Stabilization of Levansucrase, Modulation of Its Specificity and Search for Improved Synthesis of Novel Fructooligosaccharides and Levans

by

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SUGGESTED SHORT TITLE

SYNTHESIS OF FRUCTOOLIGOSACCHARIDES AND LEVAN THROUGH STABILIZED AND NOVEL LEVANSUCRASES ENZYMES

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ABSTRACT

Levansucrase (LS, EC 2.4.1) is an interesting catalyst that performs the non-Lenoir transfer of fructose from a non-activated donor molecule to an acceptor molecule, producing β-(2-6)-FOSs, neoFOSs and levan. The immobilization of LS from *Bacillus amyloliquefaciens* was studied with the goal of improving its thermal stability while concurrently augmenting the enzyme's preference for transfructosylation in place of hydrolysis through a modification of the microenvironment. Modified and unmodified Eupergit C, Sepabeads, and agarose were chosen as the solid supports. The greatest retained activity was achieved with the ionic Sepabeads HA (98.8 %) and the multi-covalent glyoxyl agarose-IDA/Cu (67.0%) supports. The highest stability was experienced by the LS immobilization onto glyoxyl agarose-IDA/Cu and glyoxyl agarose with stabilization factors of 14 and 106 times that of the native enzyme. Immobilization onto Sepabeads HA was found to provide the greatest modulation of the enzymatic microenvironment with transfructosylation over hydrolysis ratio being 2.3 times higher than the native LS. Glyoxyl agarose-IDA/Cu was found to be the overall top support for the immobilization of LS with high retention of activity (67.0%), a transfructosylation over hydrolysis ratio (1.2) and great thermal stability (13.6 stabilization factor).

The factors (protein loading, buffer molarity, buffer pH, immobilization time), which affected the LS immobilization, were examined and optimized using response surface methodology (RSM). Retention of activity was found to be most influenced by the interactions between buffer molarity/immobilization time and the interactions between buffer pH and buffer molarity. In terms of the responses, the optimized immobilization conditions (pre-covalent bond formation) were found to be: protein loading of 9.09 mg protein/g support, potassium phosphate buffer molarity of 608 mM at pH 6.8 using an immobilization time of 49h. The formation of the permanent covalent bonds was replaced with the ionic polymer polyethylenimine (PEI) at a concentration of 0.1% (v/v). The resulting glyoxyl agarose-IDA/Cu/PEI had a retention of activity of 70.9% with a protein yield of 44.7% and an activity yield of 54. 69%, while exhibiting a half-life 4.7 times higher than the native free LS at 50°C.

With the enzymatic LS activity varying significantly per bacterial source, interesting catalytic activity potentially lies within undiscovered LS. Genome mining was utilized to search for new exciting LS enzymes using a reference set of 39 enzymes with the BLAST parameters (RI\>=30, RZ\>0 0.8, RL\>200) and clustering at 80% identity, resulting in 50 cloneable genes. 10 potential

LS enzymes were selected from the resulting screening of total activity, glucose and levan production. In-depth examination of the capabilities of the these LSs revealed that the LS from *G. oxydans* produced levan of a large size of 6986 kDa. High transfructosylation versus hydrolysis ratios were found for the LSs from *Vibrio natriegens*, *Streptococcus salivarius* K12 and *G. oxydans* of 1.55, 1.44 and 1.33 respectively. The LS from *Paraburkholderia graminis* was revealed to have a very high thermal stability, with a half-life of 291 minutes when heated at 50°C. The kinetic parameters of a select few high performing LSs were determined. The catalytic efficiency for transfructosylation was found to be much higher (almost double), 4058 $s^{-1}mM^{-1}$ that of the catalytic efficiency for hydrolysis 2256 $s^{-1}mM^{-1}$ for the LS from *S. salivarius* K12; while that enzyme and the LS from *B. indica subsp. indica* had higher catalytic turnover for transfructosylation than for hydrolysis. An acceptor analysis study showed that all the top enzymes selected can utilize raffinose as the sole substrate, while multiple enzymes were capable of transfructosylation utilizing alternate acceptor molecules.

LSs from *G. oxydans*, *N. aromaticivorans*, *Beijerinckia indica subsp. indica*, *P. graminis* and *V. natriegens* were further examined in terms of their product profile, acceptor specificity and active site. Both LSs from *G. oxydans* and *N. aromaticivorans* produced FOSs up to 13 units in length. Furthermore, LS from *P. graminis* produced more than double the amount of FOS (164 g/L) as compared to the rest of the enzymes, which was predominately composed of trisaccharides (120 g/L) but also contained the largest composition of tetrasaccharides (26 g/L). The largest amount of levan (84 g/L) was produced at 12 h by the LS from V. natriegens. The LSs from *V. natriegens*, *N. aromaticivorans* and *P. graminis* preferred raffinose instead of the substrate sucrose. In the acceptor specificity study, it was shown that all the enzymes were able to utilize the alditol, sorbitol, to a varying degree while the enzymes from *V. natriegens*, *N. aromaticivorans*, *P. graminis* and *B. indica subsp. indica* were able to utilize the benzene diol, catechol as an acceptor for fructose, opening up the possibility for the production of new novel transfructosylated products.

RÉSUMÉ

Levansucrase (LS, EC 2.4.1) est un catalyseur intéressant qui accomplit le transfert non-Lenoir d'un fructose à partir d'une molécule donneuse désactivée jusqu'à une molécule acceptrice, produisant ainsi β-(2-6)-FOSs, neoFOSs et un levan. À travers la modification du microenvironnement, l'immobilisation du LS produite par *Bacillius amyloliquefaciens* a été étudiée avec, pour objectifs simultanés, l'amélioration de sa stabilité thermale et l'augmentation de la préférence de cette enzyme pour la trans-fructosylation par rapport à l'hydrolyse. De l'Eupergit® C, du Sepabeads®, et de l'agarose (modifiés et non-modifiés) ont été choisis comme supports solides. La plus grande activité résiduelle a été obtenue avec le Sepabeads® HA (98.8 %) ionique et le glyoxyl agarose-IDA/Cu (67.0%) covalent. La plus grande stabilité a été observée lors de l'immobilisation du LS sur le glyoxyl agarose-IDA/Cu et sur le glyoxyl agarose avec des facteurs de stabilisation respectivement 14 et 106 fois plus élevés que l'enzyme d'origine. Il a été montré que l'immobilisation sur le Sepabeads® HA permettait la plus grande modulation du microenvironnement enzymatique avec un ratio, favorisant la trans-fructosylation au détriment de l'hydrolyse, 2,3 fois plus grand que le LS d'origine. Il a été montré que le glyoxyl agarose-IDA/Cu produisait les meilleurs résultats en termes d'immobilisation du LS avec une rétention de l'activité élevée (67%), un ratio de 1,2 favorisant la trans-fructosylation au détriment de l'hydrolyse et une bonne stabilité thermale (facteur de stabilisation de 13,6).

Les facteurs (quantité de protéines, molarité du tampon, pH du tampon, période d'immobilisation) affectant l'immobilisation du LS ont été examinés et optimisés en utilisant la méthodologie de surfaces de réponses (MSR). Il a été montré que la rétention de l'activité a été particulièrement influencée par l'interaction molarité du tampon/période d'immobilisation et les interactions entre le pH et la molarité du tampon. En termes de réponses, il a été montré que les conditions d'immobilisations optimales (formation de liens pré-covalents) étaient d'une part, une quantité de protéines de 9,09 mg de protéines/g de support et de l'autre, une molarité de tampon de 608 mM à un pH de 6,8 en utilisant un temps d'immobilisation de 49h. La formation des liens covalents permanents a été remplacée par le polymère ionique polyethylenimine (PEI) à une concentration de 0,1% (v/v). Le glyoxyl agarose-IDA/Cu/PEI résultant avait une activité résiduelle de 70,9% avec un rendement en protéines de 44,7% et un rendement en activité de 54,7%; tout en exhibant une demi-vie 4,7 fois plus élevée que le LS d'origine à 50°C.

Par ailleurs, puisque l'activité enzymatique du LS varie significativement dépendamment de sa source bactérienne, on retrouve potentiellement une activité catalytique intéressante chez un LS non découvert. L'exploration du génome (le ''genome mining'') a été utilisée pour trouver de nouvelles enzymes LS intéressantes en s'appuyant sur un ensemble de références de 39 enzymes avec les paramètres de BLAST (RI\>=30, RZ\>0 0.8, RL\>200) et une analyse par segmentation avec 80% d'identité résultant ainsi dans la découverte de 50 gènes clonables. Dix enzymes potentielles ont été sélectionnées en fonction de l'activité totale, de la production de glucose et de levan. Une examination en profondeur des capacités de ces LS a révélé que le LS provenant du *G. oxydans* produisait un levan de grande taille, soit 6986 kDa. Des ratios élevés de trans-fructosylation par rapport à l'hydrolyse ont été trouvés dans les LS provenant de *Vibrio natriegens, Streptococcus salivarius K12* et *G. oxydans* respectivement de 1,55, 1,44 et 1,33. Le LS du *Paraburkholderia graminis* a révélé avoir une stabilité thermique très élevée avec une demi-vie de 291 minutes lorsque chauffé à 50°C. Les constantes cinétiques de quelques LS hautement performants choisis ont été étudiées. Il a été montré que l'efficacité catalytique pour la trans-fructosylation était bien plus élevée (presque doublée) avec 4058 s-1mM-1 par rapport à l'efficacité catalytique de l'hydrolyse avec 2256 s-1mM-1. Par contre, les LS provenant du *S. salivarius* K12, *Novosphingobium aromaticivorans* et *P. graminis* avaient un plus grand renouvellement catalytique que le taux d'hydrolyse. Une étude de l'analyse d'accepteurs a montré que toutes les enzymes hautement performantes sélectionnées pouvaient utiliser de la raffinose comme seul substrat, tandis que plusieurs enzymes étaient capables de trans-fructosylation en utilisant des molécules acceptrices alternatives.

À partir des vérifications initiales, les LS de *G. oxydans, N. aromaticivorans, Beijerinckia indica subsp. indica, P. graminis* et *V. natriegens* ont été examinés plus en profondeur à travers leur profil de produit, leurs spécificités d'accepteurs et leur site actif. Les LS du *G. oxydans* et du *N. aromaticivorans* ont produit des FOS allant jusqu'à une longueur de 13 unités. La production de FOS provenant du LS de *P. graminis* représentait plus du double de la quantité (164 g/L) en comparaison avec le reste des enzymes. Les FOS de *P. graminis* étaient composées de façon prédominantes de trisaccharides (120 g/L), mais une grande proportion d'entre eux étaient des tetrasaccharides (26 g/L). Cela a aussi représenté la plus grande quantité de tetrassacharides produits. Les LS des *V. natriegens, N. aromaticivorans* et *P. graminis* ont préféré la raffinose au substrat de sucrose. Dans l'analyse d'accepteurs, il a été montré que toutes les enzymes étaient capables d'utiliser l'alditol, sorbitol, à un certain degré, tandis que toutes les enzymes provenant de *V. natriegens, N. aromaticivorans, P. graminis* et *B. indica subsp. indica* étaient capables

d’utiliser le benzene diol, catechol, comme accepteur pour le fructose, ouvrant ainsi la voie à la production de nouveaux produits trans-fructosylés.

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STATEMENT FROM THE THESIS OFFICE

According to the regulations of the Faculty of Graduate Studies and Research of McGill University, Guidelines for Thesis Preparation include:

Candidates have the option of including, as part of the thesis, the text of one or more papers submitted, or to be submitted, for publication, or the clearly-duplicated text of one or more published papers. These texts must conform to the "Guidelines for Thesis Preparation" and must be bound together as an integral part of the thesis.

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.

CONTRIBUTION OF AUTHORS

The present thesis is composed of eight chapters.

Chapter I serves as a general introduction to the immobilization and synthesis of fructooligosaccharides and levan by levansucrase and describes the objectives of this study.

Chapter II presents a comprehensive literature review on fructooligosaccharides and levan. It describes their current uses and their synthesis. It focuses on their enzymatic synthesis through the enzyme levansucrase in terms of catalytic properties. It also provides a description of the immobilization techniques employed for use on levansucrase.

Chapters III to VI are presented in the form of manuscripts and have been either published or they have been prepared for publication. The connecting statements provide rational associations between the chapters. Chapter III focuses on the immobilization of levansucrase for increased thermal stability, activity modulation and retention of activity. Chapter IV provides an optimisation of the immobilization conditions and well as their influence on the desired responses. This chapter also provides an alternative to covalent bond formation for stabilization. In chapter V, new levansucrases were discovered through high-throughput sequence-based screening, with their properties evaluated in terms of total activity, transfructosylation over hydrolysis, thermal stability and catalytic constants. The evaluation was continued for the top five enzymes discovered in the screening in chapter VI. The full product spectrum was characterised, along with the acceptor specificity. The results were correlated with the enzymatic sequences and active site models.

Chapter VII provides the general summary and the conclusion of the thesis.

Chapter VIII describes the contributions of this research to the field and suggests future lines of research regarding levansucrase and the synthesis of fructooligosaccharides and levan.

The present author was responsible for the experimental work and the preparations of the first draft of all the manuscripts for publication and dissertation.

Dr. Salwa Karboune, the PhD's student's supervisor, provided guidance and supervision for all of the research work and reviewed and corrected all manuscripts prior to their submission.

Dr. Cesar Mateo was the second author for manuscripts #1 and #2, chapters III and IV respectively. In both chapters he contributed to the guidance in the enzyme immobilization.

Dr. Véronique de Berardinis and Jean-Louis Petit collaborated in the genome mining, initial expression screening of the potential levansucrase enzymes described in chapter V.

Dr. Alexandre de Brevern and Tarun Narwani of DSIMB-INSERM provided the homology modeling of the levansucrase enzymes in chapter VI.

PUBLICATIONS

1. Tian, F., Karboune, S., & Hill, A. (2014). Synthesis of fructooligosaccharides and oligolevans by the combined use of levansucrase and endo-inulinase in one-step bi-enzymatic system. *Innov Food Sci Emerg Technol, 22*(0), 230-238.
2. Hill A, Mateo C, Karboune S. (2015) Immobilization and Stabilization of levansucrase biocatalyst of high interest for the production of fructooligosaccharides and levan. *J. Chem. Technol. Biotechnol*. 91(9), 2440-2448.
3. Hill, A, Karboune, S. & Mateo, C. (2017) Investigating and optimizing the immobilization of levansucrase for increased transfructosylation activity and thermal stability. *Process Biochemistry*. 61, 63-72.
4. Hill, A., Tian, F. & Karboune, S. (2017) Synthesis of levan and fructooligosaccharides by levansucrase: catalytic, structural and substrate-specificity properties. Curr Org Chem. 21(2), 149-161.
5. Hill, A., de Berardinis, Petit, J. L. & Karboune, S. (2018) Discovering new levansucrase enzymes through high-throughput screening with genome mining with interesting properties and improved catalytic activity. *To be submitted*.
6. Hill, A., Narwani, T., de Brevern, A. de Berardinis, V., Petit, J. L. & Karboune, S. (2018) Characterization of new levansucrase enzymes for improved product spectrum and acceptor specificity. *To be submitted*.

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NOMENCLATURE/LIST OF ABBREVIATIONS

AY	Activity yield
AMU	Atomic mass unit
ATCC	American type culture collection
CLEA	Cross-linked enzyme aggregates
CLEC	Cross-linked enzyme crystals
DNS	3,5-Dinitrosalicylic acid
DP	Degree of polymerization
EC number	Enzyme classification number
EDA	Ethylenediamine
ETOH	Ethanol
FPLC	Fast protein liquid chromatography
FOSs	Fructooligosaccharides
Fuc	Fucose
Fru	Fructose
Gal	Galactose
HPAEC-PAD detector	High-pressure anion exchange chromatography with a pulsed amperiometric
HPLC	High pressure liquid chromatography
IDA	Iminodiacetic acid
Kcat	Turnover number
Km	Michaelis-Menton constant
LS	Levansucrase
Man	Mannose
MANEA	Monoaminoethyl-N-ethyl agarose
MeOH	Methanol
MW	Molecular weight
n	Hill coefficient

PEG	Polyethylene glycool
PEI	Polyethylenimine
PST	Potassium sodium tartrate
PY	Protein yield
Raf	Rafinose
RPM	Rotations per minute
SCFA	Short chained fatty acid
scFOS	Short chained fructooligosaccharides (2-5 saccharide units)
SDS-PAGE	Sodium-dodecyl-sulfate polyacrylamide gel electrophoresis
Suc	Sucrose
TEA	Triethylamine
TLC	Thin layer chromatography
T/H	Transfructosylation over hydrolysis ratio
Vmax	Maximum velocity
X_n	Saccharide units
Xyl	Xylose

CHAPTER I. GENERAL INTRODUCTION

In the current trend towards more health conscience consumption, prebiotics play an important role in maintaining the gastrointestinal health. Fructooligosaccharides (FOSs) constitute an emerging class of non-digestible oligosaccharides that fulfill the criteria for prebiotic classification (Roberfroid, 2007), by "selectively stimulating the growth or activity of one or a limited number of bacteria in the colon" (Roberfroid, 2000). The growing recognition of health benefits of FOSs and the better understanding of their structure-attribute relationships have highlighted the need for efficient biocatalytic approaches to synthesize novel FOS structures. β-(2-6)-FOSs and neoFOSs have demonstrated prebiotic effects that surpass those of β-(2-1)-FOSs available for human consumption; however, only few reports deal with the synthesis of these FOSs as major fructosylation products (Bello, Walter, Hertel, & Hammes, 2001; Bersaneti, Pan, Baldo, & Celligoi, 2018). The polysaccharide levan has shown increasing techno-functional applications in food, cosmetics and pharmaceutical applications (Monsan & Ouarné, 2009). Interestingly, the potential of levan in potential medical applications is also growing (Avsar, Agirbasli, Agirbasli, Gunduz & Oner, 2018; Gomes et al., 2018; Yoon, Yoo, Cha, & Gyu Lee, 2004).

Enzymatic strategies for the synthesis of FOSs and fructose-based polysaccharides are generally based on the action of fructofuranosidases (EC 3.2.1) and fructosyltransferases (EC 2.4.1) (Lombard, 2014). Despite the broad availability of fructofuranosidases, their application for the synthesis of novel FOS and fructose-based polysaccharides structures is limited by narrow acceptor specificity, low to modest yields and poor regioselectivity (Yun, 1996). Levansucrase (EC 2.4.1.10, LS), a fructosyltransferase, has recently gained more interest because of its ability to directly use 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) or a growing fructan chain (levan polymer synthesis) (Strube et al., 2011). Although levansucrases (LSs) are reported to behave essentially as transferases, they possess a certain amount of hydrolytic activity that is regarded as the transfer of the fructosyl group to water.

Modulating the enzyme's confirmation as well as its macro- and microenvironment may afford a means for the efficient synthesis of high yields of well-defined FOSs and levans. The transfructosylation end-product profile of LS is dependent upon the acceptor molecules accessibility to the active site of LS (subsite -1) and on the enzyme's affinity for the product (Ozimek, Kralj, Kaper, van der Maarel, & Dijkhuizen, 2006). Enzyme immobilization is a practical

tool not only for the reuse and stabilization of LS against thermal denaturation, but also for the alteration of the enzyme's conformation and micro-environment to modulate its product specificity (Chiang, Wang, Chen, & Chao, 2009). There are only few examples of LS immobilization, with adsorption techniques most frequently used. Immobilization variables, such as buffer pH, buffer concentration, immobilization time and protein loading, are determinant for the immobilization efficiency and the retention of activity of the LS upon immobilization. Understanding the effects of immobilization variables and their interactions may allow the preparation of immobilized LS with high catalytic efficiency. In addition, two-step immobilization processes allow for the orientation of the enzyme on the support first through adsorption then through covalent linkages. The enzyme is orientated by the enzyme region with the highest concentration of residues available for adsorption on the support. Once the enzyme is in close enough proximity to the support multiple covalent linkages can form, providing significant stability (Bolivar, Mateo, et al., 2009).

The limitations of the catalytic potential of LS enzymes remains within their inherent structure, with enzymes from different bacterial sources behaving differently (Li, Yu, Zhang, Jiang, & Mu, 2015). With the increasing availability of bacterial genomic sequences genome mining coupled with high-throughput sequencing and large-scale screening (Ziemert , Alanjary, & Weber, 2016) comes the possibility to unlock undiscovered LS enzymes with improved or novel activity. New LS enzymes can be found using the information currently available regarding the genomic data of known LSs and their catalytic activity. With a wide-ranging set, their catalytic potential may be wider than those currently available with the possibility of utilizing new acceptor molecules. As far as the authors are aware, genome mining has never been applied to LS.

Performing computational modeling can provide significant insights into the molecular interactions, which occur between the LS and donor and acceptor substrates. This information can be used to create a library of potential LS products. It can also be used to direct LS catalyst away from hydrolysis and towards desired products. The effects of immobilization could also be examined using computational modeling, showing the regions where immobilization is most likely to occur.

The purpose of this research was to develop new immobilization approaches for the efficient synthesis of well-defined, β(2→6) FOSs, levans and novel transfructosylated products by LS enzymes and to expand this biocatalytic synthetic route by discovering new LSs with increasing

transfructosylation activity and a wide donor/acceptor specificities. This was achieved through the investigation of the immobilization of LSs on selected supports, emphasizing the relationship between the support features and the LS catalytic properties. Genome mining was used as a tool to discover new LSs and their catalytic potential will be examined both kinetically and by acceptor utilisation. An examination of the LS active site developed through homology models was used to explain the differences in activity observed.

To accomplish this research, the work was broken down into the following specific objectives:

1- Investigation of immobilization of LS from *Bacillus amyloliquefaciens* on selected modified supports, and determination of the thermal stability and catalytic efficiency of immobilized biocatalysts.
2- Modeling and optimization of the immobilization of LS from *B. amyloliquefaciens* using response surface methodology.
3- Utilization of genome mining to determine new LS enzymes with a characterization of their properties and kinetic parameters
4- Examination of the product profiles and determination of acceptor specificity of new LS enzymes with confirmation using computational homology models.

CHAPTER II. LITERATURE REVIEW

2. Introduction

Consumers wish for food which has more functional nutrition while maintaining a label with natural sounding ingredients. Fructooligosaccharides (FOSs) and fructan polysaccharides are ingredients which can provide these qualities. Enzymatic methods are a solution to finding clean methods of synthesizing unique FOSs and fructans. Directives can be taken to improve the synthesis of tailored FOSs and to improve the efficiency of the enzymatic catalysis. Of particular interest is the production of levan-type FOS and the polysaccharide levan itself, with their unique β(2→6) glycosidic linkages. These directives are increasingly studied for FOS and levan polysaccharides have gained attention for their techno-functional, nutraceutical and medical uses.

2.1. Benefits and uses of FOSs and Levan

2.1.1. FOS Prebiotics

With more informed, health conscious consumers, there is a niche for foods which serves benefits beyond flavour and sustenance. Fructooligosaccharides have attracted increasingly attention due to their prebiotic and techno-functional properties. Prebiotics are defined as "a selectively fermented ingredient that allows specific changes, both in the composition and/or activity in the gastrointestinal microbiota that confers benefits upon the host well-being and health" (Roberfroid, 2007). They are typically carbohydrates, oligo-and polysaccharides, whose osidic linkages resist hydrolysis from the host's gastric acid and pancreatic enzymes (Bello et al., 2001). FOSs are able to satisfy these criteria and were therefore among the first recognized prebiotics (Gibson & Roberfroid, 1995). As a result of their ability to stimulate the growth/activity of beneficial lactic acid bacteria in the human gastrointestine, FOSs enhance immune response and promote intestinal health (Fanaro et al., 2005; Knol et al., 2005).

Most prebiotic research has been performed using inulin-type prebiotic FOSs, linked by β-(1→2) glycosidic linkages, with little work devoted to levan-type FOSs. An *in vitro* study conducted by Marx *et al.* (1999) found that levan-type β-(2→6) FOSs resisted proteolytic and amylolytic digestion. *Bifidobacterium adolescentis, Bifidobacterium breve, Bifidobacterium longum, Bifidobacterium pseudocatenulatum* utilized these FOSs as a sole carbon source. Only *B. adolescentis* was able to hydrolyze long chain levan-type FOSs (Marx, Winkler & Hartmeier, 2000). Neokestose was found to have a much larger bifidogenic effect in comparison to commercial, P-95 FOSs, when tested using static batch-culture fermentations. There was also a

greater enhancement of the lactobacilli populations and a greater decrease in the *clostridia* population when neokestose was used in place of P-95 FOSs (Kilian, Kritzinger, Rycroft, Gibson, & du Preez, 2002). The growth of probiotic strains *Lactobacillus paracasei, B. longum* var. infantis 17930 and *B. longum* were found to grow similarly on both levan and levan-type FOSs while *Bifidobacteria bifidum, B. long*um var. infantis NRRL 4661 and *B. breve* grew better using levan-type FOSs than levan. All strains, using both substrates as a carbon source experienced a drop in pH in their growth medium (Porras-Dominguez et al., 2014).

Health claims associated with prebiotics include constipation relief, suppression of diarrhea, enhanced mineral absorption, immune modulation, reduction in the risk of osteoporosis, atherosclerotic cardiovascular disease, obesity and cancer (Monsan & Ouarne, 2009; Roberfroid, 2000). The fermentation end-products of the oligosaccharides results in short-chain fatty acids (SCFAs), butyrate, acetate and propionate, have been associated with constipation relief, cholesterol production, immune enhancement, mineral absorption and a reduction in colorectal cancer. Constipation relief occurs through osmotic pressure changes between fecal matter and the epithelial cells of the large intestine. The SCFAs act as colonic epithelium nutrients, and therefore grow and absorb more water and salt. The moisture can be transferred from the cells to fecal matter, preventing constipation. The decrease in the colonic pH is thought to aid in mineral absorption such as calcium, magnesium and iron. In ovariectomized rats that were given calcium (0.5-1%) supplements with FOSs (5-10%), there was a significant reduction of bone loss of the femur and lumbar region (Monsan & Ouarne, 2009). An in vivo study showed that prebiotics significantly increased the absorption of calcium in young males, but there was no effect on the absorption of magnesium, iron and zinc (Coudray et al., 1997).

The duration of diarrhea can be reduced through a synbiotic use of probiotics and prebiotics during oral rehydration therapy. Using a mixture *Lactobacillus acidophilus, Lactobacillus rhamnosus, B. bifidum, B. longum, Enterococcus faecium* and FOSs (0.625 g), the duration of diarrhea in children was shortened to 77.9 ± 30.5 hours from 114.6 ± 37.4 hours which the control experienced (Dinleyici et al., 2013). The immune enhancement through prebiotics is multifaceted. The large intestinal mucosa and the epithelial cells are thought to be stimulated by butyrate, which provides a defence against infection. The intestinal lymphoid tissue is another source of immune enhancement. It was found that mice with a diet of FOS had greater activity of natural killer cells and phagocytes compared to the mice on the control diet (Sangeetha, Ramesh, & Prapulla, 2005).

And as previously described, the existing gastrointestinal microbial flora make it difficult for new pathogenic bacteria to establish residence (Monsan & Ouarne, 2009). These ideas also apply to the prevention of colorectal cancer. Apoptosis is encouraged by the production of butyrate; carcinogens within feces are passed through more quickly due to increased moisture of fecal matter; the reduction of pathogens for which there is the growing evidence are associated with colorectal cancer (Castellarin et al., 2011; Kostic et al., 2011; Monsan & Ouarne, 2009).

Bifidobacteria and *Lactobacilli*, through the consumption of prebiotics, have been shown to decrease the pH of the colon significantly (Van Meer et al., 2008). The use of prebiotics in rats increased the population of *Bifidobacteria* and *Lactobacilli* by 344-366% in the colon, 139-282% in the feces. These increases led to a drop in pH by 1.0 point in the colon and 0.5 points in the feces (Van Meer et al., 2008).

The effects on cardiovascular disease are much debated. The total blood serum levels in rats were lowered when placed upon a prebiotic diet. A meta-analysis of fifteen studies over a period of ten years concluded that there is a reduction in serum triacylglycerides in blood by 7.5% (Monsan & Ouarne, 2009). In rats, the serum cholesterol levels were found to decrease but there wasn't a reduction on the amount of dietary cholesterol absorbed (Roberfroid, 2000). Van Meer et al. (2008) found the use of prebiotics did not affect the amount of bile salts produced by the liver. These bile salts contribute to the absorption of dietary lipids and cholesterol homeostasis. The prebiotic supplementation did not affect the amount of serum triglycerides or heptic triglycerides measured (Van Meer et al., 2008).

Interestingly, relationships have been shown to exist between the gut and the brain, important for the function of the neuroendocrine system, development of the immune system and the central nervous system (Cryan & Dinan, 2012). Interactions between the gut microbiota and pain perception, learning capacity, mood, emotion, temperament, stress management, dietary behavior and social interactions have been demonstrated through studies using germ-free animals, pathogen exposure and probiotics (Liang, Wu & Jin, 2018). Modulation of the gastrointestinal microbiota, through fecal microbiota transplants, probiotics, diet and lifestyle may impart significant changes (Liang, Wu & Jin, 2018), such as the use of probiotic related to the development of brain fogginess (Rao et al., 2018)

2.1.2. Commercial uses of FOSs and Levan

Non-digestible carbohydrates, which include FOSs, have technological uses within the food industry besides their being prebiotics. These properties will depend upon their degree of polymerization which affects water solubility, viscosity, water retention, and capacity to form a cream-like texture (Roberfroid, 2000). They are of low caloric value and non-cariogenic. Specifically, short-chained fructooligosaccharides (scFOSs) are water soluble, non-viscous and have approximately 30% the sweetness of sucrose. The caloric value is approximately 1.5-2.0 kCal/g, significantly lower than that of sucrose. FOSs are used as sucrose replacers due to these properties this makes them ideal for use in low-calorie foods and in the diets of diabetics (Alles et al., 1999). They are also used to give a fatty-mouth feel to food, replacing fats and adding texture (Monsan & Ouarne, 2009; Roberfroid, 2000). The freezing temperature of foods can be altered by their use and they can help retain moisture to prevent the drying of a food product. Since oligosaccharides are non-reducing sugars and thus, are unable to react with amino acids and can be used to control browning during baking due to Maillard reactions (Nantel, 1998; Monsan & Ouarne, 2009). FOSs can be added to a variety of products, including bakery products, infant formulas, salad dressings, soups, spreads, cereals, processed meat, canned fish and animal feed (Monsan & Ouarne, 2009; Roberfroid, 2000).

Levan is completely non-toxic, with no negative interactions with the skin or eyes and producing no allergenic response. It is soluble in both water and oil, which may need to be heated, but is insoluble in all organic solvents (Srikanth, Reddy, Siddartha, Ramaiah, & Uppuluri, 2015). It has been used in pharmaceuticals, medical applications, food applications, cosmetic products and animal feed due to its low viscosity, emulsifying, moisturizing, film-forming, encapsulating and stabilizing properties (Bersaneti et al.; Oner, Hernandez, & Combie, 2016). Levan has a film forming capability (Han, 1990) which lends its use in hair care products within mousses, sprays, fixatives and conditioners (Oner et al., 2016).

Sulfated levan was used to create multi-layer films with high adhesive properties with high tensile and shear strength. These properties were thought to arise from the extensive cross-linking present, the application being a replacement for sutures in medical applications (Gomes et al., 2018). Levan has also been shown to be an effective coating for Se, Fe_3O_4 and Co_3O_4 nanoparticles (NPs). The coating produced from *Pseudomonas syringae* reduced the toxicity of the Se NPs and eliminated the toxicity of the Co_3O_4 NPs. The stability of the NP dispersions was also improved by the levan coating (Bondarenko, 2016). In a similar lane, the levan produced by *Halomonas smyrnensis* was

used to synthesize fibrous nano and micro sized scaffolds for tissue engineering (Avsar et al., 2018). Highly-branched levan a has been even shown to have some anti-tumour activity (Yoo, Yoon, Cha, & Lee, 2004).

2.1.3. FOSs and Levan uses in Nature

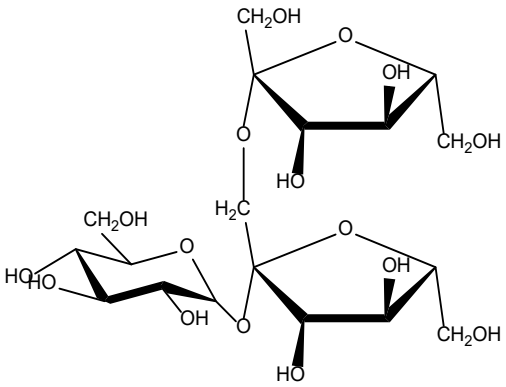
Within bacteria, levans are produced for multiple reasons: carbohydrate storage, drought protection, cell-to-cell adhesion and protection against virulent agents (Oner et al., 2016). It was proposed that in soil dwelling bacteria, *Bacillus subtilis*, levan was used for the storage of sucrose in the high sucrose environment of the rhizosphere caused by plant root exudate. Conversion of sucrose to levan allows *B. subtilis* to store the sucrose for its own use and will be available in times when sucrose is unavailable (Dogsa, Brloznik, Stopar, & Mandic-Mulec, 2013). With *Streptococcus mutans*, a bacterium which contributes to dental caries, levan aids in the bacteria adhesion onto the dental surfaces (Chambert & Petit-Glatron, 1993). Fructan production within plants has been related to protection against drought, salt and cold stress through interactions with the plants' phospholipids (Ritsema & Smeekens, 2003). Alternatively, levans production was found to be essential for the pathogenicity of *Erwinia amylovora*, causing fire blight in Rosaceous plants (Caputi et al., 2013).

2.2. Structures of FOSs and Levans

2.2.1. FOSs Structures

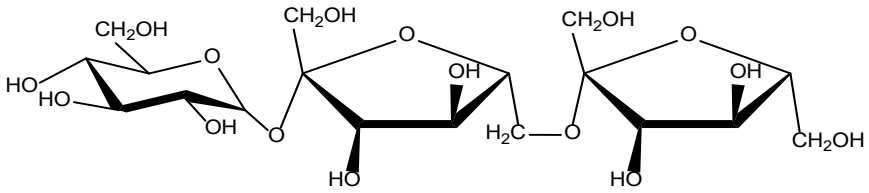
FOSs are emerging class of non-digestible oligosaccharides. They are made from 3 to 10 fructose units linked by β-(2→1) and/or β-(2→6) glycosidic linkages and contain a terminal D-glucose group. There are four major classifications of FOSs: inulin-, levan-, mixed levan- and neoseries-types (Monsan, 2009).

Inulin-type FOSs ($G_{1\text{-}2}F_{1\text{-}2}F_n$) (Scheme 1) are composed of β-(2→1) linked D-fructofuranosyl units with a D-glucose terminal head (Westhuizen, 2008). Commercially available inulin-type FOSs consists of 1-kestose (Glc-Fru2), nystose (Glc-Fru3) as well as fructofuranosylnystose (Glc-Fru4) (Plou et al., 2007).



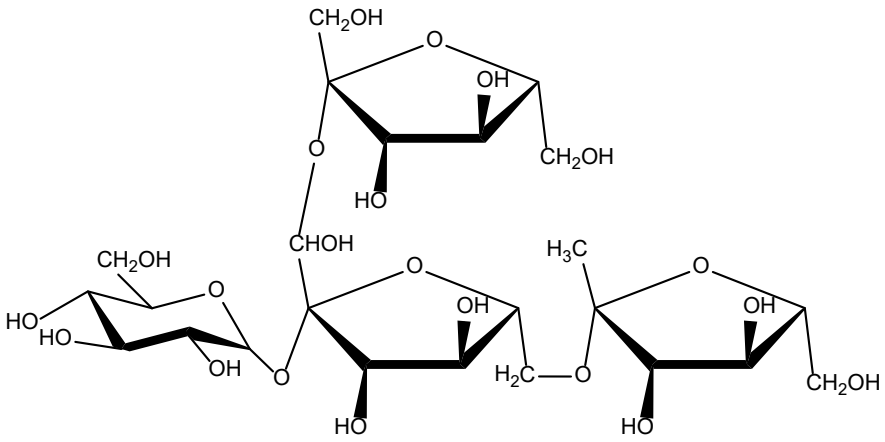
Scheme 1: Inulin-type FOSs, 1-kestose

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 (Scheme 2) is the smallest of this category (Westhuizen, 2008).



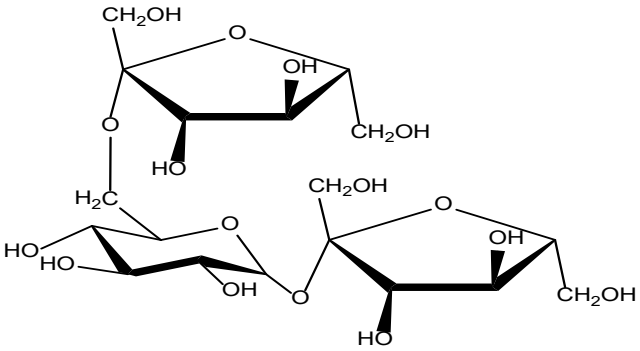
Scheme 2: Levan trisaccharide, 6-kestose

Mixed levan-type FOSs contains both β-(2→1) and β-(2→6) linked D-fructofuranosyl units. In this subclass of FOSs, the tetrasaccharide bifurcose (Scheme 3) is the smallest in which the fructosyl moiety of sucrose is β(2→6)-linked to the glucose portion of 1-kestose (Monsan & Ouarne, 2009).



Scheme 3: Mixed levan tetrasaccharide, bifurcose

Lastly, there is the inulin and levan neoseries. The neoseries contain fructose units bonded on the C1 and C6 carbons of glucose from sucrose. In the inulin neoseries (Scheme 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 & Ouarne, 2009; Westhuizen, 2008).



Scheme 4: Neo-Inulin-type FOSs, trisaccharide neokestose

2.2.2. Levan Structures

Levan is a polysaccharide consisting of β(2→6)-linked fructosyl units. There are a variety of molecular weights of levan, depending upon the source of the polysaccharide. Microbial levan comes in a variety of sizes and branching, while plant levan is limited in its size and consists of a more linear structure. Both low molecular weight levan (LMW) and high molecular weight (HMW) levan were produced by microbial sources, such as *Bacillus megaterium* producing levan found to be 2711 kDa, with some β(2→6) branching (Homann, Biedendieck, Goetze, Jahn, &

Seibel, 2007), while *Bacillus methylotrophicus* produced levan 4-5 kDa in weight (Zhang et al., 2014). The levan produced by *Aerobacter levanicum* was highly branched. It contained 66% branching, with branches linked by β(1→2) glycosidic linkages occurring approximately every 9 units on the basal structure, while being of 2-10 degrees of polymerization (DP) in length. Less branched levan was produced by *Microbacterium laevaniformans* KCTC 9732 contained 12.3% branching (Yoon et al., 2004).

2.3. Production of Levan and Fructooligosaccharides

2.3.1. Extraction from Natural Sources

In typical flowering plant species, fructans make up approximately 15 % of the composition (Ritsema & Smeekens, 2003). Inulin-type fructan are more common in nature, being found in the Asterales order such as Liliales and Compositae plants, examples of such are leeks onion, garlic, Jerusalem artichokes and chicory, with contents ranging from 1.1 to 20.5 g/100g (fresh weight) (Bosscher, 2009). Chicory derived inulin is from 3 to 70 units in length and are near linear in structure (Bosscher, 2009; Monsan & Ouarné, 2009). Low molecular weight levan (LMW) can be found naturally found in the Poaceae family in fodder grasses such as Dactylis glomeratoa, Pheleum pretense (Versluys, Kirtel, Toksoy Oner, & Van den Ende, 2018). Sizes range from 3 – 55 DP in length (Suzuki, 1993). They are limited in their structures and yields, consisting of mostly linear structures and yields up to 20% (Monsan & Ouarné, 2009; Ritsema & Smeekens, 2003).

Transgenic tabacco plants containing the genetic sequence for *Gluconacetobacter diazotrophicus* LS have been recently used for the production of levan (above 200 kDa), with yields of 10-70% dry weight found in the mature leaves (Banguela et al., 2011).

2.3.2. Chemical Methods

The traditional approach to the production of organic compounds is chemical synthesis. The synthesis provides many challenges. For stereo- and regiospecific chemical synthesis of oligosaccharides, the saccharides must be properly protected before coupling (Palcic, 1999). This is a labour-intensive process, which produces a good deal of waste and will require thorough purification practices. Another synthetic approach, which has been proven successful, is the acidic hydrolysis of polysaccharides to produce oligosaccharides. The problem of coproduced brown products was rectified by Warrand and Janssen (2007) in the production of malto-oligosaccharides

by the acidic hydrolysis of amylose under microwave heating. The downside to the chemical hydrolysis of a polysaccharide is its random specificity, resulting in a wide variety of products which will require purification (Warrand & Janssen, 2007). The highest yield of β-(2,6)-FOSs achieved by Marx et al. (2000) from acid hydrolysis of levan occurred with 5% (w/v) levan solution with 0.38 M sulphuric acid for 4 mins at 95°C. Higher concentrations of acid resulted in complete hydrolysis of levan to monosaccharide units. The resulting products were neutralized and separated by cation-exchange chromatography (Marx et al., 2000).

2.3.3. Enzymatic Synthesis of FOSs and Levan

2.3.3.1.B-Fructofuranosidases-Catalyzed Synthesis of FOSs

β-fructofuranosidases (EC 3.2.1.26) are powerful biocatalysts whose's natural function is to catalyze the hydrolysis of a glycosidic bond from the non-reducing end of sucrose, liberating fructose and glucose. β-fructofuranosidases also exhibit transfructosylating activity, where they catalyze the transfer of fructose to another acceptor molecule other than water (Alvaro-Benito et al., 2007). β-Fructofuranosidases belong to glycosyl-hydrolase (GH) family 32 and have a high degree of sequence homology (Lombard, 2014). Commercial β-fructofuranosidases are currently from *Aspergillus niger, Aspergillus oryzae, Penicillium nigricans* and *Saccharomyces cerevisiae* (Kurakake et al., 2010). The ratio of transfructosylating to hydrolytic activity of β-fructofuranosidases relies on the thermodynamic equilibrium of the reaction and on the capacity of the enzyme to bind to the acceptor with high specificity as compared to water. It is depended upon the microbial source of the enzyme, sensitive to sucrose concentration, pH and temperature (L'homme, Arbelot, Puigserver, & Biagini, 2003). The synthetic reaction catalyzed by β-fructofuranosidases may be favored over the hydrolytic one by high substrate concentration, elevated temperatures and the use of organic co-solvents or an acceptor (Plou, de Segura, & Ballesteros, 2007). The hydrolytic activity of β-fructofuranosidases can be also disfavored by the constant elimination of the transfructosylation end-products by crystallization, selective adsorption to carriers or coupling through another enzymatic reaction (Plou et al., 2007). The β-D-fructosyltransferase from *Rhodotorula sp*. was found to produce FOSs at high sucrose concentration while it had a hydrolytic nature when the sucrose concentration was low (Alvarado-Huallanco & Filho, 2011; Ghazi et al., 2007). Ghazi *et al*. (2007) separated the k_{cat} for hydrolysis and transfructosylation for the fructosyltransferase enzyme from *Aspergillus aculeatus*. The high

k_{cat} (1.62 x 10^4 s^{-1}) for the transfructosylation reaction signified that as the sucrose concentration was increased (1M), the rate of transfructosylation would be ~20-fold higher than that for hydrolysis (Ghazi et al., 2007). Yields from β-fructofuranosidases vary, where the enzyme from *S. cerevisae* yielded 8 %, *A. aculeatus* 61% (Monsan & Ouarné, 2009), *A. oryzae* KB 58.3 % (Kurakake et al., 2010), *Schwanniomyces occidentalis* 16.4 % (Alvaro-Benito et al., 2007). Although some of the yields of the β-D-fructosyltransferases are moderately high, the enzymes do suffer from narrow acceptor specificity and poor regioselectivity (Plou et al., 2007).

2.3.3.2.LS/Fructanase-Catalyzed Synthesis of FOSs and Levans

Besides β-fructofuranosidase, fructansucrases, including inulosucrase (EC 2.4.1.9) and levansucrase (EC 2.4.1.10, LS), catalyze the transfer of fructose to an acceptor molecule by directly using the free energy of cleavage of donor, most typically sucrose (Lombard., 2014; Van Hijum, Kralj, Ozimek, Dijkhuizen, & Van Geel-Schutten, 2006). Inulosucrase catalyzes the synthesis of the inulin and inulin-type FOSs. Inulin is a polysaccharide composed dominantly of β-(1→2)-linked β-D-fructofuranose units with some β-(2→6) branching (Anwar et al., 2010). The systematic name for LS is sucrose:α-D-glucosyl-(1→2)-(2→6)-β-D-fructan 6-β-D-fructosyltransferase (Lombard, 2014). Synthesis of levan and levan-type FOSs are catalyzed by LS. Both products are headed with a D-glucose group which results from the first acceptor molecule being sucrose. Microbial LSs can exist as a monomer or as a multiple subunit enzyme (Hettwer, Gross, & Rudolph, 1995; Ohtsuka et al., 1992; Pabst, 1977). There are typically four regions of this protein: (1) an N-terminal signal peptide; (2) a variable region; (3) the enzymatic active domain; and (4) a C-terminal cell wall anchoring region (Waldherr, Meissner, & Vogel, 2008). Calcium was found to be essential for LS synthesis, where all LS activity was lost after incubation with EDTA (Ozimek, Kralj, van der Maarel, & Dijkhuizen, 2006; Waldherr et al., 2008).

Bi-enzymatic systems, based on the combined use of LS and fructanases, are another approach to the synthesis of FOSs. Fructanases, including levanases (EC 3. 2. 1. 65) and inulinases (EC 3. 2. 1. 7), are glycoside hydrolylases capable of hydrolyzing the β-(2→6) and β-(1→2) glycosidic bonds. Low molecular weight levan (8.3 kDa) produced by LS from *B. subtilis*, incubated in the presence of recombinant endo-levanase from *Bacillus licheniformis IBt1*, produced FOSs, mainly levanbiose (Porras-Dominguez et al., 2014). Endo-levanase isolated from *Bacillus sp*. L7 produced

levantriose from levan (not described) at a yield of approximately 24% (Miasnikov, 1997). The hydrolysis of levan from *Bacillus amyloliquefaciens* using separately endo-inulinase from *A. niger* resulted in the production of scFOSs (2 – 5 saccharide units) and oligolevans (DP ≥ GF_5, F_6), LMW and HMW levan (in different ratios). Fructanase®, the commercial endo-inulinase from *A. niger* resulted in larger amounts of fructose and oligolevans (Tian, Karboune, & Hill, 2014). Optimization of sucrose concentration, reaction time and LS to endo-inulinase ratio resulted in the high yield (57-65% w/w) of FOSs and oligolevans. Initial products were mainly composed of scFOSs while the mid-reaction products consisted of medium chained FOSs (Tian, Khodadadi, & Karboune, 2014).

2.4. LS-Catalyzed Reactions

2.4.1. Reactions catalyzed by LS

LS can catalyze four different reactions: exchange, hydrolysis, transfructosylation and polymerization. The degree to which the enzyme performs this reaction depends upon the microbial source of the LS (Strube et al., 2011). It can perform these different reactions due to the nature of the active site of the enzyme. These reactions will produce glucose, fructose, varying FOSs and levan.

2.4.2. Active Site and Mechanism of LS

The mechanistic properties of LS enzymes have been partially elucidated through mutagenesis studies, amino acid alignment and X-ray crystal structures. Based upon the sequential information, LS belongs to glycoside hydrolase family 68, along with inulosucrase and β-fructofuranosidase (Henrissat, 1991).

LS catalyzes reactions via a double displacement or a “ping-pong” mechanism with a covalent fructosyl-enzyme intermediate (van Hijum, Kralj, Ozimek, Dijkhuizen, & van Geel-Schutten, 2006). There are currently only 4 bacterial sources of LS for which there are high-resolution crystal structures obtained by x-ray crystallography, the LSs from: *B. subtilis* (G. Meng & Fütterer, 2008; Meng & Futterer, 2003; Strube et al., 2011), *B. megaterium* (Anwar et al., 2010) as well as the gram-negative *G. diazotrophicus* (Martinez-Fleites et al., 2005) and *E. amylovora* (Wuerges et al., 2016). With each new structure comes more mechanistic information on LS. Site-directed mutagenesis along with amino acid sequence alignment has also provided a substantial amount of

structure-function information on LS. The structure of the catalytic core categorizes LS as being of clan GH-J. This is a description of its fivefold β-propeller topology, which contains four anti-parallel β-strands in the classic "W" topology (Strube et al., 2011). The central, negatively charged pocket, containing the catalytic triad, is highly conserved sequence among fructansucrase enzymes (Van Hijum et al., 2006). Knock-out studies have identified the essential amino acids constituting the catalytic triad of LS from *B. megaterium* (Asp^{95}, Asp^{257} and Glu^{352}), *B. subtilis* (Asp^{86}, Asp^{247} and Glu^{342}) and *E. amylovora* (Asp^{46}, Asp^{203} and Glu^{287}) (Strube et al., 2011; Van Hijum et al., 2006). Asp^{95} acts as a nucleophile and attacks the anomeric carbon of the glucopyranosyl unit. It forms an enzyme-intermediate with the fructosyl residue, inverting the glycosidic bond (Homann et al., 2007). The substitution of Asp^{95} with Ala resulted in the loss of LS activity (Van Hijum et al., 2006). Asp^{257} is involved in the formation of hydrogen bonds with the C3 and C4 hydroxyl groups of the fructosyl unit. This helps to stabilize the transition state of the transfructosylation reaction (Strube et al., 2011). Glu^{352} acts as an acid/base catalyst and a large decrease in activity is observed with its substitution with Ala (Van Hijum, Kralj, Ozimek, Dijkhuizen, & Van Geel-Schutten, 2006; Van Hijum et al., 2006).

Specific subsites have been identified in the LS structure. The amino acid composition of these subsites defines their affinity for certain molecules and dictates the reactions which LS can catalyze. The -1 and +1 subsites of LS are conserved in LSs from Gram-negative and Gram-positive bacteria (Van Hijum et al., 2006). Subsite -1 is highly specific for accepting the fructofuranosyl residue, whereas the +1 subsite is flexible, accepting both glucose (sucrose as donor substrate) and fructose (sucrose as acceptor substrate) (Anwar et al., 2010; Homann et al., 2007; Van Hijum et al., 2006). Sucrose first occupies the -1 and +1 subsites. A covalent intermediate will form between the enzyme and the released fructosyl unit at the -1 subsite. For a transfructosylation reaction, an acceptor molecule (e.g. sucrose) enters the active site, and binds to the +1 and +2 subsites, and reacts with the previous fructosyl-enzyme intermediate, resulting in the fructosyl transfer to the acceptor molecule (Van Hijum et al., 2006). If water enters the catalytic site, the hydrolysis of sucrose can occur, with fructose released (Ozimek et al., 2006).

Sucrose was found to be stabilized and orientated by hydrogen bonds with Try^{85}, Arg^{247}, Glu^{340}, Tyr^{411} in *B. subtilis* LS and Trp^{94}, Arg^{256}, Glu^{350}, Trp^{172} and Arg^{370} in *B. megaterium* LS (Homann et al., 2007; Strube et al., 2011). The crystal structure of the LS from *E. amylovora* showed glucose and fructose stabilized through H-bonds with Trp^{45}, Arg^{202}, Gln^{285}, Asp^{203}, Asp^{46}, Glu^{287}, His^{97},

Arg^{96}, His^{305} (Wuergeset al., 2015). Substitution of Trp^{94} in *B. megaterium* LS with Ala resulted in 9% of the original catalytic efficiency. Only 3% of the catalytic activity remained when Tyr^{421} was replaced with Ala. Trp^{94} was found to form a hydrogen bond with the C6 hydroxyl group of the fructosyl group and Tyr^{421} formed a hydrogen bond with the C2 hydroxyl group of the glucosyl residue (Homann et al., 2007). Substitutions with Ala of Arg^{370} and Arg^{360} in LSs from *B. megaterium* and from *B. subtilis,* respectively, led to unspecific substrate orientation. Arg^{370} conserved in LSs from Gram-positive bacteria is found to interact with the C2 and C3 hydroxyl groups of the glucosyl residue and to be essential for polysaccharide synthesis. With this substitution, fructosylation occurred at C6 of fructose, producing neokestose. The hydrolysis of neokestose led to the accumulation of blastose (Homann et al., 2007; Strube et al., 2011). LsdA found in LS from *G. diazotrophicus,* a Gram-negative bacterium, contains a histidine instead of Arg^{370}, where FOSs are produced rather than larger polysaccharides (Strube et al., 2011). Substitution of Ser^{173} with Ala did not change the binding of sucrose (K_m) in SacB of *B. megaterium* but did change the catalytic efficiency of the enzyme. This demonstrates that Ser^{173} is involved in catalysis but not in the binding of the substrate (Homann et al., 2007). Within *B. subtilis* LS, Asp^{312} was found on the surface of the enzyme between the two-sucrose binding boxes and forms a 180° reverse β-turn (Van Hijum et al., 2006).

LS enzymes can be distinguished by whether they perform a processive or a non-processive/disproportionate reaction. Within the LS active site, the -1 subsite is specific for the donated fructosyl group, the +1 and +2 subsites for the acceptor molecule. In the processive reaction the subsites have affinity for the new molecule formed after transfructosylation. The product initially produced is held within the active site by subsites +2 and +3, where fructosyl units are continuously added and the product is prevented from being hydrolysed (Ozimek et al., 2006). The products of the processive reaction are HMW levan polymers (Ozimek et al., 2006). LS enzymes from Gram-positive bacteria often perform the processive reaction. Examples of this can be seen from the LS from *Lactobacillus reuteri* which produced HMW levan of 2711 kDa respectively (Homann et al., 2007). On the other hand, a disproportionate reaction will occur when subsites +2 and +3 have low affinity for the product and it is released after transfructosylation (Ozimek et al., 2006). The reaction profile will be characterized by GF(n ± 1) oligosaccharides. This can occur in two fashions. If an oligosaccharide product is hydrolysed to sucrose, and then the sucrose is used as the acceptor molecule. It can also occur if the fructosyl end of an

oligosaccharide falls into subsite -1, it will be cleaved. When a fructose from another oligosaccharide enters the +1 subsite, the LS will transfer the fructose to the new molecule (Ozimek et al., 2006). The LS from *G. diazotrophicus* is an example of an enzyme with a disproportionate product profile, synthesizing predominately scFOSs (Hernandez et al., 1995a). Interestingly, Raga-Carbajal et al. (2016) found that the LS from *B. subtilis* produced both HMW (2300 kDa) and LMW levan (7.2 kDa), performing both a disproportionate and processive reaction (Raga-Carbajal et al., 2016).

Another amino acid which was found to be essential for polysaccharide growth is Asn^{252}, which is located in subsite +2 (Beine et al., 2008). When Asn^{252} was substituted with either Ala or Gly, polysaccharide synthesis was discontinued, and hydrolysis increased. It has been reported that Asn^{252}, which is retained in LSs from Gram-positive bacteria, interacts and stabilizes the third fructosyl group of the growing acceptor chain (Homann et al., 2007). Asn^{252} is retained within Gram-positive bacteria but varies in Gram-negative bacteria (Homann et al., 2007). In *E. amylovora*, a Gram-negative species, this asparagine is 5Å further than Asn^{252} in LS from *B. subtilis*, preventing the residue from playing the same role within the +2 subsite (Wuerges et al., 2015). Lys^{373} and Try^{247} of LS from *B. megaterium* were also found to affect polysaccharide synthesis. When Lys^{373}, located in loop 7, was replaced with Arg or Ala, long-chained and short-chained oligosaccharides were formed. A mixture of oligosaccharides was formed when Tyr^{247}, from loop 4, was replaced with Ala or Ile, but when replaced with Trp, the same products were observed as for the wild type. This gives evidence that the tyrosine is involved in π-π stacking mechanism. This residue, Tyr^{247}, was found not to be conserved in the LSs from *G. diazotrophicus* and *E. amylovora*, and was replaced with phenylalanine, Phe^{304} and Phe^{198} respectively (Martinez-Fleites et al., 2005; Wuerges et al., 2015). These three residues are all located on the surface of LS from *B. subtilis* (Strube et al., 2011).

2.4.3. Reaction Selectivity (Hydrolysis vs. Transfructosylation)

One limitation to the use of LS for the production of unique FOS prebiotics is its ability to catalyze the hydrolysis of sucrose. This reaction competes with the transfructosylation reaction, reducing the production of FOSs (Oseguera, Guereca, & Lopez-Munguia, 1996; Yanase et al., 1992). The ratio of transfructosylation to hydrolytic activities is dependent on the structure of the enzyme, the reaction conditions (e.g substrate concentration, interacting ions, reaction temperature, pH)

(Olvera, Centeno-Leija, Ruiz-Leyva, & Lopez-Munguia, 2012; Ortiz-Soto, Rudiño-Piñera, Rodriguez-Alegria, & Munguia, 2009). At low sucrose concentrations (e.g. 3 mM), LS from *Bacillus circulans* showed 40% transfructosylation activity which was increased to 70% when higher concentration (300 mM) were used (Oseguera et al., 1996). An increase in sucrose concentration up to 0.2 M resulted in an increase in levan formation for the LS from *B. amyloliquefaciens*. Beyond that, substrate inhibition was thought to disrupt the levan forming ability (Tian, Inthanavong, & Karboune, 2011). An increase in levan production was observed from the LS from *B. methylotrophicus* SK 21.002. It was constant until 300 g/L, then production continued to increase, but at a slower rate (Zhang et al., 2014). The inflection point for LS from *L. reuteri* 121 was 85 mM sucrose, where below that concentration, the enzyme showed predominately hydrolytic activity, while above that concentration, transfructosylating activity was preferred (Ozimek et al., 2006). LS from *Zymomonas mobilis*, in its dimeric form, was found to perform solely hydrolysis at sucrose concentrations below 250 mM. At higher concentrations, FOSs synthesis occurred (Goldman et al., 2008). LS from *B. amyloliquefaciens* experienced less hydrolysis using raffinose (9% hydrolysis) as the sole substrate as compared to sucrose (22% hydrolysis) after 12 hours reaction. The authors thought this effect was due to sucrose's high affinity to bind in both the -1 and +1 subsite on the LS (Tian & Karboune, 2012).

Optimal temperature ranges of LS enzymes from *Z. mobilis* (Yanase et al., 2002), *L. reuteri* (Van Hijum et al., 2006) and *B. megaterium* (Homann et al., 2007) were between 45 – 50°C, while the optimal temperature for *M. laevaniformans* was lower, at 30°C (Park et al., 2003). Transfructosylating and hydrolytic activity were affected by temperature. LS from thermophilic *Geobacillus stearothermophilus*, had an optimum temperature for transfructosylation at 57°C, while the hydrolytic activity was highest at 47°C (Inthanavong, Tian, Khodadadi, & Karboune, 2013). Chambert and Petit-Glatron (1993) found LS from *B. subtilis* had an optimum levan production temperature at 5°C; when the temperature was increased to 60°C, there was a significant drop in levan production and an increase in hydrolysis. Jang *et al.* (2001) noted a similar effect with LS from *Z. mobilis;* between 5-15°C, transfructosylation was preferred, while at elevated temperatures, 30-40°C, hydrolysis of sucrose was preferred. Similar was recorded for Lsc3 and LscA from *Pseudomonas syringae pv. tomato* and *Pseudomonas chlororaphis subsp. aurantiaca* where transfructosylation was higher (80 ± 2%) between 0-20°C while hydrolysis increased to 67 ± 3% and 50 ± 1% respectively, at 60°C (Visnapuu, Maee, & Alamaee, 2008).

Altering the incubation temperature can lead to change in product formation. With an increase in temperature, the formation of FOSs by LS from *Rahnella aquatilis* was preferred, while at lower temperatures, levan was formed (Kim et al., 1998). A comparison of the products formed by LS from *Z. mobilis* showed that the degree of polymerization (DP) and the yield of levan were lower when the incubation temperature was increased (Jang et al., 2001). At higher temperatures, it may be more difficult for some enzymes to retain the accepting molecule at the subsites +1, +2 and +3, resulting in the release of products with short chain length.

The optimal pH of most microbial LSs ranges from 5.0 to 6.6 (Ben Ammar, Matsubara, Ito, Iizuka, & Minamiura, 2002; Homann et al., 2007; Inthanavong et al., 2013; Takahama et al., 1991). The reaction pH was a significant factor for the transfructosylating/hydrolysis ratio of *G. stearothermophilus*. Transfructosylation was favoured over hydrolysis within the pH range of 6-6.5 (Inthanavong et al., 2013). *B. subtilis* LS had a narrow optimal pH range (5.6-6.0) for transfructosylating activity, while the pH range for hydrolytic activity was broader; from pH 5.5-7.0 (Olvera et al., 2012). pH was found to alter the 3D structure of LS from *Z. mobilis*. At pH 7, the LS was found in dimeric form and had predominately hydrolytic activity. At a lower pH (i.e. pH 5) the LS formed long insoluble fibrils. In this form, the LS had mainly transfructosylating activity. An investigation of this phenomena revealed that the transfructosylating and hydrolytic activity was dependant on the structure of the LS, not the pH of the reaction media (Goldman et al., 2008).

Hydrolysis decreased to 10% and 20% from 45% when the LS SacB gene from *B. subtilis* was inserted with a C-terminal domain and a transitional domain from *Leuconostoc citreum* inulosucrase. Transfructosylating activity was increased 90% when and a C-terminal domain and a transitional domain from *Leuconostoc mesenteroides* LS was inserted. The insertions altered the catalytic core of LS, changing the acceptor specificity of the enzyme or limiting the water activity of the active site (Olvera et al., 2012). Among 36 mutational LS variants of Lsc3 from *P. syringae* pv. *tomato* DC3000, only 7 had a similar amount of transfructosylating activity to the native enzyme. Mutants of the catalytic triad lost almost complete activity, while mutants Glu146Gln, Thr302Met and Asp333Asn had slightly more enhanced FOS production (Mardo et al., 2014).

2.4.4. Catalytic Properties of LS

The catalytic properties of different LS species vary according to their need and environment. The bacterial source of LS strongly determines its activity. Some LS enzymes direct their activity towards hydrolysis while others towards transfructosylation. *G. Stereaothermophillus* had higher activity towards transfructoslyation than it did for hydrolysis. The V_{max} and k_{cat} were 58.5 µmol/mg protein min and 53.0 s^{-1} for transfructoslyating activity, while they were 27. 7 µmol/mg protein min and 25.1 s^{-1} for hydrolytic activity, almost half. *B. amyloliquefaciens* LS had much higher values for both V_{max} and k_{cat}, they were 1196.3 µmol/mg protein min and 1136.0 s^{-1} for transfructosylating activity and 188.0 µmol/mg protein min and 178.6 s^{-1} respectively (Tian et al., 2011). When comparing the catalytic efficiency, LS from *G. stereaothermophilus* had higher catalytic efficiency for transfructosylating activity of 197.1 $M^{-1}s^{-1}$ than for hydrolytic activity, 92.5 $M^{-1}s^{-1}$ (Inthanavong et al., 2013). LS from *B. amyloliquefaciens* was in the opposite scenario, with higher catalytic efficiency towards hydrolysis, 9500 $M^{-1}s^{-1}$, than towards transfructosylation 2470.0 $M^{-1}s^{-1}$. When comparing the two bacterial sources for LS production, *B. amyloliquefaciens* had the highest activity overall, and its activity towards transfructosylation was 20 times that of *G. stereaothermophilus* (Tian et al., 2011). LS from *B. megaterium* and *L. reuteri* had 58.4% and 51.6% of its reaction products from sucrose directed towards hydrolysis products (Homann et al., 2007; Ozimek et al., 2006). The K_m for hydrolysis for LS from *B. megaterium* was considered low at 6.6 mM when compared to LS from *B. subtilis* (13.5 – 40 mM). While the k_{cat} was 2272s^{-1}, giving the LS from *B. megaterium* a high catalytic efficiency towards hydrolysis (346 $mM^{-1}s^{-1}$) (Homann et al., 2007). Table 2.1 demonstrates the optimal kinetic transfructosylating and hydrolytic parameters of LS's from various bacterial species in relation to their optimal conditions.

Table 2.1. Optimal kinetic parameters of LSs of different microbial origins

Bacterial Species	Transfructosylating activity				Hydrolytic activity				
	kcat (s^{-1})	Km (mM)	Temperature (°C)	pH	kcat (s^{-1})	Km (mM)	Temperature (°C)	pH	Reference
G. stearothermophilus	53	269	57	6-6.5	25	272	47-57	6.75	(Inthanavong et al., 2013)
B. megaterium			37	6.6	2272	6.6			(Homann et al., 2007)
Z. mobilis	379	36	15	5	64			7.4	(Crittenden & Doelle, 1994; Goldman et al., 2008)
B. subtilis	48.4	21.5		5.6-6	33.3	11.6	6	5.5-7	(Olvera et al., 2012)
R. aquatilis			40	6			50	6	(Ohtsuka et al., 1992)
P. syringae pv. *phaseolicola*			18	6.2			60	6.2	(Hettwer et al., 1995)
B. amyloliquefaciens	1137	460			178.6	18.8			(Tian & Karboune, 2012)
B. circulans			40	5-7			45	6	(Oseguera et al.,1996)
L. gasseri	53	6.9	55	3.5-4.5	242	8.3	55	3.5-4.5	(Anwar et al., 2010)
L. panis		22.5	45-50	4.0-4.6		17	45-50		(Waldherr et al., 2008)

2.4.5. Transfructosylation Product Spectrum

As can be seen from Table 2.2, LS from different microbial sources had different product spectrums in terms of FOS and levan composition. HMW levan was produced by the Gram-positive *B. licheniformis* (9.6 x 106 Da), *Bacillus natto* (2.5 x 106 Da), *L. reuteri* 121 (1.5x105 and > 2 x 106Da), *Lactobacillus sanfranciscensis* TMW (≥ 5 x 106 Da) and *P. syringae pv. Phaseolicola* (up to 107 Da) (Lu et al., 2014, Van Hijum, Szalowska, Van Der Maarel, & Dijkhuizen, 2004, Tieking, Ehrmann, Vogel, & Ganzle, 2005, Hettwer et al., 1995). Contrarily, for both *Z. mobilis* and *G. diazotrophicus* LSs, the predominate product was FOS (Crittenden & Doelle, 1993; L. Hernandez et al., 1995a)

Levan is commonly synthesized in conjunction with FOSs production. *L. mesenteroides* B-512 FMC produced levan, 1-kestose, nystose and 1,1,1-kestopentanose (Kang et al., 2005). *E. amylovora* synthesized scFOSs in the presence of sucrose such as 1-kestose, 6-kestose and neokestose, and when sucrose concentrations were high, levan and nystose were produced (Caputi et al., 2013). Whereas the LS from *B. subtilis* had a bimodial production, producing HMW levan (2300 kDa), LMW levan (7.2 kDa) as well as the FOSs: levanbiose (Fructofuanosyl-(2→6)-O-β-D-Fructose), blastose, 1-kestose, 6-kestose and neokestose. The differences between the processive and non-processive reaction were found to be affected by the enzyme concentration (Raga-Carbajal et al., 2016).

B. amyloliquefaciens LS was able to catalyze the synthesis of levan (up to 10^4 Da) and four FOSs (1-kestose, neokestose, blastose and 6-kestose) from sucrose. While when using raffinose, nystose and neokestose were synthesized at low concentrations with melibiose as the main product along with levan (Tian & Karboune, 2012). The transfructosylation product spectrum of Lsc3 from *P. syringae pv. tomato* varied upon sucrose concentration. With 300 mM sucrose, mostly levan (15.5 mg/mL) was produced; the profile was altered in favour of FOSs (104.1 mg/mL) with some levan (7.2 mg/mL) with an increase tp1200 mM sucrose. A similar trend was seen using LscA from *P. chlororaphis* (Visnapuu et al., 2011).

Table 2.3: Dominating Products of Levansucrases

Bacterial Source	Product	Reference
L. reuteri 121	HMW levan	(Ozimek et al., 2006)
L. gasseri 20077	Levan	(Anwar et al., 2010)
L. panis	Levan, small amount of FOSs	(Waldherr et al., 2008)
L. sanfranciscensis	HMW levan, FOS	(Tieking et al., 2005)
L. mesenteroides B-512	Small FOS; levan	(Kang et al., 2005)
P. syringae pv. tomato str. DC3000	Levan and FOS	(Mardo et al., 2014)
P. aurantiaca S-4380	Levan with low branching	(Jang et al., 2006)
P. fluorescens	Levan	(Jathore, et al., 2012)
B. subtilis natto	> 2000 kDa levan, 6 – 9 kDa levan	(RagazCarbajal et al., 2016; Shih et al., 2010)
B. subtilis NCIMB 11871	300 kDa	(Cheetham, Hacking, & Vlitos, 1989)
B. methylotrophicus	4-5 kDa levan	(Zhang et al., 2014)
B. circulans	10-38 kDa	(El Refai et al., 2009)
B. licheniformis	1-612 kDa	(Nakapong et al., 2013)
B. megaterium DSM 319	2711 kDa levan,1-kestose, 6-kestose, nystose, neokestose, blastose	(Homann et al., 2007)
B. amyloliquefaciens	Levan, 1-kestose, neokestose, blastose, 6-kestose	(Tian et al., 2011)
G. stearothermophilus	Levan (35% w/w); FOS (6.3% w/w)	(Inthanavong et al., 2013)
G. diatrophicus	FOS; 1-kestose	(Martinez-Fleites et al., 2005)
R. aquatilis ATCC33701	FOS	(Kim et al., 1998)
E. amylovora	FOS DP 2-6	(Caputi et al., 2013)
Corynebacterium	levan	(Henis, 1956)
Z. mobilis	> 6 x 10^6 Da levan; 1-kestose	(Goldman et al., 2008; Jang et al., 2001)

2.4.6. Donor and Acceptor Specificities

For LS to catalyze transfructosylation, it requires a fructosyl donor, typically sucrose, and a fructosyl acceptor. Besides sucrose, raffinose is a highly utilized fructosyl donor. LS enzymes from *L. sanfranciscenis*, *L. reuteri, B. subtilis, M. laevaniformans, P. syringae, Z. mobilis* were able to utilize raffinose as a fructosyl donor (Andersone, Auzina, Vigants, Mutere, & Zikmanis, 2004; Kim, Park, Sung, Park, & Cha, 2005; Park et al., 2003; Seibel et al., 2006; Tieking et al., 2005; Van Hijum et al., 2004; Visnapuu et al., 2008). The LS from *M. laevaniformas* was even capable of utilizing the tetrasaccharide stachyose as a fructosyl donor (Kim et al., 2005).

Monosaccharides D-galactose, D-xylose and D-fucose were successfully utilized as acceptor molecules to create sucrose analogs by the LSs from *B. subtilis* (Juergen Seibel et al., 2006), B. *licheniformis* (Li et al., 2015) and *M. laevaniformas* (Kim et al., 2005). Multiple sucrose analogs were also used by *B. subtilis* LS as acceptor molecules using sucrose as a donor molecule as listed in Table 2.3. It was capable of utilizing Man-Fru, All-Fru, Gal-Fru, D-Fuc-Fru and Xyl-Fru to varying degrees. Gal-Fru, D-Fuc-Fru and Xyl-Fru produced over 48% transfructosylation products, while the rest were hydrolysed. Other sucrose analogs were unsuitable as acceptors, such as All-Fru and Man-Fru, producing <1% and 5% transfructosylation products (Seibel et al., 2006). The kinetic parameters were measured and all sucrose analogues were found to have lower catalytic efficiency than sucrose (6.1 $s^{-1}mM^{-1}$), while the K_m values of both Gal-Fru and Man-Fru similar to that of sucrose (Beine et al., 2008). The C2 hydroxyl group of both xylose and galactose, in the equatorial position, is similar to that of glucose, which was found to be essential for catalysis. The equatorial position of the hydroxyl group places it in a good position to be protonated by the active site (Meng & Futterer, 2003). *B. amyloliquefaciens* LS was able to use the sucrose analogs, D-Gal-Fru and D-xyl-Fru as donor molecules. After utilizing D-galactose and D-xylose as acceptor molecules, these products were used as donor molecules themselves. The concentration of D-Gal-Fru and D-xyl-Fru decreased over time while the concentration of D-Gal-nFru and D-Xyl-nFru increased (Tian & Karboune, 2012).

Maltose, cellobiose, melibiose and lactose were identified as effective disaccharide acceptor molecules for the LSs from *B. subtilis* (Seibel et al., 2006), *B. lichenformis* (Lu et al., 2014), *M. laevaniformans* (Kim et al., 2005) and *R. aquatilis* JCM-1683 (Ohtsuka et al., 1992). Transfructosylation was the main reaction when maltose was used as an acceptor molecule for LS from *B. amyloliquefaciens*. Transfructosylation occurred at 85-90%, with the trisaccharide erlose as the main product. Contrarily, when lactose was the acceptor molecule, the main product was

Table 4.3: Acceptor molecules of LS

Bacterial source of LS	D-xylose	D-arabinose	L-arabinose	lactose	D-maltose	maltotriose	cellobiose	melibiose	xylobiose	D-fructose	D-galactose	D-mannose	D-glucose	D-ribose	D-lyxose	D-fucose	L-sorbose	L-glucose	L-rhamnose	L-galactose	L-fucose	L-xylose	3-ketoglucose	isomaltose	raffinose	lactulose	trehalose	melezitose	D-sorbitol	xylitol	D-mannitol	benzyl alcohol	isopropanol	1-pentanol	Inositol	butanol	2-deoxy-D-glucose	4-methoxyphenol	hydroquinone	catechol	resorcinol	glycerol	Man-Fru	All-Fru	Gal-Fru	D-Fuc-Fru	Xyl-Fru	D-fucose	D-galacturonic acid	glucosamine
B. licheniformis 8-37-0-1[1]	‡		‡	‡	‡		‡				+																-					o	o																	
B. amyloliquefaciens [2]	‡			‡	‡						‡		‡												‡																											
B. circulans [3]				‡	‡						‡																	+					-	o									+								
B. subtilis NCIMB 11871[4]	‡			‡	‡		‡	‡			‡	‡						+	-	-	-		-	‡													‡											‡		
B. subtilis (BS-LVS)[5]																															-			+		-	-	-	+										
B. subtilis[6]																																											-	-	‡	‡	‡			
G. stearothermophilis[7]				‡	‡						‡														‡																										
Pseudomonas chlororaphis[8]	o		o						o					o		o												o	o	o																			o	-
P. syringae pv. Tomato DC300[9]	o	o	o						o					o		o												o	o	o																			o	-
A. levanicum[10]	o			o							o																																							
R. aquatilis JCM-1683[11]	‡	‡	‡	‡	‡	‡	‡	‡		+	+	+		+	+		-								-	-	-	-																					
P. polmyxa[12]				+																																														
Z. mobilis[13]				‡																																														

+++ 100-51% transferase activity; ++ 50-20% transferase activity; + 20-10% transferase activity; - < 10% transferase activity; o enzyme was capable of using acceptor molecule, but yield was not measured. [1]Lu et al., 2014; [2]Tian & Karboune, 2012; [3]Oseguera, 1996; [4]Seibel et al., 2006; [5]Mena-Arizmedi et al., 2011; [6]Beine et al., 2008; [7]Inthanavong et al., 2013; [8-9]Visnapuu et al., 2011; [10]Hestin et al., 1955; [11]Ohtsuka et al., 1992; [12]Choi et al., 2004; [13]Han, 2009

levan. While erlose was a poor acceptor molecule, leading to its accumulation, lactose-fructose (o-β-D-galactopyranosyl-(1→4)-α-D-glucopyranosyl-(1→2)-β-D-fructofuranoside) concentration increased over 12 hours, then steadily decreased, corresponding to its usage as a fructosyl acceptor (Tian & Karboune, 2012).

LS from *G. stearothermophilus* was also able to utilize galactose, lactose and raffinose with an optimal donor to acceptor ratio for FOSs synthesis at 1.0:0.5. Maltose was an even better acceptor molecule, with a 92% conversion of the donor sucrose, had an optimal donor to acceptor ratio of 0.5:1.0 (Inthanavong et al., 2013). The activity of LS from *B. amyloliquefaciens* was directed towards polymerization instead of the production of FOSs when the fructosyl donor molecule was switch from sucrose to raffinose (Tian & Karboune, 2012).

LSs have been used to synthesize lactosyl-fructoside (O-β-D-galactopyranosyl-(1,4)-O-α-D-glucopyranosyl-(1,2)-β-D-fructofuranoside), a trisaccharide which has been used as a prebiotic ingredient in food and an artificial sweetener (Mu, Chen, Wang, Zhang, & Jiang, 2013). This results from the transfructosylation of fructose from sucrose, to lactose. The conditions for maximal production of lactosylfructoside from *B. methylotrophicus* SK 21.002, were recently determined, reaching a yield of 143 mg/L at a conversion efficiency of 36%. Similar conversion efficiency of lactosucrose was achieved by using whole *Paenibacillus polymyxa* cells containing LS (17.0% w/v) (Choi, Kim, Kim, Jung, & Oh, 2004) and *Z. mobilis* LS (43%) (Han et al., 2009).

LS from *B. subtilis* LVS was used to explore alcoholic acceptor molecules, especially phenolic molecules. Interestingly, the more nucleophilic acceptors assayed were the least productive in terms of transfructosylation. Resorcinol, hydroxyquinone, catechol and 4-methoxyphenol were efficient acceptor molecules when used with sucrose while butanol and benzyl alcohol were poor acceptors. Upon comparing butanol, benzyl alcohol and 4-methoxyphenol, the LS was more capable of utilizing secondary alcohols than the primary alcohol. 4-Hydrodxybenzyl alcohol, a molecule containing both a primary alcohol and secondary alcohol, was used to further examine this effect. The authors found that there was inverse relationship between fructoside yield and the pKa values of the hydroxyl group. Within the active site, Glu342 is responsible for the deprotonation of the acceptor molecule. Alcohols with lower pKa values, are better able to lose this hydroxyl group to Glu342, facilitating the transfructosylation reaction (Mena-Arizmendi et

al., 2011). LS from *B. licheniformis* 8-37-0-1 was capable catalyzing the transfer of fructose to isopropanol and 1-pentanose at low yields (Lu et al., 2014).

Lsc3 and LscA LSs were consistent with their acceptor profiles. Each enzyme was capable of utilizing multiple acceptor molecules (D-arabinose, L-arabinose, D-fucose, D-sorbitol, D-xylose, D-ribose, xylitol, D-mannitol, D-galacturonic acid, methyl-α-D-glucopyranoside and xylobiose) with the exception of glucosamine. Lsc3 could continue to utilize the acceptor for transfructosylation up to a DP of 5, while LscA could use the same acceptors to a DP of 4 (Visnapuu et al., 2008).

2.5. Immobilization of LS

2.5.1. Methods of Enzyme Immobilization

Protein stabilization can occur through the addition of stabilization agents such as glycerol, ethylene glycol, carbohydrates and proteins to name a few, or through the modification of the protein through immobilization or cross-linking (Ben Ammar, Matsubara, Ito, Iizuka, & Minamiura, 2002). Common methods of immobilization include adsorption, covalent binding, encapsulation, entrapment and cross-linking (Bickerstaff, 1997). Some techniques used can be a combination of those methods. Methods cannot be universally applied to all enzymes, since each enzyme will interact differently with each technique. The most appropriate method is determined though experimentation with different immobilization techniques.

Through immobilization onto a solid support, the stability and the catalytic performance of enzyme can be improved at elevated temperatures and within a wider range of pHs. In addition, immobilization may allow for an easy separation of the enzyme from its reaction products, and its repeated use for the continuous operation in a packed-bed reactor (Jang et al., 2000a; Platkova, Polakovic, Stefuca, Vandakova, & Antosova, 2006).

2.5.1.1.Immobilization of LS through Adsorption

Common methods which are non-enzyme specific are those which depend upon weak, non-covalent interactions (Bui & Haupt, 2010). Adsorption fixation occurs when the enzyme is fixed onto a solid support through non-permanent interactions. These interactions include ionic bonds, hydrogen bonds, van der Waals interactions, hydrophobic interactions and chelation (Brady &

Jordaan, 2009; Cau, 2005). Adsorption is a non-destructive technique, which causes little to no damage to the enzyme. It can change the micro-environment of the enzyme, thereby changing some of the associated properties of the catalyst (Chambert & Petit-Glatron, 1993). The enzyme is easily loaded onto the support and once this enzyme has lost all activity, it can be washed off and the support can be regenerated with fresh enzyme. This also means that the enzyme can leak from the support, causing a loss in activity. There is also the possibility that the enzyme will immobilize onto the support in an unfavourable way, reducing activity (Cau, 2005). This can be remedied through changing the method of adsorption. With adsorption, there is the possibility of site-directed immobilization, where immobilization occurs on the enzyme where there is the highest density of functional residues for interactions with the support. The support can then be tailored with specific linkers to form the correct interactions (Mateo, Bolivar, Godoy, Rocha-Martin, Pessela, Curiel, Munoz, et al., 2010).

There are a couple instances where LS is immobilized through adsorption onto hydroxyapatite (Chambert & Petit-Glatron, 1993; Jang et al., 2000b). Chambert and Petit-Glatron (1993) first investigated the immobilization of LS from *B. subtilis* onto hydroxyapatite (1993). They theorized that the immobilization was based upon the ionic adsorption of the acidic residues of LS interacting with the calcium ions on hydroxyapaptite. The reaction rate of the immobilized LS was almost two-fold that of the unbound enzyme, while the K_m of the free and the immobilized enzyme were similar. They also found that the production of levan increased significantly due to the immobilized LS binding the growing levan more strongly than the native LS (Chambert & Petit-Glatron, 1993). When LS from *Z. mobilis* was immobilized onto hydroxyapatite, stability against proteases was increased. It retained 60% activity compared to the native enzyme which retained 2%. The enzyme also retained 65% of its activity after repeated uses and maintain 67% of its original activity after forty days stored at 4°C (Jang et al., 2000b). The same authors also performed the adsorption of LS from *Z. mobilis* onto titanium-activated magnetite, achieving 70% retention of activity. The immobilized LS was also stable, retaining 61% of its original activity after 5 repeated uses (Jang et al., 2001). Cellulose triacetate, DEAE-cellulose-53, DOWEX-IXD, Sephadex A-50, alumina, asbestos, polyvinyl alcohol and chitosan were compared against each other for the immobilization of LS from *B. subtilis* mutant NRC33a. The ionic based supports, DEAE-cellulose-53 and Sephadex A-50, achieved the highest protein yield of 72.55% and 55.11% respectively (Esawy, 2008). In comparison, the supports based upon ionic adsorption achieved higher protein yield than

the other supports, which relied on physical adsorption (Esawy, 2008). Specific adsorption occurred through the immobilization of hybrid *Z. mobilis* LS onto chitin beads through the fusion of a chitin binding domain. Preliminary purification wasn't required in this instance since only substances that contain the special domain were immobilized (Chiang et al., 2009). With this method the active site will remain unobstructed and only mild conditions were required for immobilization. The best storage stability found by Plahkovà et al. (2006) in their study of adsorption materials, was with Dowex Marathon and Amberlite IRA 900. After one month, 95% and 99% of initial activity was maintained. The worst storage stability was found with Sepabeads EC-HFA, which retained only 43% initial activity after one month at 10°C (Platkova et al., 2006).

2.5.1.2.Immobilization of LS through Covalent bond

Immobilization through covalent bond formation onto a solid support is a more specific method for immobilization than adsorption. This is due to the fact that immobilization can only occur through reactive amino acid residues and reactive groups on the immobilization support (Bickerstaff, 1997). As with adsorption, the support can have effects on the enzyme by changing the surrounding microenvironment (Cau, 2005). Covalent attachment provides stability through rigidification of the enzyme, although there is a resulting loss of activity through a loss of enzyme flexibility (Mateo, Archelas, Fernandez-Lafuente, Guisan, & Furstoss, 2003). There is also the possibility of altering the molecular confirmation of the enzyme, to the point that the reaction specificity has changed (Steinberg et al., 2002). For increased stability, an immobilization process, which includes multiple covalent attachments with short spacer arms, can be employed. This reduces the flexibility of the enzyme on the support, preventing denaturation of the enzyme (Mateo et al., 2010) There must be a compromise between increased stability and retained activity during the promotion of covalent bonds. As previously stated, enzymes immobilized covalently onto solid supports benefit from increased stability, but they also do not experience leakage from the support as other methods do (Cau, 2005). Tailoring the supports for covalent immobilization is also quite feasible as the supports usually come with a reactive functional group such as an epoxide or glyoxyl groups. Reacting these groups with different linkers can completely change the effects of the immobilization (Cau, 2005).

Eupergit© C was used to covalently immobilize LS from *B. subtilis* and three other mutants, using nucleophilic attack from the supports' oxirane groups. The authors achieved protein yields ranging

from 51-59% in a two-step immobilization procedure. The stability of the LS was high in comparison to other methods used for immobilization; retaining 20% more activity than the *B. subtilis* LS CLEAs after 10 reaction cycles (Ortiz-Soto et al., 2009). LS from *B. subtilis* NRC33a was covalently immobilized with chitosan activated with 3% gluteraldehyde, achieving a protein yield of 81.5%. The multiple covalent linkages which formed rigidified the LS so that the activation energy increased from 6.62 kcal/mol to 9.27 kcal/mol. The immobilization provided pH stability against alkaline and severe acidic conditions. It was also more stable against metallic ions. After 14 repeated cycles, the LS retained 56% of its activity (Esawy, 2008).

2.5.1.3.Immobilization of LS through Entrapment

Entrapment occurs when the enzyme is immobilized within a polymeric matrix (Brady & Jordaan, 2009). This method allows for multiple enzymes or whole cells to immobilize together. It typically occurs where all the components for the immobilization are together in a solution; this includes monomers and co-monomers required to from the matrix. Polymerization of the matrix occurs through the induction with UV light, irradiation or chemicals. In order for this method to be successful, the products required for polymerization must not interfere with the activity of the enzyme (Cau, 2005). Entrapment provides a certain amount of stability to the enzyme through macromolecular crowding and limiting the enzymes' exposure to the outside environment (Brady & Jordaan, 2009; Cau, 2005). It is often a milder technique than covalent immobilization although it does suffer from serve mass diffusion limitations (Brady & Jordaan, 2009). This method can be combined with others to achieve the highest retention of activity and stability (Cau, 2005).

LS has also been immobilized through entrapment. Agarose, agar and calcium alginate were used to immobilize LS from *B. subtilis* mutant NRC33a, with agarose attaining the highest protein yield (~37%). The authors found that entrapment efficiency was highest with 1% entrapment matrix and decreased with higher amounts, up to 3% (Esawy, 2008). Entrapment in sodium alginate gel wasn't a suitable method for the immobilization of LS from *Z. mobilis*. The levan production was found to be low. The authors believed this to be due to mass transfer effects from clogging of the matrix with products from LS (Jang et al., 2001). LS from *B. subtilis natto* was successfully entrapped within calcium alginate. The polymerase activity of LS increased, with levan production increasing from 49.4 g/L to 86.3 g/L. The stability of the beads was found to degrade after 72 h. Even with

the degradation of the beads, LS retained 72% of the native's activity after 9 repeated cycles (Shih et al., 2010).

2.5.1.4.Immobilization of LS through crosslinking

Cross-linked enzyme crystals (CLECs) and cross-linked enzyme aggregates (CLEAs) are described as carrier-free immobilization techniques (Brady & Jordaan, 2009). CLECs are performed by cross-linking enzyme crystals. This technique is hindered by the requirement of highly pure enzyme. CLEAs on the other hand can be made by precipitating enzyme out of solution, forming aggregates of 50-100 μm, which can then be cross-linked (Brady & Jordaan, 2009). CLEAs offer the benefit of the easy separation of the catalyst from the reaction media, operational stability and the starting enzyme material does not need to be pure (Bickerstaff, 1997). The retention of activity of the enzyme is affected the method for precipitation, the size of the aggregate, the cross-linker used to bind the aggregates and any additives which were added to the process (Bickerstaff, 1997). The cross-linking occurs through a reaction with a cross-linker molecule. The molecule responsible for cross-linking (frequently gluteraldehyde) reacts (preferentially in this order) with: ε-amino groups, α-amino groups, guanidinyl, secondary amino groups and at neutral pH, hydroxyl groups of the enzyme (M. E. Ortiz-Soto et al., 2009). Over cross-linking can cause mass transfer limitations and/or over-rigidification of the enzyme, reducing activity (Bickerstaff, 1997; Ortiz-Soto et al., 2009).

Ortiz-Soto at al. (2009) have reported the production of cross-linked enzyme aggregates (CLEAs) with LS from *B. subtilis* and two other *B. subtilis* mutants. Cross-linked LS from *B. subtilis* exhibited higher thermal stability as compared to the free enzyme; however, these CLEAs, cross-linked with glutaraldehyde, suffered from clogging and internal diffusional limitations from the production of high MW levan (Ortiz-Soto et al., 2009). These CLEAs resulted in low yields (approximately 30%) and require the use of a large amount of enzyme (Ortiz-Soto et al., 2009). These CLEAs did provide a measure of stability, with the LS mutant, R360K, retaining 40% of the native's activity after 10 repeated cycles (Ortiz-Soto et al., 2009). *B. subtilis natto* CLEAs were also synthesized using oxidized glucomannan as the cross-linker molecule. Through this process, the specific activity of the enzyme dropped from 35U/mg at 30°C enzyme to 12U/mg at 50°C. This was due to a rigidification of the enzyme upon cross-linking. The modified enzyme did

experience increased stability in comparison to the native enzyme. The half-life of the enzyme increased from 9 mins at 50°C to 55 mins (Ammar, Matsubara, Ito, Iizuka, & Minamuira, 2002).

2.5.1.5.Immobilization through a Multi-Step Process

Two-step immobilization provides the opportunity for orientated immobilization. This is employed by first encouraging adsorption to occur so that the enzyme is in close proximity to the reactive groups on the support. The enzyme is orientated through the region on the enzyme which contains the most groups to adsorb onto the support (Grazu et al., 2005). Incubation under alkaline conditions will promote covalent attachment of the support to the enzyme (Mateo et al., 2007). Supports of this nature are usually created by performing some pre-immobilization modification to the support. This is performed so that the final product has at least two different functional groups. One group is there to promote adsorption, while the other group provides a way to form covalent bond. Common groups for covalent immobilization are epoxy rings and glyoxyl (aldehyde) groups (Cau, 2005). Immobilization in this way provides orientated, highly stabilized enzymes.

The enzyme adsorption step will happen in site-directed fashion, occurring where there are the most reactive groups to interact with the support. Immobilization supports with anionic groups such as iminodiacetic acid will attract positively charged residues such as lysine and arginine on the enzyme. Some positively charged groups include triethylamine and ethylenediamine, these will attract negatively charged residues such as aspartate and glutamate. Adsorption can also occur through groups capable of performing weaker interactions, such as hydrogen bonding and hydrophobic interactions. Hydrophobic groups can be added to an immobilization support in a variety of lengths and structures. Adsorption through hydrophobic interactions is frequently effective for the immobilization of enzymes which operate in non-aqueous conditions. Chelation is also an effective tool for immobilization. Metallic cations, such as copper, can be bond to a support by chelating residues. The metallic cation will in turn chelate with residues on the enzyme, such as histidine (Mateo et al., 2010).

Once the enzyme is in close proximity to the support through adsorption, covalent immobilization can occur in quick succession. Since there are multiple reactive sites on the enzyme and many reactive sites on the support, multiple covalent linkages will most likely form (Mateo et al., 2010).

One of the first supports capable of covalent immobilization is Eupergit© C. This support, along with the popular Sepabeads© EC, contains epoxy rings. Immobilization with epoxy rings will first react with the ε-amino group of lysine resides or the terminal amine group. To make the reacting amines more reactive and better nucleophiles, the pH conditions are increased to 8 (Mateo et al., 2003). Once this is accomplished, covalent immobilization occurs at a fast pace. Covalent immobilization through glyoxyl groups can only occur at a high pH. This is beneficial since no covalent linkages will form until the pH has been raised to pH 10 (Guisan, 1988). Covalent immobilization can also occur through disulfide linkages if there are sufficient cysteine residues on the enzyme (Mateo et al., 2010).

Often, immobilization is effective in enhancing enzyme properties and but frequently reduces some of them (Okutucu, Çelem, & Önal, 2010). A compromise is usually made between the benefits of immobilisation such as the increased stability and the downsides such as the reduced activity. The mobility and diffusivity of solid support result in an heterogeneous biocatalysis that may be limited by with internal/external diffusion of substrates and/or products (Platkova et al., 2006). An ideal compromise between the key factors that govern the efficiency of immobilized biocatalysts, including surface area, mass transfer limitations and effective enzyme loading should be considered.

2.5.2. Effects of Immobilization Parameters

Selected parameters, including protein loading, incubation time, buffer molarity and pH, can affect the immobilization yield and the retention of activity of an immobilized enzyme. In multistep immobilization procedures, these parameters determine the amount of adsorption (Zhou, Wang, Wu, Tang, & Pan, 2013).

2.5.2.1.Incubation Time

The time required for maximal immobilization to occur, with minimal loss to enzyme activity, can vary greatly. Once maximal immobilization has occurred, additional time will not increase retention of activity on the support and will possibly decrease it. With other immobilization factors already optimized, the adsorption of LS from *Z. mobilis* onto hydroxyapatite took only 4 h, with no increase in activity on the support afterwards (Jang et al., 2000b).

Stronger ionic bonds occur quicker than weaker hydrophobic interactions. A hydrophobic support (Sepabeads© EC-EP3) and a support with ionic amino groups (Sepabeads© EC-HFA) were compared for the immobilization of β-galactosidase (sourced from *A. Oryzae* and *Thermus sp.*), invertase (baker's yeast), glucoamylase (*A. niger)*, lipase (*Candida rugosa*) and glutaryl acylase (n.a). Immobilization proceeded much faster (less than 8h) with the ionic support than with the hydrophobic support (over 20 hr) and with all but the lipase, the activity yield of the ionic support was 100% (Mateo et al., 2003). If there are many reactive residues on the enzyme which are able to form interactions, then the odds increase that when the enzyme comes into contact with the support an interaction will occur (Bolivar, Mateo, et al., 2009; Mateo et al., 2003).

Once adsorption occurs, the enzyme might be in close enough proximity to form other interactions, such as covalent bond formation if the support contains the necessary linkages (Mateo et al., 2003). An increase in the rate of adsorption occurred during the immobilization of Penicillin G acylase (PGA) on a monoaminoethyl-N-ethyl agarose (MANAE). This occurred due to the formation of covalent bonds when under less favourable adsorption conditions but under favourable conditions for covalent bond formation. The covalent bonds change the adsorption equilibrium, shifting it in a favourable direction (Bolivar, Mateo, et al., 2009).

In the covalent immobilization of PGA on glyoxyl agarose, the desorption isotherms showed two types of immobilization, reversible and irreversible. Formation of one reversible immine bond would lead the enzyme to be released from the support when said bond reversed. Formation of at least two of these reversible bonds lead to a much more permanent immobilization because of the unlikelihood that both bonds would reverse at the same time (Guisan, 1988). Overtime, the desorption isotherm showed complete immobilization, with no release of enzyme. This was because all immobilizations which had occurred through slowly one bond, had reversed and the enzyme re-orientated itself in a way that more than one covalent bond could occur (Guisan, 1988).

Incubation time at conditions which promote the formation of covalent linkages can have negative effects on enzyme activity. Glutamate dehydrogenase (GDH) immobilized onto glyoxyl agarose experienced an exponential decrease in residual activity during incubation at pH 10 to promote the formation of covalent bonds. After 24 hrs, only 15% activity remained, while GDH maintained 50% activity after 48 hrs under adsorption conditions. With increasing time at pH 10, more covalent bonds form, which in turn rigidify the structure of the enzyme, decreasing activity

(Bolivar, Rocha-Martin, et al., 2009). An epoxide hydrolase from *A. niger* immobilized onto Eupergit© C/EDA was incubated at pH 9.2 to promote covalent linkages. As incubation time increase, the residual activity decreased; this coincided with an increase in thermal stability. After 72 hrs, residual activity decreased by 26%, while thermal stability was 30-times higher (Mateo et al., 2003).

2.5.2.2.Effects of Buffer Concentration on Immobilization

A low molarity buffer is required if the main adsorption interactions are ionic. High concentrations of ions from high salt solutions interact with the ionic groups on the enzyme, preventing their contact with the support. This effect was examined by Betancor et al. (2006), where they studied the method in which immobilization took place under different ionic conditions. They immobilized two different enzymes, glutaryl acylase and D-aminoacid oxidase, on three different supports: monoaminoethyl-N-ethyl (MANAE) agarose, MANAE agarose-glutaraldehyde monomer and MANAE agarose-glutaraldehyde dimmer. In the highly ionic environment, 500 mM sodium phosphate buffer, only the supports capable of covalent bonds were able to immobilize the enzyme significantly. The immobilization rates were much slower than they were in a low ionic environment. At lower concentrations, 5 mM sodium phosphate buffer, all three supports achieved similar amounts of immobilization, at similar rates. At low ionic concentrations, immobilization may have occurred via the region with the most anionic groups, while at high ionic strengths, the immobilization may have taken place in the region with the most reactive amino groups (Betancor, Lopez-Gallego, et al., 2006). Immobilization was not able to occur for three different enzymes, α-chymotrypsin type II, thermocatenulatus lipase 2 and a tannase, on a heterofunctional cationic support when high ionic concentrations of 1 M NaCl were used. It did proceed successfully when concentrations of 5 mM were used (Mateo et al., 2010). Surprisingly, opposing results were seen in the immobilization of a commercial β-fructofuranosidase preparation onto Sepabeads-EP3 and Sepabeads-EP5. The immobilization, which was relying on covalent bond formation, experienced higher yields when the buffer molarity was 300 mM as compared to 500 mM or 1 M buffer (Ghazi et al., 2007).

2.5.2.3.Effects of Buffer pH on Immobilization

The buffering pH affects the adsorption by changing which groups are ionized at a certain pH. LS from *B. subtilis* experienced higher adsorption onto Eupergit C at pH 7.0 than at pH 6.0 (Ortiz-Soto et al., 2009). More importantly, the pH can affect the reactivity of the amino acid side groups to form covalent bonds. For example, an epoxy group (oxirane) will react with carboxyl groups at slightly acidic pH, thiol groups at neutral to slight basic pH, amino groups above pH 9 and the phenolic groups of tyrosine above pH 11 (Gomez de Segura et al., 2004). When using glyoxyl based supports for the first time, Guisan (1988) attempted to immobilize PGA in buffers of pH 7, 9 and 10. Immobilization was unsuccessful at the two lower pH's but quickly occurred at pH 10 (Guisan, 1988). Due to the high pKa of the ε-amino in lysine (pKa = 10.5), reactivity with lysine must occur at high pH (Mateo et al., 2010). Another important consideration is the stability of the enzyme at the immobilizing pH. Immobilization onto glyoxyl groups requires high pH, possibly requiring a stabilizer to prevent a loss of activity. When LS from Z. *mobilis* was immobilized onto hydroxyapatite, the acidic optimum pH of the enzyme for incubation was unable to be used since it caused the degradation of the calcium phosphate support (Jang et al., 2000a).

2.5.2.4.Effect of Enzyme loading on Immobilization

Enzyme loading is an integral parameter when trying to achieve maximal enzymatic activity per gram of support. When the concentration of LS was increased, so did the amount immobilized onto the support. A diffusional limit was usually obtained, from the support being saturated resulting in a decrease in activity per gram of protein immobilized. In this scenario, the substrate would have difficulty diffusing through the multiple enzyme layers which become adsorbed. The concentration of LS from Z. *mobilis* was saturated with 20 U/g of hydroxyapaptite (Jang et al., 2000a). In some cases, the density of enzyme on the support did not change the production of levan. This was found in the case of *B. subtilis* LS immobilized upon hydroxyapatite (Chambert & Petit-Glatron, 1993). With the maximal amount of β-fructofuranosidase (300 U) immobilized by cross-linking onto a WA-30 resin, the immobilization efficiency was only 15%. By changing the immobilization conditions so that there is a greater ratio of support/enzyme, the activity decreased to 15-50 U/g of resin, but the immobilization efficiency increased to 15-40% (Kurakake et al., 2010). Enzyme loading of β-fructanfuranosidase, from *A. aculeatus* was varied from 30-200 mg/g of support, on two different supports. Although the authors were successful in immobilizing

21.1-31.4 mg/g and 17.3-54.7 mg/g of support, with both supports used, the amount immobilized had a sub-linear relationship with how much was loaded into the system (Ghazi et al., 2005). The protein loading of epoxide hydrolase was optimized on Eupergit© C/EDA. The specific activity of enzyme on the support (230 U/g support) in relation to protein loading was linear until almost 40 mg/g (175 U/g) where there was 26% decrease (Mateo et al., 2003).

2.5.2.5.Effect of Immobilization on Enzyme Micro-environment

Immobilization can alter the product specificity of an enzyme. When immobilized onto hydroxyapatite, LS from Z. *mobilis* produced a greater portion of LMW levan than the native enzyme. These results were attributed to the low binding capacity of LS to the growing levan chain. (Jang et al., 2000a). Transfructosylating activity was increased when the same LS was immobilized onto chitin beads, with 65% more levan produced (Chiang et al., 2009). The levan from the enzyme immobilized onto titanium-activated magnetite and from permeated cells had molecular weights of at least half that as the free enzyme (Jang et al., 2001). The opposite occurred with the LS from *B. subtilis*. Hydrolysis decreased by 2 factors when the LS from *B*. *subtilis* was immobilized onto hydroxyapatite. This is thought to be due to the support's microenvironment excluding water (Chambert & Petit-Glatron, 1993).

When LS was immobilized upon titanium-activated magnetite, the optimum pH shifted slightly due to more acidic conditions (Jang et al., 2001). This shift occurred as a result of the microenvironment effect of the support. More basic and a more acidic environment may be required in order to generate the right conditions near the enzyme. The optimum pH of the LS from Z. *mobilis* immobilized onto chitin beads did not change from the wild-type, but the enzyme was more stable at higher pHs (Chiang et al., 2009). For the immobilization of the FTase from *A. pullulans* on Dowex Marathon MSA, the pH at which the immobilization occurred dramatically affected the activity of the FTase immobilized (Platkova et al., 2006). The LS from *Z. mobilis* immobilized by a chitin bead was found to have a different optimal sucrose concentration. It was raised from 20% with free enzyme to 30% with immobilized (Chiang et al., 2009). It was also found when using whole *Escherichia coli* cells to express LS from Z. *mobilis,* the IPTG induction time affected the type of levan produced. After 2-4 hours of IPTG induction time, soluble levan was produced, the non-soluble form was produced at greater times (Jang et al., 2001).

2.5.2.6.Effects of pre and post-treatment on the Immobilization

Pre-immobilization treatments are performed before immobilization to condition the supports and hence beneficially affect the enzyme immobilization and microenvironment. A popular technique is the addition of spacer arms, which can promote adsorption or covalent linkages through different residues and can change the microenvironment effect of the support (Cau, 2005). Long spacer arms also contribute to the retention of some flexibility of the enzyme upon its immobilization on the support, helping to prevent steric hindrance (Cau, 2005; Garcia et al., 2011). Bolivar et al. (2009) successfully treated epoxy-activated agarose supports with different concentrations of EDA to create a support with MANAE that has the potential for cationic interactions with some epoxy groups remaining for covalent attachment. They also added epoxy groups to the existing MANAE groups to create longer arms, thus providing more flexibility and affecting the microenvironment of the support. Other pre-immobilization treatments to the support have been the addition of carboxyl groups, thiols groups, boronate, amines and metallic chelating groups (Mateo et al., 2010). This is done by reacting the support with groups such as iminodiacetic acid (IDA), IDA with a metallic cation, sodium sulphide for thiol groups and ethylenediamine (Cesar Mateo et al., 2007). Some pre-immobilization treatments can be done so prematurely that the enzyme hasn't even been expressed. A chitin binding domain was inserted into the DNA that encodes for the Z. *mobilis* LS enzyme. By doing so, the authors created an enzyme that could easily be separated and immobilized. This makes purification superfluous, lending the technique to more industrial applications (Chiang et al., 2009).

Post-immobilization treatments take place after immobilization. They are usually performed to improve the multi-covalent attachment and/or to neutralize the reactivity unbound supports. Cross-linking is a popular technique performed after conventional adsorption onto a solid support. While it is also technique used by itself for immobilization, cross-linking can also be used in conjunction with another technique to improve upon it. FTases from *A. pullulans* were immobilized through the use of anion exchange columns. Cross-linking the enzyme with the support afterwards will prevent enzyme leakage and also improve stability of the enzyme (Platkova et al., 2006). There are reports that glutaraldehyde, when used for cross-linking, can inactivate an enzyme. This occurred with the alcohol dehydrogenase from *Lactobacillus brevis*. There are other substances which can be used as cross-linkers, such as dextran polyaldehyde. The substance used for cross-

linking must be appropriate for all of the reactants (Cabirol et al., 2008). Molecular imprinting of an enzyme can be performed as a pre- or post-treatment to help the enzyme retain its most active conformation. It has proved effective for cyclodextrin glycosytransferase, in conjunction with other techniques to improve many factors (Kaulpiboon, Pongsawasdi, & Zimmermann, 2007).

2.6. High-Throughput sequence-based screening

Sequence based screenings are an ideal method for enlarging the library of LS enzymes. This method allows for an expansion of an enzymatic library with minimal effort. Sequence-based screening searches genomic databases for sequences which have homology to sequences encoding for enzymes with desired activity. This method relies on conserved sequences which are essential to functional catalytic activity, many of which have been identified for LS (Lombard., 2014; Meng & Futterer, 2003). The range and depth of the databases which will be searched are the real limiting factor in what will be discovered. Using a database, such as Genbank, UniProt, or Pfam, a BLAST (basic local alignment search tool) to search for sequences which have homology to a known reference set of LS enzymes. Parameters for the BLAST analysis can be used to ensure that only high percentage identity to the reference sequences will found. The size of the sequences is also used to restrict the hits from the BLAST analysis, to eliminate incomplete sequences. Sequenced-based screening was successfully used to discover 4 new dehalogenases which were able to hydrolyse fluoracetate (Chan et al., 2010).

Connecting Statement 1

A thorough literature review was conducted in chapter II explaining the various methodologies for the immobilization of LS and the resulting affect on enzymatic properties. Chapter III examines the immobilization of LS onto various functionalized and non-functionalized commercial and non-commercial solid supports. The effects of these immobilizations on the immobilization efficiency, thermal stability and transfructosylation versus hydrolytic activity are also described in Chapter III.

The results from this work were presented at the 2013 IFT Annual Meeting & Food Expo-Institute of Food Technologist. This presentation won 1st prize in the protein poster presentation category. The results were also presented in the journal of Chemical Technology and Biotechnology.

Hill, A., Mateo, C. & Karboune, S. (2013) Immobilization and Stabilization of Levansucrase of Great Potential for the Synthesis of Novel Fructooligosaccharides. IFT13 Annual Meeting & Food Expo, Chicago, USA, July 13-July 16, 2013

Hill A, Mateo C, Karboune S. (2015) Immobilization and Stabilization of levansucrase biocatalyst of high interest for the production of fructooligosaccharides and levan. J. Chem. Technol. Biotechnol. 91(9), 2440-2448.

3. Abstract

Levansucrase (LS)-catalyzed-transfructosylation reaction is a potential approach for the synthesis of FOSs and levan as health promoting compounds. This biocatalytic approach is hindered by low thermal stability of LS and its high rate of hydrolysis. In the present study, LS from *B. amyloliquefaciens* was immobilized onto modified and unmodified epoxy-activated supports (Eupergit®C; Sepabeads®) as well as on modified cross-linked-agarose beads, to increase its thermal stability and modulate its reaction selectivity (hydrolysis/transfructosylation). LS bound to Sepabeads® HA (98.8%) and glyoxyl agarose-IDA/Cu (67%) retained a high initial activity along with good immobilization yields. The thermal stability results indicated that glyoxyl agarose-IDA/Cu and glyoxyl agarose, provided the greatest thermal stability with factors of 14 and 106 times, respectively. Immobilization through Sepabeads® HA increased the ratio of transfructosylation/hydrolysis by 2.3 times, although it did not promote the stabilisation of LS. Immobilization on glyoxyl agarose-IDA/Cu provided a good compromise in all three properties: retention of activity (67.0%), transfructosylation/hydrolysis ratio (120%) and thermal stability (stability factor of 13.6).

The stabilization of LS through immobilization contributes to its potential use commercially. With an increasingly stable enzyme, further work will be directed towards altering LS reaction specificity towards levan and levan-type FOSs.

3.1. Introduction

In the current trend towards more health conscience consumption, prebiotics play an important role in gastrointestinal health (Bruzzese, Volpicelli, Squaglia, Tartaglione, & Guarino, 2006). FOSs are among the non-digestible oligosaccharides that fulfill the criteria for prebiotic classification (Roberfroid, 2007). Novel FOSs, levan-type FOSs and neokestose containing β-(2,6) glycosidic linkages, have demonstrated interesting prebiotic activity as compared to the commercially available inulin-type FOSs with β-(2,1)-linkages (Bello et al., 2001; Kang, Chun, & Jang, 2005; Kilian et al., 2002; Marx et al., 2000; Porras-Dominguez et al., 2014). Levan-type exopolysaccharides from *L. sanfranciscensis* were found to reduce the growth of *enterococci* and *coliforms* and enrich the amount of *Bifidobacterium spp.* in an *in vitro* experiment (Bello et al., 2001). Marx *et al.* (2000) demonstrated the production of SCFAs upon fermentation of *β*-(2-6)-FOSs by *Bifidobacterium spp.*, with *B. adolescentis* capable of metabolizing both short-chained

and long-chained FOSs. Neokestose supported an increase in biomass of *Bifidobacteria* and *Lactobacilli* while simultaneously demonstrating a decrease in *Coliforms*, *Clostridia* and *Bacteriodes* (Kilian et al., 2002). Levan, a fructan with *β*-(2-6) glycosidic linkages and some *β*-(1-2)-branching, also has applications in food and pharmaceutical industries. It can act as a stabilizer, encapsulating agent, is used in bio-films and has shown anti-tumour activities (I. Dahech, Belghith, Belghith, & Mejdoub, 2012; Han, 1990; Kim et al., 2004). With its low caloric value, levan-type FOSs and levan can also be used in the formulation of low-calorie foods (Alles et al., 1999).

The biocatalytic approach based on LS-catalyzed-transfructosylation reactions has proven to be attractive for the synthesis of well-defined FOSs and β-(2,6)-levan from sucrose as the sole substrate. LS (EC 2.4.4.10), a member of the glycoside hydrolase family 68, can catalyze four reactions: the exchange of glucose, the hydrolysis of sucrose to glucose and fructose, transfructosylation and lastly polymerization (Strube et al., 2011). One limitation to the use of LS for the production of unique FOS prebiotics and levan is its low thermal stability and its ability to catalyze the hydrolysis of sucrose into glucose and fructose. Immobilization on a solid support can increase the stability of the enzyme while providing a mean to recover the enzyme and recycle it after each usage. However, only a limited number of studies have examined the stabilization of LS through immobilization (Bekers, Laukevics, Upite, Kaminska, & Linde, 1999; Chiang et al., 2009; Esawy, Mahmoud, & Fattah, 2008; Jang et al., 2000b; Ortiz-Soto et al., 2009; Shih et al., 2010; Silbir, Dagbagli, Yegin, Baysal, & Goksungur, 2014). Ortiz-Soto *et al.* (2009) have increased the thermal stability of LS from *Bacillus subtilis*, through the formation of cross-linked enzyme aggregates and separately through the covalent immobilization on Eupergit® C support. Both immobilization techniques had similarly improved the thermal stability as compared to the free LS. An improvement in the reusability LS from *B. subtilis* natto cells (9 cycles, retained 72% of activity) upon entrapment in calcium alginate beads has been described by Shih *et al*. (2010). Nevertheless, it has been reported that mass transfer limitations are one of the drawbacks of this immobilization affecting the production of levan by LS (Tian & Karboune, 2012). Furthermore, it is hypothesized that the modulation of microenvironment of LSs through immobilization on solid supports can influence their reaction selectivity toward transfructosylation vs hydrolysis. In this context, no study has been devoted to the ability of immobilizing supports to modulate the reaction selectivity of LSs.

In our previous study, the catalytic properties of LS from *B. amyloliquefaciens* were characterized (Tian & Karboune, 2012). LS was found to be more transfructosylic than hydrolytic at its initial stage; however as the reaction was proceeded, the high affinity of sucrose to bind both sites -1 and +1 of *B. amyloliquefaciens* LS became more pronounced, inhibiting the transfructosylation activity. Our preliminary study (Tian et al., 2011) was the first to highlight the efficiency of *B. amyloliquefaciens* LS to synthesize a variety of hetero-FOSs from various fructosyl-acceptors. As part of our ongoing research, the present research work was aimed at the investigation of the immobilization of LS through multi-point attachments on selected modified and unmodified epoxy-activated supports (Eupergit® C; Sepabeads®) as well as on modified cross-linked agarose beads. Eupergit® C, an oxirane acrylic support, was easily modified to provide tailored immobilization (Katchalski-Katzir & Kraemer, 2000). Sepabeads® supports, which are methacyrilic polymer supports, known for their mechanical stability will also be examined as potential supports (Mateo, Abian, Fernandez-Lorente, Pedroche, & Fernandez-Lafuente, 2002). Agarose beads were used due to their easy functionality and for the specific bonds which form between the support and enzyme (Mateo et al., 2010). The investigated supports provide a dual functionality and promote immobilization through a two-step process. First immobilization occurs through adsorption onto the support and thereafter, conditions are altered to promote covalent bond formation (Mateo et al., 2003; Mateo et al., 2010). Pre-immobilization treatments can also add different linkers, such as carboxyl groups, thiols groups, boronate, amines and metallic chelating groups to promote adsorption through specific regions on the enzyme (Mateo et al., 2010). Linkers are added by reacting the support with iminodiacetic acid (IDA) (with/without metallic cation), sodium sulphide, triethylamine (TEA) and ethylenediamine (EDA) (Mateo et al., 2010). As far as the authors are aware, the investigation and comparison of the efficiency of selected modified and unmodified oxirane acrylic, methacyrilic polymer and agarose supports for the immobilization of LS from *B. amyloliquefaciens,* has never been reported. The supports were evaluated according to their ability to successfully immobilize LS as well as their ability to provide thermal stability. The transfructosylation versus hydrolytic activity ratio was also examined to study the effect of immobilization on reaction selectivity.

3.2. Materials and Methods

3.2.1. Materials

Sucrose, D-(-)-Fructose, D-(+)-glucose, 3,5-dinitrosalicylic acid (DNS), NaOH, polyethylene glycol (PEG) 200, potassium sodium tartrate ($KNaC_4H_4O_6$), $NaIO_4$, $NaBH_4$, ethylenediamine $C_2H_4(NH_2)_2$, iminodiacetic acid $HN(CH_2CO_2H)_2$, $CuSO_4$ and Eupergit® C beads were obtained from Sigma Chemical Co. (St. Louis, MO, USA). $CaHPO_4$, $FeSO_4{\cdot}7H_2O$, $MnSO_4{\cdot}7H_2O$, $Na_2HPO_4{\cdot}2H_2O$, $NaMoO_4{\cdot}2H_2O$, $(NH_4)_2SO_4$, K_2HPO_4, KH_2PO4, Bovine Serum Albumin and yeast extract were obtained from Fisher Scientific (Fair Lawn, NJ, USA). Agarose 10BCL was purchased from Agarose Bead Technologies. Sepabeads® EC-EP and Sepabeads® HA were generously provided by Resindion®. *B. amyloliquefaciens* (ATCC 23350) was obtained from American Type Culture Collection (Manassas,VA, USA). Bradford reagent concentrate was provided by Bio-Rad (Missasauga, ON, Canada).

3.2.2. Production and purification of LS from *B. amyloliquefaciens*

B. amyloliquefaciens, strain ATCC 23350, was stored on potato dextrose agar (39 g L^{-1}). The bacteria was pre-cultured aerobically in nutrient broth (8 g L^{-1}); afterwards 4 mL of preculture was transferred to a 1-L baffled Erlenmeyer flask containing 400 mL of the modified mineral salt medium which was composed of (g L^{-1}) $Na_2HPO_4{\cdot}2H_2O$ (2.67), KH_2PO_4 (1.36), $(NH_4)_2SO_4$ (0.5), $FeSO_4{\cdot}7H_2O$ (0.005), $MnSO_4{\cdot}H_2O$ (0.0018), $NaMoO_4{\cdot}2H_2O$ (0.0025), $CaPO_4{\cdot}2H_2O$ (0.01) and $MgSO_4{\cdot}7H_2O$ (0.2). The culture medium was induced with sucrose (10 g L^{-1}), and yeast extract (10 g L^{-1}) was added to act as an organic source of nitrogen as described previously (Tian et al., 2011)[21]. The culture was run at 35°C, 150 rpm. After 11 h, the culture medium was centrifuged (8000 rpm, 4°C) for 20 min. The centrifuged pellets were resuspended in potassium phosphate buffer (50 mM, 37.5 mL pH 6) containing 1% Triton X-100. The cells were lysed using ultrasonification for 6 min and 25 s, set at 15 kHz with 25/50 s cycles. Afterwards the mixture was mixed for 15 min at 4°C and then centrifuged (8000 rpm, 4°C) for 15 min. PEG-200 (30%) was added to the supernatant to form hydrophobic interactions with LS to encourage precipitation, the solution was stirred gently at 4°C for 14 h. The recovered protein precipitate was centrifuged (4°C, 12 000 rpm) for 45 min, after which the pellet was resuspended in potassium phosphate buffer (50 mM) at pH 6 and then dialysed against 8 changes of 4L of potassium phosphate buffer (5 mM) with a molecular weight cut-off of 6-8 kDa. The protein content of the extracts was determined using a Bradford protein assay and a bovine serum albumin as standard (Bradford, 1976). The

recovered LS was purified 15 times with a yield of 72%. It was then lyophilized to a dry powder and used as the source material for enzyme immobilization.

3.2.3. Assay of LS activity

A unit of total LS activity was defined as the amount the biocatalyst that released 1 μmol of reducing sugars from sucrose per min. One hydrolytic unit of LS was defined as the amount of the biocatalyst that produces 1 μmol of the fructose per min. On the other hand, one transfructosylation unit of LS was defined as the amount of the biocatalyst that releases 1 μmol of glucose as a result of transferring fructose, per min. Subtracting the total amount of fructose from that of glucose provides the amount of glucose resulting from transferring fructose.

The assays were carried out by adding the LS solution (250 μL, 2-200 times dilution) to a sucrose solution (1.8 M, 250 μL) in potassium phosphate buffer (50 mM, pH 6.0) and incubated at 30°C for 20 min. To quantify the reducing sugars, the enzymatic reaction was stopped with the addition of a DNS solution (750 μL), which was composed of 3,5-DNS (1% w/v) in NaOH (1.6% w/v), and by boiling the samples for 5 min. Afterwards, 250 μL of an aqueous solution of potassium sodium tartrate (50% w/v) was added to stabilize the colour. The absorbance was measured at 540 nm, and enzyme and substrate blanks were carried out in parallel with the reaction. The amount of released reducing sugars was determined from a standard curve constructed with glucose (0.0-15.0 mM).

The hydrolytic and transfructosylating activity was measured by quantifying the amount of glucose and fructose released from sucrose. After precipitating proteins and levan by methanol precipitation, the reaction mixtures were analysed for their contents in glucose and fructose by high pressure anionic exchange chromatography (HPAEC) on a Dionex (ICS-3000) chromatography system with a pulsed amperometric detector and a CarboPac PA20 column (3 x 150 nm) at 32°C. Isocratic elution was applied with 10 mM NaOH as the mobile phase at a flow rate of 0.5 mL min^{-1}. The concentration of the products was estimated by constructing standard curves for glucose and fructose and analysed using Chromeleon Software. All assays were run in duplicate.

3.2.4. Preparation and functionalization of commercial supports

The protocol for the preparation of epoxy-based supports follows the method of Mateo *et al*. (2007). Eupergit® C, Sepabeads® EC-EP or Sepabeads® HA (70 mg mL^{-1}) were washed with deionized water (10 times) followed by potassium phosphate buffer (5 times) at the desired buffer concentration and pH. Eupergit® C absorbed much of the water and increased in weight (113.1 ± 4.8 g of water per 100 g support). The Sepabeads® EC-EP support also absorbed water but to a lesser extent (89.7 ± 6.4 g of water per g support). Sepabeads® HA absorbed a negligible amount of water. Sepabeads® HA absorbed a negligible amount of water.

To prepare IDA Functionalized Supports, an IDA solution (0.5 M) with the pH adjusted to 11 was mixed with wet, epoxy-activated support, at a ratio of 1:10 (w/v). The suspension was maintained under rotation for 36 h at 25°C. The support was then washed with distilled water.

To prepare IDA-Cu^{2+} Functionalized Supports, IDA functionalized support 1% (w/v), was mixed with cupric sulfate solution (2 mg mL^{-1}) for 2 h at 25°C. The supports were then washed with 10 volumes of distilled water.

EDA Functionalized Supports were prepared by the addition of EDA solution (5% v/v), with pH adjusted to 8, to the epoxy activated support 1% (w/v). The suspension was mixed for 15 min and then washed with deionized water (10 volumes).

3.2.5. Preparation and functionalization of glyoxyl-based supports

The glyoxyl-based supports were prepared following the protocol of Mateo et al. (2010).

Epoxy-Activated Agarose. In a 1L round bottom flask on ice, $NaBH_4$ (2 g) was added to NaOH (0.656 M, 440 mL) along with acetone (160 mL). Agarose 10-BCL (100 g) and epichlorohydrin (110 mL) were added and the mixture was stirred overnight at 25°C. The support was washed with milli Q water (10 volumes).

Glyoxyl agarose-TEA. Washed epoxy activated agarose 10-BCL (1% w/v), was added to a 50:50 (v/v) solution of acetone:water. Triethylamine was slowly added to reach a final concentration of 0.1 M, and the pH was adjusted to 12.4 with concentrated HCl. The suspension was mixed with a mechanical stirrer for 48 h, after which the support was washed with distilled water (10 volumes). Remaining hydroxyl groups were oxidized with $NaIO_4$ (0.01 M) for 90 min and then washed with distilled water (10 volumes)

Glyoxyl Agarose. Epoxy activated agarose (10% w/v) was hydrolyzed with 0.5 M H_2SO_4 and mixed with a mechanical stirrer for 4 h at 25°C. The support was filtered on a sintered glass filter and washed with distilled water (10 volumes). The hydroxyl groups were oxidized with $NaIO_4$ (0.01 M, 5% v/v support:solution) for 90 min and then washed with distilled water (10 volumes).
Glyoxy agarose-IDA/Cu. An IDA solution (0.5 M), with the pH adjusted to 11, was mixed with wet, epoxy-activated agarose (10% w/v). The reaction was left to mix by rotation for 36 h at 25°C. The support was then filtered on a sintered glass filter and washed with distilled water (10 volumes). The remaining diols were oxidized with $NaIO_4$ (0.01 M, 5% v/v support:solution) for 90 min and then washed with distilled water (10 volumes). The support was then mixed with a solution of $CuSO_4$ (30 mg mL^{-1}) for 1 h at room temperature. The support was filtered and washed with distilled water (10 volumes).
Amino epoxy agarose. Ethylenediamine (0.1 M) adjusted to pH 10 with concentrated HCl on ice, was added to glyoxyl agarose (10 % w/v) and mixed for 2 h with a mechanical stirrer at 25°C. $NaBH_4$ (10 mg mL^{-1}) was added and stirred overnight. The support was then washed with 10 volumes of distilled water then subsequently with 10 volumes of NaCl (100 mM) to remove non-covalently bound EDA. The support was washed again with 10 volumes of distilled water. EDA-agarose (10g) was stirred overnight at 25°C with a mechanical stirrer in a sodium bicarbonate solution (100 mM, 80 mL, pH 9) along with acetone (12 mL). The support was filtered and washed with distilled water (10 volumes).

3.2.6. Immobilization of LS onto supports

A solution of partially purified LS (5-10 mg protein.g $support^{-1}$) in a select immobilization buffer, was added to the wet support and incubated at 4°C for selected times up to 72 h under mild stirring along with the free enzyme solution. The immobilized LS was recovered by centrifugation for 5 min at 6000 rpm, the supernatant was removed and the support was washed with potassium phosphate buffer (50 mM, pH 6). The protein content and the activity of free LS, supernatant and washed solution were quantified. The immobilized LS was resuspended in potassium phosphate buffer (50 mM, pH 6) and the activity was measured. Activity yield was estimated as the difference between the total units of the free enzyme solution and the supernatant solution, multiplied by 100 and divided by the total units of the free enzyme. Protein yield was determined by estimating the difference between the total mg of protein in the free LS solution and the supernatant, multiplied

by 100 and divided by the total mg of protein in the free enzyme solution. Retention of activity was determined by the specific activity of immobilized enzyme divided by specific activity of the free enzyme solution, multiplied by 100. The free enzyme solution was put in the same conditions as the suspension for immobilization. Therefore, any loss of activity which occurred due to external forces was accounted for. For the agarose-based supports which required reduction of the Schiff base, the activity yield, protein yield and retention of activity was measured after reduction.

3.2.7. High pH incubation of supports - formation of multipoint covalent bonds

Except for Sepabeads® HA, all immobilized LS on Sepabeads® and Eupergit®-based supports were suspended, at 4°C with gentle mixing, in sodium bicarbonate buffer at pH 9.2 (50 mM – 1 M) for 72 hours to promote the formation of multiple covalent bonds. Afterwards, the buffer was removed and replaced with 3 M glycine solution (pH 8.5) and gently mixed at 4°C for 16 h. Immobilized LS was then washed 10 times with potassium phosphate buffer (50 mM, pH 6.0). Oxirane groups on the support are the reactive groups capable of covalent bond formation.

To promote the multi-covalent attachment on glyoxyl agarose-type supports, immobilized LS was first suspended in sodium carbonate buffer (50 mM - 1M, 10 volumes) at pH 10 with 20% (v/v) glycerol. The suspension was incubated for 3 h at 4°C with gentle shaking. Sodium borohydride (1 mg mL^{-1}) was then added to the high pH suspension and gently mixed at 4°C for 30 min to reduce Schiff bases. The recovered support was washed 10 times with potassium phosphate buffer (50 mM, pH 6). On the glyoxyl agarose-type supports, aldehyde groups are the reactive groups responsible for covalent bond formation.

3.2.8. Assessment of thermal stability

Free or immobilized LS in potassium phosphate buffer (50 mM, 5 mL) was incubated at 50°C in a sand bath after the initial LS activity was measured. Every 15 min for 120 min, an aliquot was taken and the residual LS activity was measured as described previously. For increased thermal stability measurements, the procedure was repeated at 55°C.

3.3. Results and Discussion

3.3.1. Immobilization of LS on Selected Supports by Adsorption and Multi-covalent attachments

Immobilization of LS was attempted on selected modified and unmodified supports (i.e. Eupergit® C, Sepabeads® EC-EP and agarose). As far as the authors are aware, a large screening of heterofunctional supports for the immobilization of LS has never been reported. The supports were selected on the basis of their properties in terms of mechanical strength, physical stability, enzyme loading capacity and cost. Eupergit® C, composed of a co-polymer of methacrylamide, N,N'-methylen-bis(acrylamide) and oxirane containing monomers, has been identified as potential support for the enzyme immobilization through multi-point covalent attachments (Katchalski-Katzir & Kraemer, 2000). Sepabeads®, methacyrilic polymer supports, are known for their mechanical stability and their versatility (Mateo et al., 2002). Both Eupergit® C and Sepabeads® EC-EP primarily bind to proteins via their oxirane groups reacting with the ε-amino group of lysine. Covalent bonds can also occur through an enzyme's sulfhydryl or carboxylic groups (Katchalski-Katzir & Kraemer, 2000). Agarose beads were examined as potential supports for the immobilization of LS due to their high internal surface area, hydrophilicity and feasibility to be activated with a high density of groups capable to react with different amino acids (Mateo et al., 2010). Covalent bond formation using glyoxyl agarose-based supports occur via non-ionized amino groups. Lysine residues require highly alkaline conditions for reactivity (Mateo et al., 2007). Pre-immobilization treatments, aiming at the modification of some functional oxirane groups of epoxy-activated supports or glyoxyl groups with EDA, IDA, or IDA- Cu (copper), were evaluated in order to improve the adsorption of LS and promote multi-covalent attachment. Immobilization of LS from *B. amyloliquefaciens* on selected supports was performed at pH conditions between 6.0-10.0. It has been reported that the oxirane groups on the epoxy-activated supports (Eupergit® C and Sepabeads® EC-EP) can react with different nucleophile groups of enzymes at a wide range of pH values (1.0-12.0) (Katchalski-Katzir & Kraemer, 2000). Since all the supports were saturated with buffer before immobilization occurred, the hydrophilic/hydrophobic properties of the support should not have affected immobilization yields, but rather just the microenvironment that surrounds the immobilized enzyme.

Tables 3.1 and 3.2 summarize the results for the immobilization of LS on selected unmodified and modified supports. The immobilization conditions (buffer concentration and buffer pH) were

altered to influence interactions between the support and the enzyme. Using supports without ionic linkers, such as Eupergit® C, Sepabeads® EC-EP and glyoxyl agarose, the ionic strength of the immobilization buffer was maintained high (1 M) to favour the interaction between the hydrophobic regions of LS and the support. With supports where immobilization occurs first via adsorption with ionic groups (Eupergit® C-IDA, Eupergit® C-IDA/Cu, Sepabeads® HA, Sepabeads® EC-EP-IDA, Sepabeads® EC-EP-IDA/Cu, Sepabeads® EC-EP-EDA, amino epoxy agarose, glyoxyl agarose-IDA/Cu, glyoxyl agarose-TEA) a buffer with low ionic strength (5-50 mM) was used. The results show that wide ranges of activity yield (5.6-95.4%), protein yield (12.0-86.7%) and retention of activity (6.0- 129.4%) were obtained depending on the type of support.

The highest activity immobilization yield was achieved with Sepabeads® EC-EP (95.4%) using 1 M potassium phosphate buffer at pH 8. This was seen as the largest difference in the total units of LS activity in the free enzyme solution (before immobilization) and the total units found in the immobilization supernatant/washing. Subsequent to Sepabeads® EC-EP, the highest activity immobilization yields of 80.1 to 82.1% were obtained using Sepabeads® EC-EP-IDA/Cu (50 mM, pH 8), glyoxyl agarose (1M, pH 10) and glyoxyl agarose IDA/Cu (1 M, pH 8) as supports. As compared to Eupergit® C and glyoxyl-based supports, Sepabeads® EC-EP and its modified forms (Sepabeads® EC-EP-IDA/Cu; Sepabeads® EC-EP-EDA) led to the highest protein immobilization yields (75.6-86.7%). Sepabeads® EC-EP is similar to Eupergit® C in terms of functional groups, particle size and pore diameter. The main differences between them are the polymeric matrix of the support and its internal morphology, giving epoxy-Sepabeads® a more hydrophobic behaviour compared to the Eupergit® supports (Mateo et al., 2002). Agarose supports are synthesized from the cross-linked polysaccharide composed of 1,3-linked β-D-galactopyranose residues alternating with 3,6-anhydro-α-L-galactospyranose (Zhou, Wang, Ma, & Su, 2007). They are of a similar size to Eupergit® C and Sepabeads® but are more hydrophilic due to the multiple hydroxyl groups within its structure. Lower protein (12.0-33.6%) and activity (5.6-42.0%) immobilization yields were obtained with the modified Eupergit® C supports. The modification of Eupergit® C by IDA and EDA seems to significantly decrease the adsorption affinity of LS on this support. Sepabeads® EC-EP-EDA had a higher protein yield at pH 8 (75.6%) than at pH 6 (26.1%). At pH 8, more of the acidic groups on the LS may be ionized and more able to interact with the support. These results were not repeated with Eupergit® C-EDA. Additional copper modification of Eupergit® C-IDA increased the protein immobilization yield (17.7% to 30.0%) while decreasing the activity yield by

Table 3.1. Investigation of the immobilization of LS onto selected solid supports at different immobilization conditions.

Support	Buffer Concentration	pH	Activity Yield (%)[a]	Protein Yield (%)[b]	Retention of Enzyme Activity (%)[c]	Specific activity of immobilized enzyme (μmol/min. mg protein)[d]	Activity (μmol/min. g support)[e]
Eupergit® C	1 M	8	73.3	63.4	22.5	13.7 (± 1.4)	19.5 ± (2.0)
Eupergit® C-IDA	50 mM	6	21.7	17.7	6.0	3.6 (± 0.1)	1.4 ± (0.04)
Eupergit® C-IDA	50 mM	8	42.0	12.0	129.4	78.4 (± 9.8)	17.2 ± (2.1)
Eupergit® C-EDA	50 mM	6	40.3	33.6	63.8	38.7 (± 4.5)	29.0 ± (3.3)
Eupergit® C-EDA	50 mM	8	24.7	25.9	127.6	77.4 (± 7.0)	44.7 ± (4.0)
Eupergit® C-IDA/Cu	50 mM	8	5.6	30.0	110.1	66.8 (± 8.4)	44.6 ± (5.6)
Sepabeads® EC-EP	1M	8	95.4	76.7	3.7	2.3 (± 0.3)	3.9 ± (0.4)
Sepabeads® HA	50 mM	6	52.7	31.1	98.8	59.9 (± 4.2)	41.5 ± (2.9)
Sepabeads® EC-EP-IDA	50 mM	8	43.3	45.7	16.9	10.2 (± 0.3)	10.9 ± (0.3)
Sepabeads® EC-EP-IDA/Cu	50 mM	8	82.1	86.7	15.2	9.2 (± 0.3)	17.9 ± (0.5)
Sepabeads® EC-EP-EDA	50 mM	6	45.2	26.1	33.2	20.1 (± 1.3)	11.7 ± (0.8)
Sepabeads® EC-EP-EDA	50 mM	8	51.3	75.6	21.7	13.2 (± 2.2)	22.3 ± (3.8)

[a]Activity yield was calculated by the difference in the total enzymatic units used for immobilization to the total enzymatic units remaining in the immobilization supernatant, in comparison to the total enzymatic units used for immobilization, multiplied by 100.

[b]Protein yield was calculated by the total protein used for immobilization in comparison to the amount of protein remaining in the immobilization supernatant in comparison to the total protein used for immobilization, multiplied by 100.

[c]Retention of activity was based upon the specific activity of the immobilized enzyme in comparison to the specific activity of the native enzyme, multiplied by 100.

[d]The specific activity of the immobilized LS.

[e]The enzymatic activity of immobilized LS per g support

Table 3.2. Investigation of the immobilization of LS onto agarose-based solid supports at different immobilization conditions

Support	Buffer Concentration	pH	Activity Yield (%)[a]	Protein Yield (%)[b]	Retention of Enzyme Activity (%)[c]	Specific activity of immobilized enzyme (μmol/min. mg protein) [d]	Activity (μmol/min. g support)[e]
Glyoxyl Agarose	50 mM	10	61.9	26.4	48.3	29.3 (± 3.2)	16.7 (± 1.8)
Glyoxyl Agarose	1 M	10	81.0	42.0	16.8	10.2 (± 2.0)	9.0 (± 1.7)
Amino Epoxy Agarose	5 mM	7	55.0	34.3	8.7	5.3 (± 1.9)	4.0 (± 1.4)
Glyoxyl Agarose-IDA/Cu	1 M	8	80.1	36.0	67.0	40.6 (± 5.4)	32.6 (± 4.3)
Glyoxyl Agarose-TEA	5 mM	6	47.9	34.7	19.6	11.9 (± 1.0)	9.2 (± 0.8)

[a]Activity yield was calculated by the difference in the total enzymatic units used for immobilization to the total enzymatic units remaining in the immobilization supernatant, in comparison to the total enzymatic units used for immobilization, multiplied by 100.

[b]Protein yield was calculated by the total protein used for immobilization in comparison to the amount of protein remaining in the immobilization supernatant in comparison to the total protein used for immobilization, multiplied by 100.

[c]Retention of activity was based upon the specific activity of the immobilized enzyme in comparison to the specific activity of the native enzyme, multiplied by 100.

[d]The specific activity of the immobilized LS.
[e]The enzymatic activity of immobilized LS per g support.

16%. Copper modification enhanced the immobilization efficiency of LS on Sepabeads® EC-EP-IDA and on glyoxyl agarose-IDA, with activity yields of 82.1 and 80.1% respectively. Indeed, supports modified with Cu(II) form chelating interactions with neutral histidines, on which there are nine on LS from *B. amyloliquefaciens* (Tang, Lenstra, Borchert, & Nagarajan, 1990; Urrutia, Mateo, Guisan, Wilson, & Illanes, 2013). A highly concentrated immobilization buffer contributed to the high protein yield in the immobilization of LS on Eupergit® C and Sepabeads® EC-EP. Glyoxyl agarose also achieved the highest protein yield among the glyoxyl supports (42.0%) with the use of a highly concentrated immobilization buffer. The high ionic strength buffer will make the hydrophobic interactions between the support and LS more favourable.

The results also show that Eupergit® C and agarose supports led to greatest immobilization of LS at pH 8.0. This may be due to the formation of covalent bonds from the already adsorbed enzyme. This would drive the adsorption equilibrium forward as reported for the immobilization of penicillin G acylase from *E. coli* onto a monoaminoethyl-N-ethyl-agarose (MANAE) support; where a higher immobilization rate was achieved at a higher pH of 8.5 although adsorption was not as thermodynamically favoured (Bolivar, Mateo, et al., 2009). Overall, no anionically charged supports performed well in terms of protein loading. This may be due to a lack of positively charged residues on the exterior of the enzyme, or that the interactions were not strong enough to maintain the enzyme on the support. The outcome of the protein loading was a combination of pH, buffer concentration and microenvironment of the support.

Tables 3.1 and 3.2 also show that the immobilization yields of LS on the cationic Sepabeads® HA, glyoxyl agarose-TEA and amino epoxy agarose supports were similar with activity yields of 52.7%, 47.9% and 55.0% and protein yields of 31.1%, 34.7% and 34.3% respectively. Each of these supports possesses spacers with a positively charged amino functional group, and the adsorption of the enzyme on this support involves ionic interaction with negatively charged regions on the enzyme surface. Glyoxyl agarose-TEA, with a quaternary amine, will always contain a positive charge, independent of the pH, while the ionization of Sepabeads® HA and amino epoxy agarose were pH dependant. Sepabeads® HA, has no epoxy ring or a glyoxyl group capable of forming covalent bonds while glyoxyl agarose-TEA and amino epoxy agarose do. The immobilization yields from Sepabeads® HA were slightly higher than they were from Sepabeads® EC-EP-EDA at pH 6, which is capable of multiple covalent attachments. Since the interactions with Sepabeads® HA is through cationic interactions, the loss of some of these groups for epoxy rings, as in the case

with Sepabeads® EC-EP-EDA, reduces ionic adsorption power of the support. While the immobilization results from Sepabeads® HA, glyoxyl agarose-TEA and amino epoxy agarose were similar to the Sepabeads® EC-EP-EDA and Eupergit® C-EDA, they were lower than those achieved through covalent attachment on the unmodified Sepabeads® EC-EP, Eupergit® C and glyoxyl agarose. As compared to other supports, Sepabeads® EC-EP-IDA/Cu (AY of 82.1%; PY of 86.7%), Eupergit® C (AY of 73.3; PY of 63.4%), Eupergit® C-IDA (AY of 21.7%; PY of 17.7%), Eupergit® C-EDA (AY of 24.7%; PY of 25.9%) and glyoxyl agarose-TEA (AY of 47.9%; PY of 34.7%) led to similar protein (PY) and activity (AY) immobilization yields. These results demonstrate that the drop in LS activity in the supernatant is a result of the LS being immobilized onto these supports. However, the activity yields of LS immobilized on Sepabeads® EC-EP, Eupergit® C-IDA, glyoxyl agarose, glyoxyl agarose IDA/Cu were higher than the protein yields. These results may be attributed to the preferential immobilization of LS onto the supports in comparison to the contaminating proteins in the free enzyme solution, to the underestimation of protein content of the free enzyme due to its aggregation and/or to a large drop in LS activity in the supernatant or both. In the case of Eupergit® C-IDA/Cu (AY of 5.6%; PY of 30.0%) and Sepabeads® EC-EP-EDA (AY of 51.3%; PY 75.6%), the protein yield was larger as compared to the activity yield. Sepabeads® EC-EP-IDA/Cu achieved the highest known protein yield in LS immobilization studies, with the next highest reported from the immobilization of LS from *B. subtilis* onto glutaraldehyde activated chitosan (81%) (Esawy et al., 2008). Titanium-activated magnetite achieved a high protein yield of 75% upon the immobilization of LS from *Z. mobilis* at pH 4.0 (Jang et al., 2001). Retention of activity is a significant factor, which represents the specific activity of the enzyme on the support in comparison to the specific activity of the free enzyme. An activation of enzyme activity through immobilization can occur by favouring an active tridimensional conformation of the enzyme structure, a proper orientation of the enzyme active site and a better dispersion of enzyme on the support, limiting its aggregation (Mateo et al., 2007). Using the same buffer concentration of 1 M, the retention of LS activity upon immobilization on glyoxyl agarose (16.8%) and on Eupergit® C (22.5%) were within a comparable range. Using 50 mM buffer, the retention of activity of glyoxyl agarose increased (48.3%). The weaker immobilization conditions possibly allowed LS to reorient itself on the glyoxyl support until the most stable orientation was achieved (Guisan, 1988). Although the highest immobilization yields were achieved with the unmodified Sepabeads® EC-EP support, it led to the lowest retention of

activity of 3.7%. These results may be due to the rigidification of the enzyme upon its immobilization on the Sepabeads® EC-EP support and/or to the steric hindrance blocking access of the substrates to the active site of the enzyme. Through the use of pre-immobilization techniques, the supports can be functionalized with different spacer arms which could provide the immobilized LS with more flexibility and could orient the enzyme through immobilization. The functionalization of Eupergit® C and Sepabeads® EC-EP with EDA functional groups might have positively affected the orientation of enzyme on the surface of the support and resulted in a more active LS. The retention of activity of Eupergit® C increased 2.87 and 5.66 times with the functionalization of EDA at pH 6 and 8, respectively; while the retention of activity of Sepabeads® EC-EP increased 8.9 times with the addition of EDA functionality. Modification of the supports with IDA was moderately less successful. The retention of activity for Eupergit® C-IDA and Sepabeads® EC-EP-IDA was 5.7 and 4.5 times higher than the unmodified supports. Glyoxyl agarose-IDA failed to immobilize LS (data not shown), possibly due to the very hydrophilic support interfering with anionic interactions, which were required for immobilization. The addition of copper to the IDA modified Eupergit® C, Sepabeads® EC-EP and glyoxyl agarose provided the supports 4.9, 4.1 and 1.4 times increase in retention of activity. Glyoxyl agarose-IDA/Cu was the only agarose support able to achieve a retention of activity (67.0%) higher than that of glyoxyl agarose (48.3%). Addition of the quaternary amine to glyoxyl agarose supports to create glyoxyl agarose-TEA did not significantly alter the retention of activity of LS in comparison to the other supports. This was surprising since LS activity was predominately activated when immobilized onto cationically charged supports. Again, the hydrophilic agarose may be interfering with interactions which are required for adsorption. Deactivation of enzymatic activity from immobilization can take place in a few ways. Steric hindrance, caused by improper orientation of the enzyme active site upon immobilization, can lower enzyme activity. There is also the potential for a loss in flexibility, which can occur through overly intense covalent bond formation between the enzyme and the support. Supports which retained the least amount of activity include Sepabeads® EC-EP (3.7%), Eupergit® C-IDA (6.0%) at pH 6.0 and amino epoxy agarose (8.7%). The buffer pH was an important contributing factor; immobilization onto Eupergit® C-EDA at pH 8 had approximately double the retention of activity than the immobilization on the same support at pH 6. Immobilization onto glyoxyl agarose-IDA/Cu also had a similar result. Retention of activity was much higher (67.0%) when immobilization took place at pH 8 rather than at pH 6 (9.3%). The difference in buffer

concentration could have also contributed to the difference in activities. Increasing the immobilization buffer concentration from 100 mM – 1M caused a 7.2-fold increase in the retention of activity using glyoxyl agarose-IDA/Cu (Data not shown).

In the immobilization of LS onto glyoxyl agarose, glyoxyl agarose-IDA/Cu and glyoxyl agarose-TEA, a reduction step with sodium borohydride was required to reduce Schiff bases which were created. The results listed in Tables 3.2 are after the reduction, but the activity of the support was measured before reduction as well (data not shown). What was seen was a dramatic loss of activity (26-96%). There is the potential that the reducing agent caused a reduction in the amino acid residues essential for catalysis, such as the three residues which compose the LS catalytic triad, Asp^{86}, Asp^{247} and Glu^{340} (Ozimek et al., 2006).

While high retention of activity is critical for the success of the immobilization, sufficient protein must be immobilized onto the support for any real efficacy to be achieved. Although immobilization onto Eupergit® C-IDA (50 mM, pH 8) had the highest retention of activity (129.4%), the protein yield (12.0%) was low in comparison. This resulted in a moderate amount of activity per gram of support (17.2 μmol $min^{-1}g\ support^{-1}$). Eupergit® C-EDA (50mM, pH 8), Eupergit® C-IDA/Cu, Sepabeads® HA and glyoxyl agarose-IDA/Cu (1M, pH 8) supports immobilized relatively high amounts of protein while activating LS activity, thus providing the supports with the highest amount of activity per gram of support. The lack of one factor can result in a poor biocatalyst, which was the case for the support Sepabeads® EC-EP. It achieved very high protein yield yet caused a denaturation of the enzyme, resulting in poor activity per gram of support (3.9 μmol $min^{-1}g\ support^{-1}$).

3.3.2. Thermal stability of selected immobilized LSs

Stabilization of an enzyme can be achieved through rigidification by immobilization onto a solid support via short spacer arms. Immobilization may also protect particularly sensitive regions on the enzyme by shielding it from the surrounding environment (Mateo et al., 2010). The effect of immobilization on the thermal stability of LS was evaluated at 50°C using the best selected supports (Eupergit® C, Eupergit® C-IDA, Sepabeads® EC-EP-IDA, Eupergit® C-IDA/Cu, Eupergit® C-EDA, Sepabeads® HA, glyoxyl agarose, glyoxyl agarose-IDA/Cu, glyoxyl agarose-TEA and amino epoxy agarose) (Fig.3.1). Testing at 50°C was chosen due to the lability of the native enzyme at this temperature, with a measurable half-life. This temperature is similar to temperatures chosen

by other authors, 10-40°C and 30-60°C (Jang et al., 2001, Ortiz-Soto et al., 2009). The thermal inactivation of LS followed second order kinetics (data not shown, for equation see Eqn S3.1). The free LS exhibited a half-life of 16 mins (Fig. S3.1). Due to low reactivity of nucleophiles with the epoxy groups at a neutral pH, the epoxy-activated supports required high pH incubation (pH 9.2) to promote covalent bond formation through lysine residues (pKa 10.53 in peptides) and to enhance the thermal stability of LS. Nucleophilic residues are unable to react with the glyoxyl agarose-based supports below pH 10; therefore, high pH incubation (pH 10) was required to achieve any thermal stability.

The stability results (Figure 3.1) show that immobilization of LS on ionic Sepabeads® HA by adsorption was unable to provide LS with any thermal stability in comparison to the free enzyme. For all other commercial supports, except Sepabeads® EC-EP-IDA and Eupergit® C-IDA, the thermal stability increased after incubation at high pH for 72 h. Immobilization onto supports modified with IDA may have favoured an unstable LS conformation that was distorted upon incubation at high pH. Immobilization onto glyoxyl agarose-IDA/Cu and glyoxyl agarose resulted in the highest thermal stability, with stability factors of approximately 14 and 106 times as compared to the free enzyme. Glyoxyl supports are capable of performing multi-point covalent attachment to a high degree, resulting in the rigidification of the enzyme which achieves a highly stabilized enzyme (Mateo et al., 2010). These determined stability factors are very promising in comparison to other successful immobilizations of LS reported in the literature. The thermal stability of LS from *B. circulans* was improved through cross-linking the enzyme with oxidized dextrans. The modified LS had a stability factor of 2.65 at 50°C and 2.22 at 55°C as compared to the free enzyme (El Refai et al., 2009). When LS from *Z. mobilis* was immobilized onto titanium-activated magnetite, it maintained almost complete activity after heating at 45°C for 15 mins, while the free enzyme only retained 41% of its initial activity (Jang et al., 2001).

Modifications to the supports did not provide significant increased thermal stability. The stability offered by immobilization onto Eupergit® C after high pH incubation was slightly higher than that provided by Eupergit® C-EDA and Eupergit® C-IDA/Cu. Similar results were seen with the agarose supports. Glyoxyl agarose also provided more thermal stability in comparison to glyoxyl agarose-TEA, amino epoxy agarose and glyoxyl agarose-IDA/Cu. Supports are modified through the groups which are also responsible for covalent bond formation. This results in possibly better orientation of the enzyme, with higher retention of activity, but with lower thermal stability. The

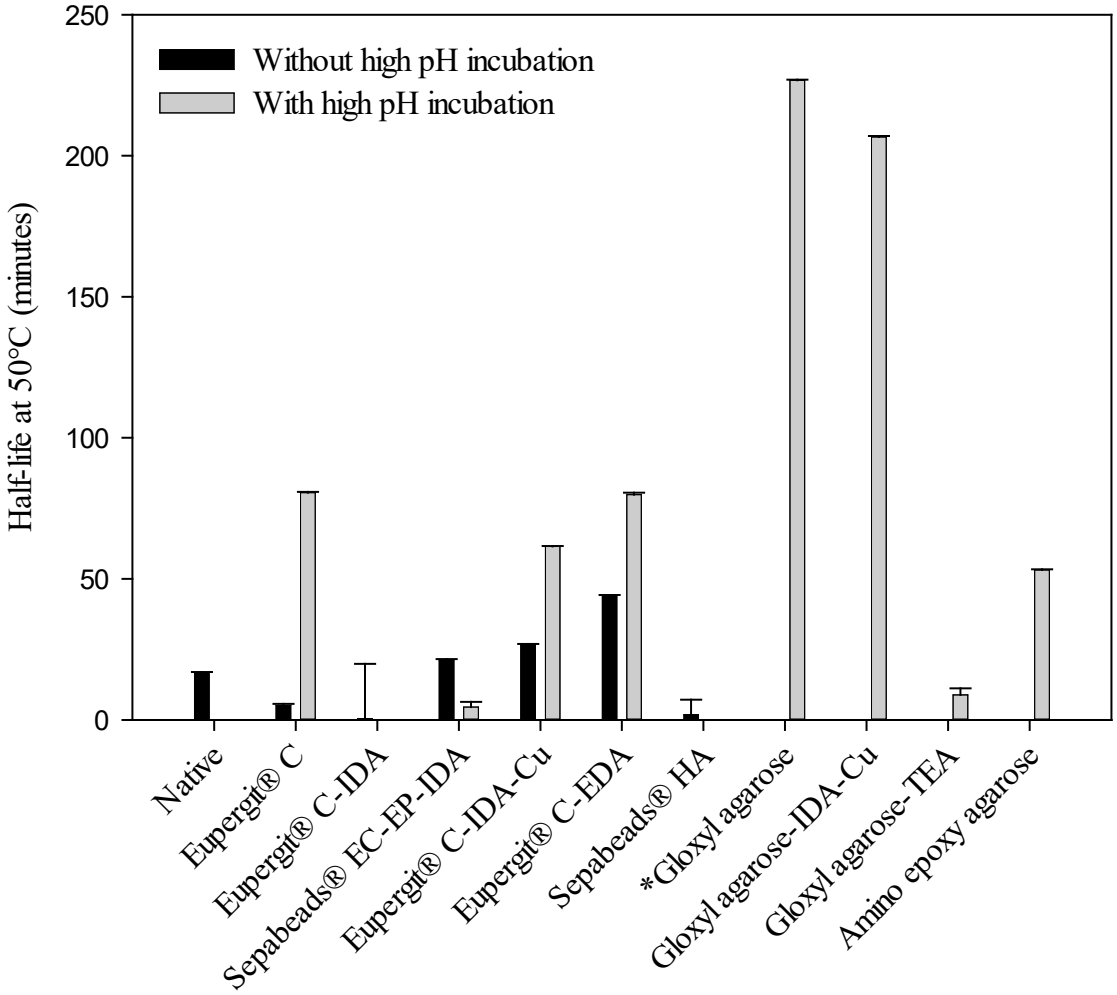


Figure 3.4: The half-lives of immobilized LS immobilized onto commercial and natural supports measured at 50°C. Sepabeads® EP-HA did not undergo high pH incubation. *It was measured at 55°C instead of 50°C.

difficulty lies in achieving a balance between high retention of activity while providing sufficient stability.

3.3.3. Investigation of the reaction selectivity (transfructosylation vs hydrolysis) of immobilized LS

The reaction selectivity of LS was reported to be dependent on its microbial source and its reaction conditions (Goldman et al., 2008). *B. amyloliquefaciens* had higher k_{catapp} for transfructosylating activity (1,136.5 ± 211.1 s^{-1}) than for hydrolytic activity (178.6 ± 8.8 s^{-1}), while the catalytic efficiency for hydrolysis (9,500 $M^{-1}s^{-1}$) is higher than that for transfructosylation (2,470.7 $M^{-1}s^{-1}$) (Tian & Karboune, 2012). It was investigated whether immobilization caused an increase in transfructosylating activity with a resulting decrease in hydrolytic activity. Indeed, immobilization may lead to better modulation of reaction selectivity of LS towards the transfructosylating reaction through changes in the enzyme's structure and its microenvironment. Such changes may affect the diffusion and the accessibility of substrates to the active site of the enzyme and its binding.

Figure 3.2 shows the effect of LS immobilization on its ratio of transfructosylation versus hydrolytic activity relative to the ratio obtained from the free LS. The highest ratio achieved upon immobilization of LS on Sepabeads® HA support, which was over two-fold higher than the free enzyme. This cationic support seems to favour an active tridimensional structure that promotes transfructosylating activity. Sepabeads® HA was also the most hydrophobic support, absorbing less water than both Sepabeads® EC-EP and Eupergit® C. The hydrophobic matrix of the support may have affected the microenvironment of the support, reducing the amount of water available for hydrolysis. Other cationic supports: better or equivalent ratios to the free enzyme except for amino-epoxy agarose. These results indicate that immobilization onto cationic supports may be altering the accessibility of acceptors to the LS subsites. Ozimek *et al.* (2006) proposed a model of the sugar-binding subsites (-1, +1, +2, +3) to better understand the reaction selectivity of LS and its oligomerizing/polymerizing activities (Ozimek et al., 2006). In the transfructosylation reaction, sucrose first occupies the -1 and +1 subsites; after the fructosyl-enzyme intermediate is formed at -1 subsite, glucose is released from the active site. A second acceptor (e.g. sucrose) enters the active site, and binds to the +1 and +2 subsites, and reacts with the fructosyl-enzyme intermediate. The increase in the transfructosylation over hydrolysis may be attributed to increased

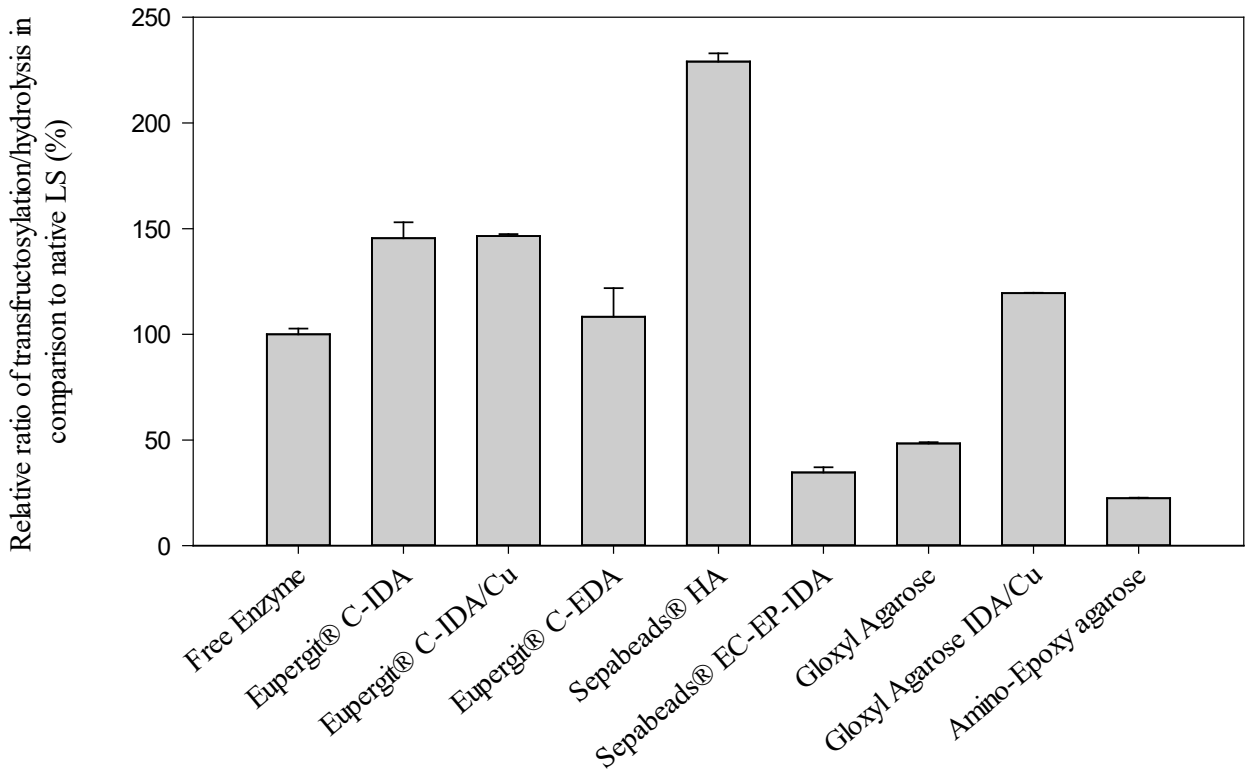


Figure 3.5:Transfructosylating activity versus hydrolytic activity on immobilized LS.

exposure of acceptors (e.g. sucrose, growing (oligo)polymer chain) to the +1 and +2 subsites (and potentially other subsites). Jang *et al*. (2001) found that they retained only 70% of their levan forming activity upon immobilization of LS from *Z. mobilis* onto titanium-activated magnetite, suggesting that the immobilized LS could not bind as strongly to the levan (Jang et al., 2001).

The low transfructosylating activity observed upon immobilization on Sepabeads® EC-EP-IDA provides further evidence that this support distorts the LS conformation in an unfavourable way. Alternatively, Eupergit® C-IDA had both higher transfructosylating and hydrolytic activity, with a resulting higher tranfructosylation versus hydrolysis ratio. The difference between Sepabeads® EC-EP-IDA and Eupergit® C-IDA may be due to their microenvironments and to their internal morphologies. Indeed, the concentration of oxirane groups on both supports differs (~Sepabeads® EC-EP: 165.0 μmol g^{-1} dry support; Eupergit® C 600 μmol g^{-1} dry support) as does the internal surface of each support. The pores in Sepabeads® EC-EP, unlike Eupergit® C, are cylindrical in shape, providing an even plane for immobilization (Mateo et al., 2002). The even plane which immobilizes LS onto Sepabeads® EC-EP may stretch the structure of LS, causing the unfavourable microconformations, while Eupergit® C, with its uneven structure and high density of functionalized groups, can cradle and stabilize the LS in a favourable way. These results differ to what was observed in the immobilization penicillin G acylase from *E. coli* onto functionalized Eupergit® C and Sepabeads® with EDA and glutaraldehyde. It was found that the ratio of synthesis/hydrolysis was slightly higher for modified Sepabeads® than it was for modified Eupergit® C (Bonomi et al., 2013). Furthermore, the activity of hydroperoxide lyase from *Penicillum camemberti* was found to be significantly affected by the pore size of its' immobilization support. It retain 3.25 times more activity when Eupergit® C250L was used, with a larger pore size, instead of Eupergit® C (Hall, Karboune, Florence, & Kermashaa, 2008).

LS immobilized onto glyoxyl agarose also experienced a low ratio (48.4%) in comparison to the free enzyme. This can be due to the damaging effects of the reducing agent on LS, required for the reduction of the Schiff base, which may have affected the transfructosylation activity more than the hydrolytic activity. Glyoxyl agarose-IDA/Cu also experienced a drop-in activity, but this drop was experienced equally by the transfructosylating and hydrolytic activity, leading to a ratio of 1.05. Immobilization of LS from *B. subtilis* onto hydroxyapatite produced similar results to the immobilization of LS onto Sepabeads® HA, where an increase in polymerase activity from 45.0% to 75.0% was obtained upon immobilization (Chambert & Petit-Glatron, 1993), while in the present experiment, the immobilization of LS from *B. amyloliquefaciens* onto Sepabeads® HA increased transfructosylating activity from 47.0% to 67.0%.

3.4. Conclusions

LS was successfully immobilized onto both commercial and modified agarose supports. The highest retention of activity was obtained through the immobilization onto Eupergit® C-IDA, while immobilization onto Sepabeads® HA and glyoxyl agarose-IDA/Cu performed well overall in terms of retention of activity, and transfructosylation versus hydrolysis ratio. LS experienced a dramatic loss in activity when sodium borohydride was used to reduce the Schiff base during the immobilization onto glyoxyl-based supports. Thermal stability at 50°C was best achieved when immobilization occurred in conditions that promoted covalent bond formation. The best overall support was determined to be glyoxyl agarose-IDA/Cu, achieving high immobilization yields, thermal stability without directing the reaction specificity of immobilized LS towards hydrolysis. Future research will involve determining the best compound to protect the active site from reduction from sodium borohydride. It will also include an examination of the kinetic properties of the immobilized LS as well as studying its reaction profile in further detail.

Connecting Statement 2

Chapter III investigated multiple potential solid supports for the immobilization of LS from *B. amyloliquefaciens*. The effects of immobilization on the enzyme's thermal stability, retention of activity and transfructosylation versus hydrolytic activity were examined. This examination found that the immobilization efficiency was dependent on multiple immobilization parameters such as immobilization time, buffer molarity and pH. A comparison was made between immobilization through adsorption and multipoint covalent attachment. Chapter IV focuses on the optimization of the immobilization of LS onto glyoxyl agarose-IDA/Cu using both chelation and covalent bond formation. A detailed examination of the effects of immobilization parameters was performed. Additional stabilization methods were also employed in this study.

The results from this study were presented at the 2013 & 2015 Biotrans conference, the Prostab Conference as well as in the journal Process Biochemistry.

Hill, A., Mateo, C., Wilson, L. & Karboune, S. (2013) The Optimization of the Immobilization of Levansucrase for the Production of Prebiotic Fructooligosaccharides on Agarose Supports using Copper Chelation. Biotrans 2013, Manchester, UK, July 21- July 25, 2013.

Hill, A., Mateo, C., Tian, F. & Karboune, S. (2014) Optimizing the immobilization of levansucrase on gloxyl agarose-ida-Cu^{2+} for higher thermal stability. Prostab 2014, Lake Maggiore, Italy, July 5 – July 9, 2014.

Hill, A., Sooyoun S., Mateo, C. & Karboune, S. (2015) Heterologous expression of levansucrase from *Bacillus amyloliquefaciens* and its immobilization. BioTrans 2015, Vienna, Austria, July 26 – July 30, 2015

Hill, A, Karboune, S. & Mateo, C. (2017) Investigating and optimizing the immobilization of levansucrase for increased transfructosylation activity and thermal stability. *Process Biochemistry*. 61, 63-72.

4. Abstract

Levansucrase (LS) represents a key enzyme in glycoside synthesis of novel prebiotics and β-2,6-levan. The study of the immobilization parameters on LS, produced from *B. amyloliquefaciens*, onto glyoxyl agarose-iminodiacetic acid/Cu (glyoxyl agarose-IDA/Cu) by response surface methodology revealed significant interactive effects. Retention of activity was altered by interactive effects from buffer molarity/time and buffer pH/buffer molarity. The optimized immobilization conditions were identified to be a protein loading of 9.09 mg protein/g support, a buffer concentration of 608 mM at pH 6.8 and an incubation time of 49h. Normally a reducing agent is applied to the immobilized enzyme in order to promote the formation of covalent bonds. This step was replaced with a post-immobilization crosslinking treatment with the ionic polymer polyethylenimine (PEI), which provided a better compromise between retained activity and thermal stability of the immobilized LS. Indeed, LS immobilized onto glyoxyl agarose-IDA/Cu/PEI had a retention of activity of 70.91% with a protein yield of 44.73% and an activity yield of 54.69%, while exhibiting a half-life 4.7 times higher than that of the free LS at 50°C.

4.1. Introduction

FOSs constitute a class of functional ingredients, whose potential health benefits in terms of supporting intestinal health and reducing the risk of cancers are increasingly being recognized (Roberfroid et al., 2010). Besides acting as prebiotics (Kilian et al., 2002; Marx et al., 2000; Porras-Dominguez et al., 2014), FOSs can be used as non-cariogenic sweetener replacing agent in food production (Monsan & Ouarne, 2009). On the other hand, β-(2,6)-levan polysaccharides have shown antitumor and antidiabetic activities in addition to their stabilizing, formulation aid, encapsulating agent and flavour carrier capacities relevant to the pharmaceutical, cosmetic and chemical industries (Dahech et al., 2011; Han, 1990; Kim et al., 2004; Yoo et al., 2004). Levansucrase (EC 2.4.1.10, LS) has been studied by our group and others as a potential biocatalyst for synthesizing FOSs and β-(2,6)-levan polysaccharides (Hill, Karboune, & Mateo, 2015; Inthanavong, 2011; Inthanavong et al., 2013; Tian et al., 2011; Tian & Karboune, 2012; Tian, Karboune, et al., 2014; Tian, Khodadadi, et al., 2014). Belonging to glycoside hydrolase family 68, LS contains a five-fold, β-propeller typology (Lombard, 2014). The LS active site contains subsites, which orient and stabilize the fructosyl and glucosyl residues of sucrose as it enters the LS active site (Wuerges et al., 2015). The amino acid composition of the subsites of LSs defines their substrate affinity and the predominant reactions, exchange, hydrolysis, oligomerization and polymerization (Homann et al., 2007; Wuerges et al., 2015). LSs from different microbial sources differ with respect to their reaction selectivity (hydrolysis/transfructosylation) and oligo-/polymerization ratio (Homann et al., 2007). Recently, some

hypotheses and structural features have been put forward to describe the reaction selectivity and the polymerizing activity of LSs (Homann et al., 2007; Strube et al., 2011; Wuerges et al., 2015).

Few studies (Chambert & Petit-Glatron, 1993; Goldman et al., 2008; Kim et al., 1998), including our own (Hill et al., 2015; Inthanavong et al., 2013), revealed that modulating LS's macro/microenvironments may afford means for favoring its reaction selectivity toward transfructosylation. For instance, immobilization may help modulate the hydrophilic/hydrophobic balance of LS microenvironment and hence optimize the reaction selectivity. In addition, immobilization of enzymes can allow for the easy reuse of the biocatalyst and can promote enzyme stabilization (Polizzi, Bommarius, Broering, & Chaparro-Riggers, 2007). Site-directed immobilization orients the enzyme on the support by having immobilization occur through specific regions on the enzyme. Modifications of solid supports can limit immobilization to where there is the highest density of reactive residues on the enzyme (Mateo et al., 2010). Multipoint covalent attachments via short spacer arms can also increase the stability of an enzyme by reducing flexibility. Immobilization parameters may also affect not only the enzyme orientation, but also the immobilization rate and the homogeneous distribution of enzyme on the supports (Betancor, López-Gallego, et al., 2006; Jang et al., 2000b).

Previous immobilization of LS had been performed using various supports including hydroxyapatite, titanium-activated magnetite, chitin beads, chitosan modified with glutaraldehyde as well as the production of CLEAs (Chambert & M. F. Petit-Glatron, 1993; Chiang et al., 2009; Esawy, 2008; K.-H. Jang et al., 2001; Jang et al., 2000b; M. E. Ortiz-Soto et al., 2009); yet no study performed a through optimization of the immobilization conditions of LS. In our previous studies, glyoxyl-agarose-IDA/Cu support was identified as the most appropriate support for the immobilization of LS from *B. amyloliquefaciens*. This support was identified upon a screening performed using modified and unmodified Eupergit® C, Sepabeads® and glyoxyl agarose supports as well as unmodified Sepabeads® HA (Hill et al., 2015). However, compromising between the retained LS activity upon immobilization on glyoxyl agarose-IDA/Cu support, the stability and the reaction selectivity has to be addressed. In this present study, the effects of immobilization parameters, including protein loading, immobilization buffer concentration, pH and immobilization time, on the retention of LS activity, immobilization yield, activity yield, and transfructosylating/hydrolytic activity ratio were investigated using response surface methodology (RSM)**.** RSM allows the development of mathematical models to assess the statistical significance of the variables being studied and their combined effects upon the system as a whole (Huang & Akoh, 1996). Understanding of the interactive effects of immobilization parameters are expected to allow the optimization of the retained LS, but also a better modulation of its reaction selectivity. The use

of sodium borohydride to reduce Schiff bases and stabilize LS immobilized on heterofunctional glyoxyl agarose-IDA/Cu had caused a decreased in the retained LS activity. Alternative stabilization of the immobilized LS by crosslinking with glutaraldehyde or through interactions with polyethylenimine (PEI), which does not require the use of a reducing agent, was successfully examined.

4.2. Materials and Methods

4.2.1. Materials

Sucrose, D-(-)-fructose, D-(+)-glucose, 3,5-dinitrosalicylic acid, NaOH, polyethylene glycol (PEG) 200, potassium sodium tartrate ($KNaC_4H_4O_6$), $NaIO_4$, $NaBH_4$, iminodiacetic acid ($HN(CH_2CO_2H)_2$), glutaraldehyde ($OHC(CH_2)_3CHO$), polyethylenimine ($H(NHCH_2CH_2)_nNH_2$) and $CuSO_4$ were obtained from Sigma Chemical Co. (St. Louis, MO). $CaHPO_4$, $FeSO_4 \cdot 7H_2O$, $MnSO_4 \cdot 7H_2O$, $Na_2HPO_4 \cdot 2H_2O$, $NaMoO_4 \cdot 2H_2O$, $(NH_4)_2SO_4$, K_2HPO_4, KH_2PO_4, $NaHCO_3$, glycerol, bovine serum albumin (BSA) and yeast extract were obtained from Fisher Scientific (Fair Lawn, NJ). Agarose 10BCL was purchased from Agarose Bead Technologies. Low molecular weight levan was produced by the procedure described by Tian et al. Orafti®-P95 was provided by Beneo Inc. (Morris Plains, NJ) *B. amyloliquefaciens* (ATCC 23350) was obtained from American type culture collection (Manassas, VA, USA). Bradford reagent concentrate was provided by Bio-Rad (Missasauga, ON, Canada).

4.2.2. Production of LS from *B. amyloliquefaciens*

Production of LS from *B. amyloliquefaciens,* strain ATCC 23350, followed the protocol described by Tian et al. (2011). *B. amyloliquefaciens*, pre-cultured aerobically in nutrient broth (8 g/L), was transferred (4 mL) to a 1-L baffled Erlenmeyer flask, which contained 400 mL of modified mineral salt medium consisting of (g/L) $Na_2HPO_4 \cdot 2H_2O$ (2.67), KH_2PO_4 (1.36), $(NH_4)_2SO_4$ (0.5), $FeSO_4 \cdot 7H_2O$ (0.005), $MnSO_4 \cdot H_2O$ (0.0018), $NaMoO_4 \cdot 2H_2O$ (0.0025), $CaPO_4 \cdot 2H_2O$ (0.01), $MgSO_4 \cdot 7H_2O$ (0.2) and yeast extract (10). Sucrose was used to induce the production of LS. The media was incubated at 35°C at 150 rpm for 11 h, afterwards the solution was centrifuged (8000 rpm, 20 min) to retrieve the pellet. Potassium phosphate buffer (50 mM, 37.5 mL), pH 6 containing 1% Triton X-100 was used to resuspend the bacteria cells. Ultrasonification was applied to the cells for 6 min and 25 s, set at 15 kHz with 25/50 s cycles. The cellular debris was separated by centrifugation (8000 rpm, 4°C) for 15 min after which PEG 200 (30% v/v) was added to the supernatant to partially purify LS. The solution was slowly stirred at 4°C for 14 h. The solution was centrifuged (12000 rpm, 4°C) for 45 min, the supernatant was removed, and the pellet was resuspended in a minimum volume of potassium phosphate buffer (50 mM) pH 6. The resuspended pellet was dialysed against potassium phosphate buffer (5 mM, 30 L) with a molecular weight cut-off of

6-8 kDa, then lyophilized until dry. The protein content of the powder was determined using the Bradford protein assay, using bovine serum albumin as a standard (Bradford, 1976).

4.2.3. LS Activity assays

The total, transfructosylating and hydrolytic activities of LS were assessed. The total LS activity is expressed as the μmol of reducing sugars released per min per mL of enzyme. LS (250 μL) in potassium phosphate buffer (50 mM) pH 6, was added to sucrose solution (1.8 M, 250 μL), and the reaction mixture was incubated at 30°C for 20 min. 3, 5-Dinitrosalicyclic acid (DNS) assay was used to measure the total reducing sugars (Miller, 1959). The hydrolytic activity was estimated as the μmol of free fructose released per min per mL of enzyme. The transfructosylating activity was calculated as the μmol of fructose transferred to an acceptor molecule per min per mL of enzyme. This was measured by subtracting the amount of glucose from the amount of free fructose. The monosaccharides were quantified by high-pressure-anionic-exchange-chromatography with a pulsed amperometric detector (HPAEC-PAD, Dionex) using a CarboPac PA-20 (3 x 150 mm) column and analysed using Chromeleon Software. The products were separated using isocratic elution with 10 mM NaOH at a flow rate of 0.5 mL/min and maintained at 32°C.

4.2.4. Functionalization of glyoxyl agarose-IDA/Cu

The glyoxyl-based supports were prepared following the protocol of Mateo et al. (2010).
Epoxy-Activated Agarose. $NaBH_4$ (2 g) was added to NaOH (0.656 M, 440 mL) and acetone (160 mL), which was kept on ice. Agarose 10-BCL (100 g) and epichlorohydrin (110 mL) were added and the mixture was stirred 14 h at 25°C. The support was washed with deionized H_2O (10 volumes).
Glyoxyl agarose-IDA/Cu. An IDA solution (0.5 M) adjusted to pH 11, was mixed with wet, epoxy-activated agarose (10% w/v). The reaction was mixed for 36 h at 25°C. The support was then filtered on a sintered glass filter and washed with deionized H_2O (10 volumes). $NaIO_4$ (0.01 M, 5% v/v support:solution) was mixed with the support for 90 min and then washed with deionized H_2O (10 volumes). The support was then mixed with a solution of $CuSO_4$ (30 mg/mL) for 1 h at room temperature. The support was filtered and washed with deionized H_2O (10 volumes).

4.2.5. Immobilization of LS

LS (9.09 mg protein/g wet support) solution was added to glyoxyl agarose-IDA/Cu support in potassium phosphate buffer (608 mM, pH 6.8). The mixture was gently mixed at 4°C for 49 h. The LS activity and the protein content of the recovered supernatant were measured. A sodium bicarbonate buffer (608 mM, 1 mL) at pH 10 containing 20% (v/v) glycerol was added to immobilized LS, and the resulted suspension

was incubated for 3 h at 4°C to promote the formation of covalent bonds. To reduce the Schiff bases, $NaBH_4$ (1 mg/mL) was added to the high pH suspension and gently mixed at 4°C for 30 min. Afterwards, the supernatant was recovered and tested for protein content. The support was washed with 10 volumes of potassium phosphate buffer (50 mM, 1 mL), at pH 6.

The immobilized LS was resuspended in potassium phosphate buffer (50 mM) and the activity was measured. The immobilized LS activity reported for RSM was measured before high pH incubation and reduction, since it was performed to study the adsorption of LS onto glyoxyl agarose-IDA/Cu. Other measurements of immobilized LS activity were measured after high pH incubation and reduction. Activity yield was calculated as the difference between the total units of the native solution and those of the supernatant solution, multiplied by 100 and divided by the total units of the native solution. Protein yield was determined as the difference between the total mg of protein in the native solution and that in the supernatant, multiplied by 100 and divided by the total mg of protein in the native solution. Retention of activity was defined as the specific activity of the immobilized LS, multiplied by 100 and divided by the specific activity of the native LS solution.

Equation 1. $Activity\ yield\ (\%) = 100\ x\ \frac{total\ units\ native - total\ units\ of\ supernatant}{total\ units\ of\ native\ LS\ solution}$

Equation 2. $Protein\ yield\ (\%) = 100\ x\ \frac{total\ protein\ in\ native - total\ protein\ in\ supernatant}{total\ protein\ in\ native\ LS\ solution}$

Equation 3. $Retention\ of\ Activity\ (\%) = 100\ x\ \frac{specific\ activity\ of\ immobilized\ LS}{specific\ activity\ of\ native\ LS\ solution}$

4.2.6. Effects of immobilization parameters of LS onto glyoxyl agarose-IDA/Cu

The effects of immobilization parameters were studied using RSM and a central composite rotatable design (CCRD). Factors considered important for immobilization and worth investigating were protein loading (X_1, 2.5-35.0 mg/g support), pH (X_2, 5.5-8.0), immobilization buffer concentration (X_3, 25-1000 mM) and immobilization time (X_4, 6-60 h). A 2 level 4 factor fractional factorial design was created using Design Expert® Software (version 8.0.7) (Box & Behnken, 1960). Activity yield, protein yield, retention of activity, transfructosylating and hydrolytic activity were the quantified responses. The runs were randomised. The quadratic response surface model fitted Equation 4:

$$y_k = B_{k0} + \sum_{i=1}^{4} B_{ki} x_i + \sum_{i=1}^{4} B_{ki} x_i^2 + \sum_{i=1}^{3} \sum_{i=1}^{4} B_{ki} x_i x_j$$

Equation 4: Quadratic response surface model.

Where y_k is the response variable (retention of activity; %, activity yield; %, protein yield; %, transfructosylating activity; μmol/min*g, hydrolytic activity; μmol/min*g). B_{k01}, B_{kii}, B_{kij} and B_{kij} are

constant coefficients and x_is are independent variables. Design Expert® Software was used to fit the second order polynomial to the experimental data.

4.2.7. Evaluation of selected protecting agents

To prevent the denaturation of LS by $NaBH_4$ reducing agent, the efficiency of selected protecting agents was assessed. Sucrose, FOS-Orafti P95 and low molecular weight levan (686 Da) (102.6 g/L) were added to sodium carbonate buffer (608 mM) at pH 10 with 20% (v/v) glycerol. The high pH incubation was, then, carried out as previously described, in the presence of the protecting agents followed by the reduction of the Schiff base with $NaBH_4$.

4.2.8. LS stabilization without reduction

Instead of reduction with $NaBH_4$, **a**lternative stabilization of the immobilized LS by glutaraldehyde and PEI was attempted. After high pH incubation, a solution of glutaraldehyde (0.1-0.5% v/v) in potassium phosphate buffer (607 mM), pH 6.8 was added to the immobilized LS (1:10 v/w); the suspension was incubated for 1-3 h. On the other hand, the PEI solution (0.1-2% v/v) in potassium phosphate buffer (607 mM, pH 6.8) was added to the immobilized LS (1:10 v/w) and mixed for 1-15 h. The immobilized LS on glyoxyl agarose IDA/Cu was then washed with potassium phosphate buffer (50 mM, 10 volumes) at pH 6.

4.2.9. Assessment of thermal stability

Free or immobilized LS (500 mg immobilized LS, equivalent quantity of protein for free LS) in potassium phosphate buffer (50 mM, 5 mL) was placed in a 50°C water bath after the initial LS activity was measured. Every 15 min within 120 min, an aliquot was taken, and the residual LS activity was measured as described previously. For increased thermal stability measurements, the procedure was repeated at 55°C. The thermal degradation followed second order kinetics, which was used to calculate the half-life of the enzyme.

4.3. Results and Discussion

4.3.1. Effects of immobilization parameters of LS on glyoxyl agarose-IDA/Cu

The investigation of the effects of each immobilization parameter of LS on glyoxyl agarose-IDA/Cu one by one would be a labour-intensive process. It would also not account for interactions between immobilization parameters. RSM enables the evaluation of effects of multiple parameters, alone or in combination, on response variables, while offering the advantage of reducing the number of experiments needed by developing mathematical models (Huang & Akoh, 1996). Therefore, RSM was used to study

the effects of protein loading onto the support (X_1, 2.5-35 mg/g support), buffer pH (X_2, 5.5-8) buffer concentration (X_3, 25-1000 mM), and immobilization time (X_4, 6-60 h) on the activity yield, protein yield, retention of activity and the transfructosylating activity versus the hydrolytic activity. A two-level, four-factor fractional factorial design with five center points, eight factorial points, and eight axial points was performed (Table 4.1). The levels of the selected parameters were set based on preliminary trials, where one factor at a time was varied (data not shown). As seen in Table 4.2, a reduced cubic model was statistically significant for the description of the variations of activity yield (*F*-value of 306.79 and *p*-value of <0.0001) and the relative ratio of transfructosylating activity versus hydrolytic activity as compared to the native LS (T/H ratio, *F*-value of 16.55 and *p*-value of <0.0001). A quadratic model was statistically significant for the description of protein yield (*F*-value of 9.15 and *p*-value of 0.0018) and retention of activity (*F*-value of 21.61 and *p*-value of <0.0001). The lack of fit was not significant relative to pure error with *F*-values of 0.86 to 1.70 and *p*-values of 0.3033 to 0.5868; these results indicate a good quality of the fit and its ability to predict within a range of variables used. In addition, the closer the coefficient of determination (R^2) value is to 1.00, the better is the model to predict the response. The R^2 values of the fitted models were 0.9558 for immobilization activity yield, 0.5041 for the protein yield, 0.9982 for retention of activity and 0.9169 for the relative T/H ratio. The fitted models in terms of actual factors is given by Equations 5-8. The variables are deemed more significant if the *F*-value is bigger and the *p*-value is smaller.

Equation 5 $$\textbf{Activity Yield} = -1181.73 + 327.51X_2 + 0.56X_3 - 2.28X_4 + 0.31X_1X_4 - 0.051X_2X_3 + 0.46X_2X_4 + 4.50 * 10^{-4}X_3X_4 - 22.67X_2^2 - 2.06 * 10^{-4}X_3^2 + 3.29 * 10^{-3}X_4^2 - 2.77 * 10^{-4}X_1X_2X_4 + 1.02 * 10^{-5}X_1^2X_3$$

The effects of the immobilization parameters on activity yield are seen in Table 4.2. The most significant linear terms were buffer pH (X_2, *F*-value of 260.30, *p*-value <0.0001) and buffer molarity (X_3, *F*-value of 198.42, *p*-value <0.0001). According to equation 5, the linear terms of buffer, pH and molarity affected activity yield positively, while the linear term of immobilization time had a negative effect. The most significant quadratic effects were those of buffer molarity (X_3^2, *F*-value of 1283.48, *p*-value <0.0001) and pH (X_2^2, *F*-value of 727.38, *p*-value <0.0001).

The interactive effects of buffer pH/buffer molarity (X_2X_3, *F*-value of 263.24, *p*-value <0.0001), protein loading/immobilization time (X_1X_4, *F*-value of 86.23, *p*-value <0.0001), buffer pH/immobilization time (X_2X_4, *F*-value of 71.55, *p*-value <0.0001) and protein loading/buffer pH/immobilization time ($X_1X_2X_4$, *F*-value of 79.81, *p*-value <0.0001) were significant in the activity yield model. Negative effects on activity yield were seen from interactions from buffer pH/buffer molarity and protein

Table 4.1: Factorial experimental design and experimental results of the immobilization of LS onto glyoxyl agarose-IDA-Cu

Protein loading ($\mathbf{X_1}$, mg/g)[a]	Buffer pH ($\mathbf{X_2}$)	Buffer molarity ($\mathbf{X_3}$, mM)	Immob. time ($\mathbf{X_4}$, h)[b]	Retention of activity (%)[c]	Protein Yield (%)[d]	Activity Yield (%)[e]	Rel. T/H ratio (%)[f]
18.75	6.75	512.50	60.00	55.12	41.51	93.98	169.51
28.41	6.01	222.63	49.05	23.90	29.51	27.42	233.95
18.75	6.75	512.50	33.00	75.31	43.00	93.99	325.51
18.75	6.75	1000.00	33.00	49.75	43.49	57.56	97.12
9.09	6.01	802.37	16.95	86.54	28.10	69.75	188.97
9.09	6.01	222.63	16.95	95.78	18.85	40.01	284.64
18.75	6.75	512.50	33.00	83.16	40.84	94.03	233.74
28.41	7.49	802.37	16.95	22.79	45.43	83.70	154.12
18.75	6.75	512.50	6.00	59.43	28.76	85.89	241.78
28.41	7.49	222.63	16.95	19.43	51.94	93.79	178.63
35.00	6.75	512.50	33.00	56.30	46.67	91.17	65.76
28.41	6.01	802.37	49.05	33.75	26.68	56.30	230.95
18.75	6.75	512.50	33.00	62.99	54.75	93.89	177.37
9.09	7.49	222.63	49.05	31.05	52.97	94.75	73.46
2.50	6.75	512.50	33.00	124.62	42.36	92.49	135.91
18.75	6.75	25.00	33.00	28.18	21.28	19.63	228.26
18.75	6.75	512.50	33.00	97.00	37.64	96.06	72.17
9.09	7.49	802.37	49.05	53.68	38.62	89.17	231.84
18.75	8.00	512.50	33.00	55.62	36.22	73.83	276.85
18.75	6.75	512.50	33.00	83.16	49.24	91.36	197.15
18.75	5.50	512.50	33.00	77.96	24.77	30.39	169.51

[a]Protein loading is the amount of protein put into contact with the support .

[b]The time allowed for adsorption onto the support.

[c]Retention of activity was based upon the difference in specific activity of the immobilized enzyme to the specific activity of the native LS, multiplied by 100 and divided by the specific activity of the native LS.

[d]Protein yield was calculated by the amount of protein remaining in the immobilization supernatant in comparison to the total protein used for immobilization, multiplied by 100, divided by the total amount of protein.

[e]Activity yield was calculated on the total enzymatic units remaining in the immobilization supernatant in comparison to the total enzymatic units used for immobilization, multiplied by 100 and divided by the total enzymatic units of the native solution.

[f]Relative ratio of transfructosylating activity versus hydrolytic activity as compared to the native enzyme where transfructosylating activity is expressed μmole/min*mL of glucose released minus the amount of μmole/min*mL free fructose released and the hydrolytic activity was calculated by the μmole/min*mL of free fructose released.

Table 4.2: Analysis of variance (ANOVA) for the immobilization of LS onto glyoxyl agarose-IDA-Cu

	Immobilization Activity Yield (%)[a]		Protein Yield (%)[b]		Retention of Activity (%)[c]		Rel. TF/F ratio (%)[d]	
			F	*p*-value	*F*	*p*-value	*F*	*p*-value
Model	306.79	<0.0001	9.15	0.0018	21.61	<0.0001	16.55	<0.0001
Protein loading (mg/g) (X_1)					109.35	<0.0001	12.75	0.0038
Buffer pH (X_2)	260.30	<0.0001			9.11	0.0129		
Buffer molarity (mM) (X_3)	198.42	<0.0001			13.18	0.0046	25.25	0.0003
Time (h) (X_4)	9.04	0.0198						
X_1X_2	38.57	0.0004					13.46	0.0032
X_1X_3					4.81	0.0531		
X_1X_4	86.23	<0.0001	14.80	0.0012			7.29	0.0193
X_2X_3	263.24	<0.0001			10.35	0.0092		
X_2X_4	71.55	<0.0001						
X_3X_4	9.67	0.0171			14.03	0.0038		
X_1^2					3.56	0.0884	41.14	<0.0001
X_2^2	727.38	<0.0001	3.50	0.0777	3.37	0.0962	3.51	0.0857
X_3^2	1283.43	<0.0001			38.60	<0.0001		
X_4^2	4.81	0.0643			11.10	0.0076	17.29	0.0013
$X_1 X_2 X_4$	79.81	<0.0001						
$X_1^2 X_3$	16.69	0.0047					42.00	<0.0001
Lack of fit	1.70	0.3033	1.44	0.4008	0.86	0.5868	0.92	0.5760

[a]Activity yield was calculated on the total enzymatic units remaining in the immobilization supernatant in comparison to the total enzymatic units used for immobilization, multiplied by 100.

[b]Protein yield was calculated by the amount of protein remaining in the immobilization supernatant in comparison to the total protein used for immobilization, multiplied by 100.

[c]Retention of activity was based upon the specific activity of the immobilized enzyme in comparison to the specific activity of the native enzyme, multiplied by 100.

[d]Relative ratio of transfructosylating activity versus hydrolytic activity as compared to the native enzyme.

loading/pH/immobilization time. The other significant interactions exhibited positive/synergistic effects. Activity yield had the most terms and interactions affecting it. Activity yield was the only response in which the cubic terms, protein loading/pH/immobilization time ($X_1X_2X_4$), were significant. Equation 6 $$Protein\ Yield = 0.16 + 3.89 * 10^{-3}X_1X_4 + 0.77{X_2}^2$$

In the protein yield model, the only significant parameter was the interaction between protein loading/immobilization time (X_1X_4, *F*-value of 14.80, *p*-value of 0.0012). Equation 6 shows that this interaction positively affected protein yield. The response for protein yield was the most simplistic with the least number of parameters shaping its response.

Equation 7 $$Retention\ of Activity = -26.71 - 3.05X_1 + 65.60X_2 - 0.16X_3 - 2.40 * 10^{-3}X_1X_3 + 0.046X_2X_3 + 2.71 * 10^{-3}X_3X_4 + 0.046X_1^2 - 7.31X_2^2 - 1.65 * 10^{-4}X_3^2 - 0.024X_4^2$$

Retention of activity, the most significant response for enzyme immobilization, was significantly affected linearly by protein loading (X_1, *F*-value of 109.35, *p*-value of < 0.0001), followed by buffer molarity (X_3, *F*-value of 13.18, *p*-value of 0.0046). In terms of quadratic effects, buffer molarity (X_3^2, *F*-value of 38.60, *p*-value of <0.0001) and immobilization time (X_4^2, *F*-value of 11.10, *p*-value of 0.0076) had significant effects on the retention of activity. Among all interactive effects, the most important ones were between buffer molarity/time (X_3X_4, *F*-value of 14.03, *p*-value of 0.0038) and buffer pH/buffer molarity (X_2X_3, *F*-value of 10.35, *p*-value of 0.0092). As seen in Equation 7, protein loading and buffer molarity will negatively affect the retention of activity, while the linear term of buffer pH has positive effect. The quadratic effects of buffer molarity and immobilization time negatively affected the retention of activity, while both significant interactions (X_3X_4, X_2X_3) exhibited an additive/positive effect on the retention of activity. The interactions between protein loading and molarity (X_1X_3) had a negative effect. Retention of activity was the only response examined which was affected by the interaction between protein loading and buffer molarity. Similarly, to immobilization activity yield, the largest quadratic variable comes from buffer molarity (X_3^2). The other quadratic variables were either less significant or insignificant.

Equation 8 $$\boldsymbol{Relative\frac{T}{F}ratio = 242.37 - 19.76X_1 + 5.60 * 10^{-4}X_3 + 5.16X_1X_2 + 0.14X_1X_4 - 0.46X_1^2 - 4.05X_2^2 - 0.039X_4^2 + 4.85 * 10^{-6}X_1^2X_3}$$

The T/H ratio was compared to the same ratio as the native as reported by Hill *et al*. [16]. Looking at the results from Table 4.2, the most significant linear parameters for the T/H ratio were buffer

molarity (X_3, *F*-value of 25.25, *p*-value of 0.0003) and protein loading (X_1, *F*-value of 12.75, *p*-value of 0.0038). The significant quadratic effects include protein loading (X_1^2, *F*-value of 41.14, *p*-value of <0.0001) and immobilization time (X_4^2, *F*-value of 17.29, *p*-value of 0.0013). The significant interactive effects came from protein loading/buffer pH (X_1X_2, *F*-value of 13.46, *p*-value of 0.0032) and protein loading/immobilization time (X_1X_4, *F*-value of 7.29, *p*-value of 0.0193). There were cubic interactions resulting from protein loading/buffer molarity ($X_1^2X_3$, *F*-value of 42.00, *p*-value of <0.0001). In terms of linear effects, protein loading negatively affected the relative T/H ratio, while buffer molarity had a positive effect. All quadratic effects were negative while the interactive and cubic effects had positive effects on the activity ratio. Relative T/H ratio was more strongly affected by the cubic variable ($X_1^2X_3$) than immobilization activity yield, the only other response which was altered by this variable.

4.3.2. Investigation of the interactive effects of immobilization parameters

The 2D contour plots presented in Figure 4.1 illustrate the interactive effects of immobilization buffer pH/protein loading, time/protein loading, buffer molarity/pH, time/buffer molarity and lastly immobilization time/buffer pH on the predicted immobilization activity yield. Looking at Figure. $1a_1$ where buffer molarity and time were kept at the center points of 512.50 mM and 33 h (0, 0), pH was found to have a more significant effect on the immobilization activity yield than the protein loading. Immobilization activity yield increased as the pH increased, until a maximum was reached, and then decreased. As pH gradually increases, it surpasses the pKa for histidine (pKa 6), making it more available for chelation. Above pH 7.5, activity yield decreased. At this pH, the enzyme is negatively ionized, and it may have adsorbed onto the support via ionic interactions than by chelation. In addition, higher protein loadings at pH above 7.5 may have favored a cooperative effect with the proteins already adsorbed onto the support resulting in a high activity yield. When the buffer molarity was increased to 802.37 mM (Figure $4.1a_2$), a hyperbolic trend was seen, with a maximum activity yield reached at the protein loading of 20 mg/g and pH of 6.75. At high buffer molarity, optimum activity yield was achieved when the pH was appropriate to favor chelation; while the promoting effect of high protein loading on the cooperative binding was less significant than at low buffer molarity.

At lower buffer molarity, 222.63 mM (Figure $4.1a_3$), as pH increased, the activity yield increased. No significant effect was seen from protein loading until it reached 26 mg/g, then a steep increase

was seen as pH increased. Buffer molarity altered the contour plot in Figure 4.1b$_1$ more strongly than immobilization time when protein loading and pH were at their centre points (18.75 mg/g, 6.75). Activity yield increased as buffer molarity did until approximately 611 mM, afterwards it decreased. At high protein loading (28.41 mg/g) (Figure 4.1b$_2$), the effect of immobilization time increased, with activity yield decreasing as the immobilization time increased. With a larger amount of protein adsorbed onto the support, increased time may afford the opportunity for the enzyme to desorb from the support. Close to optimal buffer molarity, the strength of the buffer, which encourages adsorption, prevents the protein from desorbing from the support over time, decreasing the effect of immobilization time. At low protein loading (9.09 mg/g) (Figure 4.1b$_3$), the effect of immobilization time increased, with activity yield increasing as immobilization time increases. Increased immobilization time is required for low amounts of protein to adsorb onto the support since there are not inter-protein interactions to encourage adsorption.

Hyperbolic behavior was seen in Figure 4.1c when protein loading was kept at 18.75 mg/g and time was maintained at 33 h. A maximal activity yield was found at pH 7.2 and buffer molarity of 520 mM. The contour plot altered only slightly when protein loading or time shifted. As pH is increased, the capability of histidine residues to chelate with the support increases (Deschamps, Kulkarni, Gautam-Basak, & Sarkar, 2005). As buffer molarity is increased, hydrophobic interactions are encouraged, increasing activity yield (Bolivar, Mateo, et al., 2009). If the buffer concentration is too high, the ions may prevent the enzyme from interacting with the support or they may favour hydrophobic interactions with the enzyme itself, resulting in aggregation. At mid-pH and mid-buffer molarity, the pH is appropriate to promote chelation while the buffer molarity isn't too high to prevent interactions. Figure 1d shows the contour plot of buffer pH and time at protein loading (18.75 mg/g) and mid-buffer molarity (512.50 mM) at the centre points (0, 0). Buffer pH has a stronger effect on the activity yield than the immobilization time. Where pH was at an optimum, immobilization time had a more significant effect. This could be due to the immobilization occurring through chelation, with increased time, more enzyme can interact with the support.

In Figure 4.1e where pH was maintained at 6.75 and buffer molarity was 512.50 mM (0, 0), both protein loading and immobilization time affected the activity yield similarly, providing a saddle-shaped contour plot. At immobilization times shorter than 45 h, the activity yield increased as the

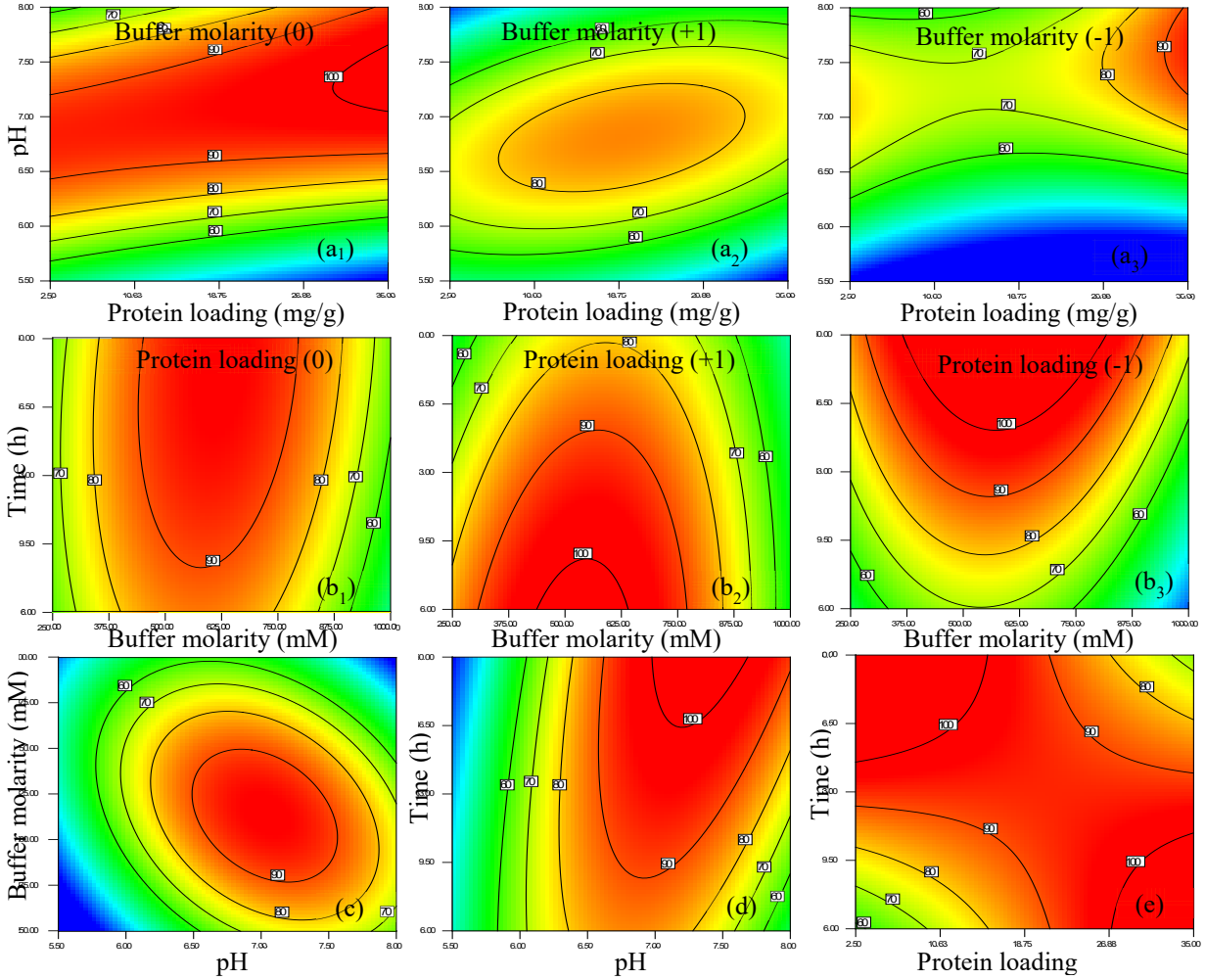


Figure 4.1: Contour plots of immobilization activity yield for the immobilization of LS onto glyoxyl agarose-IDA-Cu: as a function of protein loading/buffer pH (a) with a constant buffer molarity and immobilization time of 512.50 mM and 33 h (a1); 802.37 mM and 33 h h (a_2); 222.63 mM and 33 h (a_3); buffer molarity/immobilization time (b) with a constant protein loading and buffer pH of 18.75 mg/g and 6.01 (b_1); 28.41 mg/g and 6.01 (b_2); 9.09 mg/g and 6.01 (b_3); pH/buffer molarity (c); pH/immobilization time (d) and protein loading/immobilization time (e). Immobilization activity yield: 14.33% 96.06%.

protein loading was increased; the opposite was seen after 45 h. Similarly, at a protein loading lower than 23 mg/g, the immobilization activity yield increased as the immobilization time was increased; while the opposite trend was observed when the protein loading was above 23 mg/g. With increased immobilization time, more protein is expected to immobilize onto the support from the supernatant up until the point of saturation. The observed negative effect of immobilization time at a high protein loading may be attributed to the high desorption of proteins from weak protein/support interactions and/or to the aggregation of proteins due to the high protein/protein interaction. At low protein loading and short immobilization time, there are few effects to encourage the enzyme adsorption onto the support.

Figure 4.2 shows the contour plots of retention of activity and the relative T/F ratio. Figure 4.2a_1 indicates that the protein loading significantly affected the retention of activity more than the buffer molarity when pH (6.75) and immobilization time (33 h) were maintained at the centre points (0, 0). At high pH (7.5) (Figure 4.2a_2), the effect of buffer molarity is slightly increased while at low pH (6.0), the effect of buffer molarity was decreased slightly (Figure 4.2a_3). Similar trend was seen at high and low immobilization time (data not shown). High retention of activity at low protein loading can be explained by the low number of layers of enzyme on the support, limiting the substrate diffusional limitations. As protein loading increases, the enzyme layers will build up, with activity increasing sub-linearly (J. Garcia et al., 2011). On the other hand, buffer molarity had less of an effect on the retention of activity because the adsorption may have dominantly taken place through chelation.

The contour plot represented in Figure 4.2b_1 shows a hyperbolic curve with pH and buffer molarity having similar effect on the retention of activity, when the protein loading was 18.75 mg/g and the immobilisation time was set at 33 h. As pH increased, the retention of activity decreased until a buffer molarity optimum of approximately of 610 mM, then the retention of activity increases as pH increases. A similar trend was seen for buffer molarity, with retention of activity decreasing as buffer molarity increased until a pH maximum (~6.75) was reached, then retention of activity increased as buffer molarity increased. It was observed that mid-range buffer molarity and pH values resulted in a maximum retention of activity, revealing that the chelation favored the active conformation of LS upon immobilization. At the extreme of either buffer molarity or pH, the retention of activity significantly decreases. While at a high buffer molarity and low pH, there are two effects that may have limited the high retention of LS activity. Indeed, a high concentration of

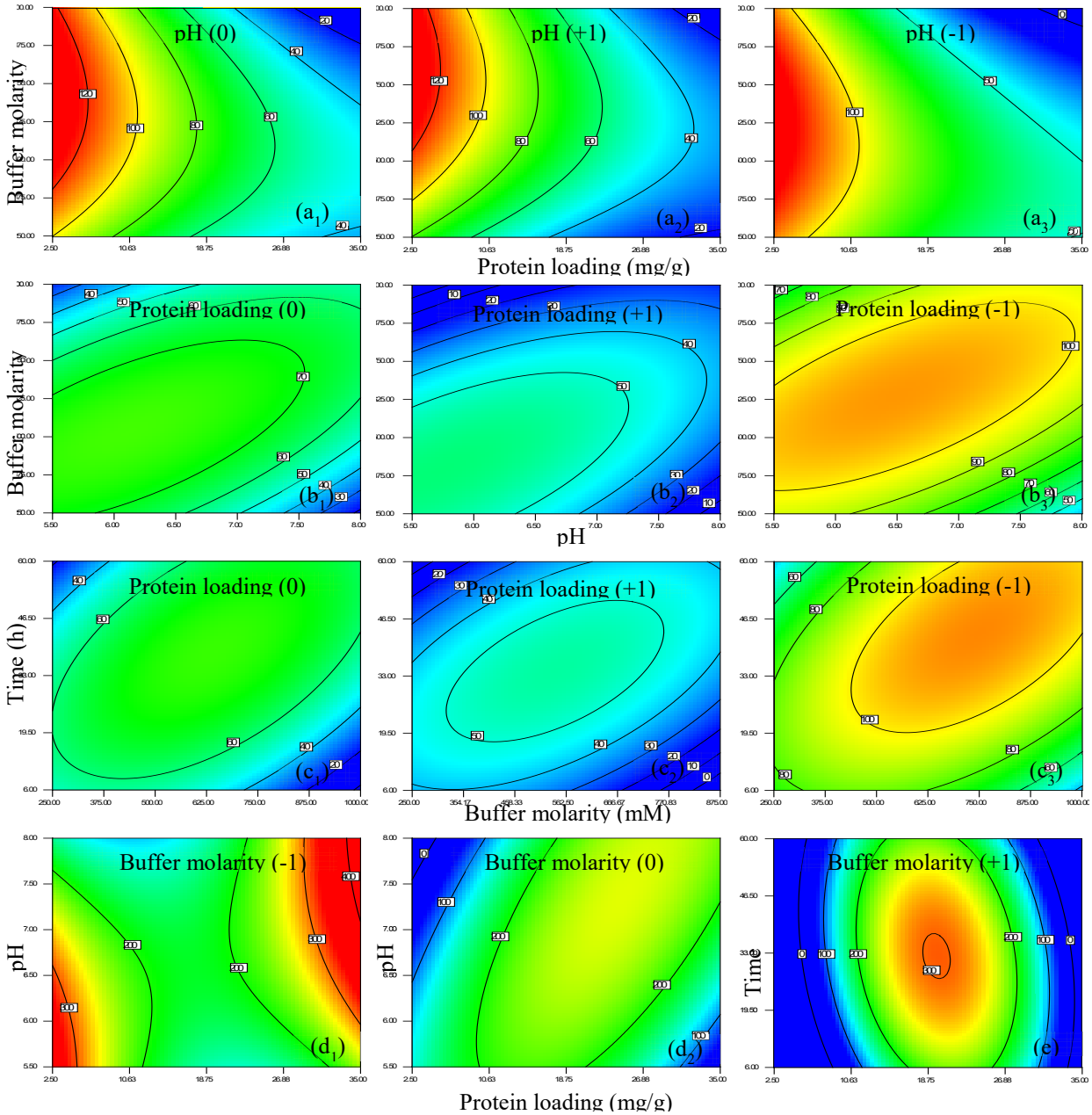


Figure 6.2. Contour plots of retention of activity as a function of protein loading and buffer molarity at an immobilization time of 33 h and pH of 6.75 (a1); pH of 7.49 (a2) and pH of 6.01 (a3). The contour plots of pH and buffer molarity when immobilization time was 33 h and protein loading was 18.75 mg/g (b_1); 28.41 mg/g (b_2); and 35 mg/g (b_3). The contour plot of immobilization time and buffer molarity on retention of activity when buffer pH was 6.75 and protein loading was 18.75 mg/g (c_1); 28.41 mg/g (c_2); and 35 mg/g (c_3). The colours inside the contour plot indicate the predicted values under different reaction conditions, 19.43% 124.65%. The relative T/H contour plot in relation to protein loading and pH when immobilization time was 33 h and buffer molarity was 222.63 mM (d_1) and 512.50 mM (d_2). The relative T/H contour plot of protein loading and immobilization time when pH was 6.75 and buffer molarity was 802.37 mM (e). The colours inside the contour plot indicate the predicted values under different reaction conditions, 65.76% 325.51**%.**

ions may have favored the immobilization to proceed via the hydrophobic interactions, rather than through site-specific orientation; this may have resulted in a large amount of unspecific immobilization, not necessarily retaining high enzymatic activity (Wheatley & Schmidt Jr, 1999). At low pH, a greater proportion of the secondary amines on the histidine residues become ionized and lose chelating potential (Casella & Gullotti, 1983). This will diminish the supports' ability to provide site-specific immobilization through chelation. At low protein loading (Figure 4.2b_3), a similar contour plot was generated but the optimum was higher, while at high protein loading (Figure 2b_2), the optimum was lower but the contour plot is steeper (data not shown). At low protein loading, there will be less crowding amongst the enzyme when it adsorbs, causing less steric hindrance and solely the formation of a monolayer upon adsorption. A similar relationship was seen in the immobilization of LS from *Z. mobilis* onto hydroxyapaptite, where the support reached saturation at 20 U/g of support (Jang et al., 2000b).

Figure 4.2c_1 shows a hyperbolic model with protein loading and buffer pH maintained at the center points (18.75 mg/g, 6.75). The range demonstrated by this plot shows an optimum retention of activity at short immobilization time/low buffer molarity to long immobilization time/high buffer molarity. At low buffer molarity, there are few ionic interferences to prevent LS from chelating with the support, therefore retention of activity can be maintained high even at short immobilization time (Bolivar, Mateo, et al., 2009). While when buffer molarity was high, higher immobilization time would be required for the enzyme to reorient itself on the support. When protein loading is high, the optimum is lower and there is a smaller range (Figure 4.2c_2). When the protein loading is kept low, the effect is the same but with a higher optimum retention of activity (Figure 4.2c_3).

Figure 2d_1 shows the contour plot of T/H ratio at the low buffer molarity (222.63 mM) and mid-immobilization time (33.0 h). Protein loading is seen to have a stronger effect on the T/H ratio than the buffer pH. The plot shows a saddle-like shape with maximums at both low protein loading/low buffer pH and high protein loading/high buffer pH. At high pH the histidines' are capable of chelating with the LS, resulting in a highly concentrated and well-oriented LS. Low protein loading at acidic pH also resulted in higher T/H ratio. At low buffer pH, less of the acidic LS residues should be ionized, contributing to a more hydrophobic environment. At the buffer molarity and immobilization time centre points (Figure 4.2d_2), the contour plot of the T/F ratio by buffer pH and protein loading is hyperbolic. The T/H ratio increased as the protein loading was increased,

until the maximum was achieved (~18.75 mg/g). This effect becomes more pronounce at high buffer molarity (data not shown). As protein loading increased, aggregates may have formed, reducing the availability of the LS' active site and hence its transfructosylating ability. Higher buffer molarity may have strengthened the effect of protein loading, promoting the formation of hydrophobic interactions (Wheatley & Schmidt Jr, 1999).

The relationship between immobilization time and protein loading on the relative T/H ratio was also hyperbolic (Figure 4.2e) when the immobilization pH was set at the mid-point (6.75) and buffer molarity was at the +1 point (802.37 mM). Protein loading was found to have a more profound effect on the T/H ratio than immobilization time. This result reveals that the mid-long immobilization time may have provided the enzyme the opportunity to reorient itself on the support for optimal conformation. The optimum was shifted from lower to higher protein loading when the pH ranged from the minimum to the maximum (results not shown). It has been reported that at low pH and at high ionic strength conditions, free LS from *Z. mobilis* would form ordered microfibrils, which preferentially produced levan with no hydrolysis observed. If LS from *B. amyloliquefaciens* behaves like the LS from *Z. mobilis*, it may immobilize in the fibril form, with sites already available to form interactions with levan (Goldman et al., 2008). When pH and buffer molarity were kept at the centre points (6.75, 512.5 mM), the relationship between protein loading and immobilization time on T/H was still hyperbolic and the optimum remained close to mid-high protein loading and the low to mid-long immobilization time.

4.3.3. Model validation and optimum immobilization conditions

Using the predictive models, the optimum parameters for the immobilization of LS with the highest activity yield, retention of activity and protein immobilization yield were determined. It predicted activity yield of 95.41% and protein yield of 49.13% and retention of activity of 95.19% when immobilization was performed with 9.09 mg LS/g support, for 49 h, at pH 6.8 and buffer concentration of 607.85 mM. The validation of the model was done by carrying out triplicate experiments under the predicted optimum conditions. The experimental value for activity yield was 83.85 ± 0.50%, protein yield was 41.35 ± 1.83% and the retention of activity was 112.15 ± 3.2%. The results correlated well with the predicted results, within a 95% confidence level, when using a 49 h immobilization time. Shorter immobilization times of 14 and 30 h were also examined, and they resulted in similar activity yields, 89.00 ± 3.52% and 92.99 ± 0.91%, and protein yields,

37.01 ± 0.09% and 41.80 ± 1.91%, while the retention of activity was significantly lower, 46.61 ± 1.93% and 40.33 ± 8.65%, respectively. Similarly, Zhou et al. in 2013 used a 2-level-10-factorial central composite RSM design for the optimization of the immobilization of β-glucosidase onto chitosan beads by cross-linking-adsorption-cross-linking method (Zhou, Pan, Wu, Tang, & Wang, 2013). These authors found that adsorption time and enzyme loading were the most significant factors contributing to retention of activity. Through their optimization, they increased retention of activity from 33% to 51%.

4.3.4. Stabilization of immobilised LS by reduction and cross-linking

To better stabilize LS immobilized on glyoxyl agarose-based supports, the reduction of Schiff bases to secondary amines is a required step for the bond to become irreversible. The reduction step with sodium borohydride caused a loss in retention of activity by 85.67 ± 6.38%. This could be due to the active site of LS containing two essential aspartate residues and a glutamate residue (Meng & Futterer, 2003). To prevent this loss of activity, attempts were made to occupy the active site of LS by substrates and/or products. It was hypothesized that this would block sodium borohydride from entering the active site and turning the carboxyl groups on the essential amino acids into aldehydes. LSs' natural substrate, sucrose, a product analog, FOSs (Orafti P95), and low molecular weight levan (686 Da) were evaluated as protecting agents as shown in Figure 4.3. All protecting agents, sucrose, FOSs and levan were found to significantly prevent a loss in activity as compared to reduction without the protecting agents. The thermal stability of the preparations were performed to asses the success of the reduction. There was more thermal stability with reduction without protection, with sucrose and with FOSs. Reduction without a protecting agent provided better thermal stability as compared to reduction with FOSs. The immobilized LS protected by FOSs had more activity compared to the preparation without a protecting agent, after reduction; though this preparation did not provide adequate thermal stability, indicating that reduction was inefficient. After 2 hours, reduction with levan had significantly more activity. Levan was found to moderately protect LS while the immobilized enzyme maintained high thermal stability. The protecting effects of levan may be explained by the polymerization reaction is the most dominant reaction-catalyzed by LS from *B. amyloliquefaciens (Tian et al., 2011; Tian & Karboune, 2012)*. By performing a processive reaction, *B. amyloliquefaciens* LS retains the growing fructan rather than releasing it after each fructosyl transfer (Homann et al., 2007). This happens because the

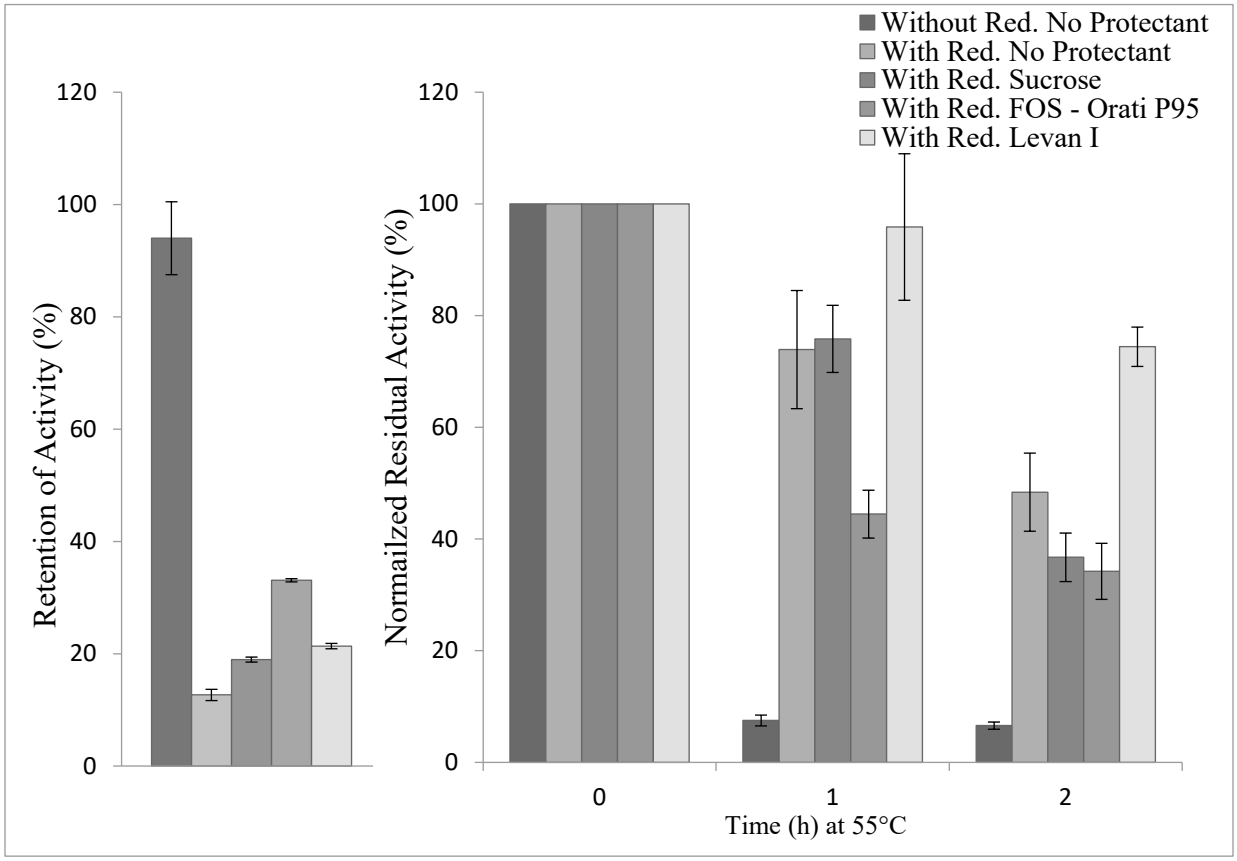


Figure 4.3: Immobilized LS protected by protecting agents. The retention of activity of immobilized LS on glyoxyl agarose-IDA/Cu after reduction with NaBH4 in the presence of protecting agents (A). Thermal stability of LS immobilized onto glyoxyl agarose-IDA/Cu with $NaBH_4$ in the presence of protecting agents (A). Thermal stability of LS immobilized onto glyoxyl agarose-IDA/Cu with reduction taking place in the presence of protecting agents (B).

subsites of this LS exhibit high affinity towards levan, which remained positioned in the +2 and +3 LS subsites (Goldman et al., 2008). On the other hand, the smaller protecting agents, such as sucrose and FOSs, do not have enough fructosyl units to interact efficiently with LS subsites and to provide a high protecting effect.

Cross-linking was sought as an alternative to stabilizing the LS on the support instead of reduction step. Two concentrations of glutaraldehyde (0.1, 0.5% v/v) were used to cross-link the immobilized LS on the glyoxyl agarose-IDA/Cu (Figure 4.4). 0.1% glutaraldehyde resulted in a higher retention of LS activity, it did not provide the expected thermal stability as upon reduction. While 0.5% glutaraldehyde led to similar thermal stability and a large loss in initial activity as compared to the reduction by sodium borohydride. PEI is an ionisable polymer, which will be cationically charged at pH 6, enabling the formation of stabilizing interactions between PEI and the immobilized enzyme (Vieira et al., 2011). Concentrations from 0.1% -2% (v/v) PEI were incubated with immobilized LS on glyoxyl agarose-IDA/Cu for 15 h, with activity checked after 1 h, 5 h and 15 h. A thermal stability assay was completed to determine whether the PEI incubation was effective at stabilizing the LS in place of forming permanent covalent bonds.

A loss in activity was only seen after 15 h of incubation with 2% PEI, the other concentrations did not significantly differ in retained activity from one another. After 2 h of heating at 50°C, the immobilized LS with 0.1% PEI (incubated for 15 h) retained the most activity. The thermal stability kinetic of LS immobilized onto glyoxyl agarose-IDA/Cu incubated with 0.1% PEI was measured along with free LS as shown in Figure 4.5. The immobilized LS had a stabilization factor of 4.7 times that of the native enzyme. Overall, the LS immobilized with glyoxyl agarose-IDA/Cu/PEI retained 70.91 ± 9.06% activity, with a protein yield of 44.73% and an activity yield of 54.69%.

4.1. Conclusion

In this work, the optimized immobilization of LS onto glyoxyl agarose-IDA/Cu was performed by successfully using response surface methodology. The understanding of the interactive effects of immobilization parameters allows their better modulation. Although permanent covalent immobilization completed through reduction with sodium borohydride provided the enzyme with high thermal stability, sodium borohydride was found to negatively affect LS activity. Protection of the enzyme from damaging effects using protecting agents proved fruitless. Alternatively, the

ionic polymer PEI (0.1% v/v) proved capable of stabilizing the LS onto the support when incubation was performed for 15 h. The immobilized LS retained a high amount of activity while achieving good thermal stability in comparison to the native enzyme.

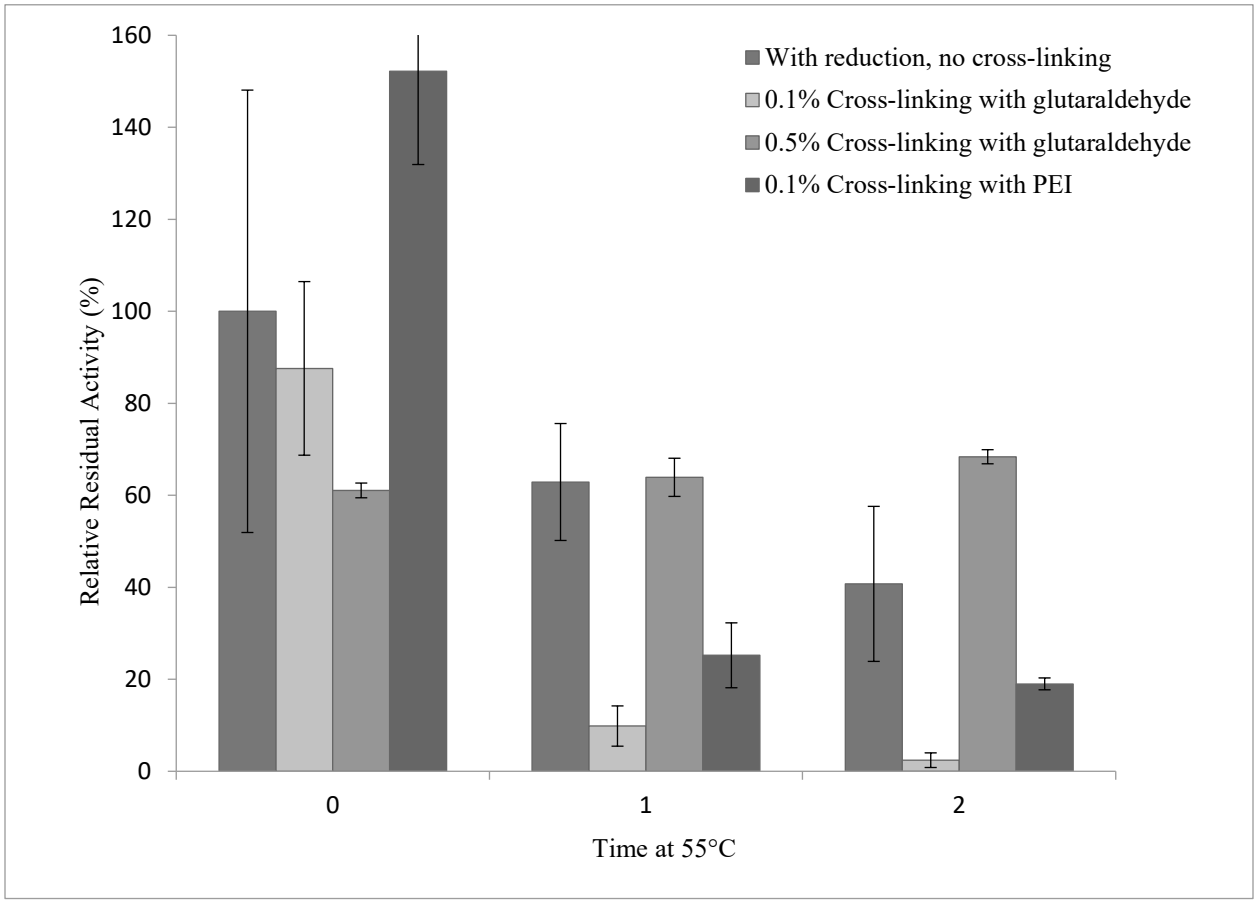


Figure 4.4: Thermal stability of immobilized LS on glyoxyl agarose-IDA/Cu with reduction with NaBH4; cross-linking with glutaraldehyde (0.1%) in place of reduction with NaBH4; cross-linking with glutaraldehyde (0.5%) in place of reduction with NaBH4; cross-linking with glutaraldehyde (0.5%) in place of reduction with $NaBH_4$; cross-linking with PEI (0.1%)

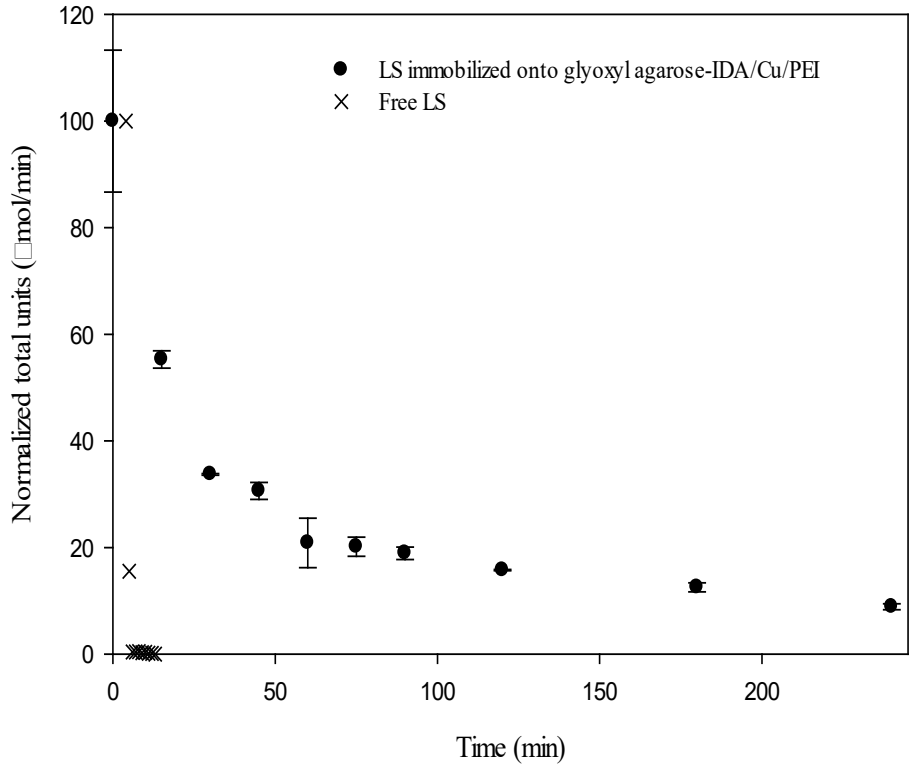


Figure 4.5: Thermal stability of LS immobilization onto glyoxyl agarose-IDA/Cu/PEI (0.1% PEI) as compared to the native free LS at 50°C.

Connecting Statement 3

Chapter IV completed our investigation into the immobilization of LS. The information generated from chapters III and IV provided immobilization strategies which can be applied to other LS enzymes. Depending upon the reason for immobilization, an increase in transfructosylation or an increase in stability, either glyoxyl agarose-IDA/Cu/PEI or Sepabeads® HA should be chosen as the basis for LS immobilization. Varying catalytic activity was seen from LSs produced by different bacterial species. To expand upon the efficient strategies of synthesizing β(2→6) FOSs and levan, the search for new, undiscovered catalysts was performed. Chapter V focuses on the exploration of new potential LS enzymes based upon their similarity to known LSs. A study of their activity, thermal stability and kinetic parameters was explored.

This work was first presented as an oral presentation and a digital poster presentation at the IFT Annual Meeting & Food Expo-Institute of Food Technologist. The intend manuscript will be submitted to the Journal of Food Chemistry

Hill, A., Chen, L., Sooyoun, S., de Berardinis, V., Mariage, A., Petit, J. L., Debard, A., Pellouin, V. & Karboune, S. (2016) Discovering new LS enzymes through high-throughput screening with genome mining for the efficient production of fructooligosaccharide prebiotics. IFT16 Annual Meeting & Food Expo, Chicago, USA, July 16 – July 19.

Hill, A., de Berardinis, Petit, J. L. & Karboune, S. (2018) Discovering new levansucrase enzymes through high-throughput screening with genome mining with interesting properties and improved catalytic activity. *To be submitted*.

5. Abstract

Levansucrases (EC 2. 4. 1. 10, LS) are of high interest for the synthesis of novel prebiotic fructooligosaccharides. Hindered by the limited availability of LS enzymes, and a propensity for performing the hydrolysis of sucrose, rather than a transfructosylation reaction, a desire was fueled to find new LSs. Genome mining was employed to explore their biodiversity using 26 characterized LSs as a reference set for a sequence driven-approach leading to a collection of 32 enzymes representative of the biodiversity for which the gene was cloned and over-expressed in *E. coli*. These enzymes underwent an initial screening process based upon total activity, transfructosylation activity and levan forming ability narrowed the candidates to 10 potential enzymes. These LS enzymes were found to have high levan production 643±68 mg levan/mg protein and able to produce very large polymers (6,986 kDa). The LS from *ParaParaburkholderia graminis* had a very high natural thermal stability with a half-life of 291 minutes at 50°C.The full kinetic parameters of the top candidates were characterized for the enzymes with the most potential. Enzymes with higher catalytic efficiency and activity for transfructosylation over hydrolysis were identified. The acceptor specificity of these new levansucrase enzymes was briefly explored. It showed wide specificity for each of the selected enzymes.

5.1. Introduction

The catalytic potential of levansucrase enzymes (EC 2. 4. 1. 10, LS), known for the synthesis of prebiotic fructooligosaccharides (FOSs), is increasingly appreciated with a growing need to easily synthesize novel products that promote the intestinal health. As a beta-2,6-fructosyltranferase, it can catalyse the formation of prebiotic FOSs and/or the polysaccharide levan by transferring the fructosyl residue from a non-activated donor molecule, such as sucrose to an acceptor molecule (Martinez-Fleites et al., 2005; Tian & Karboune, 2012). The acceptor molecule of the fructosyl residue dictates the reaction catalyzed by LS. Hydrolysis occurs when the fructosyl residue is transferred to a water molecule, resulting in the release of glucose and fructose, while the transfructosylation mechanism can be characterized as either producing FOSs or levan1. LS effectivity is hindered by its capability to hydrolyze sucrose. Indeed, high rates of hydrolysis or a preference for the hydrolysis reaction can hinder the usefulness of the enzyme, causing largely the production of undesirable monosaccharides instead of desirable FOSs and levan. The catalytic rates of hydrolysis and transfructosylation differ depending on the microbial source of the LS. The

catalytic efficiency rates for transfructosylation can vary significantly; SacB from Bacillus amyloliquefaciens (Uniprot ID: P21130) has rates of 2.47 s-1mM-1 and 9.5 s-1mM-1 2respectively, while SacB from Geobacillus steareothermophilis (Uniprot ID: P94468) has rates if 0.197 s-1mM-1 and 0.0919 s-1mM-1 (Inthanavong, Tian, Khodadadi, & Karboune, 2013).

Interestingly, the levan producing properties of each LS varies upon its microbial source, with the quantity of levan produced and its size and branching of the levan varying to a wide degree (Jang et al., 2006). Levan has many alternative uses in the food and pharmaceutical industry, for example as an additive for bio-edible films, as a flavour carrier and a fiber additive (Han, 1990). Alternatively, the levan can be hydrolysed by acidic conditions or specifically by inulinases or levanases to create larger prebiotic FOSs (Marx, Winkler, & Hartmeier, 2000; Tian, Karboune, & Hill, 2014). In spite of having similar active-site conformations, LSs from selected sources exhibit different oligomerization (FOSs) vs polymerization (levans) ratio. For instance, LS from Bacillus megaterium (Strube et al., 2011) and Bacillus subtilis (Ortiz-Soto, Rivera, Rudiño-Piñera, Olvera, & López-Munguía, 2008) were found to catalyze dominantly the synthesis of levan, whereas those from G. diazotrophicus (Martinez-Fleites et al., 2005) and Zymomonas mobilis (Bekers et al., 2002) synthesize mainly FOSs. Some structural elements of LSs that govern the oligomerization/polymerization ratio have been identified.

Also affecting the application of the enzyme is its thermal stability. Higher thermal stability provides greater industrial practicality of the enzyme to produce prebiotics. Natural thermal stability variability occurs amongst LS enzymes. Thermally stable enzymes usually derive from microbes found in warm areas, such as the LS from a Bacillus sp. found in Tunisian thermal source, which has a half-life of 1 hour at 90°C (Belghith, Dahech, Belghith, & Mejdoub, 2012). In comparison, SacB from B. amyloliquefaciens has a half-life of 16 minutes when heated at 50°C (Hill, Karboune, & Mateo, 2015).

Within the currently identified LS enzymes, each have their own specific characteristics, which may or may not be suitable to a desired process. It is therefore of high interest to discover new LS enzymes with varying properties. With the sequence of tens of thousands of bacterial genomes now available (Ziemert, Alanjary, & Weber, 2016), it is possible to explore this data for naturally occurring undiscovered LSs. There are three main techniques for discovering new biocatalysts from genomic libraries: homology-driven genome mining; substrate-induced gene expression and

activity-based analysis according to Ferrer et al. (2005). The conserved sequences found in family 68 (Meng & Futterer, 2003; Pons, Naumoff, Martínez-Fleites, & Hernández, 2004; Strube et al., 2011), make homology-driven genome mining an interesting approach. The overall objective of this study was to locate new enzymes with LS activity from a homology-driven genome mining approach. This was accomplished first through the creation of a reference set of recognized LS, a basic local alignment search tool (BLAST) using said reference set, cloning and expression of the potential new genes, an initial screening of their activities and an extensive analysis of the activities and kinetics of the top LS candidates.

5.2. Materials and Methods

5.2.1. Materials

Sucrose, D-(-)-Fructose, D-(+)-glucose, D-(+)-galactose, α-lactose, D-(+)-maltose, D-(+)-raffinose, 3,5-dinitrosalicylic acid (DNS), potassium sodium tartrate ($KNaC_4H_4O_6$), Dextran standards (50 to 670 kDa), lysoszyme from chicken egg white, Pefabloc® SC were obtained from Sigma Chemical Co. (St. Louis, MO). K_2HPO_4, KH_2PO4, NaCl, NaOH, tryptone, Bovine Serum Albumin, β-D-isothiogalactopyranoside and yeast extract were obtained from Fisher Scientific (Fair Lawn, NJ). Bradford reagent concentrate was provided by Bio-Rad (Missasauga, ON). Ampicillin was supplied by Wisent. Terrific broth was purchased by BioBasic and Lysonase was purchased by Merck. *Escherichia coli* BL21(DE3) plysE strains were supplied by Invitrogen.

5.2.2. Reference Set of LS

A review of literature for LS's enzymes corresponding to experimental data and an evaluation of the CAZY database produced the protein sequence of 24 LS and 2 inulosucrase enzymes (Lombard, 2014). The complete reference set is listed in Table 5.1.

Table 5.1: Known levansucrases used as reference set for the sequence driven analysis

Uniprot id	Characterization	Genus	Species
W8IRX0	Levansucrase	*Bacillus*	*amyloliquefaciens*
P21130	Levansucrase	*Bacillus*	*amyloliquefaciens*
H6UZK4	Levansucrase	*Bacillus*	*licheniformis*
D5DC38	Levansucrase	*Bacillus*	*megaterium*
D5E1N6	Levansucrase	*Bacillus*	*megaterium*
K2HQ21	Levansucrase	*Bacillus*	*amyloliquefaciens*
Q8GDF0	Levansucrase	*Bacillus*	*subtilis*
P05655	Levansucrase	*Bacillus*	*subtilis*
K7Q788	Levansucrase	*Burkholderia*	*pseudomallei*
Q62DJ0	Levansucrase	*Burkholderia*	*mallei*
J7JEC6	Levansucrase	*Burkholderia*	*cepacia*
A5CNK2	Putative levansucrase	*Clavibacter*	*michiganensis*
Q97I81	Levansucrase	*Clostridium*	*acetobutylicum*
Q97I79	Levansucrase	*Clostridium*	*acetobutylicum*
D4IGH9	Levansucrase	*Erwinia*	*amylovora*
P94468	Inactive levansucrase	*Geobacillus*	*stearothermophilus*
A9H664	Levansucrase	*Gluconacetobacter*	*diazotrophicus*
Q9LBX1	Levansucrase	*Komagataeibacter*	*xylinus*
D3WYW0	Levansucrase	*Lactobacillus*	*gasseri*
U5F0V5	LPXTG-domain-containing	*Lactobacillus*	*jensenii*
Q8GGV4	Levansucrase	*Lactobacillus*	*reuteri*
Q70XJ9	Levansucrase	*Lactobacillus*	*sanfranciscensis*
G2KV82	Levansucrase	*Lactobacillus*	*sanfranciscensis*
Q03WB9	Uncharacterized	*Leuconostoc*	*mesenteroides*
Q5IS34	Levansucrase	*Leuconostoc*	*mesenteroides*
Q8VW87	Beta-fructofuranosidase	*Microbacterium*	*saccharophilum*
Q9Z5E5	Levansucrase	*Paenibacillus*	*polymyxa*
Q93FU9	Levansucrase	*Pseudomonas*	*chlororaphis*
E2XQB6	Levansucrase	*Pseudomonas*	*fluorescens*
I4K143	Levansucrase	*Pseudomonas*	*fluorescens*
O68609	Levansucrase	*Pseudomonas*	*syringae*
O52408	Levansucrase	*Pseudomonas*	*syringae*
Q88BN6	Levansucrase	*Pseudomonas*	*syringae*
O54435	Levansucrase	*Rahnella*	*aquatilis*
M2M145	Levansucrase	*Streptococcus*	*mutans*
F8LMW0	Levansucrase	*Streptococcus*	*salivarius*
F8DT26	Levansucrase	*Zymomonas*	*mobilis*
P0DJA3	Levansucrase	*Zymomonas*	*mobilis*
Q55242	Levansucrase	*Streptococcus*	*salivarius*

5.2.3. Collection of LS from biodiversity

Sequence driven approach (Vergne-Vaxelaire et al., 2013) have been applied using LS experimentally described in Table 5.1. From 601 proteins from UniProtKB database, the clustering at 80% of identity allowed us to select 50 enzymes for which strains were available in the Genoscope prokaryote strain collection. Primers were chosen for the corresponding genes. Genes were cloned in a pET22b(+) (Novagen) modified for ligation independent cloning as already described (Vergne-Vaxelaire et al. 2013). All primers and strains are listed in Table S1. All the strains along with their identifiers were purchased from DSMZ collection. Each expression plasmid was transformed into *E. coli* BL21-CodonPlus (DE3)-RIPL. Cell culture, induction of protein production and cell lysis were conducted as previously published (Bastard et al., 2014) in 96-well microplates. Enzymes were purified in 96 microwells using HisLink™ 96 Protein Purification System (Promega). The elution buffer was 50 mM TRIS 50mM (pH 8), 50 mM NaCl, 250 mM imidazole and 10 % glycerol. Samples were analyzed by SDS-PAGE using the NuPAGE system (Invitrogen).

5.2.4. Sequence analysis

Multiple sequence alignment with known LSs (Table 5.1) and 4 known inulosucrases was done using website Clustal Omega online website (https://www.ebi.ac.uk/Tools/msa/clustalo/) and the Percent Identity Matrix was created by Clustal2.1. Gram coloration is indicated for each organism. Known inulosucrases: Inu (UniProt ID: Q8GP32) from *Lactobacillus reuteri* (van Hijum, van Geel-Schutten, Rahaoui, van der Maarel, & Dijkhuizen, 2002), InuJ (UniProt ID: Q74K42) from *Lactobacillus johnsonii* (Pijning et al., 2011), InuGB (UniProt: D3WYV9) from *Lactobacillus gasseri* (Díez-Municio et al., 2013) and IslA (UniProt: Q7X481) from *Leuconostoc citreum* (Olivares-Illana, López-Munguía, & Olvera, 2003) are written in orange.

5.2.5. Initial screening using micro-plates

5.2.5.1.LS initial activity screening (modified for microplates)

Total LS specific activity was quantified as the total amount of reducing sugars produced per minute per mg of protein. Purified LS samples (4 - 180 µg protein/mL) were incubated with sucrose solution (0.45 µM) in 50 mM potassium phosphate buffer (50 mM, pH 6) at 30°C for 20 minutes. 3,5-dinitrosalicyclic acid (1%, w/v) in a sodium hydroxide solution (1.6% w/v) was then added to each well to yield a ratio of 1:1.5 v/v total reaction mixture; then the plate was sealed and heated at 80°C for 5 minutes using a thermocycle. Afterwards an aqueous potassium sodium tartrate

solution (50% w/v, 1:5, v/v total reaction mixture) was added to stabilize the colour. The plates were read at 540 nm in a UV-Vis spectrophotometer plate reader. The amount of glucose was measured through using D-glucose HK assay kit by Megazyme (Ireland). The plates were read using a UV-Vis spectrophotometer plate reader.

5.2.5.2.Levan forming activity.

The levan forming reaction was initiated by adding purified LS samples (2 µL) to a sucrose solution (0.45 µM, 48 µL) in 50 mM potassium phosphate buffer (50 mM, pH 6). The reaction mixtures were incubated at 37°C. OD_{600} measurements were taken at initially and at 1 h, 3.5 h, 6 h, 21.5 h, 24 h and 29.5 h. The system was first blanked against buffer and sucrose solution (0.45 µM). The reaction mixtures were performed in triplicate. Linear regression calculations were performed to determine the increase in optical density per hour.

5.2.6. Production, recovery and purification of potential LS candidates

The *E. coli* cells, BL21(DE3) (Invitrogen), transformed with the potential LS genes, stored on LB agar plates, containing carbenicillin (100 µg/mL) were precultured with LB containing ampicillin (100 µg/mL) for 24 hours at 37°C at 250 rpm. Terrific broth, with ampicillin (100 µg/mL) was inoculated with preculture (2%) then incubated at 37°C at 250 rpm in an orbital shaker (New Brunswick Scientific Excella E24 Incubator Shaker Series). Once growth achieved an optical density of 1.2 at 600 nm (DU 800 UV/Visible Spectrophotometer, Beckman), the culture enzyme expression was induced using IPTG (1 mM). Growth of the culture was continued at room temperature for 18 hours at 250 rpm. The cells were then collected by centrifugation at 8000 rpm and then stored at -70°C.

The pellet was thawed on ice for 40 minutes and resuspended in the sonication buffer (50 mM Pipes, 300 mM NaCl, 10% Glycerol, pH 7.2; 4 mL v/w). Lysozyme (4 mg/g pellet) and DNase (2000 U/mL) were added; and the mixture was left to incubate on ice, for 30 minutes at 40 rpm. The samples were sonicated with microtip (Misonix Ultrasonic Liquid Processor S-4000) for 1 minute (10 seconds on, 60 seconds off, amplitude of 15) in a salt-ice bath. The resulting sample was centrifuged at 16000 g at 4°C for 45 minutes. The supernatant was retained and dialyzed against potassium phosphate buffer (5 mM, 12 L, pH 6.0) and lyophilized.

The LS candidates, cloned with either a His tag on the N-terminal or the C-terminal, were purified using affinity chromatography on HisTrap FF column (1 ml, GE Health-care). The lyophilized enzyme powder was solubilized in sonication buffer (500 µL), filtered and loaded onto the column. The column was washed subsequently with sonication buffer (15 mL), wash buffer (50 mM Pipes, 300 mM NaCl, 10 % glycerol, pH 6.4, 15 mL), 5 mM imidazole prepared in wash buffer (15 mL) and 10 mM imidazole prepared in wash buffer (15 mL). The enzyme was eluted using imidazole solutions ranging from 100-200 mM (3 mL) in wash buffer as the eluent. Each fraction was subjected to total LS activity and Bradford assays (Bradford, 1976), and analysed by SDS-PAGE. SDS-PAGE was performed by loading 20 µg of protein of each sample along with Bio-Rad SDS-PAGE, Low Range Standards (14400-97400 Da) to verify the purification of each sample (He, 2011). The LS fractions were pooled and dialyzed against potassium phosphate buffer (5 mM, 12 L, pH 6.0).

5.2.7. Reaction selectivity (transfructosylation vs hydrolysis) of selected LSs

Purified LS was added to sucrose substrate solution (final concentration of 0.9 M) in potassium phosphate buffer (50 mM, pH 6.0), and the mixtures were incubated for 30 minutes at 30°C. Afterwards the reactions were stopped by boiling the samples for 5 minutes. The protein and levan were precipitated through the addition of methanol (1:1, v/v). Glucose, fructose and sucrose were quantified by high-pressure anion-exchange chromatography (HPAEC) using a Dionex ICS-3000 system equipped with a pulsed amperometric detector (PAD) and a CarboPac PA20 column (3 x 150 nm). The components of reaction mixtures were eluted with an isocratic mobile phase made of 20 mM sodium hydroxide at a flow-rate 0.4 mL/min and 32°C. All assays were run in triplicate. LS activity was defined as the amount (µmol) of reducing sugars (glucose and fructose) produced per mg of LS from sucrose per minute. LS hydrolytic activity is defined as the amount of fructose (µmol) produced per minute per mg of LS. While transfructosylating activity is defined as the amount (µmol) glucose liberated per minute per mg of LS from sucrose resulting from the transferring of fructose to an acceptor molecule. This is determined by subtracting the free fructose from the amount of liberated glucose (Tian, Inthanavong, & Karboune, 2011).

5.2.8. Levan production and characterization

To produce levan, the reaction mixtures composed of sucrose substrate solution (0.9 M) and purified LS in potassium phosphate buffer (50 mM, pH 6.0) were incubated at 4°C over a period of two weeks. This was performed in triplicate. The OD_{600} of the resulting mixtures were then

measured. The quantity of levan produced was estimated using a calibration curve created using known concentrations of levan (0.018 g/mL – 0.575 g/mL). The size of the levan was determined by high-pressure size-exclusion chromatography (HPSEC) using a Waters HPLC system equipped with 1525 binary pump, refractometer 2489 detector and Breeze™ 2 software. The components of reaction mixtures were eluted with an isocratic elution of 200 mM NaCl, at a flow rate of 0.5 mL/min and resolved with (7.8 mm x 30 cm), TSKgel G3000PWXL-CP and TSKgel G5000PWXL-CP, aligned in sequence. Carbohydrate size calibration curve was constructed using dextran standards.

5.2.9. LS thermal stability assays

Purified LS (in triplicate) in potassium phosphate buffer (50 mM, pH 6.0), was incubated at 50°C for selected times (15 min to 2 hrs). The initial activity and final total LS activity were measured by DNS assay. The half-life of selected LS enzymes was estimated using second-order decay kinetics.

5.2.10. Determining LS kinetic parameters

The total, hydrolytic and transfructosylation activities of pure LS were measured at a substrate sucrose concentration ranging from 0.002 – 2.4 M as described above. Lineweaver-Burk and Michaelis-Menten plots ($1/V = 1/V_{max} + (K_m/V_{max}) \times 1/[S]$) enabled the apparent Michaelis-Menten constant (K_{mapp}) and maximum velocity ($V_{max\ app}$) for LSs to be estimated by using Sigma Plot software (Systat Software, version 12.3). Hill plots ($1/V = 1/V_{max} + (K_m^n/V_{max}) \times 1/[S]^n$) and substrate inhibitor (uncompetitive) plots ($1/V = (1+[I]/K_i)/V_{max} + (K_m/V_{max}) \times 1/[S]$) enabled the estimation of the Hill coefficient (n_H) and the inhibition constant (K_i).

5.2.11. Substrate specificity and TLC analysis

Substrate specificity of selected levansucrases was assessed following the method described by Tian et al. (2011). Purified enzymes (5-7 units/mL) were incubated with sucrose (0.9 M) and an acceptor molecule (0.45 M; galactose, glucose, maltose, lactose and raffinose) for 50 hours. The enzymes were also incubated solely with sucrose and raffinose (0.9 M). The reactions were stopped by boiling for 5 minutes. The reaction mixtures were analyzed by thin-layer chromatography (TLC) on Silica-gel 60 plates. Aliquots of 2 µL of the reaction mixtures were spotted on the Silicagel 60 plates, along with standards of all acceptor sugars (0.45 M) and donor

molecules (0.9 M) and inulin (380 g/L). They were developed using a solvent mixture of butanol/acetic acid/deionized water (5:4:1, v/v/v). After drying, the plates were sprayed with a sulfuric acid (2 % v/v) solution in methanol and then heated at 100°C for 2 hours.

5.3. Results and Discussion

5.3.1. Data-mining microbial genomes for the discovery of new LSs

In the attempts to discover new LS enzymes, a LS collection was built by a sequence driven approach (Vergne-Vaxelaire et al., 2013) using known LSs as reference. A literature survey inventoried 26 characterized LSs (Table 5.1). A preliminary analysis of this inventory showed by a multiple sequence alignment (SI Fig. S5.1) that they can be divided in two distinct groups sharing very low sequence identity (<30%). Group G1 contains LS from Gram-negative organisms such as LsdA and Lsc from *Gluconacetobacter* or *Erwinia* species, respectively, whereas group G2 contains Gram-positive bacteria as SacB from *Bacillus* species. Generally, it is assumed that LSs from Gram-positive bacteria produce high molecular weight polymers and those from Gram-negative bacteria produce FOSs (Wuerges et al., 2015). It should be noticed that only LSs from G2 show a good homology with known inulosucrases (SI Fig. S5.1). Notably, Bff and M1ft from *Microbacterium saccharophilum* and *Leuconostoc mesenteroides*, respectively, two Gram-positive organisms, that produce FOSs (Wuerges et al., 2015), are found in group G1. Nevertheless, best hits by sequence comparison of M1ft against UniprotKB are LS from *Pseudomonas* species with more than 99% of identity suggesting horizontal gene transfer between these Gram-negative organisms and *L. mesenteroides*.

Sequence comparison with UniprotKB using the reference set brought back 601 sequences, which were clusterized into putative iso-functional groups (more than 80% of identity of protein sequence inside a group), resulting in 50 selected enzymes representative of the biodiversity as far as possible based on the availability of the corresponding genomic DNA in the Genoscope strain library. Among them, 76% present less than 70% of identity with at least one LS of the reference set showing the diversity of repatriated proteins from databases. Corresponding genes were then cloned in an expression vector. Finally, 45 potential LS genes were cloned and 39 were successfully over-expressed in *E. coli*. Notably, few proteins were selected from Archaea domain for which no previous LSs have been described and specifically from Halobacteria species. Most of the 6 cloned Halobacteria genes, encode for proteins that exhibited between 35% and 39% of

identity with known LSs and especially with Mf1t from *L. mesenteroides* and LscA from *P. chlororaphis.* Cell free extracts have been prepared and enzyme purified.

5.3.2. LS Screening

The desired catalytic properties of robust LS candidates appropriate for industrial applications would include high total activity, high transfructosylating activity with abundant levan and/or FOS production coupled with high thermal stability. In the initial screening, the total LS and levan-forming activities of the LS candidates were determined for the 32 LSs that include the 8 LS identical or highly similar (>90% of identity) to the reference set (Table 5.1). The evaluation of known LSs in tandem with the identified LS candidates allows the discovery of novel capabilities. Out of them, 19 LSs exhibited a reasonably-high level of specific activity towards the release of reducing sugars. Positive results indicate the high release of reducing sugars from sucrose resulting from either the hydrolysis reaction (glucose and fructose) or the transfructosylation reaction (glucose). Furthermore, the glucose forming activity was used an indicator to assess the rate of the transfructosylation versus that of the hydrolysis reaction. Indeed, the high amount of released glucose compared to the total monosaccharides can reveal the higher transfructosylating activity than the hydrolytic one. Examples of high specific glucose forming activity coupled with high production of reducing sugars include LSs from *B. megaterium* (LS19), *Rahnella aquatilis* (LS4), *Erwinia tasmaniensis* (LS5), *Vibrio natriegens* (LS6) and *Z. mobilis* (LS20) as seen in Table 5.2. Nevertheless, the high levan production was a unique feature of the prospective LS candidates. Each LS has a unique product profile, with some being capable of producing levan and others producing mainly FOSs or both. The screening results reveal that the most efficient LS candidates in terms of levan forming activities, are LS4 from *R. aquatilis* and LS19 from *B. megaterium* but also two new LSs, LS21 from *Sporolactobacillus laevolacticus* and LS32 from *Oenococcus kitaharae* that share, respectively, only 8% and 33% of identity with the SacB from *B. subtilis*. High thermal stability of *R. aquatilis* LS, discussed in the literature, was a possible contributor to its high levan production (Kang et al., 2004), by preventing denaturation and permitting the enzyme to remain active longer at 37°C. Concurrently, this *R. aquatilis* LS was also reported to prefer levan forming activity and in general, transfructosylation over hydrolysis, with this behavior decreasing as temperature increased (Ohtsuka et al., 1992). These LSs, LS19, LS21 and LS32, except one are Gram-positive organisms and these results are not surprising since it was observed (Caputi et al., 2013; Crittenden & Doelle, 1994; Euzenat, Guibert, & Combes, 1997; Hernandez et al., 1995; Lukasz K. Ozimek, Kralj, Kaper, van der Maarel, & Dijkhuizen, 2006; Park et al., 2003)

Table 5.2*:* Screening results of potential LS enzymes

LS identity	Entry	Mass (Kda)	Genome	Total Activity[a]	Glucose Forming Activity[b]	Levan Forming Activity[c]	Best Hit with known LSs
LS1	A0A2S3U487	27,0	*Lactobacillus plantarum*	–	+	+	~ 30% Q55242
LS2	K9DHJ1	39,3	*Sphingobium yanoikuyae*	+	+	+	38% E2XQB6
LS3	Q2G754	41.5	*Novosphingobium. aromaticivorans*	++	+++	+	-
LS4	O54435*	45,9	*Rahnella aquatilis*	++++	+++	+++	100% O54435
LS5	B2VCC3	46,4	*Erwinia tasmaniensis*	++++	+++	+	90% A0A0M3KKU6
LS6	A0A1B1EI54	46,8	*Vibrio natriegens*	++	+++	+	76% Q8VW87
LS7	G8PYZ4	47,0	*Pseudomonas fluorescens*	–	+	+	93% Q5IS34
LS8	B1G3X6	47,4	*Paraburkholderia graminis*	+	++	+	79% A0A0H2WDV2
LS9	A0A0F7A902	47,7	*Pseudomonas syringae pv. syringae*	++++	++	+	97% O68609
LS10	Q5FSK0	47,9	*Gluconobacter oxydans*	++	++	+	69% Q9LBX1
LS11	Q5V249	49,7	*Haloarcula marismortui*	–	+	+	38% Q5IS34
LS12	B9LT89	49,9	*Halorubrum lacusprofundi*	–	–	+	39% Q97I81
LS13	M0E014	50,9	*Halorubrum saccharovorum*	+	+	+	42%
LS14	L9X0M4	51,4	*Natronococcus amylolyticus*	+	+	+	38% Q5IS34
LS15	D5UCP7	52,1	*Cellulomonas flavigena*	–	–	+	47% E2XQB6
LS16	A0A1Y0XLP2	52,9	*Bacillus amyloliquefaciens*	+	+	+	97% P21130
LS17	D5E2J1	53,7	*Bacillus megaterium*	+	+	+	99% D5DC38
LS18	Q65EI8	53,7	*Bacillus licheniformis*	+	++	+	79% P05655
LS19	D5DC07	53,9	*Bacillus megaterium*	++	+++	++	82% D5DC38
LS20	F8DT26*	54,1	*Zymomonas mobilis subsp. mobilis str.*	–	+++	+	100% F8DT26
LS21	V6IV04	54,1	*Sporolactobacillus laevolacticus*	–	–	++	68% P05655
LS22	B2JVY2	57,3	*Burkholderia phymatum*	+	+	+	84% A0A0H2WDV2
LS23	V6J0B5	57,9	*Sporolactobacillus laevolacticus*	++	++	+	67% P05655
LS24	B8HBC9	58.0	*Arthrobacter chlorophenolicus*	+	++	+	-
LS25	B2IF78	58,0	*Beijerinckia indica subsp. Indica*	++	++	+	72 % A0A0H2WDV2
LS26	Q9EVD6	68,3	*Actinomyces naeslundii*	–	–	+	55% Q8VW87
LS27	C2E5J0	86,3	*Lactobacillus johnsonii*	–	+	+	61% D3WYW0
LS28	A0A0K2JK54	93,7	*Lactobacillus plantarum 16*	–	–	+	30% Q70XJ9
LS29	J7TH23	104,2	*Streptococcus salivarius K12*	+	+	+	98% F8LMW0
LS30	Q03WB8	111,5	*Leuconostoc mesenteroids subsp. Mesenteroids*	–	+	+	70% Q03WB9
LS31	Q1L7R6	113,4	*Leuconostoc mesenteroids*	+	+	+	83% Q03WB9
LS32	G9WIM3	129,7	*Oenococcus kitaharae*	++	++	++	33% P05655

Low/negative results were omitted. [a]Specific activity (µmol of reducing sugars released/mg of enzyme*min): - 0 to 100, + >100 to 1000, ++ > 1000 to 10 000, +++ > 10 000 to 20 000, ++++ > 20 000. [b]Specific activity (µmol of glucose released/mg of enzyme*min): - 0 to 100, + >100 to 1000, ++ > 1000 to 10 000, +++ > 10 000 to 20 000, ++++ > 20 000. [C]Increase in OD600/hour: - 0, + > 0 to 0.01, ++ > 0.01 to 0.05, +++ > 0.05 to 0.10.

that LS enzymes from Gram-positive bacteria tend to form levan, while LSs from Gram-negative bacteria produce more FOSs. *R. aquatilis*, a gram-negative bacterium, was an exception to this. In addition, to our knowledge, this is the first time that archaea organisms were found to express LS as LS12 from *Halorubrum saccharovorum* or LS13 from *Natronococcus amylolyticus* (Table 5.2). It should be noticed that our temperature conditions of reaction are probably suboptimal for archaea LS, underestimating their actual catalytic efficiency. From the top producers of levan, we then selected 10 diverse LSs from both groups including the one from the Archea *N. amylolyticus* for further study.

5.3.3. Reaction Selectivity (transfructosylation over hydrolysis) of the 10 top LS Candidates

Potential 10 LS candidates were selected based on their total, glucose forming and levan forming activities as well as their novelty. These identified candidates were assessed for their reaction selectivity by determining the hydrolytic and the transfructosylation activities. Indeed, LS can catalyze both the transfructosylation reaction, that consists of transferring a fructosyl group to an acceptor molecule and the hydrolysis reaction, that is regarded as the transfer of the fructosyl group to water (Ozimek, Kralj, van der Maarel, & Dijkhuizen, 2006). The hydrolysis reaction may compete with the transfer reaction under certain conditions and limit FOSs/levan synthesis (Homann, Biedendieck, Goetze, Jahn, & Seibel, 2007; Ortiz-Soto et al., 2017). A high transfructosylation versus hydrolysis (T/H) ratio indicates a LS with high reaction selectivity potential. Structural elements of LS that govern its reaction selectivity have been identified by sequence alignment and mutagenesis (Martinez-Fleites et al., 2005; Wuerges et al., 2015). The mechanistic studies reported so far on the reaction selectivity have been carried out for only a few LSs from *B. megaterium*, *Microbacterium laevaniformans, Z. mobilis, Erwinia amylovora, B. subtilis*, *Aerobacter levanicum, Lactobacillus panis and L. reuteri 121* (Beine et al., 2008; Hestrin, Feingold, & Avigad, 1955; Kim, Park, Sung, & Cha, 2005; Mena-Arizmendi et al., 2011; Meng & Futterer, 2003; Ortiz-Soto et al., 2017; Ozimek et al., 2006; Senthilkumar, Busby, & Gunasekaran, 2003; Strube et al., 2011; Waldherr, Meissner, & Vogel, 2008).

In our preliminary study, it has been found a difference in the activities of selected LSs with N-terminal His-tags and C-terminal His-tags (data not shown), maybe due to the predicted signal peptide. In this regard, these LSs were also cloned with a C-terminal His-tags and both constructions were expressed, purified and studied. The differences seen in activity between

enzymes with His-tags on either the N-terminal or C-terminal has been reported for other enzymes (Booth et al., 2018; Dickson, Lee, Shepherd, & Buchanan, 2013). In literature, the extent and whether an effect is observed of the His tag, either at the C-terminal or N-terminal varies on a case-by-case basis (Dickson et al., 2013). In this case, it was prudent to study both when a difference was observed.

The transfructosylation/hydrolytic (T/H) activity ratios are listed in Table 5.3. The LSs with a high reaction selectivity towards transfructosylation are characterized by T/H ratio higher than 1. The results show that LSs from *V. natriegens*, followed by *S. salivarius*, *G. oxydans*, *B. indica* and *N. aromaticivorans* favored the transfructosylation reaction over the hydrolytic one under the investigated conditions. The T/H ratio values are in good agreement with the obtained glucose-forming activities (Table 5.2), revealing the efficiency of the initial screening. It has been described that this ratio can be affected by sucrose concentration (Oseguera, Guereca, & Lopez Munguia, 1996), substrate choice (Tian & Karboune, 2012), reaction temperature (Visnapuu, Maee, & Alamaee, 2008), reaction pH (Inthanavong et al., 2013). Therefore, altering anyone of these parameters can change the ratio. The reaction conditions set for the measurement of the T/H ratio was set at the average conditions (pH, temperature, sucrose concentration) reported in the literature. Indeed, at lower sucrose concentrations, hydrolytic activity typically predominates with a preference for transfructosylation occurs as the amount of sucrose is increased (Goldman et al., 2008). The sensitivity to the increase in T/H ratio varies by species. At low sucrose concentrations (3 mM) the LS from *Bacillus circulans* for which no protein sequence have been determined performs mostly hydrolytic reactions (Oseguera et al., 1996), while other enzymes such as SacB from *B. subtilis* and LsdA from *G. diazotrophicus* did not catalyse any transfructosylation at sucrose concentrations below 50 mM (Chambert & Petit-Glatron, 1991; Hernandez et al., 1995). Temperature plays a similar role, with a propensity for transfructosylation at lower temperatures and increased hydrolysis as temperature increases, with the effect of temperature varying depending upon the enzyme. SacB from *Z. mobilis* (Jang et al., 2001) and Lsc from *Pseudomonas syringae pv. phaseolicola* (Hettwer, Gross, & Rudolph, 1995) were more sensitive to temperature, increasing in hydrolytic activity between 30-40°C, while SacB from *B. subtilis* was less sensitive to temperature, increasing in hydrolysis only at 60°C (Chambert & Petit-Glatron, 1993). Indeed, an increase in temperature will increase the energy transferred to the enzyme, increasing in

Table 5.3: Ratio of transfructosylating activity versus hydrolytic activity of potential LS enzymes using sucrose as the substrate

LS ID (His tag in N or C-term)	Organism	Avg. T/H ratio
LS6-N	*Vibrio natriegens*	1.550
LS6-C	*Vibrio natriegens*	0.830
LS29-N	*Streptococcus salivarius*	1.435
LS10-N	*Gluconobacter oxydans*	1.330
LS10-C	*Gluconobacter oxydans*	1.035
LS25-N	*Beijerinckis indica subsp. indica*	1.207
LS3-N	*Novosphingobium aromaticivorans*	1.134
LS3-C	*Novosphingobium aromaticivorans*	0.864
LS8-N	*Paraburkholderia graminis*	0.722
LS30-N	*Leuconostoc mesenteroides subsp. mesenteroides*	0.610
LS24-N	*Arthrobacter chlorophenolicus*	0.201
LS2-N	*Sphingobium yanoikuyae*	0.121
LS14-N	*Natronococcus amylolyticus*	0.096

T/H ratio was estimated by dividing the transfructosylation activity over the hydrolytic activity

vibrations; this may make it more difficult for a larger acceptor molecule to enter the active site when compared to the smaller and ubiquitous water.

5.3.4. Levan production efficiency of the potential LS candidates

The efficiency of Levan production by the identified potential LS candidates and the respective size of levan are shown in Fig. 5.1. The produced levan ranged in size from 586 to 6984 kDa, with production efficiency ranging from 0.07 mg levan/mg protein to 643 mg levan/mg protein. Although there is high conservation of LSs' catalytic core, there is a large variability of the products produced by the different LSs (Ziemert et al., 2016). There have only been few mutagenesis experiments to elucidate the differences in catalysis (Beine et al., 2008; Homann et al., 2007; Meng & Fütterer, 2008; Meng & Futterer, 2003; Olvera, Centeno-Leija, Ruiz-Leyva, & Lopez-Munguia, 2012; Ortiz-Soto et al., 2017; Senthilkumar et al., 2003; Strube et al., 2011). LS product specificity varies according to whether the enzyme follows a processive mechanism, producing mainly levan, or a non-processive/disproportionate mechanism, releasing mainly FOSs (Ozimek et al., 2006). These mechanisms involve additional subsites (subsites +2, +3) beyond subsites -1 and +1, where the glucosyl and frucosyl residue from sucrose reside, and postulate that LSs exhibiting high affinity toward the growing polymers at subsites +2, +3 will mainly result in levan (Ozimek et al., 2006). As already mentioned, there is the tendency of LS enzymes from Gram-positive bacteria to catalyze via a processive reaction, producing predominately levan (Ozimek et al., 2006; Strube et al., 2011). Surprisingly, LS candidates did not always follow the trend of LS that follows Gram staining as already postulated for previous known LS. The LS, which produced the largest levan was from the Gram-negative *G. oxydans* (6984 kDa) that shares 70% of identity with LsxA from *Komagataeibacter xylinus* for which no levan size product was determined in the literature and 60% with SacB from *Z. mobilis* known to produce high molecular weight level (>10^6 Da) (Jang et al., 2001). The second largest was from *B. indica subsp. indica* (2128 kDa). The enzyme producing the smallest polysaccharide (586 kDa) was LS6 from the Gram-negative *V. natriegens* that presents 70% of identity with Bff from the Gram-positive *M. saccharophilum* (formerly known as *Arthrobacter* sp. K-1) (Takashi, Tamaki, Yokoi, Miyazaki, & Ichikawa, 2012). The low production of levan as seen in Fig. 5.1 is consistent with the low T/H ratio as seen in Table 5.3. However, the production of smaller levan in terms of size with lower yields indicates a poorly enzyme affinity towards transfructosylation but does not necessarily

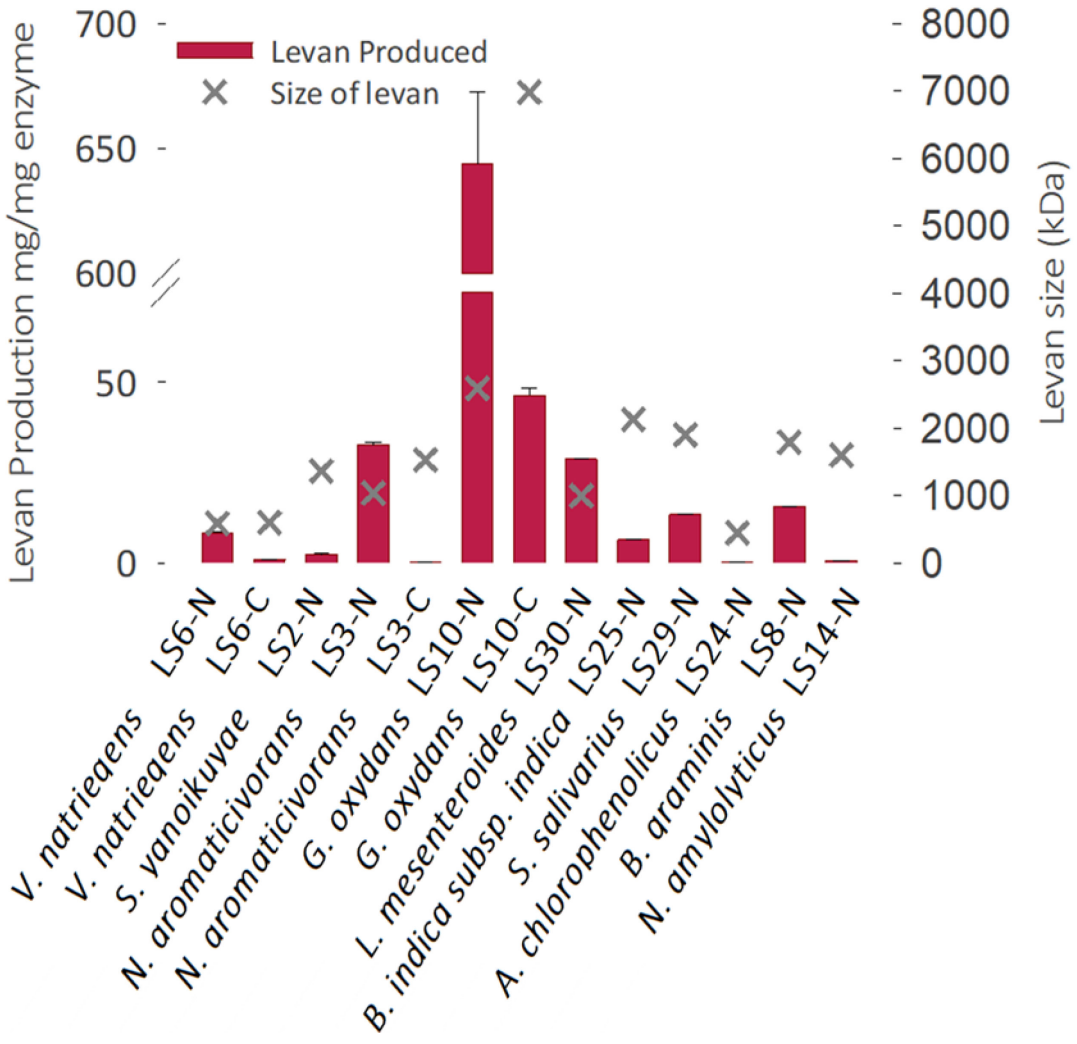


Figure 5.1: Levan production and size from top candidate LSs after 2 weeks' incubation with 0.8 M sucrose. Levan production was monitored through OD measurements. LS genes were cloned with a his tag in N-terminal (noted LSx-N) and C-terminal (noted LSx-C).

dictate whether the enzyme performed a more processive or disproportionate reaction. For instance, LS14 from *N. amylolyticus* exhibited a very low T/H ratio of 0.096, preferring hydrolysis over transfructosylation, and led to a low yield of small levan size. The relatively large levan (1381 kDa) produced at low yields indicates a processive reaction. LS6 from *V. natriegens,* which had a mid-level production yield of levan of a shorter size, was an example of an enzyme, which had less affinity for levan, possibly performing a disproportionate reaction. LS6 with the His-tag located at the N-terminal (LS6-N) showed a high transfructosylation over hydrolysis ratio of 1.55, while at the C-terminal form (LS6-C) had a ratio of 0.830. LS6-N produced more levan than LS6-C with the activity of LS6-C directed towards hydrolysis instead of transfructosylation. Alternatively, LS10 from *G. oxydans* led to a high production of high molecular weight levan, strongly indicating interactions between the growing polysaccharide and stabilizing regions on the enzyme. Similarly, Lsc from *P. syringae pv. phaseolicola* (100 kDa – 1000 kDa), SacB from *Z. mobilis* (600 kDa), SacB from *B. megaterium* (2711 kDa) and LS from *B. licheniformis* (612 kDa) were reported to produce high molecular weight levans (Hettwer et al., 1995; Homann et al., 2007; Jang et al., 2001; Nakapong, Pichyangkura, Ito, Iizuka, & Pongsawasdi, 2013). The levan produced by LS10*,* at 6986 kDa, is at a comparably large size to those levans. Several residues have been identified as crucial for product specificity (Wuerges et al., 2015). Even if the overall sequence of these strains is low, conserved residues can be identified by multiple sequence alignment (Fig. 5.2 and Fig. 5.3). All Gram-negative LS contain phenylalanine instead of a tyrosine at position 429 on *B. subtilis.* This residue, part of loop 9 (Wuerges et al., 2015), was found to be important for polymerization, forming stacking interactions between the sugar and the benzene ring (Meng & Futterer, 2003). Of the top LS candidates, only three LS (LS23, LS29, LS30) contain a tyrosine at this position (Fig. 5.2). Another residue, Arg360 on *B. subtilis*, involved in product formation and the regulation of product length, was replaced by a histidine residue in Gram-negative LS from our study (Fig. 5.3) as in Lsc from *E. amylovora* (Wuerges et al., 2015). This residue is part of the +1 subsite, which through mutational experiments, was found to alter product length, with enzymes deriving from Gram-negative bacteria containing a histidine at this position and Gram-positive bacteria containing an arginine (Meng & Fütterer, 2008). The authors showed that high polymerase activity is observed only in the presence of the R360. The LS from *E. amylovora* produces predominately FOSs instead of levan, Wuerges et al. (2015) stipulated that this was the result of a histidine residue residing at position 305 instead of R360 and changes to the loop

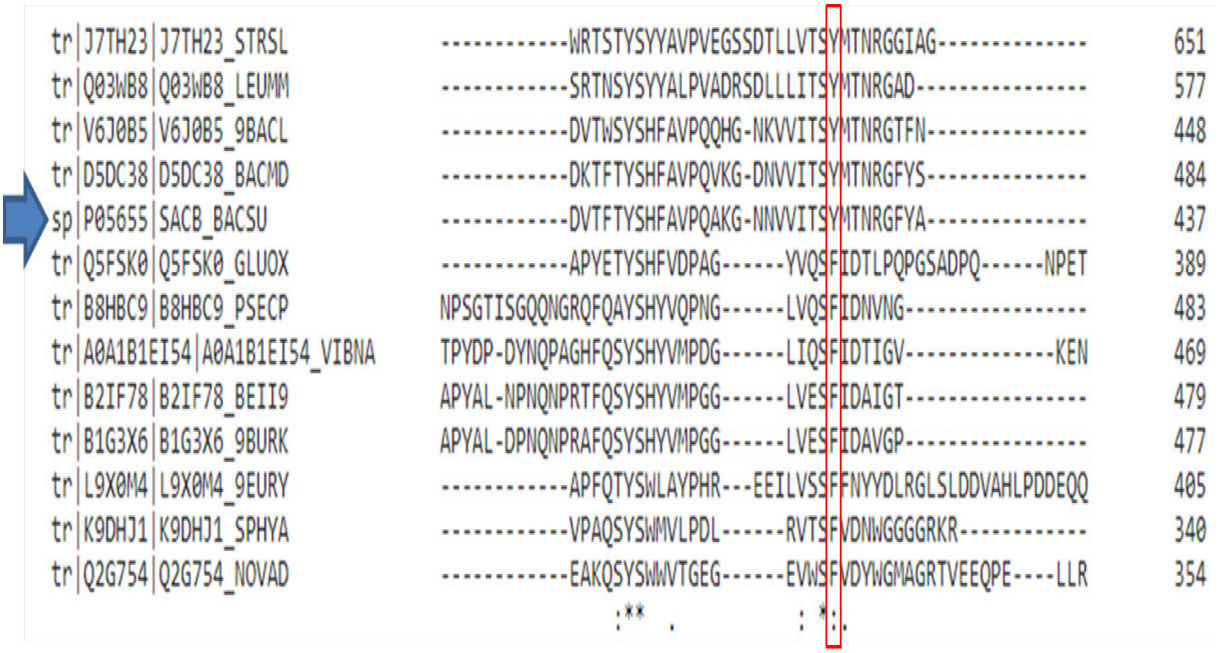


Figure 5.2 Multiple sequence alignment of the selected LS with SacB from *Bacillus subtilis***.** The red box shows the position 439 in SacB (Uniprot ID: P05655) involved in polymerization, forming stacking interactions between the sugar and the benzene ring. SacB is indicated by a blue arrow.

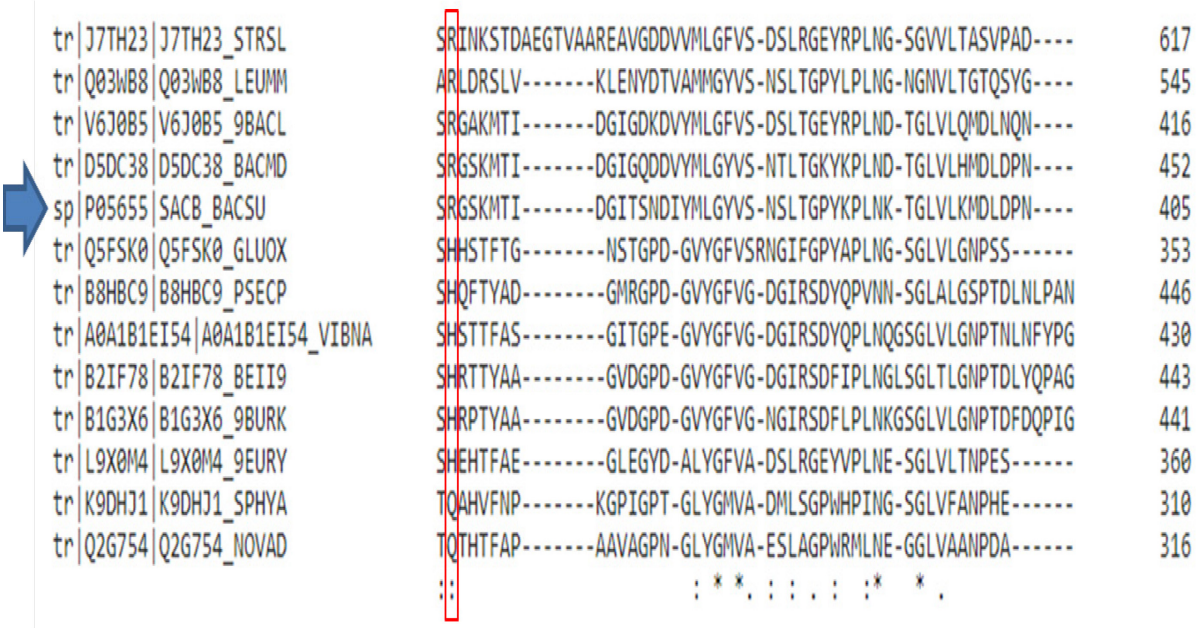


Figure 5.3 Multiple sequence alignment of top selected LS with SacB from *Bacillus subtilis*. The red box shows the position R360 in SacB (Uniprot ID:) involved in polymerization, forming stacking interactions between the sugar and the benzene ring. SacB is indicated by a blue arrow

structures (Wuerges et al., 2015). We have to note that LS2 from *Sphingobium yanoikuyae* and LS3 from *Novosphingobium. aromaticivorans* have a long chain polar residue (glutamine) at this position (Fig. 5.3). Nevertheless, LS10 from G. oxydans, the best producing levan in this study,present both a phenylalanine instead of Tyr439 and a histidine in R360. Alternative residues to those already attributed to polysaccharide synthesis must reside on these Gram-negative high levan producing enzymes. Analysis of these residues may be possible through mutagenesis studies.

5.3.5. Thermal stability of top 10 LS candidates

Highly stable enzymes are often sought for their practicality. With high thermal stability, an increased productivity can be achieved, through the ability to increase the reaction temperature and by ensuring more catalytic cycles before the enzyme inactivation. In addition, stable enzymes are usually preferred for protein engineering needed for the optimization of biocatalysts since it is assumed that they are more robust to mutagenesis procedures. The LSs identified in the initial screening were subjected to a thermal treatment at 50°C for 1 hour, with the results of the retained activity listed in Fig. 5.4. Among them, 5 LSs, including LS6-C from *V. natriegens*, LS29-N from *S. salivarius*, LS24-N from *A. chlorophenolicus*, LS8-N from *P. graminis* and LS14-N from *N. amylolyticus* retained over 20% of their initial activity; and especially LS8 from *P. graminis* seems to retain all its initial activity and LS6-C from *V. natriegens* more than 40%. The half-lives of the identified thermoresistant LSs were further determined at 50°C (Table 5.4). The thermal stability of *A. chlorophenolicus* was not further examined due to its poor catalytic efficiency in levan production and its very low transfructosylation versus hydrolysis ratio. Confirming the preliminary observations (Fig. 5.4), LS8 from *P. graminis* had very high thermal stability, with a half-life of 290.7 minutes at 50°C. Interestingly, *P. graminis* was isolated from senescent maize roots, and not a thermal source (He et al., 2014). The thermal stability of LS8 is comparable to other thermally stable LSs (Belghith et al., 2012; Ben Ammar, Matsubara, Ito, Iizuka, Limpaseni, et al., 2002; Ben Ammar, Matsubara, Ito, Iizuka, & Minamiura, 2002; Inthanavong, 2011; Inthanavong et al., 2013; Ni et al., 2018; Tian & Karboune, 2012). From the Bacillaceae family, SacB from the thermophile *Geobacillus stearothermophilus* retained activity for 6 h at 47°C (Inthanavong et al., 2013), the LS genes from *Bacillus sp. TH4-2* was stable for 1 hr at 50°C (Ben Ammar, Matsubara, Ito, Iizuka, & Minamiura, 2002), while the LS from *Bacillus sp*. found by a Tunisian thermal source had a half life of 1h at 90°C (Belghith et al., 2012). The literature also revealed that the LS

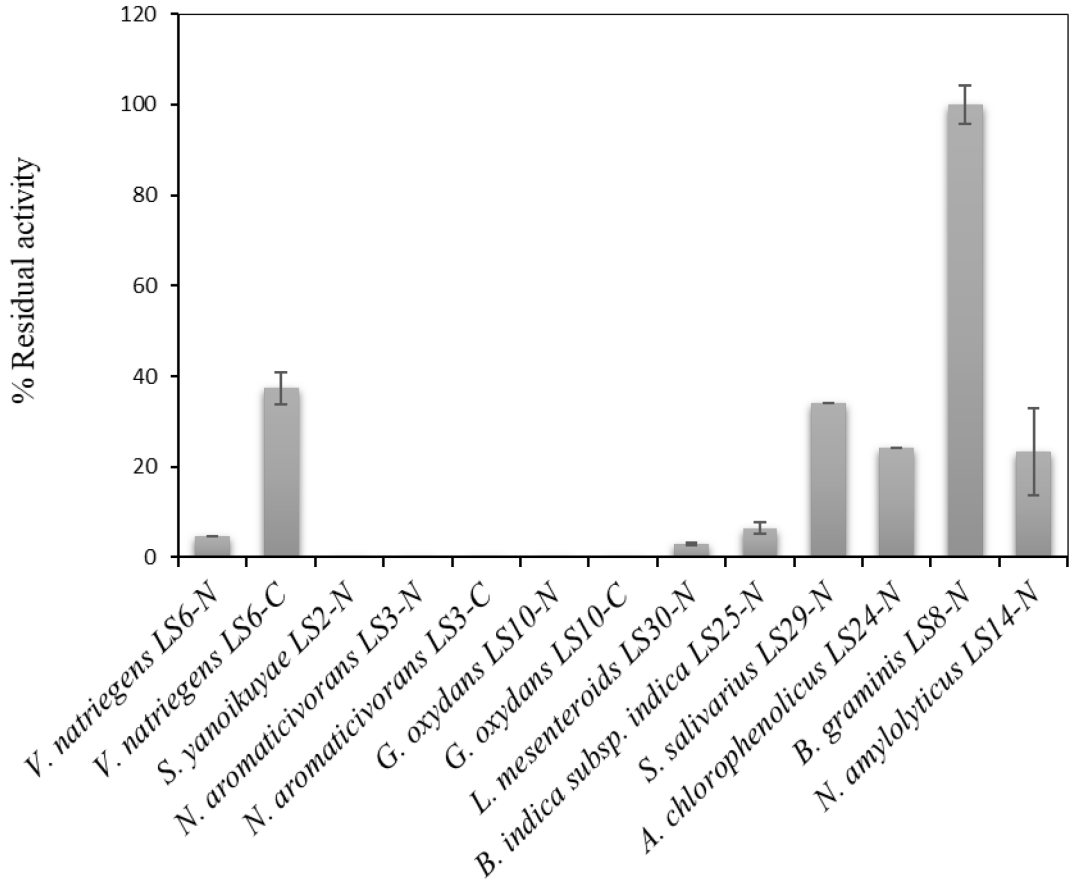


Figure 5.4: Thermal stability of potential LSs. Results list the residual activity of the LS enzymes after heating for 1 hour at 50°C**.**

Table 5.4: Half-lives of potential LSs heated at 50°C for three hours

LS Candidate	Half-life at 50°C (mins)
V. natriegens N-terminal His tag	21.2
V. natriegens C-terminal His tag	21.9
N. aromaticivorans C-terminal His tag	24.3
S. salivarius K12 N-terminal His tag	20.1
P. graminis N-terminal His tag	290.7
N. amylolyticus N-terminal His tag	11.8

The responses were measured based on total LS activity. All LSs showed 2nd order thermal decay kinetics

FtfA from *L. reuteri* LTH5448 (Uniprot ID: Q683P2) had exceptionally high thermal stability, retaining 63.8% of its activity after 12 hr at 55°C (Ni et al., 2018). 2.1.1.

5.3.6. Catalytic Efficiency of potential LS

The top candidates were narrowed by comparing the enzymes' levan production, size of the levan produced, the transfructosylation vs. hydrolysis ratio and the thermal stability. Despite the complexity of LS simultaneously catalyzing several reactions (hydrolysis, transfructosylation, and polymerization), it was considered worthwhile to determine and analyze the kinetic parameters of the transfructosylating, hydrolytic and total activities to better understand the catalytic efficiencies of the selected LS. However, the kinetic parameters for the hydrolytic and the total activity for LS30 from L. mesenteroides could not be determined with a high accuracy, as the results could not fit any kinetic model. The results of the kinetic parameters are listed in Table 5.5.

The transfructosylating, hydrolytic and total activities of the selected LSs predominately followed Michaelis-Menton kinetics, apart from the transfructosylating and total activity of LS10 from G. oxydans and the hydrolytic and total activity of LS6 from V. natriengens. Indeed, the total activity and the transfructosylating one of LS10 were found to follow Hill kinetics with Hill constant of 3.00 and 2.31, respectively. These results reveal the positive cooperativity between the sub-sites of LS10 as the substrate increased. The identified positive cooperativity feature may strongly affect the catalytic efficiency of LS10, whereby, as the enzyme starts to synthesize levan, the binding affinity of this growing levan polysaccharide to the enzyme sub-sites may increase. The results (Table 5.5) also show that the kinetic values for the hydrolytic and total activity for the LS6 followed substrate inhibitor (uncompetitive) kinetic behavior. In this regard, glucose can enter the active site (or re-enter after being released), re-form sucrose and prevent hydrolysis from occurring. By supplementing the reaction with glucose (0.28 – 1.1 M), Alvarado-Huallanco et Filho (2011) demonstrated glucose competitive inhibition with the fructosyltransferase from a *Rhodotorula sp.* (Alvarado-Huallanco & Maugeri Filho, 2011).

Furthermore, the hydrolytic activity was reported to decrease as sucrose concentration increased for the LS enzymes from L. reuteri and Z. mobilis (Goldman et al., 2008; Ozimek et al., 2006). Each investigated enzyme followed typical LS behavior, with the hydrolytic activity maximizing, then decreasing as the substrate concentration increased, apart from the LS29 from S. salivarius.

For most of the enzymes examined in this study (LS25 from *B. indica*, LS29 from *S. salivarius*, LS3 from *N. aromaticivorans*, LS6 from *V. natriegens* and LS8 from *P. graminis*), the Vmax for hydrolysis was achieved at very low substrate concentrations, as evidenced by their low Km values. After the maximum of initial velocity is achieved, it plateaus, then gradually decreases. As compared to the hydrolytic activity, the transfructosylating activity increases with the substrate concentration to reach a maximum value at higher concentrations as evidenced by higher Km values. Eventually, the two rates intersect, with the rate of hydrolysis equaling that of transfructosylation.

Highest Vmax's for transfructosylation was achieved by LS10 from *G. oxydans* (416 µmol*mg-^{1}*min^{-1}), LS3 from *N. aromaticivorans* (225 µmol*mg-^{1}*min^{-1}) and LS6 from *V. natriegens* (163 µmol*mg-^{1}*min^{-1}). These enzymes also exhibited the highest Vmax for hydrolysis (LS10: 429 µmol*mg-^{1}*min^{-1}; LS3: 254 µmol*mg-^{1}*min^{-1}; LS6: 265 µmol*mg-^{1}*min^{-1}). As a result, the highest turnover of sucrose (kcat) resulting in transfructosylation was achieved by the LS10 (332x10^{3} s^{-1}), LS3 (157x10^{3} s^{-1}) and LS6 (152x10^{3} s^{-1}), while the turnover for hydrolysis involved the same three enzymes but in a different order: LS10 (342x10^{3} s^{-1}), LS6 (247x10^{3} s^{-1}) and LS3 (176x10^{3} s^{-1}). Enzymes, which had higher Vmax for transfructosylation than for hydrolysis, were the LS29 from *S. salivarius* (19.3 vs 11.1 µmol*mg-^{1}*min^{-1}) and LS8 from *P. graminis* (9.5 vs 8.3 µmol*mg-^{1}*min^{-1}). Therefore, with these two enzymes, it is possible to modulate the LS activity towards transfructosylation by using high sucrose concentrations.

The investigated LS10 from *G. oxydans*, LS3 from *N. aromaticivorans* and LS6 from *V. natriegens* were highly active when compared with those previously described in literature. SacB from *B. amyloliquefaciens* and *Z. mobilis* were reported to have high kcat values for transfructosylation of 1137s^{-1} and 379s^{-1}, which were significantly lower than the values determined in the present study (Goldman et al., 2008; Tian & Karboune, 2012). The kcat of LS8 from *P. graminis* was some of the lowest values found in our study (4723 s^{-1} for transfructosylation). This is expected activity for this enzyme when considering its high thermal stability. Increased enzymatic stability typically requires a more rigid enzyme structure, which usually comes at a compromise to the enzyme activity at moderate temperatures (D'Amico, Marx, Gerday, & Feller, 2003).

Table 5.5: Kinetic results of top potential LSs for transfructosylating, hydrolytic and total activity

LS ID	Activity	Vmax (μmol*mg^{-1}*min^{-1})	K_M (mM)	n/K_i[d]	R^2	kcat (s^{-1})	Cat efficiency (s^{-1}.mM^{-1})
LS25 *B. indica*	Transfructosylating activity[a]	9.5 ± 1.5	467		0.954	9 x10^3 ± 1.4x10^3	20
	Hydrolytic activity[a]	8.3 ± 0.5	21		0.992	8 x10^3 ± 0.5x10^3	382
	Total activity[a]	12.5 ± 0.9	23		0.928	12 x10^3 ± 0.9x10^3	534
LS10 *G. oxydans*	Transfructosylating activity[b]	416 ± 32	674	3.00	0.992	3 x10^5 ± 25.2x10^3	493
	Hydrolytic activity[a]	429 ± 62	617		0.940	3 x10^5 ± 49.5x10^3	555
	Total activity[b]	716 ± 99	480	2.31	0.951	6 x10^5 ± 79.4x10^3	1192
LS29 *S. salivarius*	Transfructosylating activity[a]	19.3 ± 0.4	8		0.796	33.6x10^3 ± 0.7x10^3	4058
	Hydrolytic activity[a]	11.1 ± 0.3	9		0.755	19.3x10^3 ± 0.5x10^3	2256
	Total activity[a]	31.9 ± 0.7	9		0.850	55.4x10^3 ± 1.2x10^3	6124
LS3 *N. aromaticivorans*	Transfructosylating activity[a]	225 ± 22	519		0.964	157.3x10^3 ± 15.8x10^3	303
	Hydrolytic activity[a]	254 ± 15	4		0.793	176.0x10^3 ± 10.1x10^3	40735
	Total activity[a]	363 ± 27	8		0.886	317.4x10^3 ± 14.0x10^3	32461
LS6 *V. natriegens*	Transfructosylating activity[a]	163 ± 12	436		0.974	1x10^5 ± 11.5x10^3	350
	Hydrolytic activity[c]	265 ± 9	2	1.43	0.868	2x10^5 ± 8.4x10^3	100555
	Total activity[c]	289 ± 5	3	9.08	0.832	2x10^5 ± 4.6x10^3	79574
LS8 *P. graminis*	Transfructosylating activity[a]	4.96 ± 0.53	479		0.993	4.7x10^3 ± 0.5x10^3	9.87
	Hydrolytic activity[a]	5.97 ± 0.36	20		0.889	5.7x10^3 ± 0.3x10^3	289
	Total activity[a]	7.65 ± 0.66	30		0.875	7.2x10^3 ± 0.6x10^3	246
LS30 *L. mesenteroides*	Transfructosylating activity[a]	0.822 ± 0.156	440		0.864	1.5x10^3 ± 0.3x10^3	3.47

[a] Activity followed Michaelis-Menton kinetic behavior; [b] Activity followed Hill kinetic behavior [c] Activity followed Substrate Inhibitor (uncompetitive) kinetic behavior, [d] Ki (M)

The Michaelis-Menton constant (Km) of each enzyme was determined for transfructosylating, hydrolytic and total activity. The lowest Km value for transfructosylating activity was obtained with LS29 from *S. salivarius* (8.28 mM). This LS had more or less similar Km values for its hydrolytic and total activity at 8.56 mM and 9.05 mM, respectively. For the other investigated LSs, the Km values for their transfructosylation activity were in the range of 440-674 mM. The narrow range of Km values indicates that there is a standard affinity for sucrose by LS enzymes. Literature reports of the Km values of various LS enzymes show greater variability, with values ranging from 6.9 mM to 460 mM (Goldman et al., 2008; Inthanavong et al., 2013; Olvera et al., 2012; Tian & Karboune, 2012; Waldherr et al., 2008).

The catalytic efficiency for the transfructosylation, hydrolysis and total activity, for each enzyme was calculated and found to be reasonably high, ranging from 3.47-100555 $s^{-1}mM^{-1}$. The highest catalytic efficiency for transfructosylation was achieved by LS29 from *S. salivarius* (4058 $s^{-1}mM^{-1}$). LS29 did not have the highest catalytic turnover for transfructosylation, but rather a low Michaelis-Menton constant (8.28 mM). The enzyme with the highest catalytic efficiency for total activity was LS6 from *V. natriegens* (79574 $s^{-1}mM^{-1}$).

LSs exhibiting higher catalytic efficiency towards transfructosylation than hydrolysis can be described as having great catalytic potential. There was one enzyme which satisfied this criterion, LS29 from *S. salivarius (*4058 versus 2256 $s^{-1}M^{-1}$), while LS10 from *G. oxydans* had similar catalytic efficiency for the two activities (493 and 555 $s^{-1}M^{-1}$). Following this comparison, LS29 (19.3 vs 11.1 µmol*mg^{-1}*min^{-1}) and LS25 from *B. indica* (9.5 vs 8.3 µmol*mg^{-1}*min^{-1}) showed higher Vmax values for the transfructosylation than hydrolysis. By using high sucrose concentrations, a modulation of the catalytic activity towards the transfructosylating activity can be achieved. The Vmax for transfructosylation and hydrolysis were essentially the same for LS10 (416 vs 429 µmol*mg^{-1}*min^{-1}), LS3 (225 vs 254 µmol*mg^{-1}*min^{-1}), LS6 (163 vs 265 µmol*mg^{-1}*min^{-1}) and LS8 (5.0 vs 6.0 µmol*mg^{-1}*min^{-1}). Alternatively, all these LS (excepted LS10) plus LS8 had lower catalytic efficiency for transfructosylation than for hydrolysis.

5.3.7. Acceptor specificity of potential LS

LS enzymes require a fructosyl donor and a fructosyl acceptor molecule. Typically studies of LS rely on using sucrose as the fructosyl donor and acceptor molecules, synthesizing a variety of

FOSs, levan and glucose and fructose. Alternatively, it is interesting to test whether alternate fructosyl donors and fructosyl acceptors can be used by a LS. Many LSs can utilize raffinose as a fructosyl donor to a similar degree as sucrose such as SacB from *Z. mobilis*, the LS orphan of gene from *M. laevaniformans,* LevS from *Lactobacillus sanfranciscenis*, LevG from *L. reuteri,* SacB from *B. subtilis*, Lsc3 from *P. syringae* (Andersone, Auzina, Vigants, Mutere, & Zikmanis, 2004; Park et al., 2003; Seibel et al., 2006; Tieking, Ehrmann, Vogel, & Ganzle, 2005; S. Van Hijum, Szalowska, Van Der Maarel, & Dijkhuizen, 2004; Visnapuu et al., 2008*)*. The selected LSs were incubated with raffinose for 50 h and the product formation was analyzed by TLC. The spots which appeared/disappeared after the reaction mixture was developed by TLC demonstrated that all the enzymes were capable of using raffinose, see supplementary information for TLCs (Fig. S5.2) and Table S5.2 for the analysis. LS3 from *N. aromaticivorans* and LS25 from *P. graminis* largely utilized the raffinose without any polysaccharide conversion. In the reaction with the LS6 from *V. natriegens*, the raffinose spot disappeared before the sucrose spot in the reaction. Since sucrose is double the concentration of raffinose when the reaction was initiated, this unfortunately cannot provide information on which substrate was preferred. LS10, LS25, LS29 and LS6-N showed some transfructosylation action, synthesizing a polysaccharide solely from raffinose. The LS25 from *P. graminis* and LS30 from *L. mesenteroides* can utilize the acceptor galactose. Alternate acceptor molecules for the fructosyl group, such as galactose, glucose, maltose and lactose were examined. LS3 from *N. aromaticivorans* and LS10 from *G. oxydans* were confirmed to use maltose and lactose as fructosyl acceptor molecules, while the LS6 from *V. natriegens* used lactose and the LS25 looked to have used galactose. Identification of products and measurement of depleting starting material can be difficult to identify by TLC. Further characterization will be done by HPAEC-PAD.

5.4. Conclusions

Genome mining was a useful tool in exploring new potential LSs. Using *in silico* tools provides an easier method of deeply exploring genetic databases and metagenomes in a time and material efficient manner. Our exploration provided 10 LSs of high interest, with further screening narrowing that list to 5 new enzymes. Our screening revealed LSs which can be tailored to a specific reaction. If high thermal stability is needed, LS8 from *P. graminis* is the most suitable enzyme, with the LSs from *V. natriegens*, *N. aromaticivorans* and *S. salivarius* also available. If

high levan production is the desired outcome, then the LS10 from *G. oxydans* is the best LS to select. For specifically high transfructosylating activity, the LS from *S. salivarius,* with the highest kcat, and LS10, with the highest Vmax for transfructosylation are the best options. Alternatively, transfructosylation activity can be selected for by increasing the substrate concentration for the LS25 from *B. indica* and LS3 from *N. aromaticivorans*. The choice of enzyme when searching for specific acceptor specificity will depend upon the acceptor/product desired. The variety of these enzymes makes LS a catalytic tool of immense potential for diverse synthetic needs.

Connecting Statement 4

Chapter V regarded alternative LS enzymes for transfructosylation. The focus was to find new microbial sources of LS with an increased reaction selectivity towards transfructosylation as opposed to hydrolysis, with the high ability to produce a large sized levan and with improved enzymatic stability. This study discovered 5 LS enzymes with remarkable activity worth further investigation along with a comparison to the previously studied LS from *B. amyloliquefaciens*. Chapter VI continues to understand the full potential of these LS enzymes, through the characterization of their end-product spectrum with sucrose as the substrate and additional analysis of the LSs' acceptor specificity. These facts can be compared against the active site structure of the enzymes as well as the composition.

The results from this work was presented at BioTrans 2017. The intended manuscript will be submitted to Catalysis, Science & Technology.

Hill, A., de Berardinis, V., Petit, J. L. & Karboune, S. (2017) Discovery of new Levansucrase enzymes for improved catalytic activity and acceptor specificity. BioTrans 2017, Budapest, Hungary, July 9 – July 13, 2017.

Hill, A., Narwani, T., de Brevern, A., de Berardinis, V., Petit, J. L. & Karboune, S. (2017) Characterization of new levansucrase enzymes for improved product spectrum and acceptor specificity. *To be submitted*.

6. Abstract

The easy synthesis of complex oligosaccharides is highly desired for their potential as prebiotics, their role in the cosmetic industry, pharmaceutical industry and food industry. Levansucrase (LS, EC 2. 4. 1. 10) has the capacity to catalyze the synthesis of some of these compounds without the use of expensive cofactors. It is a beta-fructosyl transferase which follows a double displacement reaction, whereby it acquires a fructosyl residue from a donor molecule, and transfers it to an acceptor molecule, typically with β-(2→6)-glycosidic linkages. Depending upon the acceptor, LS can catalyze hydrolysis (water), oligomerization (sucrose) and polymerization (oligomers). The direction of LS activity varies upon its microbial source. Genome mining was used to uncover new LS enzymes with increased transfructosylating activity and wider acceptor promiscuity. An initial screening revealed 5 LS enzymes with high potential. The LSs from *G. oxydans* and *N. aromaticivorans* synthesized FOSs with up to 13 units in length. Alignment of the LS amino acid sequences and substrate docking with homology models were used to identify structural elements causing differences in their product spectra. Raffinose over sucrose, was the preferred donor molecule for the LS from *V. natriegens, N. aromaticivorans* and *P. graminis*. The LSs examined were found to have wide acceptor promiscuity, utilizing the monosaccharides galactose and xylose, the disaccharides, sucrose, maltose, lactose, the trisaccharide, raffinose and the alditol, sorbitol and the benzene-diol, catechol to a high degree.

6.1. Introduction

Biosynthetic routes for the synthesis of novel carbohydrates is an attractive course. Enzymatic glycosylation reactions can proceed via a regio- and stereo-selective manner, unlike chemical synthesis which requires the use of protecting groups (Seibel & Buchholz, 2010). Fructooligosaccharides (FOSs) have interesting properties which makes their synthesis of particular interest. They have been shown to have prebiotic properties, having a bifidogenic effect (Fahey Jr, 2010), anti-carcinogenic effects (Tanaka, Bush, Klauck, & Higgins, 1989) increased mineral absorption (Coudray et al., 1997) and their fermentation leads to the production of short-chained fatty acids (SCFAs) (Fahey Jr, 2010; Rycroft, Jones, Gibson, & Rastall, 2001). They also have interesting food applications, acting as a non-cariogenic sweetener in food (Bali, Panesar, Bera, & Panesar, 2015). The fructan polysaccharide, levan, is composed of β-(2-6)-linked fructosyl residues. It has its own functional properties for food and pharmaceutical industries and can also

be hydrolyzed to FOSs (Han, 1990; Kim et al., 2004; Tian, Karboune, et al., 2014; Yoo et al., 2004).

Levansucrase (EC 2.4.1.10, LS) is a β-fructosyl transferase capable of catalyzing the non-Lelior-type transfructosylation reaction generating prebiotic fructooligosaccharides (FOSs) and levan. LS belongs to glycosyl hydrolase family 68, along with β-fructofuranosidase (EC 3.2.1.26) and inulosucrase (EC 2.4.1.9) and is expressed by both gram positive or gram negative bacteria. Belonging to clang GH-J, along with family GH-32, their structure contains 5-fold β-propeller topology with 4 anti-parallel strands (Lombard, 2014). The physiological role of LS play in bacteria is varied, levans produced enable the binding of bacteria on dental surfaces, protects cell structures against drought conditions, provide cell defense and carbohydrate storage (Vijn & Smeekens, 1999).

LS catalyzes these reactions via a "ping-pong" or double displacement mechanism. This all occurs within a deep negative cavity on the enzyme. The fructofuranosyl group is donated by an acceptor molecule, such as sucrose, raffinose or stachyose (Kim et al., 2005). An enzyme intermediate is formed with the fructosyl group, with the remainder of the donor molecule released. LS is can catalyze different reactions, depending upon the acceptor of the fructosyl group. Hydrolysis of the donor molecule occurs when the acceptor is water, releasing fructose. Transfructosylation occurs when the fructosyl group is transferred to the acceptor molecule, typically via a β-(2-6)-glycosidic linkage. Transfructosylation can be classified as either oligomerization or polymerization (Martinez-Fleites et al., 2005).

LS from different microbial sources have varying product profiles. LSs can be characterized as having predominately a processive reaction or a disproportionate reaction (Ozimek et al., 2006). In a processive reaction, the enzyme has subsites which hold onto the growing transfructosylated product, leaving it available for another fructosyl residue. Enzymes which act in a processive manner, such as the LS from *B. subtilis* and *B. megaterium*, produce levan and larger FOSs (Strube et al., 2011). In a disproportionate reaction, the LS lacks affinity for the product, releasing it after performing the transfructosylation reaction. This results in shorter FOS products. The LS from *E. amylovora*, *Z. mobilis* and *G. diazotrophicus* all produce smaller FOS products (Crittenden & Doelle, 1993; Martinez-Fleites et al., 2005; Wuerges et al., 2015).

LS is capable of utilizing other, non-sucrose derived acceptor molecules due to the flexibility of the +1 subsite (Visnapuu et al., 2008). LS has been shown to use alternate monosaccharides to create sucrose analogues, such as D-Gal-Fru, D-Man-Fru, D-2-deoxy-D-glucose, D-Fuc-Fru and D-Xyl-Fru (Beine et al., 2008; Seibel et al., 2006; Tian & Karboune, 2012). The sucrose analogues can in turn act as acceptor molecules for further transfructosylation reactions, producing hetero-FOSs (Beine et al., 2008). Multiple disaccharides are also utilized as acceptor molecules, such as lactose, producing the prebiotic lactulose. With some LSs, the trisaccharide raffinose is preferred as a fructosyl donor and acceptor molecule as compared to sucrose (Andersone et al., 2004).

Non-carbohydrate acceptor molecules in LS-catalyzed transglycosylation reactions can be used to generate a variety of compounds, such as those used in cosmetics and pharmaceuticals (Kang et al., 2009). Mena-Arizmendi et al. (2010) fructosylated aromatic compounds as well as aliphatic alcohols using the LS from *B. subtilis*. They found that there was an inverse relationship between pKa values and transfructosylation (Mena-Arizmendi et al., 2011). Lu et al. (2014) successfully, albeit at low yields, fructosylated isopropyl and 1-pentanol, while larger alcohols were unsuccessful as acceptors with LS from *B. lichenformis*. The authors suggested that this was due to steric restrictions based on size (Lu et al., 2014).

Enzymatic β-(2→6) transfructosylation is restricted by the low number of LS enzymes available (Hill, Karboune, & Mateo, 2017). There is an interest in discovering new LS with a wide acceptor substrate specificity. Previously, genome mining was utilized to search for new LSs with novel properties (Hill *et* al., to be submitted). A reference set of 39 known LSs was subjected to a BLAST analysis (RI\>=30, RZ\>0 0.8, RL\>200) and clusterized at 80% identity which resulted in 50 cloneable genes. The LS were narrowed based upon activity, transfructosylation versus hydrolysis, levan production, size of levan produced, thermal stability and novelty. The top candidates were identified, which included the LSs from *Beijerinckia indica subsp. indica*, *Burkholderia graminis*, *Vibrio natriegens*, *Novosphingobium aromaticivorans* and *Gluconobacter oxydans*. The objective of the proposed study was to investigate the end-product profiles of reaction-catalyzed by the selected LSs and to study their acceptor specificity using mono- and di-saccharides as well as two alcohols, an aliphatic alcohol and an aromatic one. The results of their product profiles were related back to the amino acids sequence of each enzyme, to connect the enzymatic structure with LS activity. To further advance the structure-function information of LS gleamed from the amino-acid

sequence comparison, homology-based modeling was employed to map the structure of the selected LSs along with the LS for *B. amyloliquefaciens* and compared against the structure of the LS from *B. subtilis*. Docking study was performed with the established model to characterise the binding affinity of LS to substrate.

6.2. Materials and Methods

6.2.1. Materials

Sucrose, D-(-)-Fructose, D-(+)-glucose, D-(+)-galactose, α-lactose, D-(+)-maltose, D-(+)-xylose, 1,2-dihydroxybenzene (catechol), sorbitol, D-(+)-raffinose, 3,5-dinitrosalicylic acid (DNS), potassium sodium tartrate ($KNaC_4H_4O_6$), Dextran standards (50 to 670 kDa), lysoszyme from chicken egg white, Pefabloc® SC were obtained from Sigma Chemical Co. (St. Louis, MO). K_2HPO_4, KH_2PO4, NaCl, NaOH, tryptone, Bovine Serum Albumin, β-D-isothiogalactopyranoside and yeast extract were obtained from Fisher Scientific (Fair Lawn, NJ). Calibration standards 1-kestose, nystose, and 1^F-fructosylnystose were supplied by Wako Pure Chemical (Japan). Bradford reagent concentrate was provided by Bio-Rad (Missasauga, ON). Ampicillin was supplied by Wisent. Terrific broth was purchased by BioBasic and Lysonase was purchased by Merck. *Escherichia coli* BL21(DE3) plysE strains were supplied by Invitrogen.

6.2.2. Enzyme production, recovery and purification

E. coli BL21(DE3) cells by Invitrogen each containing pET22b(+) expression vector containing the genes for: *Vibio natriegens* (VIBNA, A0A0S3EPZ1), *Gluconobacter oxydans* (GOX0873, Q5FSK0), *Novosphinogbium aromaticivorans* (Saro_1879, Q2G754), *Paraburkholderia graminis* (BgramDRAFT_4066, B1G3X6) and *Beijerinckia indica subsp. indica* (Bind_2021, B2IF78).

A preculture, containing Lysogeny broth (LB) and ampicillin (100 µg/mL) was inoculated by selecting colonies grown on LB agar containing carbenicillin (1 µg/mL). The preculture was grown for 24 h at 37°C at 250 rpm. The preculture (2%) was used to inoculate the culture medium composed of terrific broth and ampicillin (100 µg/mL). The culture was grown at 37°C at 250 rpm until growth achieved an optical density of 1.2 at 600 nm (approximately 8 h). Gene expression was then induced through the addition of β-D-thiogalactopyranoside (IPTG, 1 mM), with growth continued for 18 h afterwards at room temperature at 250 rpm. The cells were then collect by centrifugation (8 000 rpm) and stored at -70°C.

Enzyme recovery was initiated by defrosting the pellet on ice for 40 mins and resuspending the cells in sonication buffer (50 mM Pipes, 300 mM NaCl, 10% glycerol v/v, pH 7.2). Lysozyme (4 mg/g pellet) and DNase (2000 U/mL) were added and the suspension was gently mixed at 40 rpm on ice for 30 mins. The cells were lysed by ultrasonication using a microtip (Misonix Ultrasonic Liquid Processor S-4000) for 1 minute (10 seconds on, 60 seconds off, amplitude of 15) in a salt-ice bath. The cellular debris was removed through centrifugation (45 mins, 14 000 rpm, 4°C). The supernatant was dialyzed against potassium phosphate buffer (5 mM, 12 L) at pH 6, then frozen and lyophilized.

The crude enzyme extracts were resolubilized potassium phosphate buffer (50 mM, 0.5 mL) at pH 6.0, filtered and loaded onto GE Healthcare Histrap FF 1 mL column. The column was washed with sonication buffer (15 mL), wash buffer (50 mM Pipes, 300 mM NaCl, 10% glycerol v/v, pH 6.4, 15 mL), 5 mM imidazole prepared in wash buffer (15 mL), 10 mM imidazole prepared in wash buffer (15 mL) and eluted with a gradient of imidazole (100 mM-200 mM, 3 mL each fraction) prepared in wash buffer. Each fraction was tested for activity by the DNS activity assay. Active fractions were pooled together, dialyzed against potassium phosphate buffer (5 mM, 12 L), frozen and lyophilized. Purity was confirmed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Bio-Rad SDS-PAGE, Low Range Standards (14 400 – 97 400 Da) were used to confirm size.

6.2.3. Time course for LS-catalyzed reactions

LS (5-7 U/mL) was incubated with sucrose (0.9 M) at 30°C in triplicate, with samples withdrawn at 2 h, 4 h, 6 h, 8 h, 10 h, 12 h, 24 h and 50 h. The samples were boiled for 5 mins to stop the reaction. To quantify the reaction products and the remaining sucrose, a sample (10 µL) of the reaction mixture was analyzed using high-pressure anion exchange chromatography (HPAEC) with a pulsed amperometric detector (PAD) using a Dionex ICS 3000 system (Thermo Fisher) eluted on a CarboPac PA200 column (3 x 250 mm). The sample was eluted with a linear gradient of 0-100% of 200 mM sodium acetate prepared in 100 mM NaOH for 25 mins at a flow rate of 0.5 mL/min at 32°C. Calibration standards of glucose, fructose, sucrose, 1-kestose, nystose, and 1^F-fructosylnystose were used.

6.2.4. Acceptor specificity

LS (5-7 U/mL) was incubated with either sucrose (0.9 M), raffinose (0.9 M) or sucrose (0.9 M) and an acceptor molecule (0.45 M), including raffinose, glucose, galactose, maltose, lactose, xylose, sorbitol and catechol. Samples were withdrawn at selected reaction times (2 h to 50 h) and boiled for 5 mins to stop the reaction. The reaction was characterized by HPAEC-PAD with same conditions previously used on the CarboPac Pa200 column. They were also analyzed using a CarboPac PA20 column, with samples eluted isocratically with 20 mM NaOH at a flow rate of 0.5 mL/min at 32°C.

6.2.5. Homology Modeling

6.2.5.1.Peptide identification for homology modeling

The LS from *B. amyloliquefaciens*, was purified by size-exclusion chromatography and was separated by SDS-PAGE gel. The LS bands were removed and sent for proteomic analysis by mass spectrometry at the Plateforme de Protéomique - Centre de Recherche du Chu de Québec (Laval, Québec). The bands were subjected to tryptic digestion using a MassPrep liquid handling robot (Waters) following the protocol by Brotherton et al. 2014 (Brotherton et al., 2014). After an initial reduction (10 mM dithiothreitol) and alkylation (55 mM iodoacetamide) the sample was digested with using 126 nM porcine trypsin (sequencing grade, Promega) at 58°C for 1 hour. The digestion products were extracted using 1% formic acid and 2% acetonitrile which was then followed 1% formic acid and 50% acetonitrile. The protein extracts were pooled, dried by vacuum centrifugation and then resuspended into 10 µL of 0.1% formic acid for analysis by electrospray ionization mass spectrometry. The peptide samples were separated by an online reverse-phase (RP) nanoscale capillary liquid chromatography (nanoLC) and analysed by electrospray mass spectrometry (ESI MS/MS) (Sheta et al., 2016). The fragments were analyzed using Scaffold software (version 4.0) (Proeome Software).

6.2.5.2.Homology model selection

PSI-BLAST (NIH) was used to search for sequences with the highest E-value and sequence identity to the amino acid sequences of the LSs from *B. amyloliquefaciens*, *G. oxydans*, *V. natriegens*, *B. indica subsp. indica*, *P. graminis* and *N. aromaticivorans*. Template models were developed using enzymes with crystal structures which had the highest homology. The LS from *B. subtillis* (PDB 1PT2), bound with sucrose with a E342A mutation was chosen as the model for *B. amyloliquefaciens*; the LS *G. diazotrophicus* (1W18) was the model for *B. indica subsp. indica*

and P. graminis; β-fructofuranosidase by *M. saccharophilum* K-1 (3VSR); the LS from *E. amylovora* (4D47) with the sucrose hydrolysis products within the active site was the model for the LS from *G. oxydans and N. aromaticivorans*.

6.2.5.3.Model development

Templates containing a substrate had them removed. After the template models were selected, they were validated using ProSa-web Protein Structure Analysis (Sippl, 1993; Wiederstein & Sippl, 2007). Alignment was performed using Pymol, to ensure that there were high Root Mean Squared (RMSD) and low TM scores. The LS being modeled were aligned with the template to ensure essential amino acids were structurally conserved. Molecular dynamics simulations (100) were executed using GROMACS (version 4.5) were performed to align the sequence to the template at a timescale of 2 fs per step, 15 forcefield, type 5 water, charge of -5. The template models with the lowest DOPE were selected for evaluation. Alignment was again performed using PyMol to review the β-factors and the flexibility of the backbone (Cα). Sequence and structure conservations was evaluated using PoseView (Stierand, Maaß, & Rarey, 2006; Stierand & Rarey, 2007).

6.3. Results and Discussion

6.3.1. Time courses for LS-catalyzed transfructosylation reaction of sucrose

The results (Figures 6.1-6.5) shows that the conversion rate of sucrose. For the LS *V. natriegens*, the consumption of sucrose was constant from 4 h – 12 h reaction leading to a total conversion 60 % of the starting material by hour 50. The conversion rate of the sucrose decreased over the time course; this can be the consequence of a lower sucrose concentration and/or product inhibition. The rate of sucrose consumption by LS from *G. oxydans*, *N. aromaticivorans*, *P. graminis* and *B. indica subsp. indica* was constant throughout the 50 h of the reaction with final consumptions of 78%, 53%, 81% and 72% used respectively. Only LS from *G. oxydans* and *P. graminis* almost depleted the sucrose as seen in Figures 2 and 4. The sucrose conversion by LS results in the release of glucose and fructose, which can be transferred to the fructosyl 6'-hydroxyl of the acceptor products (levan, FOSs). Glucose was the highest produced products for the LS *V. natriegens, N. aromaticivorans*, *P. graminis* and *B. indica subsp. indica*. Glucose was the highest product for the LS from *G. oxydans* at 12 h, then the amount of glucose decreased, possible through exchange reactions. The concentration of free fructose can be used for the determination of the extent of the hydrolysis of sucrose. Subtracting the concentration of free fructose from that of glucose provides

the transfructosylation extent of LS-catalyzed reaction. Free fructose caused by hydrolysis exceeded the fructose used for transfructosylation throughout the 50 h of measured reaction time for all of the LSs with the exception of *G. oxydans*. The amount of fructose used for transfructosylation, in the reaction with the LS from *V. natriegens*, increased to 40% from 30% from 2 h to 8 h. When the reaction was stopped at 50 h, transfructosylation had decreased to 20%; this decrease was in accordance with the hydrolysis of some end-products. When comparing the amount of transfructosylation products to the amount of hydrolysis products, the extent of the transfructosylation over the hydrolytic reactions can be assessed. There was a higher amount of transfructosylation productions in the reaction system catalysed by LS from *V. natriegens* during the first 2 h, with the amount decreasing to a minimum at 50 h. The ratio of transfructosylation products over hydrolytic products decreased from 5.1 to 0.7 over the course of this reaction. This decrease was mainly due to the release of monosaccharides; indeed, the amount of transfructosylation products only decreased from 78 g/L to 74 g/L while the amount of monosaccharides increased from 15 g/L to 100 g/L. The amount of transfructosylation products can be higher than the monosaccharides production even when the transfructosylation vs. hydrolysis ratio (T/H) is in favour of hydrolysis. This is because the transfructosylation products includes the sucrose used as the initial acceptor molecule, while the T/H just takes into account the fructose used for transfructosylation over the free fructose from hydrolysis. When there is a high amount of trisaccharides, sucrose is a high contributor. The high production of hydrolysis products at 50 h corresponded with the kinetic parameters of the LS from *V. natriegens*, where both the turnover rate (k_{cat}) and the catalytic efficiency were higher for hydrolysis (247364 s^{-1}, 100555 $s^{-1}.mM^{-1}$) than they were for transfructosylation (152369 s^{-1}, 350 $s^{-1}.mM^{-1}$).

Initially within the first 2 h of incubation with sucrose, the reaction selectivity of LS from *G. oxydans* was dominated by transfructosylation. Transfructosylation decreased after this time, gradually increasing once again to parity with hydrolysis at 50 h. Upon comparing the transfructosylating products versus the monosaccharides produced, the ratio was in favor of transfructosylation during the first 6 h. It decreased to a ratio of 0.7 by 50 h. The amount of transfructosylating products actually increased from 72 g/L to 88 g/L from 2 h to 50 h, while the monosaccharide content increased from 21 g/L to 125 g/L.

The reaction catalyzed by the LS from *N. aromaticivorans* was dominated by hydrolytic activity, with the amount of fructose used for transfructosylation reaching a maximum of 33%. The high catalytic efficiency for hydrolysis (40735 $s^{-1}.mM^{-1}$) correlated the high hydrolytic activity, being many times higher than the transfructosylation catalytic efficiency (303 $s^{-1}.mM^{-1}$). While the amount of free fructose, caused by hydrolysis, was much greater than the amount used for transfructosylation, the amount of transfructosylated products was greater than the amount of monosaccharides. The ratio between the transfructosylation over hydrolysis products started at 4.5 then decreased to 1.3 over the course of the 50 h. The amount of transfructosylated products increased from 46 g/L to 83 g/L while the amount of monosaccharides increased from 10 g/L to 62 g/L. This LS, from *N. aromaticivorans*, had a similar turnover rate for transfructosylation (157398 s^{-1}) and hydrolysis (175934 s^{-1}).

Similarly, hydrolysis was the dominate reaction over transfructosylation, with transfructosylation products making up 39 - 49% of the products over the time period from the LS from *P. graminis*. In the initial stage of the reaction catalyzed by LS from *P. graminis*, similar amounts of monosaccharide products (25 g/L) and transfructosylation products (27 g/L) were generated; as the time course proceeded, the monosaccharide content increased to 144 g/L, while the transfructosylation products increased to 82 g/L. These results makes sense with the context that the turnover rate of LS from *P. graminis* being similar for transfructosylation (4723 s^{-1}) than for hydrolysis (5687 s^{-1}), resulting in an initial high production of transfructosylation products, dropping due to the higher catalytic efficiency of hydrolysis (289 $s^{-1}.mM^{-1}$) as compared to transfructosylation (9.87 $s^{-1}.mM^{-1}$).

The transfructosylating activity from the LS from *B. indica subsp. indica* increased over the 50 h reaction time, with the amount of fructose used from transfructosylation increasing from 34 % in the first 2 h, to 45 % by 50 h. Similar to LSs from *G. oxydans* and *P. graminis*, the LS from *B. indica subsp. indica* initially produced more transfructosylation products at 2 h (48 g/L) compared to monosaccharides (16 g/L) By hour 50, the monosaccharide content increased to 120 g/L, while the amount of transfructosylation products increased to 77 g/L. Indeed, both the turnover rate for transfructosylation (9186 s^{-1}) and hydrolysis (7981 s^{-1}) were relatively similar for LS from *B. indica*, but the catalytic efficiency was much higher for hydrolysis (382 $s^{-1}.mM^{-1}$) than for transfructosylation (20 $s^{-1}.mM^{-1}$) activities.

The ratio of transfructosylation versus hydrolysis is known to be affected by sucrose concentration. Using a moderately high sucrose concentration of 0.3 M, the LS from *B. circulans* obtained 70% transfructosylating activity (Oseguera et al., 1996). Ni et al. (2016) analyzed the products of the LS from *L. reuteri* LTH5448 and reported that very high levels of levan were produced by this LS by optimizing reaction pH, reaction time, reaction temperature, sucrose concentration and enzyme loading (Ni et al., 2018). Modulating the reaction conditions may maximize the transfructosylation reactions in the investigated LS-catalyzed systems.

Figure 6.1 indicates that LS from *V. natriegens* produced levan-type polysaccharides (84.3 g/l; 74.6 % w/w total transfructosylation products) at 12 h (maximum levan production) and 18.6 % oligosaccharide (25.4 %, w/w total transfructosylation products) made of a mixture of trisaccharides (1-kestose, 6-kestose and neokestose), tetrasaccharides, pentasaccharides, octasaccharides and undecasaccharides (n=11). All products produced by LS from *V. natriegens* increased in concentration as time increased, at least until 24 h (with the exception of X_8 and X_{11}). The production of X5, X8 and X11 was detected after 2 h, while the production of tetrasaccharides was only detected after 8 h. The later detection of these products can be due to a lack of their accumulation, resulting from their use as precursors for larger molecules, i.e. levan. The trisaccharide mixture produced by LS from *V. natriegens* is composed of 1-kestose, neokestose and 6-kestose. 1-kestose is the predominant trisaccharide produced, with there being 14-times less 6-kestose. The lack of accumulation of 6-kestose can be due to it's recapture by the enzyme and utilized as a substrate for the generation of other oligosaccharides and levan. This was the case for 6-kestose when sucrose was incubated with the LS from *G. diazotrophicus* SRT4 (Hernandez, Suarez, Balmori, 1996-5). LS's have demonstrated the ability to produce similar amounts of 1-kestose as inulosucrases from the same bacterial (Ozimek et al., 2006).

The product profile of the LS from *G. oxydans* from its reaction with sucrose over 50 h was shown in Figure 6.2. Levan (22.3 %, w/w total transfructosylation products), X4 and X6 (6.0 % and 3.5 % w/w transfructosylation products) were produced almost linearly within the first 10 h. Then their production rate slowed, but it did not plateau. At 50 h, the highest production was achieved by levan, X3, X4, X6, X8 and lastly, X11. High levan production corresponds with the results previously reported in Hill et al. (to be submitted), where *G. oxydans*, with a C-terminal His tag, was found to produce the second largest amount of levan, second only to *G. oxydans* with a N-

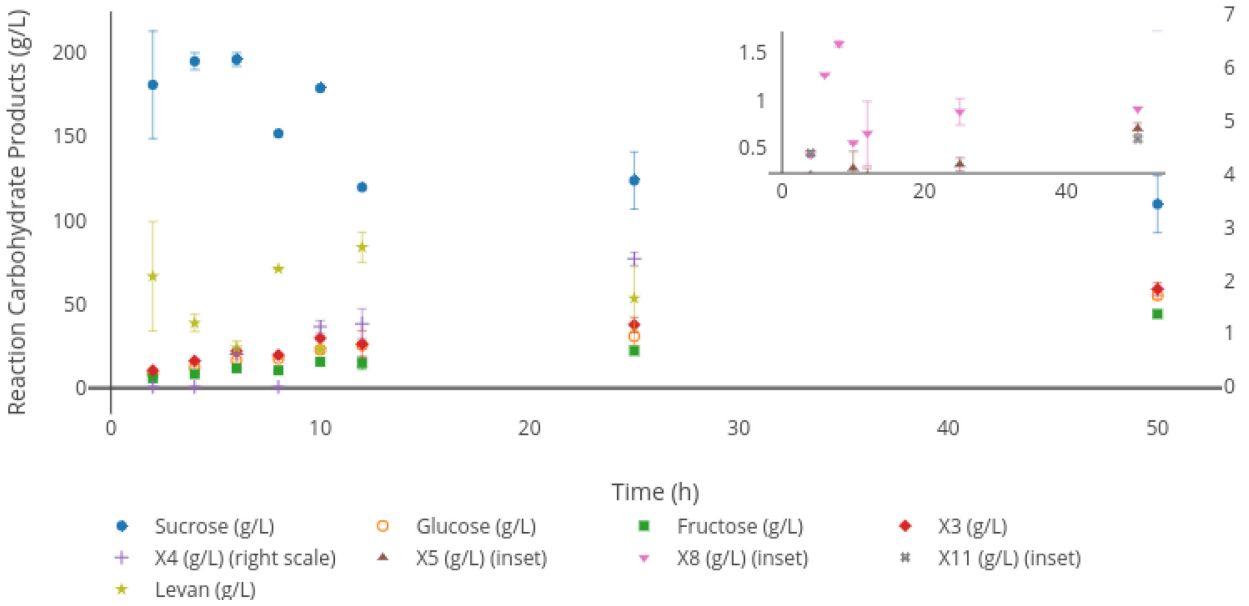


Figure 6.1: Reaction product profile of LS from *Vibrio natriegens* with sucrose for 50 h at 30°C.X3 is composed of all trisaccharides (1-kestose, neokestose, 6-kestose), X4, all tetrasaccharides quantified; X5 the pentasaccharides quantified, X8 octasaccharides, X11 undecasaccharide quatified**.**

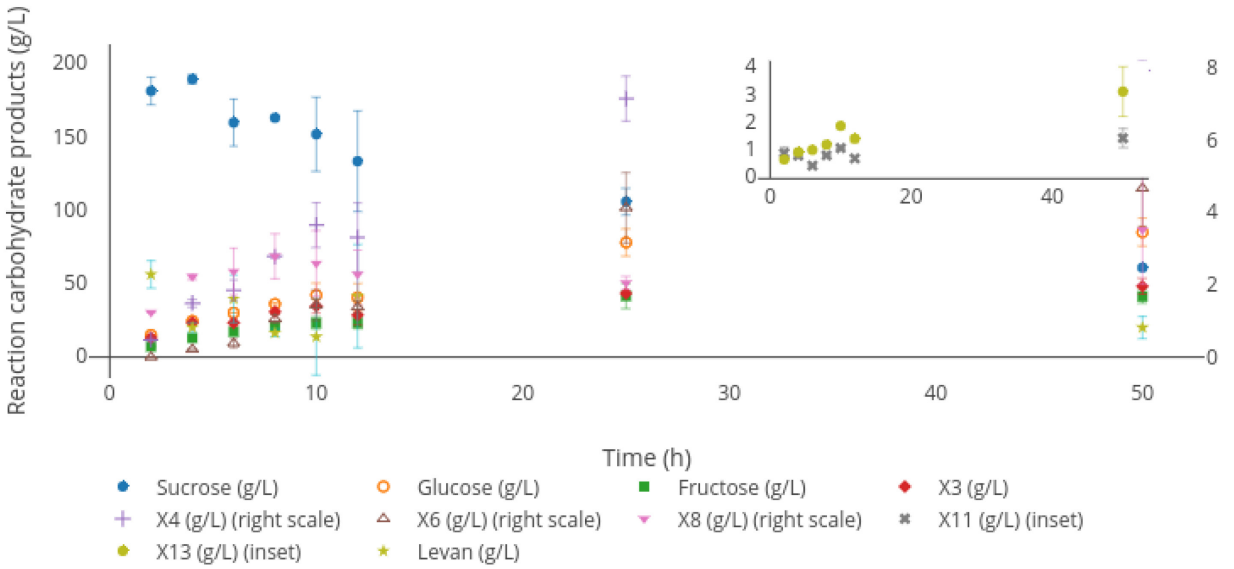


Figure 6.2: Reaction product profile of LS from *Gluconobacter oxydans* with sucrose for 50 h at 30°C. X3 is composed of all trisaccharides quantified (1-kestose, neokestose, 6-kestose), X4, all tetrasaccharides quantified; X6 the hexasaccharides quantified, X8 octasaccharides, X11 undecasaccharide q

terminal His tag of all the LSs screened. Almost half of the levan produced within 50 h was already produced after 2 h. This high level levan production reveals that the LS from *G. oxydans* predominately performs a processive reaction, retaining the product in the active site, ready to accept more fructosyl residues.

The LS from *N. aromaticivorans* utilized the least amount of sucrose after 50 h, with levan production increasing from 25 h to 50 h (33 % w/w transfructosylation products) and total oligosaccharides increasing from 2 h to 25 h, then plateauing at 50 h (66% w/w transfructosylation products). The main products produced by this enzyme were X3, X8, X4, X13 and X11, as seen in Figure 6.3. There was an accumulation of 1-kestose (36% w/w transfructosylation products), with only a small portion of the X3 products being comprised of by neokestose and 6-kestose (1 % and 9 % w/w transfructosylation products respectively) at 50 h. These products may be then utilised as an acceptor substrate for transfructosylation, resulting in higher oligosaccharides and levan. Between 25 h and 50 h the amount of 1-kestose and 6-kestose essentially stayed the same (32 g/L - 30 g/L and 8 g/L – 8 g/L) while their make-up of the total transfructosylation products decreased (63% - 36% and 16% - 9%). Production of 1-kestose and 6-kestose is linear until 10 h' reaction time (19 g/L and 5 g/L), then production plateaus, while production of neokestose remains linear until 25 h. By hour 50, more X3 was synthesized more than X4, X11 and X13.

The LS from *P. graminis* used the highest amount of sucrose amongst all the enzymes tested, shown in Figure 6.4. The high bioconversion of sucrose by LS from *P. graminis* may be attributed to it high stability and/or to the low substrate/product inhibitions. Indeed, this LS was found to have very high thermal stability, with a half-life of 291 mins at 50°C. The largest FOS released upon the transfructosylation reaction of sucrose catalyzed by LS from *B. graminins* was X7 (3% w/w total transfructosylation product) followed by X6 (2% w/w total transfructosylation product), while the largest product produced was X3 (73% w/w total transfructosylation product) and no levan was produced. In the first 2 h there is no evidence of 6-kestose while there is already an accumulation of 1-kestose, levan and X4. Again, the 6-kestose was likely utilised as an acceptor substrate, converted into larger products such as X4 and levan. By hour 12, the levan was consumed, hydrolysed to smaller FOSs and fructose.

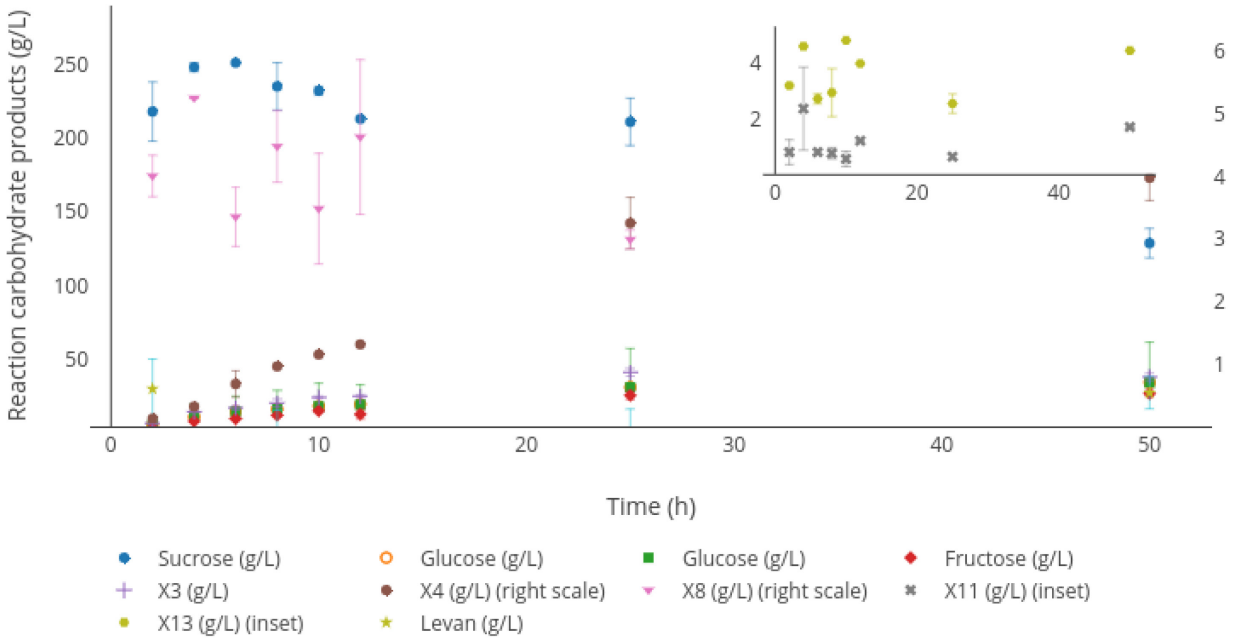


Figure 6.3: Reaction product profile of LS from *Novosphingobium aromaticivorans* with sucrose for 50 h at 30°C. X_3 is composed of all trisaccharides quantified (1-kestose, neokestose, 6-kestose); X_4, all tetrasaccharides quantified; X_8 octasaccharides quantified; X_{11} undecasaccharide quantified; X_{13} the tredecasaccharides quantified

Levan production from the LS from *B. indica subsp. indica* started high (36.7 g/L, 76% w/w transfructosylation products), with the quantity decreasing between 2 and 50 h (23.9 g/L, 31% w/w transfructosylation products) as seen in Figure 6.5. During this time the amount of transfructosylating products steadily increased (12 g/L to 53 g/L). The main products from the incubation of the LS with sucrose (Figure 5) were glucose, fructose, X3, X4, X5 and X7. In that time production of X3, X4 and X5 increased from 20%, 0.8% and 0.1% to 46%, 11% and 3% w/w of the transfructosylation products respectively. X7 also increased from 2 h to 50 h (1 g/L – 3 g/L) but it's make-up of the total transfructosylation products remained the same (3% w/w transfructosylation products).

Comparing the five enzymes amongst each other, the largest oligosaccharides of up to 13 residues were produced by *G. oxydans* (3 g/L, 4% w/w transfructosylation products) and *N. aromaticivorans* (4 g/L, 5% w/w transfructosylation products) LSs'. While the LS from *V. natriegens* produced oligosaccharides of up to 11 residues (0.6 g/L, 1% w/w transfructosylation products) and the LS from *P. graminis* and *B. indica subsp. indica* only produced oligosaccharides of up to 7 residues (5 g/L, 3% w/w transfructosylation products and 3 g/L, 3% w/w transfructosylation products). The LS from *P. graminis* (120 g/L) had the highest production of trisaccharides as compared to the other enzymes, at least double that of all the other LS studied. Comparing production of FOSs from X3-X13 by weight, the LS from *P. graminis* produced the most at 164 g/L, followed by the *G. oxydans* (68 g/L), *V. natriegens* (68 g/L), *N. aromaticivorans* (55 g/L), *B. indica subsp. indica* (53 g/L) and lastly. The order changed slightly with the comparison of total transfructosylation products (including levan). The highest amount total transfructosylation products was produced by the LS from *P. graminis* (164 g/L), *G. oxydans* (88 g/L), *N. aromaticivorans* (83 g/L), *B. indica subsp. indica* (77 g/L) with the lowest production coming from *V. natriegens* (68 g/L). The highest FOSs yields percentage wise, as compared to the initial amount of sucrose, all occurred at 50 h. The highest was achieved by the LS from *P. graminis* (60 %), which is high as compared to *B. macerans* EG-6 (33 %) (Park et al., 2003) and *Z. mobilis* (32 %) (Bekers et al., 2002). The LS from *V natriegens* produced the most levan (84.3 g/L), followed closely by *B. indica subsp. indica* (83.5 g/L), then by *G. oxydans* (55.8 g/L), *N. aromaticivorans* (30.5 g/L) and lastly the *P. graminis* (2.0 g/L). The yields from both *V.*

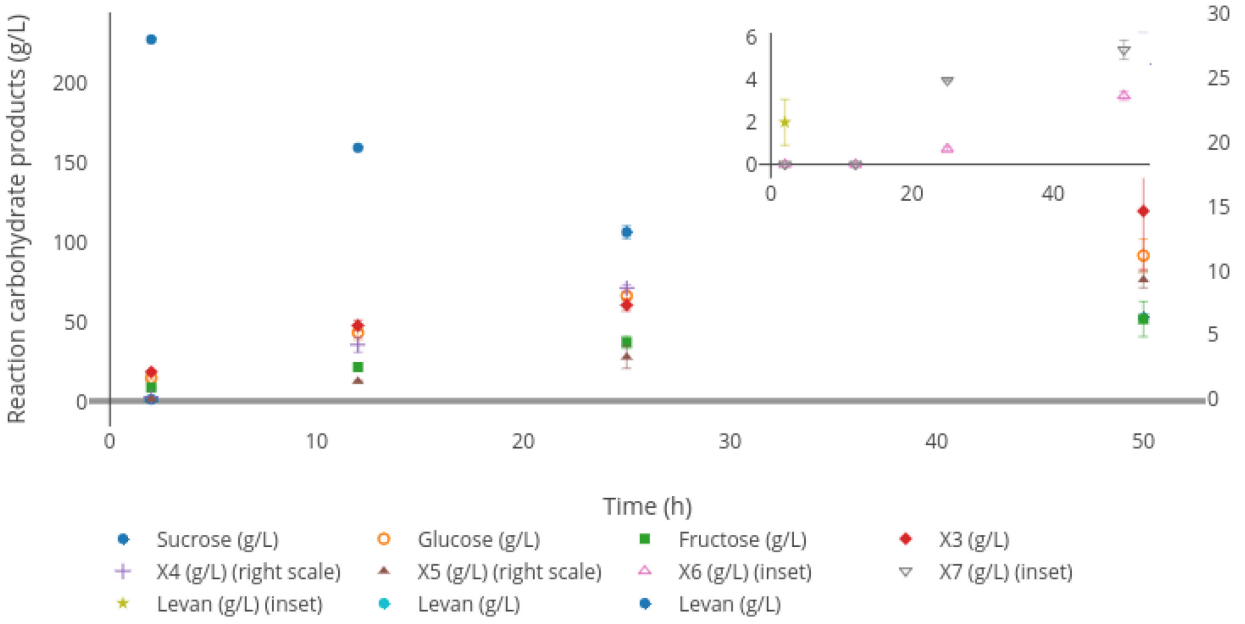


Figure 6.4: Reaction product profile of LS from *Paraburkholderia graminis* with sucrose for 50 h at 30°C. X_3 is composed of all trisaccharides quantified (1-kestose, neokestose, 6-kestose); X_4, all tetrasaccharides quantified; X_5 the pentasaccharides quantified; X_6 the hexasaccharides quantified; X_7 all heptasaccharides quantified**.**

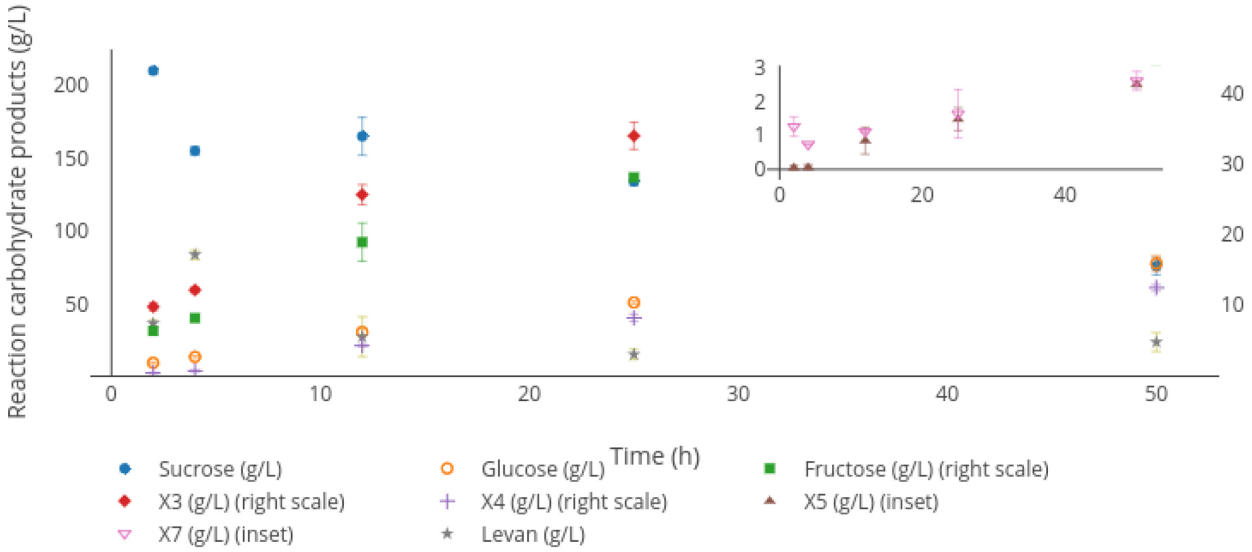


Figure 6.5: Reaction product profile of LS from *Beijerinckia indica subsp. indica* with sucrose for 50 h at 30°C. X_3 is composed of all trisaccharides quantified (1-kestose, neokestose, 6-kestose); X_4, all tetrasaccharides quantified; X_5 the pentasaccharides quantified; X_7 all heptasaccharides quantified.

natriegens, B. indica subsp. indica and *G. oxydans* are all quite high for Gram-negative bacteria. Their levan production resembles the LS from Gram positive bacteria, such as *B. subtilis* CCT7712 (111.6 g/L) (Dos Santos et al., 2013) and *B. methylotrophicus* SK 21.002 (100 g/L) (Zhang et al., 2014). The LS of *N. aromaticivorans* resembles more typical Gram-negative levan production, similar to that *G. diazotrophicus* SRT4 (L. Hernandez et al., 1995b), with levan production of 24.7 g/L or *A. xylinum* NCIM 2526 with production of 13.25 g/L (Srikanth, Siddartha, et al., 2015).

6.3.2. Amino acid sequence comparison

The amino acid sequence of each of the 5 LS enzymes were compared against the sequences belonging to LS to which there are crystal structures available. These sequences include the LS from *B. megaterium* (3OM2, mutation D257A), *B. subtilis* (1OYG), *E. amylovora* (4D47) and *G. diazotrophicus* (1W18). The amino acid sequence of the LS from *B. amyloliquefaciens*, whose characteristics have been extensively studied in our lab, was also added to the alignment. The sequence-based alignment of all the LSs' are listed in Figure 6.6. Clustal Omega (Sievers et al., 2011) was used to perform the alignment and the results were analysed using Jalview 2.10.1 (Waterhouse, Procter, Martin, Clamp, & Barton, 2009).

LS follows a ping-pong mechanism, involving three conserved amino acids which are essential to catalysis. In BS SacB, they were Glu^{342}, Asp^{86} and Asp^{247} (highlighted in red) (Homann et al., 2007; Meng & Futterer, 2003), and conserved amongst all LSs. Glu^{342} takes part in forming a H-bonds with glucosyl residue of the incoming sucrose and acts as an acid base catalyst (Meng & Futterer, 2003).

When sucrose enters the active site, it is oriented and positioned by amino acids corresponding to the -1 and +1 subsites. Trp^{85}, Arg^{246}, Trp^{163} from BS SacB (highlighted in blue; Trp^{271}, Arg^{423}, Trp^{340} in LS from *L. reuteri*; Trp^{95}, Arg^{256}, Trp^{172} in the LS from *B. megaterium*) are highly conserved within family GH68 and contribute to the -1 subsite which interacts with the fructosyl unit of sucrose (Ozimek et al., 2006). Trp^{85} forms H-bonds with the 6-OH of fructofuranoside, Arg^{246} coordinates with 3-OH, 4-OH of the fructofuranoside and the 4-OH of the glucopyranoside while Trp^{163}, is part of the +1 subsite (Homann et al., 2007). These residues were entirely conserved in the 5 LSs which were examined. This will maintain the orientation of the sucrose

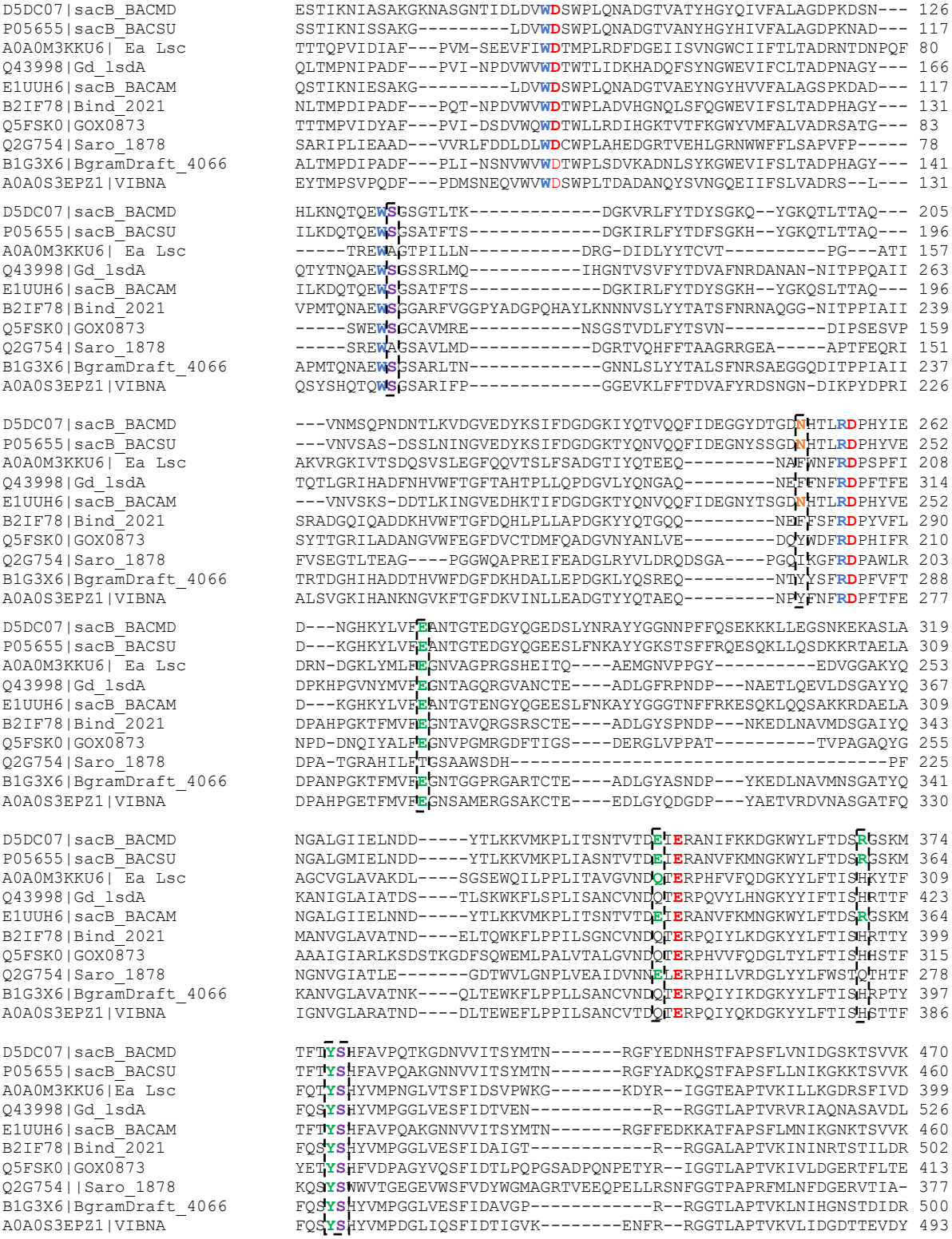


Figure 6.6: CLUSTAL O(1.2.4) multiple sequence alignment of LS enzymes with characterized crystal structures and enzymes investigated. The sequences for the LSs from *B. megaterium* (SacB_BM, D5DC07), *B. subtilis* (SacB_BS, P05655), *E. amylovora* (Ea Lsc, A0A0M3KKU6), *G. diaztrophicus* (Gd lsdA, Q43998), *B. amyloliquefaciens* (SacB_BACAM, E1UUH6), *B. indica subsp. indica* (Bind_2021, B2IF78), *G. oxydans* (GOX0873, Q5FSK0), *N. aromaticivorans* (Saro_1879, Q2G754), *P. graminis* (BgramDRAFT_4066, B1G3X6) and *V. natriegens* (VIBNA, A0A0S3EPZ1). Some residues excluded.

within the active site of each enzymes. Other amino acids (from Bs SacB) which interact with the incoming sucrose at the glucosyl residue are Glu^{340} which for forms H-bonds with the 3-OH and the 4-OH, Arg^{360} interacts with 2-OH and 3-OH groups, Tyr^{411} interacts with the 2-OH while Arg^{246} forms H-bonds with the 4-OH of the glucopyranoside residue as well as the 3-OH group of the fructofuranoside (Homann et al., 2007; Meng & Fütterer, 2008). Residues were highlighted in green. Meng et al. (2003) found that the Tyr^{411} rotated 17° downwards upon sucrose binding (Meng & Futterer, 2003). The investigated residues of corresponding to Tyr^{411} and Arg^{246} were both conserved, while Glu^{340} is maintained for the LS from *B. amyloliquefaciens* and *N. aromaticivorans* but was replaced with glutamine in the LSs' expressed by *B. indica subsp. indica*, *G. oxydans*, *P. graminis* and *V. natriegens*. The LS from *Erwinia amylovora* and *G. diazotrophicus*, both Gram-negative LSs, had glutamine residue at this position (Martinez-Fleites et al., 2005; Wuerges et al., 2015). The sucrose-binding site was found to be maintained with this substitution (Martinez-Fleites et al., 2005).

In the Bs SacB, there were two Serine residues (Ser^{164} and $Ser^{412,}$ highlighted in purple) which formed H-bonds with Asp^{86}, orientating it within the active site. Ea Lsc (*E. amylovora*) only had one Serine instead of 2 (Ser^{353}), with the other serine replaced by an alanine (Wuerges et al., 2015). Hommann et al. (2007), found that a mutation of this serine, Ser^{173} in Bm SacB (LS from *B. megaterium*) to an alanine, didn't alter the binding mode of sucrose, but did decrease catalytic activity (Homann et al., 2007). The LS from *N. aromaticivorans*, contained an Ala^{118} and a Ser^{323}. All the other enzymes contained serine residues at both positions.

Glu^{262} from BS SacB (highlighted in green), is part of the hydrogen bond network with Arg^{246}, Tyr^{411}, Arg^{360} and Glu^{342} (Meng & Futterer, 2003), and was conserved amongst all LSs currently examined with the exception of the LS from *N. aromaticivorans*, which had a Thr^{215} at this position. This hydroxyl group will group will have the potential to form hydrogen bonds, but the length of the side chain is slightly smaller than the chain from glutamate. This may decrease the intensity of the hydrogen bond network. Perhaps increasing the flexibility of the active site.

With the sucrose donor stabilized and oriented within the active site, Glu^{342} (Bs SacB) protonates the leaving group (glucose). Interactions stabilizing the glucosyl residue are withdrawn and switch to inter-enzyme interactions. Arg^{360} switches rotamer states and forms hydrogen bonds with Glu^{340}. Asp^{86} performs a nucleophilic attack on the glucopyranosyl residue, inverting the

glycosidic bond, releasing glucose and forming an enzyme intermediate with the fructofuranosyl residue (Meng & Fütterer, 2008). Asp^{247} continues to interact with the fructofuranoside, stabilizing the intermediate (Meng & Futterer, 2003). As Glu^{342} and Asp^{86} are essential for catalysis, they were both completely conserved by the 5 enzymes examined as well as *B. amyloliquefaciens* LS.

As discussed previously, LS enzymes follow a Ping-Pong mechanism. After the enzyme-intermediate is formed with the fructofuranosyl residue, an acceptor molecule must enter the active site, and be positioned properly to perform nucleophilic attack with the 6'-hydroxyl of the anomeric carbon to the C2 residue of the fructofuranoside bound to the enzyme (Homann et al., 2007).

Arg^{360} in BS SacB, essential for polysaccharide synthesis, was replaced with His in *B. indica*, *G. oxydans*, *P. graminis* and *V. natriegens* (Meng & Futterer, 2003). This substitution is typical with gram negative bacteria and leads to the formation of oligosaccharides instead of levan (Wuerges et al., 2015). Surprisingly, *G. oxydans* produced a large amount of levan while *V. natriegens* and *P. graminis* produced smaller amounts as seen in Figure 6.1, 6.2 & 6.4. This residue in–*N. aromaticivorans* LS was replaced by a Gln^{274}. While glutamine has the potential for a positive charge, it's side chain is significantly smaller than that of Arginine. *N. aromaticivorans* LS was not able to synthesize levan to any real degree.

Another amino acid which was found to be essential for polysaccharide growth was Asn^{242} (highlighted in orange), located in subsite +2 (Meng & Futterer, 2003). Mutation of *B. megaterium* SacB Asn^{252} (Asn^{242} in Bs SacB) to Alanine removes polymerase activity, while a mutation to an amino acid with a side chain, such as aspartate, maintained polymerase activity (Homann et al., 2007). This residue was conserved for the LS from *B. amyloliquefaciens* and *V. natriegens*, while it was replaced with Phe^{280} and Ile^{193} from the LS from *B. indica subsp. indica* and *N. aromaticivorans* respectively. Unlike asparagine, phenylalanine and isoleucine both have neutral side-chains and were unable to produce levan. The asparagine was maintained in both *E. amylovora* (Asn^{200}) and *G. diazotrophicus* (Asn^{306}), but structurally, was located 10 Å away and unlikely to interact with the saccharide (Wuerges et al., 2015). There was no structural alignment with any sequences at this position for the LS from *P. graminis* and *G. oxydans*.

6.3.3. Examination of LS active site

A homology-based model of LS from *B. amyloliquefaciens* was created using the crystal structure of LS from *B. subtilis* (PDB 1PT2), bound with sucrose with the mutation E342A. Homology models were created for LS from *B. indica subsp. indica* and *P. graminis* from the crystal structure of *G. diazotrophicus* (1W18), *V. natriegens* from the crystal structure of β-fructofuranosidase by *Microbacterium saccharophilum* K-1 (3VSR) and the LS *G. oxydans* and *N. aromaticivorans* using the crystal structure of *E. amylovora* (4D47) with the sucrose hydrolysis products trapped within the active site.

Autodock 4.2 was used to perform rigid docking with fixed LS models and flexible ligands, sucrose, glucose and fructose. The representation of the molecule within the active site represents the orientation with the least binding energy conformation. The models with the docking for each enzyme are represented in Figures 6.7-6.9. Docking was used to examine some of the catalytic differences between each enzyme.

Initial observations of the cavities of the enzymes tested and *B. amyloliquefaciens* LS were compared to the deep negatively charged pocket *of B. subtilis* (Figure 6.7a). The LS from *B. amyloliquefaciens*, a Gram-positive bacterium, produced dominantly high-molecular weight levan up to 10^4 kDa (Tian et al., 2011). It's kcat for transfructosylation was much higher than it was for the Bs SacB (LS from *B. subtilis*). Although the catalytic efficiency for hydrolysis (9.5 $s^{-1}mM^{-1}$) from the LS from *B. amyloliquefaciens* was much higher than it was for transfructosylation (2.5 $s^{-1}mM^{-1}$), the value for transfructosylation was very similar to the catalytic efficiency of hydrolysis (2.9 $s^{-1}mM^{-1}$) and transfructosylation (2.3 $s^{-1}mM^{-1}$) from Bs SacB (Olvera et al., 2012; Tian & Karboune, 2012).

The shape of the active site of the LS from B. *amyloliquefaciens* based upon electrostatic surface potential of the LS from *B. amyloliquefaciens* is similar to that of Bs SacB. The *B. amyloliquefaciens* LS had increased charged residues. Comparing the least binding energy conformation of sucrose (Figure 6.7b), the sucrose is turned counter-clockwise by approximately 15° and inverted. The cavity of the LS for *B. indica subsp. indica* (Figure 6.7c) was wider, with a

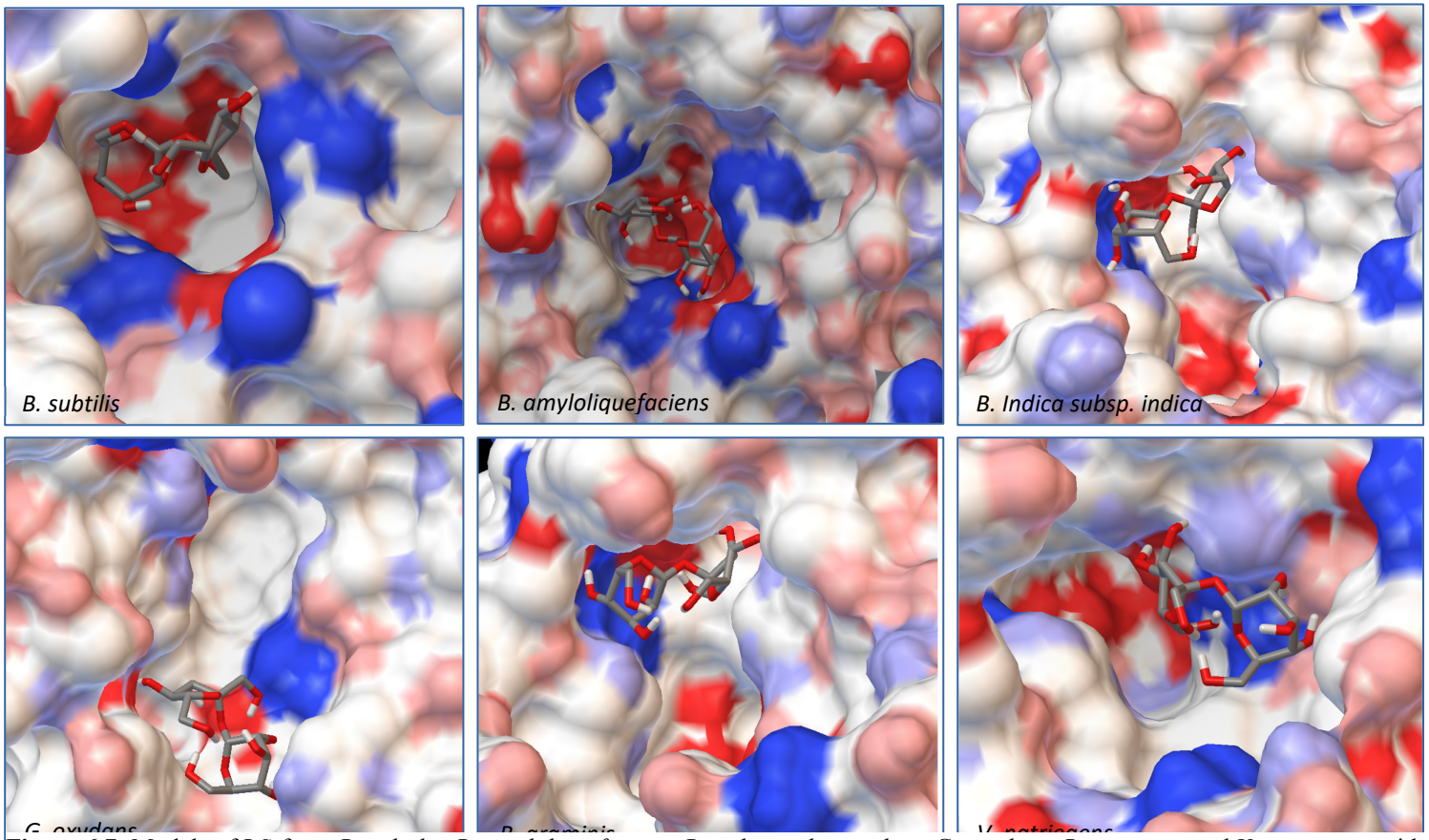


Figure 6.7: Models of LS from *B. subtilis, B. amyloliquefaciens, B. indica subsp. indica, G. oxydans, B. graminis and V. natriegens* with sucrose docked within the active site. Sucrose is docked in a position representing the least binding energy state. Colouring represents electrostatic surface potential.

deeper region and fewer charged residues. Sucrose was positioned much in the same way as Bs SacB, with the glucopyranoside residue slightly tilted backwards. The cavity of the LS from *G.oxydans* (Figures 6.7d/6.8d) was similarly shaped but less deep, with many charged residues concentrated together on the exterior of the active site. Sucrose within the active site was rotated 45° downwards. *P. graminis* LS active site (Figure 6.7e) is deeper than that of Bs SacB. It has more positive and less negative electrostatic potential, with sucrose in an extremely similar orientation but with it being less deep within the cavity. *V. natriegens* LS active site (Figure 6.7f) is wider and shallower and had more positive and less negative electrostatic potential. Like *P. graminis* LS, the sucrose was orientated in the same way as Bs SacB. The structures of these enzymes were compared to their kinetic values. *B. subtilis* LS had the lowest Km values for transfructosylating and hydrolytic activity, while the other enzymes had Km values for transfructosylation ranging from 436 mM –944 mM and values for hydrolysis from 1.116 mM – 617 mM (Hill et al, 2017). The lower the Km value, the higher the affinity of the enzyme for sucrose. The interior of the LS from *G. oxydans* had a lack of charged residues as compared to Bs SacB, correlating to a lower affinity for sucrose, which can be seen by the higher Km values for transfructosylation and hydrolysis (674 mM and 617 mM). *G. oxydans*'s LS had the highest kcat for both transfructosylating (331999 s^{-1}) and hydrolytic activity (342570 s^{-1}). The shallow pocket of the active site may allow for a faster turnover of the substrate. The concentrated residues on the exterior of the active site can interact with the growing fructan product, resulting in a processive reaction. The large levan produced by this enzyme (6986 kDa) and the high oligosaccharides (up to 13 residues in length) were evidence of this.

The LS from *V. natriegens* had the next highest kcat for transfructosylation (152369 s^{-1}) and hydrolysis (247364 s^{-1}). This enzyme also has a concentration of charged residues running along the left side of the active site; helping to contribute to levan production which was mid-level (8.41 mg/g protein) and of a relatively low size (586 kDa). There were also smaller and fewer FOSs (X11; 0.6 g/L) produced by the LS from *V. natriegens*. With sucrose orientation within the active site, the products produced would be directed towards where the line of charged residues lie**.** *V. natriegens* LS's had a high Km for hydrolysis (2460 mM), the charges located within the active site would contribute to the affinity and positioning of the sucrose for hydrolysis. Comparing the location of fructofuranoside residue in Figure 6.9f, it is completely to one side of the cavity, away

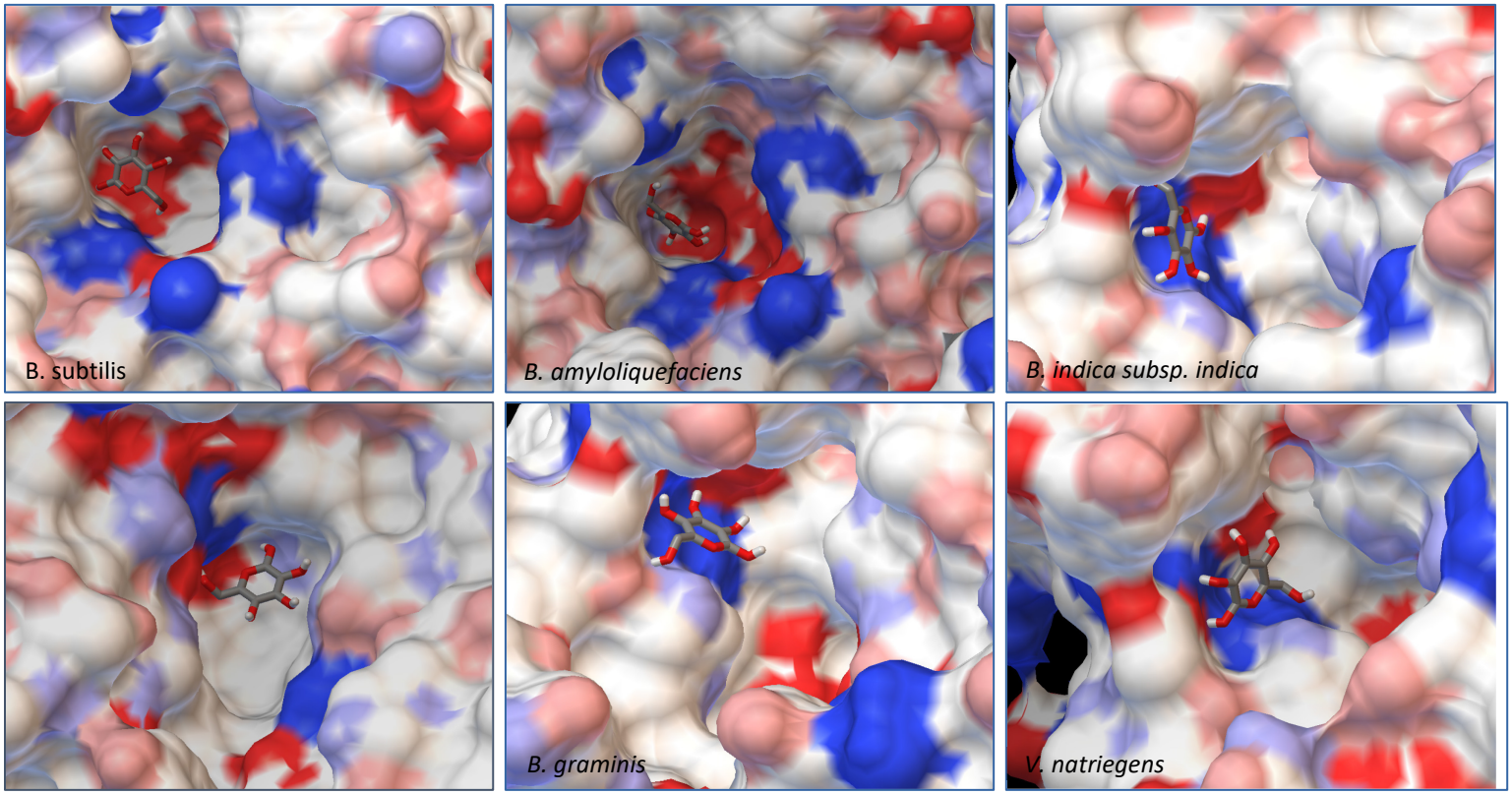


Figure 6.8: Models of LS from *B. subtilis, B. amyloliquefaciens, B. indica subsp. indica, G. oxydans, B. graminis and V. natriegens* with glucose docked within the active site. Glucose is docked in a position representing the least binding energy state. Colouring represents electrostatic surface potential.

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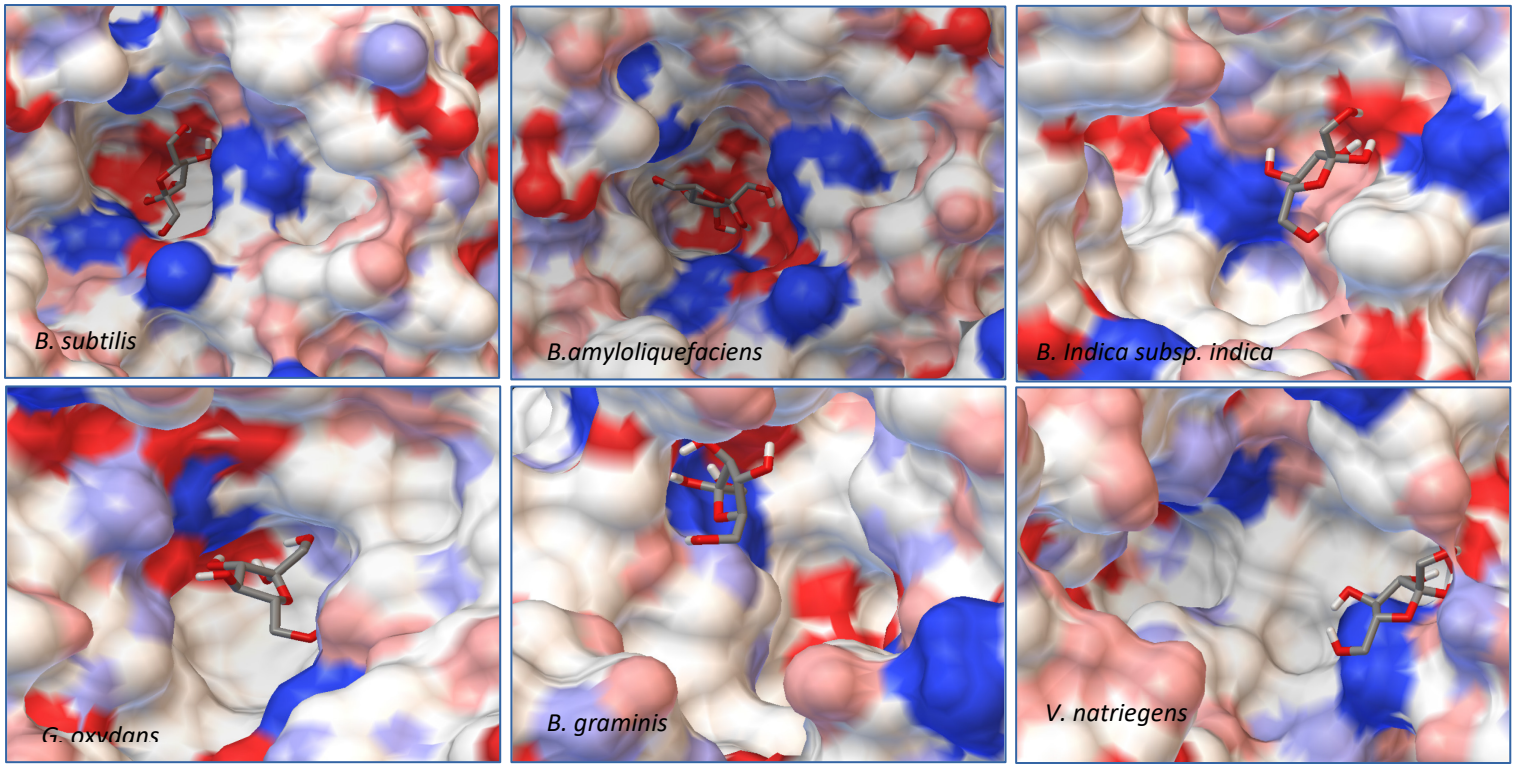

Fi**gure 6.9**: Models of LS from B. subtilis, B. amyloliquefaciens, B. indica subsp. indica, G. oxydans, B. graminis and V. natriegens with fructose docked within the active site. Fructose is docked in a position representing the least binding energy state. Colouring represents electrostatic surface potential.

from where a fructan product would interact. This may increase the likelihood of hydrolysis by decreasing the feasibility of transfructosylation. The LS from *B. indica subsp. indica* also had high activity (kcat transfructosylation 9186 s^{-1}; kcat hydrolysis 7981 s^{-1}). Similar to *G. oxydans* LS, the cavity was wide but had fewer charged residues. While this LS produces a moderate amount of levan (6.4 mg/mg protein) of a decently large size (2128 kDa), there was no concentration of charged residues on the outer rim of the active site, unlike the LS from *G. oxydans*. The lack of charged residues on the exterior of the active site corresponds to the products of this LS, being of a low degree of oligomerization, with 7 residues being the largest oligosaccharide produced. The Km for transfructosylation of LS from *P. graminis* (479 mM) was higher than the Km for hydrolysis (19.6 mM). Indicating the orientation of sucrose within the active site of *P. graminis* makes it more susceptible to hydrolysis than transfructosylation. The kcats of the two activities were very comparable ($kcat_{trans}$ 4723 s^{-1}, $kcat_{hydro}$ 5687 s^{-1}). During the time course trials, there was levan production at 2 h, but it was hydrolysed by 12 h. On previous inspection after 2 weeks incubation with sucrose, there was positive levan production (15.5 mg/mg protein, 1790 kDa).

B. amyloliquefaciens LS had a similar shaped cavity to that of *B. subtilis*, but with more charged residues. The increase in charged residues may have contributed to the elevated kcat values for transfructosylation (1137 s^{-1}) and hydrolysis (179 s^{-1}) (Tian & Karboune, 2012). The rotation of sucrose found in the active site may have also contributed to this increased activity. The Km value for transfructosylation was 20 times larger than they were for Bs SacB. Indicating that this active site has much greater affinity for the hydrolysis substrates (water) than for the transfructosylation substrates (acceptor molecules). The extra charges within the active site may also contribute to this.

6.3.4. Acceptor Specificity of Selected LSs

LS's ability to utilize alternate acceptor molecules for transfructosylation can give rise to the synthesis of multiple sucrose analogs and FOSs headed with new groups. These molecules have a variety of potential applications, and a more simplistic route to their high yield synthesis could be very advantageous. Due to the relaxed binding nature of the +1 subsite, there can be some variability of the docking of acceptor molecules (Visnapuu et al., 2011). Each LS examined was incubated with a fructosyl donor molecule (sucrose or raffinose) in excess and an acceptor molecule (sucrose, raffinose, glucose, galactose, maltose, lactose, xylose, sorbitol and catechol).

The results of acceptor reactions are reported in Figure 6.10 Comparing the percentage of each acceptor used for transfructosylation, the LS from *V. natriegens* consumed the combined largest amount of acceptor molecules, followed by LS from *P. graminis*, *G. oxydans*/*B. indica subsp. indica* and *N. aromaticivorans*.

As seen in Figure 6.10 a-c, the LSs used both sucrose and raffinose as acceptor molecules. The consumption of sucrose increases sub-linearly from 2-50 h, while the same was seen for raffinose incubated with LS from *V. natriegens*. The other LS enzymes experienced a high amount of raffinose consumption initially at 2 h, with less or the same amount used at hour 50 h. The products of transfructosylation with raffinose can later be used as the acceptor molecule for another transfructosylation reaction. These molecules can also be hydrolysed, eventually releasing a raffinose molecule and fructose molecules into the system. Similarly, the frustosyl-raffinose can also be used as a fructosyl donor molecule, creating other larger hetero-FOSs. Looking at the percentage of transfructosylation and hydrolysis (results not shown), the later situation seems to be what is occurring for the LS enzymes from *G. oxydans*, *N. aromaticivorans* and *P. graminis*. The percentage of transfructosylation products increased with incubation time. Comparing the use of sucrose and raffinose used separately, the LS from *V. natriegens*, *N. aromaticivorans* and *B. indica subsp. indica* preferred raffinose as the substrate. The LS from *Zymonanas mobilis* was also found to prefer raffinose to sucrose as a fructosyl donor (Andersone et al., 2004). An advantage of LSs ability to also catalyze the hydrolysis of the donor molecule is that LS can effectively produce meilbiose, a molecule with beneficial health properties (Xu et al., 2017), from raffinose. The LS from *N. aromaticivorans* had the highest amount of hydrolysis (48 %), compared with the other enzymes (16 – 38 %). All the LSs were able to produce a few different (1-3) trisaccharides using raffinose as the sole substrate. A greater variety of tetrasaccharides (3-4) was produced with all enzymes producing between 1-3 pentasaccharides. The LS from *P. graminis* catalyzed the synthesis multiple oligosaccharides using raffinose, a heptasaccharide (X7), two octasaccharides (X8) and a hendecasaccharide (X11). There was less diversity of oligosaccharides produced by the other enzymes but each produced a hendecasaccharide and tridencasaccharides (X13).

The synthesis of galactose-headed FOSs was desired since they can provide interesting prebiotic activity. Multiple species of LS are capable of utilizing galactose as a fructosyl-acceptor molecule

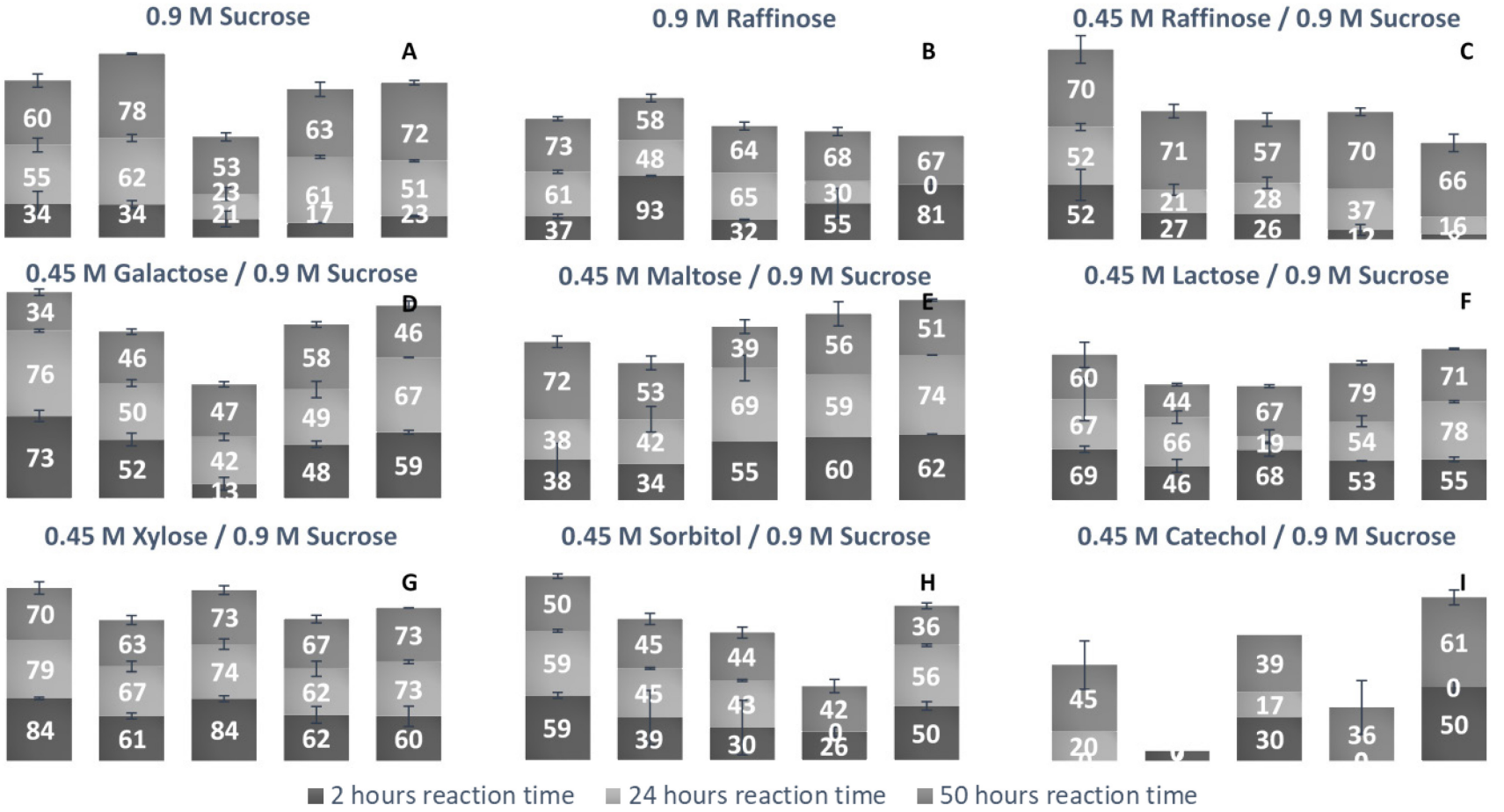

Figure 6.10: Bioconversion of various monosaccharides, disaccharides, and trisaccharides as acceptor/donor molecules by various LSs as a function of time. Column 1: LS from *V. natriegens*; column 2: LS from *G. oxydans*; column 3: *N. aromaticivorans*; column 4: LS from *P. graminis*; column 5: *B. indica subsp. indica*

(Hill, Tian & Karboune, 2017). In Figure 6.10d, the LS from *V. natriegens* consumed a large amount of galactose within the first 2 h of incubation, then by hour 50, much of the galactose was released into the system. The LS from *B. indicia subsp. indica* and *G. oxydans* followed a similar trend. The release of galactose could be due to the hydrolysis of the fructosylated galactose products. When the sucrose analogue, Gal-Fru was incubated with Bs SacB, 52% was converted into transfructosylated products, while the rest (48 %) was hydrolysed (Beine et al., 2008). At 50 h, the LS from *P. graminis* consumed the most galactose (58 %). The greatest consumption of galactose occurred within the first 2 h of incubation, then only slightly increased over the remaining 48 h. Early consumption of galactose by the LS from *V. natriegens*, *G. oxydans*, *P. graminis* and *B. indica subsp. indica* relays that the Km values of the enzymes towards galactose values are relatively small.

By regarding Figure 10e, it can be seen that all LSs tested here were able to consume maltose to a high degree. Since the utilization of maltose requires the transfructosylation of a glycopyranosyl residue, making the glycosidic bond between maltose and fructose similar to what occurs in sucrose. The LS from *Rahnella aquatilis*, *B. subtilis*, *B. amyloliquefaciens* and *Geobacillus stearothermophilis* were all able to use maltose to a high degree as an acceptor molecule (Lotthida Inthanavong et al., 2013; Ohtsuka et al., 1992; Juergen Seibel et al., 2006; Tian & Karboune, 2012). The peaks produced using with maltose and sucrose as the substrates were compared to those produced using solely sucrose. Two new trisaccharide peaks were identified from the LSs from *B. indica subsp. indica* while one new trisaccharide was produced by the LSs from *N. aromaticivorans*, *G. oxydans*, *V. natriegens* and *P. graminis*. Both *P. graminis* and *B. indica subsp. indica* catalyzed the synthesis of a pentasaccharide using maltose while both of these enzymes and *V. natriegens* were able to catalyze the synthesis of a heptasaccharide using maltose.

Another fructosyl acceptor which was widely used by all LSs was lactose as seen in Figure 10F. Each of these enzymes can be used to make the must desired prebiotic lactulosucrose (Rycroft et al., 2001). In all of the cases, lactose was predominately used within the first 2 h. With lactose, all enzymes were able to catalyze the synthesis pentasaccharides with the exception of the LS from *G. oxydans*, while this enzyme was able to produce a hexasaccharide using lactose. Both the LSs from *P. graminis* and *B. indica subsp. indica* catalyzed the synthesis of a heptasaccharide.

Looking at Figure 6.10g, all LS enzymes examined here used xylose to a high degree within the first 2 h, with a minimum 60% of xylose consumed. The LSs from *V. natriegens* and *N. aromaticivorans* had some of their xylose products hydrolysed, returning xylose. Xylose, was also found to be a good acceptor molecule for Bs SacB, yielding β-D-fructofuranosyl-α-D-xylopyranoside (Juergen Seibel et al., 2006). The reasoning for the high consumption of xylose was suspected to be due to the equatorial position of the C2-OH, which was found to be in a good position for protonation by the active site (Meng & Futterer, 2003). Using the profile with the LSs enzymes and sucrose for comparison, there were additional peaks indicating new trisaccharides (2) and tetrasaccharides (2) from all enzymes apart from *G. oxydans*. Interestingly, the LS from *G. oxydans* was able to catalyze the synthesis of hexasaccharides, along with the LS from *B. indica subsp. indica*. Both enzymes were capable of catalyzing the synthesis of levan. There is the distinct possibility that levan was produced with a xylose head group and hydrolyzed into small FOSs. Alternatively, the LSs may have catalyzed, solely through transfructosylation, the hetero-FOSs.

An alditol and a benzene diol were tested for their use as acceptor molecules as seen in Figures 6.10 h-i. The ability to use alcohols as acceptor molecules is not a common attribute to LSs (Hill, Tian, & Karboune, 2017). Catechol had a much lower signal on the PAD, which made its detection much more difficult when the quantities were low. The lowest detection limit of catechol was 10 μM, limiting its detection when the substrate was highly used. The LS from *V. natriegens* used the highest amount of sorbitol, most of it within the first 2 h. By hour 50, approximately 10% was hydrolysed. The other enzymes used approximately 40% of the sorbitol. There was a peak indicating Sor-Fru and two new trisaccharide peaks from *N. aromaticivorans* LS and the *V. natriegens* LS. Also seen was a hexasaccharide peak from *G. oxydans* and *B. indica subsp. indica*. Catechol was an efficient acceptor molecule for fructose. By hour 50, the LS from *B. indica subsp. indica* had consumed over 60% of the initial catechol. This enzyme produced new peaks indicating a trisaccharide, a heptasaccharide and a nonasaccharide. The LS from *G. oxydans* had a product profile indicating the use of catechol in a hexasaccharide and heptasaccharide while the LS from *P. graminis* catalyzed the formation of a heptasaccharide and a nonasaccharide using catechol. Mena-Arizmendi et al. (2011) found that Bs SacB was more capable of fructosylating secondary alcohols than primary alcohols, due to an inverted relationship between pKa and the ability of Glu342 to deprotonate the hydroxyl group (Mena-Arizmendi et al., 2011). While the pKa of sorbitol is 13.57, and higher than the pKa of catechol (9.48), they were both similarly used as

acceptor molecules (Sergeant, Dempsey & Boyd, 1979). The additional hydroxyl groups on sorbitol may have provided additional sites for stabilization within the active site, resulting in good yields for transfructosylation.

The *B. indica subsp. indica* showed a general trend of catalyzing the synthesis of the larger hetero-FOSs ($\geq$ 6 units). Using the example of the sucrose incubation, this enzyme had also produced a large amount of levan (84 g/L) by hour 4 of the incubation, which had decreased to 24 g/L by hour 50. This is evidence that this LS hydrolyses its produced levan. With the acceptors and sucrose incubation, the levan may be produced with the acceptor molecule at its head and later hydrolysed into the hetero-FOSs. With the LS from *G. oxydans*, most frequently the largest molecule incorporating the alternative acceptor molecule was a hexasaccharide. With sucrose as the sole substrate, this enzyme produced a large amount of hexasaccharides (5 g/L) as well as the smaller molecules (trisaccharides and tetrasaccharides). This may be a reflection of the structure of the enzyme. As seen in Figure 6.8, there is a line of charged residues capable of interacting with the products. Upon reflection of the results, these residues likely have a strong interaction up to 6 residues in length.

6.4. Conclusion

The investigated enzymes had interesting properties in terms of the production of high level FOS. The LS *P. graminis* produced the largest amount of FOSs (164 g/L), predominately smaller molecules. Two of these enzymes, the LS from *G. oxydans* and *N. aromaticivorans* were both able to produce large FOSs (X13) with 14.5% and 11.3% total FOS molar yields (68 g/L and 55 g/L) respectively. A further approach to large FOS production may include using co-solvents. Co-solvents can aide in precipitating the larger product, thereby further driving the thermodynamic equilibrium towards FOS production.

There was high conservation of the Asp^{339} among the LS residues with all but the LS from *N. aromoaticivorans* retaining aspartate. This amino acid contributed to the calcium binding of the enzyme (Meng & Futterer, 2003). Incubation in a calcium solution may be the next step to further improve the activities of the investigated enzymes.

Each enzyme investigated had a wide substrate specificity. A further examination of the reasons why this is so can occur through docking experiments with the LS models, which is the next step

in our study. The ability for these LSs to transfructosylate aromatic and aliphatic alcohols is an interesting attribute, providing more potential synthetic applications. Using substrate engineering, and potentially using protecting groups, the site of transfructosylation can be controlled (Seibel & Buchholz, 2010). With the success already achieved in these trials, there is the potential for the transfructosylation of more elaborate substrates for the easier synthesis of glycosylated compounds.

CHAPTER VII GENERAL SUMMARY & CONCLUSIONS

The main objective of this research was to investigate the biocatalytic approach, based on LS-catalysed transfructosylation reaction, for the production of β(2→6) FOSs, neolevans and levan. The main body of this research was devoted to overcoming LS's shortcomings, including the limited availability of LS enzymes, their low thermal stability and the propensity to hydrolyse the fructosyl donor molecule, namely, sucrose, as the result of the weak reaction selectivity.

To make LS more practical and to enhance their thermal stability, LS was immobilized onto modified and unmodified commercial and natural supports. Successful immobilization was achieved on a few supports. High activity per gram of support was achieved using the cationic supports Eupergit® C-EDA, Eupergit® C-IDA/Cu, Sepabeads® HA and glyoxyl agarose-IDA/Cu, but not by all the cationic supports tested. Interestingly, Sepabeads® HA provided an environment which encouraged transfructosylation over hydrolysis. Based upon the results of our study, covalent bond formation was found to be a requirement for an increase in thermal stability through immobilization. The information garnered in the screening of immobilization supports not only resulted in the identification of appropriate supports for the immobilization of LS from *B. amyloliquefaciens* but provided interesting parameters to control for the better modulation of the immobilization of LSs from other bacterial sources. The overall best support for the immobilization of LS from *B. amyloliquefaciens* was identified to be glyoxyl agarose-IDA/Cu as it achieved high immobilization yields and great thermal stability and it didn't direct the reaction selectivity of immobilized LS towards hydrolysis.

Parameters were found to affect the immobilization efficiency of LS, such as buffer molarity, pH, immobilization time and protein loading. RSM was successfully utilized to optimize the initial orientation of LS (pre-covalent bond formation) onto the support, glyoxyl agarose-IDA/Cu. This study examined the interactions between the immobilization parameters and how they affected the immobilization responses (retention of activity, protein yield and activity yield). The results can be conferred to a certain extent to other LS enzymes as well as other cationic supports. Providing a basis point for the ranges to be explored. Unfortunately, the reduction step, necessary for covalent bond formation when immobilizing with glyoxyl agarose-IDA/Cu, was found to adversely affect LS activity. The cationic polymer, PEI, proved capable of stabilizing the LS onto the support in place of covalent bond formation. This polymer can be used in other capacities in conjunction with LS.

There is a limit to the capabilities an immobilization support can accomplish. To find LS enzymes with great catalytic potential, an exploration of enzymes with similar sequences to those of known LSs was performed. A screening was completed based upon total activity, transfructosylation potential and levan production. This process may be repeated as more LS enzymes are discovered. More structures would expand upon the BLAST search. The screening can be enlarged as well, to search for alternative activities. The initial screening provided 10 enzymes with promising activity worth investigating. A few candidates stood out, such as the LS from *G. oxydans*, which produced exceptionally large LS (6984 kDa) with good yields. Alternatively, the LS from *P. graminis* remarkedly high thermal stability, with a half-life of 291 mins at 50°C. Three other enzymes had moderate thermal stability. With a study of the kinetic constants of several of the top LSs, the dominating reactions taking place can be controlled. For the LSs from *B. indica subsp. indica*, *N. aromaticivorans* and *V. natriegens*, transfructosylation can be favoured over hydrolysis by using high sucrose concentrations. An initial screening of the acceptor specificity of the LSs by TLC demonstrated the additional potential of these enzymes. Amongst the enzymes tested, there was broad utilization of multiple acceptor molecules and the donor raffinose. Tailoring of the enzymatic reactions becomes a possibility with the information gathered regarding activity, thermal stability, kinetic parameters and acceptor specificity, resulting in essentially, a LS toolbox.

To fully capitalize upon the catalytic potential of the enzymes screened from the genome mining, additional characterization was done. The product spectrum of the incubation with sucrose was analysed over the course of 50 h to reveal that the LSs from *G. oxydans* and *N. aromaticivorans* were capable of producing oligosaccharides up to 13 DP in length. Another enzyme, the highly stable *P. graminis*, was capable of producing a large amount of oligosaccharides (164 g/L), specifically trisaccharides (1-kestose, neokestose and 6-kestose) and tetrasaccharides (nystose GF3, F4). The acceptor specificity of the enzymes was examined with a wider range of molecules. Remarkedly, there was wide specificity, with all molecules utilized to a certain extent. Since the study over done over a period of 50 h, the time where maximum conversion occurs without hydrolysis of the product can be selected for, allowing for a tailoring of the reaction. The ability of the LSs from *V. natriegens*, *N. aromaticivorans*, *P. graminis* and *B. indica subsp. indica* to utilise the benzene diol catechol opens other catalytic potential for the fructosylation of interesting novel acceptor molecules. Comparison of enzymes' catalytic ability to the active site structure

provides insights into the enzymes' mechanism, which may be later used for the rational design of new LSs.

CHAPTER VIII. CONTRIBUTIONS TO KNOWLEDGE & RECOMMENDATIONS FOR FUTURE STUDIES

The major contributions of knowledge resulting from this study are:

1. A screening of immobilization supports was examined, and for a first time, supports with different functionalities (cationic, anionic, neutral) were compared for the immobilization of LS. The immobilization of LS onto glyoxyl agarose achieved one of the highest increases in thermal stability reported. Sepabeads® HA and glyoxyl agarose-IDA/Cu were found to be highly effective supports for the immobilization of LS.
2. The optimized immobilization conditions were determined for the immobilization of LS onto glyoxyl agarose-IDA/Cu. The effect of the immobilization conditions on retention of activity, activity yield, protein yield and the T/H ration were determined.
3. An alternative to the use of a reducing agent for the formation of covalent bonds was found with the ionic polymer PEI. This is the first use of this cross-linker in combination with LS and an early use of it in enzyme immobilization.
4. The first genome mining was performed for LS. This resulted in the discovery of previously unknown LSs enzymes. Characterization of their levan production, thermal stability, kinetic constants, product profile, active site structure and acceptor specificity was performed for the first time.
5. Homology models were developed for the LSs from *V. natriegens*, *G. oxydans*, *P. graminis* and *B. indica subsp. indica*. As far as the authors are aware, this is the first time that homology models were developed for LS.

Recommendations for Future Work

1. Determination of the optimal conditions (reaction temperature, buffer molarity, pH, sucrose concentration) for the LSs from *V. natriegens*, *G. oxydans*, *P. graminis*, *B. indica subsp. indica* and *N. aromaticivorans*. These enzymes showed remarkable potential using the average optimal conditions, but the results may be enhanced through optimisation.
2. Immobilization of the newly discovered LSs using the immobilization characteristics discussed in chapters III & IV. Potentially utilising Sepabeads® HA in combination with PEI for additional stability.
3. Docking simulations with the acceptors studied within the homology models (work is currently ongoing).

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Supplementary information

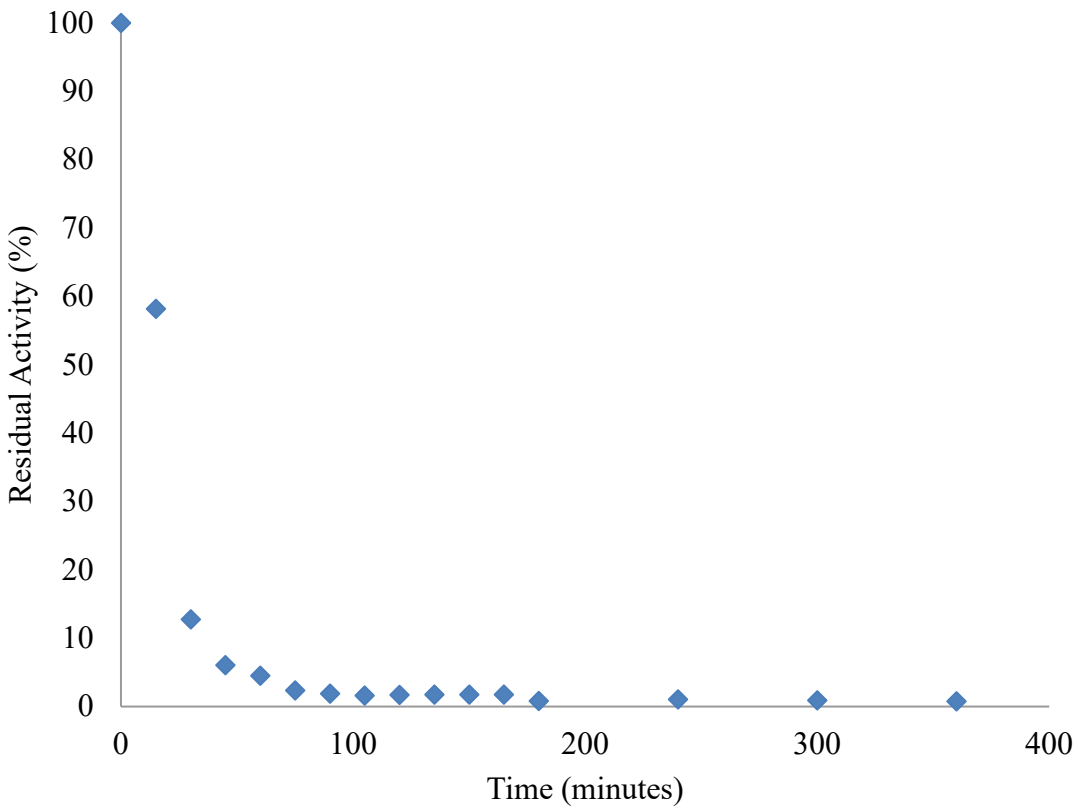


Figure S3.1: Thermal Stability of Free Levansucrase from B. amyloliquefaciens at 50°C

$$t_{1/2} = \frac{1}{k[A]_0}$$

Equation S3.1 Second-order equation to determine half-life for the decay of enzymatic activity. A_0 is the initial activity of the enzyme, while $t_{1/2}$ is the half-life of the enzyme.

Table S5.1. Primers

Uniprot	Annotation Uniprot	Genome strain	MDA	Collection Strain	5PRIM	3PRIM
A0NI59	Levansucrase	Oenococcus oeni ATCC BAA-1163	249	DSMZ 20252	AAAGAAGGAGATAGGATCATGCACAAAAAAATGTATAAATC	GTGTAATGGATAGTGATCTTAATAAGTTTTCTGACCGTCATTTAC
B3XLE9	Levansucrase (EC 2.4.1.10	Lactobacillus reuteri 100-23	163	DSMZ 20016	AAAGAAGGAGATAGGATCATGCTAGAACGCAAGGAACATAAG	GTGTAATGGATAGTGATCTTAGTTAAATCGACGCTTGTTAATTCC
C2E5J0	LPXTG-motif cell wall anc	Lactobacillus johnsonii ATCC 33200	243	DSMZ 10533	AAAGAAGGAGATAGGATCATGTTGGAAAATAAAAATCATAAAAAG	GTGTAATGGATAGTGATCTTAATTATTGCGTTTACGTTTTG
F8DRC0	Putative levansucrase	Lactobacillus reuteri (strain ATCC 55730 / SD2112)	163	DSMZ 20016	AAAGAAGGAGATAGGATCATGAACTTCTCGAAAGCTGCAAAG	GTGTAATGGATAGTGATCTTAGTTAACTCGACGTTTGTTAATTCC
D0R4Z3	Inulosucrase InuJ (EC 2.4.	Lactobacillus johnsonii (strain FI9785)	243	DSMZ 10533	AAAGAAGGAGATAGGATCATGTTGGAAAATAAAAATCATAAAAAG	GTGTAATGGATAGTGATCTTAGTTTTTGCGTTTGCGCTTC
D5DC07	Levansucrase (EC 2.4.1.10	Bacillus megaterium (strain DSM 319)	571	DSMZ 319	AAAGAAGGAGATAGGATCATGAAAATGAAACGAGTTGCTAAG	GTGTAATGGATAGTGATCTTAGTCTTCGTCTACTGTTAACTGACCTTG
D5E2J1	Levansucrase (EC 2.4.1.10	Bacillus megaterium (strain ATCC 12872 / QMB1551	571	DSMZ 319	AAAGAAGGAGATAGGATCATGAATATTAAGAAAATCGCAAAGCAAAC	GTGTAATGGATAGTGATCTTATTTATCCGTACTTAATTGCCCTTG
Q97I79	Levansucrase	Clostridium acetobutylicum (strain ATCC 824 / DSM	11	DSMZ 792	AAAGAAGGAGATAGGATCATGAAAACAAGAAAAACTTATAAAATG	GTGTAATGGATAGTGATCTTAATGTGCAGGCGTAACTACT
Q97I81	Levansucrase	Clostridium acetobutylicum (strain ATCC 824 / DSM	11	DSMZ 792	AAAGAAGGAGATAGGATCATGAATAAGTTAAAAATTGTAAAGTGC	GTGTAATGGATAGTGATCTTAATAATTATCAATAGTAAGTTG
P11701	Levansucrase	Streptococcus mutans serotype c (strain ATCC 7006	220	DSMZ 20523	AAAGAAGGAGATAGGATCATGGGTGGTAGCCACCAT	GTGTAATGGATAGTGATCTTATTTAAAACCAATGCTTACACAGAAAG
Q03WB8	Uncharacterized protein	Leuconostoc mesenteroides subsp. mesenteroides (	169	DSMZ 20343	AAAGAAGGAGATAGGATCATGAGAAAAAAACTATATAAATCA	GTGTAATGGATAGTGATCTTAACGTAAGTAATATGTGCCATC
Q1L7R6	Levansucrase	Leuconostoc mesenteroides	169	DSMZ 20343	AAAGAAGGAGATAGGATCATGAGAAAAAAGTTATATAAGGC	GTGTAATGGATAGTGATCTTAACGTAAGTAATATGTGCCATC
S5NRZ4	Levansucrase	Lactobacillus reuteri TD1	163	DSMZ 20016	AAAGAAGGAGATAGGATCATGCTAGAACGCAAGGAACATAAG	GTGTAATGGATAGTGATCTTAGTTGAATCGACGCTTGTTAATTCC
V6IV04	Levansucrase	Sporolactobacillus laevolacticus DSM 442	760	DSMZ 442	AAAGAAGGAGATAGGATCATGACTATTCAATCAACACTAAAGAAAGC	GTGTAATGGATAGTGATCTTAACGATCGATTGTTAATTGT
O54435	Levansucrase	Rahnella aquatilis (strain ATCC 33071 / DSM 4594 /	767	DSMZ 4594	AAAGAAGGAGATAGGATCATGACAAATTTAAATTATACACCG	GTGTAATGGATAGTGATCTTAATTTAAAATAATGTTTTTCATCG
A0A0F7A902	Levansucrase	Pseudomonas syringae pv. syringae HS191	285	DSMZ 10604	AAAGAAGGAGATAGGATCATGTCCAATAGCAGCTCTGCTGTAATCC	GTGTAATGGATAGTGATCTTAGCTCAGTTGCACGTCTTTCATCG
B2IF78	Levansucrase (EC 2.4.1.10	Beijerinckia indica subsp. indica (strain ATCC 9039 /	324	DSMZ 1715	AAAGAAGGAGATAGGATCATGGCAAGTCGATCGTTTAATGTTTG	GTGTAATGGATAGTGATCTTACTGGCCGTTCGTGACACC
B2VCC3	Levansucrase (Beta-D-fru	Erwinia tasmaniensis (strain DSM 17950 / CIP 10946	412	DSMZ 17950	AAAGAAGGAGATAGGATCATGTCTAACTTTAATTACAAACCCACTCCG	GTGTAATGGATAGTGATCTTATTTTAAAATAATGTCCTTCATTG
C2EU18	KxYKxGKxW signal domair	Lactobacillus vaginalis DSM 5837 = ATCC 49540	816	DSMZ 5837	AAAGAAGGAGATAGGATCATGATACTATCCCTGTTGGATTTTGA	GTGTAATGGATAGTGATCTTACTTTACGCGTTTTTGAATACCAGCAAG
A0A2S3U487	Dextransucrase	Lactobacillus plantarum subsp. Plantarum	244	DSMZ 20174	AAAGAAGGAGATAGGATCATGAATTTTAAATCACAAAATCCAA	GTGTAATGGATAGTGATCTTACGTCATGGTACTTTGATATGACGCC
D2S0N4	Beta-fructofuranosidase (	Haloterrigena turkmenica (strain ATCC 51198 / DSM	475	DSMZ 5511	AAAGAAGGAGATAGGATCATGGTAGATAAGAGTACTAATCT	GTGTAATGGATAGTGATCTTACTGATTATTTCCGTTCGTCCCC
D4E4H9	Levansucrase/Invertase (	Serratia odorifera DSM 4582	555	DSMZ 4582	AAAGAAGGAGATAGGATCATGAGTGATGACGTTTTTGTATGGGAC	GTGTAATGGATAGTGATCTTAGACGAAATCGTATCCGCCCAC
D8FQV7	Cell wall-binding repeat p	Lactobacillus delbrueckii subsp. bulgaricus PB2003/0	107	DSMZ 20355	AAAGAAGGAGATAGGATCATGAAAAAGGAAGAAAAGAAACGCGG	GTGTAATGGATAGTGATCTTAGAAGTTTGCGGACTTGATGTACTTGC
A0A1Y0XLP2	Levansucrase	Bacillus amyloliquefaciens (Bacillus velezensis)	116	DSMZ 7	AAAGAAGGAGATAGGATCATGAACATCAAAAAAATTGTAAAAC	GTGTAATGGATAGTGATCTTAGTTGACTGTCAGCTGTCC
E6U567	Glycoside hydrolase famil	Ethanoligenens harbinense (strain DSM 18485 / JCM	601	DSMZ 18485	AAAGAAGGAGATAGGATCATGAAAAAACGAGCACTTGCAC	GTGTAATGGATAGTGATCTTACCGGCGGTGCTGGTC
F3BM18	Levansucrase (EC 2.4.1.10	Pseudoalteromonas haloplanktis ANT/505	GB	INTERNE	AAAGAAGGAGATAGGATCATGAATAGTAAAATAGGTAAATCG	GTGTAATGGATAGTGATCTTATTTCTTTAGAGGCTTGATCCTGTCCACC
F8DT27	Extracellular sucrase (EC 3	Zymomonas mobilis subsp. mobilis (strain ATCC 109	64	CIP 102538	AAAGAAGGAGATAGGATCATGTTTAATTTTAATGCCAGTCGC	GTGTAATGGATAGTGATCTTATTTGCGACGATCAGGGAAAGG
F8ESF2	Beta-fructofuranosidase (	Zymomonas mobilis subsp. pomaceae (strain ATCC 2	64	CIP 102538	AAAGAAGGAGATAGGATCATGTTTAATTTCAATGCTAGTCGTTGGACG	GTGTAATGGATAGTGATCTTAACGAATACTGGGACGTCGATCTG
F8ESF3	Beta-fructofuranosidase (	Zymomonas mobilis subsp. pomaceae (strain ATCC 2	64	CIP 102538	AAAGAAGGAGATAGGATCATGTTGAATACTGTAGGTATTGCAGAATC	GTGTAATGGATAGTGATCTTAGAAAAGGTCAGCAGTTGCTTCCG
G9WIM3	Levansucrase	Oenococcus kitaharae DSM 17330	872	DSM 17330	AAAGAAGGAGATAGGATCATGCAAATTTTAAGAAAGAAACTAT	GTGTAATGGATAGTGATCTTAAGCCAAAGCGCGACGG
I0QHA9	Levansucrase	Streptococcus salivarius PS4	MB	INTERNE	AAAGAAGGAGATAGGATCATGGACAATACAGCTAAATCGCATTC	GTGTAATGGATAGTGATCTTATTTTTTAGGTAACTGAATTTTAC
J7TH23	Levansucrase (EC 2.4.1.10	Streptococcus salivarius K12	MB	INTERNE	AAAGAAGGAGATAGGATCATGGATAGTACAGTTAATTCACA	GTGTAATGGATAGTGATCTTATTTTTTTAGGCAAATGAATCTTT
K7T8J7	Levansucrase	Gluconobacter oxydans H24	49	DSMZ 2343	AAAGAAGGAGATAGGATCATGAATGCTATTTCCAGCCGAATCC	GTGTAATGGATAGTGATCTTAGGTGCGAACGTCATAGGCCAG
L9ZZ73	Beta-fructofuranosidase	Natrialba taiwanensis DSM 12281	773	DSMZ 12281	AAAGAAGGAGATAGGATCATGGGAGATAGAAGTAGTAATAT	GTGTAATGGATAGTGATCTTATTTCCCTCTTCCGTTCGATCC
M0GXL5	Beta-fructofuranosidase	Haloferax gibbonsii (strain ATCC 33959 / DSM 4427 /	813	DSMZ 4427	AAAGAAGGAGATAGGATCATGACGGAAGACAACTCCAAGCG	GTGTAATGGATAGTGATCTTATTTCGGTCCGTTGCCA
M0GZM4	Beta-fructofuranosidase	Haloferax gibbonsii (strain ATCC 33959 / DSM 4427 /	813	DSMZ 4427	AAAGAAGGAGATAGGATCATGTCGGCGAGTGGTTCTGTAGC	GTGTAATGGATAGTGATCTTAGCGTGATTGCTTCGGCCC
Q2NBK2	Levansucrase	Erythrobacter litoralis (strain HTCC2594)	81	DSMZ 8509	AAAGAAGGAGATAGGATCATGTCGCCGGAACTGGACG	GTGTAATGGATAGTGATCTTAGCCTTCTACCAGGCGCGCTTTA
Q65EI8	Levansucrase, Glycoside H	Bacillus licheniformis (strain ATCC 14580 / DSM 13 /	54	DSMZ 13	AAAGAAGGAGATAGGATCATGAACATCAAAAACATTGCTAAAAA	GTGTAATGGATAGTGATCTTATTTGTTTACCGTTAGTTGTCCCTGTTC
A0A0R1QUE4	Fructansucrase	Lactobacillus paraplantarum DSM 10667	244	DSMZ 20174	AAAGAAGGAGATAGGATCATGGAAAAGTATTCTACAACTAAG	GTGTAATGGATAGTGATCTTATTCTTTGCGCTGCCGCT
A0A0K2JK54	Cell wall-anchored adhesi	Lactobacillus plantarum	244	DSMZ 20174	AAAGAAGGAGATAGGATCATGTCAAAAGATAATCAAAAAGTGACC	GTGTAATGGATAGTGATCTTAACCCGTATTCCAGTCAATTTTTGAG
A0A1B1EI54	Levansucrase	Vibrio natriegens	761	DSMZ 759	AAAGAAGGAGATAGGATCATGGGAACAGCGGCAAGTCAG	GTGTAATGGATAGTGATCTTATTTAAGCGTTTTCTGAATCACACCG
T0TLB7	Uncharacterized protein	Lactobacillus fermentum MTCC 8711	241	DSMZ 20052	AAAGAAGGAGATAGGATCATGTATTACTACGACAAGAATGGGGTTCG	GTGTAATGGATAGTGATCTTATTTATCAGAAGCAACTGAAACTGAAT
F8DT26	Levansucrase	Zymomonas mobilis subsp. mobilis (strain ATCC 109	64	CIP 102538	AAAGAAGGAGATAGGATCATGTTGAATAAAGCAGGCATTGCAG	GTGTAATGGATAGTGATCTTATTTATTCAATAAAGACAGGGC
V6J0B5	Levansucrase	Sporolactobacillus laevolacticus DSM 442	760	DSMZ 442	AAAGAAGGAGATAGGATCATGAAGATTGGAAGACGCGTAAAACAG	GTGTAATGGATAGTGATCTTAATCTCCAACCGTCAACTGACCTTGTTC
A1R4L4	Levansucrase (Beta-D-fru	Paenarthrobacter aurescens (strain TC1)	635	ATCC BAA_1386	AAAGAAGGAGATAGGATCATGAACACGCACTCAACCCCTCAACGGC	GTGTAATGGATAGTGATCTTACTTGTAGTGGCCTTCGCCGCCAAC
B1G3X6	Levansucrase (EC 2.4.1.10	Paraburkholderia graminis C4D1M	297	DSMZ 17151	AAAGAAGGAGATAGGATCATGAACAGACTTCGATCCCCGCAATTCCC	GTGTAATGGATAGTGATCTTACTGACCGTTGTTCGCGCCACCCTG
B1SXR4	Levansucrase (EC 2.4.1.10	Burkholderia ambifaria MEX-5	157	DSMZ 16087	AAAGAAGGAGATAGGATCATGCCGAACATCCCCGCGGACTTCCC	GTGTAATGGATAGTGATCTTACGGCGCGTTGCCTCCGCCATTG
B2JVY2	Levansucrase (EC 2.4.1.10	Paraburkholderia phymatum (strain DSM 17167 / CIP	214	DSMZ 17167	AAAGAAGGAGATAGGATCATGAACGTTGGCCACCACCCGC	GTGTAATGGATAGTGATCTTACTGGCTGTTGCCGCCCTGCCC
B8HBC9	Levansucrase (EC 2.4.1.10	Pseudarthrobacter chlorophenolicus (strain ATCC 70	378	DSMZ 12829	AAAGAAGGAGATAGGATCATGCACAAGCACCCCCAATCACCCC	GTGTAATGGATAGTGATCTTACTTGTAGAGGCCTTCGCCGCCA
C7P4M9	Levansucrase (EC 2.4.1.10	Halomicrobium mukohataei (strain ATCC 700874 / D	373	DSMZ 12286	AAAGAAGGAGATAGGATCATGAGCAAGGACAGTCCTGGGAC	GTGTAATGGATAGTGATCTTAATGGCTACTATAGTACGATCCGC
D2RUR9	Uncharacterized protein	Haloterrigena turkmenica (strain ATCC 51198 / DSM	475	DSMZ 5511	AAAGAAGGAGATAGGATCATGATCGTGTCGATGGTCGCTCTGTCG	GTGTAATGGATAGTGATCTTAGCTACGCTGACGGGCTAACAGCAACG
D5UCP7	Beta-fructofuranosidase (	Cellulomonas flavigena (strain ATCC 482 / DSM 2010	424	DSMZ 20109	AAAGAAGGAGATAGGATCATGAACACTCGCACCATGCGCGC	GTGTAATGGATAGTGATCTTACCATGGGTACATCGTCATGGC
D5VEF7	Glycoside hydrolase famil	Caulobacter segnis (strain ATCC 21756 / DSM 7131 /	680	DSMZ 7131	AAAGAAGGAGATAGGATCATGTCCAGTGTCATTCCTTCAGCGGTCAG	GTGTAATGGATAGTGATCTTAGCCCTGCAGCTTGGCCTGGTCG
D8J9C2	Levansucrase	Halalkalicoccus jeotgali (strain DSM 18796 / CECT 72	551	DSMZ 18796	AAAGAAGGAGATAGGATCATGACTCCCGAGCACAGCGGGCG	GTGTAATGGATAGTGATCTTAGTACCCCCGGCGAGTTTCCT
D9X8L9	Levansucrase	Streptomyces viridochromogenes (strain DSM 40736	575	DSMZ 40736	AAAGAAGGAGATAGGATCATGTACAGAGCAAGACGAGTGGTG	GTGTAATGGATAGTGATCTTACGGAATGAAGCCGTAGTCCAACTGACC
G8PYZ4	M1ft (EC 2.4.1.10)	Pseudomonas fluorescens F113	207	DSMZ 50090	AAAGAAGGAGATAGGATCATGAAAACCACCACTGAAAAATTCGG	GTGTAATGGATAGTGATCTTACTTGAGGGTGACGTCGAGCATCGG
K9DHJ1	Uncharacterized protein	Sphingobium yanoikuyae ATCC 51230	817	DSMZ 7462	AAAGAAGGAGATAGGATCATGACGATATCCTGGACGGCTGCG	GTGTAATGGATAGTGATCTTATGCCGCCACTCCAGCAGCATCG
L9X0M4	Levansucrase	Natronococcus amylolyticus DSM 10524	770	DSMZ 10524	AAAGAAGGAGATAGGATCATGCACGACGATCCGCAGTCCGC	GTGTAATGGATAGTGATCTTAGTACTCGCCGCCGCCGTCCC
M0H859	Levansucrase	Haloferax gibbonsii (strain ATCC 33959 / DSM 4427 /	813	DSMZ 4427	AAAGAAGGAGATAGGATCATGAGTGAAAAATTCAGAGAAGGGATT	GTGTAATGGATAGTGATCTTAGTAGTCGCCGCCGCGGGTG
M5B6V8	Putative levansucrase (EC	Clavibacter nebraskensis NCPPB 2581	192	DSMZ 46364	AAAGAAGGAGATAGGATCATGACAAAGAGAATCAGGCGCGGGCTGTC	GTGTAATGGATAGTGATCTTACTGCGGCGGGTTCGCCGG
Q2G754	Levansucrase (EC 2.4.1.10	Novosphingobium aromaticivorans (strain ATCC 700	92	DSMZ 12444	AAAGAAGGAGATAGGATCATGTCCGTGGTCAATCCATCAGAACAATCG	GTGTAATGGATAGTGATCTTAGGCGATGGTGACCCGCTCG
Q43998	Levansucrase (EC 2.4.1.10	Gluconacetobacter diazotrophicus (Acetobacter dia	347	DSMZ 5601	AAAGAAGGAGATAGGATCATGGCGCATGTACGCCGAAAAGTAGCCACG	GTGTAATGGATAGTGATCTTACTGGTTCAGGAATTGGCGAACC
Q5FSK0	Levansucrase (EC 2.4.1.10	Gluconobacter oxydans (strain 621H) (Gluconobacte	49	DSMZ 2343	AAAGAAGGAGATAGGATCATGAACGCTGTTTCCAGCACGCAGAGC	GTGTAATGGATAGTGATCTTAGGAACGCTTGTCCCAGGCCGAAGAGG
Q5V249	Levansucrase (EC 2.4.1.10	Haloarcula marismortui (strain ATCC 43049 / DSM 3	50	DSMZ 3752	AAAGAAGGAGATAGGATCATGACAAACGAGGCGCTCGGCG	GTGTAATGGATAGTGATCTTACTTCCGCTCAGCCTCGGTTC
Q9EVD6	Fructosyltransferase	Actinomyces naeslundii	826	DSMZ 43013	AAAGAAGGAGATAGGATCATGACCGTGACACACACTTCGTTCCGAGC	GTGTAATGGATAGTGATCTTACTTCAGGCGCTGCGGGCGG
R0CZ62	Uncharacterized protein	Caulobacter vibrioides OR37	75	DSMZ 4727	AAAGAAGGAGATAGGATCATGTCCAGTGTCATTCCTTTAGCGA	GTGTAATGGATAGTGATCTTAGACGTCCAGTTCAGCCTGATCGCCAGC
W8S8J1	Levansucrase (EC 2.4.1.10	Roseibacterium elongatum DSM 19469	799	DSMZ 19469	AAAGAAGGAGATAGGATCATGCTGCCCAAGACATTTGTCGCC	GTGTAATGGATAGTGATCTTATTCCGACCCGGACAGCGCG
B9LT89	Glycoside hydrolase famil	Halorubrum lacusprofundi (strain ATCC 49239 / DSM	236	DSMZ 5036	AAAGAAGGAGATAGGATCATGCACGAGACGCCGGAGAGCGAC	GTGTAATGGATAGTGATCTTAAATAGCGCCGTGAGAGCCGCCG
M0E014	Glycoside hydrolase famil	Halorubrum saccharovorum DSM 1137	762	DSMZ 1137	AAAGAAGGAGATAGGATCATGCACGAGACGCCGGAGAGCGAC	GTGTAATGGATAGTGATCTTAGATCGCGCCGTGGGAACCGTC
M0NGE2	Glycoside hydrolase famil	Halorubrum lipolyticum DSM 21995	809	DSMZ 21995	AAAGAAGGAGATAGGATCATGCACGAGACGTCGGGGAGCGAC	GTGTAATGGATAGTGATCTTAAATAGCGCCGTGAGACCCGCCG

Table S5.2. TLC Results from Alternative Substrates for LSs'

	LS3-N		LS3-C		LS10-N		LS10-C		LS8-N		LS30-N		LS25-N		LS29-N		LS6-N		LS6-C		
	Distance (mm)	Rf	Distance (mm)	Rf	Distance (mm)	Rf	Distance (mm)	Rf	Distance (mm)	Rf	Distance (mm)	Rf	Distance (mm)	Rf	Distance (mm)	Rf	Distance (mm)	Rf	Distance (mm)	Rf	
Sucrose	258	0.5139	262	0.5219	269	0.5275	247	0.5393	324	0.6353	223	0.5045	235	0.5341	249	0.5659	234	0.5318	253	0.5524	F, G
	326	0.6494	322	0.6414	395	0.7745			397	0.7784	275	0.6222	275	0.625	282	0.6409	272	0.6182	281	0.6135	Suc
	384	0.7649	380	0.757	431	0.8451	351	0.7664	483	0.9471	342	0.7738	339	0.7705	337	0.7659	301	0.6841	356	0.7773	GF2
	416	0.8287	424	0.8446	456	0.8941	385	0.8406					373	0.8477	365	0.8295	332	0.7545			GF3
													398	0.9045	391	0.8886	366	0.8318			GF4
																	389	0.8841			GF5
					487	0.9549	436	0.952			429	0.9706	423	0.9614	416	0.9455	418	0.95	441	0.9629	Levan
Raffinose	246	0.49		0.494	251	0.4922	229	0.5	237	0.4647	229	0.5193	221	0.5023	225	0.5114	232	0.5273	237	0.5152	F
							347	0.7576									276	0.6273	354	0.7696	Gal+G
	408	0.8127	408	0.8127			441	0.9629	401	0.7863	372	0.8435	351	0.7977	355	0.8068	358	0.8136	445	0.9674	Raf
	436	0.8685	440	0.8765					439	0.8608			382	0.8682	381	0.8659	380	0.8636			Raf+F
					489	0.9588							421	0.9568	416	0.9455	423	0.9614			Levan
Galactose + Sucrose	254	0.506	262	0.5219	265	0.5196	249	0.5437	253	0.4961	223	0.5068	230	0.5227	227	0.5159	236	0.5364	235	0.5131	F,G, Gal
	312	0.6215	314	0.6255					312	0.6118	281	0.6386	270	0.6136	269	0.6114	275	0.625			Suc, Gal+F
									330						296	0.6727	304	0.6909			
															325	0.7386					
	384	0.7649	384	0.7649	383	0.751	349	0.762	379	0.7431	339	0.7705	337	0.7659	337	0.7659	341	0.775	349	0.762	GF2/GalF2
			416	0.8287	420	0.8235	381	0.8319					367	0.8341	363	0.825	368	0.8364			GF3/GalF3
													391	0.8886	388	0.8818	387	0.8795			GF4/GalF4
					487	0.9549	432	0.9432					421	0.9568	417	0.9477	421	0.9568	441	0.9629	Levan
Glucose + Sucrose	256	0.51	254	0.506	271	0.5314	228	0.4978	252	0.4941	282	0.6409	229	0.5205	230	0.5227	236	0.5364	283	0.6179	F, G
	312	0.6215	304	0.6056					319	0.6255	426	0.9682	270	0.6136	273	0.6205	272	0.6182			Suc
															298	0.6773	301	0.6841			
	382	0.761	380	0.757	379	0.7431	351	0.7664	389	0.7627			338	0.7682	330	0.75	335	0.7614	351	0.7664	GF2
					417	0.8176	387	0.845					363	0.825	361	0.8205	367	0.8341			GF3
													394	0.8955	386	0.8773	388	0.8818			GF4
					483	0.9471	436	0.952					425	0.9659	419	0.9523	421	0.9568	381	0.8319	Levan
Sucrose + Maltose	258	0.5139	258	0.5139	263	0.5157	7	0.5415	255	0.5	226	0.5136	229	0.5205	232	0.5273	235	0.5341	247	0.5393	F, G
									312	0.6118	279	0.6341	269	0.6114	268	0.6091	279	0.6341			Suc/Mal
							308	0.6725							304	0.6909	311	0.7068			
	382	0.761	382	0.761	378	0.7412	360	0.786	389	0.7627	338	0.7682	337	0.7659	331	0.7523	336	0.7636	351	0.7664	GF2/GGF
	410	0.8167	408	0.8127	409	0.802	379	0.8275					373	0.8477	362	0.8227	366	0.8318	385	0.8406	GF3/GGF2
													393	0.8932	386	0.8773	392	0.8909			GF4/GGF3
					480	0.9412	441	0.9629					425	0.9659	415	0.9432	421	0.9568	436	0.952	Levan

Lactose + Sucrose	262	0.5219	256	0.51	255	0.5	231	0.5044	247	0.4843	224	0.5091	233	0.5295	229	0.5205	233	0.5295	249	0.5437	F, G
									319	0.6255	279	0.6341	269	0.6114	272	0.6182	281	0.6386			*Suc/Lac*
	386	0.7689	388	0.7729	373	0.7314	348	0.7598	387	0.7588	356	0.8091	343	0.7795	340	0.7727	339	0.7705	349	0.762	GF2/LacF
			410	0.8167					425	0.8333			369	0.8386	354	0.8045	362	0.8227	381	0.8319	GF3/LacF2
													393	0.8932	384	0.8727					*GF4/LacF3*
					483	0.9471	441	0.9629					426	0.9682	414	0.9409			432	0.9432	Levan
Raffinose + Sucrose	256	0.51	256	0.51	258	0.5059	240	0.524	253	0.4961	226	0.5136	225	0.5114	234	0.5318	241	0.5477	248	0.5415	G, F
									318	0.6235	281	0.6386	277	0.6295	271	0.6159	272	0.6182			Suc
																	303	0.6886	308	0.6725	
	390	0.7769	386	0.7689	378	0.7412	348	0.7598	389	0.7627			354	0.8045	345	0.7841	347	0.7886			GF2/Raf
			414	0.8247	411	0.8059	389	0.8493	420	0.8235	366	0.8318			367	0.8341					GF3/RafF
					441	0.8647			444	0.8706			379	0.8614							GF4/RafF2
					477	0.9353	443	0.9672											436	0.952	Levan

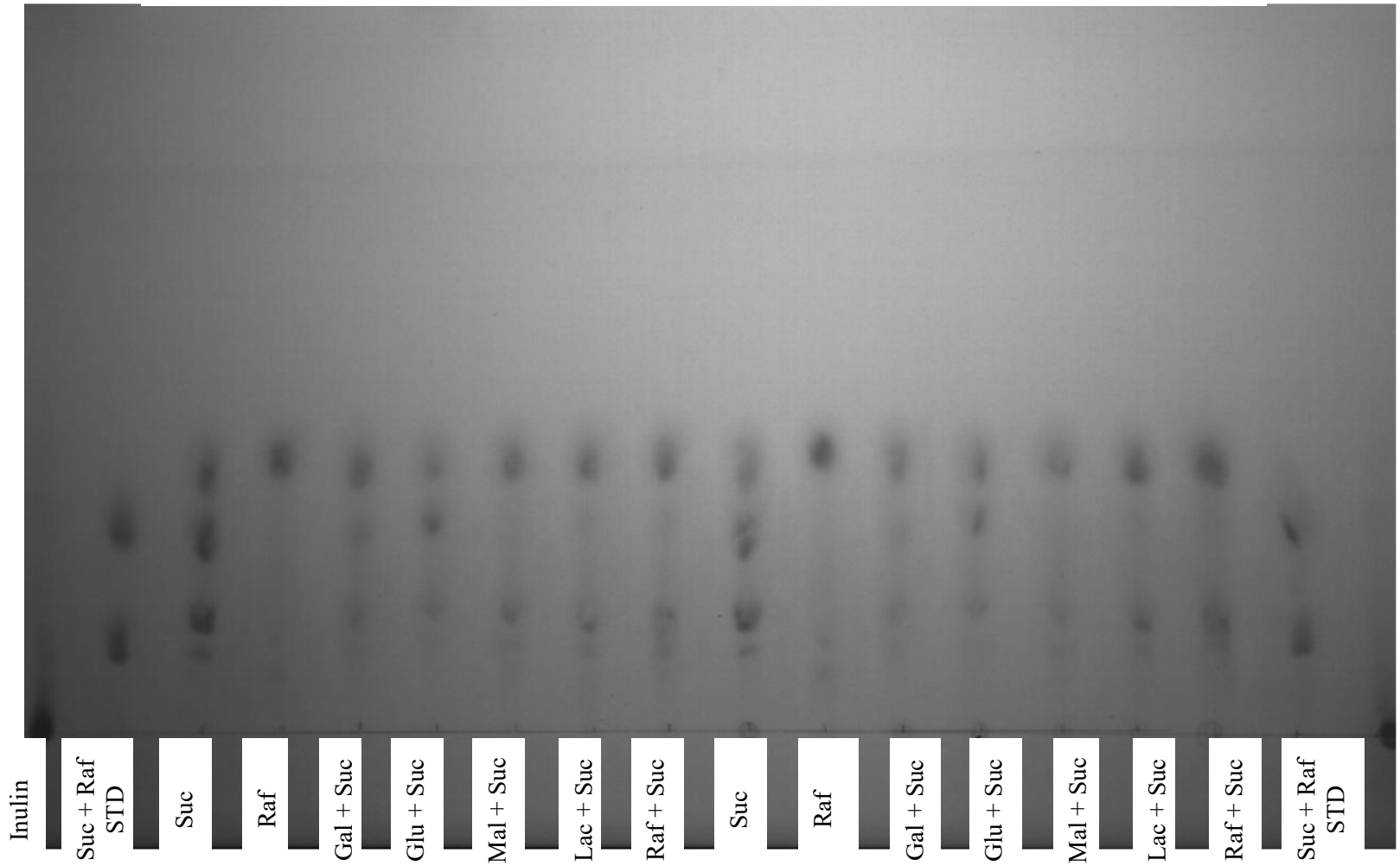
	A9H664	Q8VW87	Q5IS34	Q93FU9	A0A0M3KKU6	O54435	E2XQB6	O52408	O68609	Q88BN6	Q9LBX1	F8DT26	Q03WB9	Q9Z5E5	P21130	P05655	P94468	H6UZK4	D5DC38	D5DC07	Q7X481	A0A2I1TMP2	Q55242	P11701	Q8GGV4	Q70XJ9	D3WYW0	Q8GP32	Q74K42	D3WYV9	*Organisms*	Gram coloration	Group
A9H664	100	55	42	42	44	43	47	44	43	43	40	45	24	25	25	25	24	26	24	25	26	21	21	21	22	23	20	23	22	22	*Gluconacetobacter diazotrophicus*	-	
Q8VW87	55	100	43	42	46	45	47	45	44	43	36	44	24	24	25	25	25	25	24	24	27	22	22	22	23	23	21	23	22	22	*Microbacterium saccharophilum*	+	
Q5IS34	42	43	100	96	72	72	77	73	74	73	44	51	27	27	28	28	27	29	28	29	27	25	25	24	25	24	24	26	25	25	*Leuconostoc mesenteroides*	+	
Q93FU9	42	42	96	100	72	73	77	73	74	73	44	51	27	27	28	28	27	28	28	29	27	26	26	24	25	25	25	26	26	25	*Pseudomonas chlororaphis subsp. aurantiaca*	-	
A0A0M3KKU6	44	46	72	72	100	77	80	76	78	77	47	53	28	28	28	29	28	28	29	29	29	26	26	26	26	25	25	26	27	27	*Erwinia amylovora*	-	
O54435	43	45	72	73	77	100	81	79	79	78	46	54	28	28	29	30	29	29	29	29	29	27	27	25	26	26	26	27	27	26	*Rahnella aquatilis*	-	
E2XQB6	47	47	77	77	80	81	100	90	90	90	47	54	28	28	28	29	28	29	30	30	30	29	29	27	29	29	27	28	27	27	*Pseudomonas fluorescens*	-	G1
O52408	44	45	73	73	76	79	90	100	93	93	46	54	27	27	28	27	27	27	28	28	28	26	26	25	25	25	25	26	25	25	*Pseudomonas savastanoi pv. glycinea*	-	
O68609	43	44	74	74	78	79	90	93	100	96	45	53	26	26	26	26	25	26	27	27	27	26	26	25	25	25	24	26	25	24	*Pseudomonas savastanoi pv. Phaseolicola*	-	
Q88BN6	43	43	73	73	77	78	90	93	96	100	45	53	26	26	27	27	26	26	27	27	27	26	26	25	25	25	24	26	25	25	*Pseudomonas syringae pv. tomato*	-	
Q9LBX1	40	36	44	44	47	46	47	46	45	45	100	57	24	24	25	25	24	24	26	25	24	22	22	21	22	22	22	23	22	22	*Komagataeibacter xylinus*	-	
F8DT26	45	44	51	51	53	54	54	54	53	53	57	100	25	28	27	27	26	28	27	27	28	24	24	24	26	25	26	25	24	24	*Zymomonas mobilis subsp. Mobilis*	-	
Q03WB9	24	24	27	27	28	28	28	27	26	26	24	25	100	39	41	43	41	41	39	42	37	37	37	39	37	38	38	39	37	38	*Leuconostoc mesenteroides subsp.*	+	
Q9Z5E5	25	24	27	27	28	28	28	27	26	26	24	28	39	100	68	69	67	68	66	66	35	35	35	36	35	35	37	37	36	34	*Paenibacillus polymyxa*	+	
P21130	25	25	28	28	28	29	28	28	26	27	25	27	41	68	100	90	88	78	77	74	39	39	39	40	40	40	39	39	38	38	*Bacillus amyloliquefaciens*	+	
P05655	25	25	28	28	29	30	29	27	26	27	25	27	43	69	90	100	98	79	78	75	39	38	38	39	39	39	39	38	37	37	*Bacillus subtilis*	+	
P94468	24	25	27	27	28	29	28	27	25	26	24	26	41	67	88	98	100	77	76	73	38	37	37	39	39	38	38	38	36	36	*Geobacillus stearothermophilus*	+	
H6UZK4	26	25	29	28	28	29	29	27	26	26	24	28	41	68	78	79	77	100	78	76	37	37	37	38	38	38	38	38	39	39	*Bacillus licheniformis*	+	
D5DC38	24	24	28	28	29	29	30	28	27	27	26	27	39	66	77	78	76	78	100	82	37	36	36	37	37	38	37	36	37	38	*Bacillus megaterium*	+	
D5DC07	25	24	29	29	29	29	30	28	27	27	25	27	42	66	74	75	73	76	82	100	38	38	38	39	40	40	40	39	39	38	*Bacillus megaterium*	+	
Q7X481	26	27	27	27	29	29	30	28	27	27	24	28	37	35	39	39	38	37	37	38	100	32	33	34	35	34	35	36	33	35	*Leuconostoc citreum*	+	
A0A2I1TMP2	21	22	25	26	26	27	29	26	26	26	22	24	37	35	39	38	37	37	36	38	32	100	95	46	46	47	49	48	48	48	*Streptococcus salivarius*	+	
Q55242	21	22	25	26	26	27	29	26	26	26	22	24	37	35	39	38	37	37	36	38	33	95	100	46	46	47	49	49	48	48	*Streptococcus salivarius*	+	
P11701	21	22	24	24	26	25	27	25	25	25	21	24	39	36	40	39	39	38	37	39	34	46	46	100	48	47	48	54	52	52	*Streptococcus mutans*	+	
Q8GGV4	22	23	25	25	26	26	29	25	25	25	22	26	37	35	40	39	39	38	37	40	35	46	46	48	100	79	57	54	51	52	*Lactobacillus reuteri*	+	
Q70XJ9	23	23	24	25	25	26	29	25	25	25	22	25	38	35	40	39	38	38	38	40	34	47	47	47	79	100	55	53	50	51	*Lactobacillus sanfranciscensis*	+	
D3WYW0	20	21	24	25	25	26	27	25	24	24	22	26	38	37	39	39	38	38	37	40	35	49	49	48	57	55	100	54	61	62	*Lactobacillus gasseri*	+	
Q8GP32	23	23	26	26	26	27	28	26	26	26	23	25	39	37	39	38	38	38	36	39	36	48	49	54	54	53	54	100	63	65	*Lactobacillus reuteri*	+	
Q74K42	22	22	25	26	27	27	27	25	25	25	22	24	37	36	38	37	36	39	37	39	33	48	48	52	51	50	61	63	100	82	*Lactobacillus johnsonii*	+	
D3WYV9	22	22	25	25	27	26	27	25	24	25	22	24	38	34	38	37	36	39	38	38	35	48	48	52	52	51	62	65	82	100	*Lactobacillus gasseri*	+	

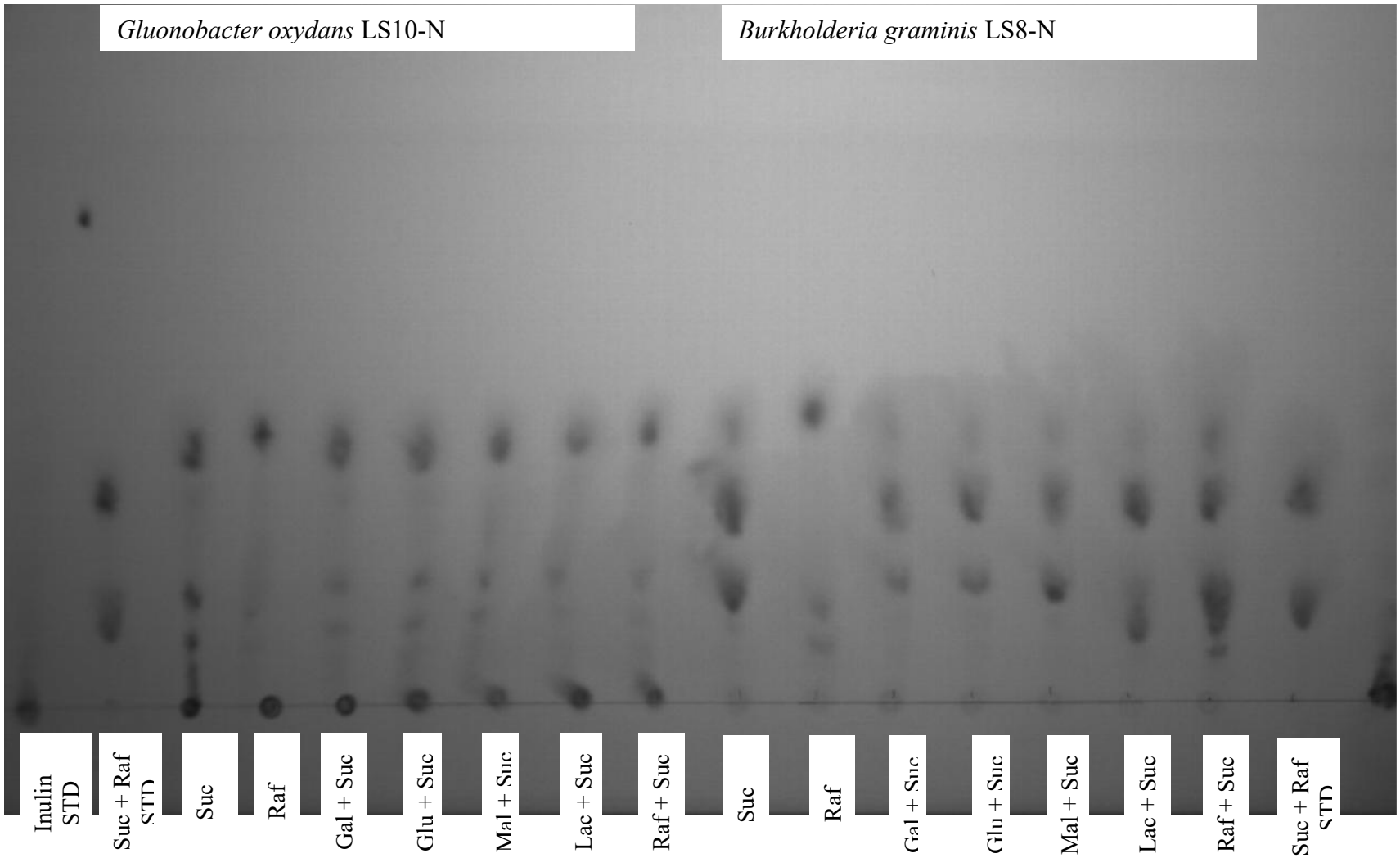
Fig. S5.1 Sequence comparison between known LS revealed two distinct groups. Multiple sequence alignment with known LSs (Table 1) and 4 known inulosucrases was done using ClustalOmega website and the Percent Identity Matrix was created by Clustal 2.1. Gram coloration is indicated for each organism. Known inulosucrases Q8GP32 from *Lactobacillus reuteri* (Van Hijum et al., 2002), Q74K42 from *Lactobacillus johnsonii* (Pijning et al., 2011), D3WYV9 from *Lactobacillus gasseri* (Diez-Municio et al., 2013) and Q7X481 from *Leuconostoc citreum* (Olivares-Illana et al., 2003) are written in orange.

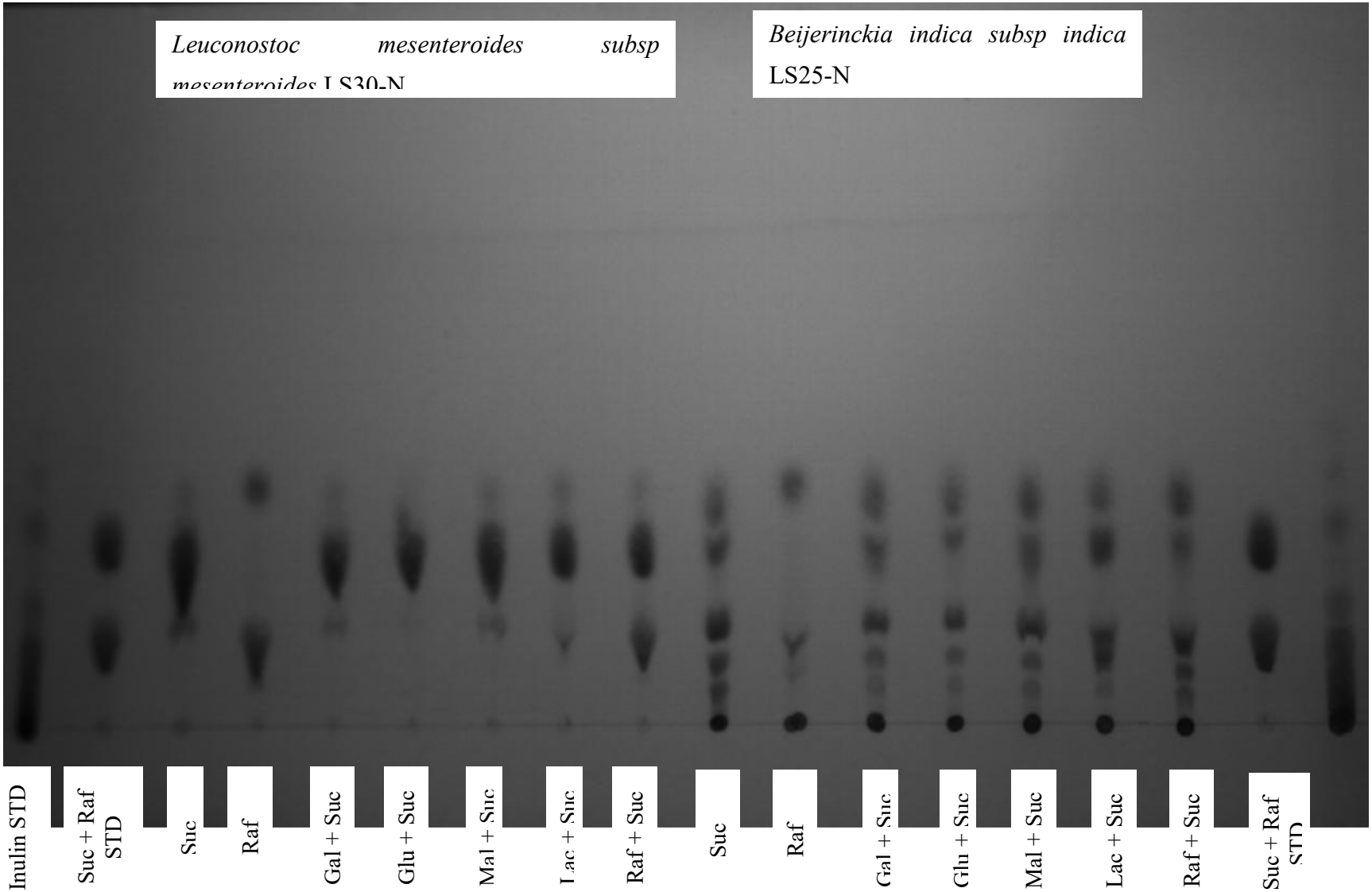
Figure S5.2. TLC Results from Alternative Substrates for LSs'

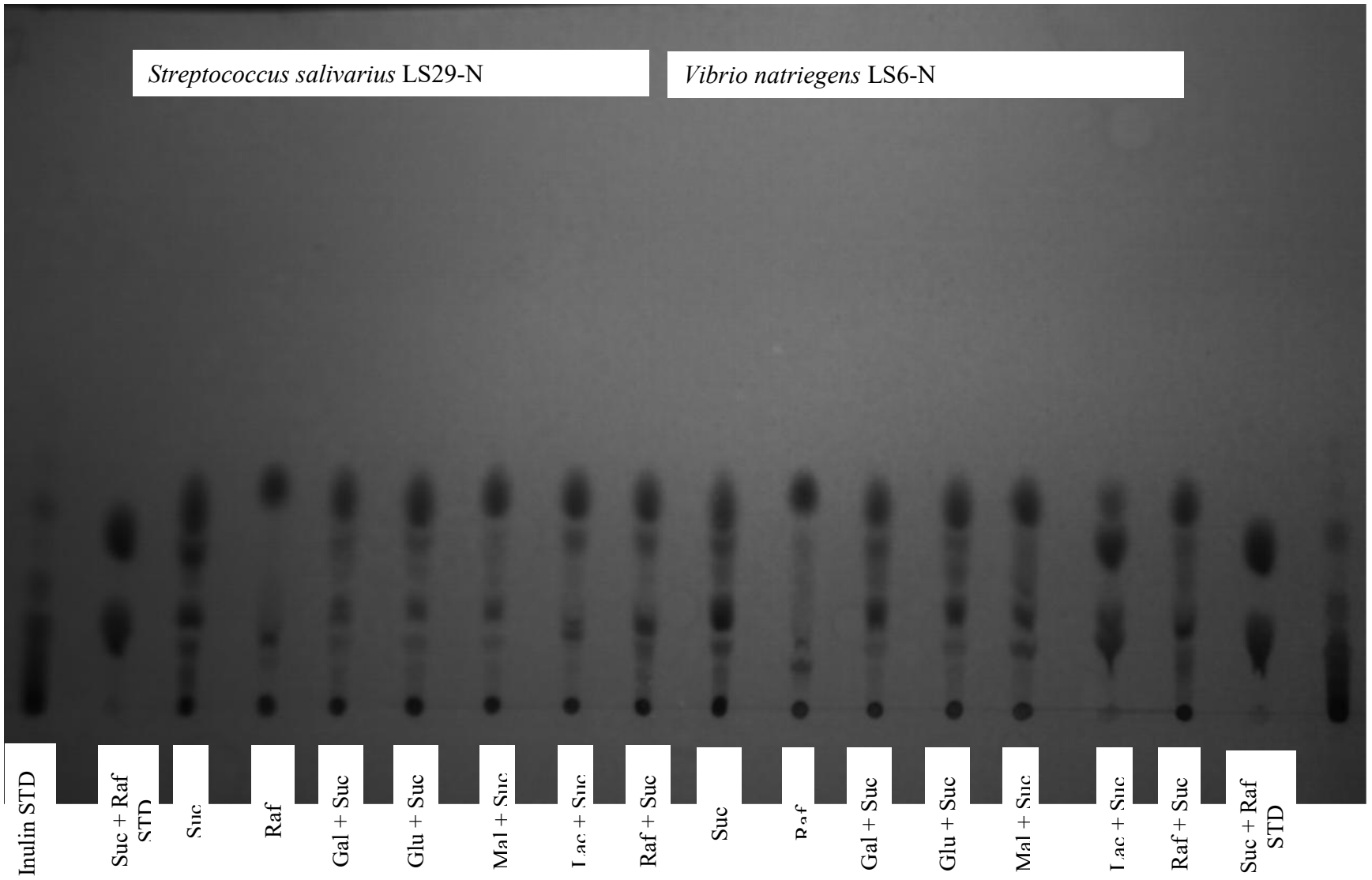
Vibrio natriegens LS6-C

Novosphinogobium aromaticivorans LS3-N

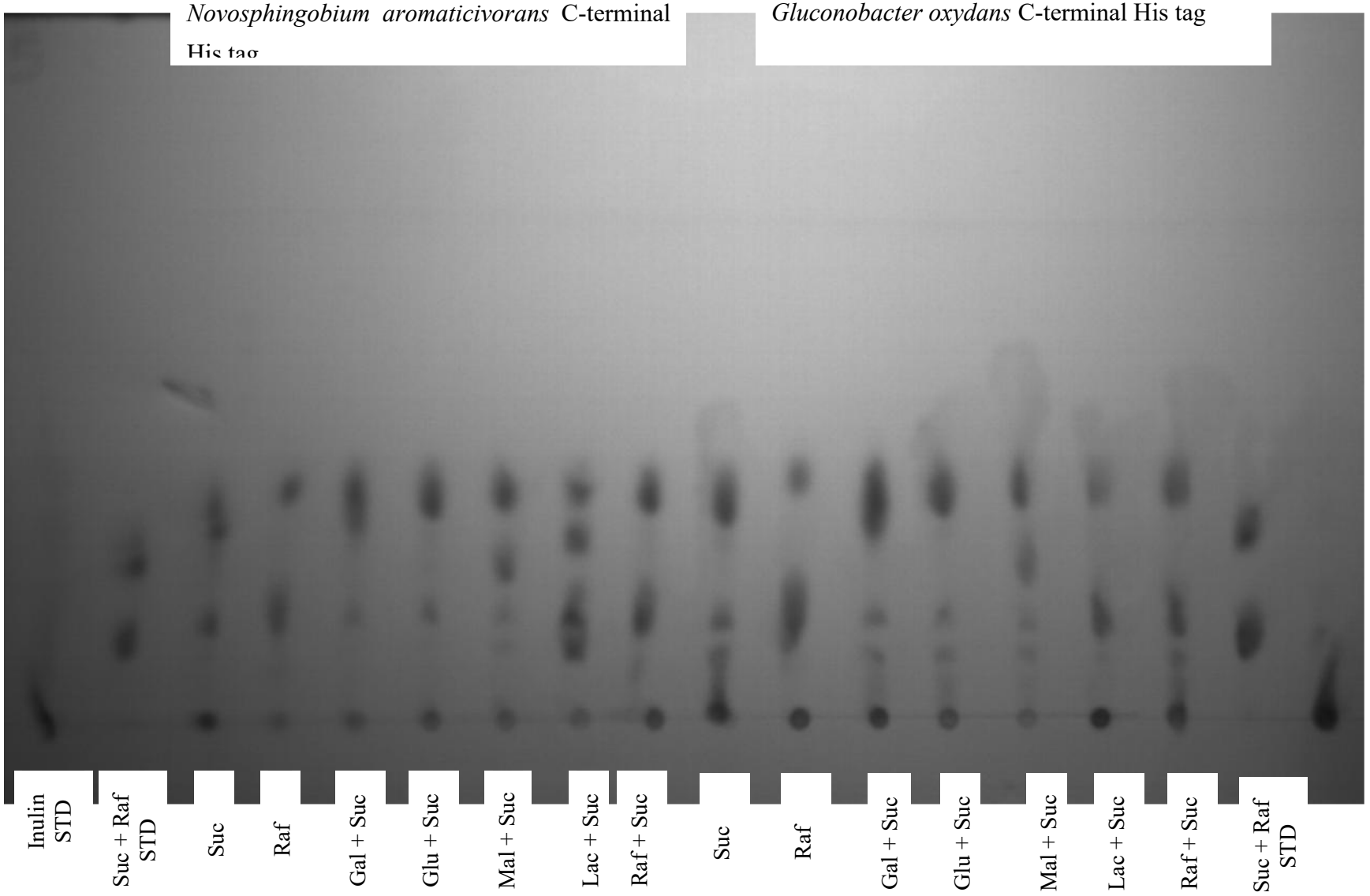



Gluonobacter oxydans LS10-N
Burkholderia graminis LS8-N
Inulin STD
Suc + Raf STD
Suc
Raf
Gal + Suc
Glu + Suc
Mal + Suc
Lac + Suc
Raf + Suc
Suc
Raf
Gal + Suc
Glu + Suc
Mal + Suc
Lac + Suc
Raf + Suc
Suc + Raf STD


Leuconostoc mesenteroides subsp mesenteroides LS30-N
Beijerinckia indica subsp indica LS25-N
Inulin STD
Suc + Raf STD
Suc
Raf
Gal + Suc
Glu + Suc
Mal + Suc
Lac + Suc
Raf + Suc
Suc
Raf
Gal + Suc
Glu + Suc
Mal + Suc
Lac + Suc
Raf + Suc
Suc + Raf STD


Streptococcus salivarius LS29-N
Vibrio natriegens LS6-N
Inulin STD
Suc + Raf STD
Suc
Raf
Gal + Suc
Glu + Suc
Mal + Suc
Lac + Suc
Raf + Suc
Suc
Raf
Gal + Suc
Glu + Suc
Mal + Suc
Lac + Suc
Raf + Suc
Suc + Raf STD

\


Novosphingobium aromaticivorans C-terminal His tag
Gluconobacter oxydans C-terminal His tag
Inulin STD
Suc + Raf STD
Suc
Raf
Gal + Suc
Glu + Suc
Mal + Suc
Lac + Suc
Raf + Suc
Suc
Raf
Gal + Suc
Glu + Suc
Mal + Suc
Lac + Suc
Raf + Suc
Suc + Raf STD