	*V. natriegens*	LMW Levan	74.91 ± 10.09	3.70	75.41	27.93	1.08
		HMW Levan	70.56 ± 8.56	4.01	71.03	23.60	0.77
LEV5-A7	*A. aurescens*	LMW Levan	122.52 ± 19.23	3.88	117.62	30.31	1.44
		HMW Levan	325.52 ± 25.07	1.13	312.50	75.67	1.48

v_{max}, measured as the release of reducing sugars from low- or high-molecular weight levan in mmol per mg enzyme per minute
K_m, the concentration (mM) of low- or high-molecular weight levan reached at ½ v_{max}
k_{cat}, the levanase turnover number, indicating the quantity of substrate conversion to product per second

Comparatively, all of the candidate levanases characterized in our work are hydrolyze levan at highly efficient rates when compared to those that have been previously reported; however, the bioconversion of substrate to end-product occurs less efficiently than the aforementioned *B. subtilis* levanase. This is indicated by the k_{cat} values in Table 4.1, which show that *C. ochracea* levanase (LEV4-D4) was most efficient in hydrolyzing levan and thereby generating FOS end-products, although its k_{cat}, 193.2 s^{-1}, is still lower than that for *B. subtilis* levanase. With respect to catalytic efficiency, LEV4-D4 levanase from *C. ochracea* is capable of generating FOS end-products most rapidly, followed by LEV5-A7, the levanase from *A. aurescens*, with enzyme turnover values of 101.15 and 75.67, respectively. The remaining top candidate levanases demonstrated substantially lower substrate turnover rates, with LEV4-H2, the levanases from *V. natriegens* being least efficient in this context. This is owing to its relatively low specific activity compared to the other candidates.

4.4.2 *Effect of pH on Levanase Activity*

The effect of pH on levanase activity was investigated between pH 3.0-9.0 using low-molecular weight levan as substrates. As indicated in Figure 4.1, the top candidate levanases displayed different pH activity profiles. In general, hydrolytic activity on low-molecular weight levan was drastically decreased below pH 5.0 and above pH 7.0. While certain levanases remained relatively stable across a broad pH range, for instance that from *C. ochracea* (LEV4-D4), the majority of the levanases investigated lost activity rapidly as pH strayed from the neutral range. Further, the levanases in our research appeared to be more drastically affected by acidic conditions than alkaline.

The pH activity profiles of LEV4-D3, LEV4-D4, LEV4-H2 and LEV5-A7 displayed common features, and were active within the range of pH 5.0-8.0. It is evident from Figure 1 that LEV4-A10 was most sensitive to fluctuations in pH, virtually having no activity below pH 5.0 and above pH 7.0, with its maximal activity being achieved sharply as the pH increases from 5.0 to 6.4. In a study which investigated the effect of pH on *Streptomyces* sp. 366L levanase, Lim *et al.* (1998) observed that maximal enzyme activity was achieved at approximately pH 7.0, and rapidly declined under conditions below and above this value. This trend is consistently observed among the levanases investigated in our work.

Interestingly, although the optimal pH for the levanase studied by Lim *et al.* is comparable to the values reported in the present work, it is evident from Figure 4.1 that the candidate levanases we investigated generally exhibit significantly higher stability against changes in pH. According to Figure 1, the optimal pH for the levanases in our research is 6.0, with the exception of *B. baltica* (LEV-A10) levanase, which achieves its maximal activity at pH 6.4. The data collected in our work concerning the effect of pH on levanase activity is also corroborated by Menéndez *et al.* (2002), who reported that for *G. diazotrophicus* SRT4 levanase, maximal enzyme activity is reached at pH 6.0. They further reported enzyme inactivation below pH 4.0 and drastically decreased activity above pH 7.0. Wanker, Klingsbichel, and Schwab (1995) have also previously examined the sensitivity of *B. subtilis* levanase to changes in its environmental pH. They concluded that the purified enzyme exhibited highest levels of activity within the broad pH range of 5.0-6.5, and decreased drastically below pH 4.0 and above pH 7.0, which is more consistent with findings in the present work.

4.4.3 *Effect of Temperature on Levanase Activity and Stability*

The effect of reaction temperature on levanase activity was also examined over a wide temperature range (20-60°C). The results show different temperature profiles for the hydrolytic activity of levanases. LEV4-D4, LEV4-H2 and LEV5-A7 achieved maximal activity at 37°C, while LEV-A10 and LEV4-H2 required higher temepratures to function at their peak capacity, 47 and 40°C, respectively. The results also show that the ascending stage of the temperature-activity profiles were dissimilar, suggesting that the activation step for catalysis determined by temperature is different for the top candidate levanases. Indeed, LEV4-D3, LEV4-D4 and LEV5-A7 reached their optimal temperatures relatively rapidly as compared with LEV4-A10 and LEV4-H2, which steadily increased in activity above 20°C. Generally, the top candidate levanases investigated in our work displayed minimal activity below 27°C. The hydrolytic activity of LEV4-D3 and LEV5-A7 decreased sharply at temperatures above 40°C, a trend also seen with LEV4-D4 above 37°C. For instance, LEV4-D4 activity decreased by 38.76% when the incubation temperature was increased from 37 to 47°C, and LEV4-D3 only retained 34.27% of its original activity at 57°C. These differences reveal the occurrence of certain conformational changes to the aforementioned levanases upon incubation at temperatures exceeding 37°C. Most notably, LEV4-A10 activity was observed at higher temperatures, as this levanase demonstrated its maximal activity at 47°C, which

is substantially higher than the other levanases we studied and those previously reported. For instance, Lim *et al.* (1998) reported 40°C to be the optimal temperature for *Streptomyces* sp. 366L levanase, while Song *et al.* (2002) determined that the levanase from *Microbacterium laevaniformans* ATCC 15953 reached its maximum rate of activity at 30°C. An average optimal temperature of 37°C has been gathered for levanases (Chaudhary *et al*., 1996; Kang *et al*., 1999, Lim *et al*., 1998; Murakami *et al.*, 1990; Song *et al.*, 2002), which is more consistent with the findings for levanases from LEV4-D3, LEV4-D4 and LEV5-A7. It is also interesting to note that *C. ochracea* (LEV4-D4) and *A. aurescens* (LEV5-A7) levanases are especially sensitive to changes in temperature, showing drastically reduced activity below 30°C and above 40°C. This may be attributed the environmental conditions in which these source bacteria naturally dwell, suggested to be within the physiological range.

The thermal stability of the selected levanases was investigated at 50°C using 1.0% (w/v) low-molecular weight levan as the substrate. Thermal inactivation of these enzymes followed second order kinetics (data not shown). It is evident from the data summarized in Table 4.2 that *B. baltica* (LEV4-A10) and *C. ochracea* (LEV4-D4) levanases exhibited the most resilience to thermal treatment, notably retaining 62.2% and 64.9% of their initial activity, respectively, after 6 hours at 50°C. In contrast, *D. fermentans* (LEV4-D3) levanase was observed to be the most sensitive to elevated temperatures, having a half-life of only 2.4 minutes at 50°C. It is interesting to note that, while the 5 candidate levanases all function optimally within the range of pH 6.0-6.4 and at 37°C (with the exception of LEV4-A10, which achieves its maximal activity at 47°C), they have highly differentiable half-lives and associated thermal inactivation constants. The lowest thermal inactivation constants are attributed to LEV4-D4 (0.0012 min^{-1}) followed by LEV5-A7 (0.0022) and LEV4-A10 (0.0037). While LEV5-A7 did not exhibit the longest half-life, it retained its activity more steadily than the other candidate levanases, for instance LEV4-D3. Similar patterns were observed with *V. natriegens* (LEV4-H2) levanase, which lost a major proportion of its original activity initially (up to 4 hours), but then showed a slower rate of activity depletion with extended thermal treatment (up to 12 hours).

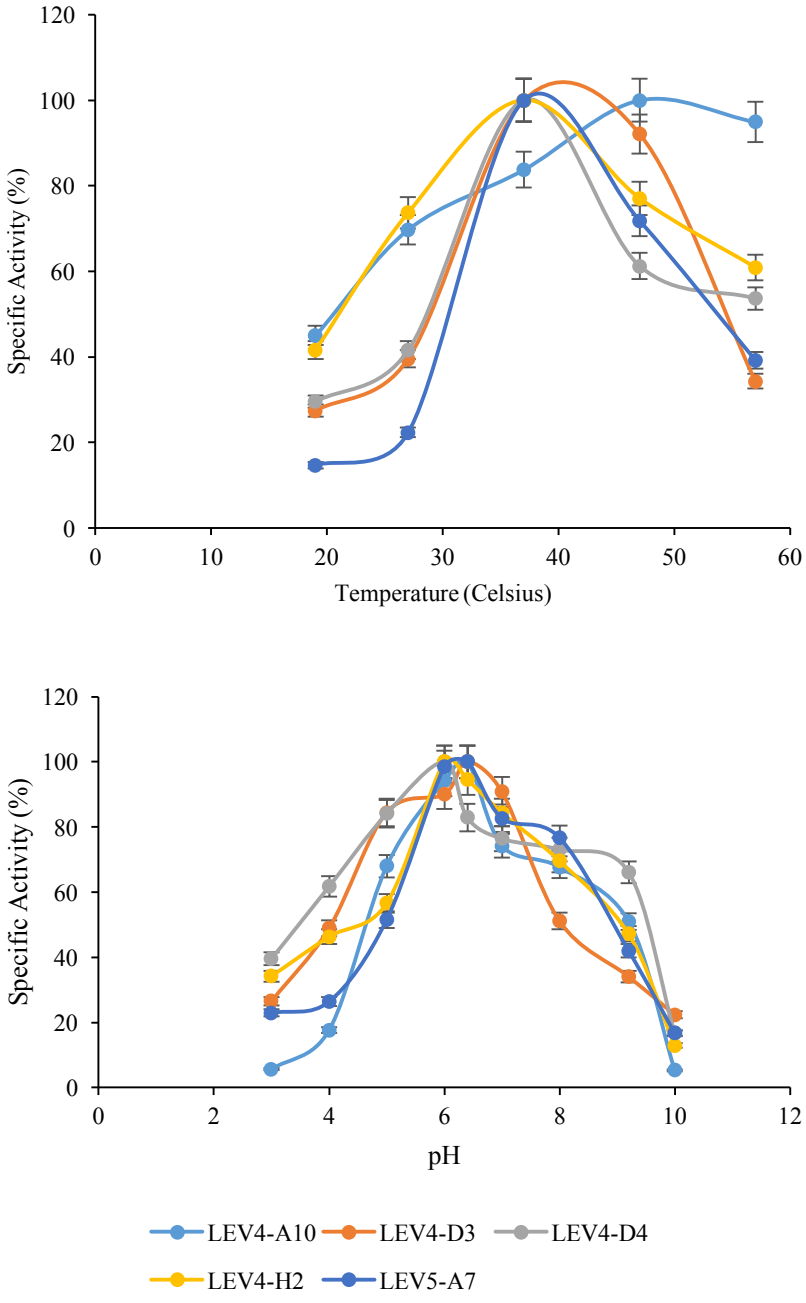


Figure 4.1 Effects of reaction parameters (pH and temperature) on the activity and stability of the top 5 levanase candidates

Levanase sources: *B. baltica* (LEV4-A10); *D. fermentans* (LEV4-D3); *C. ochracea* (LEV4-D4); *V. natriegens*

Table 4.2 Thermal stability of the top 5 levanase candidates and their optimal reaction conditions

Enzyme Code	Bacterial Source	Optimal Conditions		Half-Life (min)	Thermal Inactivation Constant (min^{-1})
		pH	T (°C)		
LEV4-A10	*B. baltica*	6.4	47	2.6×10^2	0.0037 ± 0.0007
LEV4-D3	*D. fermentans*	6.4	37	2.4	0.2847 ± 0.0024
LEV4-D4	*C. ochracea*	6.0	37	3.5×10^2	0.0012 ± 0.0004
LEV4-H2	*V. natriegens*	6.0	37	0.91×10^2	0.0042 ± 0.0005
LEV5-A7	*A. aurescens*	6.4	37	3.3×10^2	0.0022 ± 0.0005

Lim *et al.* (1998) previously studied the thermal stability of *Streptomyces* sp. 366L levanase, which was isolated from soil. They observed that the enzyme was stable up to a maximum temperature of 45°C, and was rapidly inactivated thereafter. These results are similar to those obtained by Wanker, Klingsbichel, and Schwab (1995), who reported that the levanase from *B. subtilis* was most active between 47 and 55°C, and that long-term thermal stability was maintained between 50 and 55°C. In comparison to other studies detailing the thermal stability of various characterized levanases, (Menéndez *et al.*, 2002), who investigated the levanase from *G. diazotrophicus* SRT4, reported irreversible enzyme inactivation above 60°C. Most notably in our investigation, LEV4-A10 had maximal hydrolytic activity at 47°C, which is not typical for levanases.

4.4.4 *End-product Profiles of Levanase-Catalyzed Hydrolytic Reactions*

To investigate the end-product profiles of the selected levanases, reactions were performed using low-molecular weight levan as the substrate. Figure 4.2 shows the end-product profiles at selected enzymatic units (0.2-0.6 U/mg substrate). The results show that the end-product profile and the endo/exo bioconversion ratio were dependent on the microbial source of levanase and on the quantity of enzymatic units used in the reaction. The most interesting findings made from using a range of levanase quantities on the same amount of substrate was that 0.3 U appeared to be the threshold, because at higher enzyme concentrations (i.e., 0.6 U), the reaction kinetics tended to favor the release of fructose, which is undesirable in terms of FOS synthesis. In general, the greatest proportion of endo-hydrolytic products was achieved when 0.2 U of each levanase was reacted with 1.0% (w/v) levan; however, higher absolute yields of FOSs were obtained when 0.3 U was used, which is relevant to enhancing the bi-enzymatic system for FOS production previously developed in our laboratory. Under these reaction parameters, the predominant FOS products were levantriose followed by levanbiose, although in the case of *B. baltica* (LEV4-A10) levanase, levantetraose also occurred as a dominant product. Figure 4.2 shows which levanases among the top candidates have the highest endo-hydrolytic activity as compared with exo-hydrolytic activity, from which only fructose is released. In this respect, D. fermentans (LEV4-D3) and *B. baltica* (LEV4-A10) demonstrated the highest levels of endo-hydrolytic activity, followed by *C. ochracea* (LEV4-D4) levanase, as seen in their end-products which constitute relatively high proportions of levanbiose and levantriose. These levanases produced significantly higher quantities of FOSs than

V. natriegens (LEV4-H2) and *A. aurescens* (LEV5-A7) levanases. Figure 4.2 also speaks to the biocatalytic tendencies of the top candidate levanases. As shown, our candidates preferentially synthesize short-chain FOSs from the hydrolysis of low-molecular weight levan, with the highest DP being 4 when these enzymes are employed alone. Although it appears from the proportional distributions of end-products that *D. fermentans* (LEV4-D3) levanase out-performed LEV4-D4 in terms of FOS yield, the absolute quantity obtained was lower, which can be attributed to the lower specific activity of LEV4-D3 compared to LEV4-D4. For instance, at 0.3 U, LEV4-D4 demonstrates a bioconversion rate of 85.3% on low-molecular weight levan to hydrolytic end-products, while this is lower for LEV4-D3 at 68.7%. In terms of absolute yield in this context, LEV-D4 produces 8.5 g of FOSs, while LEV4-D4 produces 6.9 g (data not shown). Ultimately, LEV4-A10 and LEV4-D4 were selected for investigation in bi-enzymatic systems with newly discovered, high levan-producing levansucrases to prospect for higher, more efficient yields of FOSs and oligolevans. This is justified by the high specific activities of these levanases, their end-product profiles (which showed the highest proportional yields of FOSs), and other pertinent considerations for industrial applications such as thermal stability.

4.4.5 *Bi-Enzymatic Synthesis of Levan-Type FOSs*

A bi-enzymatic system based on the combined use of *B. amyloliquefaciens* levansucrase and a commercial endo-inulinase was previously developed in our laboratory (Tian, Karboune, and Hill, 2013). The availability of levan, synthesized by levansucrase, at the appropriate amount and molecular weight, is a major determinant of the ultimate biocatalytic end-products. The presence of endo-inulinase was a major contributor to the formation of transfructosylation products. Notably, these authors observed that the production of FOSs and oligolevans by the hydrolysis of low-molecular weight levan was substantially higher than when high-molecular weight levan served as the substrate, justifying the use of the former in the present work.

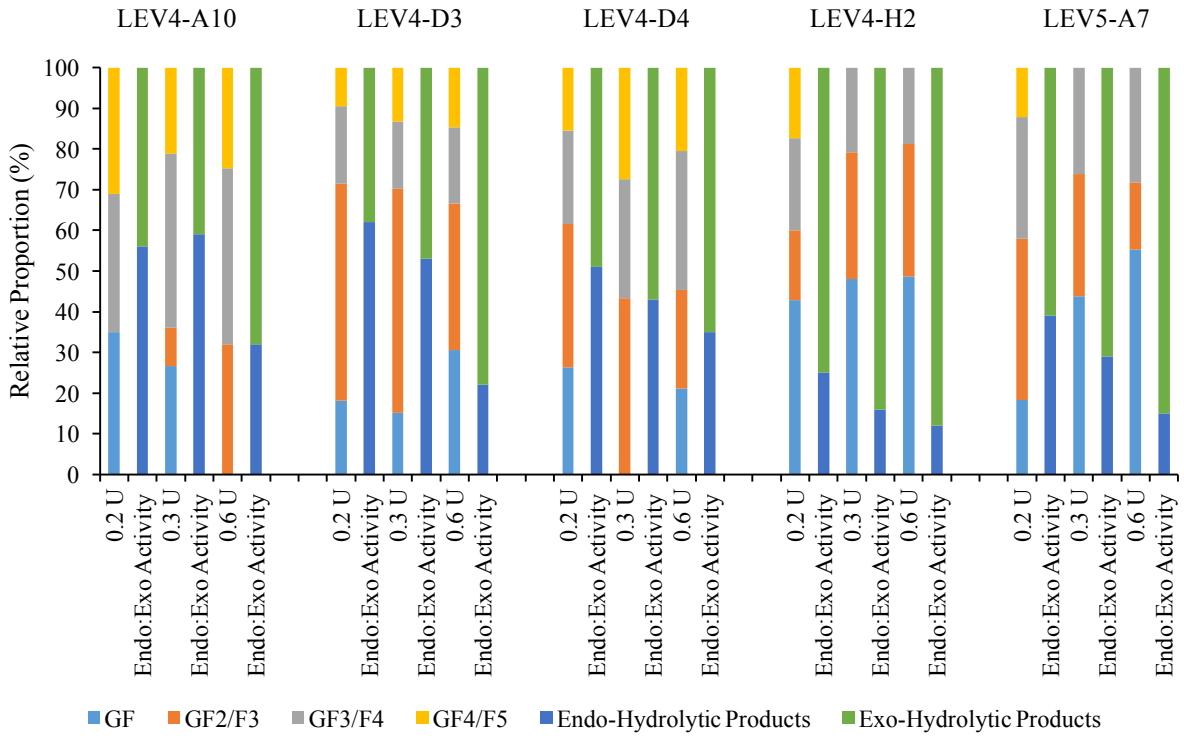


Figure 4.2 Comparison of the relative proportion of FOSs obtained from the top candidate levanases, as well as their catalytic tendencies (endo-hydrolytic vs. exo-hydrolytic) when different enzyme units are used

U: $U_{Levanase}$/mg low-molecular weight levan

Endo:Exo Activity: ratio of endo-hydrolytic products (i.e., GF, GF_2, GF_3, GF_4) to exo-hydrolytic products (i.e., fructose)

Levanase sources: *B. baltica* (LEV4-A10); *D. fermentans* (LEV4-D3); *C. ochracea* (LEV4-D4); *V. natriegens* (LEV4-H2); *A. aurescens* (LEV5-A7)

As part of the ongoing research, recombinant levansucrases from *G. oxydans* and *V. natriegens* were examined for their ability to synthesize FOSs and polymeric levan prior to pairing with the top levanases we recently identified: *B. baltica* (LEV4-A10) and *C. ochracea* (LEV4-D4) The concerted action of these enzymes was investigated in bi-enzymatic systems differing in design in order to evaluate the most efficient approach for synthesizing prebiotic levan-type FOSs and oligolevans. The one-step system entailed the simultaneous addition of levansucrase and levanase, while in the two-step system, levanase was introduced in a sequential manner 12 hours after the start of the reaction between levansucrase and sucrose (starting material).

Levansucrases were selected on the basis of high transfructosylation and polymerization activity over hydrolysis. The results (Figure 4.3**)** show that *G. oxydans* (C03) and *V. natriegens* (F03) levansucrases exhibit similar product spectra. In both cases, it is evident that mixed end-products were obtained, with a higher proportion of FOSs in the early to intermediate reaction stages and greater amounts of polymeric levan as time proceeds. However, while their product profiles were highly comparable in terms of the proportion of compounds within the same molecular weight range*, G. oxydans* levansucrase was much more efficient in synthesizing levan compared to that from *V. natriegens*. After 48 hours, *G. oxydans* levansucrase produced 97.8 mg/ml high-molecular weight levan, while *V. natriegens* levansucrase synthesized 51.8 mg/ml (data not shown). In addition, *G. oxydans* levansucrase synthesized a comparable quantity of low-molecular weight levan, 88.9 mg/ml, while *V. natriegens levansucrase* only produced 22.6 mg/ml (data not shown), suggesting this levansucrase preferentially synthesizes high-molecular weight levan over its low-molecular weight counterpart. The data presented in Figure 4.3 are consistent with findings from other authors. For instance, Santos-Moriano *et al.* (2015) recently investigated the products of *Z. mobilis* levansucrase by size-exlcusion chromatography and reported a molecular weight identical to that for commercial *Z. mobilis* levan. Levans of other bacterial levansucrases, as with *B. subtilis*, is typically at least 2,000 kDa with a minimum of 10,000 fructosyl moieties. In our investigation, the largest levans produced after 48 hours were 4221.4 kDa and 3133.6 kDa by *V. natriegens* and *G. oxydans* levansucrases, respectively. Santos-Moriano *et al.* (2015) also found that in terms of FOS production, the *Z. mobilis* levansucrase produced a mixture of FOSs, mono- and disaccharides, as determined by the concentration of sucrose provided, which is concordant with the data collected in our investigation, as visualized from Figure 4.3 from the distribution of end-products according to molecular weight. Interestingly,

Santos-Moriano *et al.* (2015) observed that more FOSs were produced at higher concentrations of sucrose, attributed to the fact that under these conditions, the role of sucrose as an acceptor of the fructosyl-enzyme intermediate is favored, allowing for the generation of short-chain FOSs. Specifically, the most abundant reaction product obtained during the beginning stages of their reaction system was 6-kestose, which reached its maximum concentration after 6 hours. Thereafter, the amount of this FOS in the reaction system was diminished owing to its use in the elongation of polymeric levan. In addition, they observed that the production of known FOSs reached a maximum after 21 hours of reaction, which is corroborated with the results obtained in the present work. Similar trends are observed in the end-products obtained from *G. oxydans* and *V. natriegens* levansucrases, as highlighted in Figure 4.3. Notably, the highest quantity of FOSs was produced after 12 hours of reaction. Decrease in the levels of FOS production thereafter can be attributed to the use of transfructosyl moieties for elongation of polymeric levan, which consistently increased in quantity as the reaction progressed. While *G. oxydans* and *V. natriegens* levansucrases have demonstrated the ability to generate proportionally high quantities of desirable end-products in terms of FOSs and levan, it was noted that these levels increased when *B. baltica* and *C. ochracea* levanases were introduced into the reaction system, either in a simultaneous or two-step manner.

As highlighted in Figure 4.4, the investigation of one-step and two-step bi-enzymatic systems involving *G. oxydans* and *V. natriegens* levansucrases and *B. baltica* and *C. ochracea* levanases produced differentiable results. In addition, data shown in Figure 4.5 indicate that the combined application of levansucrase and levanase yielded a higher diversity of end-products (i.e., more compounds of different size ranges were obtained compared to when these enzymes were employed independently). In general, the one-step bi-enzymatic systems were more favorable in terms of FOS and oligolevan yield, as exemplified by the concerted action of *G. oxydans* levansucrase and *B. baltica* levanase. In this bi-catalytic system, maximal FOS production was reached after 24 hours in a reaction employing 0.9 M sucrose as the starting material, at which point the predominant products of endo-hydrolysis consisted of FOSs of DP 2-4. Specifically, 13.7 mg/mL of levanbiose and 17.2 mg/mL levantriose were synthesized within this reaction time (data not shown). It was further noted that when employed simultaneously, this levansucrase-levanase pair demonstrated the fastest consumption of sucrose (the starting material), at a rate of 3.70 mmol/min (data not shown). These data are corroborated by Tian, Hill and Karboune (2013), who

observed that in the early and intermediate stages (up to 30 hours) of the reaction between *B. amyloliquefaciens* levansucrase and commercial endo-inulinase, the synthesis of short-chain FOSs and oligolevans by endo-inulinase was more kinetically favored than the release of fructose. The rapid bioconversion of starting material to FOS end-products by *G. oxydans* levansucrase and *B. baltica* levanase in a one-step bi-enzymatic system suggests it is highly promising for scale-up in industrial applications.

With regard to the two-step bi-enzymatic systems we investigated, in which levanase from either *B. baltica* or *C. ochracea* was introduced into the reaction system 12 hours after levansucrase from either *G. oxydans* or *V. natriegens*, it was observed that *V. natriegens* levansucrase and *B. baltica* levanase formed the most ideal pairing. The maximal production of FOS end-products was obtained after 24 hours of reaction, at which point the predominant FOS product was levantriose; however, levanbiose and levantetraose were also generated at appreciable quantities. Quantitatively, 6.9 mg/ml of levanbiose and 14.8 mg/ml of levantriose were produced during this reaction time. The lower yield of FOS products in this case can be attributed to the lower rate of sucrose consumption, of 2.76 mmol/min (data not shown). In our investigation of the previously described bi-enzymatic systems, we observed that FOSs, oligolevans, and inevitably, fructose, were continuously released at the expense of sucrose (trend not shown).The different possible pathways of sucrose expenditure provide the rationale for the various end-product profiles observed, and more interestingly, justification for the one-step bi-enzymatic systems being more efficient in producing desirable end-products than those using the two-step design. When levansucrase and levanase are introduced simultaneously in a system with sucrose, both the synthesis of FOS and polymeric levan (by levansucrase) and the hydrolysis of these compounds (by levanase) need to be taken into consideration in terms of reaction thermodynamics. As the levansucrase begins to synthesize transfructosylation and/or polymerization products, the levanase hydrolyzes these, allowing equilibrium to be reached such that the reaction system favors synthesis and hydrolysis simultaneously. However, when levanase is introduced into the reaction 12 hours into the reaction of levansucrase with sucrose, the reaction thermodynamics have to shift in order to favor hydrolysis and establish a state of equilibrium. This justifies the higher release of fructose in the two-step systems. On a more detailed level, the maximal levels of FOS products are generally achieved at intermediate stages (24 hours) in the reaction, after which they decrease. This can be explained by the fact that, in addition to the polymeric levan being generated by

levansucrase, the levanase in the bi-enzymatic system can also hydrolyze the FOS products synthesized by either itself or the levansucrase. As the reactions progress and products of higher molecular weight than sucrose begin to accumulate and ultimately reach a maximum, these are hydrolyzed by levanase to reach an equilibrated state. A study previously conducted by Dahech *et al.* (2013) on the action of a levanase isolated from a Tunisian thermal source revealed that levanbiose was the major product in the early reaction stages. Thereafter, levanoligosaccharides with degree of polymerization higher than 3 were transiently detected at the expense of polymeric levan. These authors reported that the concentration of levanbiose reached a maximum after 8 hours, which was subsequently degraded into fructose, as corroborated by results from a similar study performed by Chaudhary *et al.* (1996). In our investigation, the FOS end-products were similar to those obtained by Dahech *et al.*; however, they were obtained after a longer period of time owing to the reliance on levansucrase to synthesize FOSs and levan. Figure 4.5 not only shows this trend, but also the synthesis of oligolevans from the one-step and two-step bi-enzymatic systems previously discussed. As indicated in Figure 4.5A, the one-step pairing of *G. oxydans* levansucrase and *B. baltica* levanase resulted in the highest production of low-molecular weight levan and oligolevans after 12 hours, a trend that continued through to 48 hours of reaction. This corresponded to the production of 28.2 mg/ml of compounds within the 200 kDa range and 20.1 mg/ml of compounds within the 800 kDa range (data not shown). Similar end-products were obtained from *V. natriegens* levansucrase and *B. baltica* levanase in a two-step system (Figure 4.5B), although at lower quantities.

It should be noted that levans produced by different microbial sources vary in terms of size and structure. This affects the degree of hydrolysis by levanases and the type(s) of end-products generated. For instance, *Serratia* sp. levan is more easily hydrolyzed than *Zymomonas mobilis* levan, owing to more branching points and thus lower accessibility in the latter (Lim *et al.*, 1998). This information is relevant to future advancements on the present work, as the levans produced by *G. oxydans* and *V. natriegens* need to be structurally characterized (i.e., by nuclear magnetic resonance or mass spectroscopy) in order to analyze in greater detail the hydrolytic mechanisms of the levanases discussed.

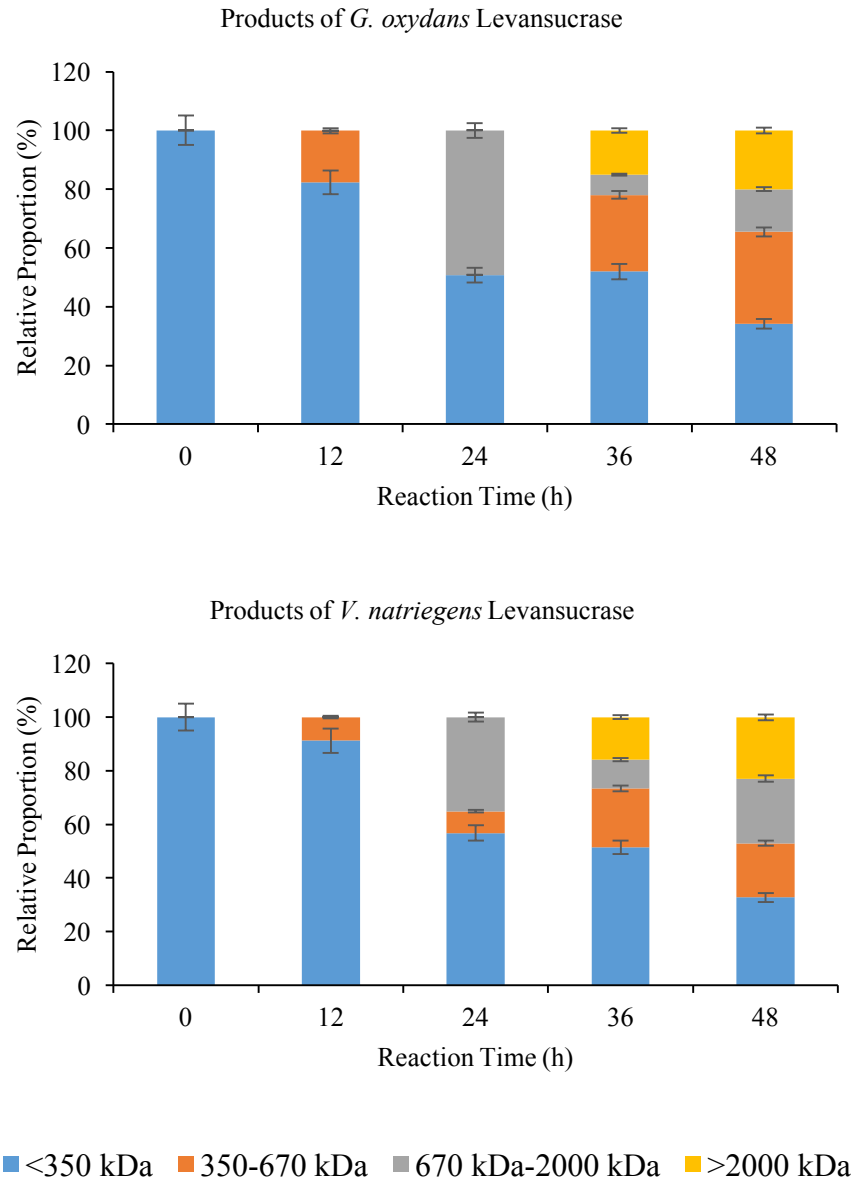


Figure 4.3 Products of *G. oxydans* and *V. natriegens* levansucrases

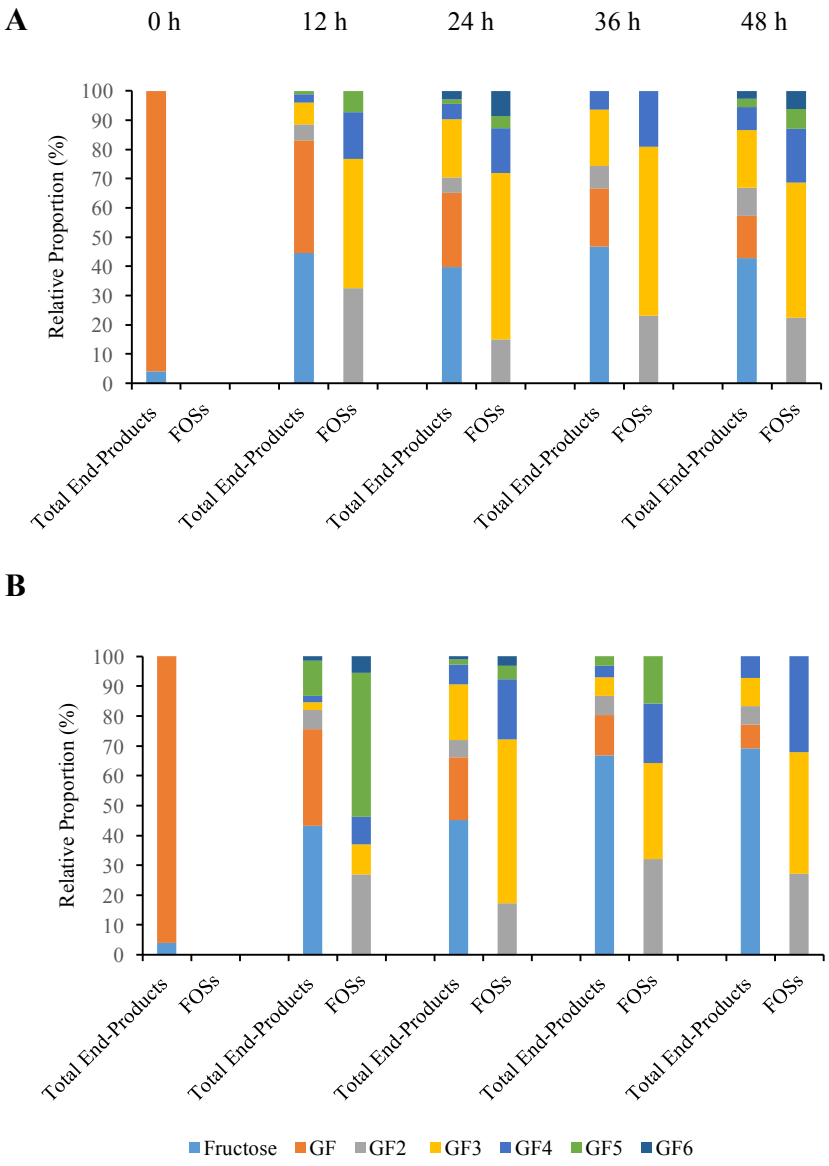


Figure 4.4 Total end-products and FOSs obtained from the A) one-step (*G. oxydans* levansucrase and *B. baltica* levanase) and B) two-step (*V. natriegens* levansucrase and *B. baltica* levanase) bi-enzymatic systems resulting in the highest generation of FOSs

C03: *G. oxydans* levansucrase; F03: *V. natriegens* levansucrase; A10: *B. baltica* levanase; D4: *C. ochracea* levanase

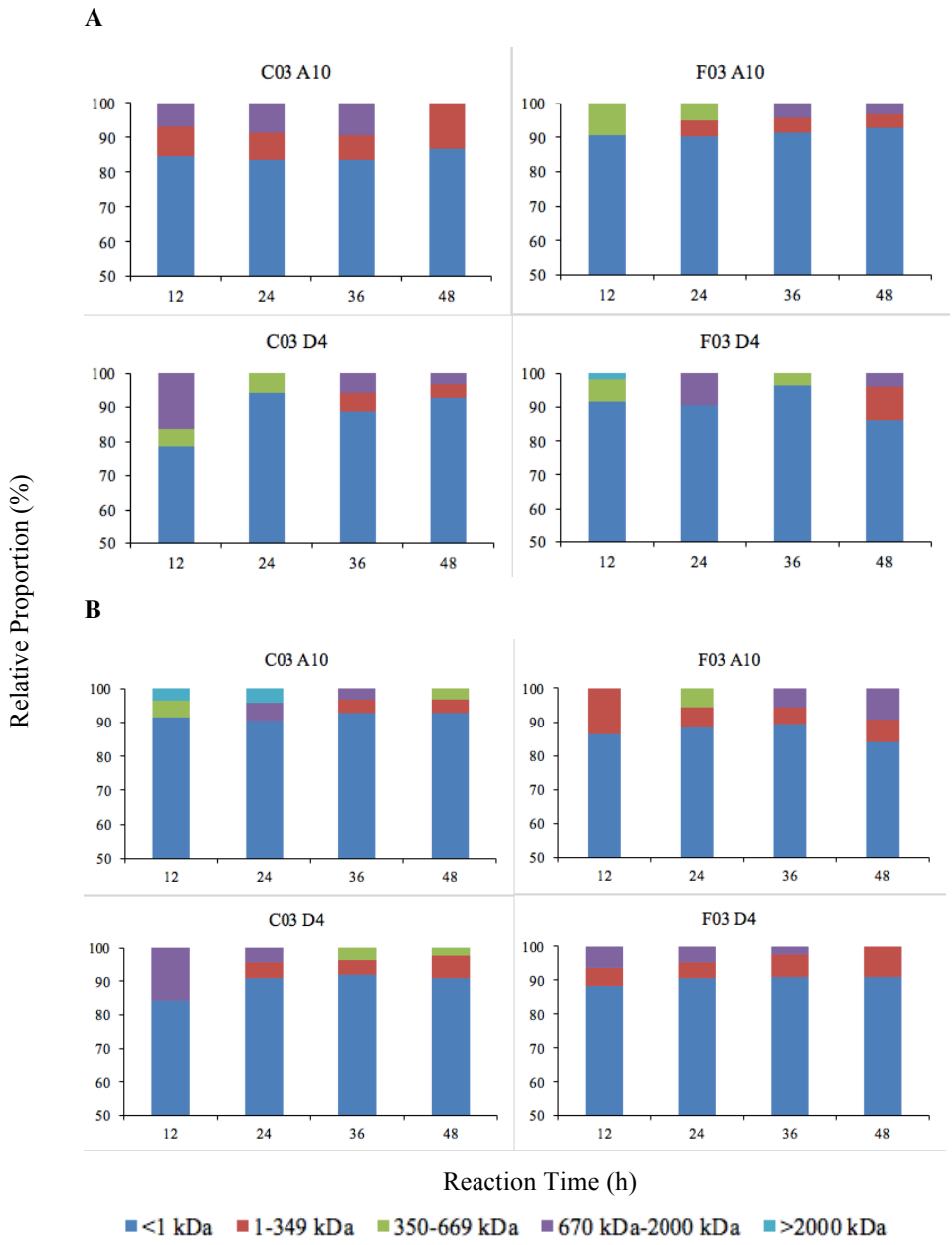


Figure 4.5 Molecular weight of end-products obtained from one-step (A) and two-step (B) bi-enzymatic systems designed for the synthesis of FOSs and oligolevans
C03: *G. oxydans* levansucrase; F03: *V. natriegens* levansucrase; A10: *B. baltica* levanase; D4: *C. ochracea* levanase

4.5 Conclusion

Characterization of top candidate levanases screened from a genetically diverse new collection identified two promising biocatalysts from *B. baltica* and *C. ochracea* for the production of FOSs. Not only did these enzymes demonstrate rapid bioconversion of starting material to high proportions of endo-hydrolytic products, they also showed high degrees of thermal stability compared to previous reports in literature. To date, few endo-levanases have been discovered and characterized, which highlights the success of the genome-mining process undertaken to produce the collection of levanases we investigated in our studies, and the novelty of the top candidates from *B. baltica*, *D. fermentans*, *C. ochracea*, *V. natriegens*, and *A. aurescens*. Most notably, they were studied in the advancement of a bi-enzymatic system previously developed in our laboratory designed to take advantage of the concerted action of levansucrase with levanase. Results from these studies show promise for industrial applications, as *G. oxydans* levansucrase and *B. baltica* levanase produced significant yields of industrially valuable oligolevans. Optimization will follow in future work to elucidate optimal reaction parameters with emphasis on the type and quantity of starting material used.

CHAPTER V.

GENERAL SUMMARY AND CONCLUSIONS

The ultimate objective of the present work was to develop an enhanced bi-enzymatic approach for the synthesis of β-(2,6)-prebiotics with functional properties surpassing those of current commercial preparations which solely constitute the β-(2,1)-type. This was accomplished through data-mining of microbial genomes for sequences bearing homology to those encoding exo- and endo-levanases, whicch produced 1902 hits amenable to cloning. Among 123 representative candidates expressed in *E. coli* BL21(DE3) and screened for levanase activity, substantial diversity was observed not solely in terms of phylogenetic origin, but also the degree to which low- and high-molecular weight levans were hydrolyzed. This resulted in the selection of 10 viable biocatalysts found in various bacterial species which exhibited preferential hydrolysis of levan, while also demonstrating varying levels of activity on inulin. In particular, candidate levanases from *B. baltica*, *D. fermentans*, *C. ochracea*, *S. parasanguinis*, *P. salivae*, *G. limnaea*, *V. natriegens*, *A. naeslundii*, and 2 different ones from *A. aurescens* demonstrated the highest levels of preferential activity on low- and high-moleular weight levan. Considering this criterion, *C. ochracea* (LEV4-D4) levanase exhibited the highest hydrolytic activity on low-moelcular weight levan (137.79 μmol/mg*min) while *A. aurescens* (LEV5-A7) levanase revealed a significantly higher ability to hydrolyze high-moleuclar weight levan (329.83 μmol/mg*min). The end-product spectra of these levanases were investigated by HPAEC-PAD, which allowed the candidate levanases to be differentiated in terms of endo- versus exo-activity, and provided an indication of the types of FOS end-products which could be obtained from this new collection of microbial levanases. Generally, levanbiose and levantriose were the major FOSs obtained from the reaction of these enzymes with low-molecular weight levan. This is promising as the majority of microbial levanases investigated to date function in an exo-hydrolytic manner, releasing fructose as the sole reaction product. Characterization of top levanases screened from the initial new collection of 123 candidates has allowed for the identification of two promising biocatalysts, those from *B. baltica* and *C. ochracea*, for the production of FOSs. Not only did these enzymes demonstrate rapid bioconversion of starting material to high proportions of endo-hydrolytic products, but they also showed high degrees of thermal stability as compared to previous reports in literature. To date, few endo-levanases have been disovered and characterized, which highlights the success of the genome-mining process undertaken to produce the collection of levanases we investigated in our studies, and the novelty of the top candidates from *B. baltica*, *D. fermentans*, *C. ochracea*, *V. natriegens*, and *A. aurescens*. Most notably, they were studied in the advancement

of a bi-enzymatic system previously developed in our laboratory designed to take advantage of the concerted action of levansucrase with levanase. Results from these studies show promise for industrial applications, as *G. oxydans* levansucrase and *B. baltica* levanase produced high yields of FOS products and oligolevans in a one-step system. Optimization will follow in future work in order to elucidate optimal reaction parameters with emphasis on the type and quantity of starting material used.

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