Investigation and Development of Innovative Biocatalytic Processes Using Levansucrase Enzymes for the Biogeneration of Functional Carbohydrates from Dairy By-Products.

By

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Synthesis of Functional Carbohydrates from Dairy By-Products Through Free and Immobilized Levansucrase Enzymes

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ABSTRACT

Levansucrase (LS, EC 2.4.1.10) is a fructosyltransferase that is garnering higher interest due its ability to synthesize novel prebiotics which promote intestinal health. LS is capable of transferring a fructose unit from a fructosyl donor molecule to a fructosyl acceptor molecule resulting in the production of various products. One notable compound where LS offers a potential attractive catalytic activity for its production is lactosucrose, which requires the use of sucrose as a fructosyl donor and lactose as a fructosyl acceptor. Lactosucrose has been increasing in popularity due to its prebiotic and technofunctional properties. Unfortunately, low quantities of lactosucrose exist naturally in foods and thus an efficient biocatalytic system is needed for its synthesis. Sucrose is the most used sweetener in the food industry, and lactose is abundant due to several million tons of it being generated in the dairy by-products known as whey and milk permeate.

The enzymatic activity of LS varies depending on its microbial source. Four LS strains from *Gluconobacter oxydans* (strain 621H) (LS1), *Vibrio natriegens* NBRC 15636 (LS2), *Novosphingobium aromaticivorans* (LS3), and *Burkholderia graminis* C4D1M (LS4) were selected and examined in three different reaction systems lactose/sucrose, whey permeate (WP)/sucrose, and milk permeate (MP)/sucrose. The catalytic efficiency of all 4 LSs demonstrated a higher transfructosylation activity as opposed to the hydrolytic one (1.29 - 7.28), with the sole exception of *V. natriegens* LS2 in the presence of MP/sucrose (0.16). Moreover, the bioconversion end-products, such as lactosucrose and fructooligosaccharides (FOSs), exhibited varying production time courses and profiles depending on the type of LS and starting material used. The *V. natriegens* LS2 resulted in the highest bioconversion giving rise to 328 g/L and 251 g/L of lactosucrose with lactose/sucrose and WP/sucrose, respectively. Contrary to other LSs, *N. aromaticivorans* LS3 synthesized the lowest amounts of lactosucrose of 39.7 g/L with lactose/sucrose, 30.6 g/L with WP/sucrose, and 2.0 g/L with MP/sucrose. *N. aromaticivorans* LS3 showed a higher product specificity toward the synthesis of FOSs, in particular kestose, nystose, and fructosyl nystose, in all investigated reaction systems. Additionally, *G. oxydans* LS1 was the sole LS that generated levan polymers when using lactose/sucrose and MP/sucrose (0.71 g/L).

Consequently, the immobilization of *G. oxydans* LS1 and *V. natriegens* LS2 by multipoint covalent attachment was studied with the main focus of augmenting the enzyme's preference for transfructosylation over hydrolysis through a modification of its microenvironment in addition to

improving the thermal stability. Modified glyoxyl-agarose, Sepabeads® EC-EP/S, and Relizyme™ EP403/S were chosen as solid supports. It was found that the iminodiacetic acid/Cu (IDA/Cu) Relizyme™ EP403/S achieved the highest retained activity with 55% for *G. oxydans* LS1 and 98% for *V. natriegens* LS2. The greatest thermal stability (a stabilization factor of 53 at 50°C) was experienced by *V. natriegens* LS2 immobilized onto Relizyme™ EP403/S-IDA/Cu that had been treated with a post-immobilization step involving a high pH incubation. Additionally, the process of immobilizing both LSs was found to provide a great modulation of the enzyme's microenvironment with transfructosylation over hydrolysis ratios being higher than in their free forms, particularly for *V. natriegens* LS2 with ratios for the sucrose/lactose reaction of 3.51 in its free form in comparison to 1637, 5, and 4, respectively, for glycoxyl agarose-IDA/Cu, Sepabeads® EC-EP/S-IDA/Cu, and Relizyme™ EP403/S-IDA/Cu. The *V. natriegens* LS2 immobilized on Relizyme™ EP403/S-IDA/Cu was found to provide the highest produced lactosucrose amounts with 96, 86, and 35 g/L respectively for lactose/sucrose, WP/sucrose, and MP/sucrose.

Ultimately, the *V. natriegens* LS2 was chosen for the optimization of the lactosucrose synthesis using the WP/sucrose substrate combination. The lactose (WP)/sucrose ratio and incubation time were the factors selected for examination and optimization using response surface methodology with both free and Relizyme™ EP403/S-IDA/Cu immobilized forms of *V. natriegens* LS2. The optimal lactose/sucrose ratio and incubation time were determined to be, respectively, 0.59 and 3.12h for the free *V. natriegens* LS2 and 0.50 and 3.08h for the Relizyme™ EP403/S-IDA/Cu immobilized *V. natriegens* LS2. Furthermore, the Relizyme™ EP403/S-IDA/Cu immobilized *V. natriegens* LS2 was efficiently reused in 3 consecutive reactions, doubling the amount of produced lactosucrose compared to that obtained with the free *V. natriegens* LS2.

RÉSUMÉ

La levansucrase (LS, EC 2.4.1.10) est une fructosyltransférase qui suscite un intérêt accru en raison de sa capacité à synthétiser de nouveaux prébiotiques qui favorisent la santé intestinale. La LS est capable de transférer une unité de fructose d'une molécule donneuse de fructosyle à une molécule acceptrice de fructosyle résultant en la production de divers produits. Un composé notable dont la production pourrait potentiellement bénéficier de l'activité catalytique de LS est la lactosaccharose, qui nécessite l'utilisation de saccharose comme donneur de fructosyle et de lactose comme accepteur de fructosyle. Le lactosaccharose est de plus en plus populaire en raison de ses propriétés prébiotiques et technofonctionnelles. Malheureusement, seulement des faibles quantités de lactosaccharose existent naturellement dans les aliments ; un système biocatalytique efficace est donc nécessaire pour sa synthèse. Le saccharose est l'édulcorant le plus utilisé dans l'industrie alimentaire, et plusieurs millions de tonnes de lactose sont générées dans les sous-produits laitiers connus sous les noms de lactosérum et de perméat de lait.

L'activité enzymatique de LS varie en fonction de sa source microbienne. Quatre souches de LS provenant de *Gluconobacter oxydans* (souche 621H) (LS1), *Vibrio natriegens* NBRC 15636 (LS2), *Novosphingobium aromaticivorans* (LS3), et *Burkholderia graminis* C4D1M (LS4) ont été sélectionnées et examinées dans trois systèmes de réaction différents lactose/saccharose, perméat de lactosérum (WP)/saccharose, et perméat de lait (MP)/saccharose. L'efficacité catalytique des 4 LS a démontré une activité de transfructosylation supérieure à celle hydrolytique (1.29 - 7.28), à la seule exception de *V. natriegens* LS2 en présence de MP/saccharose (0.16). De plus, les produits finaux de bioconversion, par exemple le lactosaccharose et les fructooligosaccharides (FOS), ont présenté cours de temps et des profils de production variables selon le type de LS et de la matière première utilisée. La *V. natriegens* LS2 a entraîné la bioconversion la plus élevée donnant lieu à 328 g/L et 251 g/L de lactosaccharose avec lactose/saccharose et WP/saccharose, respectivement. Contrairement aux autres LS, *N. aromaticivorans* LS3 a synthétisé les plus faibles quantités de lactosaccharose de 39.7 g/L avec lactose/saccharose, 30.6 g/L avec WP/saccharose et 2.0 g/L avec MP/saccharose. *N. aromaticivorans* LS3 a montré une spécificité de produit plus élevée envers la synthèse des FOS, en particulier le kestose, le nystose, et le fructosyl nystose, dans tous les systèmes de réaction étudiés. De plus, *G. oxydans* LS1 est la seul LS qui a générée des polymères de lévane lors de l'utilisation de lactose/saccharose et de MP/saccharose (0.71 g/L).

Par conséquent, l'immobilisation de *G. oxydans* LS1 et *V. natriegens* LS2 par attachement covalent multipoint a été étudiée dans le but principal d'augmenter la préférence de l'enzyme pour la transfructosylation par rapport à l'hydrolyse par une modification de son microenvironnement en plus d'améliorer la stabilité thermique. Le glyoxyl-agarose modifié, les Sepabeads® EC-EP/S, et Relizyme™ EP403/S ont été choisis comme supports solides. Il a été constaté que l'acide iminodiacétique/Cu (IDA/Cu) Relizyme™ EP403/S a permis d'atteindre l'activité retenue la plus élevée avec 55 % pour *G. oxydans* LS1 et 98 % pour *V. natriegens* LS2. La plus grande stabilité thermique (un facteur de stabilisation de 53 à 50°C) a été observée avec l'utilisation de *V. natriegens* LS2 immobilisée sur Relizyme™ EP403/S-IDA/Cu qui avait été traité avec une étape de post-immobilisation impliquant une incubation à un pH élevé. De plus, le processus d'immobilisation des deux LS s'est avéré être efficace pour une modulation du microenvironnement de l'enzyme avec des rapports de transfructosylation sur hydrolyse plus élevés que dans leurs formes libres, en particulier pour *V. natriegens* LS2 avec des rapports pour la réaction saccharose/lactose de 3.51 dans sa forme libre par rapport à 1637, 5, et 4, respectivement, pour le glycoxyl agarose-IDA/Cu, Sepabeads® EC-EP/S-IDA/Cu, et Relizyme™ EP403/S-IDA/Cu. L'utilisation de *V. natriegens* LS2 immobilisée sur Relizyme™ EP403/S-IDA/Cu a conduit à la production de quantités de lactosaccharose les plus élevées avec 96, 86, et 35 g/L, respectivement, en présence du lactose/saccharose, WP/saccharose, et de MP/saccharose.

Finalement, la *V. natriegens* LS2 a été choisie pour l'optimisation de la synthèse de lactosaccharose en utilisant la combinaison de WP/saccharose. Le rapport lactose (WP)/saccharose et le temps d'incubation ont été les facteurs sélectionnés pour l'étude et l'optimisation à l'aide de la méthodologie de surface de réponse avec à la fois la forme libre et la forme immobilisée sur Relizyme™ EP403/S-IDA/Cu de *V. natriegens* LS2. Le rapport lactose/saccharose et le temps d'incubation optimaux ont été déterminés comme étant, respectivement, de l'ordre de 0.59 et 3.12h pour la *V. natriegens* LS2 libre et de 0.50 et 3.08h pour la *V. natriegens* LS2 immobilisée sur Relizyme™ EP403/S-IDA/Cu. De plus, la *V. natriegens* LS2 immobilisée sur Relizyme™ EP403/S-IDA/Cu a été efficacement réutilisée dans 3 réactions consécutives, doublant la quantité de lactosaccharose produite par rapport à celle obtenue avec la *V. natriegens* LS2 libre.

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STATEMENT FROM THE THESIS OFFICE

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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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PREFACE AND CONTRIBUTION OF AUTHORS

This thesis includes five chapters.

Chapter I provides a general introduction to lactosucrose and its synthesis by levansucrase (LS) using lactose containing dairy by-products in addition to the benefits of enzyme immobilization. Moreover, the research objectives of the current research are outlined.

Chapter II presents a comprehensive literature review on the valorization of lactose from dairy by-products as well as on the properties of lactosuscrose and fructooligosaccharides. It describes their current uses and synthesis particularly through the transfructosylation reactions catalyzed by LS. It also provides a description of immobilization techniques employed for use on levansucrase.

Chapter III describes the results of three distinct biotransformation reactions, where the difference laid in the lactose source (lactose and two different dairy by-products), carried out by four selected LSs. The catalytic efficiency of each LS to catalyze the transfructosylation of lactose and sucrose into lactosucrose, fructooligosaccharides, and levans was investigated. Additionally, the ability of lactosucrose to be utilized as a fructosyl acceptor and/or donor by LSs was examined.

Chapter IV focuses on the results of the immobilization of two selected LSs on selected functionalized supports. The main enzyme immobilization features, such as immobilization protein yield, retained activity, and reaction selectivity (transfructosylation/hydrolysis), were discussed since they are related to the support type and LS properties. The best support was selected for the two selected LSs. Post-immobilization treatments, thermal stability, and more importantly lactosucrose synthesis using lactose and two dairy by-products were investigated. Finally, a RSM design of the most promising LS, both in its free and immobilized forms, was carried out in order to optimize lactosucrose synthesis. The resulting product profiles were described and the reusability of the immobilized LS was also studied.

Finally, Chapter V covers an overall summary and possible future of the current research results.

The present author was responsible for the experimental work and the preparation of the first draft of the thesis and publications.

Dr. Salwa Karboune, the supervisor of the current M.Sc. research project, guided the entire research framework and reviewed all the presented chapters in this thesis prior to the submission.

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NOMENCLATURE/LIST OF ABBREVIATIONS

ACN	Acetonitrile
Asp	Aspartate
BLAST	Basic local alignment search tool
BOD	Biochemical oxygen demand
BSA	Bovine serum albumin
CLEAs	Cross-linked enzyme aggregates
CLECs	Cross-linked enzyme crystals
CNS	Central nervous system
COD	Chemical oxygen demand
DNS	3,5-Dinitrosalicylic acid
EC number	Enzyme classification number
EDA	Ethylenediamine
EDTA	ethylenediaminetetraacetic acid
FAO	Food and Agricultural Organization
FOSs	Fructooligosaccharides
FOSHU	Functional Food Ingredients for Foods for Specified Health Uses
Fruf	Fructofuranosyl
Galp	Galactopyranosyl
GH	Glycosyl-hydrolase
GIT	Gastrointestinal tract
Glcp	Glucopyranosyl
Glu	Glutamate
GOSs	Galactooligosaccharides
GRAS	Generally Regarded As Safe
HPAEC	High-pressure anion-exchange chromatography
HPLC	High pressure liquid chromatography
HPSEC	High-pressure size-exclusion chromatography
IBS	Irritable bowel syndrome

IDA	Iminodiacetic acid
IPTG	β-D-isothiogalactopyranoside
ISAPP	International Scientific Association for Probiotics and Prebiotics
L	Lactose
LAB	Lactic Acid Bacteria
LB	Lysogeny broth
LBA	Lactobionic acid
LS	Levansucrase
MOPS	4-Morpholinepropanesulfonic acid
MP	Milk permeate
PAD	Pulsed amperometric detector
PEI	Polyethylenimine
PHAs	Polyhydroxyalkanoates
PLA	Polylactic acid
Q-TOF – MS	Quadrupole Time of Flight Mass Spectrometer
RPM	Rotations per minute
RSM	Response surface methodology
SCFA	Short chain fatty acid
SCP	Single cell protein
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
TAG	Triacylglyceride
TB	Terrific broth
TEA	Triethylamine
WP	Whey permeate
WPC	Whey protein concentrate
WPI	Whey protein isolate

CHAPTER I. GENERAL INTRODUCTION

As consumers are becoming increasingly health-conscious, they tend to make more healthy eating decisions. Consequently, the demand for functional ingredients and nutraceuticals has been on the rise, as evident with prebiotics whose global market value is expected to exceed $8.5 billion by 2024 (Quigley, 2019). Lactosucrose is a functional carbohydrate that has garnered increased popularity due to its prebiotic properties. Lactosucrose is a trisaccharide composed of a β-D-galactopyranosyl (Galp) unit linked via a (1→4)-α bond to a D-glucopyranosyl (Glcp) unit that in turn is connected to a α-D-fructofuranosyl (Fruf) unit via a (1→2)-β bond [β-D-Galp-(1→4)-α-D-Glcp-(1→2)-β-D-Fruf] (Duarte et al., 2017; Silvério, Macedo, Teixeria, & Rodrigues, 2015). It is certified as FOSHU (Functional Food Ingredients for Foods for Specified Health Uses) in Japan and used as a dietary supplement (Monsan & Ouarné, 2009; Xu, Liu, Yu, Zhang, & Mu, 2018). Moreover, lactosucrose provides attractive technofunctional properties (e.g. hygroscopic behavior and water holding capacity), which could improve food consistency and texture (Silvério, Macedo, Teixeria, & Rodrigues, 2015). Unfortunately, low quantities of lactosucrose exist naturally in foods, and thus an efficient process is needed for its synthesis (Mu, Chen, Wang, Zhang, & Jiang, 2013). Lactosucrose can be produced by the action of either levansucrase (LS), β-galactosidase, or β-fructofuranosidase, all of which require sucrose and lactose as substrates (Duarte et al., 2017).

LS, a fructosyltransferase, has particularly gained attention due to its ability to catalyze the transfer of a fructosyl unit from a donor molecule to an acceptor one, resulting in the synthesis of various carbohydrates through oligomerization, polymerization, or donor/acceptor reactions. For instance, in the presence of only sucrose, LS can synthesize fructooligosaccharides (FOSs) and/or levan polymers by a processive or non-processive mechanism. LS offers an attractive catalytic activity for the production of lactosucrose by catalyzing the transfer of fructosyl unit from a sucrose donor to a lactose acceptor. Sucrose is a commonly used sweetener, and lactose is abundant being the main component of several million tons of the dairy by-products known as whey and milk permeate (Mu, Chen, Wang, Zhang, & Jiang, 2013). Both whey and milk permeate are recovered upon filtration steps, where the protein portion is isolated. The generation of dairy by-products is increasing in volume where in 2016, it was estimated that whey has a worldwide production of 200 million tons/year and its disposal represents an environmental hazard (Iliada et al., 2019). Lactose constitutes up to 75-85% (w/w) of whey permeate (Boer, 2014, Ryan & Walsh, 2016).

LSs from different microbial sources differ with respect to their reaction selectivity (hydrolysis/transfructosylation) and acceptor/product specificity. Consequently, modulating LS's

microenvironment through immobilization is expected to not only modulate the reaction selectivity towards transfructosylation, but to also promote enzyme stabilization and reusability (Hill, Karboune, & Mateo, 2016). The immobilization efficiency depends on the type and properties of the support used, which include pore size, physical characteristics, chemical stability, and binding sites, that should preferably improve enzyme characteristics, such as stability, substrate specificity, inhibition prevention, and kinetics (Ricardi et al., 2018; Duarte et al., 2017). Additionally, multi-attachments by adsorption and covalent bonds can be used to modulate the orientation of enzymes and enhance its action towards a favorable and efficient synthesis of lactosucrose (Grazu et al., 2005; Hill, Karboune, & Mateo, 2016).

The objective of the present research was to develop an innovative bioconversion process for the biogeneration of highly valued functional carbohydrates, in particular lactosucrose, from dairy by-products (i.e. whey and milk permeate). This study involves examining the catalytic efficiency of LSs from *Gluconobacter oxydans* (LS1), *Vibrio natriegens* (LS2), *Novosphingobium aromaticivorans* (LS3), and *Burkholderia graminis* (LS4) towards the transfructosylation of lactose and their application in a process for the synthesis of lactosucrose. The best identified LSs were further immobilized on selected supports and the catalytic properties were discussed as they are related to the support features.

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

1. Development of a bioconversion process for the synthesis of functional carbohydrates (i.e. lactosucrose) using selected LSs and different lactose sources.
    1.1. Determine the catalytic efficiency of selected LSs.
    1.2. Characterize the end-product profiles of LS-catalyzed bioconversion reactions.
    1.3. Determine the effects of reaction parameters on the efficiency of the enzymatic process and its product profile.
2. Immobilization of selected LSs on selected supports and assessment of their catalytic efficiency and their ability to synthesize lactosucrose.
    2.1. Determine the effects of supports on immobilization yield and retained specific activity.
    2.2. Assess the thermal stability and the substrate specificity of immobilized LSs.
    2.3. Characterize end-product profiles of immobilized LS-catalyzed bioconversion reactions.
    2.4. Investigate and optimize the effects of reaction parameters on the efficiency of the enzymatic process using response surface methodology.

CHAPTER II. LITERATURE REVIEW

2. Introduction

The human gastrointestinal tract (GIT) is host to a highly diverse, interconnected, and complex microbial community that has garnered increased attention due to its significant impact on human physiology and pathology. Under healthy physiological conditions, the gut microbiome aids in nutrient digestion, drug metabolism, immune system regulation, in addition to many other vital physiological functions. However, an overall reduction in the gut microbiome population as well as lower diversity levels have been shown to be involved in various pathological states such as irritable bowel syndrome (IBS), diabetes, Crohn's disease, Parkinson's disease, and so on (Park et al., 2017; Principi, Cozzali, Farinelli, Brusaferro, & Esposito, 2018). Looking at IBS from the aforementioned conditions, it has been found to affect approximately 11% of the global human population (Canavan, West, & Card, 2014). In addition to its high global incidence, it is also a financial burden due to increasing health care costs (Fedorak, Vanner, Paterson, & Bridges, 2012). Fortunately, consumers nowadays are becoming more aware of what they are taking into their bodies and are thus making more informed health-conscious decisions. That is evident with prebiotics that are increasing in popularity with their global market expected to exceed $8.5 billion by 2024 (Quigley, 2019). Therefore, it is of great importance to develop functional ingredients and nutraceuticals to meet that growing demand in an efficient, feasible, and sustainable manner for the proper maintenance of the gut microbiome and consequently the human health.

2.1. By-product of the dairy industry (whey)

The food industry generates a significant quantity of by-products and waste; therefore, the prevention of waste generation or its exploitation is one of the main objectives of environmental management and industries (Jang et al., 2012). Yogurt, cheese, butter, milk, ice cream, and so on are just some of the few everyday consumed products from the dairy industry. However, the dairy industry also generates a green-yellow liquid by-product known as whey as part of the production of cheese or casein from milk (Ryan & Walsh, 2016). The composition and physicochemical characteristics of whey are primarily dependent on the milk used and its animal source, diet, health, and lactation stage. However, whey always has a relatively high organic load dictated by the high biochemical oxygen demand (BOD) and chemical oxygen demand (COD). Its high organic load is primarily due to the presence of milk carbohydrates followed by proteins in addition to fats, minerals, and other suspended solids (Prazeres, Carvalho, & Rivas, 2012). Whey makes up 85 -

95% of the milk volume and keeps around 55% of the nutrients from the milk. 93% of the total whey volume is water followed by total solids that are divided into 66 - 77% (w/w) lactose, 8 - 15% (w/w) proteins, and 7 - 15% (w/w) minerals (mainly sodium, potassium, calcium, and magnesium salts) with trace amounts of fats, metals, such as zinc and copper, lactic acid, citric acid, non-protein nitrogen compounds, such as urea and uric acid, and B vitamins, such as riboflavin (vitamin B2) that provides whey with its yellow color (Ryan & Walsh, 2016; Iliada et al., 2019). The minerals are primarily due to the addition of NaCl during cheese production and thus it is made up of mineral salts with NaCl and KCl making up the majority of it followed by calcium salts that are mainly phosphates (Prazeres, Carvalho, & Rivas, 2012). Moreover, the type of whey depends on the processing technique used to remove the casein from the milk with mainly two types existing which are sweet and acidic whey. Sweet whey has a pH value of 5.6 and comes from the manufacturing of most types of cheeses and certain casein products where the first step involves the addition of rennet, which is a mixture of enzymes containing the protease chymosin, to milk. The rennet induces the coagulation of the casein proteins at a pH of 6.5 forming curds that are then strained from the remaining liquid which is the sweet whey. Acidic whey has a lower pH value of 4.5 and is produced by the activity of *Lactobacilli* or the addition of organic acids, such as lactic acid, or mineral acids, such as hydrochloric or sulphuric acid, to cause the coagulation of the casein proteins and make most types of industrial caseins. Apart from their pH values, both types also differ in their mineral content and protein fraction composition that on average is made up of 50 - 55% β-lactoglobulin, 20 - 25% α-lactoglobulin, 10% immunoglobulins, 5 - 10% serum albumin, 12% protease peptone 3, 1 - 2% lactoferrin, and 0.5% lactoperoxidase with 10 - 15% of a casein peptide glycomacropeptide only present in sweet whey (Ryan & Walsh, 2016). The bulk of the whey is sweet whey, but acidic whey is on the rise as yogurt consumption is becoming more popular (Fischer & Kleinschmidt, 2018).

In 2016, it was estimated that whey has a worldwide production of 200 million tons per year and increasing (Iliada et al., 2019). The volume of whey produced is almost equal to that of the processed milk used in cheese production and therefore it is increasing at a rate equal to that of milk volumes which is >2% per year (Ryan & Walsh, 2016). Furthermore, cheese production has in fact being increasing by 3% annually and every kg of cheese requires about 10kg of milk and gives rise to around 9kg of whey (Prazeres, Carvalho, & Rivas, 2012; Ryan & Walsh, 2016). Unfortunately, treating the whey can be expensive and even difficult due to its constantly changing

composition and strict legal requirements but without treating it, it poses a serious environmental hazard particularly on aquatic life (Prazeres, Carvalho, & Rivas, 2012). In 2008, a spill of acid whey in a body of water in the state of Ohio led to eutrophication due to the nutrient-dense nature of whey, especially its nitrogen and phosphorous content. That in turn caused a depletion of dissolved oxygen and thus the death of more than 5,400 wild animals, mostly fish, and the development of bad odors. Consequently, the treatment and/or valorization of whey is necessary and since it is a source of functional proteins and peptides, lipids, vitamins, minerals, and lactose, it can be transformed into valuable commodities to be utilized in the agri-food, biotechnological, medical, and many other industries (Ryan & Walsh, 2016).

One traditional use of whey is the usual dilution of its unmodified form with potable water for use as an animal feed for pigs, sheep, and cattle. It provides them with high quality proteins, lactose as energy source, and micronutrients like calcium, phosphorous, sulphur, and water-soluble vitamins. However, excess lactose and minerals can cause harm to the farm animals and thus it must be limited. Another traditional use of whey is spreading it on the land as a fertilizer but that can lead to a build-up of compounds like salts damaging the soil and impacting plant's growth and lifecycle. Crops have been shown to perish due to excessive whey application which causes rapid oxygen consumption in the soil as a result of breaking down the lactose and proteins in the whey. The whey can also reduce the soil's redox potential and lead to the solubilization of the Fe and Mn present in the soil and thus potentially contaminating ground water supplies. Moreover, a substantial amount of salt is added alongside whey increasing the salinity of the soil and reducing crop yield. If acid whey is added, then it also lowers the pH value damaging the soil. Both the aforementioned uses have drawbacks in terms of the volumes and high transportation costs, so they are impractical considering how much whey is being manufactured nowadays. More modern used of whey as a whole include its use to make beverages with examples including fruit juices and carbonated soft drinks mixed with whey in addition to low alcoholic beverages (<1% alcohol content), whey beer, whey wine, and whey champagne. The alcoholic beverages can be synthesized through the fermentation of whey alongside additives such as sucrose and malt with *Kluyveromyces fragilis* or *Saccharomyces lactis*. Other products include whey cheeses, such as Ricotta and Mysost, whey butter, and whey cream. Unfortunately, all the aforementioned products do not have a widespread commercial appeal and thus do not present an effective manner to treat the large quantities of the manufactured whey. Finally, the whey can be spray dried to make whey

powder which extends its shelf life for possible transportation and further processing. The whey powder can also be used as an animal feed in the form of dairy nuts when mixed with molasses or soya flour but can also be used in human foods such as ice cream, baked goods, sauces, and so on (Ryan & Walsh, 2016).

Focusing on the whey components separately, whey proteins tend to come to mind due to their functional properties and thus can be used in the food industry as emulsifiers, gelling agents, foaming agents, and so on (Kaur, Sharma, Jaimni, Kehinde, & Kaur, 2020). Whey protein is actually used in several products such as soups, salad dressings, processed meats, dairy, and baked goods. Whey proteins are usually separated through various physicochemical processes such as membrane separation (ultrafiltration or diafiltration) and then treated to provide whey protein concentrate (WPC), whey protein isolate (WPI) that has a higher content of protein and almost no lactose and fat, and lastly whey hydrolysate through the enzymatic hydrolysis of WPC and/or WPI. This entire filtration process leaves behind another liquid known as whey permeate which is primarily made of lactose (75 - 85% w/w) and thus also possess a high BOD making its disposal problematic. Fortunately, it can be crystalized and purified to provide lactose or can be fermented to form ethanol, single cell protein, yeast extract, bioplastics, glycerol, and other organic compounds (Boer, 2014; Ryan & Walsh, 2016). Those physicochemical processes can also be applied to milk, such as skimmed milk, to again produce a protein portion and another portion known as milk permeate, which contains a much lower lactose content (4.7% w/w) in comparison to the whey permeate (Boer, 2014).

2.2. Whey lactose valorization

Lactose, also known as milk sugar, can be recovered from cheese whey or more commonly from whey and milk permeate (Ryan & Walsh, 2016). It has unique properties like a low solubility and sweetness (16% of the sweetness of sucrose) as well as being difficult to crystallize, tending to form super-saturated solutions, and having low hygroscopicity when properly crystallized and the ability to carry flavours and pigments (Wong & Hartel, 2014). It is used in baked goods to promote the browning of the crust through the Maillard reaction, added to cow's milk to form infant formula, and used as an excipient in the pharmaceutical industry. Lactose production from whey has been on the rise and can be utilized to form several products and compounds (Ryan & Walsh, 2016).

Some compounds that could be made from lactose include:

- Lactulose, a disaccharide composed of galactose and fructose, is formed by either the chemical or enzymatic isomerization of lactose with the alkaline isomerization being the more commonly used method (Karim & Aider, 2020). It can be used as a sweetener as it provides 48 - 62% of the sweetness of sucrose and has the advantage of being more soluble than lactose thus making it easier to apply in foods (Ryan & Walsh, 2016). Several products that it can used in include diabetic and elderly food as a sweetener, confectionary products as a sucrose substitute, and dairy products as an additive as well as be used as a laxative to treat acute and chronic constipation, to treat hyperammonemia and chronic hepatic encephalopathy, and more recently as a prebiotic (Ryan & Walsh, 2016, Karim & Aider, 2020).

- Lactitol, a sugar alcohol composed of galactose and sorbitol, is created by chemical hydrogenation/reduction of lactose. It is also utilized as a sweetener providing 40% of the sweetness of sucrose and can be used in low calorie foods and diabetic products. It can be used to treat constipation and chronic hepatic encephalopathy and as a prebiotic due to its ability to be metabolized to form short chain fatty acid (SCFA) by the gut microbiome (Ryan & Walsh, 2016).

- Lactose can be hydrolyzed into a mixture of glucose and galactose by β-galactosidase (EC 3.2.1.23). That mixture delivers greater sweetness levels since glucose and galactose offer 80% and 60% of the sweetness of sucrose, respectively. It can be applied in confectionary products, ice cream, and soft drinks to potentially replace saccharose or corn starch syrup. The glucose can be even converted into fructose by glucose isomerase to further increase the sweetness of the solution since fructose has 110% of the sweetness of sucrose (Ryan & Walsh, 2016).

- Galactooligosaccharides (GOSs) can be formed as well from lactose through the transgalactosylation activity of β-galactosidase. GOSs are non-digestible oligosaccharides composed of galactose units and a terminal glucose unit with a degree of polymerization of 2 - 9. They are primarily known for their prebiotic effects having a positive impact on human health (Iliada et al., 2019).

- Lactobionic acid (LBA) has attracted the attention of the market due to its unique physicochemical properties. It belongs to the aldobionic acid family that is comprised of a galactose unit linked to a gluconic acid unit via an ether-like linkage. It can be formed by oxidation of the free aldehyde group of lactose. LBA is a chelating agent for heavy metals in situation that ethylenediaminetetraacetic acid (EDTA) is not effective. Different salts of LBA have been produced such as calcium that has been defined as a good source of calcium in pharmaceutical preparations and food additive (Minal, Balakrishnan, Chaudharym, & Jain, 2017). This lactose derivative has emerged as a promising and versatile substance with countless applications in the cosmetic, pharmaceutical, and food industries (Bai, Li, Tai, & Wang, 2020; Alonso, Rendueles, & Díaz, 2012; Kang et al., 2020).

Other compounds include tagatose as well as lactosucrose that is gaining more interest recently.

The lactose can also act as a carbon source for the synthesis of various products through biotechnological processes.

- Ethanol production through the use of whey permeate allows for a more limited usage of food resources in more common ethanol production methods and does not require much preprocessing such as high temperature acid treatment to break down the different types of cellulose in corn for example. Several yeast strains can be used for this such as *K. fragilis*, *Kluyveromyces marxianus*, and *Candida pseudotropicalis* and some like *K. marxianus* can be even immobilized in calcium alginate to improve ethanol production with a maximum productivity value of 6.97 g/L/h in continuous culture fermentation. Additionally, through genetic modification, lactose consuming *Saccharomyces cerevisiae* strains can be found and used in a continuous bioreactor to give rise to an ethanol productivity of about 10 g/L/h. The process of ethanol production varies, but all share the same basic principles and steps where whey permeate is fermented by efficient lactose utilizing yeast strains such as *K. marxianus*, *Streptococcus fragilis*, or *K. fragilis*. Despite the process being highly attractive, it is not as economical when compared to the production of ethanol from other sources such as sugarcane, corn, or lignocellulose. Butanol production from whey using *Clostridia* species has also been examined as it offers an advantage since it can be directly utilized in petrol engines while ethanol requires engine modifications (Ryan & Walsh, 2016).

- Hydrogen is a clean high energy source that does not produce greenhouse gases or contribute to acid rain. It can be formed from whey lactose as part of a biogas mixture alongside methane and carbon dioxide through anaerobic fermentation by using obligatory anaerobes like *Clostridium butyricum*, *Clostridium pasteurianum*, or *Clostridium beijerinkii* or facultative anaerobes such as *Enterobacter*, *Citrobacter* sp., or *Escherichia coli* (Prazeres, Carvalho, & Rivas, 2012; Ryan & Walsh, 2016).

- Aroma compounds are traditionally extracted from plants, but the method is accompanied with a low yield and high cost, and they can also be chemically synthesized which is an inexpensive method, but the compounds cannot be applied to food. Therefore, the production of natural aroma compounds through fermentation is a possible alternative. Some yeast strains are capable of producing an aroma compound found in rose petals known as 2-phenylethanol using a whey medium supplemented with other nutrients. *S. cerevisiae* was found to produce the highest concentration of 3.3 g/L (Iliada et al., 2019).

- Food colorants such as carotenoids and pulcherrimin can be produced as well. Carotenoids are a group of natural pigments consisting of β-carotene, lutein, astaxanthin, and so on that exhibit antioxidant, anti-diabetic, anti-cancer, and anti-inflammatory activities as well as health benefits such as reducing the risk of cardiovascular diseases. They are utilized as food supplements and colorants in products such as butter, margarine, cheese, confectionary products, ice cream, beverages, and so on. They can be extracted naturally, chemically synthesized, or produced through fermentation. Their fermentation can utilize various carbon sources such as glucose, sucrose, xylose and so on but low cost and renewable sources are being examined to reduce production costs. That is when whey was investigated, specifically whey permeate, and was found to provide the highest yield values among agro-industrial by-products. The enzymatic hydrolysis of whey permeate is often carried out before its fermentation as most of the microorganisms used are lactose-negative species. One example is *Blakeslea trispora* that appears to provide the highest yield with hydrolyzed whey permeate out of the tested microorganisms with 1,620 mg/L and an intracellular yield of 222 mg/g via a bubble column reactor. Also, the selection of the strains can form specific carotenoid types like obtaining more canthaxanthin through the use of *Dietzia natronolimnaea*. However, not all types can be obtained easily such as astaxanthin as some microorganism cannot assimilate

lactose and galactose. The microalgae *Chlorella zofingiensis* is one example of that and was even found to provide the lowest yield of astaxanthin with lactose from all the sugars used with it. Pulcherrimin is another pigment but is red in color and belongs to cyclodipeptides. It has anti-bacterial, anti-tumoral, and anti-inflammatory activities and can be used as a biocontrol agent against postharvest pathogens that cause spoilage. Hydrolyzed whey permeate must be used again here as the utilized microorganisms such as the yeast *Metschnikowia pulcherrima* cannot hydrolyze lactose. However, there are instances where whey lactose can be metabolized directly such as by *Bacillus licheniformis* to provide a yield of 331.7 mg/L under optimal conditions (Iliada et al., 2019).

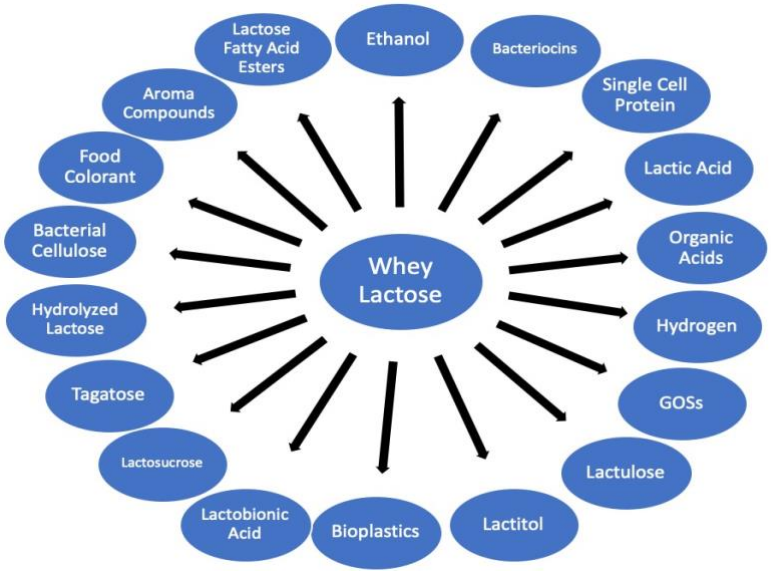
- Bacteriocins are a narrow spectrum of antimicrobial peptides with the spectrum varying based on the species producing them. Both gram positive and negative bacteria are capable of generating them to inhibit the growth of bacteria. It can be utilized by the food industry in dairy products, meat and fish products, and fruits and vegetables as well by medical, pharmaceutical, and veterinary professions. Bacteriocins can be synthesized by Lactic Acid Bacteria (LAB) like *Lactococcus lactis* which forms nisin. Nisin is industrially synthesized and approved for food use by the US Food and Drug Administration. They could play an important role against microbial infections and the whey media can provide a cheap and effective means of producing them. Nisin and other bacteriocins, such as pediocin from *Pediococcus acidilactici*, enterocin from *Enterococcus faecalis*, and bacteriocin from *B. licheniformis*, can be synthesized through supplemented whey growth media. (Ryan & Walsh, 2016).

- Bioplastics can be made from polyhydroxyalkanoates (PHAs) and polylactic acid (PLA) that in turn can be manufactured from the lactose of whey permeate. PHAs are polyesters of various hydroxyalkanoates created by many microorganisms as a carbon source and thus energy reserve for themselves. It is synthesized when essential nutrients like nitrogen or phosphorous are limited but there is plenty of carbon available. Lactose can be directly converted into PHA by using *Hydrogenophaga pseudoflava* or recombinant *E. coli* via β-galactosidase. Another way involves hydrolyzing lactose into its monosaccharides which in turn can be fermented by *Pseudomonas hydrogenovora* or *Haloferax mediterranei*. Lastly, lactose can be converted into lactic acid by LAB and then that can be used to form PHAs by

Alcaligenes latus. PLAs are formed as a result of the condensation reaction of lactic acid. Both those types of bioplastics offer advantages such as the fact that they are made from renewable sources, but regrettably most are not biodegradable. They are used in packaging for food and other material, disposable cutlery, insulation, and in the medical field for sutures, rivets, tacks, staples, screws, and surgical mesh. PLAs in particular have been used to produce loose-fill packaging, compost bags, compostable food packaging and disposable tableware, and in fibers and non-woven textiles like disposable garments, awnings, feminine hygiene products, and disposable diapers (Ryan & Walsh, 2016).

- Single cell protein (SCP) is a protein mixture from either pure or mixed cultures of algae, yeasts, fungi, or bacteria grown on agricultural waste and used as a substitute for protein rich food. It is produced by the fermentation of whole whey or whey permeate by the direct usage of lactose or its hydrolyzed form by the microorganisms. The majority of studies were done with *K. fragilis* or *K. marxianus* strains as they offer good growth yields and are classified as Generally Regarded As Safe (GRAS) (Ryan & Walsh, 2016).

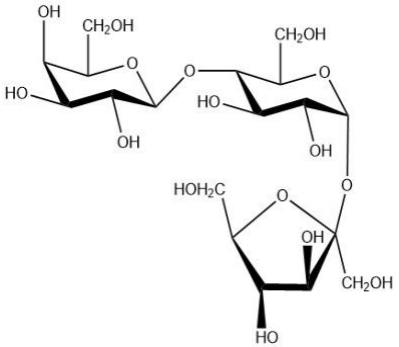
Other products that can be produced using lactose include yeasts for yeast extract production and baker's yeast, surfactants like sophorolipids, bacterial cellulose, lactic acid as the food additive E270, lactose fatty acid esters, organic acids like citric acid, acetic acid (vinegar), propionic acid, glycerol, and so on. Whey lactose can also simply act as a carbon source to support the growth of several microorganisms to harvest the produced enzymes and even for the development of functional food ingredients (Ryan & Walsh, 2016; Iliada et al., 2019).



Scheme 2.1. Possible products and compounds of whey lactose.

2.3. Lactosucrose

One product that is gaining interest is the aforementioned trisaccharide lactosucrose, which is composed of a D-galactopyranosyl (Galp) unit linked via a β-1, α-4 glycosidic bond to a D-glucopyranosyl (Glcp) unit that in turn is connected to a D-fructofuranosyl (Fruf) unit via an α-1, β-2 glycosidic bond), making it an isomer to the commonly known sugar raffinose (Duarte et al., 2017; Silvério, Macedo, Teixeria, & Rodrigues, 2015). The major difference between the two isomers is that the non-reducing end of raffinose with the galactosyl unit is in the α type configuration while that of lactosucrose is in the β type configuration. Some analogues for lactosucrose include isolactosucrose (D-Galp unit linked via a β-1, α-3 glycosidic bond to a D-Glcp unit that in turn is connected to a D-Fruf unit via a α-1, β-2 glycosidic bond), 4'-galactosyl-lactosucrose (D-Galp unit linked via a β-1, β-4 glycosidic bond to another D-Galp unit linked via a β-1, α-4 glycosidic bond to a D-Glcp unit that in turn is connected to a D-Fruf unit via a α-1, β-2 glycosidic bond), and 4'-galactosyl-lactose (D-Galp unit linked via a β-1, β-4 glycosidic bond to another D-Galp unit linked via a β-1, α/β-4 glycosidic bond to a D-Glcp unit) (Li, 2015). The α-1, β-2 glycosidic bond between the D-Glcp unit and the D-Fruf unit appears to be the most susceptible to hydrolysis as was shown through lactosucrose hydrolysis in supercritical water using a continuous flow-type reactor operating at 10 MPa with temperature values of 200, 210, and 230 °C. Under short durations, equimolar amounts of fructose and lactose were detected but with longer durations glucose and galactose molecules were also identified. Moreover, lactosucrose hydrolysis occurred at a faster rate at higher temperatures (Silvério, Macedo, Teixeira, & Rodrigues, 2015).



Scheme 2.2. Chemical structure of lactosucrose.

Comparing it to sucrose (2000 g/L at 25°C), lactosucrose (3670 g/L at 25°C) has a much higher solubility in water and ~30% of the relative sweetness thus providing a high-quality taste similar

to sucrose when compared to other oligosaccharide sweeteners. It is stable for 1h at a pH value of 4.5 and temperature of 120°C. Lactosucrose is fairly indigestible in the upper section of the human GIT and low in calories making it appropriate for use in low calorie foods (Mu, Chen, Wang, Zhang, & Jiang, 2013). Its powder form is highly hygroscopic with a relatively high-water holding capacity. The high-water holding capacity can also be applied at an industrial level as that can decrease syneresis during the storage of products such as yoghurt and cheeses. Also, that property allows it to possibly be used as a fat replacer as it could improve consistency and texture of products since it decreases syneresis (Silvério, Macedo, Teixeria, & Rodrigues, 2015). Overall, it has been used in confectionaries, desserts, sweets, bakery products, yoghurt, coffee, and tea but mainly in Japan. The annual sales volume of lactosucrose reaches several kilotons in Japan with the two major manufacturers being Ensuiko Sugar Refining Co. and Hayashibara Shoji Inc., with the product brand names of Nyuka-Origo and Newka-Oligo respectively (Mu, Chen, Wang, Zhang, & Jiang, 2013).

2.3.1. Prebiotic effects

It has a relatively high laxative threshold level compared to other lactose-based functional ingredients, such as lactulose and GOSs. Combining its high laxative threshold with its high-water holding capacity, physiologically that means that more of it can be consumed without exerting laxative effects and it will increase peristalsis and improve fecal formation and output (Silvério, Macedo, Teixeria, & Rodrigues, 2015). Moreover, lactosucrose is certified as FOSHU (Functional Food Ingredients for Foods for Specified Health Uses) in Japan and used as a prebiotic; available in dietary supplements and in foods like yoghurt (Monsan & Ouarné, 2009; Xu, Liu, Yu, Zhang, & Mu, 2018). It must be noted that prebiotics are very different from the more commonly known probiotics as there appears to be a misconception between them and what makes them distinct from each other. The International Scientific Association for Probiotics and Prebiotics (ISAPP) supported the probiotic definition offered by the Food and Agricultural Organization (FAO) that defines probiotics as “live microorganisms which when administered in adequate amounts confer a health benefit on the host.”. On the other hand, the ISAPP in 2016 defined prebiotics as “a substrate that is selectively utilized by host microorganisms conferring health benefits.” (Quigley, 2019). This new definition allows the inclusion of non-carbohydrate substances, other non-food groups, and lastly the application to body sites other than the GIT. Nonetheless, their selective

utilization by microorganisms is kept as a criterion alongside proof for positive health exerting effects through those microorganisms (Gibson et al., 2017). Lactosucrose is selectively utilized by *Bifidobacterium* and shows little hydrolysis during its passage through the GIT with most of it reaching the cecum intact to be fermented resulting in an enhancement of *Bifidobacterium* in the faecal microflora. *In vitro* studies show that the analogues isolactosucrose and 4'-galactosyl-lactosucrose present potential prebiotic activity exhibiting relatively high selectivity index values for *Bifidobacterium* and *Lactobacilli* more than lactosucrose and 4'-galactosyl-lactose. Additionally, the SCFA production was highest with isolactosucrose, specifically acetic and lactic acids, with a total concentration of 105.13 mM followed by 4'-galactosyl-lactose and 4'-galactosyl-lactosucrose with 99.82 mM and 99.28 mM respectively (Li et al., 2015). *Bifidobacterium* take up lactosucrose and its analogues to produce SCFA resulting in a drop in the pH and inhibiting the proliferation of pathogenic microorganism, especially *Clostridium*. That inhibition also causes a decrease in the formation of toxic compounds like ammonia, phenol indole, skatol, and ethylphenol. It appears to improve the intestinal microenvironment in patients with Crohn's disease and ulcerative colitis and provides a protective effect on intracolonic indomethacin-induced small intestinal ulcers in rats. Furthermore, lactosucrose has been found to promote intestinal absorption of minerals, such as calcium. Through the tracing of ^{45}Ca during its transportation and deposition, it was proven that dietary supplementation with lactosucrose may effectively increase calcium absorption from the intestine in growing rats. Also, its supplementation in single and 2-week administration in healthy men could enhance intestinal calcium absorption and in addition to that could even have more long-term effects in reducing bone resorption in healthy young women. Lastly, it seems to influence the lipid profile as it inhibits the absorption of 2-monoacylglycerol by rat brush border membrane vesicles prepared from the small intestine. As a result of being able to inhibit β-monoglyceride absorption, it can reduce the intestinal absorption of dietary fat and thus reducing the parametrial adipose tissue weight of female mice fed with a high-fat diet (Mu, Chen, Wang, Zhang, & Jiang, 2013).

2.3.2. Lactosucrose production

Lactosucrose demand has been on the rise, in 2007 it was estimated to be around 3,000 tons/year with an annual growth rate of 10% (Silvério, Macedo, Teixeria, & Rodrigues, 2015). It scarcely exists in nature and is difficult to manufacture chemically, and thus its enzymatic synthesis is

preferred (Mu, Chen, Wang, Zhang, & Jiang, 2013). It can be produced by the action of either one of the three enzymes levansucrase (EC 2.4.1.10), β-fructofuranosidase (EC 3.2.1.26), and β-galactosidase, all of which require sucrose and lactose as substrates (Duarte et al., 2017). With sucrose being the most commonly used sweetener in the food industry and several million tons of lactose being produced annually from the cheap byproduct known as whey, both disaccharides are increasingly recognized as vital inexpensive sources to produce valuable functional ingredients (Mu, Chen, Wang, Zhang, & Jiang, 2013).

The literature appears to indicate that its synthesis was first carried out by Avigad in 1957 by the levansucrase from *Aerobacter levanicum* (Mu, Chen, Wang, Zhang, & Jiang, 2013). As previously discussed, levansucrase requires sucrose as the fructosyl donor and lactose as the fructosyl acceptor (Xu et al., 2019). Some other examples of levansucrases producing lactosucrose are from *Bacillus subtilis* (Park, Choi, & Oh, 2005), *Leuconostoc mesenteroides*, *B. subtilis natto* (Li, Yu, Zhang, Jiang, & Mu, 2015), *B. licheniformis* (Lu, Lu, & Xiao, 2014), *Zymomonas mobilis* (Han et al., 2009) *Brenneria goodwinii* (Xu, Liu, Yu, Zhang, & Mu, 2018), and so on. However, the levansucrase from *B. subtilis* proved to be one of the more efficient employers of lactose as a fructosyl acceptor and sucrose as the fructosyl donor. Its optimal conditions were determined to have been at a pH of 6.0 and temperature of 55°C providing 183 g/L of lactosucrose after 10h (Monsan & Ouarné, 2009). Another efficient producer of lactosucrose is *Pseudomonas aurantiaca*, which produces 285 g/L from 510 g/L of sucrose and 360 g/L of lactose at a pH of 4.0 and temperature of 45°C for 2h. It must be stated that the increase of the ratio of sucrose to lactose aided in increasing the lactosucrose yield. The majority of lactosucrose production via transfructosylation, whether it be from the use of microorganisms or biocatalytic systems, is equal to about 100 - 200 g/L from varying concentration of sucrose and lactose (Li, Yu, Zhang, Jiang, & Mu, 2015). Moreover, one study conducted utilized industrial by-products for the formation of lactosucrose. They used a cheese and tofu whey mixture alongside a LS from *B. subtilis* CECT 39 and were able to produce a maximum lactosucrose amount of 80.1 g/L after 2h of reactions time (Corzo-Martinez, Luscher, de Las Rivas, Muñoz, & Moreno, 2015).

The industrial production of lactosucrose has been developed by Ensuiko Sugar Refining Co. and is carried out by the β-fructofuranosidase from *Arthrobacter* sp. K-1 (Mu, Chen, Wang, Zhang, & Jiang, 2013). It must be noted that through the phylogenic analysis on the 16S rDNA sequence,

the strain has been reclassified as *Microbacterium saccharophilum* K-1 (Li et al., 2015). Despite β-fructofuranosidase being widespread in nature, very few microorganisms have been shown to be able to synthesize lactosucrose with that enzyme and thus the majority of studies focus on *Arthrobacter* sp. K-1 showing that its optimum temperature and pH values are 55°C and 6.5 respectively. Additionally, it is somewhat thermostable as it retains 70% of its initial activity after being incubated for 30min at 60°C (Mu, Chen, Wang, Zhang, & Jiang, 2013). However, it is shown to be quite costly to use due to it still being easy to deactivate at high temperatures as well as exhibiting product contamination, low transfructosylation efficiency, and the glucose byproduct acting non-competitively to inhibit transfructosylation activity ultimately leading to low lactosucrose yields averaging around 25% and the need to utilize expensive purification techniques (Long, Pan, Xie, Xu, & Jin, 2019). Fortunately, the conversion ratio of sucrose into lactosucrose can be improved to over 80%, from 50% in batch processing, through the use of β-fructofuranosidase in a simulated moving bed reactor for a continuous production process (Mu, Chen, Wang, Zhang, & Jiang, 2013).

Lactosucrose can also be synthesized via the transgalactosylation action of β-galactosidase from *Bacillus circulans*, where lactose would act as the galactosyl donor molecule while sucrose would be the acceptor molecule. However, only 56 g/L of lactosucrose is produced from 300 g/L of sucrose and 300 g/L of lactose. That is a conversion ratio of about 10% making its lactosucrose production significantly lower than that of levansucrase and β-fructofuranosidase (Mu, Chen, Wang, Zhang, & Jiang, 2013).

Lactosucrose yields do not tend to be very high due to product degradation and thus several methods are employed to favor transglycosylation over hydrolysis. One method involves thermal deactivation of the enzymes to terminate the reaction before degradation can initiate. Another method involves product separation via enzyme immobilization or through process integration, which combines product synthesis and separation, both of which could reduce contact time between the enzymes and products to favor transglycosylation and/or eliminate potential inhibitors (Silvério, Macedo, Teixeria, & Rodrigues, 2015). The product could also be removed via the use of a bi-enzymatic system. In 2009, Han et al. tested that out by using levansucrase from *Z. mobilis* with glucose oxidase, which removes the glucose byproduct from the reaction mixture, resulting in a reduction of product inhibition by glucose and the elevation of lactosucrose production

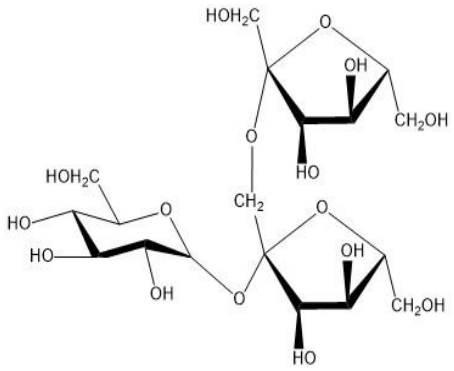
efficiency from 28.5% to 43.2% (Li, Yu, Zhang, Jiang, & Mu, 2015). Another possible method of removing the product involves the addition of a microorganism to the reaction mixture. The combination of β-fructofuranosidase from *Bacillus* sp. V230 with a sucrose unassimilable yeast, such as *S. cerevisiae*, allows the yeast to only assimilate monosaccharides, glucose in this case, without consuming or hydrolyzing the di- and oligosaccharides simultaneously increasing the overall lactosucrose content and decreasing the amount of byproducts at the end. This was similarly done through the use of β-fructofuranosidase from *Arthrobacter* sp. alongside invertase-deficient yeast. Finally, protein engineering is another approach that has been utilized as well (Silvério, Macedo, Teixeria, & Rodrigues, 2015). One example includes the levansucrase from *Sterigmatomyces elviae* ATCC 18894 that has shown an improved lactosucrose productivity of 54.3% when compared to the wild type (Li, Yu, Zhang, Jiang, & Mu, 2015).

2.4. Fructooligosaccharides

Focus is also currently placed on a class of non-digestible oligosaccharides known as fructooligosaccharides (FOSs). They belong to a water-soluble fructose-based carbohydrate family known as fructans. Fructans exist as oligosaccharides (FOSs) and polysaccharides (levan) and can be linear or branched with a degree of polymerization ranging from 3 units and up to a few hundred units (Van, 2013). FOSs are 3 - 10 fructose units long, usually composed of a terminal D-Glcp unit connected to a D-Fruf unit via an α-1, β-2 glycosidic bond with the remaining D-Fruf units attached via β (2,1) and/or β (2,6) glycosidic linkages (Monsan & Ouarné, 2009).

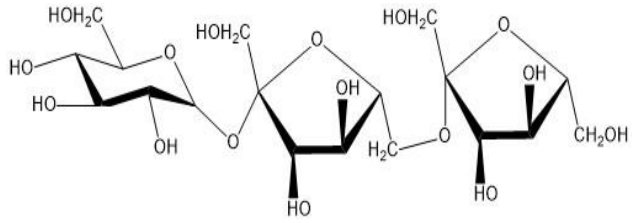
FOSs have four major classifications based on their linkages:

1) Inulin-type FOSs: They are linear in structure with β (2,1) linkages between the D-Fruf units attached to an initial sucrose base. The building block is the trisaccharide 1-kestotriose (1-kestose: Glucose-Fructose-Fructose). There are certain cases where the terminal glucose unit within the sucrose base could be substituted with fructose giving rise to a molecule composed solely of fructose termed as inulo-n-oses, where the "n" indicates the number of fructose units. Some examples include inulobiose, with 2 fructose units, and inulotriose, with 3 fructose units (Van, 2013).



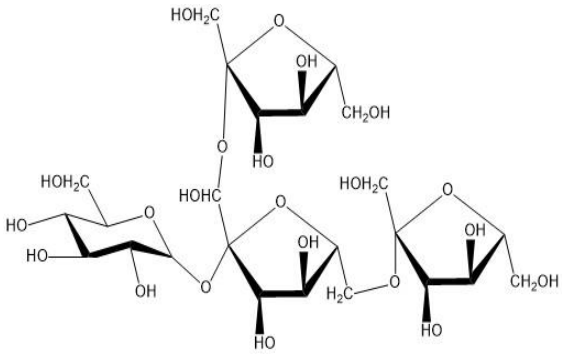
Scheme 2.3. Chemical structure of 1-kestotriose.

2) Levan-type FOSs: They are linear as well but contain β (2,6) linked D-Fruf units attached to the initial sucrose base. The building block is the trisaccharide 6-kestotriose (6-kestose: Glucose-Fructose-Fructose) (Van, 2013).



Scheme 2.4. Chemical structure of 6 kestotriose.

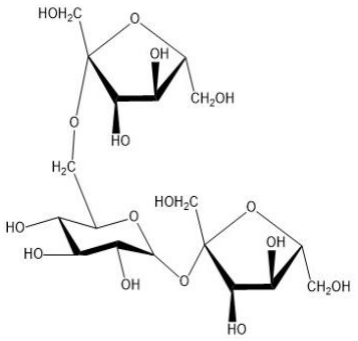
3) Mixed/Graminan-type FOSs: They contain both β (2,1) and β (2,6) linkages for the D-Fruf units bonded to the initial sucrose base (Van, 2013). The building block is the tetrasaccharide bifurcose where both β (2,1) and β (2,6) linkages are found at the D-Fruf unit within the sucrose base (Monsan & Ouarné, 2009).



Scheme 2.5. Chemical structure of bifurcose.

4) Neo-series FOSs: They are more complex FOSs where a D-Fruf unit is bonded to the C6 of the glucose unit that is found within the same initial sucrose base with any additional D-Fruf units now elongating on both ends. The building block is referred to as the 6G-

kestotriose base (neokestose: Fructose-Glucose-Fructose). There are 2 subcategories known as neo-inulin, where the predominantly found linkages are β (2,1), and neo-levan, where the predominantly found linkages are β (2,6) (Van, 2013).



Scheme 2.6. Chemical structure of 6G-kestotriose.

FOSs have functional uses within the food industry. The physicochemical properties of FOSs include that they are water-soluble, non-viscous, heat stable at food processing temperatures up to about 140°C, and stable over a pH range of 5.0 - 10.0. However, outside the aforementioned pH range and temperature levels, the FOSs will hydrolyze and lose their technofunctional and beneficial properties (Monsan & Ouarné, 2009).

FOSs are often used as fat and sugar substitutes due to their unique organoleptic properties. The use of FOSs as sugar substitutes is very common since they are considered low calorie non-cariogenic sweeteners. FOSs exhibit low sweetness levels equivalent to ~33% of the sweetness of sucrose with an energy supply of 0 - 3 kcal/g, which averages to about 40 - 50% of the energy provided by digestible carbohydrates like sucrose (Dominguez, Rodrigues, Lima, & Teixeira, 2014; Monsan & Ouarné, 2009). The sweetness level of FOSs varies with the chain length where longer chains lead to less sweetness but they can help with emulsions and provide a more neutral taste (Monsan & Ouarné, 2009; Vijn & Smeekens, 1999). Also, it can be noticed that the minimum energy supply is 0 kcal/g and that is because FOSs are not properly hydrolyzed by the human digestive enzymes and thus are not a significant source of energy or even sugar making them safe for diabetic individuals (Dominguez, Rodrigues, Lima, & Teixeira, 2014). Moreover, FOSs seem to affect the rheological properties of dough in bakery products, specifically the water absorption, elasticity, and stability. The water absorption is found to be reduced with more FOS content while the elasticity and stability of the dough increase. One example is the use of FOSs to partially replace sucrose in cookie dough where a soft texture was developed, and the nutritional value of the cookies was enhanced as well (Kumar, Sripada, & Poornachandra, 2018). Another advantage

of using FOSs in heat-processed foods, such as bakery products, is to minimize the browning levels occurring due to the Maillard reaction since they contain a non-reducing sucrose end that would not react with amino acids (Monsan & Ouarné, 2009). However, they can also be added to frozen foods in order to bring down the freezing temperature (Kumar, Sripada, & Poornachandra, 2018). Furthermore, FOSs can elevate aromas and mask off-flavors that are provided with some intense sweeteners (Monsan & Ouarné, 2009). Lastly, FOSs are incorporated as fat substitutes into several water-based products like dairy products, dressings, frozen desserts, and meat products. It appears that a ratio of 0.35 g of FOSs is sufficient to replace 1 g of fat (Kumar, Sripada, & Poornachandra, 2018).

2.4.1. Prebiotic effects

FOSs are gaining interest as they meet the prebiotic classification criteria (Quigley, 2019). They cannot be absorbed through the GIT and cannot be hydrolyzed due to humans lacking the required digestive enzymes for that (Monsan & Ouarné, 2009). That somewhat makes them similar to dietary fibers, such as cellulose for example that is not considered a prebiotic, but a major difference is that cellulose stimulates the growth of a wider range of microorganisms while FOSs allow for a narrower and more selective proliferation of primarily *Lactobacillus* and *Bifidobacterium* at the expense of potential pathogenic ones, such as *Clostridium* (Quigley, 2019). These two microorganisms can further suppress pathogens through the use of the lactic acid they produce to induce immunomodulatory molecules with antagonistic effects against those pathogens (Dorna et al., 2019). The high selectivity of those two microorganisms to FOSs is because they are some of the microorganisms that contain β-fructosidase enzymes capable of degrading FOSs and they even appear to prefer those chain lengths. It must be mentioned that both *Lactobacillus* and *Bifidobacterium* do not produce gas and so do not lead to uncomfortable gas distension, which is an undesired consequence (Gibson et al., 2017). Also, due to differences in length and solubility of FOSs and cellulose, the time for their metabolism and the location of their actions differ. The shorter and more soluble FOSs require a shorter time for their metabolism and are taken up by the microorganisms around the ileum and ascending colon, more proximal in the GIT, while the less soluble cellulose would require a longer time and would be partially utilized by the microorganisms in the distal colon due to the higher microbial density and the lower transit time (Holscher, 2017). Other than altering the proliferation of specific microorganisms, FOSs also

affect the gut microbiome metabolism. Their metabolism by the gut microbiome leads to a higher production of SCFAs, which include lactic acid, butyric acid, and propionic acid (Dorna et al., 2019). SCFAs are known to possess antimicrobial activity in addition to promoting homeostasis in the GIT and butyric acid in specific seems to influence intestinal epithelia development (Quigley, 2019; Dorna et al., 2019). Moreover, SCFAs have the ability to diffuse through the enterocytes of the GIT and thus are not limited to only act locally on the GIT but can affect distant organs leading to various effects on the human body (Dorna et al., 2019).

FOSs have been shown to decrease various conditions such as diet-induced obesity, diabetes, hepatic steatosis, and inflammation through changes in the gut microbiome population and function. Rat studies show that FOSs could normalize characteristic metabolic traits seen in insulin resistant obese rats and even decrease the incidence of obesity in their offsprings. The prebiotic effects and SCFA production might be associated with the augmented secretion of satiety hormones peptide YY and glucagon-like peptide-1 (Gibson et al., 2017). The FOSs with levan-type β (2,6) linkages appear to have anti-tumor activity and more bifidogenic properties than the inulin-type β (2,1) FOSs. (Yoo, Yoon, Cha, & Lee, 2004). Additionally, IBS symptoms have been shown to be positively influenced through the consumption of 5 g/day of FOSs for 6 weeks (Paineau et al., 2008).

One of the important effects of FOSs is on the immune system. Studies involving FOSs show an improvement of antibody response to viral vaccines with lower side effects. Additionally, they can reduce antibiotic use and disease duration (Dorna et al., 2019). A study carried out on healthy adult volunteers supplemented with β (2,1) fructans showed an up-regulation in circulating levels of the cytokine interleukin 4 (IL-4) as well as improved levels in CD282+/TLR2+ myeloid dendritic cells and toll-like receptor 2-mediated immune responses. This was carried out over a 28-day period to show moderate results but suggests that a longer duration of supplementation would possibly lead to greater effects on the human immune system (Clarke et al., 2016).

Prebiotics can alter the blood lipid profile of an individual and thus affecting their cardiovascular health. Due to the ability of FOSs to be converted into SCFAs, they can have varying effects. Acetic acid can be converted into acetyl-CoA to be used to synthesize fatty acids in the liver and in turn possibly increase serum triacylglyceride (TAG) and cholesterol levels. On the other hand, butyric and propionic acids can have the opposite effect as propionic acid can inhibit the

lipogenesis initiated by acetic acid thus decreasing TAG, LDL, and total cholesterol levels (Dorna et al., 2019). In fact, FOSs have been shown to be able to reduce TAG by an average rate of 7.5% through a meta-analysis of 15 studies between 1995 and 2005 (Brighenti, 2007).

Lastly, an intriguing relationship exists between the GIT and the central nervous system (CNS) as they are part of the "gut-brain axis". However, further research must be carried out in order to determine how significant these interactions are in affecting the endocrine pathway, mood, memory and neurodegenerative diseases. Fortunately, one promising outcome suggests that FOSs could affect the neural pathway as a result of the products that are formed due to their metabolism by the gut microbiome. They might have a regulatory effect on the molecules that help with the growth, survival, differentiation, and regulation of the CNS which would include brain-derived neurotrophic factors, neurotransmitters, and synaptic proteins (Dorna et al., 2019).

2.4.2. Fructooligosaccharides and levan production

2.4.2.1. Natural sources

Fructans are naturally found in numerous fruits, vegetables, and even honey. Some examples of plants in which they can be found as oligosaccharides and polysaccharides include asparagus, sugar beet, garlic, chicory, onion, Jerusalem artichoke, wheat, tomato, banana, and so on. FOS concentrations vary tremendously between these various sources where it can reach up to 20% (w/w) in Jerusalem artichoke and yacon (Dominguez, Rodrigues, Lima, & Teixeira, 2014; Monsan & Ouarné, 2009). In addition to the variable concentrations, the forms that are found in each source vary as well. Fructans from the agave plant for example exhibit both β (2,1) and β (2,6) linkages but not only provide graminan/mixed types as they also contain the neo-series types of fructans that have come to be named agavins. Plant fructans have been shown to have a maximum of around 200 monomer units (Kumar, Sripada, & Poornachandra, 2018). Unfortunately, the concentration of FOSs is still not significant enough to provide the previously mentioned technofunctional and beneficial effects (Dorna et al., 2019). There are natural extraction methods for FOSs from chicory roots, which contain 5 - 10% FOSs. That method involves harvesting, slicing, primary washing, hot water diffusion, purification, and drying. Any byproducts, such as glucose, fructose, and unreacted sucrose, can be separated by chromatography (Kumar, Sripada, & Poornachandra, 2018). However, a natural extraction method would still theoretically prove too slow and inefficient.

Microorganisms such as bacteria and fungi also contain fructans. Several fungal examples include *Xanthophyllomyces dendrorhous*, *Aspergillus japonicus*, *Rhizopus stolonifera*, and so on. Similarly to plants, the fructans vary in concentration and form between the various microorganisms. It is important to add that the fructans from bacterial sources shows greater branching and can have a length of up to 100,000 fructose units (Kumar, Sripada, & Poornachandra, 2018).

In nature, fructans can be found in nature as they are present in around 36,000 plants and various microorganism, such as bacteria and fungi (Quigley, 2019). Plants appear to primarily utilize fructans in its various structures as a storage form for carbohydrates in the stems, tubers, or rap roots (Kumar, Sripada, & Poornachandra, 2018). However, they appear to provide additional functions in the form of protecting the plants against water stress in response to drought or cold temperatures (Vijn & Smeekens, 1999). It is still not known why plants use the many different forms of fructans, but it is speculated to be due to physiological needs and/or as a result of varying evolutionary ancestors for its synthesis. In regards to the microorganisms, the simple reason is because some of them, such as B. subtilis, are able to synthesize the enzymes responsible for the production and hydrolysis of fructans and thus providing them with nutrients and energy as well as storing sucrose for later use when needed (Monsan & Ouarné, 2009). Additionally, fructans, specifically the polysaccharide levan, interact with other exopolysaccharides to contribute to the microbial biofilm structural integrity. The incorporation of fructans into the biofilm of B. subtilis in its soil habitat allows the fructans to protect them from moisture loss and other competing microorganisms as well as improve their adhesion to the environment. Another example is when Gluconacetobacter diazotrophicus uses the same polysaccharide form of fructans in its biofilm but this time the fructans help establish a microaerobic environment necessary for nitrogen fixation as it acts as a barrier for oxygen diffusion (Oner, Hernandez, & Combie, 2016).

2.4.2.2. Chemical synthesis

Chemically synthesizing FOSs is another possible option to obtain them. There is an acidic hydrolysis approach where the polysaccharides are broken down into the oligosaccharides with the use of microwave heating in order to reduce the coproduction of brown products. Further tests need to be carried out specifically on fructans as that was carried out on amylose to convert it into malto-oligosaccahrides by Warrand and Janssen in 2007. Additionally, this approach lacks

specificity giving rise to numerous products requiring further steps such as purification (Warrand & Janssen, 2007). Fortunately, the chemical synthesis of FOSs has been demonstrated by Marx et al. in 2000 via the acidic hydrolysis of levan to predominantly provide levan-type FOSs with β (2,6) linkages. This was carried out by the combination of a 5% (w/v) levan solution with 0.38 M sulphuric acid for 4min at 95°C followed by the use of cation-exchange chromatography for purification (Marx, Winkler, & Hartmeier, 2000). Using the same acidic hydrolysis principle, FOSs can be synthesized from agave fructans, but this method is still not as preferred as the enzymatic methods due to the poorer specificity and selectivity of the chemical approach (Nascimento et al., 2019).

2.4.2.3. Enzymatic synthesis

There are several enzymes that are used in the production of FOSs, which can be used separately or in combinations to form multi-enzymatic systems. Enzymes offer to be an attractive approach for FOS synthesis as they allow for more control of the regioselectivity and stereochemistry of the products (Duarte et al., 2017). Even though some of the enzymes can be found in animals and plants, the predominate sources are microbial and obtained through submerged and solid-state cultivation techniques (Nascimento et al., 2019).

β-Fructofuranosidase, also known as invertase, is one enzyme used in FOS production (Nascimento et al., 2019). Depending on their source, they can be part of the glycosyl-hydrolase (GH) 32, 68, or 100 family (Xu et al., 2019). They mainly catalyze the hydrolysis of the glycosidic linkage from the non-reducing end of the disaccharide sucrose to release an equimolar mixture of the monosaccharides glucose and fructose; this mixture is referred to as invert sugar. This enzyme is highly utilized in the food industry with a major application involved in non-crystallizable/invert sugar syrup formation (Nascimento et al., 2019). However, invertase can also exhibit transfructosylation activity where it catalyzes the transfer of a fructosyl group from sucrose to an acceptor molecule, such as another sucrose molecule to provide FOSs and a free glucose molecule (Monsan & Ouarné, 2009). In fact, some invertases exhibit a much higher transfructosylation activity than hydrolytic activity. That is most likely due to differences in subunit structures, molecular weight, degree of glycosylation, chemical susceptibility, and substrate specificity from one source to another. Furthermore, the ratio of transfructosylation to hydrolysis is controlled by the thermodynamic equilibrium that is affected by substrate and product concentrations in addition

to the pH and temperature values. It has been shown that higher sucrose concentrations tend to favour transfructosylation while higher glucose concentrations appear to suppress it (Dominguez, Rodrigues, Lima, & Teixeira, 2014). Since residual sucrose and glucose concentrations play a critical role in its activity, a continuous means to remove these substances has been investigated in order to increase FOS yields. One approach utilized glucose oxidase (EC 1.1.3.4) alongside invertase. Invertase would convert the sucrose into FOSs releasing glucose into the mixture. That is where glucose oxidase comes into play to convert the glucose byproduct into gluconic acid that can in turn be removed by ion-exchange resins or coagulation with calcium carbonate. This bi-enzymatic system has been found to increase the FOS yield up to 98% (g of FOSs/g of sucrose). Another approach investigated the use of nanofiltration means to remove glucose, which increased the FOS yield to up to 90% (g of FOSs/g of sucrose) (Dorna et al., 2019). Invertases are commercially available from fungal sources such as *Aspegillus niger*, *Aspergillus oryzae*, *Penicillium nigricans*, and *S. cerevisiae* (Kurakake et al., 2010).

Another important enzyme in FOS production is fructosyltransferase that catalyzes the hydrolyses of sucrose and transfers the fructosyl group to sucrose and/or FOSs for the production of a mixture of molecules (Nascimento et al., 2019). The mixture is mainly composed of the FOSs 1-kestose, nystose, and fructofuranosyl nystose in addition to free glucose and fructose molecules (Dominguez, Rodrigues, Lima, & Teixeira, 2014). However, it can also simply transfer the fructosyl unit to water, which simply means hydrolysis has taken place. Similar to invertases, substrate and product concentrations as well as the pH and temperature values regulate the thermodynamic equilibrium affecting the ratio of transfructosylation to hydrolysis. Fructosyltransferase can be classified as inulosucrase (EC 2.4.1.9) or levansucrase (Kumar, Sripada, & Poornachandra, 2018). Inulosucrase is responsible for synthesizing inulin-type fructans with β (2,1) linkages while levansucrase primarily gives rise to levan-type fructans with β (2,6) linkages. These enzymes appear to only belong to the GH 68 family. Inulosucrases can be principally found in lactic acid bacteria while levansucrases can be detected in both gram positive and negative bacteria (Xu et al., 2019).

2.5. Levansucrase

2.5.1. Reactions-catalyzed by levansucrase

As mentioned, levansucrase is a fructosyltransferase used by microorganisms to hydrolyze sucrose and simultaneously synthesize levan-type fructans with a chain length ranging from 20 kDa to several MDa (Lammens et al., 2009). Overall, levansucrase is capable of carrying out four different reactions with various acceptors:

1) Hydrolysis, where the acceptor is water (Strube et al., 2011)
2) Transfructosylation, where the acceptor is sucrose, other appropriate sugars, and FOSs (Strube et al., 2011)
3) Polymerization, where the acceptor is levan (Strube et al., 2011)
4) Exchange, where the acceptor is a monosaccharide (Strube et al., 2011)

2.5.2. Active site and mechanism

Levansucrase functions via a double displacement mechanism with a covalent fructose-enzyme intermediate. It is a simple 2-step reaction of glycosylation and de-glycosylation. The first part of the reaction starts with a nucleophilic attack on the anomeric carbon of glucose by the carboxylate group of the amino acid in the active site. The result is a covalent fructose-enzyme intermediate. The reaction continues as an acid/base catalyst donates a proton to the glycosyl leaving group. The second part of the reaction is where the acid/base catalyst now removes a proton from an incoming fructosyl acceptor, which could be water (hydrolysis) or a proper sugar acceptor (synthesis), leading to the breakdown of the fructose-enzyme intermediate (Lammens et al., 2009).

X-ray crystal structures allow for a better understanding of structure-function relationships and thus providing further details as to how the enzymes operate. The first apo-form (ligand free) high resolution crystal structure of levansucrase was provided from *B. subtilis* with later information provided by *G. diazotrophicus*, *Bacillus megaterium*, and *Erwinia amylovora* (Xu et al., 2019).

By looking at the active site of levansucrase, it can be classified as being part of the clan GH-J enzyme group. The active site shows a five-bladed β-propeller catalytic domain with 3 conserved amino acids in the N-terminal. Each blade contains 4 antiparallel β-strands in a classical ‘W’ topology causing the central negatively charged area of the active site to be enveloped. That area

contains the 3 conserved amino acids, which are 2 aspartates (Asp) and 1 glutamate (Glu), known as the "catalytic triad" (Lammens et al., 2009). In *B. subtilis*, they are identified as Asp46, Asp203, and Glu342 (Strube et al., 2011). The first Asp is part of the WMNDPNG motif acting as the nucleophile. The second Asp is found in the RDP motif and allows for substrate binding and acts as a transition-state stabilizer since it forms hydrogen bonds with the C3 and C4 hydroxyls of fructose. Finally, the Glu is in the EC motif and is identified as the acid/base catalyst (Lammens et al., 2009).

It must be emphasized that there are other amino acids throughout levansucrase important for its function. With the help of a holo-form (sucrose-bound) 3D structure of *B. subtilis* levansucrase, that can be further examined. It appears that -1 subsites bind fructose while the +1 subsites bind glucose. However, the +1 subsites do show some variability in regards to what they bind as they also bind fructose while -1 subsites appear to only show specificity to fructose. This might explain the sucrose-dependent enzyme activity between hydrolysis and polymerization. At low sucrose concentration levels below 250mM, the sucrose can still bind to the high affinity -1 subsites to cause hydrolysis while polymerization is promoted at higher sucrose concentrations due to the binding at the +1 and +2 subsites (Lammens et al., 2009). Glu340 and Arg360, equivalent to Asn399 and His419 in *G. diazotrophicus*, are found in the +1 subsite and create hydrogen bonds with glucose to place it in a specific orientation for the reaction to take place. Focusing more on Arg360, it seems to be essential for polymerization in *B. subtilis*. That is because when Arg360 is mutated into lysine, serine, or leucine, levansucrase loses its ability to carry out polymerization. Furthermore, this amino acid appears to regulate the specificity and efficiency of transfructosylation. The Arg360 is conserved in levansucrases in gram-positive bacteria but gram-negative bacteria replace it with the amino acid histidine at equivalent positions; His419 in *G. diazotrophicus* and His296 in *Z. mobilis* are some examples. Looking at the His296 of *Z. mobilis*, its mutation to Arg prevents the formation of microfibrils needed for polymerization and thus levan is not synthesized. Another example is the role of Arg370 and Asn252 of *B. megaterium*, equivalent to the Arg360 and Asn242 of *B. subtilis*, in levansucrase reactions. A mutation of the Asn into alanine or glycine completely prevents polymerization while a change into an aspartate simply lowers polymerization levels. Other examples include the importance of Arg360, Tyr429, and Arg433 in acceptor substrate specificity as their mutation into Ser360, Asn429, and Ala433 hinders polymerization reactions due to a possible reduction in polymer affinity to the binding

sites (Lammens et al., 2009). Moreover, looking at the His321 and Thr302 located at the +1 subsites of *Pseudomonas syringae*, their mutation alters the polymerization reaction by focusing more on short-chain FOSs, kestose, and nystose (Ithanavong, Tian, Khodadadi, & Karboune, 2013). Lastly, the amino acids Asn252, Lys373, and Tyr247 from *B. megaterium* impact the fructan product spectrum of levansucrase by stabilizing the acceptor fructan chains (Strube et al., 2011).

Metal co-factors are not required for levansucrase but the one from *B. subtilis* exhibits low-affinity calcium binding at Asp339 for upholding the fold stability. These sites appear to be conserved in most gram-positive bacteria but are absent in gram-negative bacteria. Interestingly, a disulphide bridge in gram-negative bacteria may offer a similar role as the calcium binding sites (Lammens et al., 2009). Furthermore, the thermal stability of levansucrase seems to have been improved by the tetramerization of Fe^{2+}, Al^{3+}, and Zn^{2+} (Xu et al., 2019).

2.5.3. Reaction selectivity (hydrolysis vs transfructosylation)

As discussed, levansucrase can carry out 4 reactions, one of which is hydrolysis, which would limit its ability to efficiently synthesize FOSs. Therefore, this ratio between hydrolysis and transfructosylation must be somehow modulated to favor transfructosylation. The ratio between the two reactions depends on various factors that include the source of the enzyme and reaction conditions, which comprise substrate and enzyme concentrations, temperature, pH, ionic strength, and the presence of water-miscible organic solvents (Olvera, Centeno-Leija, Ruiz-Leyva, & Lopez-Munguia, 2012; Oner, Hernandez, & Combie, 2016).

First, depending on the microbial source from which the levansucrase is found, it will exhibit diverse structural features and consequently different catalytic parameters. Variations exist in the levansucrases extracted from different sources such as molecular weight, optimal conditions, thermal stability, and as previously mentioned even the amino acid sequences. The levansucrase from *B. subtilis* has a molecular weight of 39 ± 1.5 kDa and performs optimally in a pH range of 5.8 - 6.0 while *Bacillus amyloliquefaciens* has a levansucrase with a molecular weight of 52kDa and an optimum pH range of 6.0 - 6.2. Those traits are more or less similar, but the two aforementioned levansucrases differ in their total specific activity and thermal stability, both of which are greater for *B. amyloliquefaciens* (Xu et al., 2019). The levansucrase from *B. subtilis* is even found to favor levan synthesis while that of *G. diazotrophicus* produces more FOSs. A

levansucrase from *Rahnella* was inspected and found to have a molecular weight of 120kDa, which is much larger than the average of 45 - 55kDa found in other bacteria (Ohtsuka et al, 1992). Another interesting example to look at is the levansucrase obtained from the thermophilic *Geobacillus stearothermophilus*. Due to the habitat in which this microorganism grows, its levansucrase exhibits superior thermal stability when compared to levansucrase produced by the mesophilic *B. subtilis* for example (Ithanavong, Tian, Khodadadi, & Karboune, 2013). The pH was even found to be capable of altering the 3D structure of the levansucrase from *Z. mobilis*. It is found in its dimeric form at a pH of 7 with hydrolytic activity but is altered at lower pH values to form insoluble fibrils and having transfructosylation activity take over. This is a noteworthy example since the pH caused the change in the structure but the actual ratio of those two reactions is directly affected by the structure and not the pH (Goldman et al., 2008).

The substrate concentration, pH, and temperature values are variables that could affect the levansucrase reactions and thus must be inspected. Starting with the concentration of the substrate, which is sucrose, a general pattern appears to be that the higher the sucrose concentration, the more transfructosylation is favored. Levansucrase from *B. subtilis* hydrolyzes sucrose at lower sucrose concentration, which are less than 250mM, but with higher sucrose concentrations, that are above 250mM, it tends to favor synthesis reactions (Lammens et al., 2009). Additionally, at a sucrose concentration of about 3mM, the transfructosylation activity of the levansucrase from *B. circulans* is at 40% but is then increased to 70% when the sucrose concentration is raised to 300mM (Oseguera, Guereca, & Lopez Munguia, 1996). Also, when exposed to sucrose concentrations less than 250mM, the dimeric form of the levansucrase from *Z. mobilis* only hydrolyzes the sucrose but a shift occurs at higher concentrations that lead to FOS synthesis (Goldman et al., 2008). Another example includes the levansucrase from *B. amyloliquefaciens* where an increase in sucrose concentration till 0.2M causes a further increase in levan synthesis. However, sucrose concentrations above 0.2M start to have an opposite effect as they hinder levan synthesis (Tian, Inthanavong, & Karboune, 2011). That suggests that there could possibly be some substrate inhibition where the effect of further increasing sucrose concentration could cause an opposite effect by decreasing transfructosylation activity. Furthermore, enzyme concentration appears to play a role as well. A low concentration of around 0.1 U/mL of levansucrase from *B. subtilis* caused more high molecular weight levans to be produced. That could be due to the promotion of polymerization of the fructan chains with little to no effect from the initial substrate concentration.

Heavy molecular weight levan synthesis was also favored in the presence of organic solvents such as ethanol, polyethylene glycol, and acetonitrile but that was shifted to low molecular weight levan synthesis when the ionic force of the solution was elevated. One example is the levansucrase from *B. licheniformis* that usually synthesizes heavy molecular weight levans, but when NaCl was added to the mixture to increase the ionic strength, low molecular weight levans were being more prominently synthesized (Oner, Hernandez, & Combie, 2016).

Transfructosylation and hydrolysis activities are affected by temperature and/or pH as well. Optimal pH and temperature values for levansucrases in general range between 5.0 - 6.6 and 25 - 60°C, respectively. Overall, there seems to be a direct correlation between hydrolysis and temperature, meaning as the temperature is increased, the rate of hydrolysis increases over transfructosylation. Levansucrases from both *Z. mobilis* and *P. syringae pv. phaseolicola* show an increase in hydrolysis with increasing temperatures at around 30 - 40°C while *B. subtilis* shows less sensitivity and increases hydrolysis at about 60°C (Hill, Tian, & Karboune, 2017). The ratio of transfructosylation to hydrolysis of the levansucrase from *G. stearothermophilus* is relatively high and is not affected by temperature changes as the highest hydrolytic and transfructosylation activities are at 57°C but it does show dependence on pH levels with a higher transfructosylation activity at pH levels of 6.0 - 6.5 (Ithanavong, Tian, Khodadadi, & Karboune, 2013). The levansucrase from *B. subtilis* has an optimum pH range for transfructosylation at 5.6 - 6.0 that is fairly narrow and even falls within the optimum pH range of 5.5 - 7.0 for hydrolysis (Olvera, Centeno-Leija, Ruiz-Leyva, & Lopez-Munguia, 2012).

2.5.4. Donor and acceptor specificity

In order for the transfructosylation reaction to take place, both a fructosyl donor and acceptor are needed. The most commonly used fructosyl donor by levansucrase is sucrose but raffinose has also been shown to be used. Raffinose is a non-digestible water-soluble trisaccharide made up of galactose and the two monosaccharides found in sucrose, glucose and fructose, making it a sucrose derivative (Li, Yu, Zhang, Jiang, & Mu, 2017).

The fructosyl acceptor list is far broader and includes sucrose again but encompasses other monosaccharides, disaccharides, and trisaccharides such as D-galactose, D-fructose, D-xylose, maltose, lactose, lactulose, cellobiose, melibiose, isomaltose, D-arabinose, L-arabinose, maltotriose, and raffinose (Li, Yu, Zhang, Jiang, & Mu, 2017; Park et al., 2003; Ohtsuka et al.,

1992). The acceptor specificity depends on a variety of factors, some of which are the enzyme source as usual in addition to the structure of the acceptor. It appears that the sugars with a pyranose ring, such as galactose, maltose, lactose, and so on, are better fructosyl acceptors than the ones with furanose ring, which include arabinose, xylose, and raffinose. Additionally, disaccharides are more favorable fructosyl acceptors than monosaccharides (Park et al., 2003). In 2003, Park et al. determined with the use of levansucrase from *Microbacterium laevaniformans* that the presence of a free C1 hydroxyl group on the glucose unit of three effective fructosyl acceptors (maltose, melibiose, and cellobiose) is needed as that is not available in the glucose unit of an ineffective fructosyl acceptor (trehalose). Furthermore, alkyl alcohols can be fructosyl acceptors and include glycerol, sorbitol, ethanol, ethylene glycol, propylene glycol, and aromatic alcohols like hydroquinone, resorcinol, and catechol (Xu et al., 2019; Kang et al., 2009).

2.6. Immobilization

2.6.1. Enzyme immobilization

When enzymes are used in their free form, that can prevent reuse and recovery. Immobilization helps resolve that issue as it allows for easier separation of the enzyme from the reaction mixture for possible reuse and thus reducing cost. The performance depends on the type and properties of the support used, which include pore size, physical characteristics, chemical stability, and binding affinity, that should preferably improve enzyme characteristics, such as stability, substrate specificity, inhibition prevention, and kinetics (Ricardi et al., 2018; Duarte et al., 2017). Also, the use of support systems allows for a possible development of a continuous operating system which would allow for the implementation at an industrial scale. It involves organic and inorganic supports with methods including adsorption, entrapment, encapsulation, crosslinking, and covalent binding (Ricardi et al., 2018).

1) Adsorption: involves relatively weak intermolecular forces leading to the accumulation of the enzyme on a surface. It is highly dependent on those intermolecular forces, which include ionic bonds, hydrogen bonds, van der Waals forces, hydrophobic interactions, and chelation, and thus the charges on the enzyme and polarity must be taken into account (Minteer, 2017; Brady & Jordaan, 2009; Cao, 2005). It is considered to be a mild immobilization method that has some leaching of the enzyme with time (Minteer, 2017).

2) Entrapment and encapsulation: both occur within a polymeric matrix.

- Entrapment involves trapping the enzyme by having a monomer or low molecular weight polymer polymerize around it (Minteer, 2017). It can be carried out with the so-gel method with traditional or electropolymerized polymers such as polyacrylamide, gelatin, alginate, carrageenan, polyurethane, and so on (Minteer, 2017; Bersaneti, Baldo, & Colabone, 2019). Several drawbacks include the chemical environment where polymerization is occurring and how it will affect the pore size and interconnectivity as well as the enzyme itself. It does not offer high improvements in stability against heat and organic solvents, but it has not been found to significantly decrease enzyme activity. It is common to have the enzyme leach out and thus the entrapment method and enzyme loading must be done carefully (Minteer, 2017).

- Encapsulation involves the same general principle but contains "pores" to immobilize the enzyme in. An example includes micellar polymers that swell and the enzyme intercalates into the "pores" as the solvent evaporates. They provide the enzyme with temperature, pH, and organic solvent stability by creating a microenvironment similar to the one found in the cell (Minteer, 2017).

3) Crosslinking: leads to the formation of cross-linked enzyme aggregates (CLEAs) and cross-linked enzyme crystals (CLECs). CLECs require a highly purified enzyme mixture while CLEAs involve precipitating the enzyme out of a solution to form 50 - 100 μm crosslinked aggregates (Brady & Jordaan, 2009). Despite increasing enzyme stability, crosslinking can decrease enzyme activity (Minteer, 2017).

4) Covalent bonding: involves the formation of covalent bonds between surfaces and the side chains of the amino acids of the enzyme (Bersaneti, Baldo, & Colabone, 2019). It usually requires the use of a bifunctional cross-linking reagent such as glutaraldehyde (Punekar, 2018). The supports can be the inner walls of bioreactors or polymeric beads in a packed-bed reactor (Minteer, 2017). Through covalent bonding, the enzyme tends to increase in stability and does not experience leaching as the previous methods do (Cao, 2005).

Additionally, multi-step methods can be used to have more control over the orientation of the enzymes. The first step is when adsorption is encouraged to bring the enzyme close to the reactive

groups of the support and then the second step involves the formation of covalent bonds (Grazu et al., 2005).

The use of immobilization techniques could be highly advantageous as it would allow for the formation of a microenvironment around the enzyme. That would in turn allow for more control of the process especially in the case of transglycosylation, as three different reactions are being controlled; 1) The synthesis of the product, 2) the hydrolysis of the glycosyl donor, and 3) the hydrolysis of the product (Duarte et al., 2017).

2.6.2. Levansucrase immobilization

Levansucrase has been immobilized through the various methods of adsorption, covalent bonding, entrapment, encapsulation, and crosslinking using different material (Bersaneti, Baldo, & Colabone, 2019).

Levansucrase from *B. subtilis* has been adsorbed onto hydroxyapatite via the ionic bonds of acidic residues. That reaction rate was increased by 2 folds and the levan production was higher as well. The levansucrase from *Z. mobilis* immobilized onto hydroxyapatite showed increased stability and maintained 60% of its activity compared to the 2% of its soluble form. Its adsorption onto titanium-activated magnetite also showed enhanced stability and even more activity retention equal to 70% (Hill, Tian, & Karboune, 2017). It was shown that the levansucrase from *B. subtilis* NRC33a immobilized through covalent bonding on chitosan beads activated with 3% glutaraldehyde provided the highest immobilization yield of 81.51% when compared to its adsorption, ionic bonding, and entrapment (Esawy, Fattah, & Mahmoud, 2008) Chitosan, which is a cationic polysaccharide made up of linearly linked N-acetylglucosamine and glucosamine units, has been shown to have good operational results for levansucrase and could be activated with glutaraldehyde to form covalent bonds. The use of chitosan beads to immobilize levansucrase from *B. licheniformis* produced 7.35 g/L of FOSs corresponding to a yield of 29.4% and was able to be used for 5 consecutive cycles (Bersaneti, Baldo, & Colabone, 2019).

Agarose, agar, and calcium alginate were also used to immobilize levansucrase from *B. subtilis* mutant NRC33a via entrapment. From all of the three polymers, agarose showed the highest protein yield of around 37%. The entrapment of the levansucrase from *Z. mobilis* in a sodium alginate gel was not found to be favorable as the levan production was low, which was speculated

to be due to the clogging of the matrix with the products. Another example is of the levansucrase from *B. subtilis natto* that was entrapped in calcium alginate which increased the stability and polymerase activity of levansucrase (Hill, Tian, & Karboune, 2017).

The use of CLEAs crosslinked with glutaraldehyde on levansucrase from *B. subtilis* and 2 other *B. subtilis* mutants provided increased thermal stability but ended up causing clogs and limited internal diffusion due to the produced high molecular weight levans. Overall, the CLEAs provided a low yield of about 30% and necessitated the use of a large quantity of enzyme (Hill, Tian, & Karboune, 2017). The levansucrase from *B. subtilis natto* was exposed to CLEAs crosslinked with oxidized glucomannan. That resulted in a rigidification of the enzyme causing the specific activity to decrease from 35 U/mg at 30°C to 12 U/mg at 50°C. However, there was an increase in the enzyme's thermal stability up to 45°C where more than 90% of its activity was retained after 15min and in the half-life from 9min to 55min at 50°C (Ben Ammar, Matsubara, Ito, Iizuka, & Minamiura, 2002).

Finally, a two-step method for immobilizing levansucrase from *B. amyloliquefaciens* was carried out on modified and unmodified epoxy-activated supports known as Eupergit® C, an oxirane acrylic support, and Sepabeads®, an aremethacrylic polymer support, respectively. Also, agarose beads were included for their easy functionality and the bonds they are capable of forming between the levansucrase and the support. Specific linkers were added as well by reacting the support with iminoacetic acid (IDA), with and without Cu, sodium, sulphide, triethylamine (TEA), and ethylenediamine (EDA). The first step involved the adsorption of the levansucrase close to the support in specific regions determined by the linkers so that the second step can occur where covalent bonds form. Glyoxyl agarose-IDA/Cu was the best overall support found among the rest, providing a high immobilization yield, thermal stability, and transfructosylation to hydrolysis ratio (Hill, Karboune, & Mateo, 2016). Further modification on glyoxyl agarose-IDA/Cu was done by the addition of the ionic polymer polyethylenimine (PEI) to improve protein and activity yields (44.73% and 54.69% respectively), activity retention, and a longer half-life at 50°C (Hill, Karboune, Mateo, 2017).

CHAPTER III. INVESTIGATION OF LACTOSUCROSE AND FRUCTOOLIGOSACCHARIDE PRODUCTION FROM DAIRY BY-PRODUCTS USING LEVANSUCRASE

Connecting Statement 1

A thorough literature review was conducted in chapter II providing the background information regarding the mechanism of levansucrase and its use in lactosucrose production. Chapter III examines the catalytic efficiency and end-product profile of various levansucrase from selected strains with the use of lactose and other dairy by-products, particularly whey and milk permeate.

The results from this study were presented at the 2020 consortium for research, innovation, and transformation of agrifood (RITA) at McGill university.

3. Abstract

In the present study, the catalytic efficiency of selected levansucrases (LSs) to catalyze the transfructosylation of lactose and sucrose into lactosucrose and fructooligosaccharides (FOSs) was investigated. Additionally, dairy by-products, including whey permeate (WP) and milk permeate (MP), were assessed for their effectiveness as lactose sources. LSs from *Gluconobacter oxydans* (LS1), *Vibrio natriegens* (LS2), *Novosphingobium aromaticivorans* (LS3), and *Burkholderia graminis* (LS4) were utilized in three selected biotransformation reactions that combined sucrose with either lactose, WP, or MP. Substrate activation was observed with *B. graminis* LS4, particularly in the presence of MP (805.80 μmol/mg protein•min) and WP (623.44 μmol/mg protein•min) compared to lactose. By examining the catalytic efficiency, it was found that all LSs demonstrated a higher transfructosylation activity than hydrolytic one, with the sole exception of *V. natriegens* LS2 in the presence of sucrose and MP/sucrose. Furthermore, the bioconversion efficiency of lactose/sucrose into lactosucrose and FOSs exhibited varying time courses and end-product profiles depending on the type of LS and starting materials used. *V. natriegens* LS2 resulted in the highest bioconversion yields (87 - 100%), giving rise to 328 and 251 g/L of lactosucrose with lactose/sucrose and WP/sucrose, respectively. Contrary to other LSs, *N. aromaticivorans* LS3 showed a higher product specificity toward the synthesis of FOSs, in particular kestose, nystose, and fructosyl nystose, in all investigated reaction systems. *G. oxydans* LS1, *V. natriegens* LS2, and *B. graminis* LS4 were proven to be able to use lactosucrose as a fructosyl acceptor; while *V. natriegens* LS2 could use lactosucrose as a fructosyl donor to form transfructosylation products. Our results revealed the potential of LS-catalyzed transfructosylation for the biotransformation of dairy by-products into value-added prebiotic lactosucrose.

3.1. Introduction

Yogurt, cheese, butter, milk, ice cream, and so on are just some of the few everyday consumed products from the dairy industry. However, the dairy industry generates a green-yellow liquid by-product known as whey from the production of cheese or casein (Ryan & Walsh, 2016). Whey makes up 85 - 95% of the milk volume and is nutritious as it keeps around 55% of the nutrients from the milk. For every kg of cheese, 10 kg of milk is required, giving rise to 9 kg of whey. In 2016, it was estimated that whey has a worldwide production of 200 million tons per year with an expected annual increase by 3% (Iliada et al., 2019). Consequently, the treatment and/or valorization of whey is necessary. Indeed, the solid content of whey is made up of 66 - 77% (w/w) lactose, 8 - 15% (w/w) proteins, and 7 - 15% (w/w) minerals (mainly sodium, potassium, calcium, and magnesium salts) with trace amounts of fats, metals, such as zinc and copper, lactic acid, citric acid, non-protein nitrogen compounds, such as urea and uric acid, and B vitamins, such as riboflavin (vitamin B2) (Ryan & Walsh, 2016; Iliada et al., 2019). Whey proteins, in the form of concentrate (WPC), isolate (WPI), and hydrolysate, are well-established value-added ingredients and are usually separated from whey through various physicochemical processes such as membrane separation (ultrafiltration or diafiltration). These separation processes generate a whey permeate (WP) by-product, which is primarily made of lactose (75 - 85% w/w) and thus possesses a high biochemical oxygen demand (BOD) making its disposal problematic. Fortunately, it can be crystalized and purified to provide lactose or can be fermented to form ethanol, single cell protein, yeast extract, bioplastics, glycerol, and other organic compounds (Boer, 2014; Ryan & Walsh, 2016). These separation processes can also be applied to milk, such as skimmed milk, generating milk permeate (MP), which contains a much lower lactose content (4.7% w/w) in comparison to the WP (Boer, 2014).

Lactose production from WP and MP has been on the rise and can be used to form several compounds and products (Ryan & Walsh, 2016). Examples of lactose-based value-added ingredients include lactulose, lactitol, galactooligosaccharides, lactobionic acid, and one compound that is gaining increased attention is lactosucrose (Ryan & Walsh, 2016, Karim & Aider, 2020, Iliada et al., 2019). Lactosucrose is a trisaccharide, which is composed of a β-D-galactopyranosyl (Galp) unit linked via a (1→4)-α glycosidic bond to a D-glucopyranosyl (Glcp) unit that in turn is connected to a α-D-fructofuranosyl (Fruf) unit via a (1→2)-β glycosidic bond

[β-D-Galp-(1→4)-α-D-Glcp-(1→2)-β-D-Fruf] (Duarte et al., 2017; Silvério, Macedo, Teixeria, & Rodrigues, 2015). Lactosucrose is known for its prebiotic and technofunctional properties, such as its water holding capacity and fat replacer ability (Silvério, Macedo, Teixeria, & Rodrigues, 2015). It scarcely exists in nature and is difficult to manufacture chemically, and thus its enzymatic synthesis is preferred (Mu, Chen, Wang, Zhang, & Jiang, 2013). Lactosucrose can be produced by the action of either one of these three enzymes, levansucrase (LS, EC 2.4.1.10), β-fructofuranosidase (EC 3.2.1.26), or β-galactosidase (EC 3.2.1.23), all of which require sucrose and lactose as substrates (Duarte et al., 2017). With sucrose being the most commonly used sweetener in the food industry and several million tons of lactose made available annually from the production of abundant dairy by-products, such as WP and MP, both of these disaccharides are increasingly recognized as vital inexpensive sources to produce valuable functional ingredients (Mu, Chen, Wang, Zhang, & Jiang, 2013). Although β-galactosidase has the ability to synthesize lactosucrose, it exhibits a lower catalytic efficiency toward the lactosucrose production than β-fructofuranosidase and LS (Mu, Chen, Wang, Zhang, & Jiang, 2013). Moreover, β-fructofuranosidase has been proven to be valuable in lactosucrose synthesis but its application is subject to many key limitations, including (i) narrow acceptor specificity for the synthesis of lactosucrose, (ii) limited thermal stability at higher temperatures, (iii) low transfructosylation efficiency, and (iv) non-competitive inhibition of transfructosylation activity by the glucose by-product (Mu, Chen, Wang, Zhang, & Jiang, 2013; Long, Pan, Xie, Xu, & Jin, 2019). These shortcomings may potentially be overcome using LS. In our previous study, genome mining was carried out via a BLAST (basic local alignment search tool) analysis starting with 601 sequences, from the UniProtKB database clustered with 80% of identity, of which 50 strains of LSs were cloned and transformed into *Escherichia coli* BL21 before assessing their transfructosylation activities and thermal stabilities (Hill et al., 2019). Among these LSs, LS1 from *Gluconobacter oxydans*, LS2 from *Vibrio natriegens* (NBRC 15636), LS3 from *Novosphingobium aromaticivorans*, and LS4 from *Burkholderia graminis* (Hill et al., 2019) were selected. The objective of the present study was to assess the catalytic efficiency and the reaction specificity (hydrolysis/transfructosylation) of the selected LSs using sucrose and three different lactose sources, including lactose, WP, and MP, as substrates. The time courses and the end-product profiles of the biotransformation reactions were characterised. The best biocatalytic systems, leading to the highest productivity and yield of lactosucrose, were identified.

3.2. Materials and Methods

3.2.1 Materials

Sucrose, D-(-)-fructose, D-(+)-glucose, D-(+)-galactose, α-lactose, myo-inositol, 3,5-dinitrosalicylic acid (DNS), potassium sodium tartrate ($KNaC_4H_4O_6$), yeast extract, carbenicillin disodium salt, lysozyme from chicken egg white, DNase I, imidazole, $C_2H_3NaO_2$, C2H7NO2, NH4HCO3, NaOH solution, and dextran standards (12 – 670 kDa) were obtained from Sigma-Aldrich (Oakville, ON). Fructooligosaccharide (FOS) standards (i.e.1-kestose, nystose, and 1^F-fructofuranosylnystose) and lactosucrose were supplied by FUJIFILM Wako Chemicals U.S.A. Corporation (Richmond, VA). Bradford reagent concentrate and sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) low range standards were purchased from Bio-Rad (Missasauga, ON). K_2HPO_4, KH_2PO_4, NaOH (Pellets/Certifies ACS), acetonitrile (ACN) HPLC grade, water optima LC/MS grade, bovine serum albumin (BSA), tryptone, NaCl, β-D-isothiogalactopyranoside (IPTG), PIPES, glycerol, tris hydrochloride, and tris-glycine-SDS 10x solution were provided by Fisher Scientific (Fair Lawn, NJ). Terrific broth (TB) and lysogeny broth (LB) agar powder were acquired from Bio Basic (Markham, ON). *E. coli* BL21(DE3) plysE strains were supplied by Invitrogen. WP (96.02% total solids made up of 11.55% protein, 7.57% ash, and a minimum of 76.09% lactose by weight) and MP (5.65% total solids made up of 0.25% protein, 0.60% ash, and a minimum of 4.80% lactose by weight) were obtained from a local dairy cooperative.

3.2.2. Expression and purification of selected levansucrases

The genes corresponding to LS1 from *G. oxydans* (strain 621H), LS2 from *V. natriegens* NBRC 15636, LS3 from *N. aromaticivorans*, and LS4 from *B. graminis* C4D1M were transformed into *E. coli* BL21-CodonPlus (DE3)-RIPL (Invitrogen) and precultured into LB media also containing carbenicillin (1 µl/ml). The preculture was incubated in an orbital shaker for 8 - 10 h at 37°C under 250 rpm. The preculture (2% v/v) was added to the TB with carbenicillin (1 µl/ml), which was then incubated at 37°C under 250rpm. When growth was achieved an optical density of 1.2 - 1.6 at 600nm (DU 800 UV/Visible Spectrophotometer, Beckman), the enzyme expression was induced using IPTG (1mM). Growth of the culture was continued at room temperature for 18h under 250rpm. The cells were then collected by centrifugation at 4°C under 8,000rpm and then stored at -80°C. The recovered pellets were resuspended in the sonication buffer (50mM PIPES, 300mM

NaCl, and 10% glycerol; pH of 7.2; 4 ml/g). Lysozyme (4 mg/g) and DNase (4 µl/g) were added the suspensions, and the mixtures were incubated at 18°C under 50rpm for 1h. The suspensions were thereafter sonicated with a microtip (Misonix Ultrasonic Liquid Processor S-4000) for six cycles (10 seconds on and 60 seconds off at 15kHz) in an ice bath. The supernatants containing the enzymes were recovered by centrifugation at 4°C under 14,000RPM for 1h and then dialyzed against potassium phosphate buffer (5mM; pH of 6) using a membrane with a molecular weight cut-off of 6 - 8 kDa. The LSs were purified via immobilized metal affinity chromatography on a HisTrap^{TM} FF column (5ml, GE Healthcare). After loading, the column was subsequently washed with sonication buffer (9 volumes), wash buffer (50mM PIPES, 300 mM NaCl, and 10% glycerol; pH of 6.4; 9 volumes), 5mM imidazole-wash buffer (9 volumes), and 10mM imidazole-wash buffer (9 volumes). The LS enzyme was then eluted with 100mM and 200mM imidazole-wash buffers (2 volumes), respectively. Finally, the purity of the LSs was confirmed upon the SDS-PAGE electrophoresis analysis at 120V using 15% SDS polyacrylamide gels and a 10x diluted Tris/Glycine/SDS buffer.

3.2.3. Levansucrase activity assay

One unit of the total LS activity was expressed as a quantity of biocatalyst that released 1µmol of reducing sugars, glucose and fructose, from sucrose per min. The total LS activity includes both hydrolytic and transfructosylation activity. The LS hydrolytic unit was expressed as the amount of biocatalyst that generated 1µmol of the fructose per min, while one transfructosylation unit was expressed as the amount of biocatalyst that produced 1µmol of glucose, due to the transfer of fructose, per min. Subtracting the total amount of fructose from that of glucose offers the amount of glucose resulting from transferring fructose. All assays were run in duplicates or triplicates.

The total LS activity assay was initiated by mixing purified LS solution with a sucrose substrate solution (1.8M) in potassium phosphate buffer (50mM; pH of 6) at a ratio of 1:1 (v/v). After 20 min of incubation at 30°C under 50rpm, the reaction was terminated by adding the DNS reagent [1% (w/v) 3,5-DNS and 1.6% (w/v) NaOH] at a ratio of 1:1.5 (v/v). The mixtures were then placed in boiling water for 5 min and thereafter, the potassium sodium tartrate (50% w/v) was added to stabilize the colour at a ratio of 1:0.2 (v/v). The absorbance was measured at 540nm and the reducing sugar concentration was quantified using a standard curve constructed from glucose (0 - 20 mM).

For the hydrolytic and transfructosylation activities, purified LS solution was added to a sucrose solution to yield a final concentration of 0.9 M in potassium phosphate buffer (50 mM, pH 6.0). 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.

The protein content of the LS solution was quantified using the Bradford protein assay with a BSA standard (1 - 20 μg/ml). The specific enzyme activity was expressed as the unit of biocatalyst in μmol of reducing sugar per min per mg of protein.

3.2.4. Enzymatic biotransformation reactions

To produce lactosucrose, the enzymatic biotransformation reactions were carried out using sucrose and lactose or lactose containing dairy by-products as substrates (WP and MP). Sucrose and lactose solutions were prepared at a ratio of 2:1 to reach a final concentration of 0.9M:0.45M. To initiate the biotransformation reactions, purified LS (5 U/ml) was added to the substrate solution. The biotransformation reactions were carried out at the optimal conditions for each LS, corresponding to 30°C/pH 4 (50mM sodium acetate buffer) for *G. oxydans* LS1, 45°C/pH 8 (50mM tris-HCl buffer) for *V. natriegens* LS2, 45°C/pH 6 (50mM potassium phosphate buffer) for *N. aromaticivorans* for LS3, and 30°C/pH 4 (50mM sodium acetate buffer) for *B. graminis* LS4. All reactions were done in duplicates under 50rpm. A blank where no enzyme was added was run in parallel for each LS. The biotransformation reactions were carried out over a time course of 48h where aliquots were taken, placed in boiling water for 3 min to stop the reaction, and then stored at -20°C until further analysis.

3.2.5. Reaction selectivity (hydrolysis vs transfructosylation)

The reaction selectivity was assessed in the presence of sucrose alone or with lactose or lactose-containing dairy by-products (WP and MP) as substrates using the four selected LSs. After the enzymatic biotransformation reactions, the remaining sucrose and lactose as well as the released galactose, glucose, and fructose were quantified by HPAEC using a Dionex ICS-3000 system equipped with a PAD and a CarboPac PA20 column as described above. The hydrolysis extent of

sucrose was quantified from the concentration of released fructose and taken as a percentage of the initial sucrose concentration, while that of lactose was expressed as the galactose concentration as a percentage of the initial lactose concentration. The extent of sucrose transfructosylation was based on the difference between the concentrations of fructose and glucose as a percentage of the initial sucrose concentration, whereas the extent of lactose transfructosylation was calculated from the difference between lactose and galactose concentrations over time as a percentage of the initial lactose concentration.

3.2.6. Levansucrase specificity towards lactosucrose

To assess the donor-acceptor specificity of LSs towards lactosucrose, biotransformation reactions were carried out in the presence of lactosucrose alone (0.45M) and lactosucrose/sucrose (0.45M/0.45M) substrates. The biotransformation reactions were initiated by the addition of *G. oxydans* LS1 (30°C/pH 4), *V. natriegens* LS2 (45°C/pH 8), *N. aromaticivorans* for LS3 (45°C/pH 6), or *B. graminis* LS4 (30°C/pH 4) at a concentration of 5 U/ml. Each reaction was carried out in duplicates and a reactions blank, without a LS enzyme, ran in parallel. At selected time intervals, aliquots were taken, placed in boiling water for 3 min to stop the reaction, and stored at -20°C until end-product characterization.

3.2.7. End-product profile characterization

The end-products of the biotransformation reactions were analyzed using an Agilent 1290 II liquid chromatography system coupled to an Agilent 6560-ion mobility Q-TOF – MS. The analytes were separated with an InfinityLab Poroshell 120 HILIC-Z column (2.1 x 100 mm, 2.7μm). Mobile phase A was LC-MS grade water with 0.3% NH_4OH and mobile phase B was ACN with 0.3% NH_4OH. Flow rate was set at 0.4 ml/min with a column temperature of 35°C. The constructed gradient started off with 85% B (0.0 to 0.5 min) that had a linear decrease to 30% B (0.5 to 9.0 min) where it was held (9.0 to 13.0 min) and then increased to 85% B (13.0 to 15.0 min), followed by a 3 min post-run. The mass spectrometer was equipped with a Dual AJS ESI ion source operating in negative ionization mode. MS conditions for ESI were as follows: drying gas temperature of 150°C and flow rate of 11 L/min, sheath gas temperature of 350°C and flow rate of 12 L/min, pressure on the nebulizer of 30psig, capillary voltage of 4000V, fragmentor voltage of 200V, skimmer voltage of 30V, and nozzle voltage of 2000V. Full scan MS data was recorded at mass-to-charge ratios (*m/z*) from 80 to 1100 at a scan rate of 2 spectra/s and was collected at

both centroid and profile mode. Reference ions (*m/z* at 112.985587 and 1033.988109 for ESI-) were used for automatic mass recalibration of each acquired spectrum. The quantification was performed using Quantitative Analysis 10.0 from Agilent MassHunter Workstation Software. The samples were prepared by dilution in 50:50 ACN:Water (v/v) with the addition of myo-inositol (5 - 30 ppm) to serve as an internal standard.

Furthermore, the production of oligomers and levans was assessed by high-pressure size-exclusion chromatography (HPSEC) utilizing a Waters HPLC system equipped with a 1525 binary pump, refractometer 2489 detector, and TSKgel G5000PWXL-CP column (7.8mm x 30cm, 5µm). An isocratic mobile phase made up of 200mM NaCl was set at a flow rate of 0.5 mL/min and the molecular weight distribution was determined using a standard curve constructed with dextrans of different molecular weights (12, 50, 270, and 670 kDa).

3.3. Results and Discussion

3.3.1. Catalytic efficiency of selected levansucrases

The catalytic efficiency of selected LSs was investigated in the presence of sucrose, lactose/sucrose, WP/sucrose, and MP/sucrose substrates. Sucrose can act as a fructosyl donor and acceptor, while lactose behaves mainly as a fructosyl acceptor. Table 3.1 shows the total, hydrolytic, and transfructosylation activities of selected LSs. The hydrolytic activity of LS resulted in the release of glucose and fructose, whereas the transfructosylation activity of LS led to the transfer of fructose to an acceptor or to a fructose growing chain, resulting in the formation of lactosucrose, FOSs, and/or levan. The hydrolytic activity was estimated from the free fructose concentration, while the transfructosylation activity was determined by subtracting the concentration of free fructose from that of glucose.

Table 3.1 shows that *B. graminis* LS4 exhibited the highest total activity (543.60 - 805.80 µmol/mg protein•min) when lactose, WP, or MP were used as acceptor substrates; it was also the only LS to provide a higher total activity with lactose source substrates than when sucrose was used by itself. Contrary to *B. graminis* LS4, a higher total activity was exhibited by *G. oxydans* LS1 (3.60 µmol/mg protein. min), *V. natriegens* LS2 (78.02 µmol/mg protein•min), and *N. aromaticivorans* LS3 (142.31 µmol/mg protein•min) when sucrose was the sole substrate than when it was added alongside lactose, WP, or MP (0.31 – 0.46, 41.25 – 68.16, and 46.08 – 63.29 µmol/mg protein•min, respectively).

Table 3.1. Catalytic efficiency of each levansucrase (LS) using sucrose, lactose/sucrose, whey permeate (WP)/sucrose, and milk permeate (MP)/sucrose

Enzyme	Substrate	Total Activity (μmol/mg protein•min)[a]	Hydrolytic Activity (μmol/mg protein•min)[b]	Transfructosylation Activity (μmol/mg protein•min)[c]	Transfructosylation / Hydrolysis Ratio[d]
G. oxydans LS1	Sucrose	3.60 (±0.01)	1.04 (±0.01)	2.57 (±0.01)	2.46
	Lactose/Sucrose	0.31 (±0.01)	0.05 (±0.003)	0.27 (±0.004)	5.87
	WP/Sucrose	0.32 (±0.01)	0.05 (±0.001)	0.28 (±0.01)	5.77
	MP/Sucrose	0.46 (±0.02)	0.12 (±0.01)	0.35 (±0.01)	6.74
V. natriegens LS2	Sucrose	78.02 (±4.24)	53.25 (±3.10)	24.77 (±0.11)	0.47
	Lactose/Sucrose	68.16 (±0.01)	21.79 (±0.01)	46.38 (±0.004)	2.13
	WP/Sucrose	41.25 (±0.52)	4.98 (±0.23)	36.27 (±0.29)	7.28
	MP/Sucrose	68.05 (±0.04)	58.66 (±0.04)	9.39 (±0.001)	0.16
N. aromaticivorans LS3	Sucrose	142.31 (±10.30)	68.80 (±5.40)	73.51 (±5.30)	1.07
	Lactose/Sucrose	46.08 (±0.73)	16.53 (±2.50)	29.55 (±1.77)	1.79
	WP/Sucrose	63.29 (±0.87)	17.40 (±1.31)	45.89 (±0.41)	2.64
	MP/Sucrose	59.56 (±0.08)	22.20 (±2.81)	37.36 (±2.73)	1.68
B. graminis LS4	Sucrose	101.19 (±2.30)	46.60 (±1.21)	54.60 (±3.21)	1.17
	Lactose/Sucrose	543.60 (±17.29)	98.16 (±6.93)	445.44 (±10.36)	4.54
	WP/Sucrose	623.44 (±19.12)	113.87 (±2.06)	509.57 (±17.06)	4.48
	MP/Sucrose	805.80 (±32.85)	352.07 (±15.58)	453.73 (±17.27)	1.29

[a] Total activity was calculated by taking the slope of glucose in mmol/ml•min and multiplying it by the total reaction volume and dividing that by the enzyme content.

[b] Hydrolytic activity was calculated by taking the slope of fructose in mmol/ml•min and multiplying it by the total reaction volume and dividing that by the enzyme content.

[c] Transfructosylation activity was calculated by taking the difference between the total activity and hydrolytic activity.

[d] The ratio of transfructosylation to hydrolysis of each LS.

When comparing the lactose sources, the use of MP resulted in the highest total activity for *G. oxydans* LS1 and *B. graminis* LS4 with *V. natriegens* LS2 also falling under the same trend as it had very close total activities in the presence of lactose or MP. However, *N. aromaticivorans* LS3 had the highest total activity when WP was present. Substrate activation was observed with *B. graminis* LS4, particularly with MP (805.80 μmol/mg protein•min) followed by WP (623.44 μmol/mg protein•min) instead of lactose. Such results can be attributed to the protective effect of dairy by-product components on the enzyme activity and stability. Looking through the literature, there was a lack of reported LS total activity values in the presence of a lactose source. Hill et al. (2019) have reported that LSs from similar microbial sources, *N. aromaticivorans*, *V. natriegens* and *G. oxydans*, showed total activity values of 363, 289, and 716 μmol/mg protein•min, respectively, with sucrose substrate. The total activity values found in the literature were in fact higher due to differences in reaction conditions, but their hydrolytic activities were higher than their transfructosylation activities. In our present study, the reaction conditions were modulated in order to favor the transfructosylation reaction over the hydrolytic one. Looking at other LSs from different microbial sources (Hill et al., 2019), it was found that the total activity expressed by *Paraburkholderia graminis* LS (7.7 μmol/mg protein•min), *Streptococcus salivarius* LS (31.9 μmol/mg protein•min), and *Beijernickis indica* LS (12.5 μmol/mg protein•min) were lower than the total activity values exhibited by *V. natriegens* LS2, *N. aromaticivorans* LS3, and *B. graminis* LS4 in the presence of sucrose, lactose/sucrose, WP/sucrose, and MP/sucrose. However, they were higher than the total activity values exhibited by *G. oxydans* LS1 with sucrose, lactose/sucrose, WP/sucrose, and MP/sucrose.

It can also be seen from the results that most of LSs exhibited higher transfructosylation activity than hydrolytic one. Despite *G. oxydans* LS1 having the lowest total activity among the enzymes with all substrate combinations, its transfructosylation to hydrolysis ratios were some of the highest. Indeed, the transfructosylation to hydrolysis ratio of *G. oxydans* LS1 was estimated at 2.46, 5.87, 5.77, and 6.74 when sucrose, lactose/sucrose, WP/sucrose, and MP/sucrose were used as substrates, respectively. These results reveal the high catalytic efficiency of *G. oxydans* LS1 towards the transfructosylation reaction. Contrary to other selected LSs, *V. natriegens* LS2 exhibited the lowest transfructosylation to hydrolysis ratios (0.16 – 2.13) in the presence of sucrose, lactose/sucrose, and MP/sucrose substrates, but achieved the highest observed ratio of 7.28 when WP/sucrose substrate was used. Additionally, the ratio of transfructosylation to

hydrolysis appeared to have increased when sucrose was used alongside a lactose source in all cases except for *V. natriegens* LS2 in the presence of MP/sucrose substrate (0.16). The substrate combination of WP/sucrose provided the highest transfructosylation to hydrolysis ratio for *V. natriegens* LS2 (7.28) and *N. aromaticivorans* LS3 (2.64). Furthermore, the highest ratio for *B. graminis* LS4 (4.48 – 4.54) was achieved in the presence of lactose/sucrose and WP/sucrose substrates. However, *G. oxydans* LS1 exhibited the highest ratio of transfructosylation to hydrolysis in the presence of MP/sucrose (6.74).

The transfructosylation to hydrolysis ratio values shown by Hill et al. (2019) for LSs from *N. aromaticivorans* (0.86 - 1.13), *V. natriegens* (0.83 - 1.55), *G. oxydans* (1.04 - 1.33), with sucrose substrate, were within the range of 0.83 to 1.55. Hill et al., (2019) have also reported similar ratios for LSs from *S. salivarius* (1.44) and *B. indica* subsp. *indica* (1.21). Moreover, using sucrose alone, higher transfructosylation to hydrolysis ratio of 2.7 and 2.2 was reported for LS from *Brenneria* sp. EniD312 (Xu, Ni, Yu, Zhang, & Mu, 2018) and from *Bacillus subtilis* (Raga-Carbajal, López-Munguía, Alvarez, & Olvera, 2018), respectively. Higher transfructosylation to hydrolysis ratio of 6.36 was reported for LS from *Bacillus amyloliquefaciens* in the presence of sucrose (Tian & Karboune, 2012). In the present study, the transfructosylation to hydrolysis ratios of *N. aromaticivorans* LS3 (1.07), *B. graminis* LS4 (1.17) and *G. oxydans* LS1 (2.46) in the presence of only sucrose were within the reported range in the literature. On the other hand, the obtained ratio for *V. natriegens* LS2 ratio (0.47) was lower. However, the addition of lactose substrate source enhanced the transfructosylation to hydrolysis ratio for most of studied LSs.

3.3.2. Time courses for the transfructosylation and hydrolysis reactions

Over the time course of the 48h biotransformation reactions of lactose/sucrose, sucrose/WP, and sucrose/MP, a shift in the thermodynamics of the reactions may occur, affecting the extents of transfructosylation and hydrolysis (Figure 3.1). Focusing first on sucrose biotransformation, the lactose/sucrose reaction catalyzed by *G. oxydans* LS1 exhibited a higher transfructosylation extent than hydrolysis one throughout the entire reaction time course. However, the *G. oxydans* LS1-catalyzed WP/sucrose and MP/sucrose biotransformation reactions showed the dominance of the sucrose transfructosylation extent at the early stage; as the reaction progressed, the hydrolysis extent became greater, reaching maximum values at 48h.

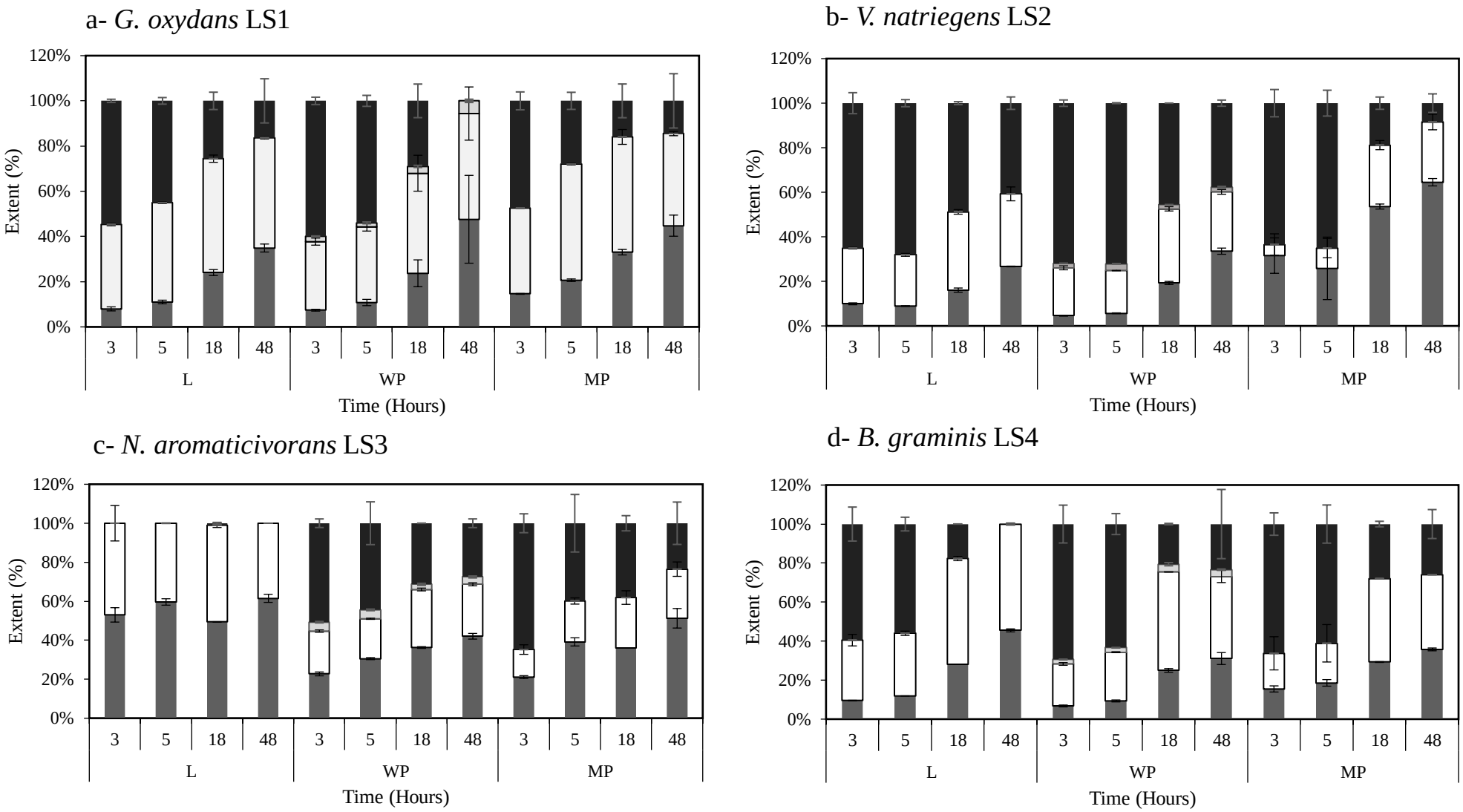


Figure 3.1. a-d. Time course for hydrolysis vs transfructosylation extent in the presence of lactose (L)/sucrose, whey permeate (WP)/sucrose, and milk permeate (MP)/sucrose for each levansucrase (LS): extent of sucrose hydrolysis (■); extent of sucrose transfructosylation (□); extent of lactose hydrolysis (■); extent of lactose transfructosylation (■).

The *V. natriegens* LS2-catalyzed lactose/sucrose reaction displayed a pattern where the transfructosylation extent was higher than that of hydrolysis over the entire time course of the reaction. However, the transfructosylation extent of the WP/sucrose reaction catalyzed by *V. natriegens* LS2 was initially greater than that of hydrolysis, but then at the end of the 48h reaction time, the extent of hydrolysis became greater. Contrary to *V. natriegens* LS2-catalyzed lactose/sucrose and WP/sucrose reactions, the hydrolysis extent of sucrose was predominant in the MP/sucrose reaction throughout the entire reaction time course. This is in agreement with the catalytic efficiency results shown in Table 3.1 where the hydrolytic activity of *V. natriegens* LS2 was greater than its transfructosylation when MP/sucrose was used. *N. aromaticivorans* LS3-catalyzed lactose/sucrose, WP/sucrose, and MP/sucrose reactions appeared to display more or less similar hydrolysis and transfructosylation extents of sucrose at the early stage, but then the hydrolysis extent took over for the remainder of the reaction time courses. *B. graminis* LS4-catalyzed lactose/sucrose, WP/sucrose, and MP/sucrose reactions all exhibited a higher transfructosylation extent than hydrolysis one throughout the entire reaction time course. The results (Figure 3.1) also show that the highest extent of transfructosylation of sucrose was achieved at around 18h with all 4 LSs with the sole exception being at 5h with *G. oxydans* LS1 for the MP/sucrose reaction. *G. oxydans* LS1 in fact delivered the highest sucrose transfructosylation extents for lactose/sucrose, WP/sucrose, and MP/sucrose reactions, estimated at 54.66%, 45.73%, and 47.08%, respectively.

Examining lactose biotransformation, no hydrolysis took place with all 4 LSs when lactose/sucrose and MP/sucrose substrates were used. Only a limited hydrolysis extent of lactose (<5%) was observed at time 0h in the presence of WP/lactose substrate (Figure 3.1). No significant change in the hydrolysis extent of lactose was obtained throughout the reactions, revealing that LSs did not contribute to the initial observed hydrolysis of lactose in the WP/lactose reaction. For the extent of lactose transfructosylation, a similar pattern was observed for *G. oxydans* LS1 and *B. graminis* LS4, where the transfructosylation extent started off high to reach maximum values at 3 - 5h but decreased steadily thereafter over the reaction time course. The lactose/sucrose reaction-catalyzed by *N. aromaticivorans* LS3 showed no significant transfructosylation of lactose. However, the extent of lactose transfructosylation in the *N. aromaticivorans* LS3-catalyzed WP/sucrose and MP/sucrose reactions varied between 23.72 - 29.81% and 18.53 - 34.79%, respectively, with a peak at 18h. The *V. natriegens* LS2 showed a high extent of lactose transfructosylation (56.82 -

72.32%) at the start (3h) of the lactose/sucrose, WP/sucrose, and MP/sucrose reactions that decreased over the time course of the reactions.

The results (Figure 3.1) also indicate that the use of *G. oxydans* LS1 and *V. natriegens* LS2 resulted in the highest extents of lactose transfructosylation at 3h and 5h in all their investigated reactions. Furthermore, *B. graminis* LS4 had its highest lactose transfructosylation extent at 5h in lactose/sucrose reaction and 3h in WP/sucrose and MP/sucrose reactions. The highest extents of lactose transfructosylation were obtained with *V. natriegens* LS2 in lactose/sucrose (61.22%, 5h) and WP/sucrose (72.32%, 3h) reactions. The highest lactose transfructosylation extent of 60.93% for the MP/sucrose reaction was achieved with *B. graminis* LS4 at 3h.

3.3.3. End-product specificity of selected levansucrases and production efficiency

The end-product profiles of the biotransformation reactions were characterized by Q-TOF – MS. Figure 3.2 shows the MS-MS spectra conducted in order to confirm the identity of the selected end-products where the fragmented compounds in the samples were compared to the fragmented compounds in the standards. The precursor ion for kestose and lactosucrose was *m/z* 503 and they shared a good portion of the resulting fragments with the major ones being *m/z* 341, 323, 179, 161, 119, and 101. The most easily identifiable fragments were *m/z* 341 for the sucrose unit formed by losing a fructose or galactose unit, m/z 323 for a hexose derivate, *m/z* 179 for possibly fructose, *m/z* 161 for a hexose structure of the monosaccharides, *m/z* 119 and 101 as fragments of glucose (Fruehwirth et al., 2021). The main differences were seen at the lower end with *m/z* 85 and 95 for kestose but *m/z* 83 and 97 for lactosucrose. The nystose had a precursor ion of *m/z* 665 with major fragments at *m/z* 503 and 485, while fructosyl nystose had a precursor ion at *m/z* 827 that fragments into *m/z* 665 and 647 with the remaining fragments matching those that can be seen in kestose.

Figure 3.3 demonstrates the end-product profiles of lactose/sucrose, WP/sucrose, and MP/sucrose reactions for each LS. The *G. oxydans* LS1 was able to catalyze the synthesis of both lactosucrose and kestose, achieving the highest contents within the first 5h of lactose/sucrose, WP/sucrose, and MP/sucrose reactions. In the lactose/sucrose and WP/sucrose reactions, *G. oxydans* LS1 synthesized high amounts of both of these compounds with a predominant product specificity towards lactosucrose (108.4 - 129.5 g/L) over kestose (86.0 - 94.6 g/L), while the MP/sucrose reaction resulted in the synthesis of higher amount of lactosucrose (84.3 g/L) than kestose (9.2 g/L).

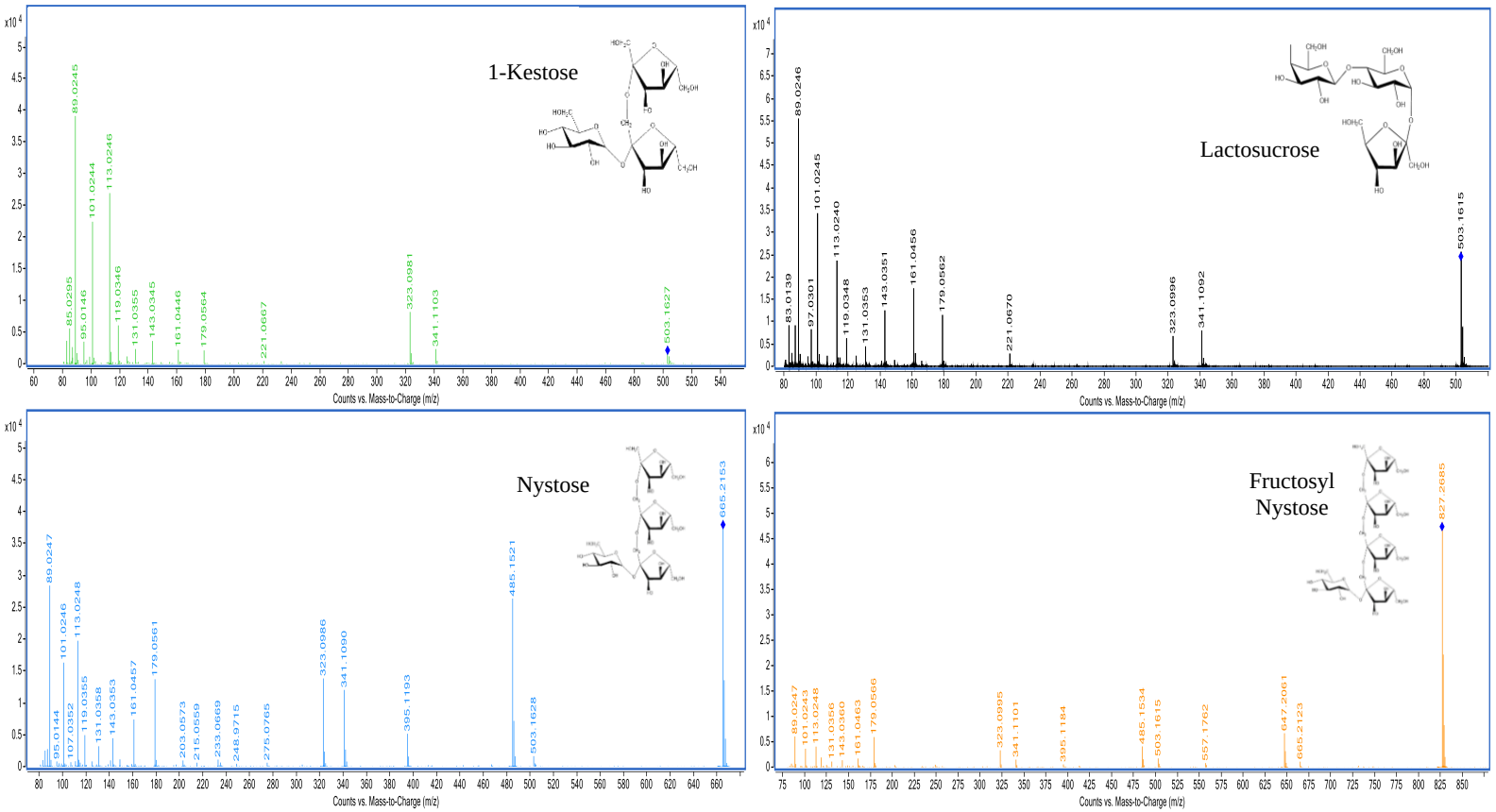


Figure 3.2. MS-MS fragmentation spectra of biotransformation end-products.

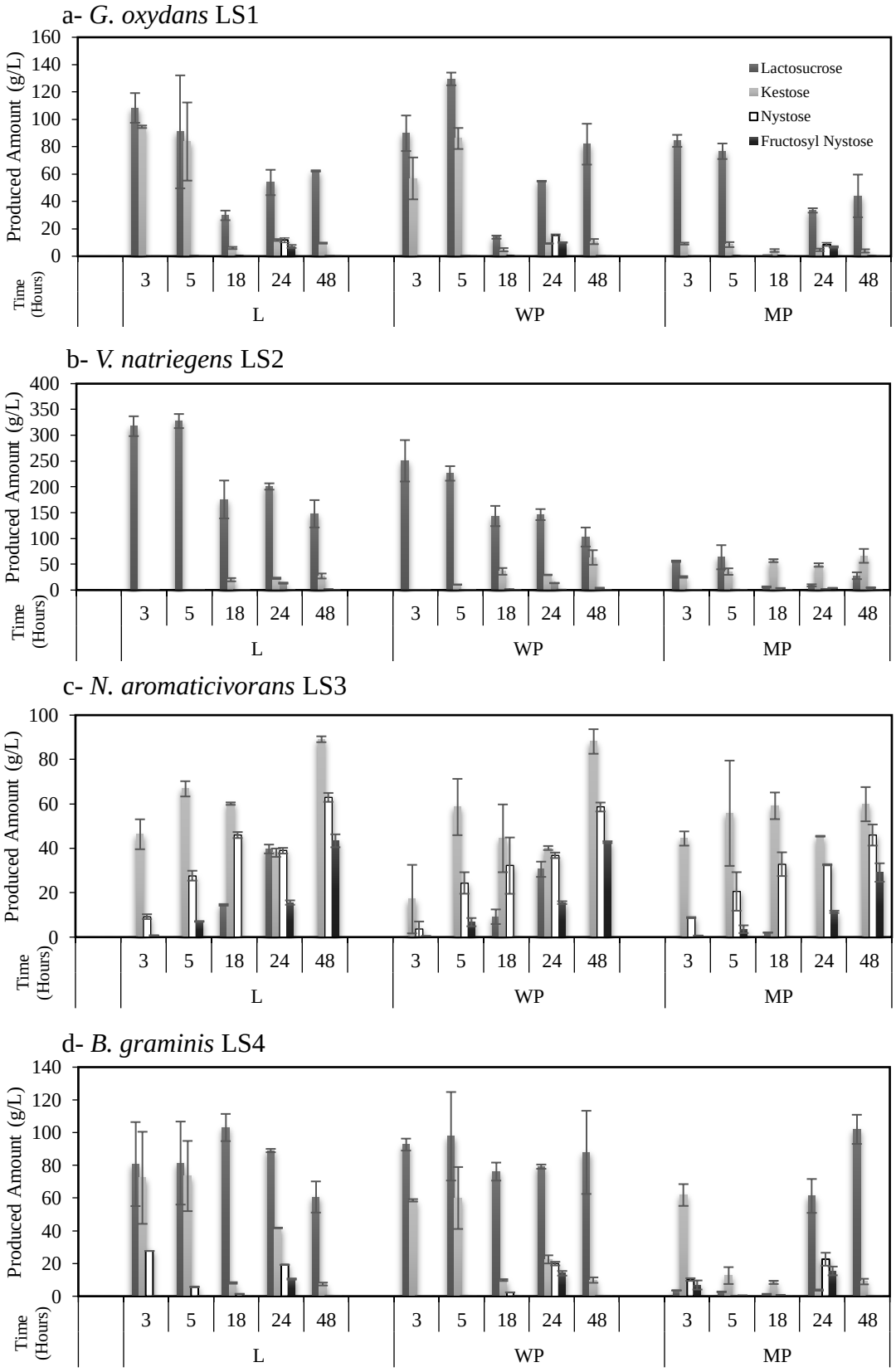


Figure 3.3. Biotransformation end-products in the presence of lactose (L)/sucrose, whey permeate (WP)/sucrose, and milk permeate (MP)/sucrose for each levansucrase (LS).

B. graminis LS4 was able to produce a constant amount of lactosucrose throughout the time course of WP/sucrose (97.8 g/L) and lactose/sucrose (103.1 g/L) reactions up to 5h and 18h, respectively. The production of kestose by *B. graminis* LS4 in lactose/sucrose and WP/sucrose reactions took primarily place within the first 5h of reaction, leading to a maximum amount of 73.5 and 60.0 g/L, respectively. The *B. graminis* LS4-catalyzed MP/sucrose biotransformation reaction favored the kestose synthesis at the start, with 61.9 g/L produced at 3h, which decreased over the time course of the reaction. A quantity of lactosucrose of 61.3 g/L eventually appeared at 24h with the *B. graminis* LS4 in the MP/sucrose reaction and continued to increase to reach 102.0 g/L at 48h. Nonetheless, an overall decrease in both lactosucrose and kestose quantities was displayed in the reactions catalyzed by *G. oxydans* LS1 and *B. graminis* LS4, which was attributed to the potential hydrolysis, possibly with lactosucrose, and/or to the elongation of end-products into oligomers and polymers, which was suggested with the emergence of nystose and fructosyl nystose.

The *V. natriegens* LS2-catalyzed lactose/sucrose and WP/sucrose reactions have primarily produced lactosucrose and very little kestose showing a high affinity of this LS towards lactose as an acceptor substrate (Figure 3.3). The highest amount of lactosucrose was estimated to be 327.6 g/L at 5h with lactose/sucrose reaction and 250.5 g/L at 3h with WP/sucrose reaction. Also, focusing on *V. natriegens* LS2, lactosucrose (63.7 g/L) over kestose (35.9 g/L) production was favored in the MP/sucrose reaction at the start but then kestose production took over at 18h till the end of the reaction time (66.3 g/L). These results support the trends observed in Figure 3.1 in which a high lactose transfructosylation extent was obtained at the first 5h and shifted thereafter towards a high sucrose transfructosylation extent. The predominance of the hydrolysis in the *V. natriegens* LS2-catalyzed MP/sucrose reaction as previously shown in Table 3.1 can explain the overall small amount of end-products observed in this reaction.

The results (Figure 3.3) also show that *N. aromaticivorans* LS3 displayed an opposite pattern compared to other LSs as there was little lactosucrose and primarily kestose and other FOSs being formed. Despite Table 3.1 showing a relatively high total activity of *N. aromaticivorans* LS3 in the presence of all lactose sources, *N. aromaticivorans* LS3 appeared to have a low acceptor specificity towards lactose. This is further supported by the observed low lactose transfructosylation extent (Figure 3.1) where it had the overall lowest lactose transfructosylation values among the LSs, especially in the lactose/sucrose reaction. Consequently, *N. aromaticivorans* LS3 was able to produce the highest amount of kestose, nystose, and fructosyl

nystose, corresponding respectively to 89.1, 62.9, and 43.4 g/L with lactose/sucrose, 88.1, 58.6, and 42.8 g/L with WP/sucrose, and 59.9, 46.0, and 29.1 g/L with MP/sucrose. However, the highest amount of lactosucrose produced by *N. aromaticivorans* LS3 was estimated at 39.7 g/L with lactose/sucrose, 30.6 g/L with WP/sucrose, and 2.0 g/L with MP/sucrose.

Reviewing the literature, it was found that in general the majority of lactosucrose production via transfructosylation, using microorganisms or biocatalytic systems, is equal to about 100 - 200 g/L from varying concentrations of sucrose and lactose (Li, Yu, Zhang, Jiang, & Mu, 2015). For instance, LS from *B. subtilis* was reported to exhibit high specificity towards lactose as the fructosyl acceptor and sucrose as the fructosyl donor. Using optimal conditions, 183 g/L of lactosucrose was produced by LS from *B. subtilis* after a 10h reaction (Monsan & Ouarné, 2009). LS from *Leuconostoc mesenteroides* B-512 led to a maximal amount of 224 g/L after a 1h reaction under the optimized conditions (Li et al., 2015). Another efficient biocatalyst for lactosucrose production was identified to be LS from *Pseudomonas aurantiaca,* which produced 285 g/L after 2h of reaction time (Li, Yu, Zhang, Jiang, & Mu, 2015). Xu, Liu, Yu, Zhang, & Mu (2018) have reported that LS from *Brenneria goodwinii* could effectively catalyze the formation of lactosucrose with a maximum of 100 g/L achieved when the reaction reached equilibrium. Moreover, one study conducted by Corzo-Martinez, Luscher, de Las Rivas, Muñoz, & Moreno (2015) utilized industrial by-products to produce lactosucrose. They used a cheese and tofu whey mixture alongside a LS from *B. subtilis* CECT 39 and were able to produce a maximum amount of 80.1 g/L of lactosucrose after 2h (Corzo-Martinez, Luscher, de Las Rivas, Muñoz, & Moreno, 2015). In our present study, *V. natriegens* LS2 was able to produce a higher amount of lactosucrose of 328 g/L and 251 g/L with lactose/sucrose and WP/sucrose, respectively, than those reported in the literature. This reveals that *V. natriegens* LS2 is a more promising candidate for the lactosucrose production at the industrial level.

The initial lactose concentrations in lactose/sucrose and WP/sucrose biotransformation systems were comparable. However, as MP was composed of a lower lactose concentration, it was difficult to reach the desired 0.45M lactose concentration without a drying step for removing the water. Therefore, to compare the production efficiency, the bioconversion yields of lactosucrose and kestose were calculated. For each LS and substrate used, the time point where there was a maximum production for each of kestose and lactosucrose was identified (Figure 3.4). As a sucrose to lactose ratio of 2:1 was used, the estimated lactosucrose bioconversion yield (calculated based

on the initial lactose concentration) was shown to be higher than that of kestose (calculated based on the initial sucrose concentration). Figure 3.4 shows that the bioconversion yield of kestose was highest for *G. oxydans* LS1 with lactose/sucrose (19%). However, *G. oxydans* LS1 has the lowest kestose bioconversion yield with MP/sucrose (2%), while the other LSs have somewhat similar kestose bioconversion yields with MP/sucrose (11 - 13%). *N. aromaticivorans* LS3 has the highest kestose bioconversion yield with WP/sucrose (17%). The highest lactosucrose bioconversion yields with lactose/sucrose and WP/sucrose are provided by *V. natriegens* LS2 (100% and 88%, respectively). The results also show that, except for *N. aromaticivorans* LS3, all LSs led to the production of the same maximum lactosucrose bioconversion yield with MP/sucrose.

The ability of LSs to produce longer chained oligomers and polysaccharides, particularly levans, was examined using HPSEC. Figure 3.5 depicts the amount of oligomers and levans produced by each LS for each substrate combination. All LSs generated oligomers where *B. graminis* LS4 produced the highest amounts of 6.55 and 5.38 g/L in the presence of lactose/sucrose and MP/sucrose substrates, respectively. *G. oxydans* LS1 produced the highest amount of oligomers of 7.28 g/L in the WP/sucrose reaction, whereas lower oligomers amounts of 3.02 - 3.64 g/L were obtained in the lactose/sucrose and MP/sucrose reaction systems. *G. oxydans* LS1 was the only LS able to produce levans in these lactose/sucrose (0.72 g/L) and MP/sucrose (0.71 g/L) reaction systems, indicating its polymerization ability. The literature shows that the amount of levan produced is much higher when only sucrose is present by itself. It was reported by Hill et al. (2019) that among the investigated LSs, the highest amount of levan produced was with LS from *N. aromaticivorans* (33g of levan per mg of protein) where only sucrose was added in the reaction (Hill et al., 2019). Another example includes crude LS from *Bacillus methylotrophicus* SK 21.002 was able to synthesize 100 g/L of levan after 16h under optimum reaction conditions with only sucrose (Zhang et al., 2014). An even higher amount of levan was produced (200 g/L) with crude LS from *Bacillus* sp. after 24h in the presence of only sucrose (Belghith, Dahech, Belghith, & Mejdoub, 2012).

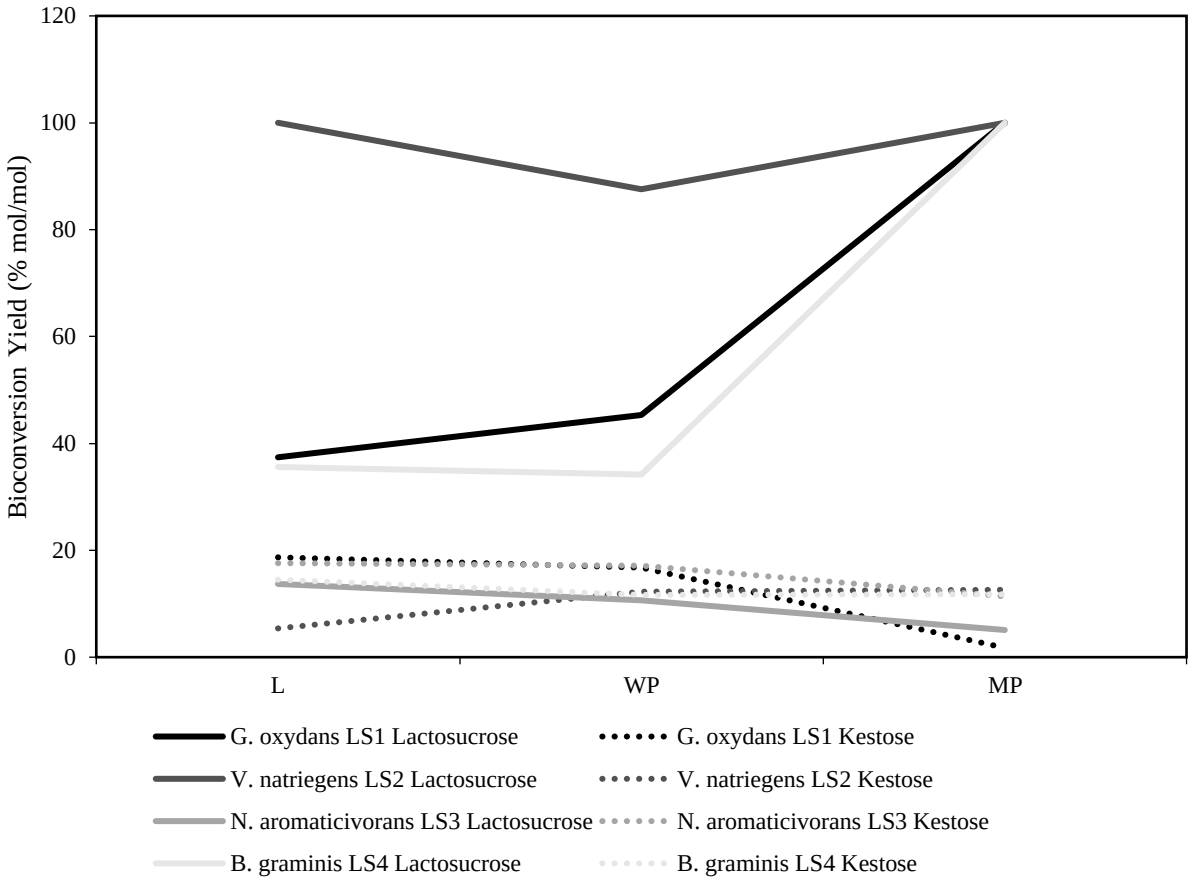


Figure 3.4. Maximum bioconversion yields of lactosucrose and kestose in the reaction system catalyzed in the presence of lactose (L)/sucrose, whey permeate (WP)/sucrose, and milk permeate (MP)/sucrose for each levansucrase (LS).

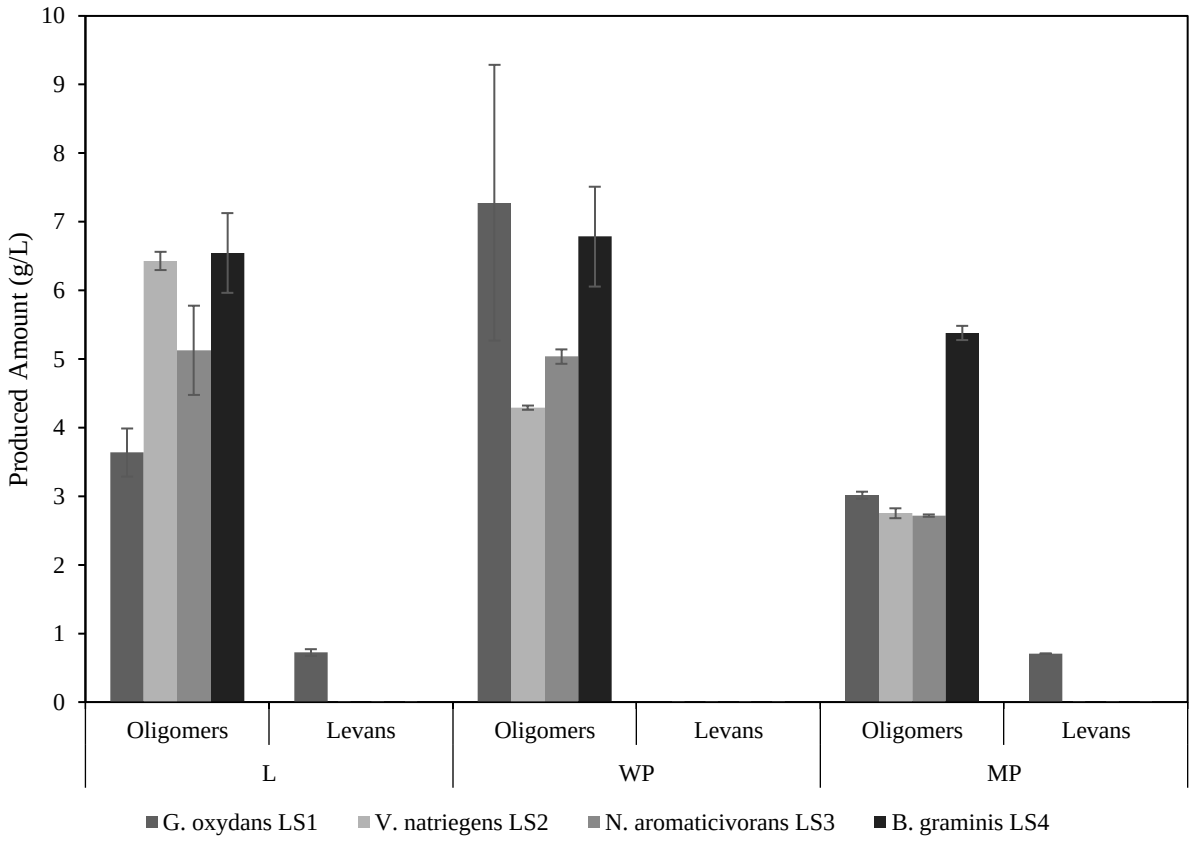


Figure 3.5. Oligomer (5 - 20 kDa) and levan (up to 5000 kDa) production in the presence of lactose (L)/sucrose, whey permeate (WP)/sucrose, and milk permeate (MP)/sucrose for each levansucrase (LS).

3.3.4. Catalytic actions of selected levansucrases on lactosucrose as a substrate

In order to confirm whether lactosucrose has the ability to be a fructosyl acceptor and/or donor, lactosucrose alone or with sucrose was utilized as a substrate in the LS-catalyzed biotransformation reactions. By looking at the percent of converted substrates, it can be seen that both lactosucrose and sucrose/lactosucrose reactions catalyzed by *G. oxydans* LS1, *N. aromaticivorans* LS3, and *B. graminis* LS4 showed increasing bioconversions of substrates up to 85 - 99%. The percent of converted substrates with *V. natriegens* LS2 provided a different pattern as it reached its highest value at 3h but then decreased with reaction time from 46% to 23% with lactosucrose alone and 59% to 51% with the sucrose/lactosucrose.

The aforementioned complete conversion of lactosucrose substrate by *G. oxydans* LS1, *N. aromaticivorans* LS3, and *B. graminis* LS4 is predominantly due to its hydrolysis, particularly with *N. aromaticivorans* LS3 where it even reached 100% after 48h. On the other hand, the sucrose/lactosucrose reaction-catalyzed by *G. oxydans* LS1 showed an extent of transfructosylation (48.7%) that somewhat matched that of hydrolysis (48.9%) at 18h before the hydrolysis extent took over and dominated at 48h reaching 63.8%. The higher percent of transfructosylation extent at 18 with *G. oxydans* LS1 was probably due to the elongation of the growing chain of fructose units as the concentrations of released lactose and fructose were similar in pattern and magnitude in lactosucrose and sucrose/lactosucrose reactions.

The results (Table 3.2) also show that the transfructosylation extent was higher than that of hydrolysis and was ascending throughout the sucrose/lactosucrose reactions catalyzed by *N. aromaticivorans* LS3 and *B. graminis* LS4, achieving 49% and 84%, respectively. The sucrose/lactosucrose reaction catalyzed by *B. graminis* LS4 exhibited an extent of transfructosylation that was constantly higher than that of hydrolysis, while no increase in either lactosucrose, kestose, nystose or fructosyl nystose was observed. These results suggest that lactosucrose may have acted as a fructosyl acceptor by *B. graminis* LS4. Lastly, the *V. natriegens* LS2 reaction with only lactosucrose was able to denote that lactosucrose could be used as a fructosyl donor but the use of lactosucrose as a fructosyl acceptor should be further examined by looking at its possible elongation products. Furthermore, the concentration of the released lactose decreased in both lactosucrose and sucrose/lactosucrose *V. natriegens* LS2 reactions, while that of lactosucrose increased.

Table 3.2. The catalytic actions of each levansucrase (LS) on lactosucrose and sucrose/lactosucrose substrates.

Enzyme	Substrate	Time (Hours)	Converted Substrate(s) (%)[a,b]	Transfructosylation extent (%)[c,d]	Hydrolysis extent (%)[e,f]	Released Lactose (M)[g]	Release Fructose (M)[h]
G. oxydans LS1	Lactosucrose	0	0.00 (±0.00)	0.00 (±0.00)	0.00 (±0.00)	0.00 (±0.00)	0.00 (±0.00)
		3	67.68 (±3.53)	43.01 (±2.51)	24.67 (±1.02)	0.28 (±0.02)	0.16 (±0.01)
		18	98.83 (±0.002)	34.01 (±0.78)	64.82 (±0.77)	0.47 (±0.001)	0.33 (±0.003)
		48	99.53 (±0.01)	21.00 (±3.51)	78.53 (±3.49)	0.45 (±0.01)	0.36 (±0.00)
	Sucrose/ Lactosucrose	0	0.00 (±0.00)	0.00 (±0.00)	0.00 (±0.00)	0.00 (±0.00)	0.00 (±0.00)
		3	38.25 (±1.38)	18.46 (±1.12)	19.79 (±0.26)	0.21 (±0.001)	0.15 (±0.001)
		18	97.56 (±0.33)	48.69 (±0.55)	48.87 (±0.88)	0.49 (±0.004)	0.39 (±0.01)
		48	98.68 (±0.12)	34.92 (±8.28)	63.76 (±8.40)	0.48 (±0.03)	0.52 (±0.07)
V. natriegens LS2	Lactosucrose	0	0.00 (±0.00)	0.00 (±0.00)	0.00 (±0.00)	0.00 (±0.00)	0.00 (±0.00)
		3	46.14 (±0.95)	11.10 (±0.49)	35.04 (±0.46)	0.30 (±0.01)	0.28 (±0.01)
		18	34.95 (±3.67)	20.30 (±0.81)	14.66 (±2.86)	0.15 (±0.01)	0.11 (±0.01)
		48	23.42 (±2.56)	30.47 (±0.10)	0.00 (±2.46)	0.11 (±0.003)	0.08 (±0.003)
	Sucrose/ Lactosucrose	0	0.00 (±0.00)	0.00 (±0.00)	0.00 (±0.00)	0.00 (±0.00)	0.00 (±0.00)
		3	58.70 (±1.75)	12.86 (±0.89)	45.84 (±0.86)	0.32 (±0.01)	0.45 (±0.01)
		18	53.32 (±5.27)	28.02 (±3.97)	25.31 (±1.31)	0.21 (±0.01)	0.25 (±0.01)
		48	51.31 (±13.35)	31.20 (±9.22)	20.11 (±4.13)	0.18 (±0.03)	0.20 (±0.03)
N. aromaticivorans LS3	Lactosucrose	0	0.00 (±0.00)	0.00 (±0.00)	0.00 (±0.00)	0.00 (±0.00)	0.00 (±0.00)
		3	44.85 (±6.65)	0.19 (±0.70)	44.66(±5.95)	0.15 (±0.001)	0.15 (±0.002)
		18	81.08 (±0.51)	0.00 (±1.07)	85.06 (±0.56)	0.33 (±0.01)	0.36 (±0.02)
		48	94.66 (±1.40)	0.00 (±2.79)	100.00 (±1.39)	0.38 (±0.07)	0.43 (±0.08)
	Sucrose/ Lactosucrose	0	0.00 (±0.00)	0.00 (±0.00)	0.00 (±0.00)	0.00 (±0.00)	0.00 (±0.00)
		3	44.87 (±8.72)	28.34 (±3.04)	16.54 (±5.68)	0.01 (±0.002)	0.21 (±0.05)
		18	63.85 (±3.64)	33.51 (±0.33)	30.33 (±3.97)	0.12 (±0.01)	0.38 (±0.03)
		48	85.23 (±3.77)	49.15 (±1.80)	36.09 (±1.98)	0.23 (±0.01)	0.46 (±0.02)

B. graminis LS4	Lactosucrose	0	0.00 (±0.00)	0.00 (±0.00)	0.00 (±0.00)	0.00 (±0.00)	0.00 (±0.00)
		3	33.44 (±10.50)	23.51 (±2.25)	9.93 (±8.25)	0.12 (±0.01)	0.04 (±0.001)
		18	76.73 (±8.81)	23.59 (±15.39)	53.14 (±6.57)	0.39 (±0.09)	0.11 (±0.03)
		48	99.19 (±0.14)	23.89 (±7.43)	75.31 (±7.29)	0.39 (±0.04)	0.15 (±0.01)
	Sucrose/ Lactosucrose	0	0.00 (±0.00)	0.00 (±0.00)	0.00 (±0.00)	0.00 (±0.00)	0.00 (±0.00)
		3	41.49 (±4.40)	37.95 (±4.25)	3.54 (±0.14)	0.09 (±0.002)	0.05 (±0.00)
		18	63.04 (±2.60)	53.15 (±2.35)	9.90 (±0.25)	0.30 (±0.003)	0.14 (±0.001)
		48	96.70 (±2.42)	84.29 (±1.65)	12.41 (±0.77)	0.41 (±0.01)	0.18 (±0.01)

[a] Only lactosucrose was a substrate in this reaction and thus the percent of converted substrate was equal to the lactosucrose quantity at a specified time point as a percentage of the initial lactosucrose quantity.

[b] Both lactosucrose and sucrose were substrates in this reaction and thus the percent of converted substrates was equal to 100 minus the sucrose and lactosucrose quantities at a specified time point as a percentage of the initial sucrose and lactosucrose quantities.

[c] Only lactosucrose was a substrate in this reaction and the transfructosylation percent was calculated by taking subtracting the fructose quantity from lactose quantity at a specified time point divided by the difference between the lactosucrose quantity at the same specified time point and its initial quantity and multiplying the result by 100.

[d] Both lactosucrose and sucrose were substrates in this reaction and the transfructosylation percent was calculated by subtracting the percent hydrolysis from the percent of converted substrates at a specified time point.

[e] Only lactosucrose was a substrate in this reaction and the hydrolysis percent was calculated by subtracting the percent transfructosylation from the percent of converted substrates at a specified time point.

[f] Both lactosucrose and sucrose were substrates in this reaction and the hydrolysis percent was calculated by dividing the fructose quantity at a specified time point by the initial sucrose and lactosucrose quantities multiplied by 100.

[g] The quantity of released fructose was calculated by taking the fructose quantity at a specified time point and subtracting it from the initial fructose quantity.

[h] The quantity of released lactose was calculated by taking the lactose quantity at a specified time point and subtracting it from the initial lactose quantity.

These results indicate that both lactosucrose and/or sucrose may have been used as the fructosyl donors by *V. natriegens* LS2. Focusing more on the lactosucrose *V. natriegens* LS2 reaction, the concentration of free lactose was higher than that of fructose across the reaction time course, indicating the use of lactose as a fructosyl acceptor and of lactosucrose as a fructosyl donor. The targeted MS-MS analyses of 48 h-*G. oxydans* LS1, *V. natriegens* LS2, and *B. graminis* LS4 reactions where lactosucrose was present by itself revealed the presence of a tetrasaccharide, frustosyl-lactosucrose. This indicates that lactosucrose could be utilized as a fructosyl acceptor by *G. oxydans* LS1, *V. natriegens* LS2, and *B. graminis* LS4. However, the structure of frustosyl-lactosucrose should be further confirmed via NMR.

3.4. Conclusion

It could be concluded that the biotransformation reactions were occurring at a rapid rate and overall, most of the reaction mixtures were enriched with both lactosucrose and kestose. The *V. natriegens* LS2 appeared to be the most promising candidate as it led to the highest ratio of transfructosylation to hydrolysis particularly in the presence of WP/sucrose substrates. Additionally, *V. natriegens* LS2 was able to produce the highest amount of lactosucrose. While *G. oxydans* LS1 led to the production of a mixture of lactosucrose and kestose. *G. oxydans* LS1 was the only enzyme that catalyzed the synthesis of levan polymers in lactose/sucrose and MP/sucrose reactions. On the other hand, *N. aromaticivorans* LS3 did not favor lactosucrose production because of its low acceptor specificity towards lactose and ended up primarily producing kestose as well as the highest amounts of nystose and fructosyl nystose. The use of lactosucrose alone or with sucrose in the LS-catalyzed biotransformation reactions revealed the ability of LSs to use lactosucrose as a fructosyl donor and/or acceptor. *G. oxydans* LS1, *V. natriegens* LS2, and *B. graminis* LS4 were proven to be able to use lactosucrose as a fructosyl acceptor. While *V. natriegens* LS2 used lactosucrose as a fructosyl donor to transfer fructose into lactose and produce lactosucrose. Further studies are needed in order to modulate the reaction parameters towards a maximum production of lactosucrose.

CHAPTER IV. THE IMMOBILIZATION AND OPTIMIZATION OF LEVANSUCRASE BIOCATALYST OF HIGH INTEREST FOR THE PRODUCTION OF LACTOSUCROSE

Connecting Statement 2

The information generated from chapter III was instrumental for the identification of the levansucrases that offered the highest lactosucrose production, including LSs from *Gluconobacter oxydans* (strain 621H) (LS1) and *Vibrio natriegens* NBRC 15636 (LS2). Chapter V investigates the immobilization of *G. oxydans* LS1 and *V. natriegens* LS2 onto various functionalized commercial and non-commercial solid supports. Additionally, the effects of the immobilization on the enzymes' thermal stability, retention of activity, transfructosylation versus hydrolytic activity, and end-product profile were examined. This study then further focused on the optimization of lactosucrose production using the free and immobilized forms of the optimal lactosucrose producing levansucrase.

The results from this study were presented at the 2021 Biotrans conference and the 2021 consortium for research, innovation, and transformation of agrifood (RITA) at McGill university.

4. Abstract

The synthesis of novel prebiotics, such lactosucrose, has gained attention due to a greater interest in promoting intestinal health. In this study, lactosucrose was produced by levansucrase (LS, EC 2.4.1.10) catalyzing the transfructosylation of sucrose and lactose. Whey permeate (WP) and milk permeate (MP) were also utilized as lactose sources. Modulating the microenvironments of LS through immobilization is expected to modulate the reaction selectivity towards transfructosylation and promote enzyme stabilization. Selected functionalized supports were investigated for the immobilization of LSs from *Gluconobacter oxydans* (LS1) and *Vibrio natriegens* (LS2) by multipoint covalent attachment. The highest immobilization protein yields of 94% and 87% and retained activities of 55% and 98%, respectively, were achieved upon the immobilization of LS1 and LS2 on Relizyme^{TM} EP403/S functionalized with iminodiacetic acid (IDA)-Cu. A greater thermal stabilization of immobilized LSs was achieved after post-immobilization treatments, especially for high pH post-immobilized *V. natriegens* LS2 with a thermal stability factor of 53. The LS immobilization enhanced the reaction selectivity towards transfructosylation, and the Relizyme^{TM} EP403/IDA-Cu immobilized *V. natriegens* LS2 led to the highest produced amount of lactosucrose with lactose/sucrose, WP/sucrose, and MP/sucrose (35 - 95 g/L). The optimal lactose/sucrose ratio and incubation time for the synthesis of lactosucrose with high selectivity by free and immobilized LS2 were identified to respectively be 0.586 and 3.117h and 0.503 and 3.083h. These optimal conditions were found to provide 117 g/L with the free *V. natriegens* LS2 and 101 g/L with the Relizyme^{TM} EP403/S-IDA/Cu immobilized *V. natriegens* LS2. Lastly, the Relizyme^{TM} EP403/IDA-Cu immobilized *V. natriegens* LS2 was successfully reused up to 3 consecutive times generating a total of 247 g/L of lactosucrose.

4.1. Introduction

The human gastrointestinal tract is the host to a highly diverse, interconnected, and complex microbial community that has a significant impact on human physiology and pathology. Under healthy physiological conditions, the gut microbiome participates in many vital activities. A reduction in the gut microbiome population and a lower diversity level were found to be involved in various pathological states (Park et al., 2017; Principi, Cozzali, Farinelli, Brusaferro, & Esposito, 2018; Canavan, West, & Card, 2014; Fedorak, Vanner, Paterson, & Bridges, 2012). Fortunately, consumers are nowadays making more informed health conscience decisions, resulting in an increased popularity in prebiotics (Quigley, 2019).

The trisaccharide lactosucrose, which is made up of galactose, glucose, and fructose, has gained appreciation for its health benefits, in particular its prebiotic property being selectively utilized by *Bifidobacterium*. Lactosucrose was certified as FOSHU (Functional Food Ingredients for Foods for Specified Health Uses) in Japan and is used as a prebiotic dietary supplement and in foods like yoghurt (Monsan & Ouarné, 2009; Xu, Liu, Yu, Zhang, & Mu, 2018). Moreover, lactosucrose exhibits attractive technofunctional properties such as water holding capacity and ability to be used as a fat replacer (Silvério, Macedo, Teixeria, & Rodrigues, 2015).

Lactosucrose can be produced in a regio and stereo controlled manner by the action of either one of the three enzymes, levansucrase (LS, EC 2.4.1.10), β-fructofuranosidase (EC 3.2.1.26), or β-galactosidase (EC 3.2.1.23), all of which require sucrose and lactose as substrates (Duarte et al., 2017). Furthermore, the use of lactose-rich dairy by-products to synthesize lactosucrose by LS has attracted a high interest. Indeed, upon the recovery of proteins from whey, a whey permeate (WP) by-product is generated and made up of lactose (75 - 85% w/w). The ultrafiltration process applied to milk to recover the proteins results in another by-product known as milk permeate (MP), which also contains lactose (4.7%, w/w) (Boer, 2014; Ryan & Walsh, 2016). Overall, the generation of dairy by-products is increasing in volume where in 2016, it was estimated that whey for example has a worldwide production of 200 million tons per year and cannot be simply discarded as it is considered a serious environmental hazard (Iliada et al., 2019). Both WP and MP by-products can be used as abundant lactose sources alongside sucrose to produce lactosucrose (Mu, Chen, Wang, Zhang, & Jiang, 2013).

In our previous study, LSs from *Gluconobacter oxydans* (strain 621H) (LS1) and *Vibrio natriegens* NBRC 15636 (LS2) were identified as potential biocatalysts for the bioconversion of lactose and sucrose into lactosucrose. The efficient use of LSs at the industrial level to synthesize lactosucrose requires high reaction selectivity (hydrolysis vs transfructosylation) and acceptor/product specificity. These catalytic characteristics can be achieved through the modulation of LS's microenvironment upon immobilization, which can also promote enzyme stability and reusability. In previous studies, several methods, based on cationic, anionic, covalent, chelating, and multi-point attachments, were investigated for the immobilization of LSs (Ben Ammar, Matsubara, Ito, Iizuka, & Minamiura, 2002; Esawy, Mahmoud, & Fattah, 2008; Hill, Karboune, & Mateo, 2016; Sangmanee, Nakapong, Pichyangkura, Kuttiyawong, 2016). One example includes the immobilization of LS from *Bacillus amyloliquefaciens* on modified and unmodified epoxy-activated supports, Eupergit© and Sepabeads®, and on modified cross-linked agarose beads. The *B. amyloliquefaciens* LS immobilized on glyoxyl agarose, functionalized with iminodiacetic acid (IDA) covered with cupric ions as chelating agents, resulted in the greatest ratio of transfructosylation to hydrolysis of 120%, retained activity of 67%, and thermal stability factor of 13.6 as compared to other tested supports (Hill, Karboune, & Mateo, 2016). Among agarose, agar, and calcium alginate supports, the use of agarose resulted in the highest immobilization protein yield of 37% upon the immobilization of LS from *Bacillus subtilis* mutant NRC33a via entrapment (Esawy, Mahmoud, & Fattah, 2008). Additionally, the immobilization of *B. subtilis* NRC33a LS through covalent bonding on chitosan beads activated with 3% glutaraldehyde crosslinking led to a high immobilization protein yield of 81.51% when compared to its adsorption, ionic bonding, and entrapment (Esawy, Mahmoud, & Fattah, 2008). Similarly, Sangmanee, Nakapong, Pichyangkura, Kuttiyawong (2016) have used chitosan beads to immobilize LS from *Bacillus licheniformis*, which produced 7.35 g/L of fructooligosaccharides (FOSs) corresponding to a yield of 29.4%. On the other hand, LS from *B. subtilis natto* was immobilized as cross-linked enzyme aggregates (CLEAs) using oxidized glucomannan crosslinker; compared to the free LS, CLEAs showed higher thermal stabilization but lower specific activity as a result of the LSs rigidification (Ben Ammar, Matsubara, Ito, Iizuka, & Minamiura, 2002). As far as the authors are aware, the immobilization of LSs from *G. oxydans* LS1 and *V. natriegens* LS2 has not been investigated so far. In addition, the examination of lactosucrose production by an immobilized LS biocatalyst or cells is very limited. Only one study was carried out by Lee et al. (2007) where the whole cells of

a mutant strain of *Sterigmatomyces elvia* ATCC 18894 containing LS were immobilized in calcium alginate and were able to synthesize 192 g/L of lactosucrose (Lee et al., 2007).

The objective of the present study was to immobilize *G. oxydans* LS1 and *V. natriegens* LS2 on the glyoxyl agarose, Sepabeads® EC-EP/S R and Relizyme™ EP403/S R supports and assess their catalytic efficiency and ability to synthesize lactosucrose. Additionally, the effects of various pre- and post-immobilization treatments were investigated. After which, the better lactosucrose producing LS was identified. The effects of reaction parameters on the efficiency of the enzymatic process for lactosucrose production were studied and optimized using the selected LS in both free and immobilized form.

4.2. Materials and Methods

4.2.1 Materials

Sucrose, D-(-)-fructose, D-(+)-glucose, D-(+)-galactose, α-lactose, myo-inositol, 3,5-dinitrosalicylic acid (DNS), potassium sodium tartrate ($KNaC_4H_4O_6$), yeast extract, carbenicillin disodium salt, lysozyme from chicken egg white, DNase I, imidazole, $C_2H_7NO_2$, NH_4HCO_3, NaOH solution, 4-morpholinepropanesulfonic acid (MOPS), epichlorohydrin, H_2SO_4, $NaIO_4$, IDA, $CuSO_4$, NaH_2PO_4, Na_2HPO_4, and polyethylenimine (PEI) were obtained from Sigma-Aldrich (Oakville, ON). FOS standards (i.e.1-kestose, nystose, and 1^F-fructofuranosylnystose) and lactosucrose were supplied by FUJIFILM Wako Chemicals U.S.A. Corporation (Richmond, VA). Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) low range standards were purchased from Bio-Rad (Missasauga, ON). KH_2PO_4, K_2HPO_4, NaOH (Pellets/Certifies ACS), acetonitrile (ACN) HPLC grade, water optima LC/MS grade, bovine serum albumin (BSA), tryptone, NaCl, β-D-isothiogalactopyranoside (IPTG), PIPES, glycerol, tris-glycine-SDS 10x solution, $NaBH_4$, acetone, triethylamine (TEA), and Pierce™ Coomassie Plus (Bradford) assay kit were provided by Fisher Scientific (Fair Lawn, NJ). Terrific broth (TB) and lysogeny broth (LB) agar powder were acquired from Bio Basic (Markham, ON). 10% BCL agarose bead standard (50 - 150μm) was purchased from Agarose Bead Technologies (Doral, FL). *Escherichia coli* BL21(DE3) plysE strains were supplied by Invitrogen. Sepabeads® EC-EP/S R and Relizyme™ EP403/S R were obtained from Resindion® (Binasco, Lombardy, Italy). WP (96.02% total solids made up of 11.55% protein, 7.57% ash, and a minimum of 76.09% lactose by weight) and MP

(5.65% total solids made up of 0.25% protein, 0.60% ash, and a minimum of 4.80% lactose by weight) were obtained from a local dairy cooperative.

4.2.2. Expression and purification of selected levansucrases

The genes corresponding to LS1 from *G. oxydans* (strain 621H) and LS2 from *V. natriegens* NBRC 15636 were transformed into *E. coli* BL21-CodonPlus (DE3)-RIPL (Invitrogen) and precultured into LB media also containing carbenicillin (1 µl/ml). The preculture was incubated in an orbital shaker for 8 - 10 h at 37°C under 250rpm. The preculture (2% v/v) was added to the TB with carbenicillin (1 µl/ml), which was then incubated at 37°C under 250rpm. When the growth has achieved an optical density of 1.2 - 1.6 at 600nm (DU 800 UV/Visible Spectrophotometer, Beckman), the enzyme expression was induced using IPTG (1mM). Growth of the culture was continued at room temperature for 18h under 250rpm. The cells were then collected by centrifugation at 4°C under 8,000rpm and then stored at -80°C. The recovered pellets were resuspended in the sonication buffer (50mM PIPES, 300mM NaCl, and 10% glycerol; pH of 7.2; 4 ml/g). Lysozyme (4 mg/g) and DNase (4 µl/g) were added the suspensions, and the mixtures were incubated at 18°C under 50rpm for 1h. The suspensions were thereafter sonicated with a microtip (Misonix Ultrasonic Liquid Processor S-4000) for six cycles (10 seconds on and 60 seconds off at 15kHz) in an ice bath. The supernatants containing the enzymes were recovered by centrifugation at 4°C under 14,000rpm for 1h and then dialyzed against potassium phosphate buffer (5mM; pH of 6) using a membrane with a molecular weight cut-off of 6 - 8 kDa. The LSs were purified via immobilized metal affinity chromatography on a HisTrap™ FF column (5ml, GE Healthcare). After loading, the column was subsequently washed with sonication buffer (9 volumes), wash buffer (50mM PIPES, 300 mM NaCl, and 10% glycerol; pH of 6.4; 9 volumes), 5mM imidazole-wash buffer (9 volumes), and 10mM imidazole-wash buffer (9 volumes). The LS enzyme was then eluted with 100mM and 200mM imidazole-wash buffers (2 volumes), respectively. Finally, the purity of the LSs was confirmed upon the SDS-PAGE electrophoresis analysis at 120V using 15% SDS polyacrylamide gels and a 10x diluted Tris/Glycine/SDS buffer.

4.2.3. Levansucrase activity assays

One unit of total LS activity was expressed as a quantity of biocatalyst that released 1µmol of reducing sugars, glucose and fructose, from sucrose per min. The total LS activity includes both hydrolytic and transfructosylation activity. The LS hydrolytic unit was expressed as the amount of

biocatalyst that generated 1μmol of the fructose per min, while one transfructosylation unit was expressed as the amount of biocatalyst that produced 1μmol of glucose, due to the transfer of fructose, per min. Subtracting the total amount of fructose from that of glucose provides the amount of glucose resulting from transferring fructose. All assays were run in duplicates or triplicates.

The total LS activity assay was initiated by mixing purified LS solution with a sucrose substrate solution (1.8M) in potassium phosphate buffer (50mM; pH of 6) at a ratio of 1:1 (v/v). After 20 min of incubation at 30°C under 50rpm, the reaction was terminated by adding the DNS reagent [1% (w/v) 3,5-DNS and 1.6% (w/v) NaOH] at a ratio of 1:1.5 (v/v). The mixtures were then placed in boiling water for 5 min and thereafter, the potassium sodium tartrate (50% w/v) was added to stabilize the colour at a ratio of 1:0.2 (v/v). The absorbance was measured at 540nm and the reducing sugar concentration was quantified using a standard curve constructed from glucose (0 - 20 mM).

For the hydrolytic and transfructosylation activity assays, glucose, fructose, and sucrose of the reaction mixtures 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.

The protein content of the LS solution was quantified using the Bradford protein assay with a BSA standard (1 - 20 μg/ml). The specific enzyme activity was expressed as the unit of biocatalyst in μmol of reducing sugar per min per mg of protein.

4.2.4. Pre-immobilization treatment of glyoxyl agarose-based supports

The protocol for the preparation of glyoxyl-based supports followed the method described by Hill, Karboune, & Mateo (2016).

Epoxy-Activated Agarose. A round bottom flask was placed on ice where NaOH (0.656M; 440ml), $NaBH_4$ (2g), acetone (160ml), and washed 10% BCL Agarose (100g) were added. Epichlorohydrin (110ml) was then introduced to the mixture and stirred overnight at 25°C. The support was finally washed with deionized water (10 volumes).

Glyoxyl Agarose/TEA. The epoxy-activated agarose (10% w/v) was then suspended in the acetone:water solution (1:1 v/v). TEA was slowly added to reach a final concentration of 0.1M and if needed, the pH was adjusted to reach a value within the range of 12.5 - 13. The mixture was

stirred for 48h and washed afterwards with distilled water (10 volumes). The remaining hydroxyl groups were oxidized with $NaIO_4$ (0.01M; 5% v/v) for 90 min and then the support was recovered by filtration and washed with distilled water (10 volumes).

Glyoxyl Agarose/IDA. The epoxy-activated agarose (10% w/v) was suspended in an IDA solution (0.5M; pH of 11) for 36h at 25°C. The IDA-functionalized glyoxyl agarose support was then recovered by filtration and washed with distilled water (10 volumes). The remaining hydroxyl groups were oxidized with $NaIO_4$ (0.01M; 5% v/v) for 90 min and then the mixture was filtered to recover the modified support, which was washed with distilled water (10 volumes).

Glyoxyl Agarose/IDA-Cu. The support was functionalized using the same method as previously detailed in "*Glyoxyl Agarose/IDA*" with the additional steps of suspending in $CuSO_4$ (30 mg/ml) solution for 1h at room temperature. The IDA-Cu functionalized glyoxyl agarose support was recovered by filtration and washed with distilled water (10 volumes).

4.2.5. Pre-immobilization treatment of commercial supports

The protocol for the preparation of the *IDA-Cu functionalized* epoxy-based supports (Sepabeads® EC-EP/S R and Relizyme™ EP403/S R) was based on the method described by Tamayo-Cabezas & Karboune (2020). The dry support (1:6 w/v) was suspended in an IDA solution (1.8M; pH of 11) and incubated for 5h at room temperature at 55rpm. The mixture was filtered and washed with distilled water (8 volumes). A solution of $CuSO_4$ (46 g/L; 30ml) was then added and the mixture was incubated for 2h at room temperature at 55rpm. Finally, the support was filtered and initially washed with distilled water (8 volumes) then with MOPS (0.02M; pH of 6; 5 volumes).

4.2.6. Immobilization of levansucrase onto selected supports

The optimal pre-immobilization treatment and immobilization time were determined by suspending the glyoxyl agarose/TEA, glyoxyl agarose/IDA, or glyoxyl agarose/IDA-Cu (200 g/L) in potassium phosphate buffer (600mM; pH of 6.8) containing *G. oxydans* LS1. Each support mixture (0.5ml) was added to the *G. oxydans* LS1 solution (1ml; 1g/L) in order to immobilize 10 mg of protein per g of support. These mixtures were carried out in triplicates alongside blanks, where the buffer substituted the support into the LS solution, and were incubated at 4°C and 80rpm. The mixtures were centrifuged at 4 different time points (0, 24, 48, and 72 h) in order to take a 100µl aliquot. The protein content of these aliquots was determined as previously described and

based on the percent difference in protein content over time, the pre-immobilization treatment with IDA-Cu support after 5h of immobilization time were selected for future immobilization.

G. oxydans LS1 and *V. natriegens* LS2 were respectively immobilized for 5h into the 3 selected supports (Glyoxyl agarose/IDA-Cu, Sepabeads® EC-EP/IDA-Cu, and Relizyme™ EP403/IDA-Cu). The immobilized LSs were recovered by centrifugation for 2 min at 8,600rpm and then the supernatants were removed while the supports were left to be washed and resuspended with potassium phosphate buffer (50mM; pH of 6; 10 volumes). The protein content of the blanks, supernatants, and washes were quantified to determine the immobilization protein yield; while the retained activity was estimated from the specific activity of free and immobilized LS.

$$Immobilization\ Protein\ Yield\ (\%) = \frac{Free\ Enzyme\ Solution\ Protein\ Content - (Supernatant + Wash\ Protein\ Content)}{Free\ Enzyme\ Solution\ Protein\ Content} x\ 100$$

$$Retained\ Activity\ (\%) = \frac{Immobilized\ Enzyme\ Specific\ Activity}{Free\ Enzyme\ Specific\ Activity} x\ 100$$

The free enzyme solutions as blanks were exposed to the same conditions as the mixtures with the supports and thus any loss of activity resulting from external forces was accounted for.

4.2.7. Reaction selectivity (hydrolysis vs transfructosylation) of immobilized levansucrases

The reaction selectivity was assessed in the presence of sucrose alone or with lactose as substrates using *G. oxydans* LS1 and *V. natriegens* LS2 immobilized on Glyoxyl agarose/IDA-Cu, Sepabeads® EC-EP/IDA-Cu, and Relizyme™ EP403/IDA-Cu. After the enzymatic biotransformation reactions, the remaining sucrose and lactose as well as the released galactose, glucose, and fructose were quantified by HPAEC using a Dionex ICS-3000 system equipped with a PAD and a CarboPac PA20 column as described above. The hydrolysis was quantified from the concentration of released fructose while the transfructosylation was estimated based on the difference between the concentrations of fructose and glucose.

4.2.8. Post-immobilization treatments

The Relizyme™ EP403/IDA-Cu immobilized *G. oxydans* LS1 and *V. natriegens* LS2 (1% v/v) were separately suspended in a high pH sodium phosphate buffer (100mM; pH of 8.5) and

incubated for 24h at 18°C and 80rpm. This post-immobilization treatment was carried out in triplicates alongside the blanks, where the immobilized LSs were suspended in neutral pH potassium phosphate buffer (600mM; pH of 6.8). The high pH-treated immobilized LSs were recovered upon centrifugation for 2 min at 8,600rpm, washed and resuspended in potassium phosphate buffer (50mM; pH of 6; 10 volumes).

PEI solution (0.1% v/v) was added to the high pH-treated immobilized LSs and incubated overnight at 4°C and 80rpm. The treatment was carried out in triplicates along with the blanks which were suspended in potassium phosphate buffer (50mM; pH of 6) instead of the PEI treatment. The PEI-treated immobilized LSs were recovered upon centrifugation for 2 min at 8,600rpm, washed and resuspended in potassium phosphate buffer (50mM; pH of 6; 10 volumes). The protein content and activity of the blanks, supernatants, and washes were quantified to assess the immobilization protein yield and retained activity of the immobilization LSs upon post-immobilization treatments. The blanks ran in parallel to each treatment and were thus experiencing the same conditions as the treated supports and thus any loss of activity resulting from external forces was taken in account.

4.2.9. Assessment of thermal stability

To assess the thermal stability, free as well as high pH and PEI-treated immobilized *G. oxydans* LS1 and *V. natriegens* LS2 on Relizyme™ EP403/IDA-Cu in potassium phosphate buffer (50mM; pH of 6) were incubated at 50°C and 50rpm. Aliquots were taken at selected incubation times, and the residual LS activities were quantified and compared to the initial ones.

4.2.10. Time course for biotransformation reactions

To produce lactosucrose, the enzymatic biotransformation reactions were carried out using sucrose and lactose or lactose containing dairy by-products as substrates (WP and MP). Sucrose and lactose solutions were prepared at a ratio of 2:1 to reach a final concentration of 0.9M:0.45M. To initiate the biotransformation reactions, immobilized LS (5 U/ml) was added to the substrate solution. The biotransformation reactions were carried out at the optimal conditions for each immobilized LS on Relizyme™ EP403/IDA-Cu, corresponding to 30°C/pH 4 (50mM ammonium acetate buffer) for immobilized *G. oxydans* LS1 and 45°C/pH 8 (50mM ammonium bicarbonate buffer) for immobilized *V. natriegens* LS2. All reactions were done in duplicates under 50rpm. A blank with no enzyme was run in parallel for each reaction. The biotransformation reactions were carried out

over a time course of 24h where aliquots were taken, placed in boiling water for 3 min to stop the reaction, and then stored at -20°C until further analysis.

4.2.11. End-product profile characterization

The end-products of the biotransformation reactions were analyzed using an Agilent 1290 II liquid chromatography system coupled to an Agilent 6560-ion mobility Q-TOF – MS. The analytes were separated with an InfinityLab Poroshell 120 HILIC-Z column (2.1 x 100 mm, 2.7μm). Mobile phase A was LC-MS grade water with 0.3% NH_4OH and mobile phase B was ACN with 0.3% NH_4OH. Flow rate was set at 0.4 ml/min with a column temperature of 35°C. The constructed gradient started off with 85% B (0.0 to 0.5 min) that had a linear decrease to 30% B (0.5 to 9.0 min) where it was held (9.0 to 13.0 min) and then increased to 85% B (13.0 to 15.0 min), followed by a 3 min post-run. The mass spectrometer was equipped with a Dual AJS ESI ion source operating in negative ionization mode. MS conditions for ESI were as follows: drying gas temperature of 150°C and flow rate of 11 L/min, sheath gas temperature of 350°C and flow rate of 12 L/min, pressure on the nebulizer of 30psig, capillary voltage of 4000V, fragmentor voltage of 200V, skimmer voltage of 30V, and nozzle voltage of 2000V. Full scan MS data was recorded at mass-to-charge ratios (*m/z*) from 80 to 1100 at a scan rate of 2 spectra/s and was collected at both centroid and profile mode. Reference ions (*m/z* at 112.985587 and 1033.988109 for ESI-) were used for automatic mass recalibration of each acquired spectrum. The quantification was performed using Quantitative Analysis 10.0 from Agilent MassHunter Workstation Software. The samples were prepared by diluting them in 50:50 ACN:Water (v/v) with the addition of myo-inositol (5ppm) to serve as an internal standard.

4.2.12. Optimization of the biotransformation reaction to produce lactosucrose

The effects of the biotransformation reaction parameters were investigated using response surface methodology (RSM). Free and Relizyme™ EP403/IDA-Cu immobilized *V. natriegens* LS2 were selected, and the WP dairy by-product was used as a lactose substrate. The investigated reaction parameters included the incubation time (3 – 15 h) and substrate ratio of lactose to sucrose (0.5 - 2) while the other conditions, such as enzyme units (5 U/ml), buffer (50mM ammonium bicarbonate; pH of 8), and temperature (45°C), were kept constant. A five-level, two variable central composite rotatable design was created using Design Expert® Software. The full designs consisted of 4 factorial points, 4 axial points, and 3 center points and the levels of the parameters

were determined based on the preliminary trials. The amount produced (g/L) and yield (%) of lactosucrose, kestose, nystose, and fructosyl nystose were the quantified responses.

4.2.13. Reusability of immobilized levansucrase

The reusability of the immobilized *V. natriegens* LS2 on Relizyme^{TM} EP403/IDA-Cu was evaluated by performing 6 consecutive biotransformation reactions using the same immobilized *V. natriegens* LS2 under the determined optimal conditions. After the appropriate reaction time, the immobilized *V. natriegens* LS2 was recovered and washed with ammonium bicarbonate buffer (50mM; pH of 8) before it was reused under the same optimal conditions. The amount of produced lactosucrose (g/L) was quantified at each run.

4.3. Results and Discussion

4.3.1. Levansucrase Immobilization

The various pre-immobilization treatments were carried out to modify the glyoxyl agarose support with IDA, IDA-Cu, and TEA, to promote anionic, chelating, and cationic interactions, respectively, and contribute to the multi-covalent attachment of LS on the modified glyoxyl agarose supports. The IDA functionalized glyoxyl agarose support primarily promotes an initial step of physical adsorption through the ionic interactions between the negatively charged IDA groups on the support and positively charged amino acid residues, such as lysine, histidine, and arginine, on the LS's surface. The lysine groups were identified as the most likely amino acid residues that can create reversible imine covalent linkages. The IDA-Cu functionalized glyoxyl agarose support can act as a chelating support and bind to the LS through the chelation of sulfhydryl or amine groups on the LS's surface with the cupric ions on the support. The TEA functionalized glyoxyl agarose support predominantly promotes ionic interactions between the positively charged TEA groups on the support and negatively charged aspartate and glutamate residues on the LS (Mateo et al, 2010). The completed pre-immobilization treatments can be seen in Table 4.1 where the functional groups for each treatment were characterized.

Figure 4.1 displays the immobilization attempts of *G. oxydans* LS1 on the aforementioned selected functionalized glyoxyl agarose supports. Unfortunately, it was found that both IDA and TEA functionalized glyoxyl agarose supports failed to significantly immobilize the *G. oxydans* LS1 even after 72h of incubation. However, within 5h of incubation, the IDA-Cu functionalized

glyoxyl agarose support was able to immobilize 89.7% (w/w) of the loaded *G. oxydans* LS1 protein content. This immobilized LS1 protein content was maintained after 24h of incubation. Therefore, the IDA-Cu functionalization was selected as the pre-immobilization treatment for the immobilization of LSs on the glyoxyl agarose and epoxy activated supports. An incubation time of 5h was applied for the immobilization of LSs.

The effects of the polymeric properties and functional group density of selected immobilization supports on LS immobilization were evaluated using IDA-Cu functionalization with the glyoxyl agarose support and the two epoxy activated supports Sepabeads® EC-EP and Relizyme™ EP403. As shown in Table 4.1, these supports differ in their pore size where the glyoxyl agarose support is composed of agarose microspheres with no pores crosslinked for a macroporous structure, while the mesoporous Sepabeads® EC-EP support has the smaller pore size (10 - 20nm) in comparison to the macroporous Relizyme™ EP403 support (40 - 60nm). For the epoxy activated supports, the oxirane group density of the supports is inversely proportional to their pore size, since supports with a small pore size have a larger surface area. Furthermore, looking at the bound IDA group content, it was found to be similar across the glyoxyl agarose/IDA-Cu and Sepabeads® EC-EP/IDA-Cu supports (128 - 141 µmol/g of beads) while the Relizyme™ EP403/IDA-Cu was significantly higher (181 µmol/g of beads). However, the bound copper content was different for all three supports as it was much higher for the Sepabeads® EC-EP/IDA-Cu (417 µmol/g of beads) followed by Relizyme™ EP403/IDA-Cu and then the glyoxyl agarose/IDA-Cu and (169 and 145 µmol/g of beads, respectively). Spadoni Andreani, Li, Ronholm, & Karboune (2021) have subjected the epoxy supports (Sepabeads® EC-EP and Relizyme™ EP403) to the same pre-immobilization treatments for the immobilization of feruloyl esterase from *Humicola insolens*. They have reported lower bound IDA group contents (12 - 48 µmol/g of beads), but similarly to our study, Relizyme™ EP403/IDA support had a higher bound IDA content in comparison to the Sepabeads® EC-EP/IDA support. Contrary to our supports, the bound copper content was found to be higher in the Relizyme™ EP403/IDA-Cu support (201 µmol/g of beads) than in the Sepabeads® EC-EP/IDA-Cu one (66 µmol/g of beads) (Spadoni Andreani, Li, Ronholm, & Karboune, 2021). These differences reveal the importance of the solid/liquid ratio in modifying the epoxy supports. However, similarly to our supports (3.3 and 0.9), the ratio of bound copper to IDA was higher in the Sepabeads® EC-EP/IDA-Cu support (5.5) in comparison to the Relizyme™ EP403/IDA-Cu one (4.2) (Spadoni Andreani, Li, Ronholm, & Karboune, 2021).

Table 4.1. Technical properties of modified supports.

	Glyoxyl-agarose	Sepabeads® EC-EP/S R	Relizyme™ EP403/S R
Average pore diameter (nm)[m]	-	10 - 20	40 - 60
Particle size range (µm)[m]	50 - 150	100 - 300	100 - 300
Minimum oxirane content (µmol/g wet)[m]	-	100	30
Minimum epoxy group density (µmol/g dry)[m]	-	200	100
Introduced epoxy group (µmol/g of beads)	214.02 (±2.14)[b]	266.34 (±0.25)[a]	192.35 (±1.58)[c]
Bound triethylamine (TEA) (µmol/g of beads)	69.23 (±4.30)	-	-
Bound iminodiacetic acid (IDA) (µmol/g of beads)	140.99 (±5.97)[b]	128.42 (±11.96)[b]	180.87 (±14.15)[a]
Bound copper (µmol/g of beads)	144.78 (±1.59)[c]	417.29 (±6.99)[a]	168.53 (±2.82)[b]

[m] Data acquired from manufacturer.

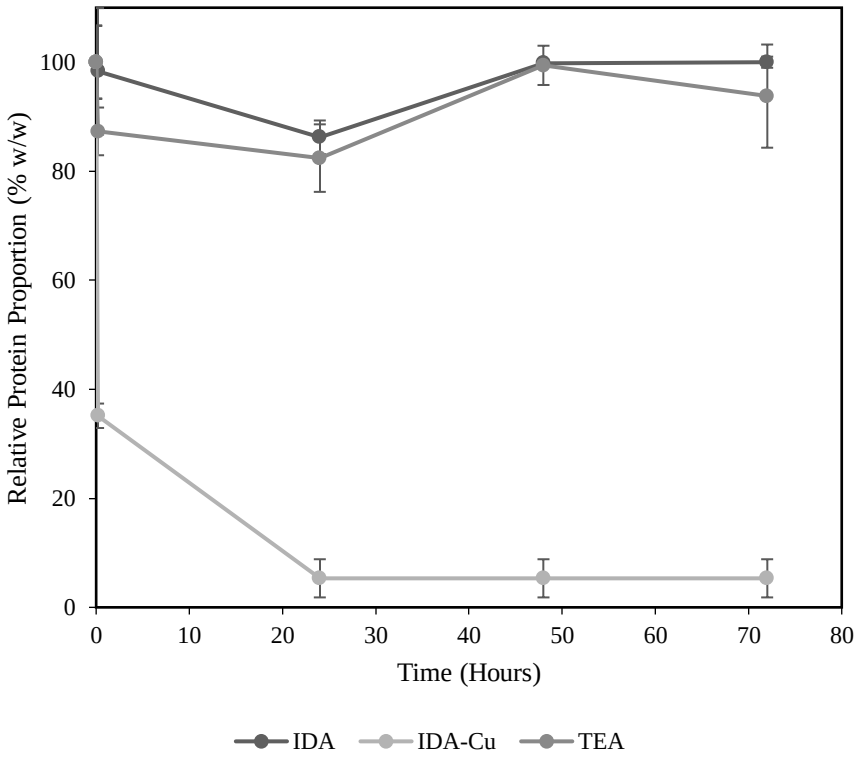


Figure 4.1. *G. oxydans* LS1 immobilization on iminodiacetic acid (IDA), IDA-Cu, and triethylamine (TEA) glyoxyl agarose supports.

The immobilization results of *G. oxydans* LS1 and *V. natriegens* LS2 on the three selected supports functionalized with IDA-Cu are shown in Table 4.2 All immobilization supports resulted in immobilization protein yields within the range of 86 - 94% (w/w). These results reveal that the differences in pore diameter and group density of immobilization supports did not affect the immobilization protein yield. In contrast to LS1 and LS2, Hill, Karboune, & Mateo (2016) have obtained an immobilization protein yield of 36% and 87% (w/w) for *B. amyloliquefaciens* LS immobilized on glyoxyl agarose/IDA-Cu and Sepabeads® EC-EP/IDA-Cu, respectively. Similarly to LS, the most promising immobilization support for *H. insolens* feruloyl esterase was identified to be a functionalized IDA-Cu epoxy-activated support (Tamayo-Cabezas & Karboune, 2019). However, contradictory immobilization protein yield results have been reported for *H. insolens* feruloyl esterase immobilized on Relizyme™ EP403/IDA-Cu (with larger pore diameter) and Sepabeads® EC-EP/IDA-Cu (with smaller pore diameter) supports by multi-covalent attachment. Tamayo-Cabezas & Karboune (2020) have reported a higher immobilization protein yield for *H. insolens* feruloyl esterase immobilized on Sepabeads® EC-EP/IDA-Cu (66% w/w) support than Relizyme™ EP403/IDA-Cu (45% w/w). In contrast, Spadoni Andreani, Li, Ronholm, & Karboune (2021) have immobilized the same feruloyl esterase and obtained a higher immobilization protein yield with Relizyme™ EP403/IDA-Cu support (90% w/w) in comparison to Sepabeads® EC-EP/IDA-Cu (73% w/w). The difference between the two last studies of multi-covalent attachment can be attributed to the use of different mg enzyme per g support. On the other hand, there are results reported in the literature in which larger pore size benefited enzyme physical adsorption onto mesoporous supports (Caldas et al., 2017; Thörn, Gustafsson, & Olsson, 2011; Li & Takahashi, 2000). Indeed, the immobilization of a feruloyl esterase containing enzymatic mixture by adsorption onto a mesoporous silica support with larger pore size (9nm) was found to have greater immobilization protein yield and specific activity than the one with the smaller pores (5nm) (Thörn, Gustafsson, & Olsson, 2011). Additionally, Caldas et al. (2017) have found that increasing the pore size of a mesoporous carbon ceramic material, composed of silica and graphite, increased the amount of immobilized glucose oxidase with the highest amount being achieved at the highest pore size (21nm) (Caldas et al., 2017).

Table 4.2 indicates that the difference between the investigated immobilized supports laid in the retained activity. The lowest values were obtained with the glyoxyl agarose/IDA-Cu support (26.9 - 32.2%) followed by the Sepabeads® EC-EP/IDA-Cu support (34.7 - 42.1%), while the

Relizyme™ EP403/IDA-Cu support provided the highest retained activity values of 55% and 98% for *G. oxydans* LS1 and *V. natriegens* LS2, respectively.

These results may be due to the occurrence of protein-protein and protein-support interactions at different sites and extents with the three aforementioned supports. The high retained activity obtained upon immobilizing LSs on Relizyme™ EP403/IDA-Cu may be due to the net charge on the surface of the support as a result of the significant modification of the oxirane groups by IDA (181 µmol/g of beads). The IDA group reacts with the oxirane group found in the epoxy activated supports and forms a functional group with two carboxylate anions that will interact with the positively charged amino on the LS, hence promoting physical adsorption (Tamayo-Cabezas & Karboune, 2019). Moreover, the larger pore size of the Relizyme™ EP403/IDA-Cu may have aided in the diffusion of substrates and products to and from the active site of LS. On the other hand, the small pore diameter and the high copper density (417 µmol/g of beads) of Sepabeads® EC-EP/IDA-Cu seem to have favored a high extent of protein-protein and protein-support interactions, resulting in LS denaturation and low retention of activity. The use of the glyoxyl agarose/IDA-Cu, which has the lowest copper density (145 µmol/g of beads) and no pores, may have led to LS denaturation and/or active site steric hindrance. Similarly, Tamayo-Cabezas & Karboune (2020) and Karboune, Neufeld, & Kermasha (2005) have reported that protein-protein and protein-support interactions were favored with the use of a support with smaller pore size. Contrarily, a higher retained activity of *B. amyloliquefaciens* LS was reported when immobilized on glyoxyl agarose/IDA-Cu (67%) than on Sepabeads® EC-EP/IDA-Cu (15%) (Hill, Karboune, & Mateo, 2016). Moreover, the macroporous Relizyme™ EP403/IDA-Cu support led to the lowest retained activity value of 70% for the immobilized *H. insolens* feruloyl esterase, while the highest ones were obtained with the mesoporous supports Relizyme™ EP113/S R and Sepabeads® EC-EP with 92% and 88%, respectively (Tamayo-Cabezas & Karboune, 2020).

Even though the retained activity value for the Relizyme™ EP403/IDA-Cu immobilized *G. oxydans* LS1 was lower in comparison to immobilized *V. natriegens* LS2, immobilized LS1 exhibited higher specific and enzyme activity values. This can be attributed to the high activity of the free LS1. Consequently, the Relizyme™ EP403/IDA-Cu was utilized to immobilize the LSs that were assessed in the biotransformation reactions.

Table 4.2. The immobilization efficiency of *G. oxydans* LS1 and *V. natriegens* LS2 on each support functionalized with iminodiacetic acid (IDA)-Cu.

Enzyme	Support	Immobilization Protein Yield (% w/w)[a]	Retained Activity (%)[b]	Specific Activity of Immobilized Enzymes (μmol/mg protein*min)[c]	Immobilized Enzyme Activity (μmol/g immobilized enzyme*min)[d]
G. oxydans LS1	Glyoxyl Agarose/IDA-Cu	89.73 (±1.67)	32.16 (±0.76)	93.85 (±2.22)	691.37 (±16.35)
	Sepabeads®EC-EP/IDA-Cu	85.95 (±1.31)	34.71 (±2.51)	91.62 (±6.62)	621.77 (±44.94)
	Relizyme™ EP403/IDA-Cu	94.23 (±0.70)	55.13 (±2.09)	205.23 (±7.76)	1633.55 (±61.80)
V. natriegens LS2	Glyoxyl Agarose/IDA-Cu	93.09 (±0.41)	26.90 (±0.99)	21.88 (±0.80)	200.92 (±7.37)
	Sepabeads®EC-EP/IDA-Cu	92.97 (±1.10)	42.12 (±3.91)	38.41 (±3.56)	367.61 (±34.09)
	Relizyme™ EP403/IDA-Cu	87.42 (±1.35)	97.48 (±13.77)	87.09 (±12.30)	795.13 (±112.32)

[a] Immobilization protein yield was calculated by combining the levansucrase protein content in the supernatant and wash and subtracting that from the total levansucrase protein content in the free enzyme solution and then dividing that result by the total levansucrase protein content in the free enzyme multiplied by 100.

[b] Retained activity was calculated as the ratio of the specific activity of the immobilized enzyme divided by the specific activity of the free enzyme solution multiplied by 100.

[c] The specific activity of the immobilized levansucrase was calculated by dividing the levansucrase activity by the immobilized levansucrase content.

[d] The activity of the immobilized levansucrase per gram of support.

4.3.2. Catalytic efficiency of selected immobilized levansucrases

Table 4.3 summarizes the total, hydrolytic, and transfructosylation activities of free and immobilized LSs with sucrose and lactose/sucrose substrates. The hydrolytic activity was estimated from the fructose content as it is expected to be solely the result of sucrose hydrolysis into glucose and fructose. Conversely, the glucose content provides the total activity corresponding to the hydrolysis of sucrose and the transfructosylation of the fructose unit of sucrose to an acceptor molecule. Therefore, the difference between the glucose and fructose contents is used to estimate the transfructosylation activity.

When sucrose was the sole substrate, all supports immobilizing *G. oxydans* LS1 provided higher total activities (94.96 - 323.28 µmol/mg protein min) than the free LS1; while only *V. natriegens* LS2 immobilized on Relizyme^{TM} EP403/IDA-Cu showed a higher total activity (101.63 µmol/mg protein min) than its corresponding free form. The results also indicate that in the presence of lactose/sucrose substrates, the total activities of immobilized *G. oxydans* LS1 and *V. natriegens* LS2 were lower in comparison to their free forms.

By comparing the ratio of total activity with sucrose and sucrose/lactose, the effect of immobilization on the substrate specificity can be inferred. A significant shift of the substrate specificity of *G. oxydans* LS1 towards sucrose than sucrose/lactose was observed upon immobilization. No change in the substrate specificity of *V. natriegens* LS2 was observed upon immobilization on Sepabeads® EC-EP/IDA-Cu exhibiting a similar ratio of total activity with sucrose and sucrose/lactose (0.85 - 0.89). However, other immobilized LS2 exhibited higher substrate specificity towards sucrose than sucrose/lactose. The highest total activity with lactose/sucrose was achieved with *G. oxydans* LS1 immobilized on Relizyme^{TM} EP403/IDA-Cu (60.36 µmol/mg protein min) and *V. natriegens* LS2 immobilized on Sepabeads®/IDA-Cu (60.35 µmol/mg protein min). Furthermore, LS2 immobilized on Relizyme^{TM} EP403/IDA-Cu and LS1 immobilized on Sepabeads® EC-EP/IDA-Cu led to more or less similar total activities (52.82 - 55.51 µmol/mg protein min) in the presence of lactose/sucrose.

Table 4.3. The catalytic efficiency of free and immobilized levansucrases (LS) on each support functionalized with iminodiacetic acid (IDA)-Cu.

Enzyme	Substrate	Form	Total Activity (µmol/mg protein min)[a]	Hydrolytic Activity (µmol/mg protein min)[b]	Transfructosylation Activity (µmol/mg protein min)[c]	Transfructosylation / Hydrolysis Ratio[d]
G. oxydans LS1	Sucrose	Free	3.60 (±0.01)	1.04 (±0.01)	2.57 (±0.01)	2.46
		Glyoxyl Agarose /IDA-Cu	94.96 (±9.65)	15.14 (±4.68)	79.82 (±4.97)	5.27
		Sepabeads®EC-EP/IDA-Cu	134.82 (±13.87)	18.73 (±3.00)	116.09 (±10.87)	6.20
		Relizyme™ EP403/IDA-Cu	323.28 (±20.19)	120.81 (±4.32)	202.47 (±15.87)	1.68
	Lactose / Sucrose	Free	291.57 (±4.83)	18.41 (±6.72)	273.17 (±1.89)	14.84
		Glyoxyl Agarose /IDA-Cu	3.19 (±1.59)	0.01 (±0.001)	3.19 (±1.59)	318.50
		Sepabeads®EC-EP/IDA-Cu	55.51 (±9.77)	19.89 (±6.97)	35.62 (±2.80)	1.79
		Relizyme™ EP403/IDA-Cu	60.36 (±9.05)	0.01 (±0.001)	60.36 (±9.05)	6035.80
V. natriegens LS2	Sucrose	Free	78.02 (±4.24)	53.25 (±3.10)	24.77 (±0.11)	0.47
		Glyoxyl Agarose /IDA-Cu	68.16 (±1.58)	32.85 (±3.07)	35.31 (±1.49)	1.07
		Sepabeads®EC-EP/IDA-Cu	53.62 (±4.63)	13.82 (±1.38)	39.80 (±3.25)	2.88
		Relizyme™ EP403/IDA-Cu	101.63 (±8.86)	19.32 (±1.47)	82.31 (±7.39)	4.26
	Lactose / Sucrose	Free	91.71 (±5.15)	20.34 (±0.53)	71.37 (±4.62)	3.51
		Glyoxyl Agarose /IDA-Cu	16.37 (±1.38)	0.01 (±0.001)	16.37 (±1.38)	1636.80
		Sepabeads®EC-EP/IDA-Cu	60.35 (±8.47)	10.01 (±2.02)	50.35 (±6.45)	5.03
		Relizyme™ EP403/IDA-Cu	52.82 (±3.39)	9.80 (±4.90)	43.02 (±1.51)	4.39

[a] Total activity was calculated by taking the slope of glucose in mmol/ml•min and multiplying it by the total reaction volume and dividing that by the enzyme content.

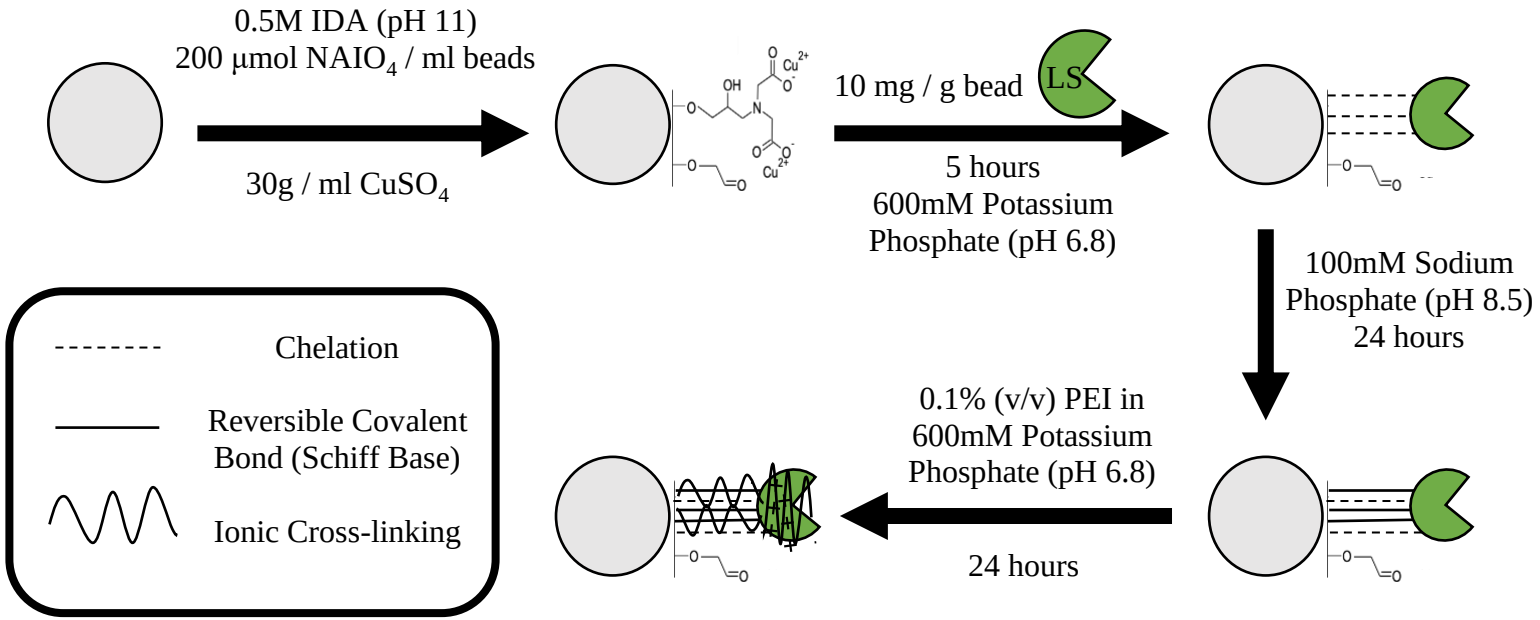
[b] Hydrolytic activity was calculated by taking the slope of fructose in mmol/ml•min and multiplying it by the total reaction volume and dividing that by the enzyme content.

[c] Transfructosylation activity was calculated by taking the difference between the total activity and hydrolytic activity.

[d] The ratio of transfructosylation to hydrolysis of each LS.

It can be seen from the results that the immobilized LSs had higher transfructosylation activities than hydrolytic ones, which is desired in order to produce more lactosucrose and FOSs and limit the sucrose hydrolysis side reaction. However, the transfructosylation to hydrolysis ratio was dependent on the type of support and the substrate used. When *G. oxydans* LS1 was immobilized on glyoxyl agarose/IDA-Cu and Sepabeads® EC-EP/IDA-Cu, it led to high transfructosylation to hydrolysis ratios (5.3 - 6.2) with sucrose substrate than the corresponding free form of LS1. On the other hand, the immobilized *V. natriegens* LS2 exhibited higher transfructosylation to hydrolysis ratios than the free LS2 with the highest ratio being achieved with immobilized LS2 on Relizyme™ EP403/IDA-Cu (4.26). The ratio of transfructosylation to hydrolysis was higher when lactose was present for most of immobilized LSs with the sole exception being the immobilized *G. oxydans* LS1 on Sepabeads® EC-EP/IDA-Cu support showing a decrease in the ratio from 6.20 to 1.79. In the presence of lactose/sucrose, glyoxyl agarose/IDA-Cu immobilized *V. natriegens* LS2 and Relizyme™ EP403/IDA-Cu immobilized *G. oxydans* LS1 exhibited the highest transfructosylation to hydrolysis ratios of 1636.8 and 6035.8, respectively. These high ratios may be attributed to the favorable partitioning of lactose, sucrose, and their transfructosylation products at macro/microenvironments of immobilized LSs, promoting their transfructosylation rather than their hydrolysis. The changes in the tridimensional structures of LSs upon immobilization may have also contributed to the modulation of their reaction selectivity. Moreover, as Table 4.1 shows, both Relizyme™ EP403/IDA-Cu and glyoxyl agarose/IDA-Cu supports have close bound copper content within the range of 144.8 - 168.5 µmol/g of beads; while the Sepabeads® EC-EP/IDA-Cu support was characterized with a smaller pore size and a higher copper density of 417.3 µmol/g of beads.

The literature shows that the immobilization of *B. amyloliquefaciens* LS on Sepabeads® HA support resulted in the highest ratio of transfructosylation to hydrolysis in the presence of sucrose alone, which was 2.3 times higher than that of the free form (Hill, Karboune, & Mateo, 2016). The same *B. amyloliquefaciens* LS immobilized on glyoxyl agarose/IDA-Cu showed a decrease in its total activity with the sucrose substrate but the transfructosylation to hydrolysis ratio remained similar to that of the free enzyme (Hill, Karboune, & Mateo, 2016).



Scheme 4.1. Pre-immobilization (iminodiacetic acid (IDA)-Cu) and post-immobilization treatments (high pH and polyethylenimine (PEI)) as well as the levansucrase (LS) immobilization on Relizyme^{TM} EP403.

4.3.3. Post-immobilization treatment of levansucrase

As shown in Scheme 4.1, post-immobilization treatments were carried out to better stabilize the LSs immobilized on the functionalized Relizyme^{TM} EP403/IDA-Cu support. One of the post-immobilization treatments encompassed the construction of reversible covalent bonds, particularly Schiff bases, by exposing the complex to high pH conditions for the formation of stronger intramolecular interactions between the LS and the epoxy activated support. Another step included the addition of the polyaminated crosslinker PEI, which is a hydrophilic and cationic polymer at pH 6 allowing for an ionic coating of the immobilized LS by its stronger attachments to negatively charged surfaces, such as the aspartate and glutamate residues on the LS (Vieira et al., 2011). However, the alkaline conditions required for the promotion of multipoint covalent attachment and the ionic coating by PEI may affect the activity of the immobilized enzyme. Figure 4.2 shows that the pH exposure caused a 26% and 58% decrease in activity for immobilized *G. oxydans* LS1 and *V. natriegens* LS2, respectively. Consequently, the immobilized *G. oxydans* LS1 had a higher immobilized enzyme activity value (39.1 µmol/g immobilized enzyme. min) after high pH exposure in comparison to that of the immobilized *V. natriegens* LS2 (9.7 µmol/g immobilized enzyme. min). Further treatment with the PEI crosslinker caused additional decrease, providing retained activity of 10.5% for immobilized *G. oxydans* LS1 and 35.5% for immobilized *V. natriegens* LS2. As a result, both *G. oxydans* LS1 and *V. natriegens* LS2 ended up with a similar immobilized enzyme activity of 3.2 - 3.3 µmol/g immobilized enzyme. min, when the PEI crosslinker was introduced. The results also reveal that contrary to the immobilized *V. natriegens* LS2, the activity of the immobilized *G. oxydans* LS1 was more affected by the PEI post-treatment as it had an overall steeper decline in activity.

It has been reported that high pH incubation followed by a reduction step with sodium borohydride caused 86% decrease in the retained activity of the *B. amyloliquefaciens* LS immobilized on glyoxyl agarose/IDA-Cu (Hill, Karboune, & Mateo, 2017). The reduction step was applied by these authors in order to form permanent covalent bonds and further improve enzyme stability, but it resulted in a high decrease in activity that was attributed to the denaturation effect of sodium borohydride (Hill, Karboune, & Mateo, 2017). Furthermore, the PEI crosslinker was examined at a concentration range of 0.1 - 2 % (v/v) for *B. amyloliquefaciens* LS immobilized on glyoxyl agarose/IDA-Cu. Only the 2% PEI showed a loss in activity after 15h of incubation while the lower

concentrations did not show significant differences in the retained activity (Hill, Karboune, & Mateo, 2017). The incubation at a high pH for immobilized *H. insolens* feruloyl esterase on Sepabeads® EC-EP/IDA-Cu support was carried out at pH 8 and 10 for 4h and led to an increase in the retained specific activity by 10.5% and 7.8%, respectively (Tamayo-Cabezas & Karboune, 2020).

However, when the high pH incubation time was extended to 14h, the pH 8 provided a retained specific activity increase of 16.2%, while the pH 10 led to an almost complete deactivation of the enzyme. Additionally, with a post-immobilization treatment of 4h at pH 10, the *H. insolens* feruloyl esterase on Relizyme™ EP403/IDA-Cu showed a higher specific activity retention than that with Sepabeads® EC-EP/IDA-Cu (Tamayo-Cabezas & Karboune, 2020).

The thermal stability factor was estimated as the ratio of the retained activity of the post-immobilization treated LS (high pH and PEI) to that of the free LS after both were incubated at 50°C at selected times. Figure 4.3 shows the thermal stabilization of Relizyme™ EP403/IDA-Cu immobilized *G. oxydans* LS1 increase by a factor of 2.5 and 2.7 at 20 and 40 min incubation upon the high-pH and PEI post-immobilization treatments, respectively. However, after 60 min of incubation at 50°C, activity of immobilized *G. oxydans* LS1 was unfortunately not detectable regardless the type of post-immobilization treatment. On the other hand, the Relizyme™ EP403/IDA-Cu immobilized *V. natriegens* LS2 that was subjected to high pH post-immobilization treatment showed a more promising thermal stability with a factor of 53.1 after 40 min incubation at 50°C; while PEI post-immobilized LS2 led to the highest value of 36.6 after 20 min.

Looking at the literature, *B. amyloliquefaciens* LS immobilized on glyoxyl agarose/IDA-Cu that has undergone high pH incubation and sodium borohydride reduction showed a high thermal stability after 60 min at 55°C (Hill, Karboune, & Mateo, 2017). The thermal stability of the same *B. amyloliquefaciens* LS immobilized on glyoxyl agarose/IDA-Cu incubated with 0.1% (v/v) PEI was improved by a stabilization factor of 4.7 at 50°C after 120 min and there was still residual activity even after 250min of incubation at 50°C (Hill, Karboune, & Mateo, 2017). The Sepabeads® EC-EP/IDA-Cu immobilized *H. insolens* feruloyl esterase that was subjected to high pH post-immobilization treatment at pH 10 for 4 h was reported to have a higher thermal stability than the free enzyme after thermal incubation at 50°C for 2h (Tamayo-Cabezas & Karboune, 2020).

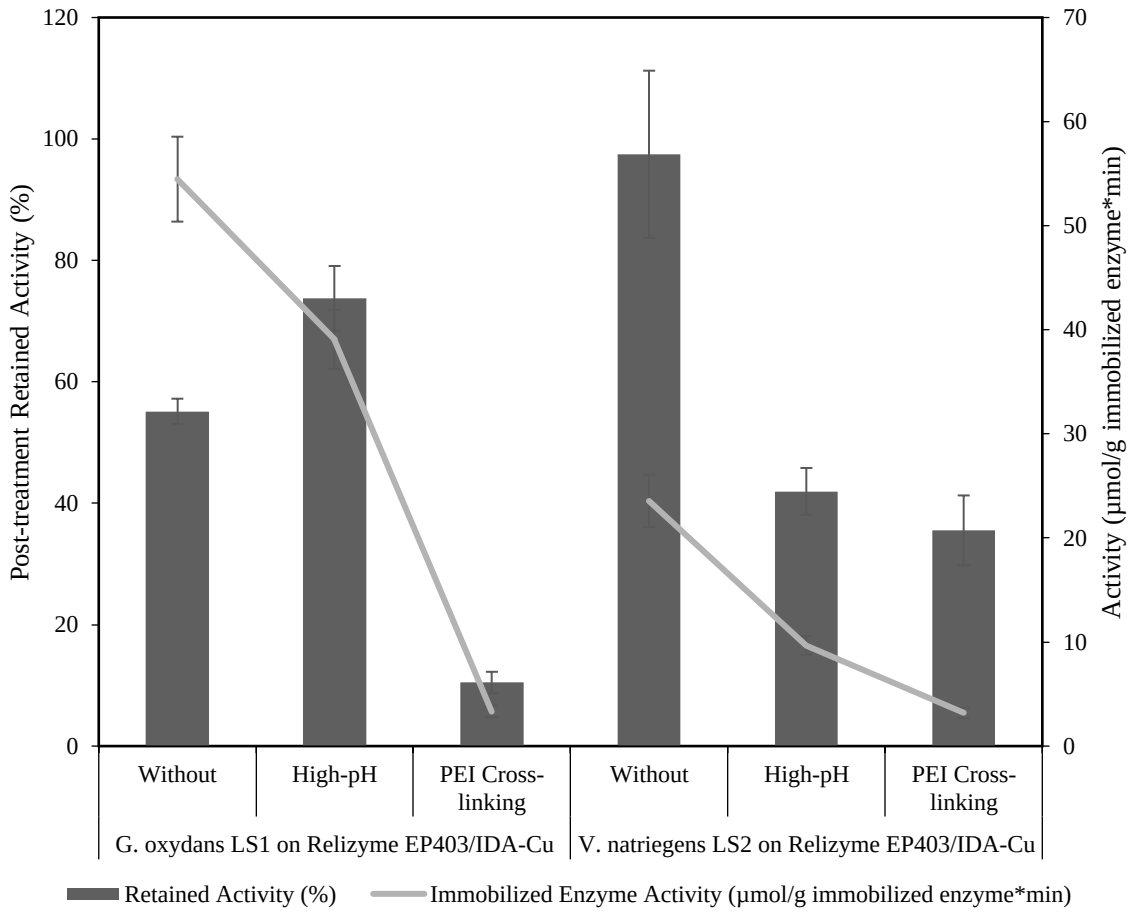


Figure 4.2. Post-immobilization treatments (high pH and polyethylenimine (PEI)) for *G. oxydans* LS1 and *V. natriegens* LS2 immobilized on Relizyme^{TM} EP403 functionalized with iminodiacetic acid (IDA)-Cu.

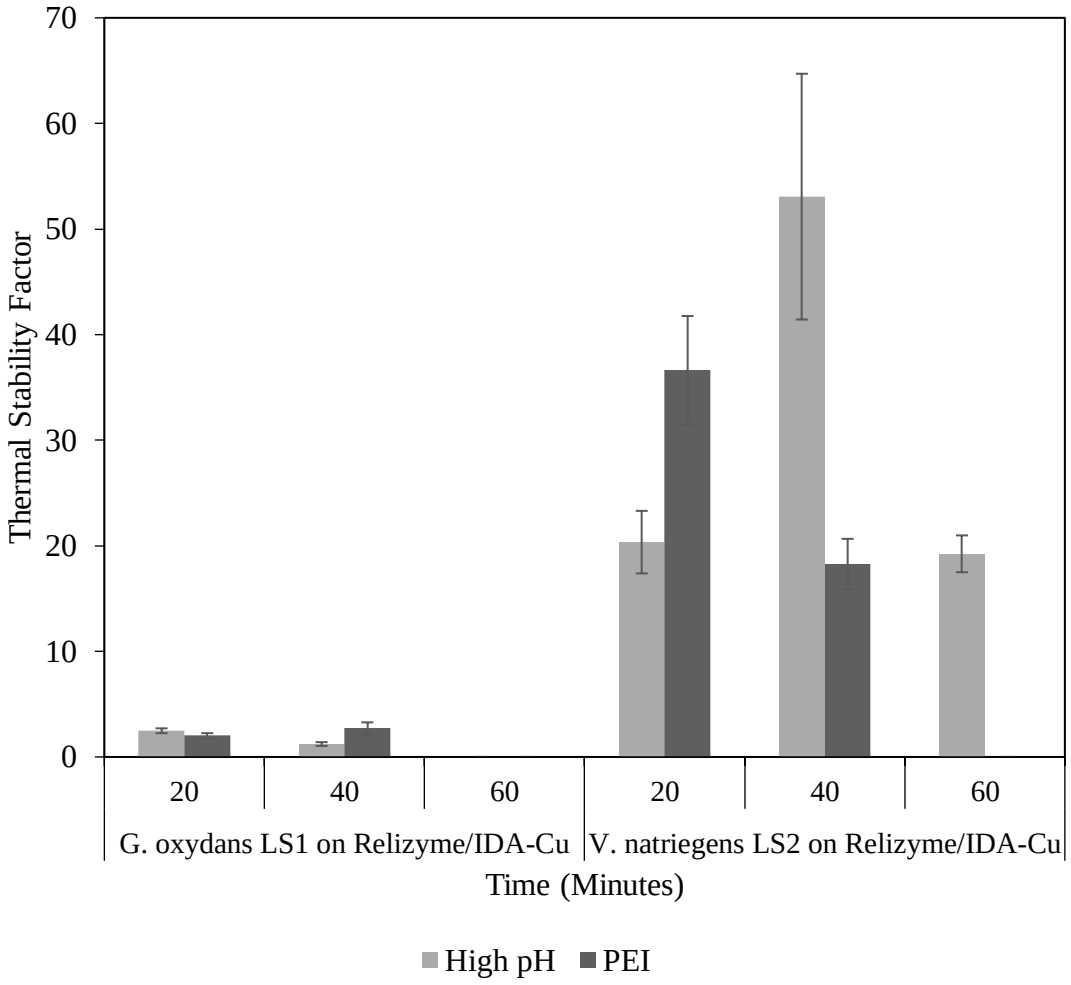


Figure 4.3. The effect of post-immobilization treatments (high pH and polyethylenimine (PEI)) on the thermal stability of *G. oxydans* LS1 and *V. natriegens* LS2 immobilized on Relizyme^{TM} EP403 functionalized with iminodiacetic acid (IDA)-Cu.

4.3.4. Biotransformation of lactose and dairy by-products by immobilized levansucrases

The time course for the biotransformation of lactose and dairy by-products in the presence of sucrose by immobilized LSs was investigated. Four end-products, lactosucrose, kestose, nystose, and fructosyl nystose, were quantified using an ion mobility Q-TOF – MS system. The investigated dairy by-products as sources of lactose included WP and MP.

Figure 4.4 shows that the Relizyme™ EP403/IDA-Cu immobilized *G. oxydans* LS1 favored the production of kestose rather than lactosucrose at the beginning of the reaction time course with lactose/sucrose and WP/sucrose. However, after 5h of reaction time, the amount of produced lactosucrose reached the highest amount of 66 - 79 g/L, which decreased thereafter and remained at a range of 18 - 29 g/L at the end of the reaction time course (9 - 24h). Contrary to the Relizyme™ EP403/IDA-Cu immobilized *G. oxydans* LS1, the Relizyme™ EP403/IDA-Cu immobilized *V. natriegens* LS2 exhibited a higher specificity towards the synthesis of lactosucrose (86 - 95 g/L), which was most dominant at the 5h and 9h reaction time with WP/sucrose and lactose/sucrose, respectively; thereafter, kestose and nystose increased in production and were synthesized within a range of 26 - 28 and 32 - 36 g/L, respectively, but lactosucrose was still the most dominant transfructosylation product (35 - 79 g/L). The decrease in the lactosucrose amount at the end of the reaction time course can be attributed to a shift of the reaction towards the hydrolysis of lactosucrose.

Contrary to lactose/sucrose and WP/sucrose, lactosucrose synthesis was predominant throughout the entire reaction time when MP/sucrose was utilized. However, the total quantity produced from MP/sucrose was smaller than that with other substrates as the initial lactose of MP was limited to 4.8 % (w/w). The highest lactosucrose amounts of 18 and 35 g/L were achieved after 18h with Relizyme™ EP403/IDA-Cu immobilized *G. oxydans* LS1 and 9h with Relizyme™ EP403/IDA-Cu immobilized *V. natriegens* LS2 catalyzed reactions, respectively. With MP/sucrose, the production of lactosucrose by Relizyme™ EP403/IDA-Cu immobilized *G. oxydans* LS1 remained predominant as the reaction progressed. However, lactosucrose, kestose, and nystose were produced at equal amounts by Relizyme™ EP403/IDA-Cu immobilized *V. natriegens* LS2 with the MP/sucrose.

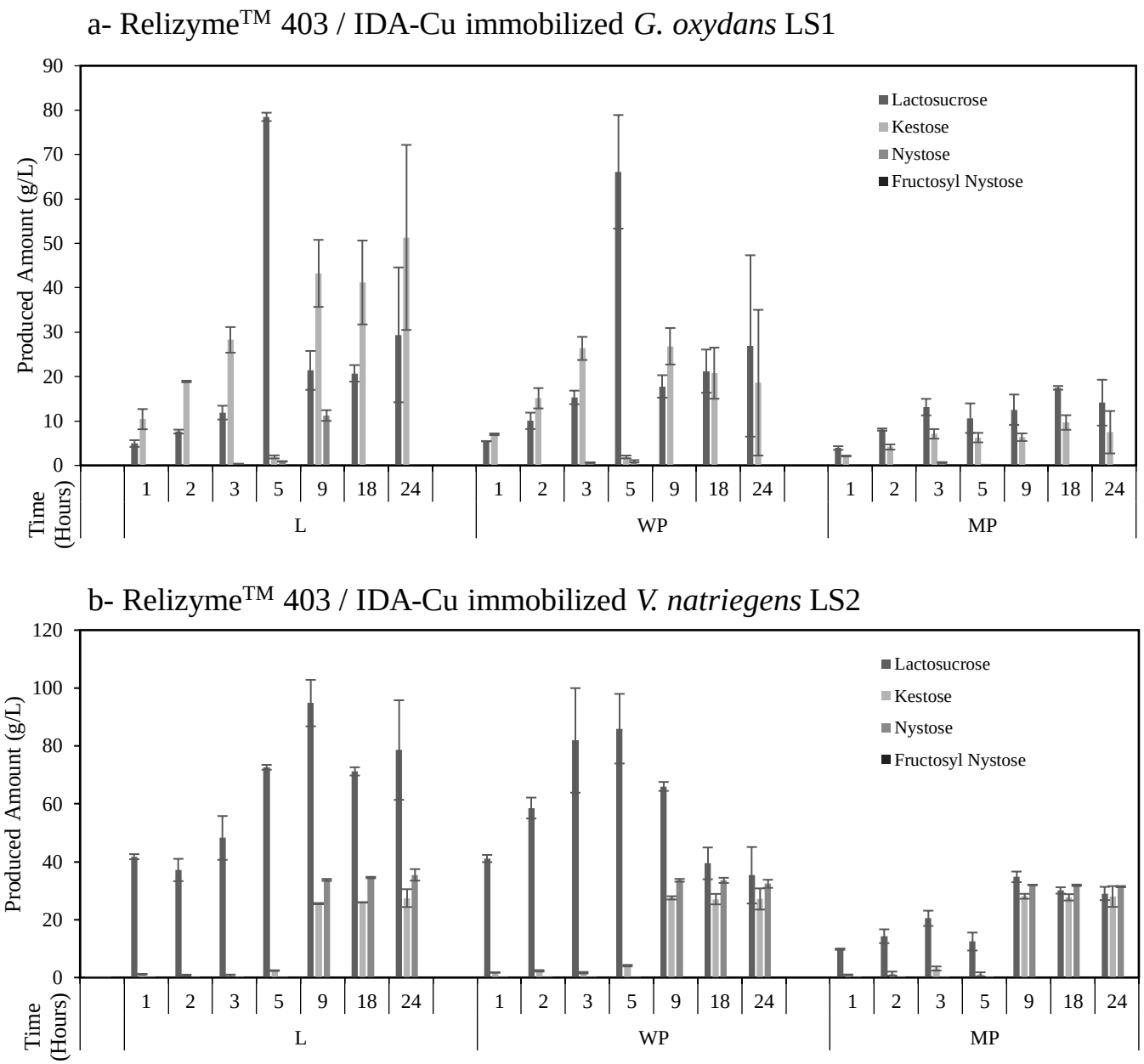


Figure 4.4. Quantified biotransformation end-products for *G. oxydans* LS1 and *V. natriegens* LS2 immobilized on Relizyme™ EP403 functionalized with iminodiacetic acid (IDA)-Cu in the presence of lactose (L)/sucrose, whey permeate (WP)/sucrose, and milk permeate (MP)/sucrose.

Figure 4.5 shows the maximum bioconversion yield of lactosucrose and kestose in all investigated reaction systems. The bioconversion yield of lactosucrose was shown to be higher than that of kestose. The bioconversion yield of kestose was the same for both immobilized LSs when WP/sucrose was utilized (6.7 - 6.9% mol/mol); however, higher yields were obtained with Relizyme^{TM} EP403/IDA-Cu immobilized *G. oxydans* LS1 in the presence of lactose/sucrose (12.5% mol/mol) and with Relizyme^{TM} EP403/IDA-Cu immobilized *V. natriegens* LS2 using MP/sucrose (6.6% mol/mol). However, the highest lactosucrose bioconversion yields were acheieved with Relizyme^{TM} EP403/IDA-Cu immobilized *V. natriegens* LS2 for all three difference lactose sources (30.0 - 87.8% mol/mol). Consequently, *V. natriegens* LS2 was determined to be the optimal candidate for lactosucrose production optimization.

4.3.5. Effects of biotransformation parameters catalyzed by free and immobilized levansucrase

The effects of biotransformation parameters were carried out via RSM using WP by-product and sucrose substrates. *V. natriegens* LS2 was identified as the top candidate for lactosucrose production both in its free and immobilized form on the Relizyme^{TM}/IDA-Cu support. The two reaction parameters, the incubation time (3 – 15 h) and the ratio of lactose to sucrose (0.5 – 2.0), were varied while the enzyme units (5 U/ml), buffer (50mM ammonium bicarbonate; pH of 8), and temperature (45°C) were kept constant. The end-products, lactosucrose, kestose, and nystose, were quantified using the 6560-ion mobility Q-TOF – MS system and their respective amounts (g/L) and yields (% mol/mol) were estimated as the responses (Table 4.4).

Table 4.5 shows the analysis of variance (ANOVA) for each response. The *F-value* indicates the degree of differentiation amongst the various reaction conditions with larger values showing greater statistically significant differences between conditions. The *p-value* provides a level of statistical confidence in the validity of the test, where small values (ideally less than 0.05) disprove the null hypothesis, which would state that the reaction conditions did not produce a perceivable difference. Contrary to that, the alternative hypothesis would state that the reaction conditions could be perceived as different (Bower, 2013). For the free form of *V. natriegens* LS2, the best models that were found to be significant for lactosucrose production were linear for the yield and quadratic for the amount produced.

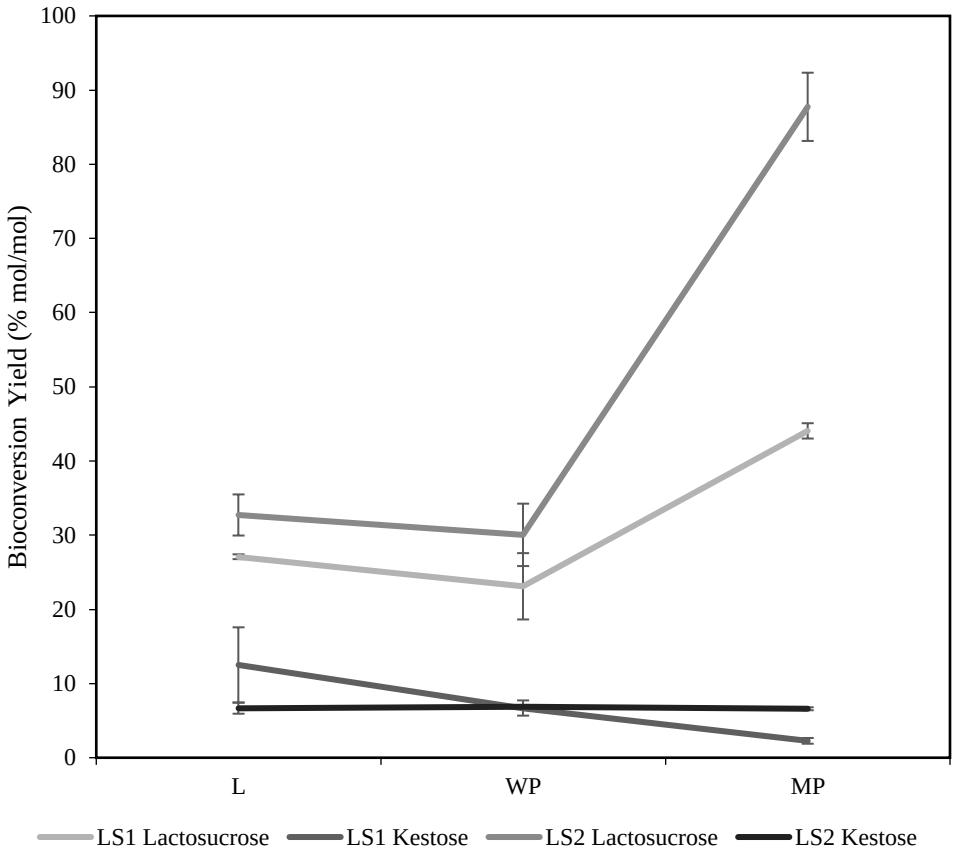


Figure 4.5. Maximum bioconversion yields of lactosucrose and kestose in the reaction systems catalyzed by *G. oxydans* LS1 and *V. natriegens* LS2 immobilized on Relizyme^{TM} EP403 functionalized with iminodiacetic acid (IDA)-Cu in the presence of lactose (L)/sucrose, whey permeate (WP)/sucrose, and milk permeate (MP)/sucrose.

Table 4.4. Experimental design factors and responses for *V. natriegens* LS2 in its free form and immobilized form on Relizyme™ EP403 functionalized with iminodiacetic acid (IDA)-Cu.

Reaction	Factor 1	Factor 2	Response 1	Response 2	Response 3	Response 4	Response 5	Response 6
	Ratio Lactose/Sucrose	Time (Hours)	Lactosucrose (g/L)	Kestose (g/L)	Nystose (g/L)	Lactosucrose Yield (% mol/mol)	Kestose Yield (% mol/mol)	Nystose Yield (% mol/mol)
Free *V. natriegens* LS2								
1	0.72	4.76	237.17 (±19.54)	0.00	1.05 (±0.06)	100 (±1.14)	0.00	0.16 (±0.01)
2	1.25	15.00	115.88 (±24.03)	0.00	6.88 (±1.95)	40.84 (±10.01)	0.00	1.68 (±0.47)
3	1.25	9.00	137.57 (±11.50)	0.00	1.89 (±0.67)	48.48 (±4.43)	0.00	0.46 (±0.16)
4	1.78	4.76	201.43 (±)	0.00	0.00	49.84 (±)	0.00	0.00
5	1.25	9.00	131.80 (±7.03)	0.00	1.78 (±0.38)	46.45 (±2.48)	0.00	0.43 (±0.09)
6	0.50	9.00	90.97 (±26.78)	0.00	0.59 (±0.47)	40.08 (±11.80)	0.00	0.09 (±0.07)
7	2.00	9.00	29.61 (±16.99)	0.00	0.00	6.52 (±4.49)	0.00	0.00
8	1.25	3.00	165.92 (±)	0.00	0.00	58.47 (±)	0.00	0.00
9	1.78	13.24	134.29 (±41.55)	0.00	2.69 (±1.37)	33.23 (±12.73)	0.00	0.69 (±0.35)
10	1.25	9.00	111.82 (±59.11)	0.00	1.85 (±2.53)	39.41 (±24.38)	0.00	0.45 (±0.62)
11	0.72	13.24	171.87 (±43.16)	0.36 (±0.51)	11.85 (±5.42)	75.71 (±19.56)	0.07 (±0.10)	1.81 (±0.86)
Immobilized *V. natriegens* LS2 on Relizyme™ EP403 /IDA-Cu								
1	0.72	4.76	148.05 (±35.96)	4.39 (±0.37)	19.97 (±0.01)	65.22 (±19.92)	0.88 (±0.08)	3.04 (±0.001)
2	1.25	15.00	27.76 (±4.62)	2.82 (±0.56)	15.51 (±0.61)	9.78 (±1.63)	0.91 (±0.18)	3.78 (±0.15)
3	1.25	9.00	26.71 (±6.27)	1.18 (±0.15)	14.72 (±0.18)	9.41 (±2.44)	0.38 (±0.05)	3.58 (±0.04)
4	1.78	4.76	103.97 (±11.95)	2.02 (±0.66)	17.17 (±0.85)	25.73 (±2.96)	0.68 (±0.22)	4.39 (±0.22)
5	1.25	9.00	32.15 (±9.39)	1.57 (±0.17)	14.71 (±0.27)	11.33 (±4.13)	0.51 (±0.06)	3.58 (±0.07)
6	0.50	9.00	141.05 (±31.77)	8.16 (±1.23)	18.85 (±1.26)	62.14 (±15.84)	1.63 (±0.25)	2.85 (±0.19)
7	2.00	9.00	58.48 (±19.71)	5.16 (±0.42)	20.81 (±0.99)	12.88 (±5.25)	2.11 (±0.17)	6.45 (±0.31)
8	1.25	3.00	74.89 (±5.58)	0.88 (±0.26)	13.56 (±0.48)	26.39 (±1.97)	0.28 (±0.10)	3.30 (±0.12)
9	1.78	13.24	41.11 (±5.36)	1.98 (±0.48)	17.27 (±0.11)	10.17 (±1.54)	0.67 (±0.16)	4.42 (±0.03)
10	1.25	9.00	49.10 (±8.90)	2.39 (±0.28)	16.01 (±0.48)	17.31 (±3.98)	0.77 (±0.09)	3.90 (±0.12)
11	0.72	13.24	72.75 (±22.34)	7.86 (±0.94)	23.56 (±0.39)	32.05 (±12.34)	1.58 (±0.19)	3.59 (±0.06)

With the linear model, it can be seen that both the ratio of lactose to sucrose (*F-value* of 91.66; *p-value* of < 0.0001) and reaction time (*F-value* of 16.75; *p-value* of 0.0011) were significant factors but a higher effect of the ratio was exhibited due to the higher *F-value* and lower *p-value*.

For the amount of lactosucrose produced, there was no significant interactive effect between the two factors (*F-value* of 0.8583; *p-value* of 0.3725) but the linear effect of reaction time seemed to play a more substantial effect (*F-value* of 12.05; *p-value* of 0.0046). The kestose yield and amount produced were equal to zero and thus were not analyzed in relation to the substrate ratio and reaction time parameters. Lastly, the nystose yield and amount produced both fell under a quadratic model and showed no significant interactive effect between the factors (*F-value* of 0.7364; *p-value* of 0.4043 and *F-value* of 3.04; *p-value* of 0.1016, respectively). Moreover, reaction time appeared to be a more imperative factor for both the nystose yield and amount produced (*F-value* of 7.7; *p-value* of 0.0141 and *F-value* of 7.5; *p-value* of 0.0153, respectively) (Table 4.5).

For the Relizyme^{TM} EP403/IDA-Cu immobilized form of *V. natriegens* LS2, the amount produced and yield of lactosucrose both significantly fit the quadratic model. Both the yield and amount produced displayed no interactive effect between the two factors (*F-value* of 1.99; *p-value* of 0.1777 and *F-value* of 0.1456; *p-value* of 0.7078, respectively) but both unveiled a more important effect of the ratio of the substrates in the linear and quadratic forms (*F-value* of 27.5; *p-value* of < 0.0001 and *F-value* of 27.36; *p-value* of < 0.0001, respectively). The yield and amount produced for kestose and nystose all followed a quadratic model, and only the amount of kestose produced showed an interactive effect between the two factors (*F-value* of 10.07; *p-value* of 0.0059) while predictive kestose and nystose yields and amounts models have not shown any interactive effects (*F-value* of 2.62; *p-value* of 0.1251, *F-value* of 0.7979; *p-value* of 0.3849, and *F-value* of 1.54; *p-value* of 0.233, respectively). The ratio of lactose to sucrose seemed to be the significant factor for the kestose yield (*F-value* of 40.43; *p-value* of < 0.0001) and amount produced (*F-value* of 108.08; *p-value* of < 0.0001) in addition to the nystose yield (*F-value* of 11.9; *p-value* of 0.0033) and amount produced (*F-value* of 24.32; *p-value* of 0.0002) (Table 4.5).

Figure 4.6 shows the contour plots of the predictive models. Figure 4.6 a_1 reveals that the substrate ratio does not affect the lactosucrose production by free *V. natriegens* LS2 at the initial stage of the reaction, but its effect became more prominent at the middle and the end of the reaction time course.

Table 4.5. Analysis of variance (ANOVA) for *V. natriegens* LS2 in its free form and immobilized form on Relizyme™ EP403 functionalized with iminodiacetic acid (IDA)-Cu.

Form		Lactosucrose Yield (% mol/mol)		Lactosucrose (g/L)		Kestose Yield (% mol/mol)		Kestose (g/L)		Nystose Yield (% mol/mol)		Nystose (g/L)	
		F	*p-value*	*F*	*p-value*	*F*	*p-value*	*F*	*p-value*	*F*	*p-value*	*F*	*p-value*
Free *V. natriegens* LS2	Model	54.2	<0.0001	6.39	0.0041	-	-	-	-	15.9	<0.0001	13.8	<0.0001
	A- Ratio Lactose/Sucrose	91.66	<0.0001	6.71	0.0236	-	-	-	-	2.17	0.1618	5.9	0.0282
	B- Time (Hours)	16.75	0.0011	15.89	0.0018	-	-	-	-	59.21	<0.0001	51.76	<0.0001
	AB	-	-	0.8583	0.3725	-	-	-	-	0.7364	0.4043	3.04	0.1016
	A^2	-	-	4.38	0.0583	-	-	-	-	5.93	0.0278	2.4	0.1422
	B^2	-	-	12.05	0.0046	-	-	-	-	7.7	0.0141	7.5	0.0153
	Lack of Fit	1.22	0.3741	4.03	0.052	1.43	0.2765	1.43	0.2765	1.02	0.4171	2.95	0.757
Immobilized *V. natriegens* LS2 on Relizyme™ EP403/IDA-Cu	Model	20.23	<0.0001	12.94	<0.0001	11.22	<0.0001	41.41	<0.0001	19.44	<0.0001	6.42	0.0019
	A- Ratio Lactose/Sucrose	54.97	<0.0001	17.44	0.0007	0.4911	0.4935	63.68	<0.0001	77.42	<0.0001	2.53	0.131
	B- Time (Hours)	16.7	0.0009	19.75	0.0004	6.32	0.023	15.54	0.0012	2.29	0.15	2.64	0.124
	AB	1.99	0.1777	0.1456	0.7078	2.62	0.1251	10.07	0.0059	0.7979	0.3849	1.54	0.233
	A^2	27.5	<0.0001	27.36	<0.0001	40.43	<0.0001	108.08	<0.0001	11.9	0.0033	24.32	0.0002
	B^2	2.4	0.1407	2.89	0.1087	0.2657	0.6132	0.007	0.9342	1.17	0.2958	0.2116	0.6517
	Lack of Fit	1.35	0.3006	2.03	0.1589	11.49	0.0006	3.35	0.0526	27.65	<0.0001	37.23	<0.0001

The free form of *V. natriegens* LS2 has a lactosucrose yield that fits a linear model while the lactosucrose amount produced and nystose yield and amount produced all fit a quadratic model.

The immobilized *V. natriegens* LS2 on Relizyme™ EP403/IDA-Cu lactosucrose, kestose, and nystose yields and amounts produced all fit a quadratic model.

A higher amount of produced lactosucrose by free *V. natriegens* LS2 was achieved at a shorter reaction time, regardless of the substrate ratio. Figure 4.6 a_2 displays the lactosucrose bioconversion yield and shows that both a lower substrate ratio and reaction time were needed for a higher lactosucrose bioconversion yield. The results from Figure 4.6 a_3 indicate that the longer is the reaction time, the higher is the amount of nystose that is produced in the free *V. natriegens* LS2 reaction system. Figure 4.6 a_3 also demonstrates that a lower substrate ratio is needed to produce more nystose by the free *V. natriegens* LS2. Therefore, both the reaction time and the substrate ratio influenced the free *V. natriegens* LS2 reaction system, but the reaction time had a narrower range making it more critical. The bioconversion yield for nystose shown in Figure 4.6 a_4 gave similar results as presented with the amount of produced nystose in Figure 4.6 a_3; therefore, a higher nystose bioconversion yield with free *V. natriegens* LS2 requires a longer reaction time and lower substrate ratio.

In the Relizyme^{TM} EP403/IDA-Cu immobilized *V. natriegens* LS2 biocatalytic system, both lactosucrose bioconversion yield and amount exhibited similar predictive patterns requiring lower substrate ratios and shorter incubation times for maximal lactosucrose synthesis as can be seen in Figures 4.6 b_1 and b_2. The predictive models of kestose bioconversion yield and amount produced (Figures 4.6 b_3 and b_4) also followed similar patterns of lower substrate ratios and longer reaction times. A similar predictive pattern to that of kestose is shown by the amount of nystose produced in Figure 4.6 b_5 requiring a lower substrate ratio and longer reaction time. However, the nystose bioconversion yield displayed in Figure 4.6 b_6 indicates that the substrate ratio was the vital factor, as previously confirmed with the ANOVA table (Table 4.5), and appeared to require a higher substrate ratio for a higher nystose bioconversion yield.

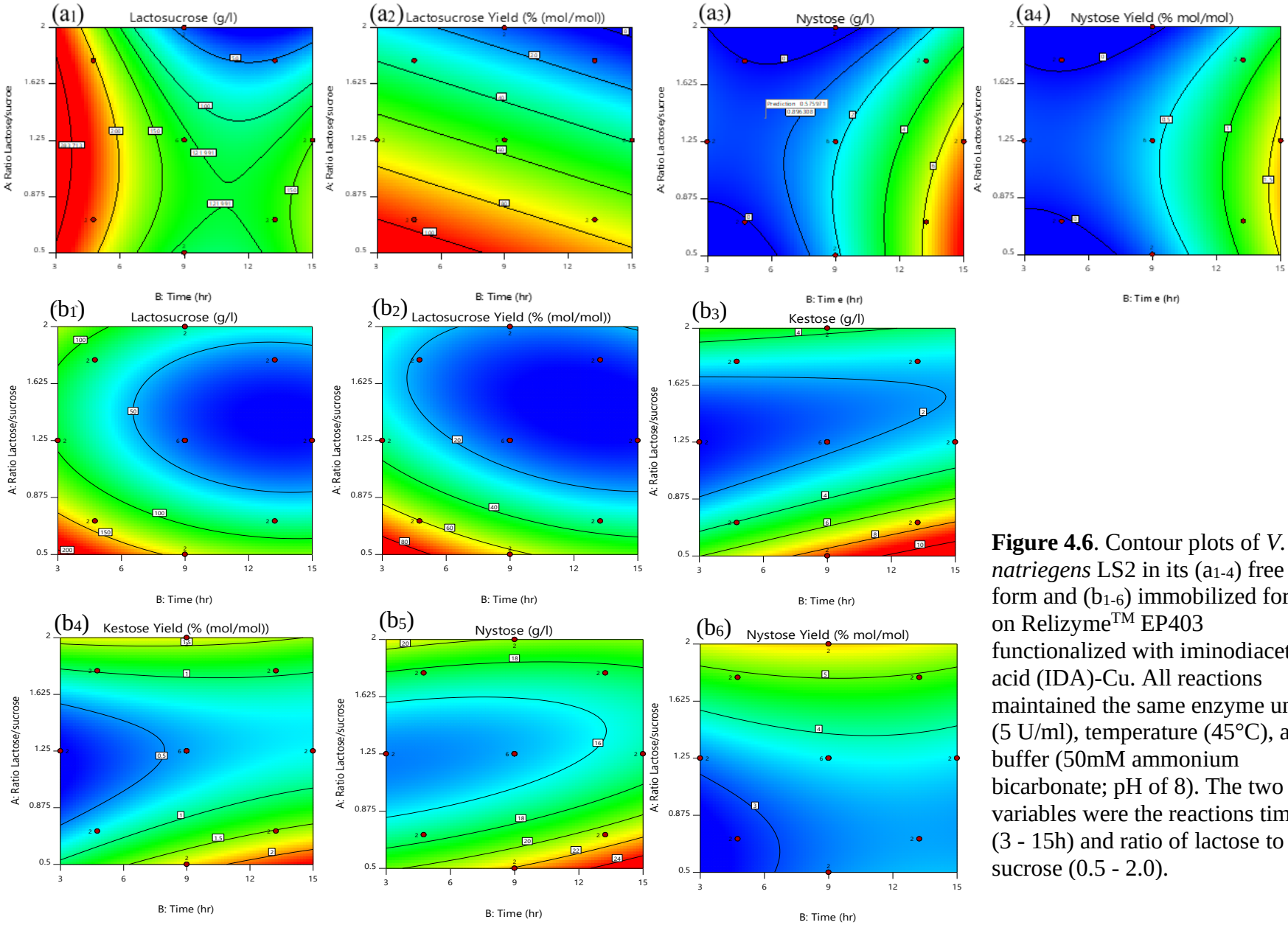


Figure 4.6. Contour plots of *V. natriegens* LS2 in its (a_{1-4}) free form and (b_{1-6}) immobilized form on Relizyme^{TM} EP403 functionalized with iminodiacetic acid (IDA)-Cu. All reactions maintained the same enzyme units (5 U/ml), temperature (45°C), and buffer (50mM ammonium bicarbonate; pH of 8). The two variables were the reactions time (3 - 15h) and ratio of lactose to sucrose (0.5 - 2.0).

4.3.6. Selected biotransformation parameters and reusability of immobilized levansucrase

The selected biotransformation parameters to maximize the selectivity of free and Relizyme™ EP403/IDA-Cu immobilized *V. natriegens* LS2 towards lactosucrose synthesis were determined from the predictive models. Table 4.6 summarizes the identified conditions, predictive responses as well as the experimental ones. The experimental lactosucrose bioconversion yield and produced amount fitted the confidence interval for both free and Relizyme™ EP403/IDA-Cu immobilized *V. natriegens* LS2, revealing the significance of the developed predictive models. Both a shorter reaction time and lower lactose/sucrose ratio are needed to achieve a high selective production of lactosucrose. The free *V. natriegens* LS2 was able to synthesize 117 g/L of lactosucrose while the Relizyme™ EP403/IDA-Cu immobilized *V. natriegens* LS2 produced 101 g/L of lactosucrose.

One of the major advantageous features of immobilizing enzymes is the reusability, which is dependent on several factors such as the enzyme's thermal stability and stability from being leached from the immobilization support. The Relizyme™ EP403/IDA-Cu immobilized *V. natriegens* LS2 was reused up to 5 times but after only 3 times, the amount of produced lactosucrose reached a plateau as seen in Figure 4.7. The initial biotransformation reaction provided 101 g/L of lactosucrose while the first time the complex was reused provided an even higher amount with 119 g/L of lactosucrose. Afterwards, the second time the immobilized LS2 was reused offered 25 g/L of lactosucrose and the third and last only gave 2 g/L of lactosucrose. Therefore, the final combined amount of lactosucrose produced was 247 g/L after 4 consecutive cycles before there was no more lactosucrose synthesis. Another LS from *B. licheniformis* immobilized on chitosan beads was used for FOS production and was reused for 5 consecutive cycles (Sangmanee, Nakapong, Pichyangkura, Kuttiyawong, 2016). A fructosyltransferase immobilized in chitosan beads was able to produce an average of 40.74% (w/w) of FOSs for 3 consecutive cycles but experienced a significant loss in its transfructosylation activity afterwards (Ganaie, Rawat, Wani, Gupta, & Kango, 2014).

Table 4.6. Responses of the optimal conditions for lactosucrose production with *V. natriegens* LS2 in its free form and immobilized form on Relizyme^{TM} EP403 functionalized with iminodiacetic acid (IDA)-Cu.

	Reaction	Ratio Lactose/ Sucrose	Time (Hours)	Lactosucrose Yield (% mol/mol)	Lactosucrose (g/L)	Confidence Interval for Lactosucrose Yield (% mol/mol)	Confidence Interval for Lactosucrose (g/L)
Predicted Responses	Free	0.586	3.117	87.096	176.067	59.5 - 94.68	46.95 - 185.095
	Immobilized	0.503	3.083	51.91	100.257	43.275 - 72.415	77.71 - 153.718
Experimental Responses	Free	0.586	3.117	51.373 (±9.165)	116.615 (±20.804)	-	-
	Immobilized	0.503	3.083	44.394 (±6.962)	100.773 (±15.803)	-	-

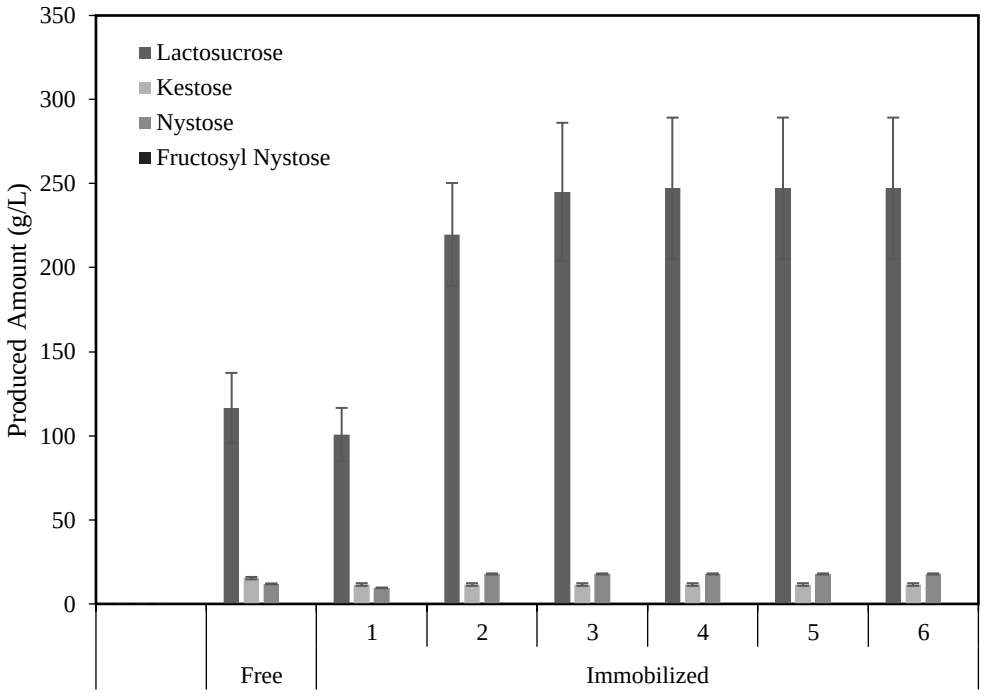


Figure 4.7. Responses of the optimal conditions for lactosucrose production with *V. natriegens* LS2 in its free form and immobilized form on Relizyme^{TM} EP403 functionalized with iminodiacetic acid (IDA)-Cu with 5-time reusability.

4.4. Conclusion

Out of all the tested immobilization supports and functionalizations, the Relizyme™ EP403 support functionalized with IDA-Cu led to the highest immobilization protein yields and retained activities for *G. oxydans* LS1 and *V. natriegens* LS2. Furthermore, the post-immobilization treatments were found to decrease the activities with the Relizyme™ EP403/IDA-Cu immobilized *G. oxydans* LS1 being less impacted by high pH incubation than the Relizyme™ EP403/IDA-Cu immobilized *V. natriegens* LS2. However, both LSs did end up reaching a similar relatively low immobilized enzyme activity value with the addition of the PEI crosslinker. Nonetheless, the aforementioned post-immobilization treatments appeared to have provided further thermal stabilization of LSs, particularly the Relizyme™ EP403/IDA-Cu immobilized *V. natriegens* LS2 treated with high pH. Similar to their free forms, the immobilized LSs favored the transfructosylation reactions that occurred at a rapid rate. The Relizyme™ EP403/IDA-Cu immobilized *V. natriegens* LS2 was able to catalyze the production of the highest lactosucrose amount with all substrate combinations lactose/sucrose, WP/sucrose, and MP/sucrose. Therefore, *V. natriegens* LS2 was the LS of choice for the optimization of the lactosucrose synthesis. Predictive models were developed for the biotransformation WP/sucrose systems, catalyzed by free and immobilized LS2, and revealed the significance of reaction time and substrate ratio in modulating the reaction selectivity. Finally, the Relizyme™ EP403/IDA-Cu immobilized *V. natriegens* LS2 afforded the additional advantage of reusability where it was successfully reused.

CHAPTER V. GENERAL CONCLUSION AND FUTURE WORK

Functional carbohydrate (e.g. lactosucrose) production by free and immobilized levansucrase (LS) from dairy by-products has been the main objective of the present research. Previous work in our laboratory involved genome mining via a BLAST (basic local alignment search tool) analysis where 50 strains of LSs were clones and transformed into *Escherichia coli* BL21 before assessing their transfructosylation activities and thermal stabilities. Among these LSs, LS1 from *Gluconobacter oxydans*, LS2 from *Vibrio natriegens* (NBRC 15636), LS3 from *Novosphingobium aromaticivorans*, and LS4 from *Burkholderia graminis* were selected for the purposes of this research.

The first part of the research focused on assessing the catalytic efficiency, reaction specificity (hydrolysis/transfructosylation), and end-product profiles of the selected LSs using sucrose and three different lactose sources, including lactose, whey permeate (WP), and milk permeate (MP), as substrates. These biotransformation reactions were found to be occurring at a rapid rate and overall, most of the reaction mixtures were enriched with both lactosucrose and kestose. The *V. natriegens* LS2 appeared to be the most promising candidate as it led to the highest ratio of transfructosylation to hydrolysis particularly in the presence of WP/sucrose substrates. Additionally, *V. natriegens* LS2 was able to produce the highest amount of lactosucrose. *G. oxydans* LS1 was also a promising candidate as it provided overall high ratios of transfructosylation to hydrolysis in the presence of all substrate combinations. Moreover, *G. oxydans* LS1 led to the production of a mixture of lactosucrose and kestose and was the only enzyme that catalyzed the synthesis of levan polymers in lactose/sucrose and MP/sucrose reactions. On the other hand, *N. aromaticivorans* LS3 did not favor lactosucrose production because of its low acceptor specificity towards lactose and ended up primarily producing kestose as well as the highest amounts of nystose and fructosyl nystose. Furthermore, LS-catalyzed biotransformation reactions involving the use of lactosucrose by itself or with sucrose revealed the ability of LSs to use lactosucrose as a fructosyl donor and/or acceptor. *G. oxydans* LS1, *V. natriegens* LS2, and *B. graminis* LS4 were proven to be able to use lactosucrose as a fructosyl acceptor. While *V. natriegens* LS2 used lactosucrose as a fructosyl donor to transfer fructose into lactose and produce lactosucrose.

The second part of the present research focused on the immobilization of *G. oxydans* LS1 and *V. natriegens* LS2 and the assessment of their catalytic efficiency and ability to synthesize

lactosucrose. Enzyme immobilization has been selected as a potential strategy to modulate enzyme selectivity to favor transfructosylation over hydrolysis in addition to increase enzyme stabilization and allow for the reusability of the biocatalyst. Functionalized agarose and epoxy-based supports were investigated as potential supports for the preparation of immobilized LSs. Out of all the tested immobilization supports and functionalizations, the Relizyme™ EP403 support functionalized with iminodiacetic acid (IDA)-Cu led to the highest immobilization protein yields and retained activities for *G. oxydans* LS1 and *V. natriegens* LS2. Furthermore, the post-immobilization treatments were found to decrease the activities with the Relizyme™ EP403/IDA-Cu immobilized *G. oxydans* LS1 being less impacted by high pH incubation than the Relizyme™ EP403/IDA-Cu immobilized *V. natriegens* LS2. However, both LSs did end up reaching a similar relatively low immobilized enzyme activity value with the addition of the polyethylenimine (PEI) crosslinker. Nonetheless, the aforementioned post-immobilization treatments appeared to have provided further thermal stabilization of LSs, particularly the Relizyme™ EP403/IDA-Cu immobilized *V. natriegens* LS2 treated with high pH. Similar to their free forms, the immobilized LSs carried out the biotransformation reactions at a rapid rate and favored transfructosylation over hydrolysis. The Relizyme™ EP403/IDA-Cu immobilized *V. natriegens* LS2 was able to catalyze the production of the highest lactosucrose amount with all the substrate combinations lactose/sucrose, WP/sucrose, and MP/sucrose. Therefore, the second part of research continued with its focus on *V. natriegens* LS2, both in its free and immobilized forms, as the LS of choice for the optimization of lactosucrose synthesis from WP/sucrose. The effects of the biotransformation reaction parameters were investigated using response surface methodology (RSM). Predictive models were developed for the selected biotransformation reactions, revealing the significance of reaction time and substrate ratio in modulating the reaction selectivity towards lactosucrose synthesis. The resulting optimized conditions showed that both a shorter reaction time and lower lactose/sucrose ratio are needed to maximize the selective production of lactosucrose by the free and Relizyme™ EP403/IDA-Cu immobilized *V. natriegens* LS2. Finally, the Relizyme™ EP403/IDA-Cu immobilized *V. natriegens* LS2 afforded the additional advantage of reusability where it was successfully reused.

Considering that *V. natriegens* LS2, both in its free and immobilized forms, resulted in highly favorable lactosucrose synthesis from dairy by-products, it could have a high potential in being scaled up and utilized in industry for lactosucrose production. However, more research needs to

be conducted to further optimize the biotransformation reaction parameters for even greater lactosucrose production using *V. natriegens* LS2. Also, the possibility of immobilizing *V. natriegens* LS2 on novel supports can aid in increasing its stability and reusability while maintaining its high immobilization protein yield and retained activity.

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