DEVELOPMENT OF A BIOCATALYTIC PROCESS FOR THE ENDOGENOUS BIOGENERATION OF FUNCTIONAL INGREDIENTS IN DAIRY PRODUCTS

By

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TABLE OF CONTENTS

ABSTRACT

Due to the promising physiological effects of levan and levan-type fructooligosaccharides, levansucrase (LS, EC 2.4.1.10) has garnered much interest in the food and pharmaceutical industries in recent years. LS is a fructosyl-transferase that can catalyze the synthesis of complex oligosaccharides, by acquiring a fructosyl residue from a donor molecule and performing a non-Leloir transfer to an acceptor molecule. The mechanism of action of LS on various carbohydrates has been well documented, and increasing interest is being drawn to the ability of alkyl and phenolic compounds to act as acceptor substrates for LS-catalyzed transfructosylation reactions. With these advances, the possibility of applying LS in food processing has gradually been gaining momentum.

The first objective of this study was to characterize the acceptor specificity of selected LSs on various carbohydrates, alcohols and phenolic compounds using sucrose as a fructosyl donor. Four LS strains from *Gluconobacter oxydans* (strain 621H) (LS1), *Vibrio natriegens* NBRC 15636 (LS2), *Novosphingobium aromaticivorans* (LS3), and *Paraburkholderia graminis* C4D1M (LS4) were selected. *V. natriegens* LS2 was overall the most efficient biocatalyst for the transfructosylation of phenolic compounds. Catechol, catechin and epicatechin were distinguishably the most versatile acceptors, being also significantly transfructosylated by other LSs like *N. aromaticivorans* LS3 or *P. graminis* LS4. This study also revealed that more than one fructosyl unit could be attached to the glycosylated phenolic compounds. As for carbohydrates, the transfructosylation yield was more dependent on the acceptor type than the LS source. Maltose, cellobiose and lactose successfully acquired a fructosyl group from sucrose. No transfructosylation activity was reported with sorbitol. LS2 was more selective towards the fructosylation of disaccharides, while LS1, LS3 and LS4 simultaneously produced fructosylated trisaccharides and fructooligosaccharides (FOSs). Following the characterization of the acceptor specificity of selected LSs, their ability to generate functional ingredients from dairy products was investigated in the second part of this study. First, the effect of pH and temperature on the transfructosylation of lactose to produce lactosucrose was examined. *V. natriegens* LS2, *N. aromaticivorans* LS3, and *P. graminis* LS4 were found to be promising biocatalysts to endogenously produce functional ingredients in dairy products. LS2 had the highest potential,

with high lactosucrose production even at the pH of milk (pH 6.6) and at a low temperature of 10 °C. LS3 instead favored FOS formation over that of lactosucrose. Finally, *G. oxydans* LS1 was found to be more suitable for moderately acidic food systems. Then the possible effects of enriching reconstituted milk formulations with lactose and cocoa powder on LS-catalyzed reactions were investigated. No significant changes were observed in the reaction selectivity, sucrose conversion, lactosucrose and FOS production with additional cocoa powder. Finally, levan produced from *G. oxydans* LS1 proved to be a potential stabiliser of great interest in chocolate milk production. Less than 1% (w/w) of high molecular weight (HMW) levan or less than 0.5% (w/w) of mixed low and high molecular weight (MIX) levan was sufficient to bring the viscosity of fortified chocolate milk equivalent to that of commercial chocolate milk.

Ultimately, *V. natriegens* LS2 was selected for the optimization of the endogenous biogeneration of lactosucrose in chocolate milk. The following parameters were optimized: enzyme concentration, sucrose concentration and lactose concentration. A three-variable central composite rotatable design was created, and response surface methodology (RSM) was used for formulation optimization. Lactose concentration was found to be the critical factor for the conversion of lactose to lactosucrose, relative transfructosylation extent, colour difference and apparent viscosities at 50 s^{-1}. Sucrose concentration dictated the sucrose conversion to lactosucrose, sucrose conversion to oligolevan/levan, and LS concentration was the most important factor for the lactosucrose production. Finally, the biotransformation parameters to maximize the selectivity of *V. natriegens* LS2 towards lactosucrose synthesis were determined. A bio-transformed chocolate milk, fortified with 21.22 to 35.56 g/L lactosucrose was obtained.

RÉSUMÉ

En raison des effets physiologiques prometteurs du lévan et des fructooligosaccharides de type lévan, la lévansucrase (LS, EC 2.4.1.10) a suscité beaucoup d'intérêt dans les industries pharmaceutique, cosmétique et alimentaire ces dernières années. La LS est une fructosyl-transférase qui peut catalyser la synthèse d'oligosaccharides complexes, en acquérant un résidu fructosylé à partir d'une molécule donneuse et en effectuant un transfert non-Leloir vers une molécule accepteuse. Le mécanisme d'action de la LS envers divers hydrates de carbone a été bien documenté, et un intérêt croissant est porté à la capacité des composés alkyles et phénoliques d'agir en tant que substrats accepteurs pour les réactions de transfructosylation catalysées par la LS. Avec ces avancées, la possibilité d'appliquer la LS dans le traitement des aliments gagne progressivement du terrain.

Le premier objectif de cette étude était de caractériser la spécificité des accepteurs de LS sélectionnés vis-à-vis de divers hydrates de carbone, alcools et composés phénoliques en utilisant le saccharose comme donneur de fructosyl. Quatre souches de LS de *Gluconobacter oxydans* (souche 621H) (LS1), *Vibrio natriegens* NBRC 15636 (LS2), *Novosphingobium aromaticivorans* (LS3) et *Paraburkholderia graminis* C4D1M (LS4) ont été sélectionnées. *V. natriegens* LS2 s'est avérée être le biocatalyseur le plus efficace pour la transfructosylation des composés phénoliques. Le catéchol, la catéchine et l'épicatéchine étaient les accepteurs les plus polyvalents, étant également significativement transfructosylés par d'autres LS tels que *N. aromaticivorans* LS3 ou *P. graminis* LS4. Cette étude a également révélé que plus d'une unité fructosylée pouvait être attachée aux composés phénoliques glycosylés. En ce qui concerne les glucides, le rendement de transfructosylation dépendait du type d'accepteur et non de la source de LS. Le maltose, la cellobiose et le lactose ont réussi à acquérir un groupe fructosylé à partir du saccharose. Aucune activité de transfructosylation n'a été signalée avec le sorbitol. LS2 était plus sélectif envers la fructosylation des disaccharides, tandis que LS1, LS3 et LS4 produisaient simultanément des trisaccharides fructosylés et des fructooligosaccharides (FOSs). Après la caractérisation de la spécificité des accepteurs des LS sélectionnées, leur capacité à générer des ingrédients fonctionnels à partir de produits laitiers a été étudiée dans la deuxième partie de cette étude. Tout d'abord, l'effet du pH et de la température sur la transfructosylation du lactose

pour produire du lactosaccharose a été examiné. *V. natriegens* LS2, *N. aromaticivorans* LS3 et *P. graminis* LS4 se sont avérés être des biocatalyseurs prometteurs pour produire endogènement des ingrédients fonctionnels dans les produits laitiers. LS2 avait le plus grand potentiel, avec une production élevée de lactosaccharose même au pH du lait (pH 6,6) et à une température basse de 10 °C. LS3 favorisait plutôt la formation de FOSs par rapport à celle du lactosaccharose. Enfin, *G. oxydans* LS1 s'est révélé être plus adapté aux systèmes alimentaires modérément acides. Ensuite, les effets possibles de l'enrichissement des formulations de lait reconstitué avec du lactose et de la poudre de cacao sur les réactions catalysées par LS ont été étudiés. Aucun changement significatif n'a été observé dans la sélectivité de la réaction, la conversion du saccharose, la production de lactosaccharose et de FOS avec l'ajout de poudre de cacao. Enfin, le lévan produit à partir de *G. oxydans* LS1 s'est révélé être un stabilisateur potentiel d'un grand intérêt dans la production de lait au chocolat. Moins de 1 % (w/w) de lévan de poids moléculaire élevé (HMW) ou moins de 0,5 % (w/w) de lévan de poids moléculaire mixte bas et élevé (MIX) était suffisant pour amener la viscosité du lait au chocolat fortifié à un niveau équivalent à celui du lait au chocolat commercial.

Finalement, *V. natriegens* LS2 a été sélectionné pour l'optimisation de la biogénération endogène de lactosaccharose dans le lait au chocolat. Les paramètres suivants ont été optimisés : concentration en enzyme, concentration en saccharose et concentration en lactose. Un plan central composite à trois variables a été créé, et la méthodologie de surface de réponse (RSM) a été utilisée pour l'optimisation des formulations. La concentration en lactose s'est avérée être le facteur critique pour la conversion du lactose en lactosaccharose, l'extension relative de transfructosylation, la différence de couleur et les viscosités apparentes à 50 s^{-1}. La concentration en saccharose dictait la conversion du saccharose en lactosaccharose, la conversion du saccharose en oligolévan/lévan, et la concentration en LS était le facteur le plus important pour la production de lactosaccharose. Enfin, les paramètres de biotransformation pour maximiser la sélectivité de *V. natriegens* LS2 envers la synthèse de lactosaccharose ont été déterminés. Un lait au chocolat bio-transformé, enrichi de 21.22 à 35.56 g/L de lactosaccharose, a été obtenu.

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STATEMENT FROM THE THESIS OFFICE

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As manuscripts for publication are frequently very concise documents, where appropriate, additional material must be provided in sufficient detail to allow a clear and precise judgement to be made of the importance and originality of the research reported in the thesis.

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PREFACE AND CONTRIBUTION OF AUTHORS

This thesis consists of the following six chapters:

CHAPTER I provides a short introduction and describes the research rationale as well as the main and specific objectives of the study.

CHAPTER II is a literature review that begins by providing a brief overview of the microbial production of levansucrases. It then focuses on their catalytic properties and explores the various LS-catalyzed reaction products. Finally, a comprehensive discussion of its applications in food systems is presented.

CHAPTER III reports the acceptor specificity of selected levansucrases towards various carbohydrates, alcohols, and phenolic compounds. The reaction time courses of each acceptor substrate are described, and their corresponding end-product profiles are characterized.

CHAPTER IV focuses on the application of levansucrases in dairy products for the endogenous biogeneration of functional ingredients. It first describes the effect of pH and temperature on the transfructosylation of lactose. Then, the application of levansucrase to sweetened milk and chocolate milk is demonstrated. Finally, the potential of levan as a stabilizer in chocolate milk is depicted.

CHAPTER V presents the optimization results of the most promising biocatalytic process in chocolate milk, using *Vibrio natriegen* levansucrase. The critical factors affecting the physical properties, reaction selectivity and end-product profile are identified. At last, the results of the predictive models, which were used to maximize the selectivity of *V. natriegens* LS towards lactosucrose synthesis, are illustrated.

CHAPTER VI provides a general conclusion to the thesis with a summary of major findings.

Muriel Yok Kam Wong Min, the author, was responsible for the experimental work and the preparation of the first draft of the manuscripts for publication.

Dr Salwa Karboune, the MSc student's supervisor, guided and supervised all research and critically revised the manuscripts prior to their submission

LIST OF FIGURES

CHAPTER II

CHAPTER III

CHAPTER IV

CHAPTER V

LIST OF TABLES

CHAPTER II

CHAPTER III

Supplementary Table 1. Time course for the conversion of sucrose and substrate with carbohydrates as acceptor substrates.

CHAPTER IV

Table 4.1. Power law parameters for levan-enriched chocolate milk at selected temperatures

Supplementary Table 4.1. Effect of pH and temperature on levan/oligolevan production

Supplementary Table 4.2. Formulations of reconstituted milk

Supplementary Table 4.3a. Effect of milk components and cocoa powder on sucrose conversion and reaction selectivity with *G. oxydans* LS1

Supplementary Table 4.3b. Effect of milk components and cocoa powder on sucrose conversion and reaction selectivity with *V. natriegens* LS2

Supplementary Table 4.3c. Effect of milk components and cocoa powder on sucrose conversion and reaction selectivity with *N. aromaticivorans* LS3

Supplementary Table 4.3d. Effect of milk components and cocoa powder on sucrose conversion and reaction selectivity with *P. graminis* LS4

CHAPTER V

Table 5.1. Experimental design factors and responses of end-product profile characterization and reaction selectivity

Table 5.2. Analysis of variance (ANOVA) for the end-product profile characterization and reaction selectivity. All reactions maintained the same temperature (10 °C) and reaction time (6 h).

Table 5.3. Experimental design factors and responses of pH and color measurements

Table 5.4. Power law parameters for chocolate milks at selected temperatures

Table 5.5. Analysis of variance (ANOVA) for the color difference and rheological properties. All reactions maintained the same temperature (10 °C) and reaction time (6 h).

Table 5.6. Responses of the optimal conditions for lactosucrose production with *V. natriegens* LS in chocolate milk

Supplementary Table 5.1. Experimental design factors

NOMENCLATURE/LIST OF ABBREVIATIONS

BLAST	Basic local alignment search tool
BSA	Bovine serum albumin
DNS	3,5-Dinitrosalicylic acid
EDTA	Ethylenediaminetetraacetic acid
FOSs	Fructooligosaccharides
FOSHU	Food for Specified Health Uses
HPAEC	High-pressure anion-exchange chromatography
HPLC	High-pressure liquid chromatography
IPTG	β-D-isothiogalactopyranoside
LB	Lysogeny broth
PAD	Pulsed amperometric detector
Q-TOF – MS	Quadrupole Time of Flight Mass Spectrometer
RPM	Rotations per minute
RSM	Response surface methodology
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
TB	Terrific broth

CHAPTER I. GENERAL INTRODUCTION

The rising interest in prebiotics due to their various health benefits is evident. The global prebiotics market size was valued at USD 6.05 billion in 2021 and is expected to grow at a compound annual growth rate (CAGR) of 14.9% till 2030 (Grand View Research, 2022). Levansucrase (LS, EC 2.4.1.10) is a fructosyl-transferase, capable of catalyzing the synthesis of a diverse range of fructose-based products with prebiotic properties such as levan, fructooligosaccharides (FOSs) and lactosucrose trisaccharides (Hill et al., 2020). These products are formed when LS acquires a fructosyl residue from a donor molecule and performs a non-Leloir transfer to an acceptor molecule. LS can catalyze four types of reactions: polymerization, oligomerization, transfructosylation, and hydrolysis (Hill et al., 2019; Li et al., 2015). The product spectrum of LSs from diverse microbial sources and their catalytic properties are greatly dependent on their microbial source (Hill et al., 2019). Significant interest has been drawn to understanding and improving the reaction mechanism and selectivity (transfructosylation/hydrolysis) of LS to enhance the production of these valuable prebiotic compounds. Efforts have been directed towards three main strategies: discovering new LSs, modifying the amino acid sequences of LS by direct mutagenesis or fine-tuning the reaction conditions (Ortiz-Soto et al., 2017; Visnapuu et al., 2015).

Besides carbohydrate acceptor substrates, alkyl and phenolic compounds have recently emerged as potential fructosyl acceptors of LS-catalyzed reactions (Núñez-López et al., 2019). Phenolic compounds are prized for their antitumor, antioxidant, antibacterial, and anti-inflammatory properties, while also showing great potential in preventing and treating cardiovascular or cerebrovascular diseases (Manach et al., 2005; Xu et al., 2016). The enzymatic glycosylation of phenolic compounds is indeed seen as an attractive means to change their aqueous solubility, stability, and bioavailability. It is an interesting alternative to chemical glycosylation that requires multistep synthetic routes and results in low overall yields (Desmet et al., 2012; Zhu & Schmidt, 2009).

With the advanced developments in enzyme technology, the focus of LS-related studies has in recent years been expanding to the possibility of applying this enzyme in food processing. LS-reaction products are not only prized for their health-promoting effects but also for their ability to act as techno-functional ingredients. In bakery products, levan has brought along the

benefits of improving bread texture and retarding staling (Jakob et al., 2013; Jakob et al., 2012). In dairy products like yogurt, levan has been shown to increase water-holding capacity and system stability (Xu et al., 2022). Films based on levan, FOS or nystose have demonstrated their capabilities to increase the quality and shelf-life of food products (Bersaneti et al., 2021; Mantovan et al., 2018). Lastly, LS application has brought a new approach to agro-industrial by-product valorization. The production of lactosucrose from whey permeate has proven to be feasible (Bahlawan & Karboune, 2022). Further exploration in the domain of application of LS in food processing is no doubt to follow, given the seemingly endless possibilities of applying this enzyme in diverse food systems.

The first main objective of this research work was to characterize the acceptor specificity of selected LSs, towards phenolic substrates and disaccharides. Four LS strains from *Gluconobacter oxydans* (strain 621H) (LS1), *Vibrio natriegens* NBRC 15636 (LS2), *Novosphingobium aromaticivorans* (LS3), and *Burkholderia graminis* C4D1M (LS4) were selected based on the genome mining carried out in our previous work (Hill et al., 2019). Then, the investigation of the biogeneration of functional ingredients in dairy products was carried out. Chocolate milk was selected for its high sugar content and interesting phenolic compound profile.

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

1. Characterization of the acceptor specificity of selected levansucrases
    1.1. Study the acceptor specificity of selected levansucrases using sucrose as a donor and selected carbohydrates (e.g., lactose, cellobiose), alcohols (e.g., sorbitol) and phenolic compounds (e.g., catechin, epicatechin) as acceptors.
    1.2. Determine the reaction time courses for the acceptor reactions and characterization of the end-product profiles by HPLC, HPAEC-PAD and MS-ion mobility-QTOF.
2. Investigation of the endogenous biogeneration of functional sweeteners and stabilizers in dairy products.
    2.1. Evaluate the effect of pH and temperature on the transfructosylation of lactose.
    2.2. Examine the application of levansucrase to reconstituted sweetened milk and chocolate milk formulations.

2.3. Assess the potential of levan in chocolate milk as a stabilizer.

3. Optimization of the endogenous biogeneration of lactosucrose by *Vibrio natriegens* levansucrase in chocolate milk.

3.1. Optimize the concentrations of levansucrase, sucrose and lactose.

3.2. Assess the pH, colour difference, rheological properties, reaction selectivity and lactosucrose production.

3.3. Use predictive models to maximize the selectivity of *V. natriegens* LS towards lactosucrose synthesis in chocolate milk.

CHAPTER II: LITERATURE REVIEW

LEVANSUCRASES: A REVIEW OF THEIR CATALYTIC PROPERTIES, PRODUCT CHARACTERIZATION AND APPLICATIONS IN FOOD SYSTEMS

2. Abstract

Due to the promising physiological effects of levan and levan-type fructooligosaccharides, levansucrase (LS, EC 2.4.1.10) has garnered much interest in recent years for pharmaceutical, cosmetic and food applications. LS is a fructosyl-transferase that can catalyze the synthesis of complex oligosaccharides, by acquiring a fructosyl residue from a donor molecule and performing a non-Leloir transfer to an acceptor molecule. The mechanism of action of LS toward various carbohydrates has been well documented, and increasing interest is being drawn to the ability of alkyl and phenolic compounds to act as acceptor substrates for LS-catalyzed transfructosylation reactions. Many studies have also focused on improving the production of LSs and their product spectrum, whether by increasing their overall activity or by shifting their selectivity towards a particular reaction. With these advances, the possibility of applying LS in food processing has gradually been gaining momentum. This review provides a comprehensive discussion of the catalytic properties of LS and a full characterization of its reaction products. It then discusses some applications of microbial LS for the biogeneration of functional ingredients in food systems.

2.1. Introduction

Glycosyltransferases catalyze the transfer of a glycosyl group from a donor to an acceptor molecule. They are important in the biosynthesis and degradation of numerous biological compounds including polysaccharides, oligosaccharides, saponins, antibiotics, glycolipids, glycoproteins, proteoglycans, and peptidoglycans (Zechel & Withers, 1999). Levansucrase (LS, EC 2.4.1.10) is a glycosyltransferase, more specifically a fructosyltransferase. LS can be expressed in many microorganisms that produce levan as an energy reserve or/and a structural component for defence (Benigar et al., 2014; Daudé et al., 2012). LS transfers a fructosyl residue from a donor molecule to an acceptor molecule via β-(2→6)-glycosidic linkages, performing a non-Leloir transfer (Hill et al., 2019). LS has been demonstrated to synthesize a wide range of products with prebiotic properties, such as levan and various fructooligosaccharides (FOSs). Prebiotics have received much attention in recent years for their effects on the gut microbiome. Indeed, it is now firmly established that the gut microbiome has an enormous impact on human health and that the microbiome composition and functions are greatly affected by one's diet (Ercolini & Fogliano, 2018). A prebiotic was first defined as a 'non-digestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon, and thus improves host health' (Gibson & Roberfroid, 1995). This definition was later revised to a prebiotic being 'a selectively fermented ingredient that allows specific changes, both in the composition and/or activity in the gastrointestinal microflora that confers benefits upon host wellbeing and health'(Gibson et al., 2004).

From a mechanistic perspective, LS can catalyze four types of reactions: polymerization, oligomerization, transfructosylation, and hydrolysis, as depicted in Fig 2.1. (Li et al., 2015). During polymerization and oligomerization, sucrose acts as both the acceptor and donor substrate forming levan and FOSs respectively. During polymerization, the growing fructan chain acts as the acceptor, synthesizing β-(2,6) linked oligofructans to form or elongate levan. If this processive reaction mechanism is not adopted, FOSs are instead formed via a non-processive/distributive reaction given the enzyme's lack of affinity for the synthesized product (Caputi et al., 2013; Öner et al., 2016; Strube et al., 2011). Transfructosylation involves other saccharides acting as reaction substrates. For instance, glucose as an acceptor leads to the synthesis of sucrose or blastose,

while the reaction with fructose forms inulobiose or levanbiose (Li et al., 2015). Recent studies have also introduced alkyl and phenolic compounds as promising fructosyl acceptors (Núñez-López et al., 2019). Finally, water is the acceptor molecule during hydrolysis, releasing glucose and fructose (Li et al.,2015; Öner et al., 2016).

LS is a very complex enzyme. Its optimal conditions for production and catalytic properties are greatly dependent on its microbial source. As such, researchers have explored the potential of LSs produced from a multitude of sources, optimizing their production, investigating their reaction mechanism and reaction selectivity, and expanding knowledge of their substrate specificity. Advanced developments in enzyme technology have also opened doors for the application of LS in food processing. Microbial enzymes in particular have been favored over animal or plant sources for being economical and allowing consistent production (Ramli et al., 2022). The benefits of enzymes in food processing range from enabling high consistent production yields of safe and high-quality food products, to being an alternative to environmentally hazardous chemical processes with multistep synthetic routes (Mishra et al., 2017). With LS-catalyzed reactions offering a diversity of end products, the usage of LS in the food industry could cater to produce ingredients with both health and techno-functional properties.

Although LSs have been extensively studied over the years, to the authors' knowledge, few reviews have been published regarding the substrate specificity and application of LSs, and none are specifically targeted to food applications (Hill et al., 2016; Li et al., 2015; Öner et al., 2016; Xu et al., 2019). This review will begin by providing a brief overview of the microbial production of LSs. It will then focus on their catalytic properties and explore the various LS-catalyzed reaction products. Finally, a comprehensive discussion of its applications in food systems will be presented.

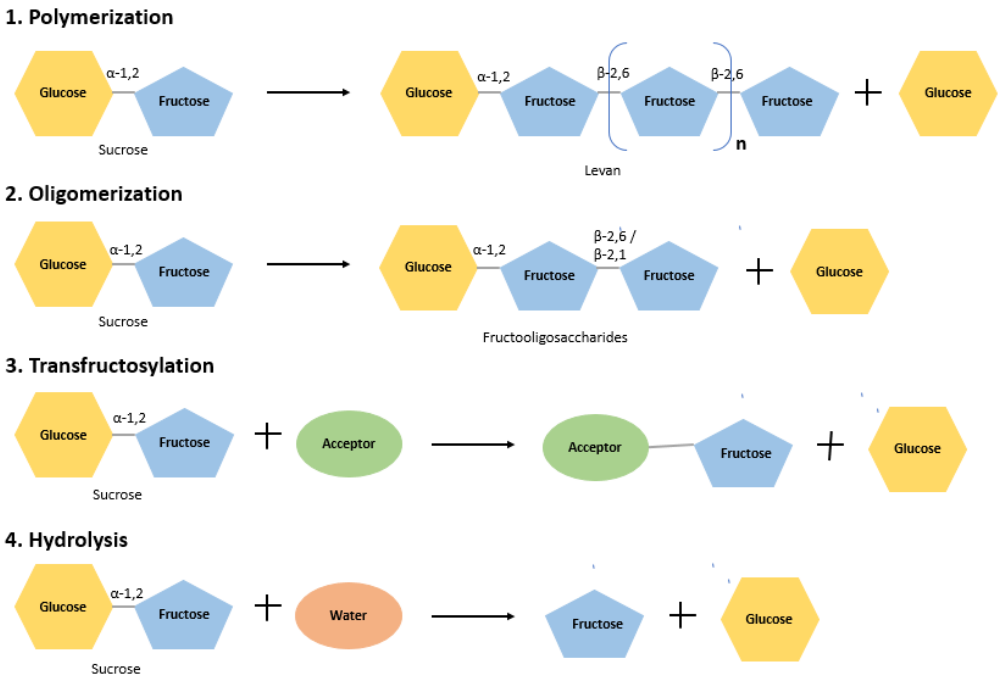


Fig 2.1. Types of reactions catalysed by levansucrase

2.2. Microbial production of Levansucrase

LSs can be expressed by diverse microorganisms, whether from gram-positive or gram-negative bacteria. The list even extends to anaerobic bacteria or those producing halophilic enzymes. The first characterized LS isolated from an anaerobic bacterium was from *Clostridium acetobutylicum*, and the full characterization of the first halophilic GH-J clan enzyme was a LS from *Halomonas smyrnensis* AAD6^T (Gao et al., 2017; Kirtel et al., 2018). Microbial production of LS is affected by various factors: the carbon source, the nitrogen source, the medium's pH and temperature, and the presence of metal ions (Li et al., 2015).

For some microorganisms, the carbon source is essential to produce a reasonable yield of LS. Sucrose is usually the most effective carbon source. As an example, for *Bacillus sp.,* it was found that sucrose is the best inducer for cell growth and LS activity rates, compared to glucose and fructose (Belghith et al., 2012). Belghith et al. (2012) also found that 300 g/L of sucrose was ideal for maximum LS production. Another study showed that sucrose was the carbon source of choice among sucrose, fructose, glucose, glycerol and raffinose for producing LS from *Geobacillus stearothermophilus*. Glucose, fructose, and sucrose-containing media had the highest microbial growth, and the media with sucrose had the highest LS activity. The addition of sucrose to glycerol and glucose also increased the enzyme activity by 2-fold (Inthanavong et al., 2013). Sucrose also resulted in the highest LS production from *Klebsiella sp.* strain L1, compared to glucose, galactose, lactose or maltose as a carbon source (Desai & Patel, 2019). On the other hand, LS production by *Bacillus subtilis* NRC 33a was not significantly different using sucrose or glucose, with resulting LS activity of 14.5 and 14.1 U/mL, respectively. 10% glucose was however chosen in that study to produce LS free of levan, avoiding problems of high viscosity of the culture broth (Abdel-Fattah et al., 2005). In another instance, with LS from *Acetobacter diazotrophicus*, glycerol, followed by sucrose, significantly increased bacterial growth while sorbitol and mannitol enhanced LS activity (Hernández et al., 1999). Optimization of LS production from *B. subtilis* NRC revealed that 5 g/L of starch was the optimal carbon source. Other carbon sources tested included arabinose, glucose, lactose, maltose, xylose, fructose, and cellulose (Salama et al., 2019).

The nitrogen source is also a crucial factor for LS production. Abdel-Fattah et al., (2015) evaluated various nitrogen sources: soybean, corn steep-liquor, baker's yeast, wheat bran, peptone, yeast extract, casein urea and ammonium sulfate. Baker's yeast with a 0.02% nitrogen concentration was the ideal nitrogen source for *B. subtilis* NRC33a, leading to the highest LS activity of 17.5 U/mL (Abdel-Fattah et al., 2005). Baker's yeast was also preferred over peptone, soybean, urea, and ammonium sulfate for *B. subtilis* NRC LS (Salama et al., 2019). In another study, yeast extract was found to be the best nitrogen source for *Bacillus sp.* LS while ammonium sulfate and ammonium nitrate limited enzyme production. The effect of tryptone, casein, urea and sodium nitrate were also analyzed (Belghith et al., 2012). Similarly, yeast extract was selected for *Klebsiella sp.* strain L1 LS production over peptone, beef extract, tryptone, and $(NH_4)_2SO_4$ (Desai & Patel, 2019). In contrast, a medium comprised of peptone or tryptone outperformed yeast extract supplementation for LS production by *G. stearothermophilus* (Inthanavong et al., 2013). All three nitrogen sources increased microbial growth and LS production. However, compared to peptone and tryptone, yeast extract did not reduce the lag phase of microbial growth and produced a low level of intracellular LS. A severe decline in LS production was nevertheless recorded earlier with the peptone-containing media at 8-9 h, while only after 16 h of culture was a decline observed in the yeast and tryptone-supplemented media. The inhibitory effect of ammonium sulfate was also reported (Inthanavong et al., 2013). Finally, *A. diazotrophicus* LS production and bacterial growth were not much affected by tryptone and yeast extract supplementation while NH_4Cl reduced bacterial growth three-fold (Hernández et al., 1999).

The pH of the fermentation medium also has a notable impact on LS production. An optimal pH range of 5.0 to 6.5 usually applies for most LS. With LS from *Bacillus sp.*, an initial pH of 6.5 resulted in the highest enzyme activity (Belghith et al., 2012). Desai & Patel (2019) reported an optimum pH of 5.0 for *Klebsiella sp.* strain L1 LS production. Interestingly, *A. diazotrophicus* LS production had the highest yield at pH 8.0 though optimal growth occurred under acidic conditions. The enzyme was highly stable between pH 4.0 to 8.0 (Hernández et al., 1999). Then, the optimal temperature also varies depending on the microbial source. 50 °C was optimal for *Bacillus sp* LS which retained 100% of its initial activity for more than 1 h (Belghith et al., 2012).

LS from *Klebsiella sp.* strain L1 had the highest production yield at 40 °C (Desai & Patel, 2019). Yet, for LS from *A. diazotrophicus,* enzyme production was the highest at 30 °C and bacterial growth was inhibited at temperatures above 35 °C (Hernández et al., 1999).

Lastly, supplementation of the medium with an ion source can in addition affect LS production. Addition of $MgSO_4$ was the most favorable for *B. subtilis* NRC 33a and *Bacillus circulans,* at 0.15 g/L and 0.2 g/L respectively (Abdel-Fattah et al., 2005; Oseguera et al., 1996). Abdel-Fattah et al. (2005) also found that 5.0 g/L of K_2HPO_4 was ideal for producing extracellular LS, while 3 g/L was preferred for LS production from *Erwinia herbicola* (Han & Clarke, 1990). For *Bacillus sp* LS, 50 mM Fe^{2+} increased the enzyme's activity by about four times the control, while 100 mM Mg enhanced the activity by three times. Zn^{2+}, Cu^{2+}, SDS and DTT on the other hand decreased the LS activity (Belghith et al., 2012). $AlCl_3$ improved the productivity of LS from *B. subtilis* NRC (Salama et al., 2019). Finally, NaCl at 2% had a noteworthy impact on bacterial growth and LS production from *Klebsiella sp.* strain L1 (Desai & Patel, 2019).

2.3. Mechanism of action and Reaction selectivity of Levansucrases

LSs belong to the glycosyl hydrolase (GH) family 68 (http://www.cazy.org) and consist of a 5-fold β-propeller topology with four anti-parallel strands (Lombard et al., 2013). They are β-retaining enzymes operating via a ping-pong type mechanism, which is a double displacement mechanism involving the formation and hydrolysis of a covalent glycosyl–enzyme intermediate (Hernandez et al., 1995). Although LSs are known for their conserved active site, they differ significantly in terms of kinetic properties, biochemical properties, and resulting products. The difference in reaction products is attributed to the enzyme's catalytic mechanism: processive or distributive. While in a processive mechanism, the product remains bound to the enzyme after a catalytic step, in a distributive (non-processive) mechanism, the intermediate product separates from the enzyme after each step of catalysis (Caputi et al., 2013). The structural determinants outside the core active site structure are believed to dictate the mechanism adopted by an enzyme (Anwar et al., 2012; Ozimek et al., 2006). Sucrose first binds in the −1 and + 1 subsites; the fructosyl moiety occupies the −1 subsite while the glucosyl unit occupies the + 1 subsite. A covalent fructosyl-enzyme intermediate with nucleophile D86 is then formed following glycosidic

linkage cleavage. The reaction is completed with the nucleophilic attack of the acceptor substrate onto this intermediate, eventually leading to the synthesis of new products such as FOSs (Meng & Fütterer, 2003). It was suggested that the docking of acceptor molecules could be variable because of the +1 subsite's relaxed binding nature. The +1 subsite participates in both donor and acceptor binding (Meng & Fütterer, 2008; Ozimek et al., 2006; Visnapuu et al., 2011).

Meng & Fütterer (2003) have found three amino acids, Asp86, Glu342 and Asp247, in the central pocket, essential for catalysis in *B. Subtilis* LS. It has been suggested that Asp86 functions as the nucleophile, Glu342 as a general acid and Asp247 stabilizes the transition state (G. Meng & Fütterer, 2003). Similarly, the catalytically active triad for *Erwinia amylovora* LS consists of Asp46, Asp203 and Glu287, for *Brenneria* sp. EniD312 LS are residues Asp68, Asp225 and Glu309, for *Bacillus megaterium* LS are amino acids Asp95, Glu352 and Asp257 and for *Brenneria* sp. EniD 312 LS are Asp68, Asp225 and Glu309 (Strube et al., 2011; Wuerges et al., 2015; Xu et al., 2022; Xu et al., 2018). Most fructansucrases are made up of a single catalytic domain. Few of them from gram-positive bacteria, particularly those from the Lactobacillales order, have however been shown to be multidomain. With an N-terminal domain, a conserved catalytic domain, and a C-terminal domain. The role of these additional domains has recently been investigated by García-Paz et al. (2022) on *Leuconostoc mesenteroides* B-512F LS. They found that the N-terminal region is mostly essential for stability. The N- and C-terminal domain conjunctions are vital for stability, specificity, and polymerization processes. Lastly, the transition region of the C-terminus is essential for the transfructosylation and polymer elongation (García-Paz et al., 2022).

During polymerization, high molecular weight, differently sized polysaccharides are produced. The high degree of polymerization of levan results from the growing fructan chain that stays bound to the enzyme as it is elongated via a processive reaction mechanism (Ozimek et al., 2006). Upon depletion of the original substrate sucrose, the produced polymer acts as the fructosyl donor. The β-(2,6) linkages of the levan chain are then cleaved by LS in an exo-type manner. The terminal fructose is constantly released until a branching point is reached and the reaction stops (Méndez-Lorenzo et al., 2015). On the other hand, during oligomerization, a non-processive/distributive reaction occurs given the enzyme's lack of affinity for the product. This results in shorter FOSs as the transfructosylated product is released without the formation of a

fructan chain (Caputi et al., 2013; Strube et al., 2011). A recent study on the non-processive levan elongation mechanism identified five substrate-binding subsites, −1, +1, +2, +3, and +4, in the presence of a levanhexaose molecule in the central catalytic cavity. The topological differences in these subsites play a significant role in the product specificities of the Bacillaceae family and gram-positive LSs, regarding the size of short FOSs and levan. An additional oligosaccharide-binding site 20 Å away from the catalytic pocket, that could be involved in the elongation mechanism, was also identified (Raga-Carbajal et al., 2021). Besides sucrose, other compounds can also be used as fructosyl acceptors due to the flexibility of the +1 subsite (Visnapuu et al., 2011). LS can catalyze the transfructosylation of various saccharides, alkyl and phenolic compounds, producing sucrose analogues, fructosylated trisaccharides, alkyl and phenolic fructosides (Li et al., 2015; Núñez-López et al., 2019). The acceptor specificity of LS will be covered in detail, in later sections of this review.

The difference in reaction selectivity depends on the microbial source. Generally, LS from gram-positive bacteria processes via a processive mechanism, producing levan. In contrast, gram-negative bacteria produce oligofructans, that undergo a non-processive reaction. They consequently have a lower levan yield (Öner et al., 2016). However, given the seemingly identical active-site architecture of LSs when analyzing their three-dimensional structures, it is unclear which structural features exactly dictate whether polymerization or oligomerization is favored by a LS (Ozimek, Kralj, van der Maarel, & Dijkhuizen, 2006). Hence, some researchers have been focusing on the amino acid sequences of the enzyme instead.

Arg360 in *B. Subtilis* LS, as well as Arg370 and Asn252 in *B. megaterium,* have been shown to be essential for levan synthesis (Homann et al., 2007; Meng & Fütterer, 2003). Okuyama et al. (2021) identified Asn84 and Ser345 to determine the regioselectivity of *Zymomonas mobilis* LS. Furthermore, Phe189 was proposed to be responsible for the chain length of levan (Okuyama et al., 2021). LS from gram-negative bacteria *Gluconacetobacter diazotrophicus* which contains a histidine instead of Arg370 at the equivalent position (419) favors FOS formation (Homann et al., 2007). His 305 in *E. amylovora* LS is responsible for the enzyme's product length (Wuerges et al., 2015). Wuerges et al. (2015) also proposed that the amino acids of loop 8 in *G. diazotrophicus* LS and *Microbacterium saccharophilum* β-fructofuranosidase play a role in the product spectrum of

these two enzymes. For *Brenneria* sp. EniD312 LS the residues in positions 154 and 327 were found to be significant factors that determine the ratio of hydrolysis to transfructosylation activity (Xu et al., 2018). For *Bacillus licheniformis* 8-37-0-1 LS, residues Tyr246, Asn251, Lys372, and Arg369, found on the surface of the substrate-entering channel of the enzyme, proved to play a significant role in polymerization and product linkage specificity (Kirtel et al., 2018). For *Sphingobium chunbukense* DJ77 LS, Arg 77, Ser112, Arg 195, Asp196, Glu257, and Gln275 participate in sucrose binding, splitting and transfructosylation reaction. Interestingly, Gln275 was suggested to accommodate the enzymes for a broad pH resistance (pH 5-10) by coordinating a favorable substrate binding environment (Le et al., 2018).

Other studies have subsequently been focusing on modifying the amino acid sequence to shift or promote LS activity towards a particular reaction to obtain the desired reaction product. For instance, Ortiz-Soto et al (2017) modified the nucleophile-coordinating network to increase *B. megaterium* LS's transferase activity. More specifically, variants of Ser173, Tys421 and Ser422 enhanced transfructosylation and altered the product spectra (Ortiz-Soto et al.,2017). N251A and N251Y mutations in the active site of *B. licheniformis* RN-01 LS disrupted the polymerization activity of the enzyme. Hence, this information could be used to design new LS favoring levan oligosaccharides production (Sitthiyotha et al., 2018). Modifications at position Arg370 and Lys373 altered the FOS product profile of *B. megaterium* LS (Possiel et al., 2019). Furthermore, residues around loop 1, loop 3, and loop 4 of LS from *E. amylovora* were modified producing mutants of G98E, V151F, and N200T which significantly increase molecular mass and yield of high-molecular-mass levan (Zhang et al., 2023).

Reaction selectivity is also greatly dependent on initial reaction conditions. A temperature of 50–60 °C usually favors sucrose hydrolysis, while lower temperatures of 10–40 °C and high initial sucrose concentration of 300 mM or more, mainly result in polymerization and/or transfructosylation (Chambert & Gonzy-Treboul, 1976; Vigants et al., 2013; Visnapuu et al., 2015). Some exceptions, however, exist since optimum temperature ranges vary depending on the enzyme source. For instance, LS from *Brenneria goodwinii* had optimum temperatures for transfructosylation, sucrose hydrolysis, and total activity of 35, 45, and 40 °C, respectively. High sucrose concentration also favored polymerisation (Liu et al., 2017). Furthermore, high

temperature and high sucrose concentration favored oligomerization of LS from *Z. mobilis*, while polymerization was favored when the reversed conditions were applied (Santos-Moriano et al., 2015). LS from *Pseudomonas orientalis*, with maximum activity at 65 °C, produced mainly high molecular weight levan, but as the temperature was reduced to 35 °C, favored FOS production (Guang et al., 2023). As sucrose nears depletion, hydrolysis was found to be predominant, unless NaCl and organic solvents were added to create a water-restricted environment promoting transfructosylation (Castillo & López-Munguía, 2004; Chambert & Petit-Glatron, 1989).

The optimum pH of LS is usually around 5–7 and in general, pH changes do not affect the rate of hydrolase and transferase activities (Homann et al., 2007; Visnapuu et al., 2015). Yet, like temperature, the effect of pH on reaction selectivity varies for different LSs. For instance, transfructosylation was favoured over hydrolysis within the pH range of 6-6.5 (Inthanavong et al., 2013). Enzyme concentration has been shown to affect the synthesized levans' molecular weight, with low enzyme concentration resulting in a high normal molecular weight distribution by LS from *B. subtilis* 168 (Porras-Domínguez et al., 2015). The same results were confirmed by another study and revealed that substrate concentration and enzyme activity did not affect the elongation mechanism of *B. subtilis* SacB (Raga-Carbajal et al., 2015). Enzyme cofactors also affect reaction selectivity. The presence of 2.5 mM Mn^{2+} ions resulted in a 100% increase in transferase activity while 0.5 mM of Fe^{3+} ions increased the hydrolytic activity by four times of LS from *B. subtilis* DSM 347 (Szwengiel et al., 2016). For LS from *Bacillus methylotrophicus* SK21.002, Cu^{2+}, Fe^{2+}, Zn^{2+}, and Ni^{2+} inhibited transfructosylation and hydrolysis, Mn^{2+} only disrupted hydrolysis, while Ca^{2+} and Mg^{2+} stimulated both transfructosylation and hydrolysis (Li et al., 2015).

2.4. Donor/acceptor specificities of Levansucrases

2.4.1. Fructosyl donor specificity

Sucrose is the most commonly used fructosyl donor in LS-catalyzed reactions. However, some studies have shown that other saccharides can as well act as donor substrates. The list includes raffinose, stachyose and some sucrose analogues. Table 2.1 and Table 2.2 provide some examples with sucrose, raffinose and stachyose as donor substrates. The transfructosylation

action mechanism of *Microbacterium laevaniformans* LS was studied by Kim et al. (2005). Levan and FOS were successfully synthesized from sucrose, raffinose, and stachyose. While more than 50% of activity was recorded with raffinose, stachyose recorded less than 30% of activity. No activity was recorded when other sugars, like D-glucose, D-galactose, cellobiose, or maltose, were used as donors. This suggests that LS cleaves the α-glucose-(1,2)-β-fructose linkage. 1-ketose and nystose containing this linkage were however not cleaved, indicating that only saccharides with sucrose as terminal residues can be hydrolyzed to produce levan (Kim et al., 2005). A thin-layer chromatography analysis also confirmed that raffinose can be used as fructosyl donors in *Bacillus amyloliquefaciens* LS-catalyzed reactions. At the early stage of the reaction, it was mainly levan that was synthesized, but after 36h and 63h of reaction FOSs production was detected (Tian et al., 2011). Similarly, LS from *L. mesenteroides* MTCC 10508 was able to catalyze reactions with raffinose and stachyose, producing melibiose, free fructose and levan with raffinose, while forming manninotriose and free fructose with stachyose (Jadaun et al., 2019).

Sucrose, raffinose, and a mixture of the two oligosaccharides were used as donors in reactions catalyzed by LS from *Vibrio natriegens, Paraburkolderia graminis, Gluconobacter oxydans,* and *Beijerinckia indica subsp. Indica* and *Novosphingobium aromaticivorans* (Hill et al., 2020). Higher bioconversion percentages were overall obtained when raffinose was used as a fructosyl donor, instead of sucrose, for LS from *V. natriegens, N. aromaticivorans,* and *B. indica subsp. Indica.* Different trisaccharides, tetrasaccharides, and pentasaccharides were synthesized when raffinose was used as the sole substrate, i.e., as both the acceptor and donor of fructosyl groups. LS from *P. graminis* also synthesized multiple oligosaccharides using raffinose: a heptasaccharide, two octasaccharides, and a hendecasaccharide (Hill et al., 2020). Furthermore, levan was also formed from raffinose by LS from *Z. mobilis*, releasing a non-catabolized melibiose into the medium (Andersone et al., 2004). Andersone et al. (2004) found that raffinose was the fructosyl donor of choice, over sucrose, resulting in higher reaction velocities, particularly at low substrate concentrations. The levan produced from raffinose had a smaller average molecular mass, higher intrinsic viscosity, and a smaller Huggin's constant than levan from sucrose.

Raffinose was also used in acceptor reactions of LS from *B. subtilis* with L-sugars of L-glucose, L-rhamnose, L-galactose, L-fucose and L-xylose to produce sucrose analogues (Seibel et al., 2006).

Finally, sucrose analogues D-Gal-Fru, D-Xyl-Fru, D-Man-Fru, and D-Fuc-Fru were found to synthesize new FOSs using an affinity-tagged *Lactobacillus reuteri* LS produced by *B. megaterium* (Biedendieck et al., 2007). With D-Man-Fru as substrate, only one mannosyloligofructoside was synthesized. With D-Gal-Fru, D-Xyl-Fru, and D-Fuc-Fru, levan and FOSs were formed, namely, two galactosyloligofructosides, two fucosyloligofructosides, and at least three different xylosyloligofructosides respectively (Biedendieck et al., 2007). It was proven via kinetic and docking studies that the sucrose derivatives bind in a mode similar to sucrose (Seibel et al., 2006). Seibel et al. (2006) also found that D-Gal-Fru is a better substrate than sucrose for LS from *B. subtilis* given the stronger binding forces attributed to Arg360, Tyr411, Glu342, Trp85, Asp247, and Arg24. However, allosucrose with an axial orientation of its 3-OH was an inefficient substrate. This was explained by the binding mode being unfavorable for catalysis given the absence of hydrogen bridges between Arg360 and 3-OH and 2-OH (Seibel et al., 2006).

2.4.2. Acceptor specificity

Various studies have demonstrated the broad substrate specificity of LSs from different microbial sources. Table 2.1, Table 2.2 and Table 2.3 illustrate the acceptor specificity of LS from *Lactobacillus sanfranciscensis, M. laevaniformans, B. subtilis, B. licheniformis, B. circulans, B. amyloliquefaciens, G. Stearothermophilus, B. indica subsp. indica, G. oxydans, N. aromaticivorans P. graminis,* and *V. natriegen* towards saccharides and alcohols. The transferase properties of *B. circulans* LS to different acceptors were investigated by Oseguera et al. (1996). The enzyme had high transferase activity with maltose and galactose, moderate activity with lactose, and methanol, and low activity towards sorbitol and glycerol. No transfructosylation was observed with inositol. It was also suggested that methyl fructoside might have been obtained via alcoholysis when water is replaced by methanol as the nucleophile in the reaction medium (Oseguera et al., 1996). Tieking et al. (2005) identified the different heterooligosaccharides formed in *L. sanfranciscensis* LS-catalyzed reactions. With sucrose as the only substrate, 1-kestose, nystose, and other FOSs with a degree of polymerization of 5 or greater were observed,

while with raffinose as the sole substrate, melibiose, kestose, nystose and some tetra-, penta-, and hexasaccharides were present, with 1^F- β -fructosylraffinose as the major product. When maltose was used as fructosyl acceptors, 1-kestose, erlose and possibly nystose were identified. As for the reaction with maltotriose, the assignment of heterooligosaccharides was unambiguous due to the overly complex mixture formed, but 1^F- β -fructofuranosylmaltotriose was no doubt formed. Then with xylose and arabinose as acceptors, xylsucrose and arabsucrose were present along with fructosylxylsucrose and fructosylarabsucrose being the major products formed respectively. Oligosaccharides with a degree of polymerization of 3 or greater were as well observed (Tieking et al., 2005).

The action mechanism of transfructosylation catalyzed by *M. laevaniformans* LS was investigated by Kim et al. (2005). Melibiose, cellobiose, maltose and lactose recorded the highest transfructosylation activity of above 50% while other sugars such as arabinose, xylose, lactulose, levanbiose and sophorose had less than 30% activity. From the acceptor specificity test, it was deduced that reducing saccharides were better acceptors than non-reducing saccharides. Although their methyl group blocked their reducing group, non-reducing sugars, such as methyl α-D-glucoside and methyl α-D-galactoside, were also able to produce transfer products. Unlike monosaccharides galactose, mannose was not a good acceptor having also a pyranose ring (Kim et al., 2005). This was suggested to be due to the axial hydroxyl group at C2 which caused a steric hindrance around the incoming fructosyl group of the donor molecule. Indeed, equatorial hydroxyl groups at C2 and C3 on chair conformation were found to be essential for acceptors *of Arthrobacter sp.* β-fructofuranosidase (Fujita et al., 1990). Finally, sugar alcohols xylitol, lactitol, and mannitol were shown to be unfavorable acceptors resulting from a marked conformational difference (Kim et al., 2005).

The acceptor and donor specificity of *B. subtilis* LS was determined by Seibel et al. (2006). A wide range of sugars, with varying positions (position 2, 3, 4 and 6) of their hydroxyl groups, were used as acceptors, with sucrose and raffinose as donors, to determine the mechanism of reaction of the enzyme. Acceptor studies at positions 4 and 6 yielded overall good results. Monosaccharides galactose, xylose and fucose all resulted in a conversion yield of about 60% when sucrose was used as a fructosyl donor. Disaccharides isomaltose, maltose and melibiose

had a high transferase activity of around 48% while cellobiose and lactose had a moderate activity of around 32% with donor sucrose. Acceptor studies at positions 2 and 3 however had a significantly lower yield reported. Mannose, 2-deoxy-glucose, allose and 3-ketoglucose had exceptionally low transferase activity, below 6%, while sophorose was not an acceptor. Finally, with raffinose as a donor, L-sugars of glucose, galactose and xylose showed low to moderate transferase activity while rhamnose and fucose had very low activity (Seibel et al., 2006).

Tian & Karboune (2012) also carried out a substrate specificity study of *B. amyloliquefaciens* LS. Sucrose and raffinose were both successfully used as sole substrates, with raffinose having a thermodynamically more favoured reaction than sucrose. When sucrose was used as a donor substrate, disaccharides showed more potential than monosaccharides as acceptors, with transfructosylation of monosaccharides not yielding a quasi-equilibrium state. This might be due to the sucrose analogues formed acting as fructosyl donors. Maltose presence also resulted in the lowest levan production compared to the other saccharides, indicating that maltose binds to the LS with lower energy but higher specificity, compared to FOSs or levan growing chain. The reaction of sucrose as a fructosyl donor and raffinose as an acceptor revealed that raffinose also acted as a donor (Tian & Karboune, 2012).

The product spectrum of *G. Stearothermophilus* LS was characterized with sucrose as the sole substrate, and the substrate specificity was investigated with galactose, lactose, raffinose, and maltose as fructosyl acceptors, and sucrose as a donor by Inthanavong et al. (2013). The limited amount of sucrose analogues formed with galactose, compared to sucrose consumed, suggests a rapid quasi-equilibrium of the reaction and/or the use of Gal-Fru as a donor. As for lactose, the low sucrose conversion was attributed to the possible inhibitory effect of lactose. Similar to previous studies, raffinose was again suggested to act as both a fructosyl acceptor and donor (Inthanavong et al., 2013). Lu et al. (2014) investigated the transglycosylation capability of recombinant *B. licheniformis* 8-37-0-1 LS. When using sucrose as a donor, the enzyme was able to catalyze reactions with various sugars, including galactose, cellobiose, xylose, maltose, lactose, arabinose, and trehalose, and with alcohols isopropanol and 1-pentanol. Only trace glycoside products were detected with alcohols. The fructosyl moiety was added via β-(2→1) glycosidic bonds to all sugar acceptors (Lu et al., 2014).

Finally, Hill et al. (2020) investigated LS from five different microbial sources: *B. indica subsp. indica, G. oxydans, N. aromaticivorans P. graminis,* and *V. natriegen.* The LS with the highest combined acceptor molecules consumed was from *V. natriegens*, followed by *P. graminis, G. oxydans, B. indica subsp. indica,* and *N. aromaticivorans*. LS from *V. natriegens, G. oxydans, P. graminis,* and *B. indica subsp. Indica* consumed much galactose after a few incubation hours, indicating that their Km values towards the monosaccharide are relatively small. Maltose, xylose and lactose were good fructosyl acceptors for all tested LSs. The equatorial position of the C2-OH of xylose was suggested to be the factor that led to its high transferase activity. Both sorbitol and catechol also proved to be efficient acceptors (Hill et al., 2020). Mena-Arizmendi et al. (2011) suggested an inverted relationship between pKa and the ability of Glu 342 to deprotonate the alcohol acceptor's hydroxyl group. However, Hill et al. (2020) found similar transferase activity of sorbitol and catechol despite their differing pKa. They suggested that the additional hydroxyl group of sorbitol might have provided additional stabilization sites with the enzyme's active site.

Table 2.1. Acceptor specificity of levansucrase- di, tri and tetrasaccharides as acceptors

Donor/Acceptor	End-products	Microbial Sources of LS	Bioconversion Yield (%)	Transferase activity Level	References
Sucrose/Maltotriose	1F- β -fructofuranosyl-maltotriose	- *L. sanfranciscensis*		- DNR	- (Tieking et al., 2005)
Stachyose/Stachyose		- *M. laevaniformans*		- +	- (Kim et al., 2005) [d]
Raffinose/Raffinose	β -fructosylraffinose	- *L. sanfranciscensis*		- DNR	- (Tieking et al., 2005)
		- *M. laevaniformans*		- +++	- (Kim et al., 2005) [d]
		- *B. amyloliqueficien*	- 92		- (Tian & Karboune, 2012) [e]
		- *G. stearothermophilus*	- 50		- (Inthanavong et al., 2013) [f]
		- *B. indica subsp. Indica*	- 81		- (Hill et al., 2020) [c]
		- *G. oxydans*	- 93		- (Hill et al., 2020) [c]
		- *N. aromaticivorans*	- 65		- (Hill et al., 2020) [c]
		- *P. graminis*	- 68		- (Hill et al., 2020) [c]
		- *V. natriegens*	- 73		- (Hill et al., 2020) [c]
Raffinose & Sucrose/ Raffinose & Sucrose		- *B. amyloliqueficien*	- 79		- (Tian & Karboune, 2012) [e]
		- *B. indica subsp. Indica*	- 66		- (Hill et al., 2020) [c]
		- *G. oxydans*	- 71		- (Hill et al., 2020) [c]
		- *N. aromaticivorans*	- 57		- (Hill et al., 2020) [c]
		- *P. graminis*	- 70		- (Hill et al., 2020) [c]
		- *V. natriegens*	- 70		- (Hill et al., 2020) [c]
Sucrose/Cellobiose	Cellobiose-Fru	- *M. laevaniformans*		- +++	- (Kim et al., 2005) [d]
		- *B. subtilis*	- 30%		- (Seibel et al., 2006) [b]
		- *B. licheniformis*	- 35%		- - (Lu et al., 2014) [b]
Sucrose/Isolmaltose	Isomaltose-Fru	- *B. subtilis*	- 53%		- (Seibel et al., 2006) [b]
Sucrose/Lactose	Lactosucrose	- *B. circulans*		- ++	- (Oseguera et al., 1996) [a]
		- *M. laevaniformans*		- +++	- (Kim et al., 2005) [d]
		- *B. subtilis*	- 34		- (Seibel et al., 2006) [b]
		- *B. amyloliqueficien*	- 60		- (Tian & Karboune, 2012) [e]
		- *G. stearothermophilus*	- 37		- (Inthanavong et al., 2013) [f]
		- *B. licheniformis*	- 26		- (Lu et al., 2014) [b]
		- *B. indica subsp. Indica*	- 78		- (Hill et al., 2020) [c]
		- *G. oxydans*	- 66		- (Hill et al., 2020) [c]
		- *N. aromaticivorans*	- 68		- (Hill et al., 2020) [c]
		- *P. graminis*	- 79		- (Hill et al., 2020) [c]
		- *V. natriegens*	- 69		- (Hill et al., 2020) [c]
Sucrose/Lactulose	Lactulose-Fru	- *M. laevaniformans*		- +	- (Kim et al., 2005) [d]
Sucrose/Levanbiose	Levanbiose-Fru	- *M. laevaniformans*		- +	- (Kim et al., 2005) [d]
Sucrose/Maltose	Erlose	- *B. circulans*		- +++	- (Oseguera et al., 1996) [a]
		- *L. sanfranciscensis*		- DNR	- (Tieking et al., 2005)

		- *M. laevaniformans*		- +++	- (Kim et al., 2005) [d]
		- *B. subtilis*	- 45		- (Seibel et al., 2006) [b]
		- *B. amyloliqueficien*	- 68		- (Tian & Karboune, 2012) [e]
		- *G. stearothermophilus*	- 92		- (Inthanavong et al., 2013) [f]
		- *B. licheniformis*	- 38		- (Lu et al., 2014) [b]
		- *B. indica subsp. Indica*	- 74		- (Hill et al., 2020) [c]
		- *G. oxydans*	- 53		- (Hill et al., 2020) [c]
		- *N. aromaticivorans*	- 69		- (Hill et al., 2020) [c]
		- *P. graminis*	- 60		- (Hill et al., 2020) [c]
		- *V. natriegens*	- 72		- (Hill et al., 2020) [c]
Sucrose/Melibiose	Raffinose	- *M. laevaniformans*		- +++	- (Kim et al., 2005) [d]
		- *B. subtilis*	- 45		- (Seibel et al., 2006) [b]
Sucrose/Palatinose	Palatinose-Fru	- *M. laevaniformans*		- +	- (Kim et al., 2005) [d]
Sucrose/Sophorose	Sophorose-Fru	- *M. laevaniformans*		- +	- (Kim et al., 2005) [d]
Sucrose/Sucrose	Fructooligosaccharides	- *L. sanfranciscensis*		- DNR	- Tieking et al., 2005)
	(e.g., kestose, nystose)	- *M. laevaniformans*		- +++	- (Kim et al., 2005) [d]
		- *B. amyloliqueficien*	- 95		- (Tian & Karboune, 2012) [e]
		- *B. indica subsp. Indica*	- 72		- (Hill et al., 2020) [c]
		- *G. oxydans*	- 78		- (Hill et al., 2020) [c]
		- *N. aromaticivorans*	- 53		- (Hill et al., 2020) [c]
		- *P. graminis*	- 63		- (Hill et al., 2020) [c]
		- *V. natriegens*	- 60		- (Hill et al., 2020) [c]
Sucrose/Trehalose		- *B. licheniformis*	- 9		- (Lu et al., 2014) [b]

[a] Transferase activity was defined as the amount of enzyme producing 1 gmol glucose/min. More than 50 %, +++; 20-50 %, ++; Below 20 %, +

[b] Yields were calculated from donors at peak product concentration.

[c] Peak percent bioconversion of oligosaccharide as acceptor/donor molecules at 2-, 24- or 50-hours reaction time.

[d] Transfer efficiency was expressed as the fructosyl product area/sucrose area determined by thin-layer chromatography densitometry. More than 50 %, +++; 30 to 50 %, ++; 10 to 30 %, +; Below 10%, +/-

[e] Total conversion yield reported for sucrose/raffinose as sole substrate; Optimum yield reported for acceptor specificity reactions

[f] The product yield from sucrose represents the conversion yield of sucrose into fructosylated products.

DNR detected but yield not reported

Table 2.2. Acceptor specificity of levansucrase- Monosaccharides as acceptors

Donor/Acceptor	End-products	Microbial Sources of LS	Bioconversion Yield (%)	Transferase activity Level	References
Sucrose/Arabinose	Fructosylarabsucrose, arabsucrose	- *L. sanfranciscensis*		- DNR	- (Tieking et al., 2005)
		- *M. laevaniformans*		- +	- (Kim et al., 2005) [d]
		- *B. licheniformis*	- 37		- (Lu et al., 2014) [b]
Sucrose/Allose	Allosucrose	- *B. subtilis*	- <0.01		- (Seibel et al., 2006) [b]
Sucrose/Fucose	Fucose-Fru	- *B. subtilis*	- 62		- (Seibel et al., 2006) [b]
Raffinose/Fucose	Fucose-Fru	- *B. subtilis*	- 4		- (Seibel et al., 2006) [b]
Sucrose/Galactose	Galactose-Fru	- *B. subtilis*	- 61		- (Seibel et al., 2006) [b]
		- *B.circulans*		- +++	- (Oseguera et al., 1996) [a]
		- *M. laevaniformans*		- ++	- (Kim et al., 2005) [d]
		- *B. amyloliqueficien*	- 46		- (Tian & Karboune, 2012) [e]
		- *G. stearothermophilus*	- 50		- (Inthanavong et al., 2013) [f]
		- *B. licheniformis*	- 10		- (Lu et al., 2014) [b]
		- *B. indica subsp. Indica*	- 67		- (Hill et al., 2020) [c]
		- *G. oxydans*	- 52		- (Hill et al., 2020) [c]
		- *N. aromaticivorans*	- 47		- (Hill et al., 2020) [c]
		- *P. graminis*	- 58		- (Hill et al., 2020) [c]
		- *V. natriegens*	- 76		- (Hill et al., 2020) [c]
Raffinose/Galactose	Galactose-Fru	- *B. subtilis*	- 10		- (Seibel et al., 2006) [b]
Raffinose/Glucose	Glucose-Fru	- *B. subtilis*	- 11		- (Seibel et al., 2006) [b]
Sucrose/2-deoxyglucose	2-deoxyglucose-Fru	- *B. subtilis*	- 0.4		- (Seibel et al., 2006) [b]
Sucrose/3-ketoglucose	3-ketosucrose	- *B. subtilis*	- 6		- (Seibel et al., 2006) [b]
Sucrose/Mannose	Mannose-Fru	- *B. subtilis*	- 0.3		- (Seibel et al., 2006) [b]
Sucrose/Methylgalactoside	Methylgalactoside-Fru	- *M. laevaniformans*		- +	- (Kim et al., 2005) [d]
Sucrose/Methylglucoside	Methylglucoside-Fru	- M. laevaniformans		- +	- (Kim et al., 2005) [d]
Raffinose/Rhamnose	Rhamnose-Fru	- *B. subtilis*	- <0.1		- (Seibel et al., 2006) [b]
Sucrose/Xylose	Fructosylxylsucrose, xylsucrose	- *B. subtilis*	- 56		- (Seibel et al., 2006) [b]
		- *L. sanfranciscensis*		- DNR	- (Tieking et al., 2005)
		- *M. laevaniformans*		- +	- (Kim et al., 2005) [d]
		- *B. amyloliqueficien*	- 55		- (Tian & Karboune, 2012) [e]
		- *B. licheniformis*	- 32		- (Lu et al., 2014) [b]
		- *B. indica subsp. Indica*	- 73		- (Hill et al., 2020) [c]
		- *G. oxydans*	- 67		- (Hill et al., 2020) [c]
		- *N. aromaticivorans*	- 84		- (Hill et al., 2020) [c]
		- *P. graminis*	- 67		- (Hill et al., 2020) [c]
		- *V. natriegens*	- 84		- (Hill et al., 2020) [c]
Raffinose/Xylose	Xylose-Fru	- *B. subtilis*	- 16.7, 26.9		- (Seibel et al., 2006) [b]

[a] Transferase activity was defined as the amount of enzyme producing 1 gmol glucose/min. More than 50 %, +++; 20-50 %, ++; Below 20 %, +

[b] Yields were calculated from donors at peak product concentration.

[c] Peak percent bioconversion of oligosaccharide as acceptor/donor molecules at 2-, 24- or 50-hours reaction time.

[d] Transfer efficiency was expressed as the fructosyl product area/sucrose area determined by thin-layer chromatography densitometry. More than 50 %, +++; 30 to 50 %, ++; 10 to 30 %, +; Below 10%, +/-

[e] Total conversion yield reported for sucrose/raffinose as sole substrate; Optimum yield reported for acceptor specificity reactions

[f] The product yield from sucrose represents the conversion yield of sucrose into fructosylated products.

DNR detected but yield not reported

Table 2.3. Acceptor specificity of levansucrase- Alcohols as acceptors

Donor/Acceptor	Microbial Sources of LS	Bioconversion Yield (%)	Transferase activity Level	References
Sucrose/Catechol	- *B. indica subsp. Indica*	- 61		- (Hill et al., 2020) [c]
	- *G. oxydans*	- 7		- (Hill et al., 2020) [c]
	- *N. aromaticivorans*	- 39		- (Hill et al., 2020) [c]
	- *P. graminis*	- 36		- (Hill et al., 2020) [c]
	- *V. natriegens*	- 45		- (Hill et al., 2020) [c]
Sucrose/Glycerol	- *B.circulans*		- +	- (Oseguera et al., 1996) [a]
Sucrose/Isopropanol	- *B. licheniformis*		- DNR	- (Lu et al., 2014) [b]
Sucrose/Lactitol	- *M. laevaniformans*		- (+/-)	- (Kim et al., 2005) [d]
Sucrose/Methanol	- *B.circulans*		- ++	- (Oseguera et al., 1996) [a]
Sucrose/1-pentanol	- *B. licheniformis*		- DNR	- (Lu et al., 2014) [b]
Sucrose/Sorbitol	- *B.circulans*		- +	- (Oseguera et al., 1996) [a]
	- *B. indica subsp. Indica*	- 56		- (Hill et al., 2020) [c]
	- *G. oxydans*	- 45		- (Hill et al., 2020) [c]
	- *N. aromaticivorans*	- 44		- (Hill et al., 2020) [c]
	- *P. graminis*	- 42		- (Hill et al., 2020) [c]
	- *V. natriegens*	- 59		- (Hill et al., 2020) [c]
Sucrose/Xylitol	- *M. laevaniformans*		- (+/-)	- (Kim et al., 2005) [d]

[a] Transferase activity was defined as the amount of enzyme producing 1 gmol glucose/min. More than 50 %, +++; 20-50 %, ++; Below 20 %, +

[b] Yields were calculated from donors at peak product concentration.

[c] Peak percent bioconversion of oligosaccharide as acceptor/donor molecules at 2-, 24- or 50-hours reaction time.

[d] Transfer efficiency was expressed as the fructosyl product area/sucrose area determined by thin-layer chromatography densitometry. More than 50 %, +++; 30 to 50 %, ++; 10 to 30 %, +; Below 10%, +/-

DNR detected but yield not reported

2.5. End products of LS-catalyzed reactions

An interesting property of LS is its ability to catalyze various types of reactions. This eventually leads to the production of a wide range of products including levan, sucrose analogues, fructooligosaccharides (FOS), fructosylated trisaccharides, and some alkyl and phenolic fructosides.

2.5.1. Levan

Besides inulin, levan is also one of the major naturally occurring fructans/homopolymers of fructose. β-(2,6) linked fructofuranosyl rings make up the main chain of levan. Fructofuranosyl rings can form branches to the main chain via β-(2,1) linkages (Arvidson et al., 2006). Levan has gained interest because of its emulsifying and encapsulating properties, texture-forming abilities, flavor, and color fixative effect and as a fat substitute. Furthermore, its medicinal benefits include being a prebiotic, blood plasma substitute, an immunomodulator, an antitumor, a sorbent of cholesterol, and it can prolong drugs' effects (Bekers et al., 2001; Calazans et al., 2000; de Oliveira et al., 2007; Queiroz Santos et al., 2014; Srikanth et al., 2015).

More recently, *B. subtilis* MT453867 levan has shown high potential as an adjunct to pancreatic-anticancer agents with chemoprotective properties (Gamal et al., 2021), and levan extracted from bacterial honey isolates had a curative effect in peptic ulcer (Ragab et al., 2020) and levan from *B. subtilis var.* natto exhibited in vitro apoptotic activity against neuroblastoma cancer cells line SH-SY5Y through caspase 3/7 pathway (Vieira et al., 2021). *Enterococcus faecalis* Esawy levan displayed antivirus properties against the Newcastle disease virus (Gamal et al., 2020). Nano-sized levan from *Pseudomonas mandelii* has also shown great potential in biotechnological applications due to its antimicrobial and antibiofilm effect on pathogenic microorganisms, and it can act as an inducer of cytotoxicity breast cells (Koşarsoy Ağçeli & Cihangir, 2020). Then, studies suggested the potent immunomodulatory effects on RAW264.7 macrophage cells of levan from *Tanticharoenia sakaeratensis* (Aramsangtienchai et al., 2020), levan from *B. subtilis* AF17 was demonstrated to be a promising source of antihypertensive agents (Bouallegue et al., 2020) and levan from *B. licheniformis* showed potential as an antioxidant and antibacterial agent (Hertadi et al., 2021). Levans *from B. amyloliquefaciens* and

G. oxydans significantly reduced the production of LPS-triggered pro-inflammatory cytokines in differentiated Caco-2 cells, demonstrating their anti-inflammatory properties (Sahyoun et al., 2024).

Some studies have also developed new approaches to enhance the health benefits of levan. Hertadi et al. (2020) investigated the enhancement of the antioxidant activity of levan from *B. licheniformis* through the formation of nanoparticle systems with metal ions. Levan–Fe^{2+} and levan–Cu^{+} nanoparticles exhibited about 33%–40% higher antioxidant activity than levan alone (Rukman Hertadi, Amari, & Ratnaningsih, 2020). Levan from *B. licheniformis* FRI MY-55 was phosphorylated via microwave-assisted synthesis and showed enhanced antioxidant and antitumor activity compared to native levan or levan phosphorylated using traditional long-term heating (Huang, Huang, Tsai, & Su, 2021).

As for the cosmetics industry, levan produced from *B. subtilis* natto KB1 was confirmed to be non-cytotoxic and non-hemolytic when used at a concentration range of 0.01 to 1.00 mg/ml. Moreover, the produced levan demonstrated antioxidant properties (Domżał-Kędzia et al., 2019). *B. subtilis* levan has also shown great potential as a natural active bio-nanocarrier in cosmetics due to its antioxidant properties and ability to penetrate the skin (Lewińska et al., 2023). The physicochemical properties of *E. amylovora* levan were compared to commercial gels of xanthan, guar, carrageenan and Arabic gums, and the results indicated that the levan could be used as a novel water-soluble micro gel in the food, medicinal and cosmetic industries (Peng et al., 2019). Another study focused on how levan produced from *Gluconobacter albidus* TMW 2.1191 is functionally diverse depending on its size. It was determined that the rheological properties of levan are dictated by its size and polydispersity instead of the amount of levan used or its structural composition (Hundschell et al., 2020). Sahyoun et al. (2024) also demonstrated how the size of levans dictates their techno-functional properties by showing varying foaming capacity and stability, emulsion stability, water and oil-holding capacities, gelling properties, and rheological behaviors.

Various levan-producing LSs from different microorganisms have been identified in completely purified (Inthanavong et al., 2013), partially purified (Dahech et al., 2012), crude (Tian

et al., 2011), recombinant (Rairakhwada et al., 2010), or immobilized forms (Chiang et al., 2009). Table 2.4 summarizes the levan production by different microorganisms and biocatalysts. The yield of levan production, the molecular weight, the number of branches, and the degree of polymerization of the produced levan differ depending on the microbial sources and the reaction conditions. These include enzyme concentration, ionic strength, temperature, and the presence of water-miscible organic solvents. LSs have different optimum polymerization and sucrose splitting temperatures, with polymerization usually favored at low temperatures. Temperature also affects the size of the levan formed. Sucrose concentration too affects the molecular weight of formed levan, and a higher sucrose concentration usually favors levan production. Furthermore, higher ionic strength in general leads to the production of lower-molecular-weight levan (Li et al.,2015; Öner et al., 2016).

Besides optimizing reaction conditions, other studies have focused on new technologies to enhance levan biosynthesis. Shang et al. (2021) have developed two types of surface-displayed LSs as an alternative to enzyme immobilization that often faces cost production issues. This technology allows the protein of interest to be auto-immobilized on the surface of microbial cells, which in this case was *Saccharomyces cerevisiae* EBY100. Over 50% and 60% of initial activities were retained after six cycles of reuse (Shang et al., 2021). Zhang et al. (2021) opted for a fusion enzyme approach to enhance the thermostability of polymerization and production of high molecular weight *B. subtilis* SacB -T305A levan, by adding an extra C-terminus of the trehalose-6-phosphate synthase. The optimum temperature of levan polymerisation of the fused enzyme was 15 °C higher than that of free SacB-T305A, with the proportion of high molecular weight levan to total polysaccharides significantly increasing from 4% to 91% (Zhang et al., 2021). In a recent study, to improve *Z. mobilis* ATCC 31821 levan production, osmotic pressure stress was applied, via Adaptive Laboratory Evolution, by gradually increasing KCl concentration. The results showed that the production of levan in the high osmotic pressure-adapted strains increased by 10 times compared to the ancestor strain (Bagoghli et al., 2023).

Table 2.4. Levan production by different microorganisms and biocatalysts

Biocatalyst	Microorganism	Production	References
Purified enzyme	*G. stearothermophilus* ATCC 7953	Optimum conditions: pH 6.75; Temperature 57 °C	(Inthanavong et al., 2013)
Partially purified enzyme	*B. licheniformis*	Levan with antitumor activity against some tumor cell lines produced in vitro	(Dahech et al., 2012)
Crude enzyme	*B. amyloliquefaciens* ATCC 23350	Optimum temperature: Intracellular levan- 25–30 °C Extracellular levan- 40 °C Levan was produced as the main product in the early stage of the reaction.	(Tian et al., 2011)
Purified recombinant enzyme	*B. amyloliquefaciens*	Maximum levan conditions: pH 8.0; Temperature 4 °C; Time after 24 h of reaction	(Rairakhwada et al., 2010)
Immobilized crude recombinant enzyme	*Z. mobilis* ATCC 10988	83 g/L levan produced with 20 % (w/v) sucrose. 480 g/L levan was produced in total after recycling the enzyme seven times.	(Chiang et al., 2009)

2.5.2. Sucrose analogues

Sucrose analogues are β-(2–1)- α -linked disaccharides, in which the glucosyl residue, from sucrose, is replaced by another aldosyl residue (Baciu et al., 2005). Various sucrose analogues can be synthesized when monosaccharides are used as acceptors in LS-catalyzed reactions. For instance, Gal-Fru is obtained from D-galactose, Xyl-Fru is derived from D-xylose, and Fuc-Fru is synthesized from D-fucose (L et al., 2014; Seibel et al., 2006). Furthermore, Seibel et al. (2006) even found that levansucrase from *B. subtilis* is more susceptible towards the transfructosylation of some monosaccharides than disaccharides. For instance, D-galactose resulted in a 61% yield from sucrose while only a 34% yield was obtained with lactose (Seibel et al., 2006).

These sucrose analogues can subsequently act as acceptor molecules and undergo further transfructosylation reactions to produce hetero-fructooligosaccharides or hetero-levans (Beine et al., 2008). Beine et al. (2008) used LS SacB of *B. subtilis* and sucrose analogues, α-Xyl-1,2-β-Fru or α-Gal-1,2-β-Fru, to produce a range of new higher oligosaccharides or polysaccharides (xylooligofructosides and galactopolyfructosides) of varying sizes instead of levan. Furthermore, random mutagenesis was performed on the LS gene SacB for the synthesis of short-chain FOS and to prevent the production of polymers. This enzyme was then used to synthesize a 6-kestose analogue (α-Xyl-1,2-β-Fru-2,6-β-Fru) from Xyl-Fru (Beine et al., 2008).

2.5.3. Fructooligosaccharides

In addition to levan, most microbial LSs can produce fructooligosaccharides (FOS). FOS are produced by LS from sucrose via oligomerization. FOS are one of the most used prebiotics, besides inulin and galactooligosaccharides, in food products like yogurts, bread, baby foods, and creamy milk (Rößle et al., 2011). They are prized sweeteners given their non-cariogenic nature and prebiotic properties (Lima et al., 2018). They have also been shown to improve calcium absorption and the immune response (Le Bourgot et al., 2014; Morohashi et al., 1998).

Compared to inulin-type FOS (β-(2,1)-linkages), which can also be synthesized from inulin by endo-inulinase or from sucrose by sucrose 1-fructosyltransferase (Kim et al., 1997; Sangeetha et al., 2004), levan-type FOS (β-(2,6)-linkages) are believed to exhibit greater prebiotic effects (Kilian et al., 2002). Hence, LS is an attractive alternative for FOS synthesis since it can produce

both levan-type and inulin-type FOS as demonstrated by numerous studies such as the one by Santos-Moriano et al. (2015). The degree of polymerization of FOS also affects their biological activities. FOS with a lower degree of polymerization showed a more significant differential effect on the number of lactic acid bacteria, mucosal immune functions, and IgA secretion in rat cecum, hence suggesting greater benefit on the health of the gastrointestinal tract than long-chain FOS (Ito et al., 2011).

Just like levan, the formation of FOS depends on the microbial source of the enzyme and the reaction conditions like sucrose concentration and reaction time (Li et al., 2015). For instance, *B. amyloliquefaciens* LS produced mainly FOS while *B. subtilis* LS favored long-chain levan synthesis (Caputi et al., 2013; Tian et al., 2011). Increasing the sucrose concentration has been shown to increase FOS formation while pH and temperature were not significant factors (Waldherr et al., 2008). Tian et al. (2011) have also demonstrated the effect of reaction time. Levan was mainly produced at the early stage of the reaction while FOS were predominant after 36 hours from *B. amyloliquefacien* LS.

As mentioned, LSs from gram-positive bacteria usually favor levan formation over FOS synthesis. Kanjanatanin et al. (2019) successfully redesigned and engineered the active site of LS from *B. licheniformis* RN-01 to control the chain length of levan-type FOS, via computational protein design, docking and molecular dynamics. The approach used aimed at blocking the oligosaccharide binding track of the LS-FOS (with 3 fructosyl units) complex with large aromatic residues. Levan-type FOS with a degree of polymerization up to five were synthesized with the new enzymes, N251W and N251W/K372Y mutants (Kanjanatanin et al., 2019). Molecular dynamics and computational protein design were also used to enhance the thermal stability of the Y246S mutant of *B. licheniformis* RN-01 LS that has previously been shown to effectively produce levan-type FOS (Surawut et al., 2016). Klaewkla et al. (2020) redesigned the enzyme by rigidifying highly flexible residues present on the enzyme's surface. The new mutant K82H/N83R had higher thermostability than the original mutant with a 1.7-fold increase. Its characteristics, product patterns and secondary structures were not drastically altered (Klaewkla et al., 2020).

Novel strategies of fusing enzymes have also been developed for the synthesis of levan-type FOS. Porras-Domínguez et al., (2017) developed a new enzyme by fusing LS from *B. subtilis* and an endolevanase from *B. licheniformis.* The results were compared to using the individual enzymes in a one-pot reaction. Similar reaction evolution, product profile and reaction yield were obtained with the fused and unfused enzymes. The FOS produced included 6-kestose, levanbiose and blastose. The fusion enzyme could hence avoid having to perform the production, purification, and application procedures of the two enzymes separately (Porras-Domínguez et al., 2017). Instead of an *Escherichia coli* strain, a recombinant *Pichia pastoris* strain expressing the levansucrase-endolevanase fusion enzyme was later investigated by Ávila-Fernández et al. (2023). This was done so that the expression of enzymes was done in a GRAS microorganism, in addition to the elimination of glucose following its selective consumption by *P. pastoris.* The *P. pastoris* cultures were successfully used to simultaneously produce the enzyme and the enzymatic synthesis of levan-type FOS (Ávila-Fernández et al., 2023). In another study, Charoenwongpaiboon et al. (2022) investigated the potential of a recombinant levansucrase-inulosucrase fusion protein. Strains used for LS were from *B. amyloliquefaciens* KK9 and for inulosucrase were from *L. reuteri* 121. Previous studies conducted have shown that using LS and inulosucrase in a one-pot reaction could successfully enhance the yield of levan-type FOS (Tian et all., 2014; Wangpaiboon et al., 2022). The fusion enzyme did not affect the optimum pH and temperature of the reaction, but slightly affected the kinetic parameters while being stable for extended periods at 30°C (Charoenwongpaiboon et al., 2022). More recently Charoenwongpaiboon et al. (2023) introduced a novel cross-linked enzyme aggregates based on *B. licheniformis* RN01 LS and N543A variant of *L. reuteri* 121 inulosucrase. Compared to the free enzyme, the co-immobilized enzyme aggregates produced higher amounts of levan-type FOS, had better stability and could be used for several reaction cycles (Charoenwongpaiboon et al., 2023).

2.5.4. Fructosylated trisaccharides

Due to its wide acceptor specificity, LS can catalyze the fructosyl transfer of sucrose from various disaccharides including maltose, lactose, melibiose, cellobiose, and isomaltose to produce different heterooligosaccharides as shown in Fig. 2.2. (Li et al., 2015).

One of the most important heterooligosaccharides, catalyzed by LS, is lactosucrose (O-β-D-galactopyranosyl-(1,4)-O-α-D-glucopyranosyl-(1,2)-β-D-fructofuranoside). This trisaccharide is derived from lactose (fructosyl acceptor) and sucrose (fructosyl donor) which are the cheapest and most common disaccharides found in nature (Mu et al., 2013). Lactosucrose has been valued for its potential prebiotic effects (Ohkusa et al., 1995), intestinal mineral absorption properties (Teramoto et al., 2006), and the ability to reduce body fat accumulation (Kimura et al., 2002). It is an approved functional food ingredient for foods for specified health uses (FOSHU) and is widely used in Japan (Mu et al., 2013). The significant water-holding capacity of lactosucrose makes it an interesting ingredient for the food industry, particularly for the production of fermented milk products like yogurts or cheese, to reduce syneresis or serum separation and act as a fat replacer (Krasaekoopt et al., 2003; Silvério et al., 2015). Lactosucrose is a rare trisaccharide that barely exists in nature and is difficult to manufacture chemically. It can however be synthesized through transgalactosylation reaction by β-galactosidase from *B. circulans* or transfructosylation reaction by β-fructofuranosidase from *Arthrobacter* sp. K-1 and different LSs including those from *Aerobacter Levanicum, Bacillus* natto*, B. subtilis, B. goodwinii, L. mesenteroides, G. oxydans and V. natriegens* (Bahlawan & Karboune, 2022; Li et al., 2015; Mu et al., 2013; Xu et al., 2018). Using raffinose as substrate and lactose as fructosyl acceptor in a reaction catalyzed by *L. mesenteroides* B-512 FMC LS has shown to produce melibiose, a disaccharide prized for its various health benefits, as the main product, in addition to lactosucrose and fructose (Xu et al., 2017).

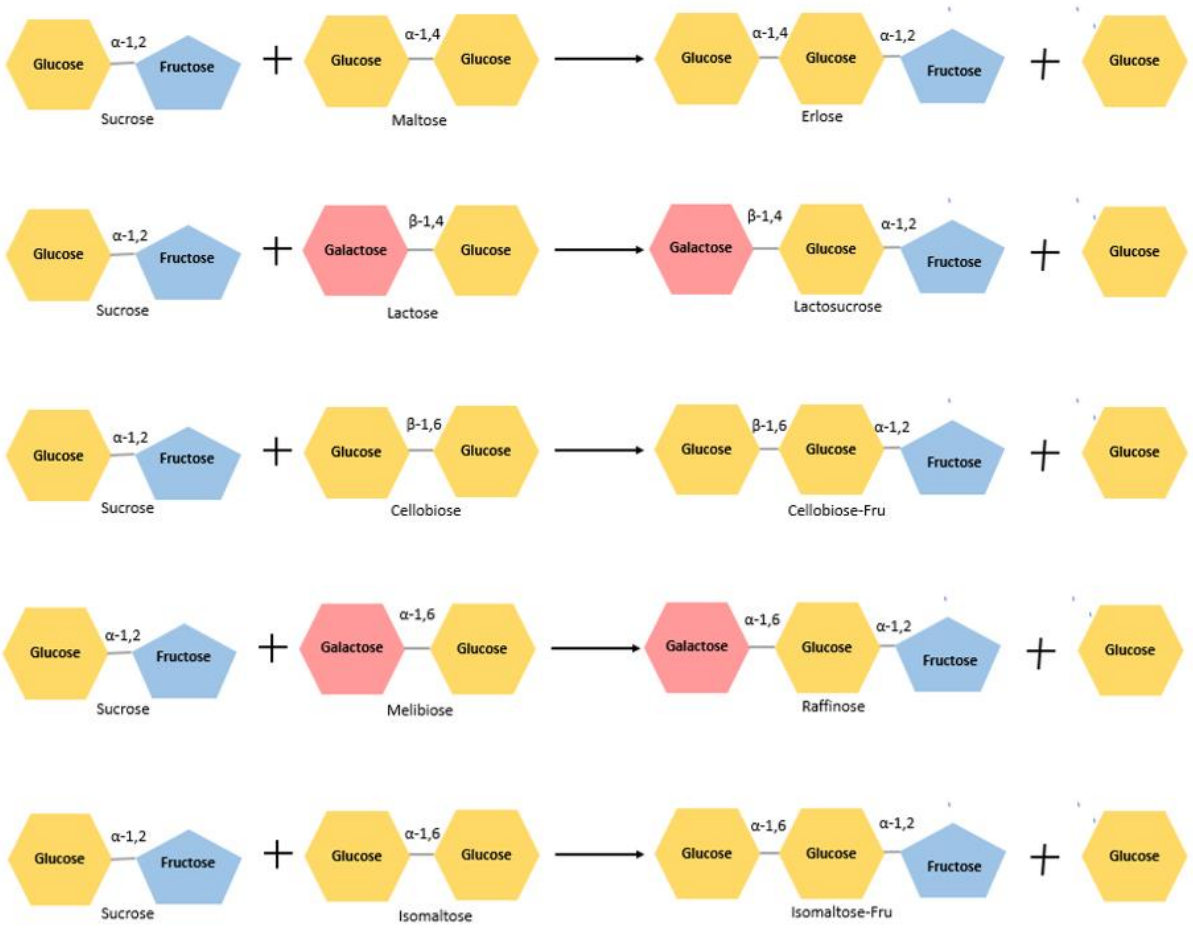


Fig 2.2. Heterooligosaccharides formation from disaccharides by levansucrase (modified from Li et al., 2015)

Another interesting trisaccharide is the non-reducing trisaccharide erlose (O-β-D-fructofuranosyl-(1,2)-O-α-D-glucopyranosyl (1,4)- α-D-glucopyranoside) for its sucrose-like taste and anticarious properties (Taga et al., 1993). Studies have shown that it can be synthesized using inulosucrase from *Lactobacillus gasseri* DSM 20604 (Díez-Municio et al., 2013) as well as various LSs from *B. Subtilis* (Canedo et al., 1999), *B. amyloliquefaciens* (Tian & Karboune, 2012), *B. indica subsp. indica, G. oxydans, N. aromaticivorans, P. graminis,* and *V. natriegen* (Hill et al., 2020). The synthesis of trisaccharide raffinose, known for its prebiotic properties, was optimized using recombinant LS from *Clostridium arbusti* SL206 and melibiose as fructosyl acceptor (Mishra et al., 2017). Finally, the synthesis and structural characterization of theanderose/isomaltosucrose, a potential sweetener and stabilizer, synthesized through the transfructosylation reaction catalyzed by LS from *B. subtilis* CECT 39 and acceptor isomaltose was assessed (Ruiz-Aceituno et al., 2017).

The trisaccharides formed can also be used to further produce new compounds. This was demonstrated by Miranda-Molina et al. (2017). *B. subtilis* LS was first used to transfer a fructosyl group to trehalose forming O-β-d-Fruf-(2↔6)-trehalose, using sucrose or levan as a fructosyl donor. Trehalase enzyme was then utilized to hydrolyze O-β-d-Fruf-(2↔6)-trehalose to yield blastose. Blastose is a β-D-fructofuranosyl-(2↔6)-D-glucopyranose sucrose analogue and is the basis of the neo-fructooligosaccharide which has been gaining interest for their superior bifido-stimulating effect, chemical and thermal stability (Miranda-Molina et al., 2017). A study by Díez-Municio et al. (2015) presented the potential of a bi-enzymatic system of levansucrase–inulosucrase. In this system, *L. gasseri* DSM 20604 inulosucrase performed a transfructosylation reaction on lactosucrose, obtained via *B. subtilis* CECT 39 LS, to eventually synthesise potentially bioactive lactosyl-oligofructosides (Díez-Municio et al., 2015). These lactosyl-oligofructosides might be more effective prebiotics than lactosucrose given longer carbohydrate chains are known to ferment at slower rates (Perrin et al., 2002).

2.5.5. Alkyl and phenolic fructosides

Finally, LS can also catalyze the formation of some alkyl and phenolic fructosides via the transfructosylation reaction of some short-chain alkylalcohols and aromatic alcohols/phenolic compounds, respectively. Fructosylation of phenolic compounds represents a potential way to increase their health benefits by improving their stability, solubility, and bioactivity (Núñez-López et al., 2019; Xu et al., 2016). Enzymatic glycosylation is an interesting alternative to chemical methods that are often labour-intensive procedures requiring multistep synthetic routes that produce low overall yields. In addition, they use toxic catalysts and solvents and produce significant waste (Desmet et al., 2012).

Butanol and two aromatic alcohols, namely hydroquinone and benzyl alcohol, were successfully used as fructosyl acceptors when LS from *B. subtilis* was used (Mena-Arizmendi et al., 2011). The aromatic alcohols, over aliphatic alcohols, were preferentially fructosylated. The optimum conditions for the fructosylation of hydroquinone were found to be an acceptor concentration of 500 mM and an enzyme concentration of 5 U/mL. Rapid hydrolysis was observed with higher enzyme concentration. The addition of organic co-solvents, which usually increases glycosylation of hydrophobic molecules, however, caused a reduction in hydrolysis and fructosylation (Mena-Arizmendi et al., 2011). The fructosylation of hydroquinone was also investigated with LS from *L. mesenteroides* (Kang et al., 2009). Kang et al. (2009) successfully synthesized a hydroquinone fructoside, 4-hydroxyphenyl-β-D-fructofuranoside, which is a potential alternative skin whitening agent to hydroquinone which often results in skin inflammation (Pieroni et al., 2004).

In another study, the acceptor specificity of LS from *B. circulans* was explored by Oseguera et al. (1996). The LS had high transferase activity with methanol and moderate activity towards sorbitol and glycerol. No activity was recorded with inositol. Furthermore, when methanol replaces water as the nucleophile in the reaction medium, methyl fructoside may be produced via alcoholysis (Oseguera et al., 1996). Then, isopropanol and 1- pentanol were as well able to act as fructosyl acceptors using *B. licheniformis* 8-37-0-1, producing isopropyl and pentyl fructosides respectively (Lu et al., 2014). Similar glycosides are used as biosurfactants in

cosmetics and household chemicals production or as building blocks in the pharmaceutical industry and molecular biology. No transferase activity was recorded with longer chained alcohols, and this might be due to the increasing hydrophobicity of those acceptors, making them unavailable to the hydrophilic enzyme (Lu et al., 2014). More recently, Polsinelli et al. (2022) investigated the *Erwinia tasmaniensis* LS- 1,2,4-butanetriol complex. It was confirmed that the enzyme favored the (S)-enantiomer of polyalcohols. Additionally, a structural comparison with inulosucrase suggested a difference in fructose binding mode of fructosyltransferases from gram-positive and gram-negative bacteria (Polsinelli et al., 2022).

Finally, Núñez-López et al. (2019) investigated the fructosylation of various phenolic compounds by LS from *G. diazotrophicus*. The phenolic compounds that were successfully transglycosylated included ferulic acid, caffeic acid, rosmarinic acid, methyl gallate, resveratrol, mangiferin, catechin, neohesperidin, puerarin, coniferyl alcohol and vanillin. Isoflavone puerarin and phenol coniferyl alcohol were the most efficient fructosyl acceptors, with conversion rates of 93% and 25.1%, respectively. They both led to the synthesis of mono-, di-, and trifructosides. Furthermore, compared to puerarin, the water solubility of fructosyl-β-(2→6)-puerarin increased significantly by 23 folds and its antioxidant capacity was only decreased by 1.25-fold (Núñez-López et al., 2019). Further studies were conducted on the fructosylation of puerarin by LS from *B. subtilis, G. diazotrophicus, L. mesenteroides* and *Z. mobilis* (Núñez-López et al., 2020). LS from *G. diazotrophicus* formed β-D-fructofuranosyl-(2→6)-puerarin and linear oligofructosides, while LS from the other sources synthesized puerarin-4'-O-β-D-fructofuranoside as major product and β-D-fructofuranosyl-(2→6)-puerarin in trace amount. LS from *B. subtilis* best elongated β-D-fructofuranosyl-(2→6)-puerarin resulting in a linear series of water-soluble puerarin polyfructosides reaching at least 21 fructosyl units. Finally, simultaneous, or sequential use of LS from *G. diazotrophicus* LS and *B. subtilis* LS led to an 82–92 % acceptor conversion range. This bi-enzymatic cascade synthesis of puerarin polyfructosides in the same reactor could allow industries to avoid isolating the intermediate product β-D-fructofuranosyl-(2→6)-puerarin (Núñez-López et al., 2020). The enzymatic synthesis of phlorizin fructosides by LS was also reported. The fructosylation of phlorizin, a low-soluble dihydrochalcone with interesting pharmacological properties, could enhance the water solubility of the compound, and

consequently its bioavailability. *G. diazotrophicus* LS was 6.5–fold more efficient than invertase from *Rhodotorula mucilaginosa*, while no activity was recorded with 1-fructosyltransferase from *Schedonorus arundinaceus.* A series of phlorizin mono- di- and tri-fructosides with up to 73 % conversion efficiency were obtained (Herrera-González et al., 2021).

2.6. Applications of Levansucrases in Food Systems

The application of LS for the biogeneration of food ingredients has garnered interest in recent years for the formation of diverse levansucrase-catalyzed reaction products. These products can positively impact food product development in terms of both added functional properties and health benefits. LS can either be used for the endogenous biogeneration of food ingredients, i.e., the enzyme is directly added to the food product, or LS can be used to exogenously biogenerate a specific ingredient which can then be added to food products (Karboune et al., 2022; Renuka et al., 2009). Endogenous biogeneration makes use of substrates already available in a food system. This can help to reduce undesired prominent levels of sucrose and can be less labour-intensive than exogenous biogeneration (Charoenwongpaiboon et al., 2021). Conversely, exogenous biogeneration brings the advantage of selectively producing a particular reaction product by varying some factors such as temperature or substrate concentrations (Santos-Moriano et al., 2015). Table 2.5 summarizes some applications of LS in food.

2.6.1. Bakery

In the bakery category, Korakli et al., (2001) demonstrated that levan and 1-kestose were produced during the growth of *L. sanfranciscensis* in wheat sourdoughs, with concentrations up to 5 and 20 g/kg, respectively. The levan produced has previously been shown to significantly improve bread texture and volume (Brandt, 2003). Tieking et al., (2005) further investigated the formation of heterooligosaccharides by *L. sanfranciscensis* in wheat sourdough. The oligosaccharide patterns of the fermented dough at 10% sucrose were compared to dough fermented with isogenic, levansucrase-positive, and levansucrase-negative strains, TMW 1.392 and TMW 1.392Δlev. Additionally, the aqueous extracts of wheat dough were treated with yeast invertase to eliminate FOS originating from flour. The results showed that 1-kestose, nystose,

arabsucrose and erlose were produced (Tieking et al., 2005). The effect of levan produced from *Gluconobacter frateurii* TMW 2.767, *Gluconobacter cerinus* DSM 9533T*, Neoasaia chiangmaiensis* NBRC 101099, *Kozakia baliensis* DSM 14400 were also evaluated in wheat bread. An increase in bread volume, anti-staling effect and reduction in crumb hardness were reported. Furthermore, high molecular weight levan was suggested to act as a hydrocolloid agent, enforcing intramolecular interaction (Jakob et al., 2013; Jakob et al., 2012).

Besides wheat-based bread, buckwheat sourdoughs were also found a promising food system for levan biosynthesis (Ua-Arak et al., 2016; Ua-Arak et al., 2017). Strains of *G. albidus* TMW 2.1191, *K. baliensis* NBRC 16680 and *N. chiangmaiensis* NBRC 101099 were incorporated in a dough with sugarcane molasses as a sucrose source. 109 CFU/g dough at 48 h was achieved by all three strains, with 16–20 g fructans/kg flour. *G. albidus* recorded the best results and its levan produced was further examined for its molecular mass and size determination. A positive relationship between the concentration of molasses and the amount of levan produced was observed at the concentration up to 35% (flour base). No statistical difference was recorded with inoculum size on levan production. Finally, there was a significantly lower quantity of levan in the firmest doughs (Dough yield 250) compared to the more liquid doughs (Ua-Arak et al., 2016). The sourdoughs with strains *G. albidus* TMW 2.1191, *K. baliensis* NBRC 16680 were then analyzed for sensory properties. The positive effect of levan on bread quality was confirmed. Upon 24 hours of fermentation, the bread's sensory properties and quality, including higher specific volume and lower crumb hardness, were significantly improved. Sensory evaluation of the bread showed that for both strains, the sourdough bread at 24 and 30 h was significantly more accepted than the control and 48 h sourdough bread. Natural acidification during fermentations however partly counteracted the positive effects of levan (Ua-Arak et al., 2017). These studies show the high potential of cereal-based, functional ingredients for the food industry.

2.6.2. Beverages

More studies were carried out on beverage products, particularly fruit juices. Sugarcane juice was a good carbon source for the synthesis of levan and FOS by *B. licheniformis* ANT 179 LS. The optimal conditions were a medium with sugarcane juice at 20 % (v/v) and casein peptone at

2 % (w/v) at an initial pH of 7.0 at 35 °C for 48 h. A levan concentration of up to 50.25 g/L on wet weight was obtained along with inulin-type FOS, kestose and neokestose (Xavier & Ramana, 2017). Ninchan & Noidee, (2021) also analyzed the production of oligofructans, with potential prebiotic properties, from the fermentation of fresh sugarcane juice using *B. subtilis* TISTR 001 that produced LS. Sugarcane juice was the chosen beverage given that it has sucrose as the main component. The sterilized sugar cane juice, adjusted to pH 6.8, was fermented with 10% (v/v) of prepared inoculum of *B. subtilis* TISTR 001, at 30 °C and 150 rpm, over a time course of 96 h. The highest oligofructans content, with prebiotic properties, was recorded at 84 h in the form of free fructose of 2.57% (w/v) with a production yield of 0.17 g/g reducing sugar, at a maximum LS activity of 1.57×10^{6} U/mL. In addition, the growth of prebiotic *Bifidobacterium bifidum* TISTR 2129 and the inhibition of pathogens, *E. coli* TISTR 073 and *Salmonella* serovar *Enteritidis* S003 were observed in the fermented juice (Ninchan & Noidee, 2021). Further studies were conducted with the same LS, comparing sucrose, sugarcane juice and molasses as carbon sources (Noidee et al., 2023). All substrates were found to be suitable, synthesizing oligofructans kestose, nystose, 1-fructofuranosyl-D-nystose, and levan. Sugarcane juice recorded the best results, with a concentration of 30 °Brix producing the highest LS activity of 2.81×107 U/mL at 48 h and the highest oligofructans content of 87.6 g/L at 60 h (Noidee et al., 2023)

Charoenwongpaiboon et al. (2021) studied the application of immobilized cross-linked enzyme aggregates of Y246S variant LS from *B. licheniformis* RN-01 in fruit juices. The immobilized enzyme had a broader pH range for catalysis and a higher optimum temperature than the free enzyme. Immobilization improved the enzyme's stability, increasing its melting temperature and operational duration. More than 50% of the initial activity was retained after six cycles of reuse. Finally, commercial fruit juices (apple, orange, strawberry, and guava), with 50 g/L supplemented sucrose, were incubated with 10 U/mL of cross-linked enzyme aggregates at 20 °C for 24 h (Charoenwongpaiboon et al., 2021). These moderately acidic juices, with a pH range of about 3.0–5.0, were chosen since the sucrase enzyme, which has previously been shown to successfully synthesize oligosaccharides in fruit juices, can have limited activity at low pH (Johansson et al., 2016; Nguyen et al., 2015). The results showed that up to 65%–75% of total sucrose was successfully transformed in moderately acidic fruit juices by the immobilized LS

(Charoenwongpaiboon et al., 2021). Zhang et al. (2022) developed a fusion tag composed of His-tag, intein, and elastin-like polypeptide tag fused with LS SacB gene from *Paenibacillus durus*. Following $CaHPO_4$-based biomimetic mineralization, the new recombinant enzyme showed good reusability, storage stability and enhanced levan yield production. It was then used to catalyze the reaction with substrate sucrose in various juices of orange, strawberry, apple, and grape. 65%–75% of sucrose was efficiently converted after reaction at 30 °C for 24 h (Zhang et al., 2022).

Then sweet sorghum juice with a sucrose concentration of about 114 g/L was treated with LS from *L. mesenteroides* MTCC 10508 (Jadaun et al., 2019). About 97% sucrose was converted yielding about 40 g/ L fructans in the juice. Short-chain FOS with degrees of polymerization 3 to 7 and levan were produced (Jadaun et al., 2019). In another study, Han et al. (2015) investigated the growth of *Leuconostoc citreum* BD1707 and the levan production in tomato juice supplemented with different carbon sources. Sucrose was the carbon source of choice among other sources such as cellobiose, amygdalin, and fructose. More than 28 g/liter of levan was obtained in the tomato juice supplemented with sucrose medium after incubation at 30 °C for 96 h (Han et al., 2015). Renuka et al., (2009) demonstrated that the fortification of pineapple, mango, and orange juice with FOS to partially substitute sucrose was possible, without significantly affecting the overall quality. The fruit juice beverages were supplemented with FOS, synthesized via the transfructosylation of sucrose, using FTase enzyme from *Aspergillus oryzae* MTCC 5154. Although in this case, LS enzyme was not used, a similar syrup could be prepared with LS for exogenous biogeneration of functional ingredients.

2.6.3. Syrups and molasses

Syrups and molasses were also investigated as possible reaction systems for LS. The LS activity of *B. amyloliquefaciens* was assessed in 15, 30 and 66 °Bx maple syrups at 8 and 30 °C. Maple syrup was chosen for its high sucrose content. At 30 °C, the highest LS activity was recorded in the 30 °Bx sample while the highest converted sucrose concentration was observed in the 66 °Bx sample. The selected degree Brix and reaction temperature were found to be significant factors of LS-catalysed reaction. Oligolevans were the major products in 30 °Bx syrup and 30 °C 66 °Bx syrup while levans were the major products in 8 °C 66 °Bx syrup. Additionally, a

wide range of hetero-fructooligosaccharides was synthesized in disaccharides-enriched 15 °Bx and 30 °Bx syrups. Lactose was the most effective fructosyl acceptor compared to cellobiose and melibiose (Li et al., 2015). Further studies were conducted for bioprocess optimization and prebiotic activity assessment of LS-catalyzed 30 and 66 °Bx maple syrups (Karboune et al., 2022). The predictive model used allowed the assessment of the most influential reaction parameter (LS units, pH and reaction time), the interactive effect between them and the overall optimal conditions. The prebiotic activity was then compared to inulin-type commercial FOSs. Higher counts of probiotic strains, *Lactobacillus acidophilus* and *Bifidobacterium lactis,* were recorded with fermentation with oligolevans/FOSs from maple syrup and more short-chain fatty acids like lactic acid were produced (Karboune et al., 2022).

Levan production from *M. laevaniformans* in date syrup was also investigated (Moosavi-Nasab et al., 2010). The effect of fermentation time, pH and sugar concentration were evaluated. It was found that increasing the fermentation time decreased levan production at any date syrup concentration tested. After 48 h incubation of the LS at 37 °C, 10.48 g/L of levan was produced in a date syrup medium while a concentration of 48.8 g/L was produced in a sucrose medium. The optimal conditions were a fermentation time of 48 h, a sucrose concentration of 25%, and a pH was 6.0. TLC and FT-IR spectroscopy revealed that the levan was mainly made up of fructose residues and Thermo Gravimetric Analysis showed that the onset of levan decomposition was 51 °C in date syrup (Moosavi-Nasab et al., 2010).

de Oliveira et al. (2007) examined levan production from *Z. mobilis* in commercial sucrose, sugarcane molasses, and sugarcane syrup. The sugar concentration was adjusted to 250 g/L and a fermentation time of 24 h was chosen as per the previous optimization experiment. Sugarcane molasses resulted in a significantly lower levan production of 2.533 g/L, compared to 21.685 g/L in commercial sucrose and 15.456 g/L in sugarcane syrup. The low levan concentration with molasses was suggested to be due to the presence of salts or trace metals that can prevent cell growth and production of metabolites. Comparing sugarcane syrup to commercial sucrose, although less levan was produced in the former, the biomass production was however 2.76 times greater with sugarcane syrup as a carbon source. Furthermore, the supplementation of the syrup with yeast extract and $MgSO_4$ was shown to increase the amount of levan formed (de Oliveira et

al., 2007). Sugarcane molasses was also treated with LS from *L. mesenteroides* MTCC 10508 leading to a conversion of about 99% of the sucrose present. FOS and levan were synthesized, with a concentration of about 5 g fructans/L of the diluted cane molasses (Jadaun et al., 2019).

Taştan et al. (2019) successfully used *Z. mobilis* LS to produce 6-kestose in carob molasses. 700 mL of 17% sucrose content carob juice was incubated with 1500 U LS at 35 °C for 6 h and then concentrated at 65 °C for 4 h to form a 65°Bx carob molasses. Storage at 20 °C for 4 months resulted in overall decent quality molasses with stable kestose. The colour, non-enzymatic browning index and titratable acidity of LS-catalyzed molasses did not differ much from the control sample. Furthermore, compared to the control sample that recorded a significant increase in 5-hydroxymethylfurfural after three and four months of storage (due to sucrose degradation), a reduction in 5-hydroxymethylfurfural was recorded in LS-catalyzed molasses given the decreased amount of sucrose (Taştan et al., 2019).

Pretreated sugar beet molasses and starch molasses were also proven as good substitutes for sucrose for *Halomonas sp.* AAD6 LS (Küçükaşik et al., 2011). Different pretreatment methods were applied, both individually and in combination. Tricalcium phosphate pretreatment effectively removed iron and zinc from the molasses while activated carbon pretreatment was favourable for nickel removal. On the other hand, clarification and pH adjustment pretreatment yielded low levan production given the retention of undesirable heavy metals and impurities which affected the growth of the microorganism. Overall, the combined pretreatment of Sulfuric Acid-Activated carbon and Tricalcium Phosphate-Sulfuric Acid-Activated carbon pretreatment led to the highest levan yields. About 70% more levan was produced with beet molasses when compared with starch molasses (Küçükaşik et al., 2011). The production of levan by *B. licheniformis* NS032 in a sugar beet molasses-based medium was also studied by Gojgic-Cvijovic et al. (2019). The maximum levan yield of 53.2 g/L was obtained with conditions of 62.6% sucrose originating from molasses, a total sugar concentration of 200 g/L, 4.66% phosphate and an initial pH of 7.2. Sulfuric acid and activated carbon pre-treatment of the molasses eased the isolation of the levan produced. Faster bacterial growth and shorter time intervals to achieve maximum levan production were obtained with the molasses-optimized medium compared to controls with 200 and 400 g/L sucrose (Gojgic-Cvijovic et al., 2019).

Finally, LS from *L. mesenteroides* MTCC 10508 was used to treat table sugar and jaggery (a non-centrifugal traditional unrefined sugar obtained by concentration of sugarcane juice) to transform the in-situ sucrose present into high-value prebiotic fructans. The catalytic conversion of 99% sucrose in the table sugar led to the production of levan and FOS with degrees of polymerization 3 to 7 and a concentration of about 303 g fructans/kg of table sugar. Similar results were obtained with jaggery with about 305 g fructans/kg of jaggery (Jadaun et al., 2019)

2.6.4. Dairy and industrial by-products

Xu et al., (2022) explored the potential of LS in yogurt. Levan was produced in vitro by recombinant BM-2 levansucrase from *Bacillus Velezensis.* Its structure and properties were characterized by FTIR spectroscopy, GC-MS, NMR spectroscopy, scanning electron microscopy, thermogram analysis and differential scanning calorimetry, among others. These analyses allow the prediction of some functional properties to levan-added products such as increased water holding capacity and thermal stability. Yogurt samples were then prepared with varying concentrations of levan and FOS. The addition of levan significantly increased the water-holding capacity of yogurt and exhibited superior system stability than FOS-added yogurts. The supplementation of levan also increased the growth and sustainability of probiotics *Lactobacillus bulgaricus* and *Streptococcus thermophilus* which were added to the fermented yogurt (Xu et al., 2022).

The application of LS in agro-industrial by-products, cheese whey permeate and tofu whey, was explored by Corzo-Martinez et al. (2015). Lactosucrose was efficiently synthesized using the enzyme LS SacB from *B. subtilis* CECT 39. Different reaction mixtures were prepared where sucrose, raffinose, stachyose or tofu whey were used as fructosyl group donors, and lactose or cheese whey permeate as acceptors. Cheese whey permeate is rich in lactose while tofu whey has a significant amount of raffinose and stachyose. The mixtures were incubated with 0.5 U/mL LS at 37 °C, pH 6.0 and at 1,350 rpm. Both by-products were suitable substrates, with the highest production of 80.1 g/L lactosucrose recorded after 120 min. The resulting high yield of lactosucrose provides a solution to valorize these two abundant and inexpensive agro-industrial by-products (Corzo-Martinez et al., 2015).

Recent studies on the synthesis of lactosucrose by LS from *G. oxydans*, *V. natriegens*, *N. aromaticivorans* and *P. graminis* using whey and milk permeate as lactose sources, were also conducted (Bahlawan et al., 2023). Sucrose and lactose solutions at concentrations of 0.9 M and 0.45 M were respectively used with 5 U/mL LS at the corresponding optimal temperature and pH of each LS. All LSs exhibited a higher transfructosylation activity than hydrolytic ones, except for *V. natriegens*, in the presence of milk permeate. *V. natriegens* LS yielded the highest lactosucrose production of 251 g/L with whey permeate. The LS-catalyzed transfructosylation also produced significant FOSs from the biomasses (Bahlawan et al., 2023). Bahlawan & Karboune (2022) also investigated the immobilization of *G. oxydans* and *V. natriegens* LS by different functional supports. The use of RelizymeTM EP403/S functionalized with iminodiacetic acid (IDA)-cupric ions (Cu^{2+}) led to the highest immobilization protein yields and retained activities. Reaction selectivity towards transfructosylation was enhanced following immobilization. Further post-immobilization treatments of alkaline pH incubation and polyethylenimine cross-linking were found to lower the retained activities but increase thermal stabilization. Up to 117 and 101 g/L of lactosucrose were respectively synthesized by free and immobilized LS from *V. natriegens* when whey permeate was used as a lactose source. The immobilized LS could be successfully reused 3 consecutive times (Bahlawan & Karboune, 2022).

2.6.5. Edible and packaging films

Finally, the use of LS from *B. subtilis* natto in the production of edible starch films was explored. Bersaneti et al., (2016) first evaluated edible films based on cassava starch and FOS produced by the LS. The FOSs were estimated to be composed of about 79.42% nystose and 20.58% 1-kestose. The solubility of the FOSs allowed easy manipulation of the filmogenic solutions. Different concentrations of FOSs, from 1 to 10 g/100 g solids, were tested and all formulations had a good appearance and texture. The addition of FOSs exerted a plasticizing effect, decreased the glass transition temperature and water vapour permeability, and increased the solubility and elongation of the films (Bersaneti et al., 2016). Mantovan et al., (2018) then investigated the potential of levan synthesized by LS from *B. subtilis* natto in Cassava starch edible film. Different starch: levan proportions (100:0, 90:10, 80:20 and 70:30) were evaluated. All formulations resulted in films with good appearance and texture. Levan increased the films'

solubility, tensile strength and elongation while decreasing their water vapour permeability (Mantovan et al., 2018)

Another study assessed the prebiotic activities of nystose and the starch-nystose film by using them as the sole source of energy for the growth of *Bifidobacterium* and *Lactobacillus* strains (Bersaneti et al., 2019). The prebiotic activity was assessed via analysis of their biomass production, final pH of fermentation media, production of organic acids and cell viability. The growth of the bacteria and the production of their organic acid, lactic and acetic acids, confirmed that nystose and the edible film can be used as prebiotics (Bersaneti et al., 2019). Finally, the quality and the shelf life of blackberries coated with the starch-nystose film were evaluated (Bersaneti et al., 2021). The blackberries were stored at 4 °C for 20 days. Starch-nystose-coated fruits had a significantly lower count of psychrotrophic microorganisms, moulds, and yeasts than the control and starch-only coated fruits, with counts still in the recommended consumption range after 7 days. The starch and starch-nystose coatings delayed the increase in pH and loss of the weight, firmness, and anthocyanin content of the blackberries. Moreover, starch-nystose coated fruits obtained a good acceptance and purchase intent score, without significant differences with the control fruit in a sensory evaluation (Bersaneti et al., 2021)

Wang et al. (2022) investigated a levan-chitosan blend film where the levan was produced from *B. subtilis* ZW019. The levan-chitosan films had higher mechanical strength than the control chitosan films, with a ratio of 1:1 levan-chitosan having the best tensile strength. Levan addition increased the UV light absorption and water contact angle, and a reduction in water swelling and water vapor permeability. Application of the film as packaging material for fresh pork retarded the quality loss of pork, as demonstrated by a delay in pH. The addition of levan did not affect the antimicrobial properties of the film but improved its mechanical and physical properties (Wang et al., 2022).

Table 2.5. Summary of applications of levansucrase in food system

	Applications	Microbial source of LS	End Products	Functional Properties	References
Bakery	Wheat sourdough	*L. sanfranciscensis*	Levan 1-Kestose Nystose Arabsucrose Erlose	Enhancement of bread texture and volume	(Brandt, 2003; Korakli et al., 2001; Tieking et al., 2005)
	Wheat bread	*G. frateurii* TMW 2.767, *G. cerinus* DSM 9533T, *N. chiangmaiensis* NBRC 101099, *K. baliensis* DSM 14400	Levan	Increase in bread volume, anti-staling effect and reduction in crumb hardness	(Jakob et al., 2012; Jakob et al., 2013)
	Buckwheat sourdough	*G. albidus* TMW 2.1191 *K. baliensis* NBRC 16680 *N. chiangmaiensis* NBRC 101099	Levan	Increase in specific bread volume and lower crumb hardness	(Ua-Arak et al., 2016, 2017)
Juices	Fruit juice (apple, orange, strawberry, and guava)	*B. licheniformis* RN-01	NR	Decrease in high sucrose content	(Charoenwongpaiboon et al., 2021)
	Sugarcane juice	*B. licheniformis* ANT 179	Kestose Neokestose Levan		(Xavier & Ramana, 2017)
	Sugarcane juice	*B. subtilis* TISTR 001	Kestose Nystose 1-fructofuranosyl-D-nystose Levan		(Ninchan & Noidee, 2021; Noidee et al., 2023)
	Sorghum juice	*L. mesenteroides* MTCC 10508	FOS Levan		(Jadaun et al.,2019)
	Tomato juice	*L. citreum* BD1707	Levan		(Han et al., 2015)
	Fruit juices (Orange, strawberry, apple, and grape)	*P. durus* SacB	Levan		(Zhang et al., 2022)

Syrups	Maple syrup	*B. amyloliquefaciens*	Levan Oligolevan FOS		(Karboune et al.,2022; Li et al., 2015)
	Date syrup	*M. laevaniformans*	Levan		(Moosavi-Nasab et al., 2010)
	Sugarcane syrup	*Z. mobilis* ATCC 31821	Levan		(de Oliveira et al.,2007)
Molasses	Sugarcane molasses	*Z. mobilis* ATCC 31821	Levan		(de Oliveira et al., 2007)
	Sugarcane molasses	*L. mesenteroides* MTCC 10508	FOS Levan		(Jadaun et al., 2019)
	Carob molasses	*Z. mobilis*	6-Kestose	Reduction in 5-hydroxymethylfurfural	(Taştan et al.,2019)
	Sugar beet molasses	*Halomonas sp.* AAD6	Levan		(Küçükaşik et al., 2011)
	Sugar beet molasses	*B. licheniformis* NS032	Levan	Faster bacterial growth and shorter time interval to achieve maximum levan production	(Gojgic-Cvijovic et al., 2019)
	Starch molasses	*Halomonas sp.* AAD6	Levan		(Küçükaşik et al., 2011)
Other sweeteners	Table sugar	*L mesenteroides* MTCC 10508	FOS Levan		(Jadaun et al., 2019)
	Jaggery	*L. mesenteroides* MTCC 10508	FOS Levan		(Jadaun et al., 2019)
Dairy	Yogurt	*B. Velezensis BM-2*	Levan	Increase of water holding capacity and superior system stability	(Xu et al., 2022)
By-products	Cheese whey permeate and Tofu whey	*B. subtilis* CECT 39	Lactosucrose		(Corzo-Martinez et al., 2015)
	Whey permeate and Milk permeate	*G. oxydans, V. natriegens, N. aromaticivorans* and *P. graminis*	Lactosucrose		(Bahlawan & Karboune, 2022; Bahlawan et al., 2023)
Films	Starch edible film	*B. subtilis* natto	FOS	Plasticizing effect Decrease in the glass transition temperature and water vapor permeability. Increase in solubility and elongation of the films	(Bersaneti et al., 2016)
	Starch edible film	*B. subtilis* natto	Levan	Increased in the films' solubility, tensile strength and elongation Decreased in their water vapor permeability	(Mantovan et al., 2018)
	Starch edible film	*B. subtilis* natto	Nystose		(Bersaneti et al., 2019)

Starch edible film for blackberries	*B. subtilis* natto	Nystose	Increase in quality and the shelf life of blackberries	(Bersaneti et al., 2021)
Chitosan blend film for pork packaging	*B. subtilis* ZW019	Levan	Increase in UV light absorption and water contact angle. Reduction in water swelling and water vapor permeability	(Wang et al., 2022)

2.7. Conclusion

Increasing trends in healthy eating have generated significant interest in new food products with prebiotic properties. Levansucrase presents immense potential for the synthesis of functional ingredients, owing to its wide substrate specificity. It can catalyze the polymerization reaction of sucrose to produce levan, oligomerization reaction synthesizing fructooligosaccharides, and the transfructosylation of various saccharides, alcohols, and phenolic compounds. Researchers have for years investigated the product spectrum of LSs from diverse microbial sources. Much attention has been drawn to its reaction mechanism and selectivity, in view of enhancing the production of these valuable prebiotic compounds. Three approaches have particularly been adopted: discovering new LSs, modifying the amino acid sequences of LS by direct mutagenesis or fine-tuning the reaction condition. Given the high production cost of LSs, constant research is also being done to increase enzyme production. With the advanced developments in enzyme technology, the focus of LS-related studies has in recent years been expanding to the possibility of applying this enzyme in food processing. LS-reaction products are not only prized for their health-promoting effects, but also for their ability to act as techno-functional ingredients. In bakery products, levan has brought along the benefits of improving bread texture and retarding staling. In dairy products like yogurt, levan has been shown to increase water-holding capacity and system stability. Lastly, films based on levan, FOS or nystose have demonstrated their capabilities to increase the quality and shelf-life of food products. Further exploration in the domain of application of LS in food processing is no doubt to follow, given the seemingly endless possibilities of applying this enzyme in diverse food systems.

CHAPTER III. INVESTIGATING THE POTENTIAL OF PHENOLIC COMPOUNDS AND CARBOHYDRATES AS ACCEPTOR SUBSTRATES FOR LEVANSUCRASE-CATALYZED TRANSFRUCTOSYLATION REACTION

Connecting statement 1

A thorough literature review was conducted in chapter II providing background information regarding the catalytic properties of levansucrase and its various reaction products. Chapter III examines the catalytic efficiency of levansucrases from selected strains towards various phenolic compounds and carbohydrates.

The results from this study were presented at the 16th International Symposium on Biocatalysis & Biotransformations, BIOTRANS 2023, held in La Rochelle, France.

Wong Min, M; Liu, L. & Karboune, S. (2024). Investigating the potential of phenolic compounds and carbohydrates as acceptor substrates for levansucrase-catalyzed transfructosylation reaction (under review).

3. Abstract

This study characterizes the acceptor specificity of selected levansucrases (LSs) from *Gluconobacter oxydans* (LS1), *Vibrio natriegens* (LS2), *Novosphingobium aromaticivorans* (LS3), and *Paraburkholderia graminis* (LS4) using sucrose as a fructosyl donor and selected phenolic compounds and carbohydrates as acceptors. Overall, LS from *V. natriegens* LS2 proved to be the best biocatalyst for the transfructosylation of phenolic compounds. LC-MS analysis further confirmed that more than one fructosyl unit could be attached to the glycosylated phenolic compounds. The transfructosylation of Epicatechin by LS4 resulted in the most diversified products, with up to five fructosyl units transferred. In addition to the LS source, the acceptor specificity of LS towards phenolic compounds and their transfructosylation products were found to greatly depend on their chemical structure: the number of phenolic rings, the reactivity of hydroxyl groups and the presence of aliphatic chains or methoxy groups. Similarly, for carbohydrates, the transfructosylation yield was dependent on both the LS source and the acceptor type. The highest yield of fructosylated trisaccharides obtained was Erlose from the transfructosylation of maltose when catalyzed by LS2, with production reaching almost 200 g/L. LS2 was more selective towards the transfructosylation of phenolic compounds and carbohydrates, while reactions catalyzed by LS1, LS3 and LS4 also produced significant yields of fructooligosaccharides, such as kestose, nystose and fructosyl nystose. This study suggests the high potential for the application of selected LSs in the glycosylation of phenolic compounds and carbohydrates.

3.1. Introduction

Due to the promising physiological effects of levan and levan-type fructooligosaccharides (FOSs), levansucrase (LS, EC 2.4.1.10) has garnered much interest in the pharmaceutical, cosmetics and food industries. LS is a fructosyl-transferase that can catalyze the synthesis of complex oligosaccharides, by acquiring a fructosyl residue from a donor molecule and performing a non-Leloir transfer to an acceptor molecule (Hill et al., 2019; Hill et al., 2020; Tian et al., 2011; Tian & Karboune, 2012; Tian et al., 2014). The catalytic mechanism of selected LSs and their donor/acceptor specificities toward various carbohydrates have been documented (Okuyama et al., 2021; Ortiz-Soto et al., 2020; Zhang et al., 2023). However, given that the microbial source of enzymes heavily impacts their catalytic properties, constant research is necessary for newly discovered LSs.

The differences in the reaction selectivity of LSs are attributed to their amino acid sequences, which dictate their active subsite structure (Hill et al., 2020; Klaewkla et al., 2024). The cavities of LS from *G. oxydans* (LS1), *V. natriegens* (LS2) and *P. graminis* (LS4) were compared to that of the deep negatively charged pocket of *Bacillus subtilis* LS. The cavity of the LS1 had a similar shape but with less depth, and with many charged residues concentrated together on the exterior of the active site. The active site of LS2 was wider and shallower and had more positive and less negative electrostatic potential. LS4's active site was deeper and had more positive electrostatic potential. Comparing the least binding energy conformation of sucrose with the LSs, sucrose was rotated 45° downwards with LS1 but was orientated in the same way as the *B. subtilis* LS with LS2 and LS4 (Hill et al., 2020). In addition, while previous studies have reported that the fructose residue of the donor sucrose molecule binds the -1 subsite with high affinity, it was suggested that the docking of acceptor molecules could be variable because of the +1 subsite's relaxed binding nature. The +1 subsite can participate in both donor and acceptor binding (Meng & Fütterer, 2008; Ozimek et al., 2006; Visnapuu et al., 2011).

In addition to the carbohydrate acceptors, aromatic compounds and aliphatic alcohols can be utilised as acceptor molecules. Butanol, hydroquinone and benzyl alcohol were successfully used as fructosyl acceptors by LS from *B. subtilis* (Mena-Arizmendi et al., 2011). The

fructosylation of hydroquinone was also investigated with LS from *Leuconostoc mesenteroides*, synthesizing a hydroquinone fructoside, 4-hydroxyphenyl-β-D-fructofuranoside (Kang et al., 2009). Then, isopropanol and 1- pentanol were as well able to act as fructosyl acceptors using *Bacillus licheniformis* 8-37-0-1, producing isopropyl and pentyl fructosides, respectively (Lu et al., 2014). In-depth studies are needed to investigate and modulate the ability of phenolic compounds to act as acceptor substrates for LS-catalyzed transfructosylation reactions. The functional benefits of phenolic compounds have been intensely studied over the years and are prized for their antitumor, antioxidant, antibacterial, and anti-inflammatory properties, while also showing great potential in preventing and treating cardiovascular or cerebrovascular diseases (Manach et al., 2005; Xu et al., 2016). Yet, despite the validated importance of phenolic compounds, their poor solubility in water and easy degradation by light irradiation in aqueous solutions have limited their applications (Sato et al., 2000). One approach suggested to cater for these drawbacks is glycosylation (Xu et al., 2016). The positive influence of glycosylation of phenolic compounds on drug properties, including pharmacokinetics, pharmacodynamics, solubility, mechanism and potency has been confirmed (Gantt et al., 2011). Enzymatic glycosylation has further shown to be an interesting alternative to chemical methods that are often labour-intensive procedures requiring multistep synthetic routes that produce low overall yields. Furthermore, the use of toxic catalysts and solvents, and the production of significant waste could be avoided using an enzymatic approach (Desmet et al., 2012; Zhu & Schmidt, 2009). The enzymatic glycosylation of phenolic compounds is indeed still of great interest. For instance, a self-sufficient biocatalyst was developed by co-immobilization of a glycosyltransferase, a sucrose synthase and the cofactor UDP and it successfully glycosylated piceid, phloretin and quercetin, with in situ regeneration of the UDP-glucose (Trobo-Maseda et al., 2023). Another study enzymatically glucosylated citrus flavonoids, via a cyclodextrin glucosyltransferase, to enhance their bioactivity and taste as new food additives (Liu et al., 2022). Various classes of carbohydrate-active enzymes can catalyze glycosylation reactions: 'Leloir' glycosyl transferase, transglycosidase, glycoside phosphorylase and glycoside hydrolase (GH) (Desmet et al., 2012). Nonetheless, few studies have been done regarding the potential of LSs to glycosylate phenolic compounds.

The objective of the present study was to characterize the acceptor specificity of LSs from *Gluconobacter oxydans* (strain 621H) (LS1), *Vibrio natriegens* NBRC 15636 (LS2), *Novosphingobium aromaticivorans* (LS3), and *Paraburkholderia graminis* C4D1M (LS4), towards various phenolic compounds and carbohydrates using sucrose as a fructosyl donor. LS from these strains were selected following a genome mining carried out in our previous study (Hill et al., 2019). Both the bioconversion of substrates and end-product profiles were investigated. Furthermore, the reaction selectivity of selected LSs towards transfructosylation and hydrolysis was assessed.

3.2. Materials and methods

3.2.1. Materials

Sucrose, D-(-)-fructose, D-(+)-glucose, (+)-catechin hydrate, gallic acid, vanillic acid, chlorogenic acid, rosmarinic acid, (-)-epicatechin, caffeic acid, D-(+)-Maltose monohydrate, D-Sorbitol, D-(+)-Cellobiose, α-Lactose monohydrate, myo-inositol, 3,5-dinitrosalicylic acid (DNS), potassium sodium tartrate (KNaC4H4O6), yeast extract, carbenicillin disodium salt, lysozyme from chicken egg white, DNase I, imidazole, C2H7NO2, NH4HCO3, NaOH solution were obtained from Sigma-Aldrich (Oakville, ON). Catechol, KH2PO4, K2HPO4, 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, acetone, and Pierce™ Coomassie Plus (Bradford) assay kit were provided by Fisher Scientific (Fair Lawn, NJ). Coniferyl alcohol was obtained from Thermo Fisher Scientific Inc. (Fair Lawn, NJ). 1-kestose, nystose, and 1F -fructofuranosylnystose were obtained from FUJIFILM Wako Chemicals U.S.A. Corporation (Richmond, VA). Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) standards were purchased from Bio-Rad (Mississauga, ON). 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 (Waltham, MA).

3.2.2. Production and purification of levansucrases

LSs from *G. oxydans* (strain 621H) (LS1), *V. natriegens* NBRC 15636 (LS2), *N. aromaticivorans* (LS3) and *P. graminis* C4D1M (LS4) were produced and purified as described in our previous studies (Hill et al., 2019). *Escherichia coli* BL21(DE3) cells (Invitrogen) were first transformed with the LS genes of selected strains. The cells, plated on LB agar containing 100 µg/mL carbenicillin, were precultured in an LB media also containing 100 µg/mL carbenicillin for 8-10 h at 37 °C under 250 rpm. Terrific broth containing 2% v/v of the preculture and 100 µg/mL carbenicillin was then incubated at 37 °C under 250 rpm for around 4 h, until bacterial growth turbidity of optical density of 1.2–1.6 at 600 nm was achieved. The enzyme expression was induced using 1mM IPTG and the growth of the culture was resumed at room temperature for 18 h under 250 rpm. To collect the cells, centrifugation at 4°C under 8000 rpm was carried out. The recovered pellets were resuspended in a sonication buffer (50 mM PIPES, 300 mM NaCl, and 10 % glycerol; pH of 7.2; 4 ml/g). 4 mg/g lysozyme and 4 µL/g DNase were added to the suspensions which were then incubated at 18 °C under 50 rpm for 1 h. The cells were lysed by ultrasonication using a microtip (Misonix Ultrasonic Liquid Processor S-4000, Farmingdale, NY, USA) for 1 minute (10 s on, 60 s off, amplitude of 15) in an ice bath. The supernatants containing the enzymes were recovered after centrifugation at 4 °C under 14,000 rpm for 1 h, dialyzed against potassium phosphate buffer (5 mM; pH of 6) using a membrane with a molecular weight cut-off of 6–8 kDa, and then lyophilized. The LSs were purified via immobilized metal affinity chromatography on a HisTrap™ FF column (5 ml, GE Healthcare). After loading the resolubilized crude enzyme, the column was subsequently washed with sonication buffer, wash buffer (50 mM PIPES, 300 mM NaCl, and 10 % glycerol; pH of 6.4), 5 mM imidazole, and 10 mM imidazole. LS enzyme was then eluted with 100 mM and 200 mM imidazole. SDS-PAGE electrophoresis analysis at 120 V using 15% SDS polyacrylamide gels and a 10x diluted Tris/Glycine/SDS buffer was performed to confirm the purity of the LSs.

The total specific activity of the purified enzyme was quantified as the total amount of reducing sugars produced per minute per mg of protein using a DNS test as described by Hill et al. (2019). The LS fractions with the highest purity and specific activity were pooled and stored at − 80 °C.

3.2.3. Enzymatic biotransformation reactions.

3.2.3.1. Transfructosylation of phenolic compounds

For the LS catalyzed transfructosylation reaction of phenolic compounds, 5 U/mL of LS was incubated with 0.9 M sucrose and 0.03 M phenolic acceptor molecules in 10% DMSO at optimal temperature and pH of the selected LSs (LS1 and LS4- 50 mM ammonium acetate at pH 4/30 °C; LS2- 50 mM ammonium bicarbonate at pH 8/45 °C; LS3- 50 mM ammonium bicarbonate at pH 6/45 °C). The phenolic compounds studied included catechol, catechin, epicatechin, coniferyl alcohol, gallic acid, caffeic acid, chlorogenic acid, rosmarinic acid and vanillic acid. All reactions were performed in duplicates under 50 rpm. The biotransformation reactions were carried out over a time course of 48 h where aliquots were taken, placed in boiling water for 5 min to stop the reaction, and then stored at -20 °C until further analysis. The bioconversion yields of phenolic acceptors were quantified via HPLC. The separation was performed on an Agilent Zobrax SB-C18 reversed-phase column (250 mm × 4.6 mm, 5 µm), using a Beckman HPLC system equipped with an autosampler (Model 508), a UV/VIS DAD (Model 168) with computerized data handling and integration analysis (32 Karat, version 8). The samples were prepared by diluting them in 10:90 acetonitrile: water (v/v). They were analyzed using either of the two following gradient of water/formic acid 0.05% (v/v) and acetonitrile/formic acid 0.05% (v/v) (90/10 at 0 min, 50/50 at 20 min, 5/95 at 20.1 min and 90/10 at 35 min) or (90/10 at 0 min, 5/95 at 5 min, 90/10 at 25 min and 90/10 at 30 min) at a flow rate of 0.700 mL/min. The phenolic compounds were quantified using UV detection at 254 nm. The bioconversion yields were calculated as the difference between the initial and final concentrations of phenolic compounds as a percentage of the initial concentration of phenolic compounds.

3.2.3.2 Transfructosylation of selected carbohydrates

For the LS-catalyzed transfructosylation reaction of carbohydrate and sugar alcohol acceptors, maltose, cellobiose, lactose and sorbitol, 5 U/mL of LS was incubated with 0.9 M sucrose and 0.45 M acceptor substrate. The biotransformation reactions were carried out at optimal temperature and pH of the selected LSs (LS1 and LS4- 50 mM ammonium acetate at pH 4/30 °C; LS2- 50 mM ammonium bicarbonate at pH 8/45 °C; LS3- 50 mM ammonium bicarbonate

at pH 6/45 °C). All reactions were performed in duplicates under 50 rpm over a time course of 48 h where aliquots were taken, placed in boiling water for 5 min to stop the reaction, and then stored at -20 °C until further analysis.

3.2.4. Reaction Selectivity (hydrolysis vs transfructosylation)

After the enzymatic biotransformation reactions, the remaining sucrose as well as the released glucose and fructose 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 of 20 mM sodium hydroxide at a flow rate of 0.4 mL/min and 32 °C. The hydrolysis extent of sucrose was quantified as the concentration of released fructose taken as a percentage of the initial sucrose concentration, while the extent of sucrose transfructosylation was based on the difference between the concentrations of released fructose and glucose as a percentage of the initial sucrose concentration.

1) $$Transfructosylation\ yield\ (\%) = \frac{Concentration\ of\ released\ glucose - Concentration\ of\ released\ fructose}{Initial\ sucrose\ concentration} \times 100$$
2) $$Hydrolysis\ yield\ (\%) = \frac{Concentration\ of\ released\ fructose}{Initial\ sucrose\ concentration} \times 100$$

3.2.5. End-product profiles characterization

To characterize the phenolic fructosides produced, the reaction mixtures were analyzed by LC-MS using an Agilent 1290 Infinity II LC system coupled to the 6560-ion mobility Q-TOF -MS (Agilent Technologies, Santa Clara, USA). The samples were prepared by diluting them in 50:50 acetonitrile: water. The LC separation was conducted on a Poroshell120 EC-C18 analytical column (Agilent Technologies; 2.7 µm × 3 mm × 100 mm) connected with a Poroshell120 EC-C18 guard column (Agilent Technologies; 2.7 µm × 3 mm × 5 mm). The mobile phase A was HPLC water with 0.1% formic acid and the mobile phase B was acetonitrile with 0.1% formic acid. HPLC parameters were as follows: an injection volume of 4 µL, a flow rate of 0.3 mL/min and a column temperature set to 30 °C. The mobile phase profile used for the run-in negative ion mode was 2% B (0 to 1.0 min), 2%-20% B (1.0 to 4.0 min), 20%-100% B (4.0 to 8.0 min), 100% B (4.0 to 8.0 min), hold at

100% B (8.0-13.0 min), decrease to 2% B (13.0.0 to 13.5 min) and hold 2% B (13.5 to 14 min). The mass spectrometer was equipped with a Dual AJS ESI ion source operating in negative ionization mode. MS conditions were as follows: for ESI-, the drying gas temperature was 200 °C, the drying gas flow rate was 12 l/min, the sheath gas temperature was 250 °C, the sheath gas flow rate was 12 L/min, the pressure on the nebulizer was 35 psi, the capillary voltage was 4000 V, the fragmentor voltage was 240 V, and the nozzle voltage was 1000 V. Full scan MS data were recorded between mass-to-charge ratios (m/z) 100 and 1700 at a scan rate of 2 spectra/s, and were collected at both centroid and profile mode. Reference ions (m/z at 112.9856 and 1033.9881 for ESI-) were used for automatic mass recalibration of each acquired spectrum. Targeted MS/MS fragmentation (collision energy = 10, 20 V) were performed for selected reaction products and compared with fragmentation pattern of analytical standards of phenolic compounds. Data treatment was conducted using Quanlitative Analysis B.10.0 and Quantitative Analysis B.10.0 from Agilent MassHunter Workstation Software.

The sucrose concentration and end-product profiles of fructosylated trisaccharides and FOSs were characterized using an Agilent 1290 II liquid chromatography system coupled to an Agilent 6560-ion mobility Q-TOF –MS. The samples were prepared by diluting them in 50:50 acetonitrile: water (v/v) with the addition of myo-inositol (5 ppm) to serve as an internal standard. 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 acetonitrile with 0.3% NH_4OH. The 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: a drying gas temperature of 150 °C and a flow rate of 11 L/min, a sheath gas temperature of 350 °C and a flow rate of 12 L/min, a pressure on the nebulizer of 30 psig, a capillary voltage of 4000 V, a fragmentor voltage of 200 V, a skimmer voltage of 30 V, and a nozzle voltage of 2000 V. Full scan MS data were recorded at mass-to-charge ratios (m/z) from 80 to 1100 at a scan rate of 2 spectra/s and were 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. Targeted MS/MS fragmentation (collision energy = 10, 20 V) were performed for selected oligosaccharide products. Retention time and fragment pattern were compared with that of oligosaccharide analytical standards. Data treatment was conducted using Quanlitative Analysis B.10.0. The quantification was performed using Quantitative Analysis 10.0 from Agilent MassHunter Workstation Software.

3.3. Results and discussion

3.3.1. The transfructosylation of phenolic acceptors catalysed by selected levansucrases

3.3.1.1. Time courses for the bioconversion of phenolic acceptor substrates

Fig 3.1. depicts the time courses for the bioconversion of phenolic compounds through LS-catalyzed transfructosylation reactions. *G. oxydans* LS1, *V. natriegens* LS2, *N. aromaticivorans* LS3 and *P. graminis* LS4 exhibited different acceptor specificities towards the investigated phenolic compounds. Besides vanillic acid, all tested phenolic compounds, including catechol, catechin, epicatechin, coniferyl alcohol, gallic acid, caffeic acid, chlorogenic acid and rosmarinic acid, were successfully fructosylated by at least one of the selected LSs.

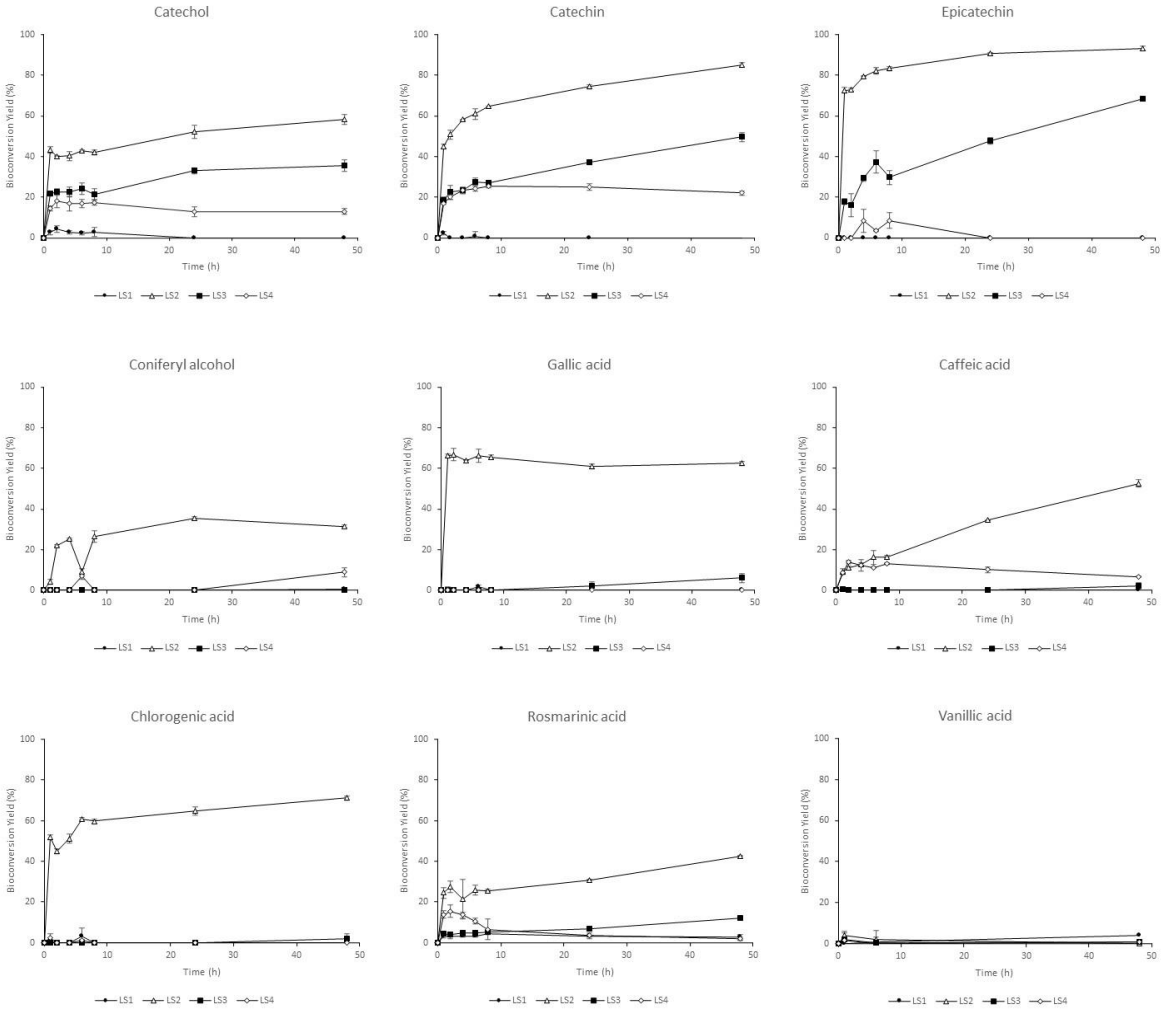


Fig 3.1. Time course for the bioconversion of phenolic compounds through levansucrase-catalysed transfructosylation (*G. oxydans* LS1, *V. natriegens* LS2, *N. aromaticivorans* LS3, and *P. graminis* LS4)

Except for *G. oxydans* LS1, all three other LSs were able to catalyse the transfructosylation of catechol, leading to a maximum bioconversion yield of 58% with *V. natriegens* LS2, 36% with *N. aromaticivorans* LS3 and 18% with *P. graminis* LS4. The results also show that the time courses for the transfructosylation of catechol were similar for all LSs with a steady increase in the bioconversion after 1 h of reaction, followed by a low increase rate before reaching a steady state at 24 h. The glycosylation of catechol was previously achieved by other enzymes including glucansucrase from *Lactobacillus reuteri* (te Poele et al., 2016), β-galactosidase from site-mutated *Lactobacillus bulgaricus* L3 (Lu et al., 2015), glucansucrase Gtf180-ΔN of *L. reuteri* 180 (Devlamynck et al., 2016) and β-xylosidase BxTW1 from *Talaromyces amestolkiae* (Nieto-Domínguez et al., 2017). Catechin was converted to a maximum yield of up to 85% with *V. natriegens* LS2, 50% with *N. aromaticivorans* LS3 and 25% with *P. graminis* LS4. No reaction equilibrium was observed in the 48 h time courses of LS2 and LS3-catalysed transfructosylation of catechin. However, a slight decrease in the bioconversion yield of catechin from 25% at 8 h to 22% at 48h occurred in LS4-catalysed transfructosylation of catechin. The glycosylation of catechin was reported by other enzymes like *Leuconostoc mesenteroides* sucrose phosphorylase (81%) (Kitao et al., 1993), *Aspergillus niger* cellulase (26%) and *Bacillus stearothermophilus* α-glucosidase (20%) (Gao et al., 2000), amylosucrase from *Deinococcus geothermalis* DSM 11300 (Cho et al., 2011) (97%) and β-glucosidase from *Novosphingobium* sp. GX9 () (Du et al., 2014). With Epicatechin, *V. natriegens* LS2, followed by *N. aromaticivorans* LS3, once again showed to be the best biocatalyst, reaching a steady rate at 24 h and a maximum bioconversion yield of 93% at 48 h. No equilibrium was reached with LS3 which achieved a final bioconversion yield of 68%. Low bioconversion yields were recorded with *P. graminis* LS4, in the range of 3-9%, between 4 to 8 h. Previously, a bioconversion of around 69% of epicatechin was reported with *L. mesenteroides* sucrose phosphorylase (Kitao et al., 1993). *V. natriegens* LS2 exhibited the highest catalytic transfructosylation efficiency on catechol, catechin and epicatechin, followed by *N. aromaticivorans* LS3 and *P. graminis* LS4. The higher acceptor specificity of LS2 and LS3 towards catechin and epicatechin than catechol may be attributed to the presence of multiple hydroxyl groups on the dihydropyran heterocycle, catechol and resorcinol moieties of catechin and epicatechin providing many potential sites for the transfructosylation. Contrary to LS2 and LS3,

P. graminis LS4 showed more or less similar acceptor specificity towards catechin and catechol, but significantly lower bioconversion yield with epicatechin. The difference in the configuration of catechin and epicatechin might partly explain their difference in acceptor specificity. Indeed, catechin has a trans configuration, unlike epicatechin, making the hydroxyl group on its dihydropyran heterocycle moiety potentially more accessible for transfructosylation When coniferyl alcohol was used as an acceptor, *V. natriegens* LS2 and *P. graminis* LS4 were able to transfer fructosyl groups from sucrose at a peak conversion of 35% and 9%, respectively. LS2 had an overall increasing trend that stabilized after 24 h. A significant drop was however recorded at 6 h. LS4 had negligible bioconversion yield except at 6 h and 48 h. The fluctuation in the bioconversion yield can be attributed to the reversible nature of the transfructosylation reaction. A shift toward the forward reaction can cause the transfer of fructosyl units to the phenolic compound, while a shift toward the reverse reaction can lead to the loss of fructosyl units from the fructosylated phenolic compound. Fructosides of coniferyl alcohol were previously reported by reactions catalyzed by β-fructofuranosidase from cell walls of *Cryptoccocus laurentii* (Dudíková et al., 2007). Then, gallic acid fructosides were produced at a significant level with *V. natriegens* LS2, with a bioconversion yield of up to 66%. Only a few studies have investigated the enzymatic production of gallic acid fructosides. For instance, the glycosylation of gallic acid by *Leuconostoc* dextransucrase at a bioconversion yield of 35.7% was reported (Nam et al., 2017). Caffeic acid was also successfully transfructosylated by *V. natriegens* LS2 at a bioconversion yield reaching 52%. No reaction equilibrium was observed for the transfructosylation of caffeic acid by LS2 over the 48 h time course; while it was achieved after 1 h in the presence of gallic acid acceptor after a high transfructosylation rate. These results reveal the high acceptor substrate affinity of LS2 towards gallic acid compared to caffeic acid. It can be hypothesized that the aliphatic chain of the caffeic acid may have hindered its binding on the active site of LS2. *P. graminis* LS4 slightly converted caffeic acid at a maximum yield of 14%. *N. aromaticivorans* LS3 exhibited low activity on gallic acid and caffeic acid acceptors with a maximum bioconversion yield of 6% and 2%, respectively. Caffeic acid glucosides have been produced by other enzymes such as glucansucrases from *Leuconostoc* and *Weissella* species (Nolte et al., 2019), glycosyltransferases

from *Arnebia euchroma* (Wang et al., 2021) and glycosyltransferase from *Paenibacillus polymyxa* NJPI29 (Chang et al., 2021).

As for chlorogenic acid, only *V. natriegens* LS2 was significantly favourable in fructosylating this acceptor substrate at a maximum bioconversion yield of 71%. No reaction equilibrium was achieved over the 48h time course. The esterification of caffeic acid with (-) quinic acid to yield chlorogenic acid enhanced the transfructosylation rate and yield catalysed by LS2; this result can be attributed to the number and location of the hydroxyl groups of the chlorogenic acid. The enzymatic synthesis of chlorogenic acid glucoside was performed using dextransucrase from *L. mesenteroides* with a production yield of 44.0% (Nam et al., 2017). With rosmarinic acid, the highest transfructosylation activity was obtained with *V. natriegens LS2* at a maximum bioconversion yield of 40%. At the initial stage of the transfructosylation reaction catalysed by LS2, the rosmarinic acid acceptor, caffeic acid ester with tyrosine, led to a higher bioconversion rate than caffeic acid; however, at the later stage, the reaction progressed at a lower rate with rosmarinic acid acceptor. On the other hand, *N. aromaticivorans* LS3 and *P. graminis* LS4 recorded a maximum bioconversion yield of 12% and 16%, respectively, for the transfructosylation of rosmarinic acid. *P. graminis* LS4 exhibited more or less the same acceptor specificity towards rosmarinic acid and caffeic acid with a reversible reaction trend at the later stage. Contrary to LS2 and LS4, insignificant transfructosylation of caffeic acid was recorded with LS3, revealing its esterification with tyrosine to yield rosmarinic acid may have favored its binding to LS3 active site. Only a few studies on the glycosylation of rosmarinic acid have been reported. One example involves the use of a glycosyltransferase from *A. euchroma* (Wang et al., 2021). Vanillic acid was the only phenolic compound tested that was transfructosylated to an insignificant level by all four LSs (below 4%). It is interesting to note that vanillic acid has only one hydroxyl group, while other selected phenolic compounds have more than one hydroxyl group available for transfructosylation. In addition, it can also be hypothesized that the methoxy group of vanillic acid at position 3 might have contributed to steric hindrance, preventing the transfer of a fructosyl group to the adjacent hydroxyl group.

As far as the authors are aware, out of the nine investigated phenolic compounds, only catechin, rosmarinic acid, gallic acid, caffeic acid and coniferyl alcohol were previously tested as

potential acceptor substrates of LS-catalyzed reactions (Núñez-López et al., 2019). Around 11%, 15%, 0%, 9% and 25%, respectively of acceptor substrate conversion were reported when 0.1 U/mL LS from *Gluconacetobacter diazotrophicus* was used to fructosylate 25 mM phenolic compounds (Núñez-López et al., 2019). Overall, LS from *V. natriegens* LS2 proved to be the most promising enzyme for the transfructosylation of phenolic compounds, successfully fructosylating all tested phenolic compounds besides vanillic acid. and achieving a maximum bioconversion yield of above 50% with catechol, catechin, epicatechin, gallic acid, caffeic acid and chlorogenic acid. *N. aromaticivorans* LS3 exhibited moderate transfructosylation activity towards catechol, catechin and epicatechin, while *P. graminis* LS4 fairly bioconverted catechol and catechin.

Then, LS from *G. oxydans* LS1, in general, did not favour the transfructosylation of phenolic compounds. In our previous study, substrate docking with homology models was performed for *G. oxydans* LS1, *V. natriegens* LS2 and *P. graminis* LS4 to examine their active site, compared to that of *B. subtilis* LS (Hill et al., 2020). The active site of LS1 had a lack of charged residues in its interior pocket compared to *B. subtilis* LS. This lack of charged residues, not seen with LS2 and LS4, might explain its lower affinity to phenolic compounds (Hill et al., 2020).

3.3.1.2. Reaction selectivity of selected levansucrases in the presence of phenolic acceptors

The most promising bioconversion reactions of phenolic acids catalyzed by LSs (LS2- all acceptors besides vanillic acid; LS1- epicatechin; LS3- catechol and catechin; LS4- epicatechin) were investigated in terms of their transfructosylation and hydrolysis extent, as shown in Fig 3.2. The hydrolysis extent of sucrose is regarded as the transfer of the fructosyl group to water resulting in the release of fructose and glucose, while the transfructosylation extent refers to the transfer of the fructosyl group to phenolic compound acceptor and/or to the transfructosylation products (e.g. fructooligosaccharides, levan, phenolic fructosides) (Hill et al., 2020; Tian & Karboune, 2012). With catechol and catechin, higher transfructosylation extents of 6 and 17%, respectively, were recorded with *V. natriegens* LS2 than with *N. aromaticivorans* LS3 (2% and 5%), in line with the higher bioconversion yield of these phenolic compounds obtained with LS2 (Fig 3.1). Reactions with LS2 and LS3 generally recorded low bioconversion of sucrose used in excess at 0.9 M. The results also reveal that the presence of phenolic compounds favored the acceptor transfructosylation reaction catalysed by LS2 and LS3 than oligo/polymerisation reactions. The highest level of hydrolysis extent with LS2 was achieved with catechin and gallic acid as acceptors at 29%, while with LS3 a maximum of 31% hydrolysis extent was reached with acceptor catechin. Indeed, in our previous study (Hill et al., 2020), up to 60% of bioconversion of sucrose was reported with reactions carried out in the presence of 0.8 M sucrose and 5-7 U/mL *V. natriegens* LS2. Similarly, the final sucrose consumption by *N. aromaticivoran* LS3 was higher at a rate of 53%. Contrary to LS2 and LS3, *G. oxydans* LS1 and *P. graminis* LS4 did not show drastic differences in sucrose bioconversion in the presence of epicatechin acceptor, with previously reported yields of 78% and 81% respectively without acceptor (Hill et al., 2020). To the authors' knowledge, this is the first study that suggests the potential of phenolic compounds as inhibitors in some LS-catalyzed reactions, preventing the formation of other LS-catalyzed end-products.

LS can indeed catalyze the formation of levan and various FOSs when sucrose is used as the sole substrate. During polymerization, the growing fructan chain acts as the acceptor, synthesizing β-(2,6) linked oligofructans to form or elongate levan. If this processive reaction mechanism is not adopted, FOSs are instead formed via a non-processive/distributive reaction

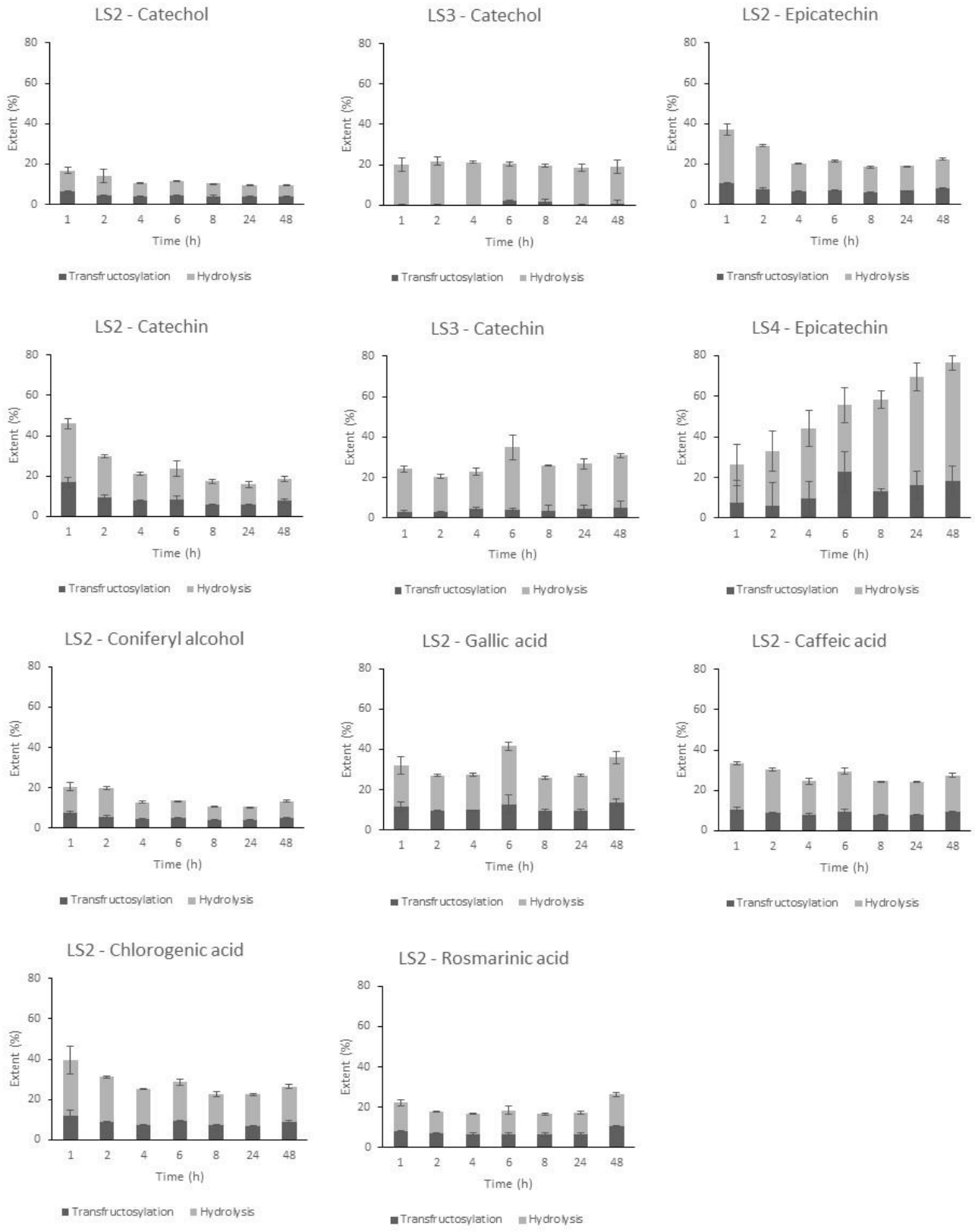


Fig 3.2. Time course for the reaction selectivity (transfructosylation/hydrolysis) of Levansucrase (LS)-catalysed reactions (*G. oxydans* LS1, *V. natriegens* LS2, *N. aromaticivorans* LS3, and *P. graminis* LS4) in the presence of phenolic compound substrates.

given the enzyme's lack of affinity for the synthesized product (Ozimek et al., 2006; Strube et al., 2011). LC-MS analysis was performed on the reaction mixtures of samples collected at 1 h, 6 h and 48 h to analyze the FOSs formed. Supplementary Figure 3.2. illustrates the MS-MS fragmentation spectra of some FOSs produced. In general, reactions with *G. oxydans* LS1 led to the highest production of FOSs such as 1-kestose, 6-kestose, 6-nystose and 6-kestose-F-F. A notable level of 6-kestose was obtained with catechin at 30.6 g/L, and reactions with coniferyl alcohol resulted in the highest concentration of 6-nystose and 6-kestose-F-F at concentrations of 52.1 g/L and 32.9 g/L, respectively. With *V. natriegens* LS2, only 1-kestose was detected at low concentrations. As for reactions with *N. aromaticivorans* LS3, the presence of trace amounts of 6G-kestose, 1-kestose and nystose were obtained. Finally, with *P. graminis* LS4, 1-kestose, nystose and fructosyl nystose were formed, with vanillic acid producing 1-kestose at an important level of 19.7 g/L. In our previous study (Hill et al., 2020), the time courses for LS-catalyzed transfructosylation reaction with 0.8 M sucrose and 5-7 U/mL LS were carried out, and the resulting FOSs were characterized. Comparing the above results with our previous ones (Hill et al., 2020), it is evident that fewer FOSs are formed when phenolic compounds are successfully transfructosylated by LSs. For instance, with LS2 around 50 g/L of 1-kestose was produced after 50 h of reaction with sucrose as sole substrate, while in the presence of phenolic compounds, insignificant levels of 1-kestose were detected. Moreover, LS1 which showed the lowest affinity to phenolic compounds produced the highest levels of FOSs. This further suggests that phenolic compounds could potentially inhibit the production of FOSs by some LSs.

3.3.1.3. Production of phenolic fructosides

The most promising reaction mixtures were also analyzed via LC-MS for the characterization of their produced phenolic fructosides. The end-product profiles characterization confirmed the successful transfructosylation of phenolic compounds catechol, catechin, epicatechin, gallic acid, chlorogenic acid, caffeic acid and coniferyl alcohol. Fig 3.3. displays the relative abundance of phenolic fructosides produced over time. The MS-MS fragmentation spectra of some fructosylated phenolic compounds obtained are shown in Fig 3.4. and Supplementary Figure 3.1. The results demonstrate that the phenolic compounds could acquire more than one fructosyl group, with up to five fructosyl groups transferred to coniferyl alcohol and epicatechin using *V. natriegens* LS2 and *P. graminis* LS4, respectively. Referring to our previous study where the substrate docking with homology models was performed for *G. oxydans* LS1, *V. natriegens* LS2 and *P. graminis* LS4 to examine their active site, compared to that of *B. subtilis* LS, the growing phenolic fructosides can be explained from the LSs subsites structure (Hill et al., 2020). Indeed, for LS2, it was found that the sucrose orientation within its active site could direct the transfructosylation products toward a concentration of charged residues running along the left side of its active site. It can hence be hypothesized that the concentrated residues on the exterior of the active site can interact with the growing fructosylated phenolic compounds, resulting in a processive reaction (Hill et al., 2020). Núñez-López et al. (2019) also obtained mono-, di-, and tri-fructosides of puerarin, coniferyl alcohol and rosmarinic acid with reactions catalyzed by LS from *G. diazotrophicus*. With mangiferin, only mono-fructosides were formed. In another study, LS from *G. diazotrophicus* synthesized phlorizin mono- di- and tri-fructosides (Herrera-González et al., 2021). Our study further confirms the hypothesis that the acceptor specificity of LS towards phenolic compounds and their transfructosylation products depends greatly on the number of phenolic rings, the presence of sugar substituents and the reactivity of the hydroxyl groups (Núñez-López et al., 2019).

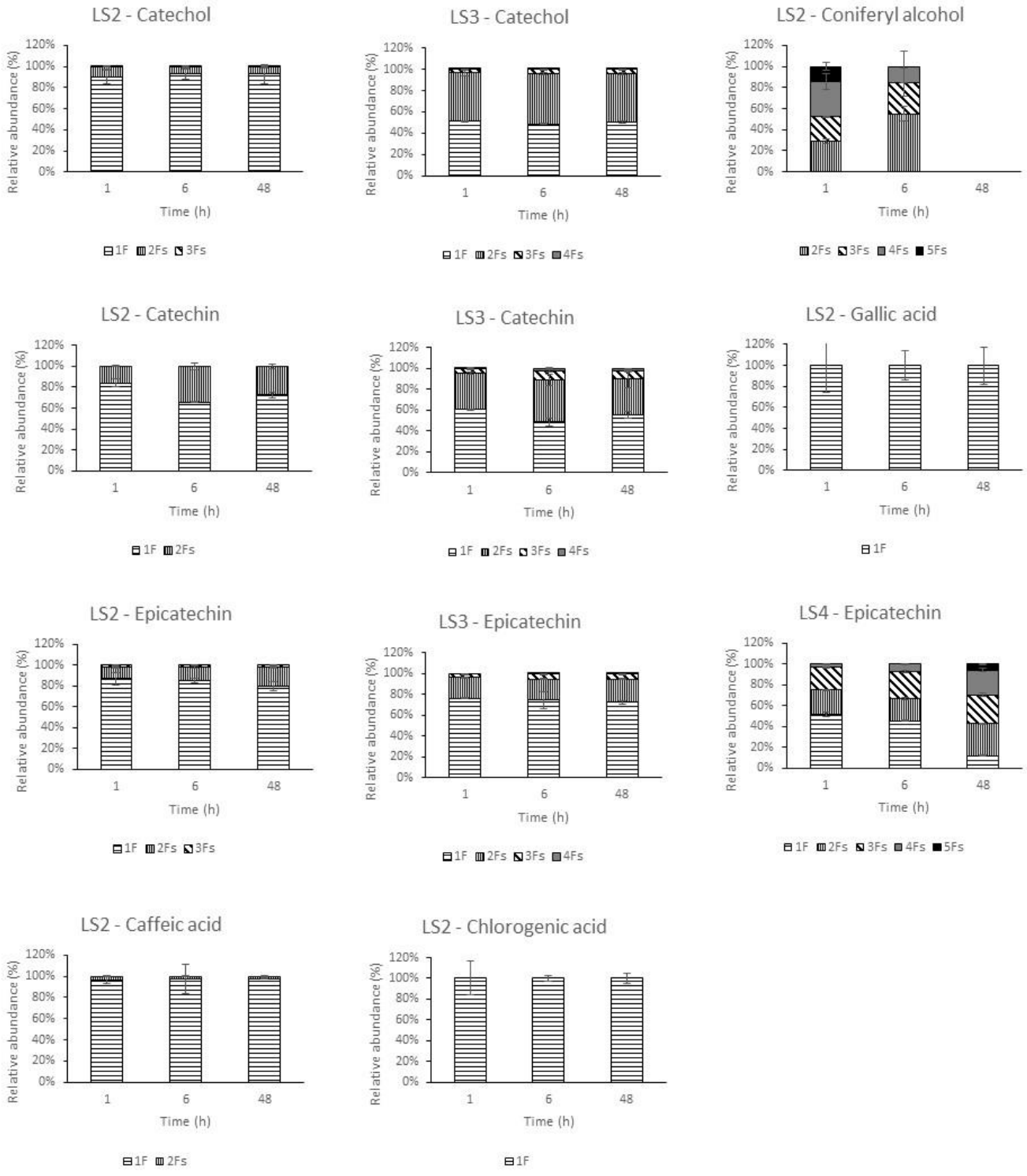


Fig 3.3. Relative abundance of phenolic fructosides where 'F' represents the number of fructosyl groups acquired by phenolic acceptors (e.g., 1F= 1 fructosyl group transferred from sucrose to acceptor substrate).

**The relative abundance was calculated as the percentage of total phenolic fructosides with the same number of acquired fructosyl groups compared to the total amount of phenolic fructosides detected*.

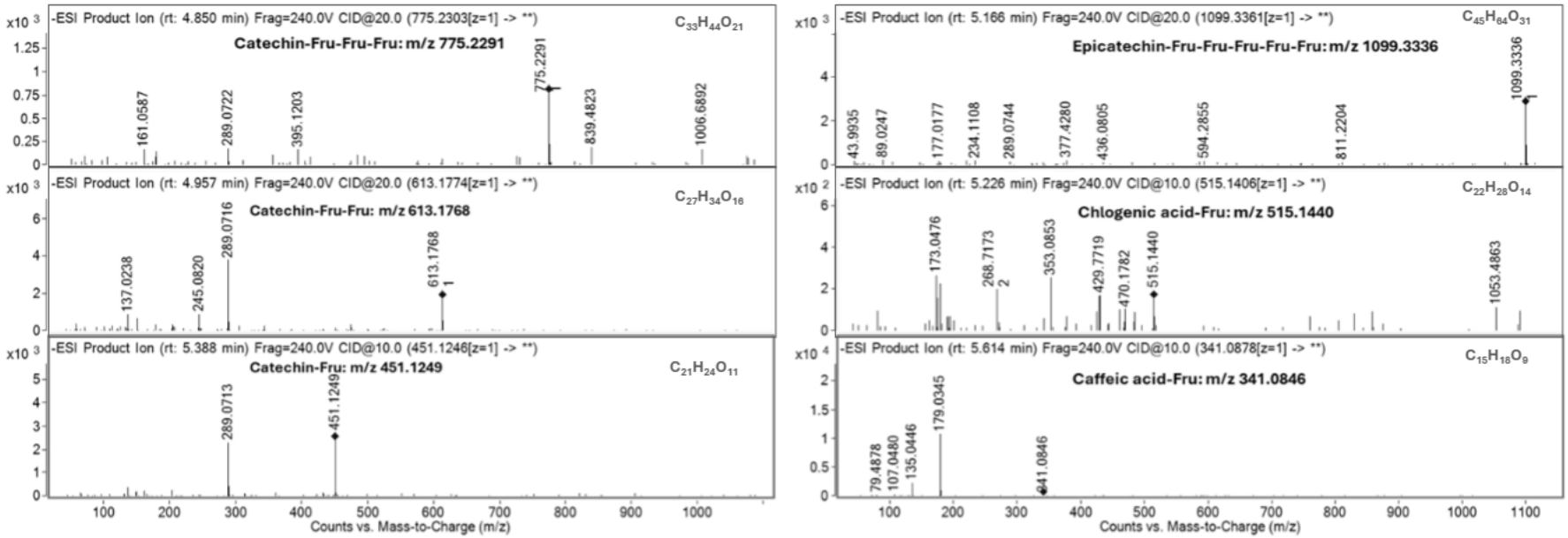


Fig 3.4. MS-MS fragmentation spectra of biotransformation end-products of LS-catalyzed transfructosylation reactions using catechin, chlorogenic acid, caffeic acid and epicatechin substrates. The major fragment for each of the end-product is its corresponding phenolic compound moiety (catechin and epicatechin moiety with m/z 289.0722, chlogenic acid moiety with m/z 353.0853 and caffeic acid moiety with m/z 179.0345). The diamond indicates parent ions.

The number of fructosyl groups acquired not only depends on the phenolic substrates but also on the source of the enzyme. For instance, with epicatechin, LS2 could acquire three fructosyl groups, LS3 four fructosyl groups and LS4 up to five fructosyl groups. On the other hand, a maximum of three and four fructosyl groups were transferred to catechol with LS2 and LS3, respectively. Then with catechin, mono- and di-fructosides were formed with LS2 as compared to mono-, di-, tri- and tetra-fructosides with LS3. Previous studies indeed demonstrated how the source of the enzyme is a key determinant in fructosides formation with LS from *B. subtilis, L. mesenteroides* and *Zymomonas mobilis* producing mainly mono-fructosides while *G. diazotrophicus* resulting in mono-, di-, tri-, tetra-, and penta-fructosides with puerarin as acceptor (Núñez-López et al., 2020). The relative abundance of each fructosylated phenolic compound as well varies with reaction time. This suggests that the fructosides formed can act as both fructosyl donors and acceptors over time. The reaction time course of the transfructosylation of phlorizin by LS from *G. diazotrophicus* also showed varying fructosides production over time (Herrera-González et al., 2021).

3.3.2. Transfructosylation of sugar acceptors catalysed by selected levansucrases

3.3.2.1. Reactions selectivity of levansucrases in the presence of sugar acceptors

The time courses for the bioconversion reaction of sucrose in the presence of sugar acceptor substrates, including maltose, cellobiose, lactose, and sorbitol were investigated (See Supplementary Table 3.1). Total sucrose bioconversion was achieved after 48 h reaction in the presence of all acceptor substrates when *G. oxydans* LS1, *N. aromaticivorans* LS3 and *P. graminis* LS4 were used as biocatalysts. With *V. natriegens* LS2, a maximum bioconversion yield of 60% was reached after 24 h. Previously, up to 78%, 60%, 53% and 81% of sucrose bioconversion were reported at 50 h with reactions carried out in the presence of 0.8 M sucrose and 5-7 U/mL LS from *G. oxydans* LS1, *V. natriegens* LS2, *N. aromaticivoran* LS3 and *P. graminis* LS4 (Hill et al., 2020). As for the bioconversion of acceptor substrates, LS1 successfully transfructosylated cellobiose and lactose; LS2 and LS4 catalysed the transfructosylation of maltose, cellobiose and lactose, and LS3 transferred a fructosyl group from sucrose to maltose and lactose. Sorbitol could not be transfructosylated by any of the selected LSs, revealing its low binding affinity to LS subsites. In general, the use of LS2 as a biocatalyst led to the highest bioconversion of acceptors

with a maximum of 86%, 80% and 34% for maltose, cellobiose and lactose transfructosylation reactions, respectively. LS4 also converted a decent amount of acceptor substrates (54%, 76% and 17% with maltose, cellobiose and lactose, respectively), while LS1 and LS3 had rather low bioconversion yields (2-4%) overall.

The transfructosylation and hydrolytic extents of the most promising reactions were then determined by quantifying the free fructose (hydrolysis) and the transferred fructose (transfructosylation). Interestingly, although only *V. natriegens* LS2 and *P. graminis* LS4 were previously found to outstandingly convert the acceptor substrates, Fig 3.5. shows that both *G. oxydans* LS1 and *N. aromaticivorans* LS3 also resulted in noteworthy transfructosylation activities. This suggests the synthesis of levan and/or FOSs by LS1 and LS3 through the transfer of the fructosyl group to the fructosyl growing chains. With maltose, LS2 did not show a particular trend in its catalytic properties, with a rather constant transfructosylation extent of around 30%. LS3 resulted in a growing transfructosylation extent, as well as overall high hydrolytic activity. The transfructosylation extent of the LS4-catalysed acceptor reaction of maltose showed a rising trend till 6 h, which then decreased at 48h, revealing the shift of the reaction towards hydrolysis. Similar trends in transfructosylation extent were observed with LS2 and LS4 in the cellobiose acceptor reactions. Furthermore, an apparent decrease in hydrolysis was altogether observed at 48h. It can be hypothesized that the release glucose may have been used as an acceptor, leading to the formation of sucrose analogues and to an apparent decrease in the hydrolysis and transfructosylation extent. With LS1 and cellobiose acceptor, a significant increase in the hydrolysis extent occurred with reaction time, while the transfructosylation extent intensified and then declined at 48h. Finally, when lactose was used as a substrate, LS1 and LS3 resulted in increasing hydrolytic and transfructosylation activities, with a higher extent recorded with LS1. LS2 tranfructosylated lactose at a declining rate while LS4 did not show any particular transfructosylation trend. Increasing hydrolysis extent could however be evident with LS4. In our previous study, similar trends were observed, with LSs showing in general higher transfructosylation activity than hydrolytic ones (Bahlawan et al., 2023)

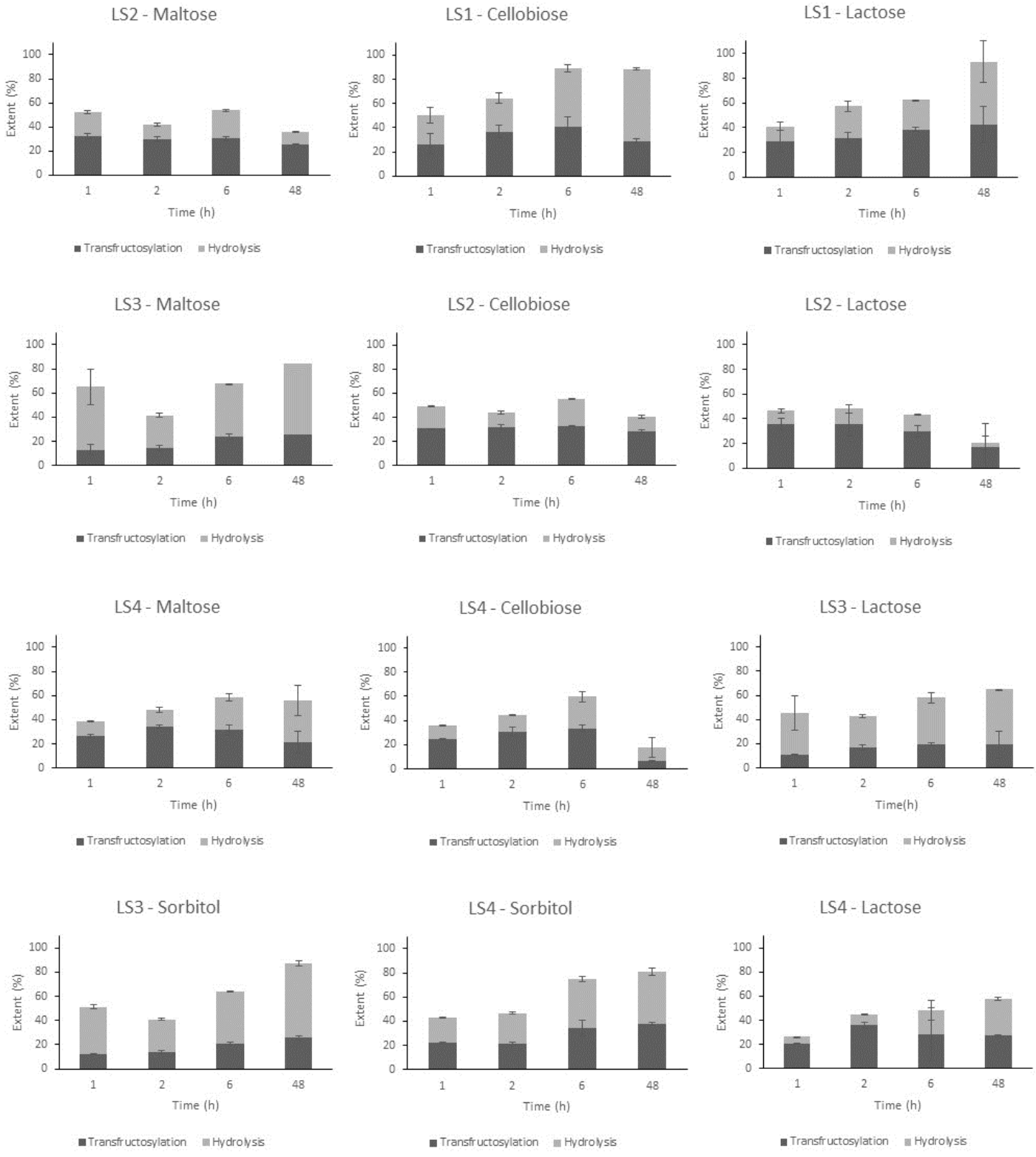


Fig 3.5. Time course for the reaction selectivity (transfructosylation/hydrolysis) of Levansucrase (LS)-catalysed reactions (*G. oxydans* LS1, *V. natriegens* LS2, *N. aromaticivorans* LS3, and *P. graminis* LS4) in the presence of sugar acceptor substrates.

3.3.2.2. Production of fructosylated trisaccharides and fructooligosaccharides

The end-product profiles of the sugar acceptor reactions were characterized via LC-MS. Overall, the fructosylated trisaccharides produced followed similar trends observed with their corresponding transfructosylation extent. In addition, the hypothesis that LS1 and LS3 produced FOSs was confirmed in Fig 3.6. Interestingly, comparing LS2 and LS4, LS2 showed a higher selectivity towards the transfructosylation of acceptor substrates maltose, cellobiose and lactose, producing only trance amounts of FOSs. Fructosylated sorbitol was not detected by LC-MS analysis, confirming that sorbitol could not act as acceptor substrates for the selected LSs. The production of FOSs in the presence of sorbitol shows that sorbitol did not have any inhibitory effect on LS-catalyzed reactions. The MS-MS fragmentation spectra of the resulting fructosylated trisaccharides and FOSs are shown in Fig 3.7. and Supplementary Figure 3.2.

When maltose was fructosylated, the non-reducing trisaccharide erlose (O-β-D-fructofuranosyl-(1,2)-O-α-D-glucopyranosyl (1,4)- α-D-glucopyranoside) was formed. Erlose is of high interest because of its sucrose-like taste and anticarious properties (Taga et al., 1993). Erlose was previously enzymatically synthesized by various LSs such as those from *B. subtilis* (Canedo et al., 1999), *Bacillus amyloliquefaciens* (Tian & Karboune, 2012), *Beijerinckia indica subsp. Indica,* as well as our selected strains (Hill et al., 2020). Results for LS from *G.oxydans* LS1are however not presented in Fig 3.7. due to insignificant concentrations of erlose detected by LC-MS. Comparing LS2, LS3 and LS4, the highest erlose amount at 200 g/L after 1 h reaction was synthesized by *V. natriegens* LS2. Overall low concentrations were obtained with LS3, with the highest of 20 g/L after 4 h of reaction. High erlose concentrations were achieved in the LS4-catalysed maltose transfructosylation reaction within the first 8 h with a maximum of 130 g/L; however, this level reduced drastically at the later stage as a result of the shift of the reaction toward the hydrolysis as shown previously with the increase in the hydrolysis extent at 48 h (Fig 3.5).

Fructosylated cellobiose was obtained in the reactions catalyzed by LS1, LS2 and LS4. The highest concentration was achieved with LS2, reaching around 180 g/L after 1 h. With LS4, around 170 g/L of fructosylated cellobiose was formed after 6 h of reaction. Finally, with LS1 a

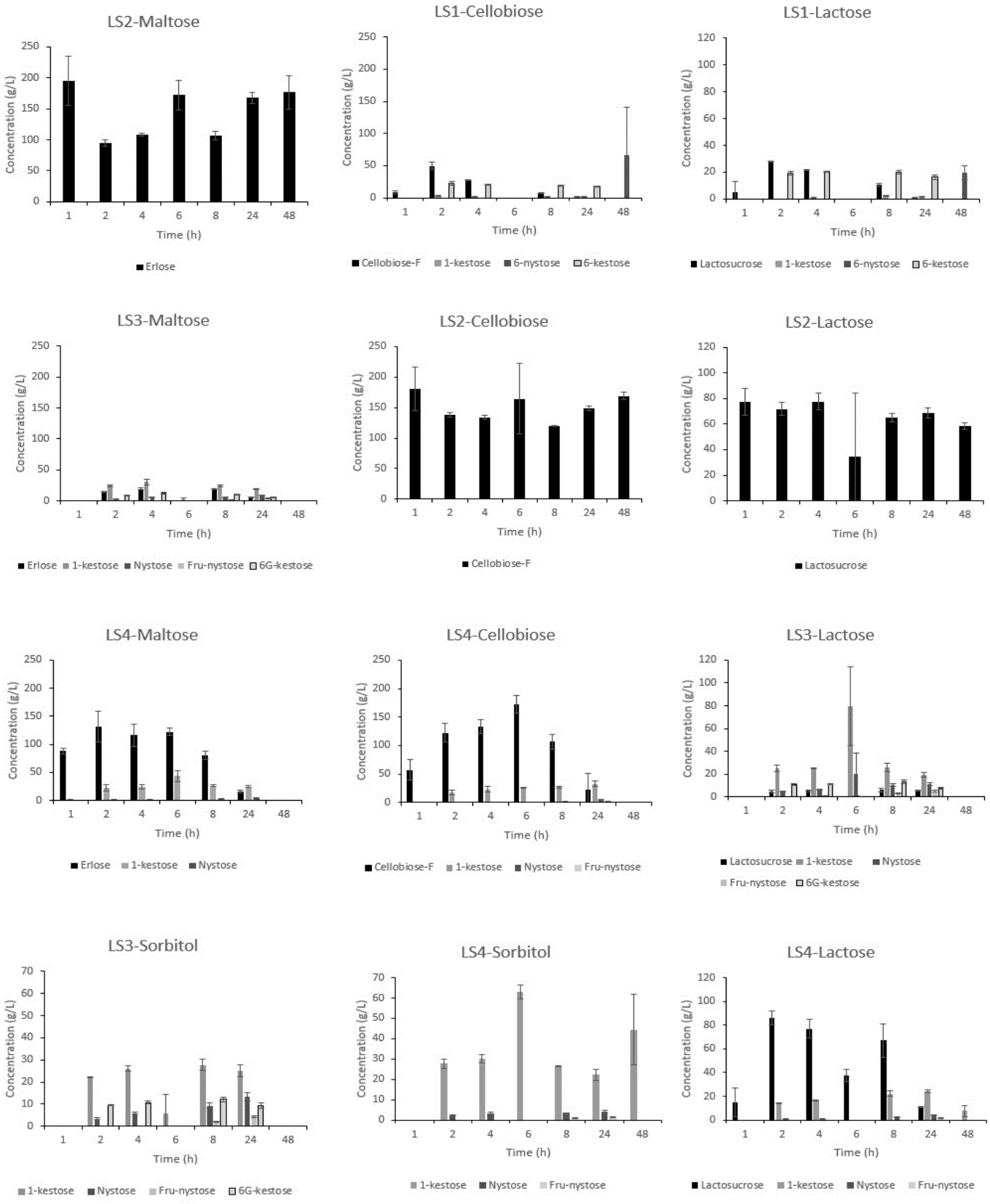


Fig 3.6. Biotransformation end-products in the presence of carbohydrate acceptors.

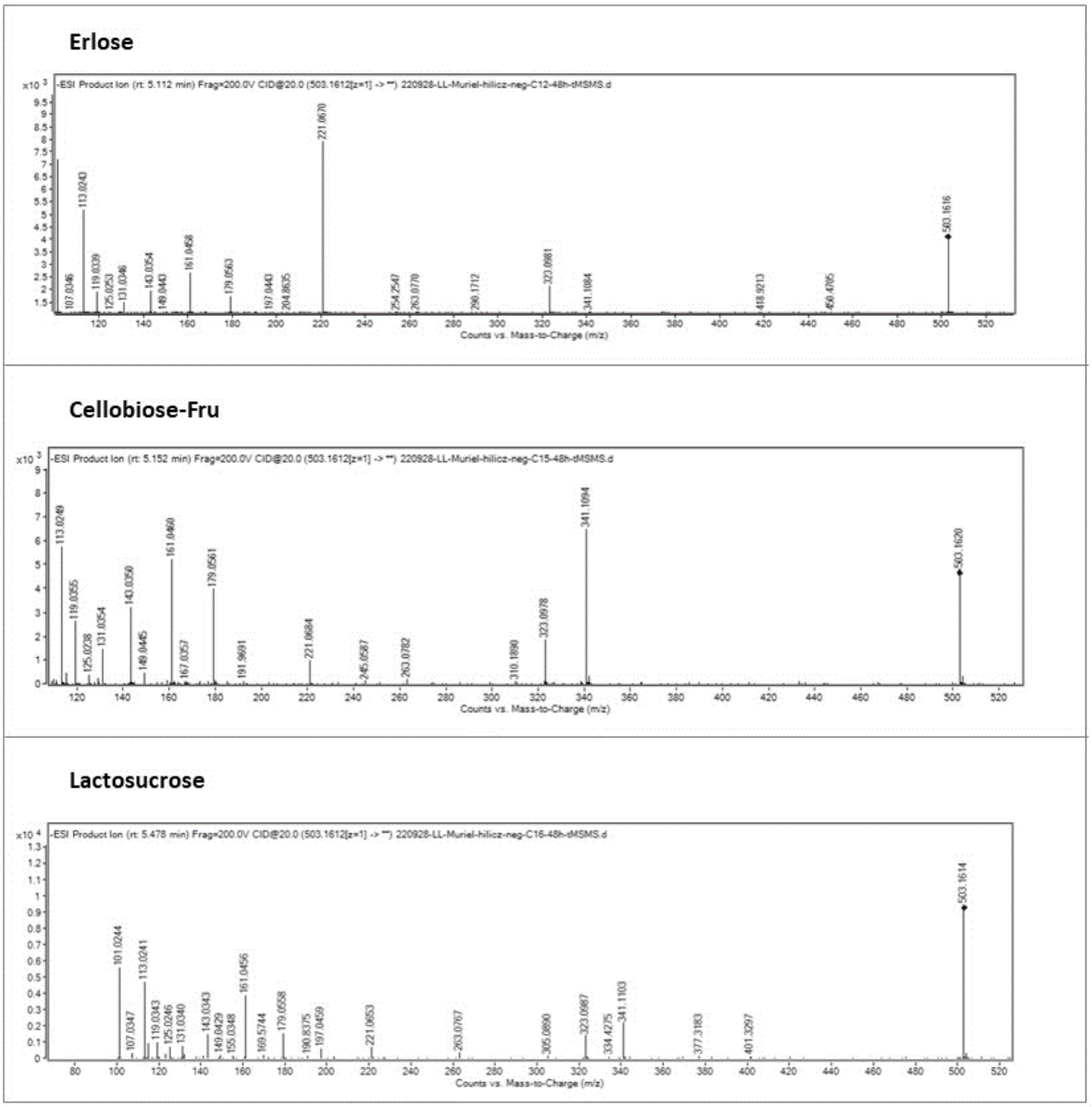


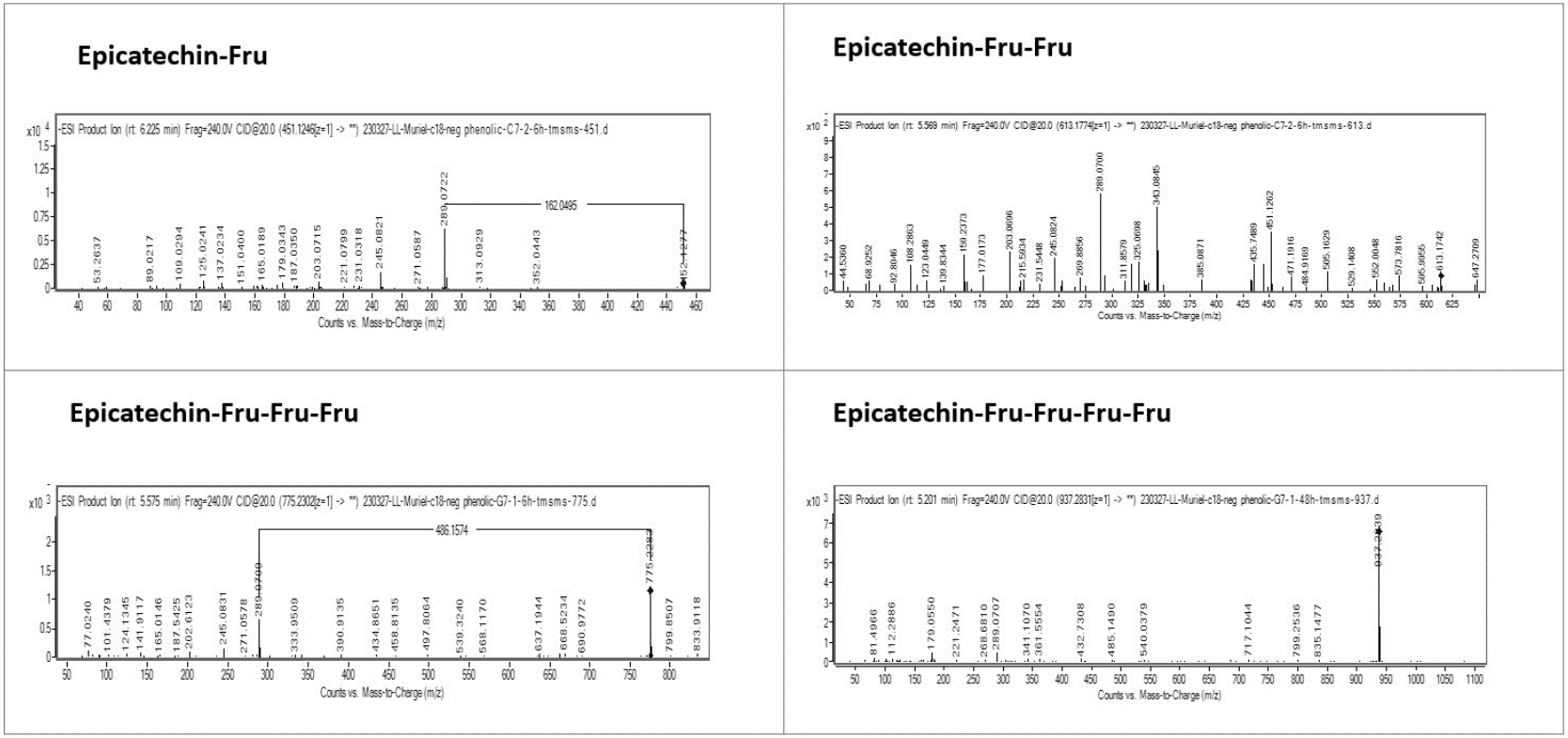
Fig 3.7. MS-MS fragmentation spectra of biotransformation end-products of LS-catalyzed transfructosylation reactions using maltose, cellobiose and lactose substrates.

maximum level of 50 g/L was reached. Besides resulting in the highest production yield, LS2 also has the advantage of being the least dependent on the reaction time. On the other hand, with LS4 or LS1, the fructosylated cellobiose concentration varies much, even drastically declining to 0 g/L at 48 h incubation. Previously, cellobiose was also fructosylated by LSs from *Halomonas smyrnensis* AAD6^T (Kirtel et al., 2018), *Microbacterium laevaniformans* (Kim et al., 2005), *B. subtilis* (Seibel et al., 2006) and *Bacillus licheniformis* 8-37-0-1 LS (Lu et al., 2014). The physiological and physicochemical properties of fructosylated cellobiose allow its use as a low-calorie sugar substitute, to replace sucrose, in food and feed industries (Biton et al., 1995).

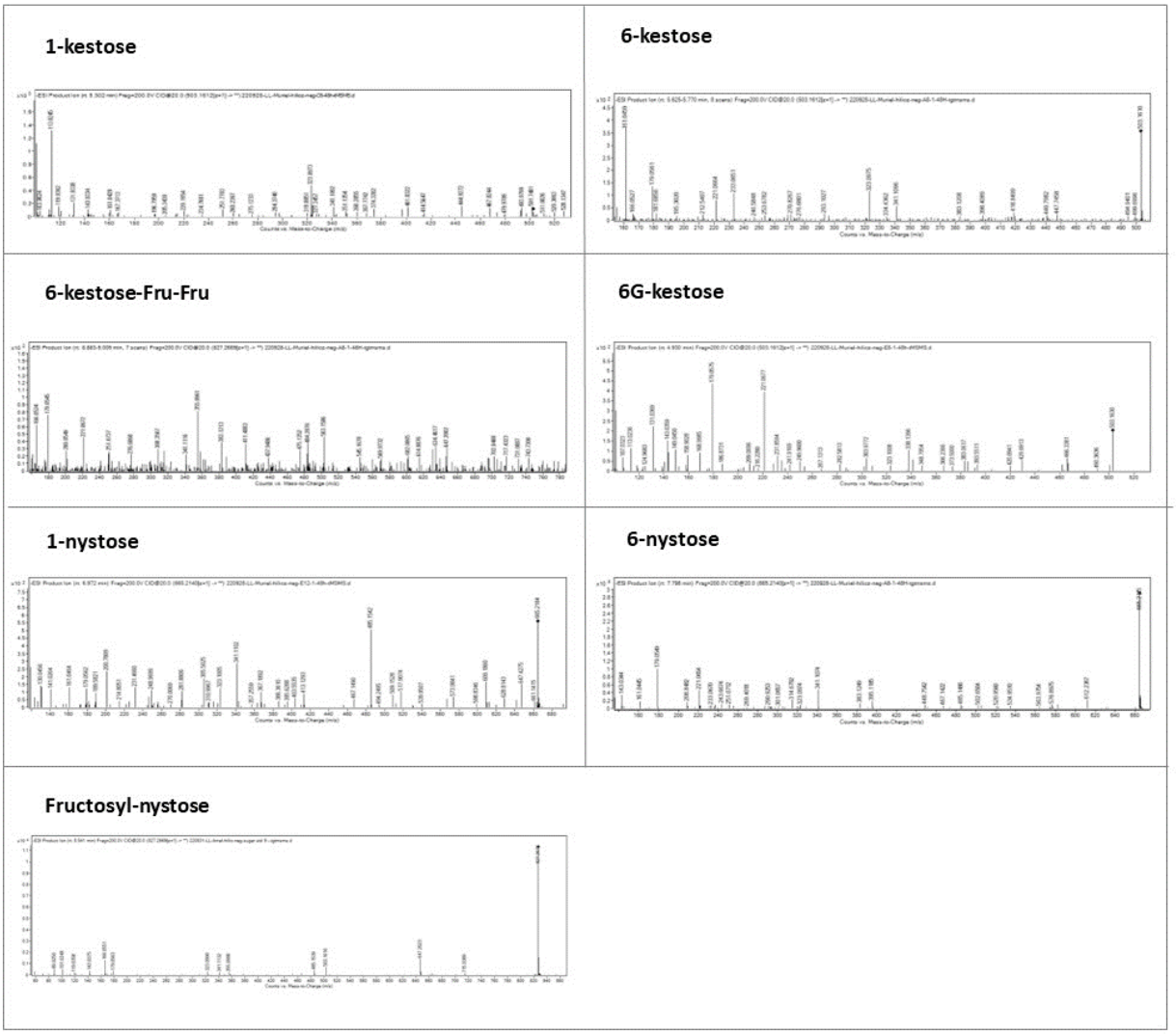
Finally, the transfructosylation of lactose was reported by all four selected LSs. The transfructosylation of lactose produces lactosucrose (O-β-D-galactopyranosyl-(1,4)-O-α-D-glucopyranosyl-(1,2)-β-D-fructofuranoside). This trisaccharide has been valued for its potential prebiotic effects (Ohkusa et al., 1995), intestinal mineral absorption properties (Teramoto et al., 2006), and the ability to reduce body fat accumulation (Kimura et al., 2002). The water-holding capacity of lactosucrose also makes it an interesting ingredient for the food industry, particularly to produce fermented milk products like yogurts or cheese, to reduce syneresis or serum separation and act as a fat replacer (Silvério et al., 2015). Although the highest lactosucrose was reported with LS4 at around 85 g/L, the production declined over time. In general, LS2 seemed to be the most reliable LS, with high and rather constant lactosucrose yields. LS1 and LS3 resulted in significantly lower production at a maximum yield of around 30 g/L and 5 g/L, respectively. Similar trends were observed in our previous study with LS2 being the best catalyst in the transfructosylation of lactose and showing great potential in the use of dairy by-products as lactose substrates (Bahlawan et al., 2023). A fluctuation in lactosucrose production was also observed. This was attributed to a possible shift of reaction towards lactosucrose hydrolysis and/or lactosucrose transfructosylation over the reaction course. In other words, lactosucrose could be utilized as a fructosyl donor and/or acceptor (Bahlawan et al., 2023). Lactosucrose was also previously found to be synthesized by LSs from *Aerobacter levanicum, Bacillus natto, B. subtilis, Brenneria goodwinii* and *L.* mesenteroides (Li et al., 2015; Mu et al., 2013; Xu et al., 2018).

3.4. Conclusion

The characterization of the acceptor specificity of selected LSs revealed that *V. natriegens* LS2 was the most efficient biocatalyst for the transfructosylation of phenolic compounds, including catechol, catechin, epicatechin, coniferyl alcohol, gallic acid, caffeic acid, chlorogenic acid, and rosmarinic acid, except vanillic acid. Catechol, catechin and epicatechin were distinguishably the most versatile acceptors, being also significantly transfructosylated by other LSs (LS3- catechol, catechin, epicatechin; LS4- catechol and catechin). LC-MS analysis further proved that more than one fructosyl unit could be attached to the glycosylated phenolic compounds, with up to five fructosyl groups transferred to coniferyl alcohol and epicatechin using LS2 and LS4, respectively. The presence of phenolic compounds was also found to act as an inhibitor, preventing the formation of other LS-catalyzed end-products (FOSs). As for carbohydrate substrates, maltose, cellobiose and lactose, they successfully acquired a fructosyl group from sucrose. No transfructosylation activity was reported with sorbitol. *V. natriegens* LS2 and *P. graminis* LS4 led to high yields of fructosylated trisaccharides. In addition, LS2 was more selective towards the fructosylation of disaccharides, while LS1, LS3 and LS4 simultaneously produced fructosylated trisaccharides and FOSs. This study highlights the potential of LS in the fructosylation of phenolic compounds and carbohydrates, resulting in the formation of functional compounds which can be of great interest to the pharmaceutical, cosmetics and food industries. Future works may include further characterization of the phenolic fructosides, determining their aqueous solubility, stability, and functional properties. The effects on levan production during the biotransformation reactions could also be investigated.



Supplementary Figure 3.1. MS-MS fragmentation spectra of biotransformation end-products of LS-catalyzed transfructosylation reactions using epicatechin as substrate.



Supplementary Figure 3.2. MS-MS fragmentation spectra of biotransformation end-products of LS-catalyzed transfructosylation reactions using phenolic compound and carbohydrate substrates.

Supplementary Table 3.1. Time course for the conversion of sucrose and substrate with carbohydrates as acceptor substrates.

	Substrate	Time (h)	Converted sucrose (%)	Converted substrate (%)
G. oxydans LS1	**Maltose**	0	0.00 ± 0.00	0.00 ± 0.05
		1	80.47 ± 1.52	0.00 ± 0.05
		6	100.00 ± 0.00	0.00 ± 0.05
		48	100.00 ± 0.00	0.00 ± 0.05
	Sorbitol	0	0.00 ± 0.00	0.00 ± 0.05
		1	57.03 ± 7.27	0.00 ± 0.05
		6	100.00 ± 0.00	0.00 ± 0.05
		48	100.00 ± 0.00	0.00 ± 0.05
	Cellobiose	0	0.00 ± 0.00	0.00 ± 0.05
		1	41.97 ± 0.55	4.06 ± 1.10
		6	100.00 ± 0.00	0.00 ± 0.05
		24	98.74 ± 0.00	1.22 ± 0.02
		48	100.00 ± 0.00	0.00 ± 0.05
	Lactose	0	0.00 ± 0.00	0.00 ± 0.05
		1	20.70 ± 4.64	2.34 ± 0.05
		6	61.80 ± 5.51	0.00 ± 0.05
		24	98.61 ± 0.05	0.59 ± 0.10
		48	100.00 ± 0.00	0.00 ± 0.05
V. natriegens LS2	**Maltose**	0	0.00 ± 0.00	0.00 ± 0.05
		1	41.18 ± 11.88	86.37 ± 17.40
		6	38.23 ± 2.89	76.15 ± 10.56
		24	50.38 ± 4.20	74.02 ± 3.92
		48	30.27 ± 0.68	78.13 ± 11.96
	Sorbitol	0	0.00 ± 0.00	0.00 ± 0.05
		1	34.49 ± 2.17	0.00 ± 0.05
		6	26.43 ± 0.05	0.00 ± 0.05
		48	29.51 ± 6.64	0.00 ± 0.05
	Cellobiose	0	0.00 ± 0.00	0.00 ± 0.05
		1	21.59 ± 2.63	79.99 ± 15.74
		6	24.86 ± 5.59	72.72 ± 25.29
		24	65.10 ± 0.13	65.90 ± 1.67
		48	19.97 ± 3.58	74.74 ± 2.78
	Lactose	0	0.00 ± 0.00	0.00 ± 0.05
		1	8.37 ± 5.70	34.12 ± 4.66
		6	36.54 ± 5.84	30.68 ± 21.69
		24	64.51 ± 0.73	30.44 ± 1.76
		48	15.53 ± 6.53	25.78 ± 1.03
	Maltose	0	0.00 ± 0.00	0.00 ± 0.05
		1	74.64 ± 7.37	0.00 ± 0.05

N. aromaticivorans LS3		6	83.11 ± 0.85	0.00 ± 0.05
		24	99.06 ± 0.03	3.48 ± 0.13
		48	100.00 ± 0.00	0.00 ± 0.05
	Sorbitol	0	0.00 ± 0.00	0.00 ± 0.05
		1	27.93 ± 9.82	0.00 ± 0.05
		6	52.51 ± 4.28	0.00 ± 0.05
		24	99.14 ± 0.08	0.00 ± 0.05
		48	100.00 ± 0.00	0.00 ± 0.05
	Cellobiose	0	0.00 ± 0.00	0.00 ± 0.05
		1	34.92 ± 2.90	0.00 ± 0.05
		6	73.38 ± 5.75	0.00 ± 0.05
		48	100.00 ± 0.00	0.00 ± 0.05
	Lactose	0	0.00 ± 0.00	0.00 ± 0.05
		1	23.68 ± 5.67	0.00 ± 0.05
		6	91.42 ± 42.68	0.00 ± 0.05
		24	98.92 ± 0.08	2.46 ± 0.19
		48	100.00 ± 0.00	0.00 ± 0.05
P. graminis LS4	**Maltose**	0	0.00 ± 0.00	0.00 ± 0.05
		1	33.09 ± 4.77	38.90 ± 2.17
		6	61.57 ± 2.46	53.85 ± 2.98
		24	95.54 ± 0.74	6.79 ± 1.08
		48	100.00 ± 0.00	0.00 ± 0.05
	Sorbitol	0	0.00 ± 0.00	0.00 ± 0.05
		1	15.34 ± 6.44	0.00 ± 0.05
		6	92.91 ± 7.56	0.00 ± 0.05
		24	98.81 ± 0.15	0.00 ± 0.05
		48	100.00 ± 0.00	0.00 ± 0.05
	Cellobiose	0	0.00 ± 0.00	0.00 ± 0.05
		1	37.41 ± 10.74	25.35 ± 8.10
		6	52.07 ± 2.22	76.18 ± 7.14
		24	94.08 ± 9.12	10.27 ± 1.29
		48	100.00 ± 0.00	0.00 ± 0.05
	Lactose	0	0.00 ± 0.00	0.00 ± 0.05
		1	23.25 ±1.73	6.49 ± 5.39
		6	45.52 ± 1.11	16.55 ± 2.22
		24	95.14 ± 0.41	4.89 ± 0.03
		48	100.00 ± 0.00	0.00 ± 0.05

CHAPTER IV. ENDOGENOUS BIOGENERATION OF PREBIOTIC FUNCTIONAL INGREDIENTS IN DAIRY PRODUCTS USING LEVANSUCRASES

Connecting Statement 2

The results from Chapter III contributed to the understanding of the acceptor specificity of selected levansucrases and the characterization of their catalytic efficiency for the biocatalytic endogenous production of functional ingredients.

Chapter IV investigates the use of levansucrase in the fortification of dairy products with functional prebiotic ingredients. This chapter starts by describing the effect of pH and temperature on the transfructosylation of lactose. Then, the application of levansucrase to reconstituted sweetened milk and chocolate milk formulations was evaluated. Finally, the assessment of levan in chocolate milk as a stabilizer was investigated.

4. Abstract

This study focuses on the application of levansucrases (LS, EC 2.4.1.10) in dairy products for the endogenous biogeneration of functional ingredients. Four LS strains from *Gluconobacter oxydans* (LS1), *Vibrio natriegens* (LS2), *Novosphingobium aromaticivorans* (LS3), and *Paraurkholderia graminis* (LS4) were selected. The effects of pH and temperature on lactose transfructosylation were first evaluated. The reaction selectivity results demonstrated that, overall, transfructosylation was predominant over hydrolysis for *G. oxydans* LS1, *V. natriegens* LS2, and *P. graminis* LS4. *V. natriegens* LS2 showed the highest potential, with high lactosucrose production even at the pH of milk (pH 6.6) and at a low temperature of 10 °C. *G. oxydans* LS1 and *P. graminis* LS4 had low lactosucrose production at pH 6.6, while *N. aromaticivorans* LS3 favoured fructooligosaccharides (FOSs) formation over that of lactosucrose. A diversified range of FOS was detected with both *N. aromaticivorans* LS3 and *P. graminis* LS4. The second part of this study focused on the possible application of LS to sweetened milk and chocolate milk. No significant changes were observed in the reaction selectivity, sucrose conversion, lactosucrose and FOS production when the chocolate milk formulations were enriched with additional cocoa powder. Finally, levan from *G. oxydans* LS1 proved to be a potential stabiliser of great interest in chocolate milk production. Less than 1% (w/w) of high molecular weight (HMW) levan or less than 0.5% (w/w) of mixed low and high molecular weight (MIX) levan was sufficient to bring the viscosity of the fortified chocolate milk equivalent to that of commercial chocolate milk.

i

4.1. Introduction

The gut microbiome has an enormous impact on human health, and its composition and functions are greatly affected by one's diet (Ercolini & Fogliano, 2018). Prebiotics have received much attention in recent years for their effects on the gut microbiome. Indeed, the global prebiotics market size was valued at USD 6.05 billion in 2021 and is expected to grow at a compound annual growth rate of 14.9% till 2030 (Grand View Research, 2022). In particular, ß-(2-6)- fructooligosaccharides (FOSs) and levans have attracted much interest as the understanding of their attributes in terms of supporting intestinal health is increasingly being recognized (Tian & Karboune, 2012; Tian et al., 2014). This has given rise to a need for efficient synthetic routes for their synthesis. Levansucrase (LS, EC 2.4.1.10) is a fructosyl-transferase, capable of catalyzing the synthesis of a diverse range of fructosyl-type prebiotics (e.g. levan, ß-2-6-FOS, lactosucrose) because of its ability to directly use the free energy of cleavage of sucrose to transfer the fructosyl to acceptors (A. Hill, Karboune, Narwani, & de Brevern, 2020). LS-catalyzed end-products, particularly levan, are not only prized for their health-promoting effects but also for their ability to act as techno-functional ingredients (Jakob et al., 2012; Xu et al., 2022).

With the advanced developments in enzyme technology, the focus of LS-related studies has in recent years been expanding to the possibility of applying this enzyme in food processing. Two different approaches can be used: LS can either be used for the endogenous biogeneration of food ingredients, i.e., the enzyme is directly added to the food product, or LS can be used to exogenously biogenerate a specific ingredient which can then be added to food products (Karboune et al., 2022; Xu et al., 2022). Endogenous biogeneration makes use of substrates already available in a food system. This can help to reduce undesired prominent levels of sugars, such as sucrose, and can be less labour-intensive than exogenous biogeneration (Charoenwongpaiboon et al., 2021; Karboune et al., 2022). Conversely, exogenous biogeneration brings the advantage of selectively producing a particular LS-catalyzed end-product by varying some factors such as temperature and substrate concentrations (Santos-Moriano et al., 2015).

Only a few studies have investigated the application of LS or LS-catalyzed end-products in selected food systems. In bakery products, levan has brought along the benefits of improving

bread texture and retarding staling (Jakob et al., 2013; Jakob et al., 2012). In dairy products like yoghurt, levan has been shown to increase water-holding capacity and system stability (Xu et al., 2022). In beverages, LS has been used to reduce sucrose content while producing prebiotic ingredients (Charoenwongpaiboon et al., 2021). Our studies and others have demonstrated the applicability of LSs in Maple syrups and molasses to endogenously biogenerate mixtures of FOS and levan (de Oliveira et al., 2007; Karboune et al., 2022; Li et al., 2015). Films based on levan, FOS or nystose have demonstrated their capabilities to increase the quality and shelf-life of food products (Bersaneti et al., 2021; Mantovan et al., 2018). Lastly, LS has brought a new approach to the valorization of agro-industrial by-products (Bahlawan & Karboune, 2022; Bahlawan et al., 2023; Corzo-Martinez et al., 2015). For instance, Bahlawan et al. (2023) have demonstrated the production of lactosucrose from whey permeate. Further exploration in the domain of application of LS in food processing is no doubt to follow, given the seemingly endless possibilities of applying this enzyme in diverse food systems.

As part of our ongoing efforts to broaden the applicability of the synthetic activity of LS in food, the objective of this study was to investigate the endogenous bioconversion of digestible sugars into non-digestible prebiotics in dairy products, with particular attention to chocolate milk. The high lactose and sucrose content of chocolate milk makes it an interesting subject. Indeed, despite its nutritional benefits being similar to regular fluid milk, one of the major concerns of chocolate milk is its high sugar content (Johnson et al., 2002; Murphy et al., 2008). The formation of lactosucrose is also of great interest since it could bring the benefits of being a prebiotic functional ingredient with both sweetening and water-holding capacity (Silvério et al., 2015). The possible production of levan should also not be overlooked. Levan has gained the attention of food scientists for its various health benefits, emulsifying and encapsulating properties, texture-forming abilities, flavour and colour fixative effect, and as a fat substitute (Bekers et al., 2001; Calazans et al., 2000; de Oliveira et al., 2007; Srikanth et al., 2015). The application of LS in beverages has so far only been explored in fruit juices with no in-depth investigation of the end-product profile characterization.

In this study, the effect of pH and temperature on the transfructosylation of lactose by LSs from *Gluconobacter oxydans* (strain 621H) (LS1), *Vibrio natriegens* NBRC 15636 (LS2),

Novosphingobium aromaticivorans (LS3), and *Paraburkholderia graminis* C4D1M (LS4) was first investigated. Then, the effects of varying the sucrose, milk powder, and cocoa powder content on reaction selectivity, sucrose conversion, lactosucrose and FOS formation were assessed on reconstituted sweetened milk and chocolate milk formulations. Finally, the rheological properties of fortified chocolate milk with levan were determined.

4.2. Materials and methods

4.2.1. Materials

Sucrose, D-(-)-fructose, D-(+)-glucose, α-Lactose monohydrate, myo-inositol, 3,5-dinitrosalicylic acid (DNS), potassium sodium tartrate (KNaC4H4O6), yeast extract, carbenicillin disodium salt, lysozyme from chicken egg white, DNase I, imidazole, C2H7NO2, NH4HCO3, NaOH solution were obtained from Sigma-Aldrich (Oakville, ON). KH2PO4, K2HPO4, 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, acetone, and Pierce™ Coomassie Plus (Bradford) assay kit were provided by Fisher Scientific (Fair Lawn, NJ). 1-kestose, nystose, and 1F -fructofuranosylnystose were obtained from FUJIFILM Wako Chemicals U.S.A. Corporation (Richmond, VA). Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) standards were purchased from Bio-Rad (Mississauga, ON). 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 (Waltham, MA). Milk powder was purchased from Fonterra Co-operative Group Limited (Auckland, New Zealand) and cocoa powder was purchased from The Hershey Company (Derry Township, PA). Two cocoa mixes (sugar, cocoa, with/without carrageenan) used in the evaluation of the rheological properties of levan were provided by a local dairy cooperative (Agropur).

4.2.2. Production and purification of levansucrases

LSs from *G. oxydans* (strain 621H) (LS1), *V. natriegens* NBRC 15636 (LS2), *N. aromaticivorans* (LS3) and *P. graminis* C4D1M (LS4) were produced and purified as described in our previous studies (Hill et al., 2019). *Escherichia coli* BL21(DE3) cells (Invitrogen) were first transformed with the LS genes of selected strains. The cells, plated on LB agar containing 100

µg/ml carbenicillin, were precultured in an LB media also containing 100 µg/ml carbenicillin for 8-10 h at 37 °C under 250 rpm. Terrific broth containing 2% v/v of the preculture and 100 µg/ml carbenicillin was then incubated at 37 °C under 250 rpm for around 4 h, until a bacterial growth turbidity of optical density of 1.2–1.6 at 600 nm was achieved. The enzyme expression was induced using 1mM IPTG and the growth of the culture was resumed at room temperature for 18 h under 250 rpm. To collect the cells, centrifugation at 4 °C under 8000 rpm was carried out. The recovered pellets were resuspended in a sonication buffer (50 mM PIPES, 300 mM NaCl, and 10 % glycerol; pH of 7.2; 4 ml/g). 4 mg/g lysozyme and 4 µl/g DNase were added to the suspensions which were then incubated at 18 °C under 50 rpm for 1 h. The cells were lysed by ultrasonication using a microtip (Misonix Ultrasonic Liquid Processor S-4000, Farmingdale, NY, USA) for 1 minute (10 s on, 60 s off, amplitude of 15) in an ice bath. The supernatants containing the enzymes were recovered after centrifugation at 4 °C under 14,000 rpm for 1 h, dialyzed against potassium phosphate buffer (5 mM; pH of 6) using a membrane with a molecular weight cut-off of 6–8 kDa, and then lyophilized. The LSs were purified via immobilized metal affinity chromatography on a HisTrap™ FF column (5 ml, GE Healthcare). After loading the resolubilized crude enzyme, the column was subsequently washed with sonication buffer, wash buffer (50 mM PIPES, 300 mM NaCl, and 10 % glycerol; pH of 6.4), 5 mM imidazole, and 10 mM imidazole. LS enzyme was then eluted with 100 mM and 200 mM imidazole. SDS-PAGE electrophoresis analysis at 120 V using 15 % SDS polyacrylamide gels and a 10x diluted Tris/Glycine/SDS buffer was performed to confirm the purity of the LSs. The total specific activity of the purified enzyme was quantified as the total amount of reducing sugars produced per minute per mg of protein using a DNS test as described by Hill et al. (2019). The LS fractions with the highest purity and specific activity were pooled and stored at − 80 °C.

4.2.3. Biotransformation reactions: Effect of pH and temperature on reaction selectivity

5 U/mL of LS was incubated with 0.9 M sucrose and 0.45 M lactose at different pH and temperatures. The biotransformation reactions were carried out at the optimal pH of each selected LS (LS1 and LS4- pH 4; LS2- pH 8; LS3- pH 6) and at the pH of milk (pH 6.6), at varying temperatures of 10, 30 and 45 °C. One unit of LS was defined as the amount of reducing sugars (glucose and fructose) produced per minute of reaction. The buffer of pH 4 was prepared using

50 mM ammonium acetate, and the buffers of pH 6, 6.6 and 8 were prepared using 50 mM ammonium bicarbonate. All reactions were done in duplicates under 50 rpm. The biotransformation reactions were carried out over a time course of 24 h where aliquots were taken, placed in boiling water for 5 min to stop the reaction, and then stored at -20°C until further analyses of reaction selectivity and end-product profile.

After the enzymatic biotransformation reactions, the remaining sucrose as well as the released glucose and fructose 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 of 0.4 mL/min and 32°C. The hydrolysis extent of sucrose was quantified from the concentration of released fructose and taken as a percentage of the initial sucrose concentration, while 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.

1) $Transfructosylation\ yield\ (\%) = \frac{Concentration\ of\ released\ glucose - Concentration\ of\ released\ fructose}{Initial\ sucrose\ concentration} \times 100$
2) $Hydrolysis\ yield\ (\%) = \frac{Concentration\ of\ released\ fructose}{Initial\ sucrose\ concentration} \times 100$

4.2.4. Biotransformation reactions: Application of levansucrases to reconstituted sweetened milk and chocolate milk

Different types of milk (a regular sweetened milk (RSM), a low sugar-sweetened milk (LSSM), a regular chocolate milk (RCM) and a low sugar chocolate milk (LSCM)) were reconstituted using milk powder, sucrose, and cocoa powder. 12.50 % (w/v) of milk powder was used which is equivalent to about 4.9 % (w/v) lactose. The regular sweetened milk and regular chocolate milk comprised 10.30 % (w/v) sucrose, while the low sugar sweetened milk and low sugar chocolate milk comprised 3.38 % (w/v) sucrose. The chocolate milk formulations had an additional 1.43 % (w/v) cocoa powder. Then from the regular sweetened milk and the low sugar sweetened milk, two more formulations were prepared with additional milk powder to reach a final concentration of 20 % (w/v) (7.83% (w/v) lactose). Similarly, from the regular chocolate milk

and the low sugar chocolate milk, two more formulations were prepared with twice the concentration of cocoa powder to reach a final concentration of 2.86 % (w/v) (See Supplementary Table 4.2).

5 U/mL of LS was incubated in the different milk formulations at optimal temperature and pH of the selected LSs (LS1 and LS4- 50 mM ammonium acetate at pH 4/30 °C; LS2- 50 mM ammonium bicarbonate at pH 8/45 °C; LS3- 50 mM ammonium bicarbonate at pH 6/45 °C). All reactions were done in duplicates under 50 rpm. The biotransformation reactions were carried out over a time course of 24 h where aliquots were taken, placed in boiling water for 5 min to stop the reaction, and then stored at -20°C until further analysis of reaction selectivity and end-product profile. The reaction selectivity was determined as previously described above.

4.2.5. End-product profile characterization of enzymatic biotransformation reactions

The sucrose concentration and end-product profiles of fructosylated trisaccharides and fructooligosaccharides (FOSs) were characterized using an Agilent 1290 II liquid chromatography system coupled to an Agilent 6560-ion mobility Q-TOF –MS. The samples were prepared by diluting them in 50:50 acetonitrile: water (v/v) with the addition of myo-inositol (5 ppm) to serve as an internal standard. 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 acetonitrile with 0.3% NH_4OH. The 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 200 V, skimmer voltage of 30 V, 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 were 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.

4.2.6. Assessment of levan as a stabilizer in chocolate milk

4.2.6.1. Levan production

Levans were produced from sucrose through LS-catalyzed transfructosylation reaction using LS from *G. oxydans* (LS1). The enzymatic reactions were initiated by adding 0.9 U/mL LS to reaction mixtures containing 0.5 M sucrose. To produce the high molecular weight levan (HMW, 1700–5700 kDa), 50 mM sodium acetate buffer of pH 5 was used. For the mix low/high molecular weight levan (MIX, 860–2700 kDa (high); 4–5 kDa (low)), 50 mM potassium phosphate buffer of pH 6 was used. The reactions were incubated at room temperature for 48 h. To recover levans, ethanol was added to the reaction mixtures at a 2:1 (v/v) ratio, left overnight, and centrifuged at 9800g for 20 min. The recovered levans were dialyzed against water through a Spectra/Por® 6 membrane with a cut-off of 1000 Da at 4 °C. Levans were then freeze-dried and stored at − 80 °C.

4.2.6.2. Rheological measurements

HMW levan (1, 3, 5% w/w) and MIX levan (0.5, 1, 2.5% w/w) were added at selected concentrations to a chocolate milk composed of 91.05% milk 1% fat, 8.13% liquid sugar (67.5% sucrose) and 0.82% cocoa mix (without carrageenan). The rheological properties of the chocolate milk formulations were determined using a stress-controlled rheometer (AR2000 Rheometer, TA Instrument, New Castle, DE) fitted with a stainless steel 60 mm cone of 2° and solvent trap. The measurement temperatures (15, 37, 60 °C) were kept using a circulating bath and a controlled Peltier system. The samples were loaded to the rheometer immediately after being homogenized at 6000 rpm for 1 min using a Fisherbrand™ 850 homogenizer (Fisher Scientific, Fair Lawn, NJ). Steady-state flow parameters including flow behavior index (n), consistency coefficient (m) and shear viscosity (η) were determined at increasing shear rates (1-100 s^{-1}). The apparent viscosity was measured as a function of shear rate ($\dot{\gamma}$). Experimental flow curves were compared to the Power-law model. The variations of consistency coefficient (m) and flow behaviour index (n) were then determined for each sample.

3) $\eta = m\dot{y}^{(n-1)}$

where n <1 for a shear-thinning fluid and n = 1 for a Newtonian fluid.

ŋ: shear viscosity

m: consistency coefficient

ẏ: shear rate

n: flow behavior index

4.3. Results and Discussion

4.3.1. Time courses for transfructosylation of lactose: Effect of pH and temperature

4.3.1.1. Reaction selectivity of levansucrase-catalyzed reactions

LS can catalyze four types of reactions: polymerization, oligomerization, transfructosylation, and hydrolysis. During polymerization and oligomerization, sucrose acts as both the acceptor and donor substrate forming levan and FOSs respectively. During polymerization, the growing fructan chain acts as the acceptor, synthesizing β-(2,6) linked oligofructans to form or elongate levan. If this processive reaction mechanism is not adopted, FOSs are instead formed via a non-processive/distributive reaction given the enzyme's lack of affinity for the synthesized product (Caputi et al., 2013; Strube et al., 2011). Transfructosylation involves other substrates besides sucrose. For instance, glucose as an acceptor leads to the synthesis of sucrose or blastose, while the reaction with lactose forms lactosucrose (Li et al., 2015). Finally, water is the acceptor molecule during hydrolysis, releasing glucose and fructose (Li et al., 2015). The reaction selectivity of LSs is greatly dependent on the type of LS and the initial reaction conditions, including temperature and pH (Inthanavong et al., 2013).

Fig 4.1a and Fig 4.1b show the transfructosylation and hydrolysis extents of the LS-catalyzed reactions over a time course of 24 h. In general, the transfructosylation of sucrose and lactose was favoured over the hydrolysis of sucrose for *G. oxydans* LS1, *V. natriegens* LS2 and *P. graminis* LS4. *N. aromaticivorans* LS3 favoured the hydrolysis of sucrose. At pH 4, *G. oxydans* LS1 showed increasing catalytic activities over reaction time, especially noticeable at 10 °C at which the transfructosylation extent (14-73%) was more dominant than the hydrolysis one (2-19%). It was also previously reported that at low temperatures transfructosylation is favored and as temperature increases, hydrolysis increases (Hill et al., 2019). This was hypothesized to be a result of increased energy transferred to the enzyme as temperature rises. This eventually may lead to an increase in vibrations which may make it more difficult for larger acceptor molecules, compared to smaller and ubiquitous water molecules, to enter the LS's active site (Hill et al., 2019). However, at pH 6.6, which is the pH of milk, very low catalytic activities were recorded for *G. oxydans* LS1, not exceeding a total of 10%. This indicates that the application of *G. oxydans* LS1 might be more suitable for moderately acidic food systems with a pH range of about 3.0–5.0.

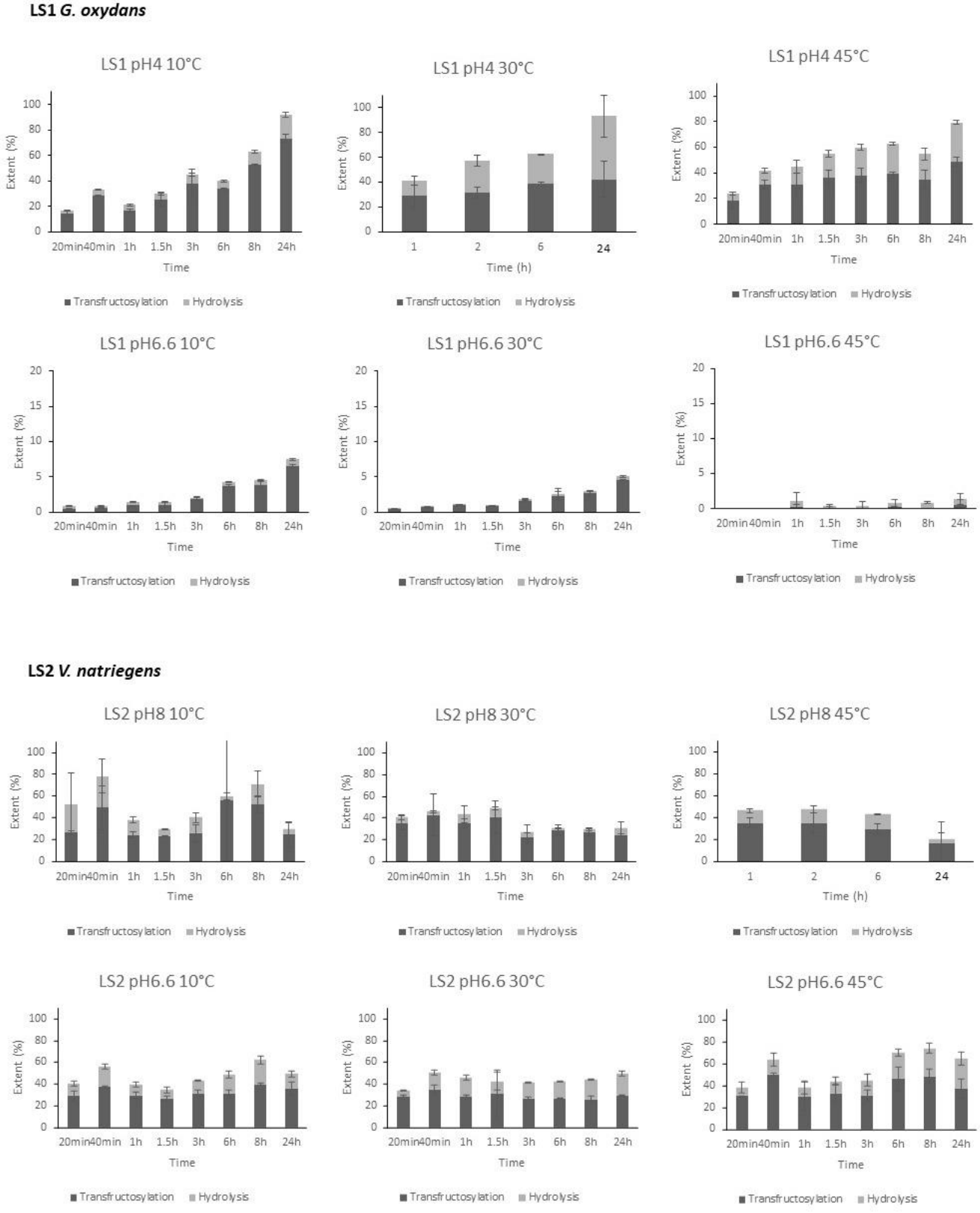


Fig 4.1a. Reaction selectivity (transfructosylation over hydrolysis) of *G. oxydans* LS1 and *V. natriegens* LS2 with varying pH and temperature in the presence of sucrose and lactose as substrates

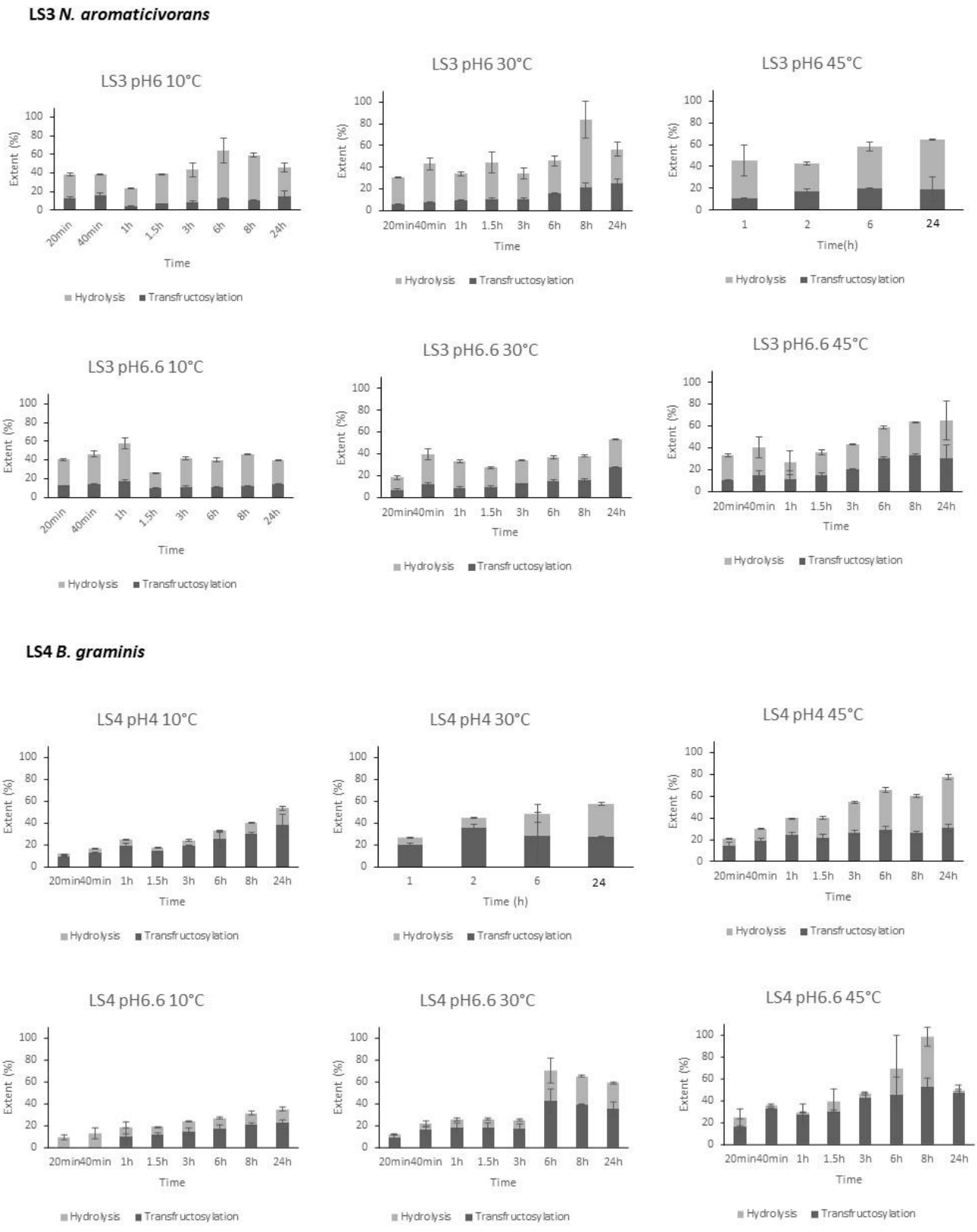


Fig 4.1b. Reaction selectivity (transfructosylation over hydrolysis) of *N. aromaticivorans* LS3 and *P. graminis* LS4 with varying pH and temperature in the presence of sucrose and lactose as substrates

Indeed, a previous study has successfully incorporated an immobilized cross-linked enzyme aggregates of Y246S variant LS from *Bacillus licheniformis* RN-01 in fruit juices, leading to up to 65%–75% of total sucrose successfully converted (Charoenwongpaiboon et al., 2021). *V. natriegens* LS2 proved to be the most promising catalyst for dairy products, with high and almost constant transfructosylation extents over time, at pH 6.6, irrespective of temperature (10°C, 30°C, 45°C) changes. At pH 8, no particular trend was also observed in the transfructosylation extents at different temperatures; however, the fluctuations were more apparent and drastic, especially at 10 °C (23-57%). The results obtained at pH 6.6 and 10 °C are of particular interest given these conditions would be ideal during the processing of dairy products. At these conditions, the transfructosylation extents remained in the 30-40% range. Out of the four selected LSs, *N. aromaticivorans* LS3 was the only one to favour the hydrolysis reaction of sucrose over the transfructosylation one. An increasing trend in transfructosylation over time is however still notable at 30 and 45 °C, increasing from around 5% to 25% at pH 6/6.6 30 °C, 10% to 20% at pH 6 45 °C and 10% to 30% at pH 6.6 45 °C. On the other hand, *P. graminis* LS4 showed increasing catalytic activities over time at both pH 4 and pH 6.6. It is also noteworthy that these activities increased with increasing temperature from 10 to 45°C. Regarding the reaction selectivity at pH 4, the transfructosylation of sucrose and lactose was more favoured at low temperatures over the hydrolysis one. However, at pH 6.6, the transfructosylation reaction was dominant at all temperatures, revealing that the ionization state of LS4 at pH 6.6 may have made the binding at the subsites temperature independent.

Previous studies have been done on the effect of pH and temperature on LS-catalyzed reactions. Although they mainly focus on the reaction selectivity in the presence of sucrose alone and not in the presence of both sucrose and lactose, these results can be still used as references for this study. From literature, a temperature of 50-60 °C has been shown to usually favour sucrose hydrolysis, while lower temperatures of 10-40 °C mainly result in polymerization and/or transfructosylation (Chambert & Gonzy-Treboul, 1976; Vigants et al., 2013; Visnapuu et al., 2015). However, this does not apply to all LSs.

For instance, LS from *Brenneria goodwinii* had optimum temperatures for transfructosylation, sucrose hydrolysis, and total activity of 35, 45, and 40 °C, respectively (Liu et

al., 2017). The optimum pH of LS is usually around pH 5-7 and in general, pH changes do not affect the rate of hydrolase and transferase activities (Homann et al., 2007; Visnapuu et al., 2015). Yet, like temperature, the effect of pH on reaction selectivity varies for different LSs. For instance, transfructosylation was favoured over hydrolysis within the pH range of 6-6.5 for LS from *Geobacillus stearothermophilus* (Inthanavong et al., 2013).

4.3.1.2. End-product profiles of levansucrase-catalyzed reactions

The end-product profiles of levansucrase-catalyzed reactions in the presence of lactose and sucrose were characterized via LCMS. The time courses for the lactosucrose synthesis are depicted in Fig 4.2. With *G. oxydans* LS1, a significantly higher amount of lactosucrose (50 g/L at 10 °C; 29 g/L at 30 °C; 60 g/L at 45 °C;) was produced at pH 4 than at pH 6.6 (14 g/L at 10 °C, 9 g/L at 30 °C; 3 g/L at 45 °C). These results are in accordance with the difference in the reaction selectivity of LS1 at pH 4 and pH 6.6 discussed above (Fig 4.1a). The production of lactosucrose also fluctuates with time. For instance, at pH 4 45 °C a maximum of 60 g/L was reached after 3 h of reaction, but a subsequent decrease to 36 g/L followed at 6h. The lactosucrose production then increased to 58 g/L after 24 h of reaction. This shift in the thermodynamic equilibrium of the transfructosylation of lactose indicates that the reaction might have shifted towards lactosucrose hydrolysis, as reported in our previous study (Bahlawan et al., 2023). In Fig 4.1a, a drastic drop at 6h in the transfructosylation extent was however not recorded, suggesting that the transfructosylation of sucrose did not undergo any shift but sucrose was instead used to transfructosylate other end-products such as levan or oligolevan (See Supplementary Table 4.1). As for *V. natriegens* LS2, its relatively higher and almost constant transfructosylation extents are undeniably reflected in the lactosucrose synthesis that for the most part remained in the 60-106 g/L range. It is also important to note that this enzyme had the overall highest lactosucrose production of 106/L at pH 6.6 and 10°C. A significant lactosucrose yield of 105 g/L was also achieved at pH 8 and 10 °C. Then, with *N. aromaticivorans* LS3 a very low amount of lactose was transfructosylated. Not much difference was observed between pH 6 and pH 6.6, with the highest concentration of lactosucrose reaching around 5 g/L. Finally, *P. graminis* LS4 had a particularly high lactosucrose production of 85 g/L at pH 4 and 30 °C, which was reached after 2 h of reaction; thereafter, a significant decrease to 38 g/L was obtained, revealing the shift in the reaction

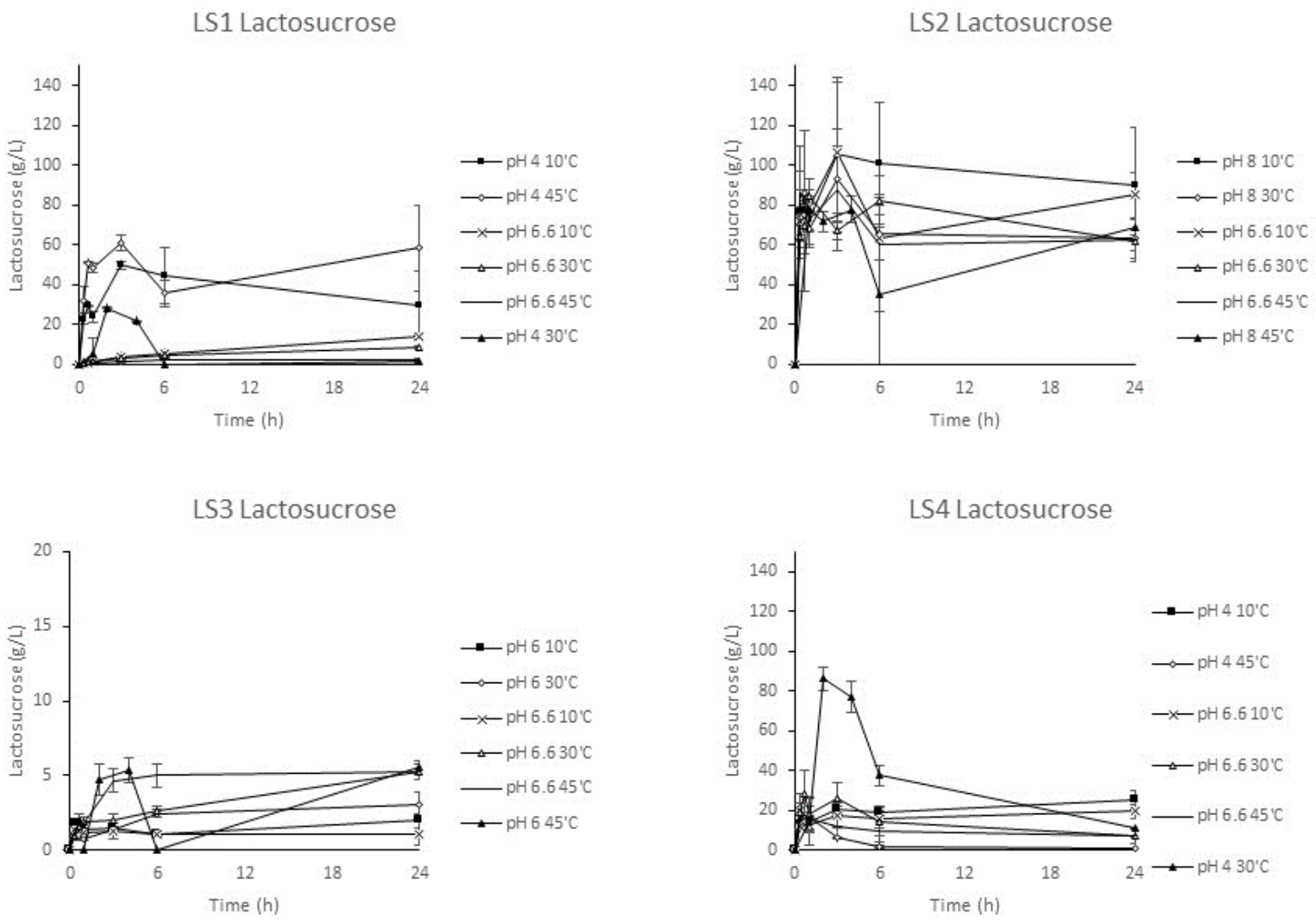


Fig 4.2. Effect of pH and temperature on lactosucrose production by *G. oxydans* LS1, *V. natriegens* LS2, *N. aromaticivorans* LS3 and *P. graminis* LS4.

equilibrium. Besides that, production did not exceed 30 g/L whether at pH 4 or pH 6.6. at the selected temperatures (10, 30 and 45 °C). The lactosucrose synthesis does not reflect the same trends, of increasing or constant transfructosylation extents, observed in Fig 4.1b. Furthermore, no particularly high transfructosylation extent was observed at pH 4 and 30 °C. This suggests that the sucrose transfructosylation may have led to the formation of other LS-catalyzed end-products such as FOS, levan and oligolevans. Indeed, Supplementary Table 4.1 indicates that high percentages of sucrose were converted to levan/oligolevan with up to 60%, 52%, 35% and 69% conversion with *G. oxydans* LS1, *V. natriegens* LS2, *N. aromaticivorans* LS3 *P. graminis* LS4, respectively.

LCMS analysis was also used to identify possible FOS formed. The results are shown in Fig 4.3. *G. oxydans* LS1 produced a notable amount of 6-kestose, reaching 20 g/L at pH 4 30 °C. Less than 5 g/L were however produced at pH 6.6. This confirms that the application of this enzyme to moderately acidic food systems would be more suitable than dairy products. *V. natriegens* LS2 favored the production of 1-kestose at pH 6.6, with a maximum level of about 14 g/L at 45 °C. 5 g/L maximum was formed at pH 8. The reactions catalyzed by *N. aromaticivorans* LS3 led to a diverse range of FOS: 1-kestose, 6G-kestose, nystose and fructosyl-nystose. Almost 80 g/L of 1-kestose was produced at pH 6 45 °C. At pH 6.6 a maximum of about 30 g/L 1-kestose was attained. Hence, the application of *N. aromaticivorans* LS3 to dairy products could result in dairy products rich in FOS. A notable amount of diversified FOS was also produced by *P. graminis* LS4. 1-kestose production was predominant, reaching around 30 g/L at both pH 4 and pH 6.6. *P. graminis* LS4 could therefore also be an interesting enzyme in the endogenous biogeneration of both lactosucrose and FOS in dairy products.

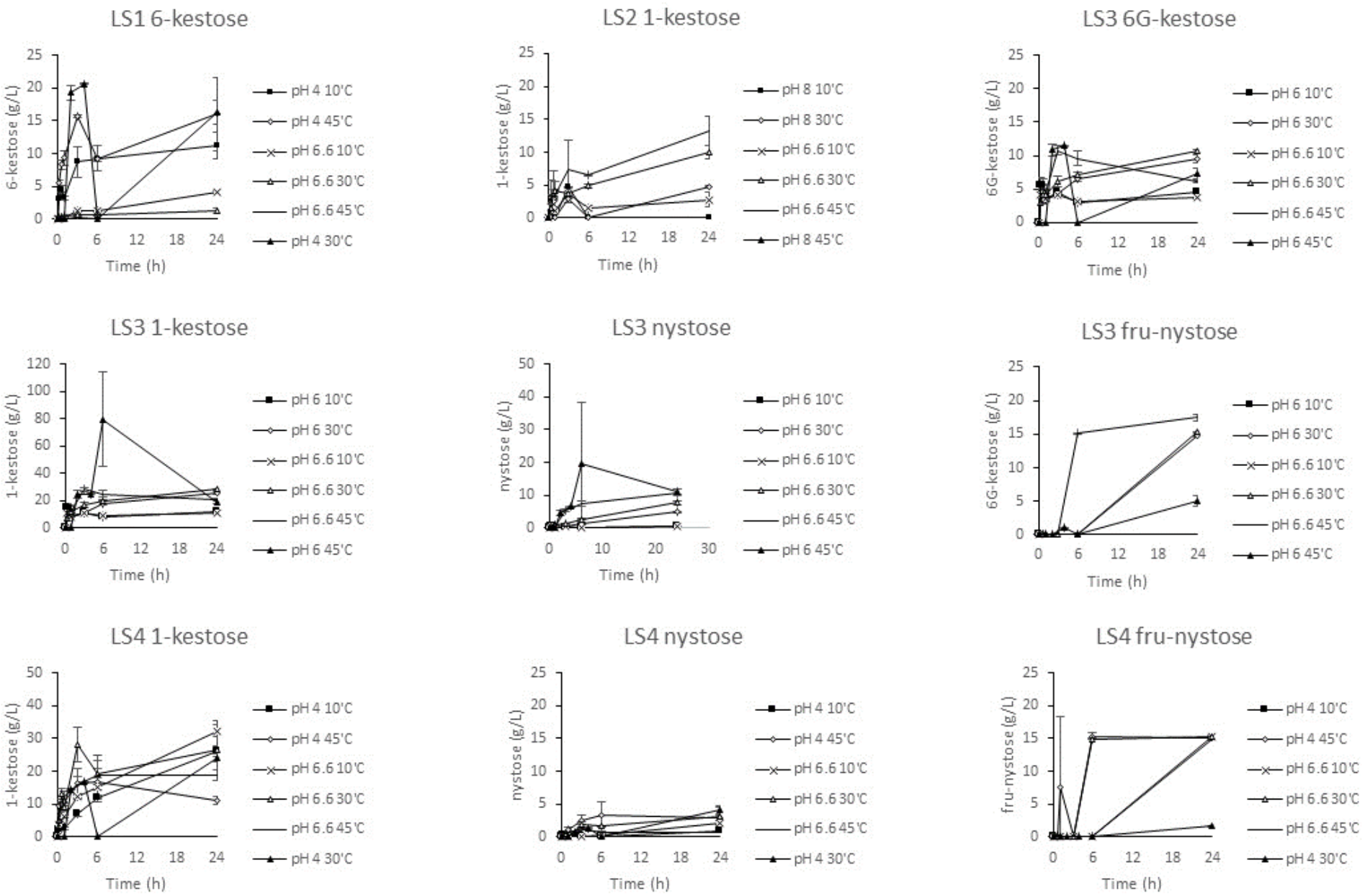


Fig 4.3. Effect of pH and temperature on FOS production by *G. oxydans* LS1, *V. natriegens* LS2, *N. aromaticivorans* LS3 and *P. graminis* LS4

4.3.2. Time courses for transfructosylation of lactose: Application of LS to reconstituted sweetened milk and chocolate milk

Different types of milk (e.g. a regular sweetened milk (RSM), a low sugar-sweetened milk (LSSM), a regular chocolate milk (RCM) and a low sugar chocolate milk (LSCM)) were reconstituted using milk powder, sucrose, and cocoa powder to study the effect of varying sucrose, milk powder (lactose) and cocoa powder on LS-catalyzed transfructosylation reactions. The reaction selectivity of selected LSs in each formulation over a time course of 24h was first determined. The results are shown in Supplementary Tables 4.3a-4.3d. Whether at 1h or 24h, no significant difference ($p > 0.05$) in the relative transfructosylation and hydrolysis extents was detected when the regular sweetened milk (RSM) and the low sugar-sweetened milk (LSSM) were enriched with milk powder. This indicates that increasing the lactose content from 4.9% to 7.83% (w/v) did not affect the reaction selectivity of the LS-catalyzed reactions. Comparing the RSM and the LSSM, the increase of sucrose, from 3.38% to 10.30% (w/v), did also not significantly alter ($p > 0.05$) the relative transfructosylation and hydrolysis extents at 1h or 24h. As for the effect of cocoa powder, no significant difference ($p > 0.05$) in reaction selectivity was detected at 1h or 24h, when comparing the regular chocolate milk (RCM) and low sugar chocolate milk (LSCM) to their cocoa powder-enriched counterparts. Hydrolysis was favored over transfructosylation for all four levansucrases. Compared to the studies on the effect of pH and temperature, LS1, LS2 and LS4 favored transfructosylation in general. This can be explained by the lower concentrations of sucrose and lactose used. Indeed, it was previously found that hydrolysis was predominant as sucrose nears depletion unless NaCl and organic solvents are added to create water-restricted environments promoting transfructosylation (Castillo & López-Munguía, 2004; Chambert & Petit-Glatron, 1989).

Fig 4.4 and Fig 4.5, show the effect of milk components and cocoa powder, respectively, on the transfructosylation of lactose to lactosucrose. The highest lactosucrose production level achieved by each formulation was compared. For the regular sweetened milk (RSM) and low sugar-sweetened milk (LSSM) formulations, a significant difference ($p < 0.05$) was detected only with *N. aromaticivorans* LS3. The lactosucrose production for the formulations enriched with lactose (RSM-L vs LSSM-L) however differed ($p < 0.05$) using *G. oxydans* LS1, *V. natriegens* LS2 or

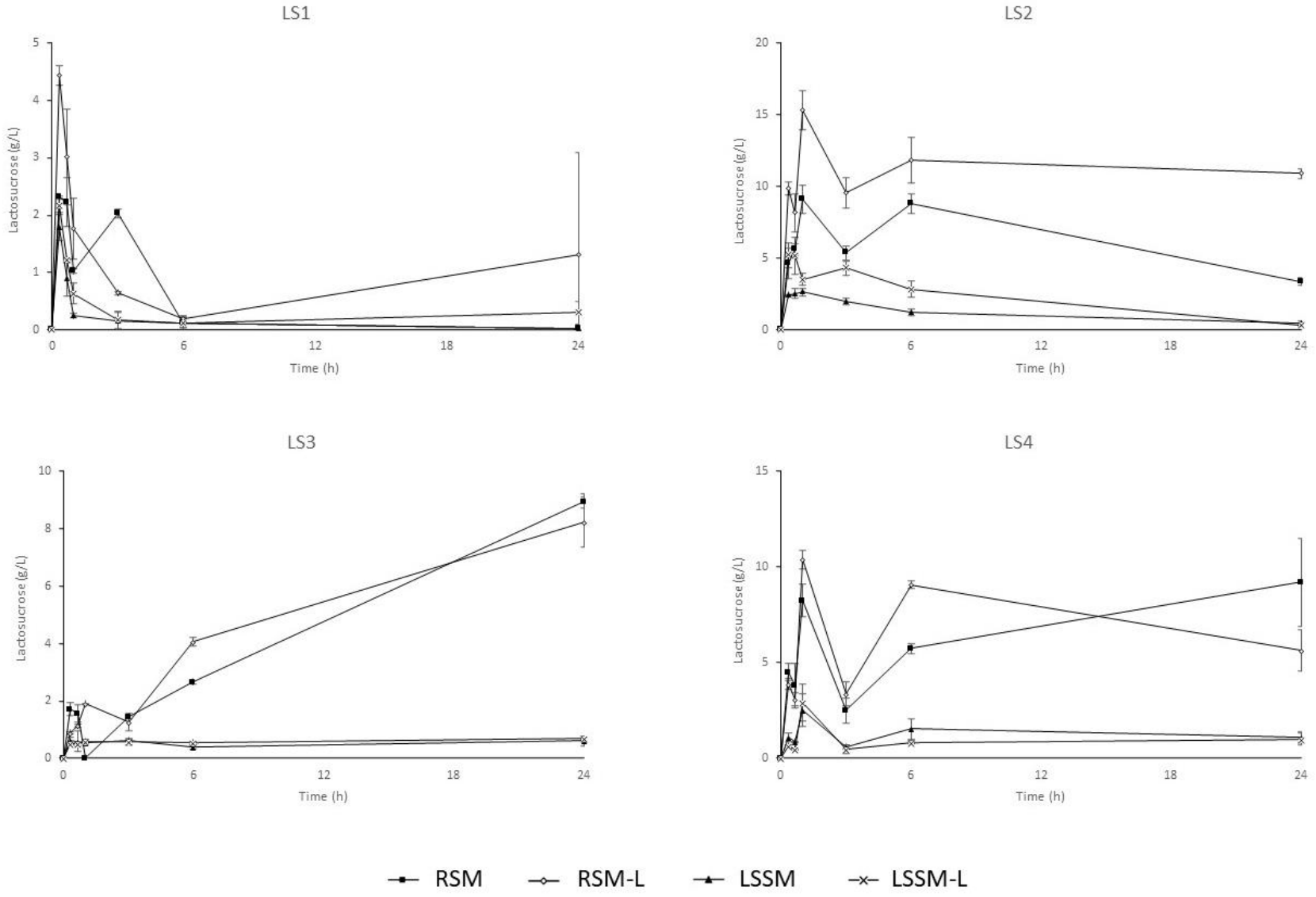


Fig 4.4. Effect of milk components on lactosucrose production (RSM- Regular sweetened milk; RSM-L- Regular sweetened milk enriched with lactose; LSSM- Low sugar sweetened milk; LSSM-L- Low sugar sweetened milk enriched with lactose)

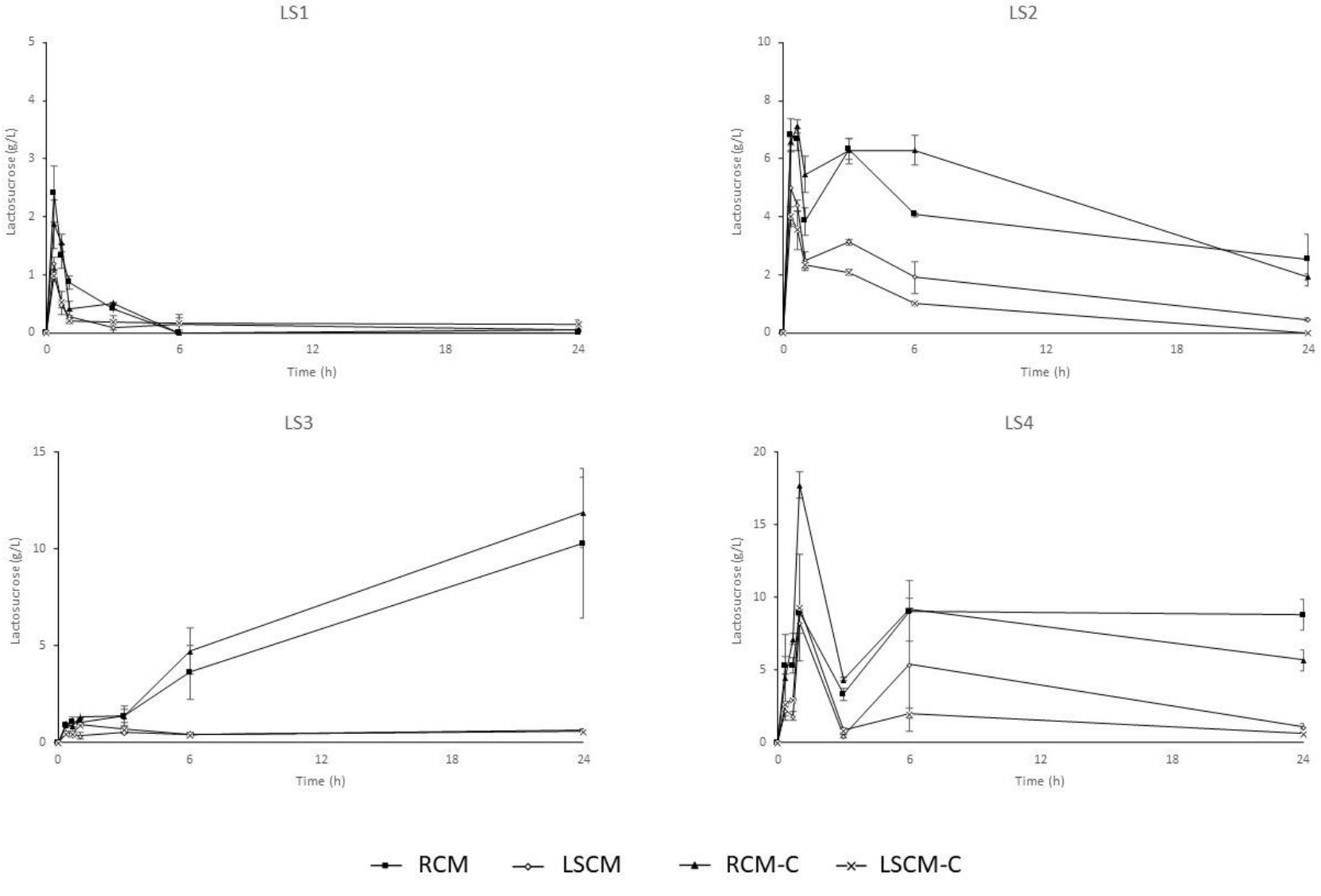


Fig 4.5. Effect of cocoa powder lactosucrose production (RCM- Regular chocolate milk; LSCM- Low sugar chocolate milk; RCM-C- Regular chocolate milk enriched with cocoa; LSCM-C- Low sugar chocolate milk enriched with cocoa)

P. graminis LS4. Focusing on the effect of additional milk powder, by comparing the RSM and LSSM formulations to their lactose-enriched counterparts, a significant difference ($p < 0.05$) was detected with *G. oxydans* LS1 and *V. natriegens* LS2 in their RSM formulations. No difference was found in their LSSM formulations. Finally, the effect of cocoa powder was insignificant for all selected LSs when comparing their regular chocolate milk (RCM) and their low-sugar chocolate milk (LSCM) to their cocoa powder-enriched counterparts. As for the effect on total sucrose conversion, significant differences were mainly found with reactions catalyzed by *V. natriegens* LS2. This can be explained by the ability *V. natriegens* LS2 to better transfructosylate lactose and produce the least amount of FOS and levan/oligolevan than the other enzymes, as previously depicted in Fig 4.2, Fig 4.3, Supplementary Table 4.1, and in our previous study (Bahlawan et al., 2023). Finally, the effect on FOS production was also determined and differences were detected only with *V. natriegens* LS2. From the reaction selectivity and end-product profile characterization results, it can be concluded that there was in general no apparent effect on the transfructosylation extent, sucrose conversion, lactosucrose production and FOS production with cocoa powder enrichment. Cocoa powder is rich in phenolic compounds, catechin and epicatechin (Maleyki & Ismail, 2010; Natsume et al., 2001). These phenolic compounds have been found to act as potential inhibitors in LS-catalyzed reactions (see Chapter III). The insignificant effect of phenolic compounds as enzyme inhibitors is even more apparent with *V. natriegens* LS2 where previously negligible sucrose conversion was achieved. This can be explained by the loss of polyphenol content during cocoa powder processing (Jalil & Ismail, 2008). Cocoa powder produced from fermented, dried, and roasted beans have been found to contain less phenolic compounds than cocoa powder produced from unfermented beans (Tomas-Barberán et al., 2007). In addition, the alkalization of cocoa powder can also reduce the polyphenol content (Adamson et al., 1999; Gu et al., 2006).

4.3.3. Rheological properties of levan-fortified chocolate milk

The application of levan in dairy products is no doubt appealing given its ability to act as both a health-promoting and techno-functional ingredient. Indeed, Xu et al. (2022) demonstrated that levan could act as a yogurt stabilizer, increasing the water-holding capacity and system

stability. The addition of LS in chocolate milk can lead to the endogenous formation of levan from the transfructosylation of sucrose. To assess the contribution of levan, the rheological properties of chocolate milk enriched with varying concentrations of HMW levan (1, 3, 5% w/w) and MIX levan (0.5, 1, 2.5% w/w) produced from *G. oxydans* LS1 were investigated at 15, 37 and 60 °C. These temperatures were selected to mimic the conditions of cold and hot chocolate milk, as well as the average normal oral temperature of 37 °C. Higher concentrations of HMW levan were tested given that our previous studies revealed that MIX levan could bring significantly higher viscosities than HMW levan (Sahyoun et al., 2024).

Table 4.1 displays the consistency coefficient (m), flow behavior index (n) and apparent viscosity at 50 s^{-1} (η50) obtained. This specific shear rate (50 s^{-1}) was chosen being commonly accepted for sensory perception analysis (Shama & Sherman, 1973). For both HMW levan and MIX levan, the consistency coefficient increased as concentration increased, but decreased with increasing temperature. For the flow behavior index, a declining trend was observed with increasing concentration, indicating a shift towards a more shear-thinning fluid ($n < 1$). This was especially more noticeable with the HMW levan. As for the apparent viscosity at 50 s^{-1} obtained, it followed a similar pattern as the consistency coefficient. Comparing the results of the chocolate milk fortified with levan to that of a commercial chocolate milk with carrageenan as stabilizer, it can be concluded that less than 1% HMW levan and less than 0.5% MIX levan would be sufficient to obtain the desired viscosity of chocolate milk. In Fig 4.6, the apparent viscosities of all chocolate milk formulations decreased significantly with increasing the shear rate, confirming the non-Newtonian shear thinning pseudoplastic behaviour. These viscosities eventually stabilized at higher shear rates. The decrease in viscosity was suggested to be attributed to the greater disentanglement of the levan chains at larger shear rates (Sahyoun et al., 2024). A psedoplastic behaviour was also obtained with levan from *Microbacterium laevaniformans* (Bae et al., 2008). With levan from *Bacillus mojavensis* and from *Bacillus sp.* a Newtonian behaviour occurred at low concentrations and shear thinning occurred at higher concentrations (Arvidson et al., 2006; Haddar et al., 2022). Haddar et al. (2022) also found that higher temperatures increased pseudoplastic behavior of levan from *B. mojavensis*.

Table 4.1. Power law parameters for levan-enriched chocolate milk at selected temperatures

	15 °C			37 °C			60 °C		
	m (mPa)	n	η50 (mPa.s)	m (mPa)	n	η50 (mPa.s)	m (mPa)	n	η50 (mPa.s)
HMW									
1%	17.64 ± 2.72	0.85 ± 0.03	9.63 ± 0.91	9.86 ± 2.55	0.86 ± 0.04	5.58 ± 0.57	4.38 ± 0.80	0.96 ± 0.03	3.67 ± 0.21
3%	606.41 ± 139.43	0.57 ± 0.06	111.63 ± 2.77	102.49 ± 11.51	0.78 ± 0.01	43.21 ± 2.93	46.42 ± 11.08	0.85 ± 0.04	25.35 ± 2.74
5%	47556.75 ± 19997.00	0.21 ± 0.02	2093.52 ± 733.46	32336.34 ± 17330.36	0.21 ± 0.03	1384.36 ± 613.83	20830.62 ± 11020.23	0.23 ± 0.04	970.92 ± 385.67
MIX									
0.50%	12.65 ± 1.75	0.86 ± 0.06	7.29 ± 0.75	4.49 ± 1.04	0.96 ± 0.05	3.82 ± 0.13	3.15 ± 0.41	0.97 ± 0.03	2.76 ± 0.10
1%	71.55 ± 1.76	0.56 ± 0.01	12.80 ± 0.39	6.28 ± 0.93	0.97 ± 0.03	5.48 ± 0.26	7.64 ± 1.42	0.83 ± 0.04	3.88 ± 0.14
2.50%	117.35 ± 4.22	0.81 ± 0.01	54.72 ± 1.76	38.59 ± 1.36	0.88 ± 0.01	23.90 ± 0.75	18.47 ± 1.11	0.93 ± 0.02	14.17 ± 0.22
Commercial chocolate milk	10.67 ± 0.12	0.87 ± 0.01	6.29 ± 0.1	3.57 ± 0.08	0.99 ± 0.01	3.37 ± 0.17	2.51 ± 0.54	0.99 ± 0.03	2.39 ± 0.25

m: Consistency coefficient

n: Flow behavior index

η50: Apparent viscosity at 50 s^{-1}

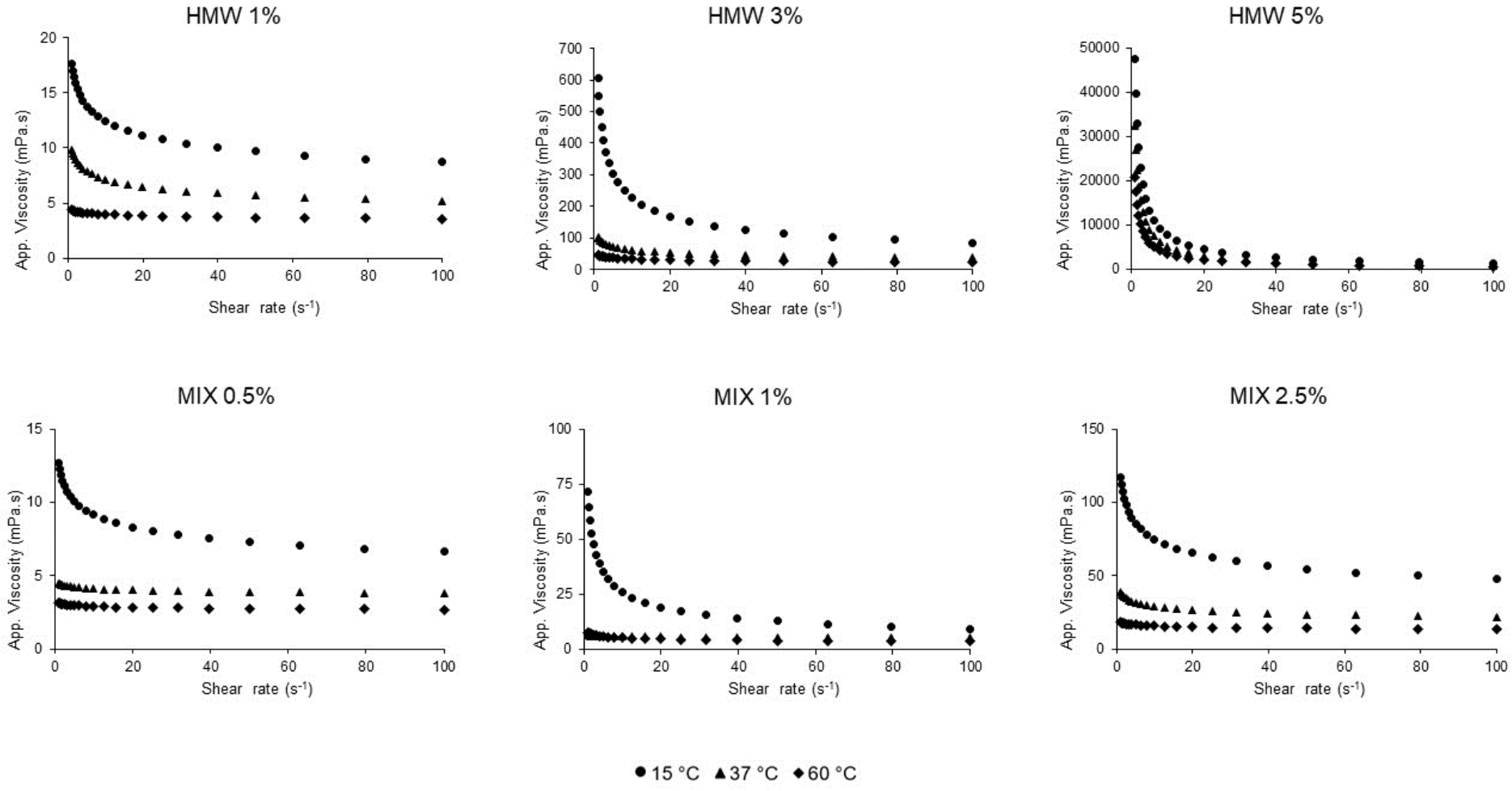
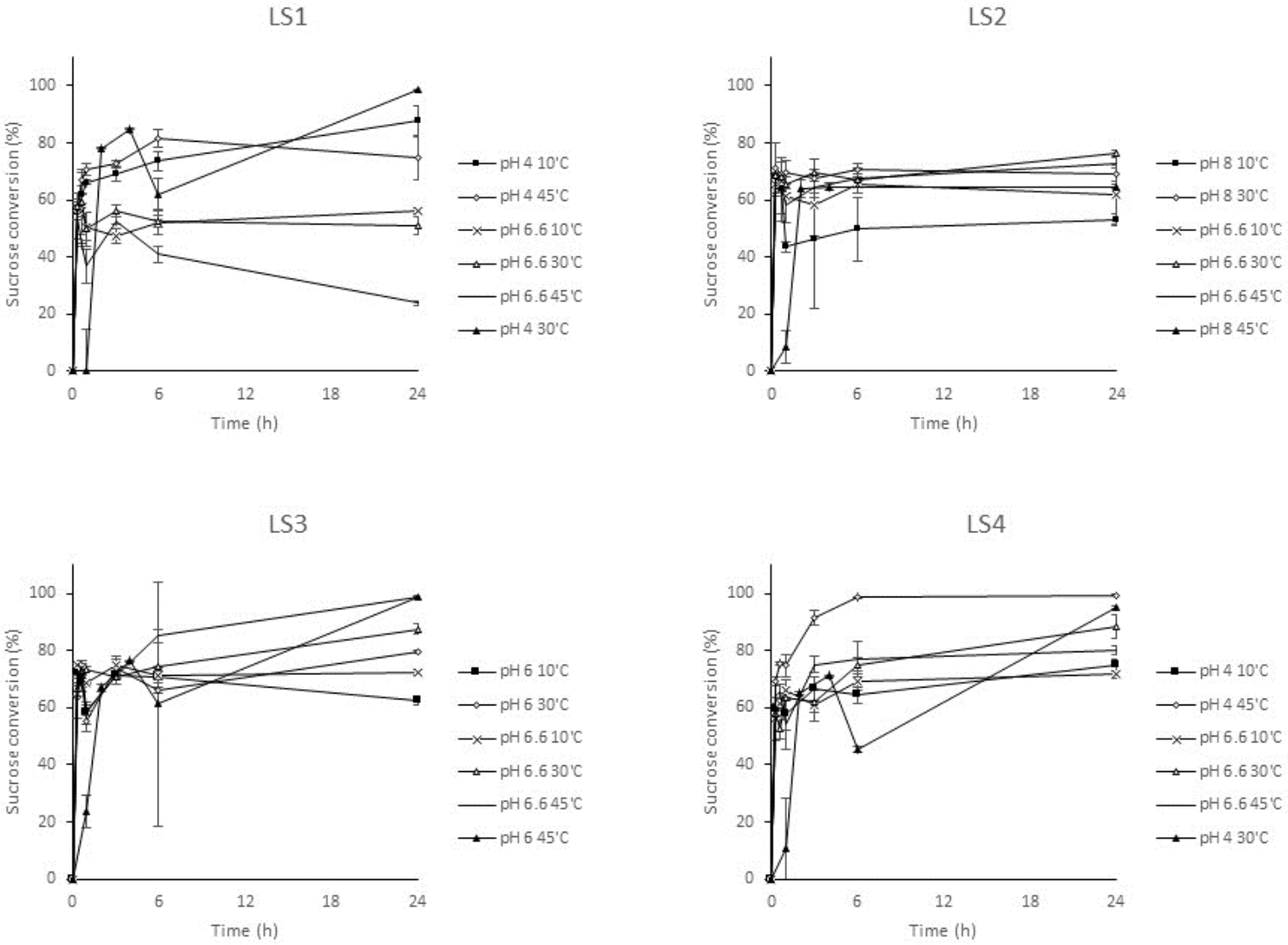


Fig 4.6. Shear-dependent viscosities of levan-enriched chocolate milk at selected temperature

4.4. Conclusion

This study demonstrated the high potential of LSs from *V. natriegens* (LS2), *N. aromaticivorans* (LS3), and *P. graminis* (LS4) as biocatalysts to endogenously produce functional ingredients in dairy products. *G. oxydans* LS1 was however found to be more suitable for moderately acidic food systems. The reaction selectivity results demonstrated that, overall, transfructosylation was predominant over hydrolysis for LS1, LS2 and LS4. In general, at low temperatures, transfructosylation was favored and as temperature increased, hydrolysis increased. The effects of pH and temperature on lactose transfructosylation showed that LS2 had the highest potential, with high lactosucrose production even at the pH of milk (pH 6.6) and at a low temperature of 10 °C. LS1 and LS4 had low lactosucrose production at pH 6.6, while LS3 favoured fructooligosaccharides (FOSs) formation over that of lactosucrose. A shift in the thermodynamic equilibrium of the transfructosylation of lactose during the 24-hour time course was observed for the four selected LSs, indicating that the reaction might have shifted towards lactosucrose hydrolysis. However, the overall transfructosylation extents did not always follow the same trend as the lactosucrose production levels recorded and sucrose was instead used to transfructosylate other end-products such as fructooligosaccharides and levan. The second part of this study focused on the possible effects of enriching reconstituted milk formulations with lactose and cocoa powder on LS-catalyzed reactions. An increase in the lactose content from 4.9% to 7.83% (w/v), and an increase in the sucrose content from 3.38% to 10.30% (w/v) did not significantly affect the reaction selectivity at 1h or 24h. No significant changes were observed in the reaction selectivity, sucrose conversion, lactosucrose and FOS production with additional cocoa powder. Finally, levan proved to be a potential stabiliser of great interest in chocolate milk production. Less than 1% (w/w) of high molecular weight (HMW) levan or less than 0.5% (w/w) of mixed low and high molecular weight (MIX) levan was sufficient to bring the viscosity of the fortified chocolate milk equivalent to that of commercial chocolate milk.



Supplementary Figure 4.1. Effect of pH and temperature on sucrose conversion

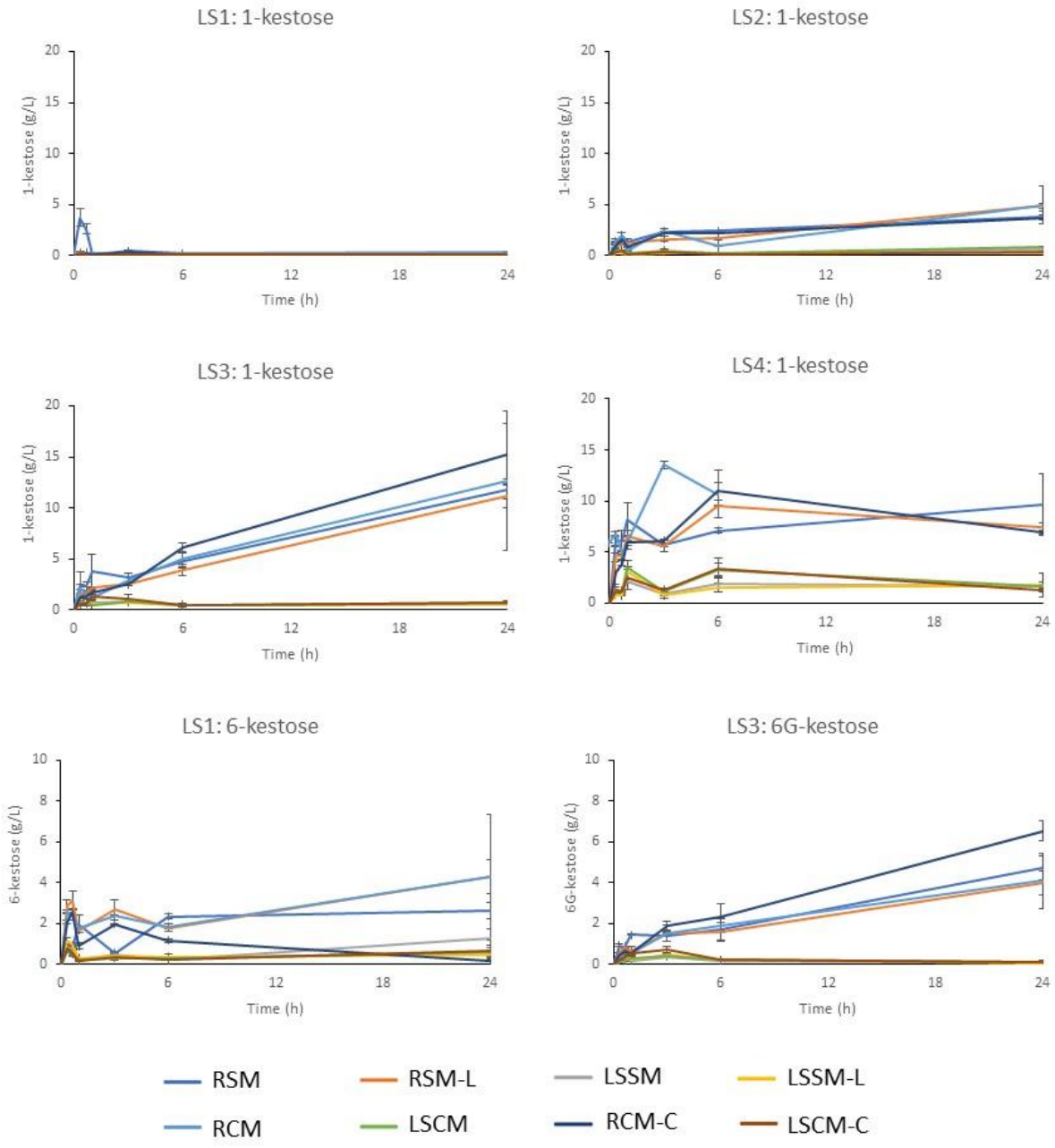
Supplementary Table 4.1. Effect of pH and temperature on levan/oligolevan production

		Conversion of sucrose to oligolevan/levan (%)			
		LS1	**LS2**	**LS3**	**LS4**
pH 6.6 10 °C	20min	30.09 ± 0.02	31.28 ± 1.54	19.49 ± 0.61	
	40min	36.40 ± 0.01	28.01 ± 0.74	18.76 ± 0.68	
	1	31.72 ± 0.03	28.34 ± 0.43	17.31 ± 0.13	35.02 ± 0.92
	3	42.21 ± 0.03	17.48 ± 8.01	16.58 ± 0.26	31.31 ± 1.30
	6	43.87 ± 0.09	28.07 ± 2.60	16.40 ± 0.05	37.71 ± 1.16
	24	45.20 ± 0.14	25.55 ± 7.70	21.14 ± 0.30	33.30 ± 1.23
pH 6.6 30 °C	20min	51.90 ± 0.05	43.58 ± 1.24	20.36 ± 0.05	40.41 ± 1.34
	40min	55.00 ± 0.08	30.76 ± 3.29	18.03 ± 1.28	30.73 ± 3.03
	1	44.35 ± 0.03	21.51 ± 1.86	15.44 ± 0.41	39.91 ± 1.95
	3	48.37 ± 0.11	29.40 ± 1.09	20.52 ± 0.68	30.37 ± 3.04
	6	46.77 ± 0.01	23.40 ± 0.76	23.36 ± 0.22	35.66 ± 2.02
	24	43.60 ± 0.26	28.68 ± 0.27	32.14 ± 0.33	42.82 ± 3.14
pH 6.6 45 °C	20min		31.51 ± 5.82	16.74 ± 0.42	38.19 ± 0.87
	40min		31.59 ± 0.96	21.96 ± 0.71	54.24 ± 0.45
	1	8.57 ± 0.03	28.54 ± 3.12	19.99 ± 0.35	44.29 ± 1.87
	3		23.60 ± 7.70	23.36 ± 0.77	63.84 ± 0.67
	6	7.28 ± 0.13	29.32 ± 7.54	31.25 ± 1.16	43.95 ± 2.66
	24	7.18 ± 0.12	24.47 ± 1.71	34.61 ± 0.51	68.96 ± 0.49
pH 4 10 °C (LS1/LS4)	20min	41.79 ± 0.71	16.67 ± 2.72	18.19 ± 0.90	44.04 ± 0.13
pH 8 10 °C (LS2)	40min	45.47 ± 0.16	22.90 ± 9.19	23.14 ± 0.95	44.75 ± 0.27
pH 6 10 °C (LS3)	1	46.07 ± 0.66	12.02 ± 1.21	7.50 ± 0.21	41.15 ± 0.87
	3	45.07 ± 1.01	5.50 ± 8.60	9.65 ± 0.12	46.94 ± 0.88
	6	50.03 ± 3.13	24.91 ± 6.77	11.25 ± 0.09	42.75 ± 0.44
	24	59.95 ± 4.34	23.56 ± 1.26	15.18 ± 0.29	42.33 ± 1.36
pH 4 45 °C(LS1/LS4)	20min	37.01 ± 1.58	45.16 ± 4.89	8.04 ± 0.48	41.89 ± 1.54
pH 8 30 °C (LS2)	40min	36.38 ± 0.28	44.45 ± 0.30	9.80 ± 0.25	42.68 ± 0.82
pH 6 30 °C (LS3)	1	35.56 ± 0.80	42.01 ± 2.10	13.01 ± 0.10	39.42 ± 2.25
	3	29.02 ± 0.97	33.04 ± 3.88	19.05 ± 0.31	38.92 ± 1.21
	6	41.29 ± 1.98	52.09 ± 0.66	16.74 ± 0.46	37.40 ± 1.88
	24	28.88 ± 6.11	40.28 ± 2.26	23.54 ± 0.54	34.48 ± 0.36
pH 4 30 °C(LS1/LS4)	1			5.57 ± 0.00	5.08 ± 2.70
pH 8 45 °C (LS2)	2	32.79 ± 0.33	31.14 ± 1.10	17.17 ± 1.16	29.94 ± 1.35
pH 6 45 °C (LS3)	6	38.07 ± 0.00			18.07 ± 1.11
	24	40.34 ± 0.51	37.27 ± 0.88	10.25 ± 0.90	35.70 ± 0.39

The conversion of sucrose to oligolevan/levan was calculated as the difference between the total percentage of sucrose converted to transfructosylated products and the percentage of sucrose converted to lactosucrose/fructooligosaccharides.

Supplementary Table 4.2. Formulations of reconstituted milk

Sample ID	Sucrose concentration % w/v	Milk powder concentration % w/v	Cocoa powder concentration % w/v
Regular sweetened milk (RSM)	10.30	12.50 (4.9% lactose)	0.00
Regular sweetened milk enriched with lactose (RSM-L)	10.30	20.00 (7.83% lactose)	0.00
Low sugar sweetened milk (LSSM)	3.38	12.50	0.00
Low sugar sweetened milk enriched with lactose (LSSM-L)	3.38	20.00	0.00
Regular chocolate milk (RCM)	10.30	12.50	1.43
Low sugar chocolate milk (LSCM)	3.38	12.50	1.43
Regular chocolate milk enriched with cocoa (RCM-C)	10.30	12.50	2.86
Low sugar chocolate milk enriched with cocoa (LSCM-C)	3.38	12.50	2.86



Supplementary Figure 4.2. Effect of milk components and cocoa powder on FOS production (RSM- Regular sweetened milk; RSM-L- Regular sweetened milk enriched with lactose; LSSM- Low sugar sweetened milk; LSSM-L- Low sugar sweetened milk enriched with lactose; RCM- Regular chocolate milk; LSCM- Low sugar chocolate milk; RCM-C- Regular chocolate milk enriched with cocoa; LSCM-C- Low sugar chocolate milk enriched with cocoa)

Supplementary Table 4.3a. Effect of milk components and cocoa powder on sucrose conversion and reaction selectivity with *G. oxydans* LS1

Sample ID	Time (h)	Sucrose conversion (%)	Time (h)	Relative transfructosylation extent (%)	Relative hydrolysis extent (%)
	0	0.00 ± 0.00	**0**	0.00 ± 0.00	0.00 ± 0.00
	20min	58.85 ± 2.69	**1**	54.97 ± 6.33 $^{a1\ b1}$	45.03 ± 6.33 $^{a1\ b1}$
	40min	78.76 ± 2.88	**6**	43.12 ± 6.29	56.88 ± 6.29
RSM	**1**	93.73 ± 1.32	**8**	38.43 ± 1.78	61.57 ± 1.78
	3	97.92 ± 0.17	**24**	30.81 ± 5.14 $^{a1\ b1}$	69.19 ± 5.14 $^{a1\ b1}$
	6	98.70 ± 0.33			
	24	99.34 ± 0.05 $^{a1\ b1}$			
	0	0.00 ± 0.00	**0**	0.00 ± 0.00	0.00 ± 0.00
	20min	46.41 ± 2.87	**1**	58.90 ± 3.67 $^{b1\ c1}$	41.10 ± 3.67 $^{b1\ c1}$
	40min	74.80 ± 3.04	**6**	44.80 ± 4.81	55.20 ± 4.81
RSM-L	**1**	93.38 ± 2.55	**8**	46.86 ± 2.84	53.14 ± 2.84
	3	97.71 ± 0.34	**24**	33.79 ± 4.76 $^{b1\ c1}$	66.21 ± 4.76 $^{b1\ c1}$
	6	98.98 ± 0.06			
	24	90.94 ± 8.99 $^{b1\ c1}$			
	0	0.00 ± 0.00	**0**	0.00 ± 0.00	0.00 ± 0.00
	20min	63.38 ± 1.29	**1**	47.35 ± 11.39 $^{a1\ d1}$	52.65 ± 11.39 $^{a1\ d1}$
	40min	89.34 ± 0.69	**6**	44.24 ± 5.15	55.76 ± 5.15
LSSM	**1**	98.42 ± 0.08	**8**	48.76 ± 0.31	51.24 ± 0.31
	3	98.95 ± 0.24	**24**	26.20 ± 11.86 $^{a1\ d1}$	73.80 ± 11.86 $^{a1\ d1}$
	6	98.94 ± 0.28			
	24	96.03 ± 2.04 $^{a1\ d1}$			
	0	0.00 ± 0.00	**0**	0.00 ± 0.00	0.00 ± 0.00
	20min	67.55 ± 1.92	**1**	50.75 ± 8.15 $^{c1\ d1}$	49.25 ± 8.15 $^{c1\ d1}$
	40min	90.65 ± 1.42	**6**	44.38 ± 8.52	55.62 ± 8.52
LSSM-L	**1**	97.55 ± 0.23	**8**	46.52 ± 2.74	53.48 ± 2.74
	3	94.89 ± 4.94	**24**	26.77 ± 5.19 $^{c1\ d1}$	73.23 ± 5.19 $^{c1\ d1}$
	6	98.65 ± 0.62			
	24	97.32 ± 0.78 $^{c1\ d1}$			
	0	0.00 ± 0.00	**0**	0.00 ± 0.00	0.00 ± 0.00
	20min	46.07 ± 0.62	**1**	62.73 ± 5.76 ^{e1}	37.27 ± 5.76 ^{e1}
	40min	77.86 ± 2.07	**6**	54.52 ± 3.12	45.48 ± 3.12
RCM	**1**	94.84 ± 0.03	**8**	57.23 ± 1.23	42.77 ± 1.23
	3	98.13 ± 0.08	**24**	30.03 ± 6.04 ^{e1}	69.97 ± 6.04 ^{e1}
	6	99.37 ± 0.03			
	24	99.44 ± 0.11 ^{e1}			
	0	0.00 ± 0.00	**0**	0.00 ± 0.00	0.00 ± 0.00
	20min	72.63 ± 0.25	**1**	55.59 ± 14.56 ^{f1}	44.41 ± 14.56 ^{f1}
LSCM	**40min**	93.52 ± 3.01	**6**	55.25 ± 12.51	44.75 ± 12.51
	1	98.12 ± 0.12	**8**	51.01 ± 10.34	48.99 ± 10.34
	3	99.44 ± 0.42	**24**	25.40 ± 8.04 ^{f1}	74.60 ± 8.04 ^{f1}

	6	98.79 ± 0.02			
	24	98.60 ± 0.10 [f1]			
	0	0.00 ± 0.00	**0**	0.00 ± 0.00	0.00 ± 0.00
	20min	56.97 ± 0.60	**1**	66.24 ± 7.63 [e1]	33.76 ± 7.63 [e1]
	40min	76.80 ± 2.02	**6**	60.32 ± 11.10	39.68 ± 11.10
RCM-C	**1**	97.57 ± 0.05	**8**	56.10 ± 1.32	43.90 ± 1.32
	3	97.99 ± 0.15	**24**	35.98 ± 14.11 [e1]	64.02 ± 14.11 [e1]
	6	99.63 ± 0.13			
	24	99.63 ± 0.10 [e1]			
	0	0.00 ± 0.00	**0**	0.00 ± 0.00	0.00 ± 0.00
	20min	76.06 ± 2.69	**1**	56.52 ± 0.67 [f1]	43.48 ± 0.67 [f1]
	40min	93.50 ± 0.38	**6**	56.23 ± 8.29	43.77 ± 8.29
LSCM-C	**1**	96.65 ± 3.49	**8**	52.92 ± 2.51	47.08 ± 2.51
	3	98.85 ± 0.01	**24**	45.63 ± 17.39 [f1]	54.37 ± 17.39 [f1]
	6	98.93 ± 0.36			
	24	91.12 ± 9.60 [f1]			

Supplementary Table 4.3b. Effect of milk components and cocoa powder on sucrose conversion and reaction selectivity with *V. natriegens* LS2

Sample ID	Time (h)	Sucrose conversion (%)	Time (h)	Relative transfructosylation extent (%)	Relative hydrolysis extent (%)
RSM	0	0.00 ± 0.00	0	0.00 ± 0.00	0.00 ± 0.00
	20min	64.51 ± 5.93	1	36.61 ± 21.37 $^{a1\ b1}$	63.39 ± 21.37 $^{a1\ b1}$
	40min	65.50 ± 0.04	6	26.36 ± 3.73	73.64 ± 3.73
	1	57.31 ± 2.33	8	30.41 ± 4.60	69.59 ± 4.60
	3	68.18 ± 6.87	24	43.59 ± 11.87 $^{a1\ b1}$	56.41 ± 11.87 $^{a1\ b1}$
	6	61.19 ± 3.67			
	24	94.52 ± 2.05 $^{a1\ b1}$			
RSM-L	0	0.00 ± 0.00	0	0.00 ± 0.00	0.00 ± 0.00
	20min	53.96 ± 5.72	1	38.60 ± 4.97 $^{b1\ c1}$	61.40 ± 4.97 $^{b1\ c1}$
	40min	56.29 ± 6.41	6	25.81 ± 26.89	74.19 ± 26.89
	1	48.81 ± 6.67	8	32.54 ± 16.12	67.46 ± 16.12
	3	55.88 ± 4.86	24	33.61 ± 6.41 $^{b1\ c1}$	66.39 ± 6.41 $^{b1\ c1}$
	6	62.22 ± 2.77			
	24	72.09 ± 2.61 $^{b2\ c1}$			
LSSM	0	0.00 ± 0.00	0	0.00 ± 0.00	0.00 ± 0.00
	20min	84.46 ± 1.16	1	31.39 ± 13.61 $^{a1\ d1}$	68.61 ± 13.61 $^{a1\ d1}$
	40min	81.41 ± 2.84	6	23.72 ± 7.05	76.28 ± 7.05
	1	86.44 ± 0.96	8	34.56 ± 5.18	65.44 ± 5.18
	3	86.64 ± 1.95	24	17.62 ± 6.74 $^{a1\ d1}$	82.38 ± 6.74 $^{a1\ d1}$
	6	93.66 ± 1.17			
	24	98.95 ± 0.11 $^{a1\ d1}$			
LSSM-L	0	0.00 ± 0.00	0	0.00 ± 0.00	0.00 ± 0.00
	20min	72.77 ± 3.38	1	37.46 ± 29.40 $^{c1\ d1}$	62.54 ± 29.40 $^{c1\ d1}$
	40min	76.23 ± 5.53	6	30.59 ± 15.03	69.41 ± 15.03
	1	88.50 ± 1.14	8	37.19 ± 2.75	62.81 ± 2.75
	3	78.48 ± 2.89	24	22.92 ± 7.89 $^{c1\ d1}$	77.08 ± 7.89 $^{c1\ d1}$
	6	90.81 ± 1.41			
	24	99.19 ± 0.01 $^{c2\ d1}$			
RCM	0	0.00 ± 0.00	0	0.00 ± 0.00	0.00 ± 0.00
	20min	45.49 ± 3.24	1	41.83 ± 7.52 ^{e1}	58.17 ± 7.52 ^{e1}
	40min	50.86 ± 2.04	6	30.98 ± 9.34	69.02 ± 9.34
	1	79.10 ± 1.98	8	33.43 ± 0.58	66.57 ± 0.58
	3	55.35 ± 1.46	24	28.43 ± 9.34 ^{e1}	71.57 ± 9.34 ^{e1}
	6	82.85 ± 0.29			
	24	93.05 ± 0.03 ^{e1}			
LSCM	0	0.00 ± 0.00	0	0.00 ± 0.00	0.00 ± 0.00
	20min	62.14 ± 7.38	1	45.30 ± 2.67 ^{f1}	54.70 ± 2.67 ^{f1}
	40min	67.56 ± 3.32	6	23.13 ± 4.89	76.87 ± 4.89
	1	83.55 ± 2.82	8	25.03 ± 3.20	74.97 ± 3.20

	3	80.01 ± 1.99	**24**	15.39 ± 5.64 [f1]	84.61 ± 5.64 [f1]
	6	90.35 ± 2.87			
	24	98.60 ± 0.11 [f1]			
	0	0.00 ± 0.00	**0**	0.00 ± 0.00	0.00 ± 0.00
	20min	42.76 ± 1.43	**1**	39.84 ± 4.22 [e1]	60.16 ± 4.22 [e1]
	40min	41.83 ± 2.22	**6**	34.17 ± 6.65	65.83 ± 6.65
RCM-C	**1**	67.00 ± 1.86	**8**	30.73 ± 8.13	69.27 ± 8.13
	3	55.84 ± 1.83	**24**	29.09 ± 10.90 [e1]	70.91 ± 10.90 [e1]
	6	68.09 ± 0.84			
	24	94.84 ± 0.17 [e2]			
	0	0.00 ± 0.00	**0**	0.00 ± 0.00	0.00 ± 0.00
	20min	66.66 ± 0.76	**1**	42.33 ± 11.69 [f1]	57.67 ± 11.69 [f1]
	40min	73.41 ± 7.94	**6**	33.37 ± 2.22	66.63 ± 2.22
LSCM-C	**1**	86.13 ± 2.12	**8**	2.61 ± 32.32	97.39 ± 32.32
	3	85.14 ± 0.23	**24**	20.62 ± 1.56 [f1]	79.38 ± 1.56 [f1]
	6	95.99 ± 0.17			
	24	97.24 ± 2.36 [f1]			

Supplementary Table 4.3c. Effect of milk components and cocoa powder on sucrose conversion and reaction selectivity with *N. aromaticivorans* LS3

Sample ID	Time (h)	Sucrose conversion (%)	Time (h)	Relative transfructosylation extent (%)	Relative hydrolysis extent (%)
RSM	0	0.00 ± 0.00	0	0.00 ± 0.00	0.00 ± 0.00
	20min	56.84 ± 1.53	1	18.37 ± 10.17 $^{a1\ b1}$	81.63 ± 10.17 $^{a1\ b1}$
	40min	56.83 ± 1.40	6	26.86 ± 5.92	73.14 ± 5.92
	1	21.54 ± 4.25	8	24.98 ± 0.98	75.02 ± 0.98
	3	65.29 ± 0.83	24	31.47 ± 4.28 $^{a1\ b1}$	68.53 ± 4.28 $^{a1\ b1}$
	6	62.86 ± 4.24			
	24	93.88 ± 1.91 $^{a1\ b1}$			
RSM-L	0	0.00 ± 0.00	0	0.00 ± 0.00	0.00 ± 0.00
	20min	47.59 ± 2.00	1	19.96 ± 6.21 $^{b1\ c1}$	80.04 ± 6.21 $^{b1\ c1}$
	40min	43.01 ± 3.51	6	20.72 ± 3.50	79.28 ± 3.50
	1	45.54 ± 7.22	8	28.47 ± 4.87	71.53 ± 4.87
	3	53.08 ± 4.95	24	29.98 ± 1.54 $^{b1\ c1}$	70.02 ± 1.54 $^{b1\ c1}$
	6	65.41 ± 10.35			
	24	93.17 ± 0.47 $^{b1\ c1}$			
LSSM	0	0.00 ± 0.00	0	0.00 ± 0.00	0.00 ± 0.00
	20min	83.50 ± 4.88	1	32.72 ± 34.69 $^{a1\ d1}$	67.28 ± 34.69 $^{a1\ d1}$
	40min	87.77 ± 1.61	6	15.31 ± 8.10	84.69 ± 8.10
	1	90.32 ± 0.37	8	19.63 ± 6.47	80.37 ± 6.47
	3	97.44 ± 0.46	24	15.63 ± 13.02 $^{a1\ d1}$	84.37 ± 13.02 $^{a1\ d1}$
	6	98.99 ± 0.24			
	24	98.85 ± 0.02 $^{a1\ d1}$			
LSSM-L	0	0.00 ± 0.00	0	0.00 ± 0.00	0.00 ± 0.00
	20min	81.89 ± 6.36	1	17.10 ± 9.18 $^{c1\ d1}$	82.90 ± 9.18 $^{c1\ d1}$
	40min	80.25 ± 2.75	6	2.08 ± 11.16	97.92 ± 11.16
	1	87.84 ± 1.38	8	12.75 ± 1.73	87.25 ± 1.73
	3	97.93 ± 0.77	24	14.40 ± 9.66 $^{c1\ d1}$	85.60 ± 9.66 $^{c1\ d1}$
	6	98.59 ± 0.54			
	24	99.00 ± 0.05 $^{c2\ d1}$			
RCM	0	0.00 ± 0.00	0	0.00 ± 0.00	0.00 ± 0.00
	20min	28.38 ± 0.03	1	27.24 ± 7.73 ^{e1}	72.76 ± 7.73 ^{e1}
	40min	28.65 ± 2.79	6	27.55 ± 3.97	72.45 ± 3.97
	1	47.79 ± 1.45	8	30.16 ± 5.17	69.84 ± 5.17
	3	43.50 ± 4.38	24	27.77 ± 6.04 ^{e1}	72.23 ± 6.04 ^{e1}
	6	57.38 ± 6.03			
	24	95.84 ± 3.17 ^{e1}			
LSCM	0	0.00 ± 0.00	0	0.00 ± 0.00	0.00 ± 0.00
	20min	3.53 ± 10.05	1	20.42 ± 3.18 ^{f1}	79.58 ± 3.18 ^{f1}
	40min	29.95 ± 5.63	6	20.83 ± 4.39	79.17 ± 4.39
	1	56.16 ± 16.60	8	22.85 ± 10.63	77.15 ± 10.63
	3	95.14 ± 2.21	24	13.27 ± 4.95 ^{f1}	86.73 ± 4.95 ^{f1}

	6	99.58 ± 002			
	24	99.08 ± 0.23 [f1]			
	0	0.00 ± 0.00	**0**	0.00 ± 0.00	0.00 ± 0.00
	20min	0.55 ± 8.34	**1**	35.12 ± 14.23 [e1]	64.88 ± 14.23 [e1]
	40min	20.00 ± 4.19	**6**	25.52 ± 2.87	74.48 ± 2.87
RCM-C	**1**	36.75 ± 0.72	**8**	26.85 ± 1.24	73.15 ± 1.24
	3	31.47 ± 0.23	**24**	26.50 ± 1.19 [e1]	73.50 ± 1.19 [e1]
	6	44.24 ± 6.98			
	24	92.71 ± 1.24 [e1]			
	0	0.00 ± 0.00	**0**	0.00 ± 0.00	0.00 ± 0.00
	20min	0.00 ± 0.00	**1**	26.11 ± 18.53 [f1]	73.89 ± 18.53 [f1]
	40min	4.43 ± 17.96	**6**	13.31 ± 6.38	86.69 ± 6.38
LSCM-C	**1**	0.00 ± 0.00	**8**	17.15 ± 12.94	82.85 ± 12.94
	3	86.98 ± 8.30	**24**	17.23 ± 13.46 [f1]	82.77 ± 13.46 [f1]
	6	99.78 ± 0.04			
	24	99.22 ± 0.04 [f1]			

Supplementary Table 4.3d. Effect of milk components and cocoa powder on sucrose conversion and reaction selectivity with *P. graminis* LS4

Sample ID	Time (h)	Sucrose conversion (%)	Time (h)	Relative transfructosylation extent (%)	Relative hydrolysis extent (%)
RSM	**0**	0.00 ± 0.00	**0**	0.00 ± 0.00	0.00 ± 0.00
	20min	92.17 ± 1.92	**1**	34.80 ± 0.29 $^{a1\ b1}$	65.20 ± 0.29 $^{a1\ b1}$
	40min	93.21 ± 2.24	**6**	36.69 ± 1.00	63.31 ± 1.00
	1	84.48 ± 0.98	**8**	28.84 ± 7.91	71.16 ± 7.91
	3	97.09 ± 0.14	**24**	25.08 ± 8.36 $^{a1\ b1}$	74.92 ± 8.36 $^{a1\ b1}$
	6	95.64 ± 0.26			
	24	97.28 ± 0.14 $^{a1\ b1}$			
RSM-L	**0**	0.00 ± 0.00	**0**	0.00 ± 0.00	0.00 ± 0.00
	20min	93.43 ± 0.27	**1**	31.60 ± 12.01 $^{b1\ c1}$	68.40 ± 12.01 $^{b1\ c1}$
	40min	94.89 ± 0.09	**6**	33.45 ± 5.53	66.55 ± 5.53
	1	84.19 ± 2.43	**8**	33.54 ± 1.59	66.46 ± 1.59
	3	96.28 ± 0.61	**24**	31.62 ± 10.82 $^{b1\ c1}$	68.38 ± 10.82 $^{b1\ c1}$
	6	93.99 ± 0.18			
	24	97.72 ± 0.22 $^{b1\ c1}$			
LSSM	**0**	0.00 ± 0.00	**0**	0.00 ± 0.00	0.00 ± 0.00
	20min	95.38 ± 0.77	**1**	31.15 ± 1.89 $^{a1\ d1}$	68.85 ± 1.89 $^{a1\ d1}$
	40min	96.88 ± 0.13	**6**	27.07 ± 3.01	72.93 ± 3.01
	1	92.18 ± 2.03	**8**	23.62 ± 3.46	76.38 ± 3.46
	3	98.25 ± 0.11	**24**	22.24 ± 1.76 $^{a1\ d1}$	77.76 ± 1.76 $^{a1\ d1}$
	6	94.53 ± 2.51			
	24	94.49 ± 0.84 $^{a1\ d1}$			
LSSM-L	**0**	0.00 ± 0.00	**0**	0.00 ± 0.00	0.00 ± 0.00
	20min	97.85 ± 0.18	**1**	31.18 ± 5.19 $^{c1\ d1}$	68.82 ± 5.19 $^{c1\ d1}$
	40min	98.28 ± 0.27	**6**	30.81 ± 5.31	69.19 ± 5.31
	1	91.15 ± 3.10	**8**	22.60 ± 2.05	77.40 ± 2.05
	3	98.58 ± 0.21	**24**	24.30 ± 2.35 $^{c1\ d1}$	75.70 ± 2.35 $^{c1\ d1}$
	6	92.88 ± 4.75			
	24	92.43 ± 1.22 $^{c1\ d1}$			
RCM	**0**	0.00 ± 0.00	**0**	0.00 ± 0.00	0.00 ± 0.00
	20min	78.85 ± 1.68	**1**	45.64 ± 3.85 ^{e1}	54.36 ± 3.85 ^{e1}
	40min	79.37 ± 0.91	**6**	41.19 ± 2.68	58.81 ± 2.68
	1	74.98 ± 3.02	**8**	30.21 ± 2.38	69.79 ± 2.38
	3	91.11 ± 0.75	**24**	36.83 ± 2.94 ^{e1}	63.17 ± 2.94 ^{e1}
	6	91.07 ± 0.86			
	24	95.80 ± 0.76 ^{e1}			
LSCM	**0**	0.00 ± 0.00	**0**	0.00 ± 0.00	0.00 ± 0.00
	20min	88.16 ± 3.09	**1**	34.52 ± 7.62 ^{f1}	65.48 ± 7.62 ^{f1}
	40min	89.97 ± 3.15	**6**	29.95 ± 6.30	70.05 ± 6.30
	1	61.50 ± 1.64	**8**	22.93 ± 1.80	77.07 ± 1.80
	3	98.40 ± 0.16	**24**	24.53 ± 5.36 ^{f1}	75.47 ± 5.36 ^{f1}

	6	93.40 ± 4.85			
	24	92.57 ± 0.34 [f1]			
	0	0.00 ± 0.00	**0**	0.00 ± 0.00	0.00 ± 0.00
	20min	68.61 ± 14.41	**1**	50.69 ± 5.16 [e1]	49.31 ± 5.16 [e1]
	40min	59.63 ± 0.06	**6**	43.29 ± 2.43	56.71 ± 2.43
RCM-C	**1**	49.90 ± 6.47	**8**	31.73 ± 4.18	68.27 ± 4.18
	3	84.34 ± 0.01	**24**	37.12 ± 2.62 [e1]	62.88 ± 2.62 [e1]
	6	91.61 ± 0.77			
	24	97.66 ± 0.05 [e1]			
	0	0.00 ± 0.00	**0**	0.00 ± 0.00	0.00 ± 0.00
	20min	82.17 ± 2.30	**1**	35.53 ± 6.40 [f1]	64.47 ± 6.40 [f1]
	40min	79.55 ± 0.82	**6**	29.32 ± 2.35	70.68 ± 2.35
LSCM-C	**1**	46.09 ± 21.97	**8**	19.77 ± 0.67	80.23 ± 0.67
	3	97.13 ± 0.23	**24**	25.70 ± 3.90 [f1]	74.30 ± 3.90 [f1]
	6	22.64 ± 61.41			
	24	96.07 ± 0.60 [f2]			

[a-f] Data with the same letter(s) were compared, a different number implies a significant difference detected at $p < 0.05$

(RSM- Regular sweetened milk; RSM-L- Regular sweetened milk enriched with lactose; LSSM- Low sugar sweetened milk; LSSM-L- Low sugar sweetened milk enriched with lactose; RCM- Regular chocolate milk; LSCM- Low sugar chocolate milk; RCM-C- Regular chocolate milk enriched with cocoa; LSCM-C- Low sugar chocolate milk enriched with cocoa)

CHAPTER V. ENDOGENOUS BIOGENERATION OF LACTOSUCROSE IN CHOCOLATE MILK USING *Vibrio natriegens* LEVANSUCRASE

Connecting Statement 3

The results from Chapter IV led to the identification of the food system, i.e. chocolate milk, to be used in the endogenous biogeneration of functional ingredients. They also helped to confirm the levansucrase of high catalytic efficiency for the biogeneration of lactosucrose in the chocolate milk in the following chapter.

Chapter V investigates the use of *Vibrio natriegens* levansucrase in the bioconversion of lactose and sucrose present in the chocolate milk into lactosucrose. This chapter focuses on the optimization of levansucrase units and the concentrations of sucrose and lactose. The following parameters were assessed as responses: pH, colour difference, rheological properties, reaction selectivity and lactosucrose production.

5. Abstract

This study focuses on the optimization of the bioconversion of lactose and sucrose to lactosucrose in chocolate milk using *Vibrio natriegens* levansucrase. A three-variable central composite rotatable design was created. The following parameters were optimized: lactose concentration, sucrose concentration and levansucrase units. The reaction selectivity, end-product profiles, and stability properties (pH, colour and rheological properties) were assessed via response surface methodology (RSM). Analysis of variance helped identify the critical parameters of each response. Lactose concentration was the critical parameter for the conversion of lactose to lactosucrose, relative transfructosylation extent, color difference and apparent viscosities at 50 s^{-1}. Sucrose concentration dictated the sucrose conversion to lactosucrose, sucrose conversion to oligolevan/levan, and LS concentration was the most important parameter for the lactosucrose production. The contour plots of the predictive models were generated. Finally, the biotransformation parameters to maximize the selectivity of *V. natriegens* LS towards lactosucrose synthesis were determined. A bio-transformed chocolate milk could contain 21.22 to 35.56 g/L lactosucrose.

5.1. Introduction

Lactosucrose (O-β-D-galactopyranosyl-(1,4)-O-α-D-glucopyranosyl-(1,2)-β-D-fructo-furanoside) is certified as a Functional Food Ingredient for Foods for Specific Health Uses (FOSHU) in Japan (Mu et al., 2013). It has indeed been valued for its potential prebiotic effects (Ohkusa et al., 1995), intestinal mineral absorption properties (Teramoto et al., 2006), and the ability to reduce body fat accumulation (Kimura et al., 2002). Being a low-digestive/non-digestible and low-cariogenic sweetener, lactosucrose has been used in recent years, in diverse foods and drinks, to improve consumers' intestinal health (Silvério et al., 2015). For instance, lactosucrose consumption in frozen yogurt, made with *Lactobacillus helveticus, Lactobacillus acidophilus, Bifidobacterium bifidum, and Streptococcus thermophilus* cultures, increased *bifidobacteria* and decreased lecithinase-negative *clostridia* levels (Hiroko et al., 2001). Additionally, faecal concentrations of ammonia, sulfide, phenol, cresol, indole, and skatol decreased significantly with lactosucrose intake (Hiroko et al., 2001). The application of lactosucrose to croissants also reported beneficial gastrointestinal effects with improvement in the frequency of defecation per week and the refreshing feeling after defecation (Ueda et al., 2000).

Besides being valued for its various health-promoting properties, lactosucrose can also be used as a techno-functional ingredient in various food systems. Its water-holding capacity makes it an interesting addition to fermented dairy products like yogurt and cheese to prevent syneresis and/or act as a fat replacer (Krasaekoopt, Bhandari, & Deeth, 2003; Silvério et al., 2015). The addition of lactosucrose to ice cream was reported to enhance viscosity, reduce hardness, and improve expansion and melting rates (Ma et al., 2021). In yogurt, lactosucrose was found to promote gel formation, increasing elasticity and viscosity . It also inhibited post-acidification and enhanced starter bacteria survival, leading to improved texture and water retention during storage (Xue et al., 2024). In baking, lactosucrose can boost bread volume and delay staling (Zhang et al., 2022).

Lactosucrose, a trisaccharide that is scarcely found in nature, poses challenges in its chemical synthesis. (Mu et al., 2013). Consequently, there has been an increasing interest in the enzymatic production of lactosucrose through transfructosylation reactions catalyzed by β-

fructofuranosidases (Chen et al., 2020) or levansucrases (LS, EC 2.4.1.10), which employ sucrose as the fructosyl donor and lactose as the acceptor. Lactosucrose was also synthesized by transgalactosylation reactions catalyzed β-galactosidases, which use lactose as the galactosyl donor and sucrose as the acceptor (Liao et al., 2023).

LS is a fructosyl-transferase that can catalyze the synthesis of complex oligosaccharides, by acquiring a fructosyl residue from a donor molecule and performing a non-Leloir transfer to an acceptor molecule (Hill et al., 2019; Hill et al., 2020; Inthanavong et al., 2013). Various LSs have shown potential in catalyzing the synthesis of lactosucrose, including LSs from *Gluconobacter oxydans, Vibrio natriegens, Novosphingobium aromaticivorans, Burkholderia graminis* (Bahlawan et al., 2023), *Bacillus subtilis* (Wu et al., 2023), and *Leuconostoc mesenteroides* (Li et al., 2015). However, limited studies applied LS directly in food systems to produce lactosucrose. LS from *Bacillus subtilis* CECT 39 could successfully synthesise lactosucrose using cheese whey permeate as a lactose source and tofu whey, which has a notable raffinose and stachyose content, as a fructosyl donor (Corzo-Martinez et al., 2015). Our previous studies also demonstrated how LS from *G. oxydans, V. natriegens, N. aromaticivorans* and *B. graminis* could be used to valorise whey and milk permeate (Bahlawan & Karboune, 2022; Bahlawan, Karboune, Liu, & Sahyoun, 2023). *V. natriegens* LS yielded the highest lactosucrose production of 251 g/L with whey permeate (Bahlawan et al., 2023). Additionally, the immobilization of *V. natriegens* LS on RelizymeTM EP403/S functionalized with iminodiacetic acid (IDA)-cupric ions (Cu2+) could produce 101 g/L of lactosucrose, using whey permeate as lactose source, and the immobilized LS could be successfully reused 3 consecutive times (Bahlawan & Karboune, 2022).

The objective of this study is to investigate and optimize the endogenous bioconversion of digestible sugars (sucrose and lactose) present in chocolate milk into non-digestible prebiotics, lactosucrose and fructooligosaccharides, using LS from *V. natriegens* NBRC 15636. A three-variable central composite rotatable design was created to optimize the lactose, sucrose and levansucrase concentrations. The reaction selectivity, end-product profiles, and stability properties (pH, colour and rheological properties) of the different chocolate milk formulations were assessed via response surface methodology (RSM). The critical parameters for lactosucrose production, lactose conversion to lactosucrose, sucrose conversion to lactosucrose, sucrose

conversion to oligolevan/levan, relative transfructosylation extent, colour difference relative to commercial chocolate milk, and apparent viscosities at 50 s^{-1} at temperatures 15, 37 and 60 °C, were identified. The contour plots of the predictive models were generated. Finally, the biotransformation parameters to maximize the selectivity of *V. natriegens* LS towards lactosucrose synthesis were determined. This biotransformation offers a less labour-intensive method than exogenous biogeneration to reduce high sugar levels in chocolate milk (Charoenwongpaiboon et al., 2021). Indeed, one of the major concerns of chocolate milk is its high sugar content (Murphy et al., 2008). Furthermore, with epidemiological survey data showing that 70% of the world's population has some degree of lactase deficiency, constant studies on producing lactose-free/low-lactose beverages are no doubt necessary to cater for the needs of lactose intolerant consumers (Li et al., 2023).

5.2. Materials and methods

5.2.1. Materials

Sucrose, D-(-)-fructose, D-(+)-glucose, α-Lactose monohydrate, myo-inositol, 3,5-dinitrosalicylic acid (DNS), potassium sodium tartrate (KNaC4H4O6), yeast extract, carbenicillin disodium salt, lysozyme from chicken egg white, DNase I, imidazole, C2H7NO2, NH4HCO3, NaOH solution were obtained from Sigma-Aldrich (Oakville, ON). KH2PO4, K2HPO4, 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, acetone, and Pierce™ Coomassie Plus (Bradford) assay kit were provided by Fisher Scientific (Fair Lawn, NJ). Coniferyl alcohol was obtained from Thermo Fisher Scientific Inc. (Fair Lawn, NJ). 1-kestose, nystose, and 1F -fructofuranosylnystose were obtained from FUJIFILM Wako Chemicals U.S.A. Corporation (Richmond, VA). Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) standards were purchased from Bio-Rad (Mississauga, ON). 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 (Waltham, MA). Lactosucrose was acquired from Pyson Biotech Co. Ltd (Shaanxi, China). Milk powder was purchased from Fonterra Co-operative Group Limited (Auckland, New Zealand). Two cocoa mixes (sugar, cocoa, with/without carrageenan) were provided by a local dairy cooperative (Agropur).

5.2.2. Production and purification of levansucrases

LS from *V. natriegens* NBRC 15636 was produced and purified as described in our previous studies (Hill et al., 2019). *Escherichia coli* BL21(DE3) cells (Invitrogen) were first transformed with the LS genes of selected strains. The cells, plated on LB agar containing 100 µg/ml carbenicillin, were precultured in an LB media also containing 100 µg/ml carbenicillin for 8-10 h at 37 °C under 250 rpm. Terrific broth containing 2% v/v of the preculture and 100 µg/ml carbenicillin was then incubated at 37 °C under 250 rpm for around 4 h, until a bacterial growth turbidity of optical density of 1.2–1.6 at 600 nm was achieved. The enzyme expression was induced using 1mM IPTG and the growth of the culture was resumed at room temperature for 18 h under 250 rpm. To collect the cells, centrifugation at 4°C under 8000 rpm was carried out. The recovered pellets were resuspended in a sonication buffer (50 mM PIPES, 300 mM NaCl, and 10 % glycerol; pH of 7.2; 4 ml/g). 4 mg/g lysozyme and 4 µl/g DNase were added to the suspensions which were then incubated at 18 °C under 50 rpm for 1 h. The cells were lysed by ultrasonication using a microtip (Misonix Ultrasonic Liquid Processor S-4000, Farmingdale, NY, USA) for 1 minute (10 s on, 60 s off, amplitude of 15) in an ice bath. The supernatants containing the enzymes were recovered after centrifugation at 4 °C under 14,000 rpm for 1 h, dialyzed against potassium phosphate buffer (5 mM; pH of 6) using a membrane with a molecular weight cut-off of 6–8 kDa, and then lyophilized. The LSs were purified via immobilized metal affinity chromatography on a HisTrap™ FF column (5 ml, GE Healthcare). After loading the resolubilized crude enzyme, the column was subsequently washed with sonication buffer, wash buffer (50 mM PIPES, 300 mM NaCl, and 10 % glycerol; pH of 6.4), 5 mM imidazole, and 10 mM imidazole. LS enzyme was then eluted with 100 mM and 200 mM imidazole. SDS-PAGE electrophoresis analysis at 120 V using 15 % SDS polyacrylamide gels and a 10x diluted Tris/Glycine/SDS buffer was performed to confirm the purity of the LSs. The total specific activity of the purified enzyme was quantified as the total amount of reducing sugars produced per minute per mg of protein using a DNS test as described by Hill et al. (2019). The LS fractions with the highest purity and specific activity were pooled and stored at − 80 °C.

5.2.3. Enzymatic biotransformation reactions: Optimization

A three-variable central composite rotatable design was created using Design Expert® Software. Eighteen chocolate milk formulations were prepared using a cocoa powder mix (0.82% w/w) without carrageenan, with lactose concentrations ranging from 3.5% to 14.0% w/w sourced from full cream milk powder (8.94 to 35.75%, w milk powder /v), and sucrose concentrations ranging from 3.5% to 24.0% w/w. To initiate the biotransformation, LS at varying concentration (from 1 to 5 U/mL) was added to chocolate milk formulation at a ratio of 0.01 to 0.05 (%, v/v). One unit of LS was defined as the amount of reducing sugars (glucose and fructose) produced per minute of reaction. See Supplementary Table 5.1 for more details on each formulation. The biotransformation reactions were carried out at 10 °C for 6h. All reactions were done in duplicates under 50 rpm. The selected reaction conditions were selected to mimic as closely as possible the conditions used during chocolate milk processing. After 6hr, the samples were placed in boiling water for 5 min to stop the reaction and then stored at -20°C until further analysis.

5.2.4. Reaction selectivity (Hydrolysis vs Transfructosylation)

After the enzymatic biotransformation reactions, the remaining sucrose as well as the released glucose and fructose 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 of 0.4 mL/min and 32°C. The hydrolysis extent of sucrose was quantified from the concentration of released fructose and taken as a percentage of the initial sucrose concentration, while 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.

1) $$Transfructosylation\ yield = \frac{Concentration\ of\ released\ glucose - Concentration\ of\ released\ fructose}{Initial\ sucrose\ concentration} \times 100$$
2) $$Hydrolysis\ yield = \frac{Concentration\ of\ released\ fructose}{Initial\ sucrose\ concentration} \times 100$$

5.2.5. End-product profile characterization of Enzymatic biotransformation reactions

The sucrose concentration and end-product profiles of lactosucrose and fructooligosaccharides (FOSs) were characterized using an Agilent 1290 II liquid chromatography system coupled to an Agilent 6560-ion mobility Q-TOF –MS. The samples were prepared by diluting them in 50:50 acetonitrile: water (v/v) with the addition of myo-inositol (5 ppm) to serve as an internal standard. 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 acetonitrile with 0.3% NH_4OH. The 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 30 psig, capillary voltage of 4000 V, fragmentor voltage of 200 V, skimmer voltage of 30 V, 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 were collected at both centroid and profile mode. Reference ions (m/z at 112.99 and 1033.99 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.

5.2.6. Assessment of stability properties of enriched chocolate milk concentrate

5.2.6.1. pH measurement

An Orion™ 3-Star Benchtop pH Meter (Thermo Scientific, Fair Lawn, NJ) was used to measure pH at room temperature. Measurements were performed in triplicate.

5.2.6.2. Color measurement

The colour parameters of the samples (L*: brightness, a*: redness, b*: yellowness) were recorded using a CR-400 chroma meter (Konica Minolta Sensing Americas, NJ USA). A sample volume of 12 mL was loaded in the 10 mm cell CM-A98. Measurements were made in triplicate.

Total colour difference (ΔE), against the commercial chocolate milk sample with stabliser (BLK 2), was calculated using the following equation:

3) $\Delta E = \sqrt{(L^* - Lo^*)^2 + (a^* - ao^*)^2 + (b^* - bo^*)^2}$

5.2.6.3. Measurement of rheological properties

The rheological properties of the chocolate milk formulations were determined using a stress-controlled rheometer (AR2000 Rheometer, TA Instrument, New Castle, DE) fitted with a stainless steel 60 mm cone of 2° and solvent trap. The measurement temperatures (15, 37, 60 °C) were kept using a circulating bath and a controlled Peltier system. The samples were loaded to the rheometer immediately after being homogenized at 6000 rpm for 1 min using a Fisherbrand™ 850 homogenizer (Fisher Scientific, Fair Lawn, NJ). Steady-state flow parameters including flow behavior index (n), consistency coefficient (m) and shear viscosity (η) were determined at increasing shear rates (1-100 s^{-1}). The apparent viscosity was measured as a function of shear rate (ẏ). Experimental flow curves were compared to the Power-law model. The variations of consistency coefficient (m) and flow behaviour index (n) were then determined for each sample.

4) $\eta = m\dot{y}^{(n-1)}$

where n <1 for a shear-thinning fluid and n = 1 for a Newtonian fluid.

η: shear viscosity

m: consistency coefficient

ẏ: shear rate

n: flow behavior index

5.3. Results and Discussion

5.3.1. Effects of biotransformation parameters on the sucrose and lactose bioconversions in reconstituted chocolate milks

The effects of the reaction parameters on the biotransformation of reconstituted chocolate milks catalyzed by *V. natriegens* levansucrase were assessed via response surface

methodology (RSM). The three reaction parameters, the enzyme units (from 1 to 5 U/mL), lactose concentration (from 3.5 % to 14.0 % w/w) and sucrose concentration (from 3.5 % to 24.0 % w/w) were varied in the reconstituted chocolate milks, while the temperature (10 °C) was kept constant. The total sucrose conversion, the conversion of lactose to lactosucrose, the conversion of sucrose to lactosucrose and the conversion of sucrose to oligolevan/levan were determined (Table 5.1). Levansucrase can also catalyze the synthesis of fructooligosaccharides (FOS), including kestose, nystose, and fructosyl nystose, when sucrose serves as both the fructosyl donor and acceptor (Tian et al., 2011; Tian & Karboune, 2012). However, negligible FOS were detected upon the biotransformation of the reconstituted chocolate milk. The results of the reaction selectivity analysis are displayed in Table 5.1.

The lowest total sucrose conversion of 22.04% was recorded with formulation #17, while the highest total sucrose conversion of 54.18% was reached with formulation #5. The lactosucrose production ranged from 15.08 to 48.68 g/L. The conversion of lactose to lactosucrose was calculated as the concentration of lactosucrose formed as a percentage of the initial lactose concentration and ranged from 10.01 to 61.90%. Then for the conversion of sucrose to lactosucrose, which was calculated as the concentration of lactosucrose formed as a percentage of the initial sucrose concentration, the lowest conversion of 7.03% and highest conversion of 34.82% were obtained with formulations #1 and #2, respectively. Conversely, formulation #2 resulted in the lowest conversion of sucrose to oligolevan/levan. The highest conversion of sucrose to oligolevan/levan was reached with formulation #5 at 35.02%. The conversion of sucrose to oligolevan/levan was calculated as the difference between the conversion of sucrose to transfructosylated products and the conversion of sucrose to lactosucrose. Finally, the relative transfructosylation varied between 74.12 to 87.77%.

Table 5.2 shows the analysis of variance (ANOVA) for each response. The F-value can be used to compare variability estimates between and within data sets. A large F-value indicates that the variability between data sets' means is larger than the variability within data sets, suggesting that there may be significant differences among the data sets. The p-value is the chance probability of a result i.e., the level of statistical confidence in the validity of the test. If the p-value is very small (typically smaller than 0.05), it suggests that the observed data is unlikely

Table 5.1. Experimental design parameters and responses of end-product profile characterization and reaction selectivity

Run	Parameter 1	Parameter 2	Parameter 3	Response 1	Response 2	Response 3	Response 4	Response 5	Response 6
	Lactose %(w/w)	Sucrose %(w/w)	LS unit (U/mL)	Sucrose conversion (%) [a]	Lactosucrose (g/L)	Conversion of lactose to lactosucrose (%) [b]	Conversion of sucrose to lactosucrose (%) [c]	Conversion of sucrose to oligolevan/ levan (%) [d]	Relative transfructosylation (%) [e]
1	11.8717	19.8447	1.81079	46.25 ± 2.22	20.54 ± 1.41	11.75 ± 0.81	7.03 ± 0.48	31.26 ± 1.51	82.73 ± 0.69
2	8.75	3.5	3	45.75 ± 3.82	17.94 ± 2.15	13.93 ± 1.67	34.82 ± 4.17	2.22 ± 3.17	80.96 ± 0.18
3	11.8717	7.65531	4.18921	50.40 ± 1.75	24.56 ± 1.69	14.05 ± 0.97	21.79 ± 1.50	17.9 ± 1.12	78.76 ± 0.51
4	8.75	13.75	3	50.07 ± 2.90	25.54 ± 2.46	19.82 ± 1.91	12.61 ± 1.22	27.12 ± 1.72	79.36 ± 1.15
5	8.75	24	3	54.18 ± 0.57	27.82 ± 0.77	21.60 ± 0.60	7.87 ± 0.22	35.02 ± 0.35	79.19 ± 0.18
6	5.62833	7.65531	1.81079	33.74 ± 2.16	21.03 ± 1.10	25.38 ± 1.33	18.66 ± 0.98	7.88 ± 1.52	78.69 ± 0.55
7	11.8717	7.65531	1.81079	28.51 ± 1.61	17.50 ± 0.10	10.01 ± 0.06	15.52 ± 0.09	7.30 ± 1.00	80.07 ± 1.00
8	5.62833	19.8447	1.81079	40.01 ± 2.27	27.62 ± 2.71	33.33 ± 3.27	9.45 ± 0.93	22.26 ± 1.99	79.27 ± 0.48
9	14	13.75	3	44.44 ± 3.07	22.15 ± 3.34	10.75 ± 1.62	10.94 ± 1.65	25.87 ± 2.48	82.80 ± 0.15
10	3.5	13.75	3	44.03 ± 0.26	31.90 ± 1.15	61.90 ± 2.22	15.76 ± 0.57	13.67 ± 0.08	66.82 ± 0.58
11	8.75	13.75	1.5	41.22 ± 1.85	19.45 ± 1.03	15.10 ± 0.80	9.61 ± 0.51	23.84 ± 1.23	81.19 ± 0.67
12	8.75	13.75	1.81079	38.82 ± 0.08	22.81 ± 0.14	17.70 ± 0.11	11.27 ± 0.07	19.73 ± 0.30	79.72 ± 0.94
13	8.75	13.75	5	33.93 ± 5.76	47.91 ± 3.49	37.19 ± 2.71	23.66 ± 1.73	3.24 ± 5.14 *	79.16 ± 1.71
14	5.62833	19.8447	4.18921	40.48 ± 0.81	46.49 ± 0.40	56.10 ± 0.48	15.91 ± 0.14	16.08 ± 0.98	78.02 ± 3.97
15	5.62833	7.65531	4.18921	26.08 ± 5.33	35.56 ± 3.27	42.92 ± 3.95	31.55 ± 2.90	*	74.12 ± 5.19
16	11.8717	19.8447	4.18921	25.96 ± 0.10	48.68 ± 0.99	27.85 ± 0.57	16.66 ± 0.34	4.33 ± 0.05 *	80.89 ± 0.12
17	8.75	13.75	1	22.04 ± 1.44	15.08 ± 2.68	11.70 ± 2.08	7.45 ± 1.33	12.00 ± 1.89	87.77 ± 2.82
18	8.75	13.75	2	31.62 ± 4.94	29.22 ± 3.95	22.68 ± 3.06	14.43 ± 1.95	13.05 ± 5.33	86.82 ± 3.30

[a] The sucrose conversion was calculated as the difference of initial and final sucrose concentration as a percentage of the initial sucrose concentration

[b] The conversion of lactose to lactosucrose was calculated as the concentration of lactosucrose formed as a percentage of the initial lactose concentration

[c] The conversion of sucrose to lactosucrose was calculated as the concentration of lactosucrose formed as a percentage of the initial sucrose concentration

[d] The conversion of sucrose to oligolevan/levan was calculated as the difference between the conversion of sucrose to transfructosylated products and the conversion of sucrose to lactosucrose. The conversion of sucrose to transfructosylated products was calculated as the percentage product of sucrose conversion and relative transfructosylation.

[e] The relative transfructosylation was calculated as the transfructosyltion extent as a percentage of the sum of transfructosylation and hydrolysis extents

*values marked were ignored in the generated model

Table 5.2. Analysis of variance (ANOVA) for the end-product profile characterization and reaction selectivity. All reactions maintained the same temperature (10 °C) and reaction time (6 h).

	Lactosucrose production (g/L)		Conversion of lactose to lactosucrose (%)		Conversion of sucrose to lactosucrose (%)		Conversion of sucrose to oligolevan/levan (%)		Relative transfructosylation (%)	
	F	***p-value***	***F***	***p-value***	***F***	***p-value***	***F***	***p-value***	***F***	***p-value***
Model	21.13	< 0.0001	33.08	< 0.0001	27.00	< 0.0001	7.88	0.0036	4.43	0.0218
A- Lactose %(w/w)	3.88	0.0690	177.38	< 0.0001	3.37	0.0877	4.77	0.0495	8.45	0.0115
B- Sucrose %(w/w)	11.36	0.0046	13.44	0.0063	46.03	< 0.0001	20.76	0.0007	0.3361	0.5713
C- LS unit (U/mL)	48.15	< 0.0001	59.84	< 0.0001	31.60	< 0.0001	0.8623	0.3714	4.50	0.0522
AB			0.2932	0.6029						
AC			3.80	0.0871						
BC			2.79	0.1334						
A^2			16.29	0.0038						
B^2			2.29	0.1690						
C^2			0.3572	0.5666						

to have occurred if the null hypothesis (which states that there is no effect or no difference between groups) were true (Bower, 2013). For the lactosucrose production, the best model that was found to be significant was linear. With the linear model, the LS concentration was the most significant parameter, with the highest F-value of 48.15 and the lowest p-value of <0.0001. The conversion of lactose to lactosucrose followed a quadratic model. No significant interactive effects between the reaction parameters were, however, observed with the highest F-value and lowest p-value obtained with lactose-sucrose concentrations, only valued at 3.80 and 0.0871, respectively. Lactose concentration seemed to be instead the most important parameter, based on both the linear and quadratic models (F-value of 177.38; p-value of <0.0001 and F-value of 16.29; p-value of 0.0038, respectively). For the conversion of sucrose to lactosucrose, a linear model was preferred over a quadratic one, with both sucrose and enzyme concentrations being critical parameters (F-value of 46.03; p-value of < 0.0001 and F-value of 31.60; p-value of < 0.0001, respectively). Then for the conversion of sucrose to oligolevan/levan, the sucrose concentration was found to be the most determinant parameter as per the linear model with an F-value of 20.76 and p-value of 0.0007. The analysis of variance for the relative transfructosylation ratio indicated that a linear model best fits the data, with lactose concentration being the most significant parameter (F-value: 8.45, p-value: 0.0115).

5.3.2. Effects of biotransformation parameters on the pH, colour and rheological properties of reconstituted chocolate milks

The results of the pH and colour measurements are shown in Table 5.3. Previous studies have shown that pH change can affect reaction selectivity (Inthanavong et al., 2013). The pH values recorded all fell within the range of 6.29 and 6.78. The difference in pH is negligible ($p > 0.05$) and it can hence be assumed that pH change will not affect the transfructosylation of lactose. The colour measurements revealed that formulation #2 had the highest L value (61.79) and colour difference relative to commercial chocolate milk, ΔE (14.89) while having the lowest a value (8.86) and b value (12.96). Conversely, formulation #10 had the lowest L value (51.15), while having the highest a value (11.22) and b value (14.28). The lowest colour difference of 6.34 was obtained with formulation #8.

Table 5.3. Experimental design parameters and responses of pH and color measurements

Run	Parameter 1	Parameter 2	Parameter 3	Response 1	Response 2	Response 3	Response 4	Response 5
	Lactose %(w/w)	Sucrose %(w/w)	LS unit (U/mL)	pH	L value	a value	b value	ΔE
1	11.8717	19.8447	1.81079	6.29 ± 0.01	55.03 ± 0.48	9.76 ± 0.66	13.53 ± 0.23	8.23 ± 0.70
2	8.75	3.5	3	6.56 ± 0.04	61.79 ± 0.25	8.86 ± 0.43	12.96 ± 0.02	14.89 ± 0.33
3	11.8717	7.65531	4.18921	6.48 ± 0.00	60.44 ± 0.12	9.00 ± 0.21	13.20 ± 0.06	13.52 ± 0.09
4	8.75	13.75	3	6.49 ± 0.03	58.44 ± 0.04	9.27 ± 0.01	13.57 ± 0.02	11.48 ± 0.03
5	8.75	24	3	6.45 ± 0.01	52.55 ± 0.30	10.6 ± 0.14	14.01 ± 0.09	6.93 ± 0.10
6	5.62833	7.65531	1.81079	6.65 ± 0.01	57.88 ± 0.06	9.69 ± 0.50	13.49 ± 0.07	11 ± 0.05
7	11.8717	7.65531	1.81079	6.38 ± 0.01	60.56 ± 0.39	9.26 ± 0.23	13.16 ± 0.04	13.61 ± 0.40
8	5.62833	19.8447	1.81079	6.54 ± 0.00	52.08 ± 0.16	10.4 ± 0.71	14.08 ± 0.06	6.34 ± 1.14
9	14	13.75	3	6.33 ± 0.01	57.19 ± 0.67	9.56 ± 0.78	13.62 ± 0.04	10.38 ± 0.54
10	3.5	13.75	3	6.78 ± 0.00	51.15 ± 0.62	11.22 ± 0.06	14.28 ± 0.27	7.85 ± 0.28
11	8.75	13.75	1.5	6.43 ± 0.00	58.23 ± 0.38	9.33 ± 0.24	13.44 ± 0.03	11.28 ± 0.39
12	8.75	13.75	1.81079	6.44 ± 0.01	57.69 ± 0.08	9.72 ± 0.16	13.53 ± 0.16	10.78 ± 0.05
13	8.75	13.75	5	6.54 ± 0.01	56.81 ± 0.40	9.59 ± 0.39	13.91 ± 0.08	9.92 ± 0.30
14	5.62833	19.8447	4.18921	6.63 ± 0.00	51.71 ± 0.11	10.98 ± 0.41	14.08 ± 0.02	7.25 ± 0.93
15	5.62833	7.65531	4.18921	6.69 ± 0.01	56.69 ± 0.08	9.95 ± 0.70	13.72 ± 0.00	10.00 ± 0.29
16	11.8717	19.8447	4.18921	6.36 ± 0.01	54.11 ± 0.12	9.90 ± 0.23	13.55 ± 0.04	7.32 ± 0.26
17	8.75	13.75	1	6.39 ± 0.01	57.62 ± 0.51	9.57 ± 0.63	13.28 ± 0.07	10.74 ± 0.43
18	8.75	13.75	2	6.44 ± 0.01	57.21 ± 0.28	9.72 ± 0.37	13.5 ± 0.1	10.32 ± 0.18
Commercial chocolate milk with stabilizer (BLK 2)					46.96 ± 0.01	9.37 ± 0.01	10.71 ± 0.01	

The rheological properties of each reconstituted chocolate milk were determined. The Power law parameters are presented in Table 5.4 and the corresponding shear-dependent viscosities are depicted in Fig. 5.1. The rheological properties of the chocolate milk formulations were investigated at 15, 37 and 60 °C. These temperatures were selected to mimic the conditions of cold and hot chocolate milk, as well as the average normal oral temperature of 37 °C. The apparent viscosity at 50 s^{-1} (η50) was also determined since this specific shear rate is commonly accepted for sensory perception analysis (Shama & Sherman, 1973). The consistency coefficient, m, and apparent viscosity at 50 s^{-1} decreased with increasing temperature for all milk formulations. The flow behaviour index, n, in general, increased from 15 °C to 37 °C and then decreased to 60 °C. In Fig. 5.1, the apparent viscosities of all chocolate milk formulations decreased significantly with increasing shear rate, confirming a non-Newtonian shear thinning pseudoplastic behaviour. These viscosities eventually stabilized at higher shear rates.

The analysis of variance for the colour difference, ΔE and the apparent viscosity at 50 s^{-1} at 15, 37 and 60 °C was evaluated (See Table 5.5). The color difference fell under a quadratic model. No significant interactive effect between the reaction parameters was detected. The lactose-sucrose concentrations interaction had the highest F-value and p-value, but the F-value was not outstandingly high (F-value of 6.64; p-value of 0.0328).

Table 5.4. Power law parameters for chocolate milks at selected temperatures

Run	Lactose %(w/w)	Sucrose %(w/w)	LS unit (U/mL)	15 °C			37 °C			60 °C		
				m (mPa)	n	η50 (mPa.s)	m (mPa)	n	η50 (mPa.s)	m (mPa)	n	η50 (mPa.s)
1	11.8717	19.8447	1.81079	48.62 ± 0.67	0.90 ± 0.00	32.88 ± 0.45	17.65 ± 0.97	0.95 ± 0.01	14.70 ± 0.61	10.01 ± 0.40	0.96 ± 0.02	8.67 ± 0.17
2	8.75	3.5	3	18.04 ± 0.38	0.85 ± 0.01	9.84 ± 0.06	5.23 ± 0.17	0.98 ± 0.01	4.77 ± 0.06	3.51 ± 0.01	0.98 ± 0.00	3.25 ± 0.01
3	11.8717	7.65531	4.18921	40.38 ± 5.77	0.91 ± 0.04	28.93 ± 8.81	13.33 ± 0.61	0.95 ± 0.03	10.76 ± 0.73	7.45 ± 0.19	0.96 ± 0.01	6.24 ± 0.02
4	8.75	13.75	3	47.14 ± 1.27	0.81 ± 0.01	22.00 ± 1.20	7.82 ± 0.26	1.00 ± 0.02	7.68 ± 0.53	5.47 ± 0.34	0.97 ± 0.00	4.86 ± 0.30
5	8.75	24	3	38.99 ± 0.08	0.89 ± 0.00	25.36 ± 0.05	13.68 ± 0.05	0.95 ± 0.00	11.25 ± 0.04	7.75 ± 0.03	0.98 ± 0.01	7.03 ± 0.22
6	5.62833	7.65531	1.81079	13.83 ± 0.53	0.81 ± 0.02	6.46 ± 0.38	3.69 ± 0.11	0.98 ± 0.01	3.38 ± 0.06	3.40 ± 0.01	0.93 ± 0.01	2.54 ± 0.08
7	11.8717	7.6553	1.8107	39.39 ± 0.47	0.86 ± 0.01	22.81 ± 1.54	13.66 ± 0.27	0.92 ± 0.01	10.12 ± 0.26	7.53 ± 0.41	0.96 ± 0.02	6.43 ± 0.18
8	5.62833	19.8447	1.81079	18.89 ± 1.24	0.86 ± 0.01	10.70 ± 0.41	5.78 ± 0.11	0.98 ± 0.01	5.29 ± 0.09	3.94 ± 0.19	0.98 ± 0.01	3.64 ± 0.03
9	14	13.75	3	121.96 ± 0.93	0.83 ± 0.04	64.02 ± 9.39	47.66 ± 3.10	0.87 ± 0.01	28.09 ± 1.05	20.39 ± 1.62	0.92 ± 0.01	15.11 ± 1.37
10	3.5	13.75	3	13.19 ± 0.51	0.80 ± 0.02	5.96 ± 0.46	3.47 ± 0.11	0.98 ± 0.01	3.24 ± 0.17	2.00 ± 0.14	1.05 ± 0.02	2.39 ± 0.03
11	8.75	13.75	1.5	23.33 ± 1.52	0.85 ± 0.01	13.16 ± 1.10	7.40 ± 0.10	0.97 ± 0.01	6.45 ± 0.09	5.93 ± 0.25	0.94 ± 0.01	4.59 ± 0.07
12	8.75	13.75	1.81079	16.94 ± 0.65	0.93 ± 0.01	12.72 ± 0.43	6.94 ± 0.40	0.98 ± 0.01	6.42 ± 0.01	6.50 ± 0.37	0.90 ± 0.01	4.34 ± 0.28
13	8.75	13.75	5	19.34 ± 0.83	0.91 ± 0.01	13.43 ± 0.80	7.84 ± 0.21	0.97 ± 0.01	6.97 ± 0.12	5.13 ± 0.12	0.98 ± 0.01	4.65 ± 0.05
14	5.62833	19.8447	4.18921	12.76 ± 0.89	0.94 ± 0.00	10.09 ± 0.70	8.27 ± 0.23	0.91 ± 0.01	5.70 ± 0.05	3.26 ± 0.16	1.03 ± 0.01	3.66 ± 0.04
15	5.62833	7.65531	4.18921	17.31 ± 2.98	0.96 ± 0.06	14.70 ± 0.71	3.81 ± 0.12	0.99 ± 0.01	3.70 ± 0.08	3.11 ± 0.09	0.97 ± 0.01	2.71 ± 0.01
16	11.8717	19.8447	4.18921	57.17 ± 1.16	0.89 ± 0.04	37.37 ± 5.42	20.89 ± 0.90	0.93 ± 0.03	15.74 ± 1.90	9.93 ± 0.60	0.97 ± 0.02	8.74 ± 0.45
17	8.75	13.75	1	19.82 ± 0.36	0.92 ± 0.03	14.56 ± 1.87	6.63 ± 0.42	1.00 ± 0.02	6.62 ± 0.05	4.33 ± 0.22	0.99 ± 0.01	4.16 ± 0.17
18	8.75	13.75	2	21.18 ± 1.07	0.89 ± 0.01	13.52 ± 1.06	6.98 ± 0.20	1.00 ± 0.01	6.97 ± 0.07	4.51 ± 0.10	0.99 ± 0.01	4.40 ± 0.11
Commercial chocolate milk without stabilizer (BLK 1)				7.81 ± 0.16	0.86 ± 0.01	4.52 ± 0.16	2.38 ± 0.10	1.02 ± 0.00	2.57 ± 0.11	1.92 ± 0.07	1.01 ± 0.01	1.95 ± 0.02
Commercial chocolate milk with stabilizer (BLK 2)				10.67 ± 0.12	0.87 ± 0.01	6.29 ± 0.1	3.57 ± 0.08	0.99 ± 0.01	3.37 ± 0.17	2.51 ± 0.54	0.99 ± 0.03	2.39 ± 0.25

m: Consistency coefficient

n: Flow behavior index

η50: Apparent viscosity at 50 s^{-1}

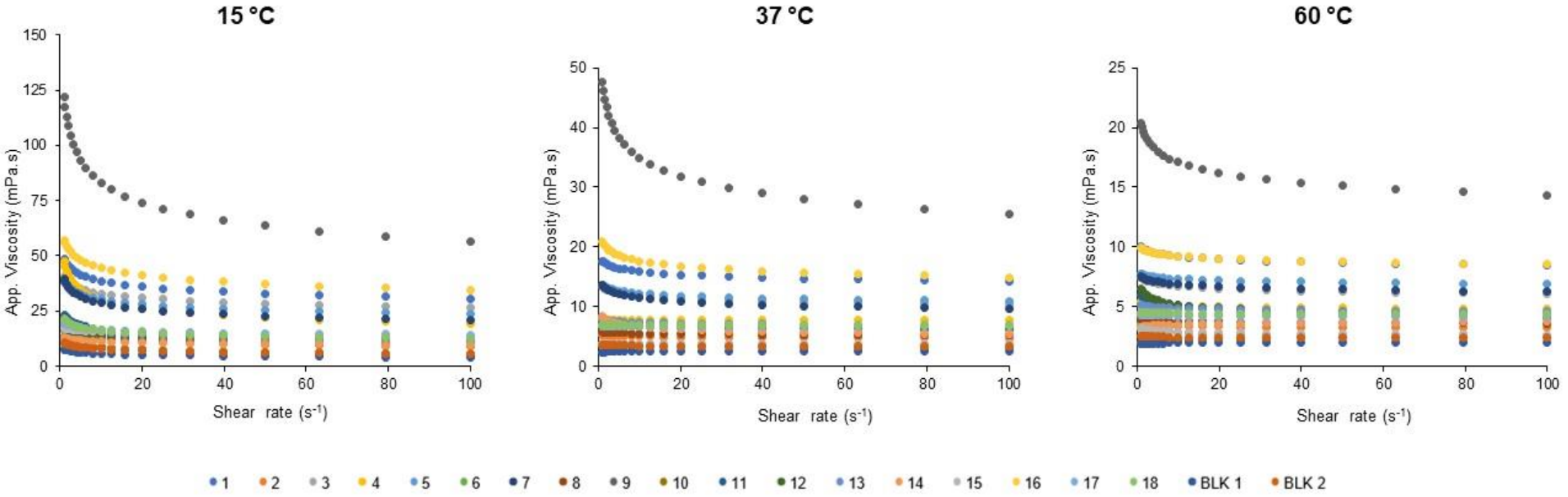


Fig 5.1. Shear-dependent viscosities of chocolate milk formulations at selected temperatures

Table 5.5. Analysis of variance (ANOVA) for the color difference and rheological properties. All reactions maintained the same temperature (10 °C) and reaction time (6 h).

	Colour difference, ΔE		η50 at 15°C (mPa.s)		η50 at 37°C (mPa.s)		η50 at 60°C (mPa.s)	
	F	***p-value***	***F***	***p-value***	***F***	***p-value***	***F***	***p-value***
Model	33.40	< 0.0001	13.23	0.0002	14.05	0.0002	15.93	< 0.0001
A- Lactose %(w/w)	34.14	0.0004	36.12	< 0.0001	37.65	< 0.0001	42.60	< 0.0001
B- Sucrose %(w/w)	235.19	< 0.0001	2.24	0.1568	3.97	0.0662	4.86	0.0447
C- LS unit (U/mL)	1.36	0.2764	1.33	0.2675	0.5325	0.4776	0.3471	0.5651
AB	6.64	0.0328						
AC	0.3108	0.5925						
BC	0.4665	0.5139						
A^2	18.66	0.0025						
B^2	0.7692	0.4060						
C^2	3.08	0.1173						

As for the most determinant parameter, it was determined to be the lactose concentration as per the quadratic model (F-value of 18.66; p-value of 0.0025). However, the linear model indicated the sucrose concentration to be the most critical (F-value of 235.19; p-value of <0.0001). The apparent viscosity at 50 s^{-1} at the different temperatures all followed a linear model. The lactose concentration was the critical parameter, regardless of the temperature (F-value of 36.12; p-value of < 0.0001, F-value of 37.65; p-value of < 0.0001 and F-valueof 42.60; p-value of < 0.0001, respectively).

5.3.3. Predictive models of biotransformation parameters

The contour plots of the predictive models are shown in Fig 5.2. Fig 5.2(A1-A3) shows that for maximizing lactosucrose production, high sucrose, and LS units, but low lactose concentrations, are needed. Similarly, for the conversion of lactose to lactosucrose (Fig 5.2(B1-B3), low concentrations of lactose maximized the conversion achieved. Sucrose and LS concentrations both increased the lactose conversion, but the LS unit had a narrower range, as seen in Fig 5.2(B2), making it a more critical parameter. Still, in accordance with the ANOVA analysis, the contour plots also indicate that the lactose concentration is the most significant parameter among the three parameters. Then for the conversion of sucrose to lactosucrose, only increasing the enzyme concentration, increased the sucrose conversion. The substrate concentrations lowered the conversion of sucrose to lactosucrose (Fig 5.2(C1-C2)). An excess in the sucrose concentration might indeed have caused a shift of the reaction towards oligomerization and/or polymerization, producing oligolevan/levan, rather than transferring a fructosyl group from sucrose to lactose. Indeed, Fig 5.2(D1) confirms that increasing the sucrose concentration increased the conversion of sucrose to oligolevan/levan. It is also important to note that the sucrose concentration is the limiting parameter when it comes to both the conversion of sucrose to lactosucrose and to oligolevan/levan.

The predictive models of the relative transfructosylation extent (Fig 5.2(E1-E2)), suggest that high lactose and sucrose concentrations can maximize the relative transfructosylation extent, with lactose being a more critical parameter. High enzyme units, however, decreased the relative transfructosylation extent, indicating a shift towards hydrolysis. Fig 5.2(F1) indicates that

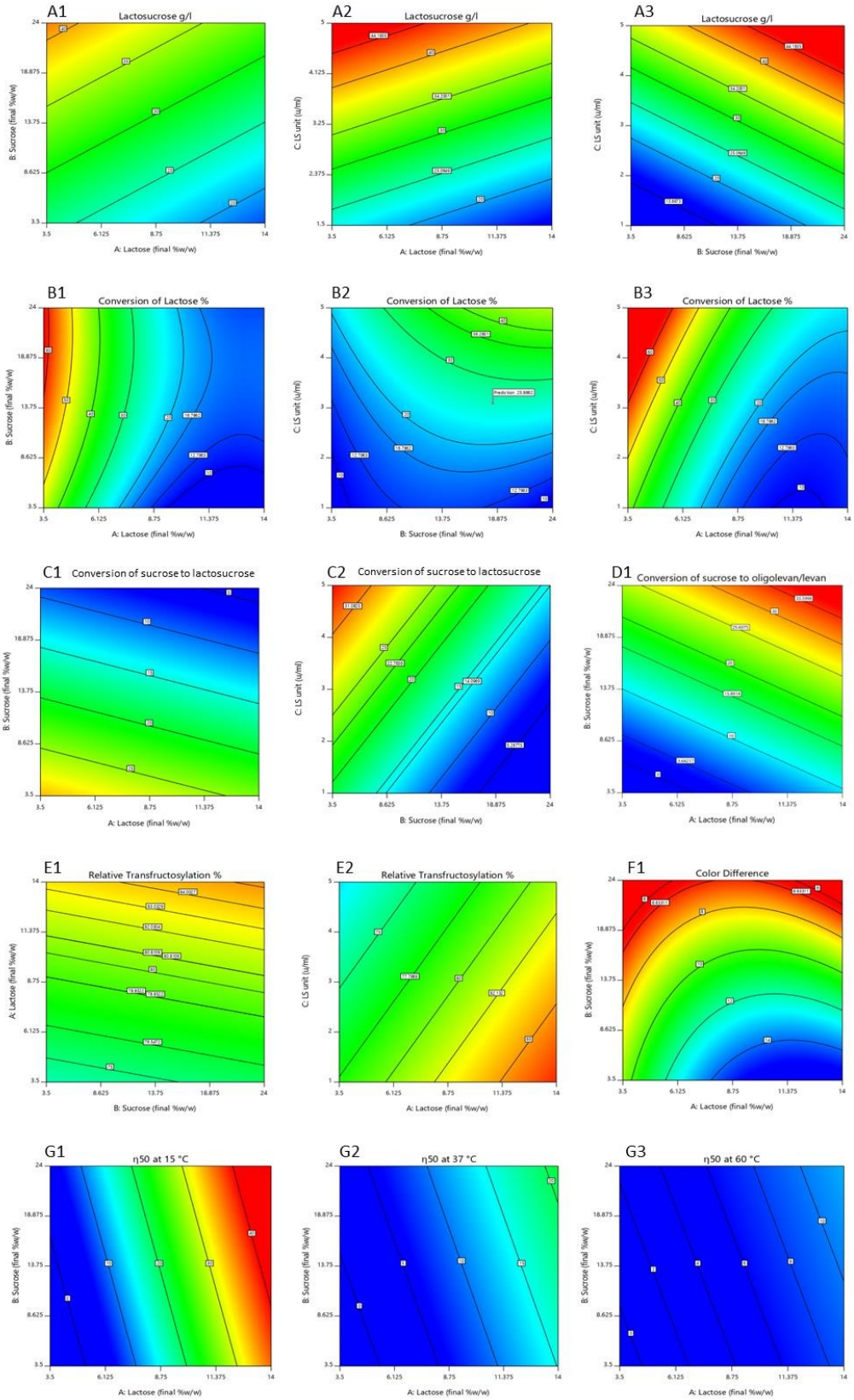


Fig 5.2. Contour plots of *V. natriegens*. All reactions maintained the same temperature (10 °C) and reaction time (6 h)

the higher the lactose concentration, the higher the colour difference relative to that of a commercial chocolate milk. This is expected, given the milk powder colour, used as a lactose source. Reversely, a high sucrose concentration decreased the colour difference. Finally, high lactose and sucrose concentrations increased the apparent viscosity at 50 s^{-1}. This is more evident at lower temperatures (Fig 5.2(G1-G3)). Lactose concentration was the most critical parameter, as previously confirmed with the ANOVA table (Table 5.2). Yet, it is important to keep in mind that the production of lactosucrose and/or levan might increase the chocolate milk formulations' apparent viscosity (Sahyoun et al., 2024; Silvério et al., 2015).

5.3.4. Selected biotransformation parameters

The biotransformation parameters to maximize the selectivity of *V. natriegens* LS towards lactosucrose synthesis were determined from the predictive models. Table 5.6 summarizes the identified conditions, i.e., the lactose, sucrose, and LS concentrations, as well as the predicted confidence interval of responses.

A low lactose concentration of 4 % (w/w) was predicted to maximize lactosucrose production. It is interesting to note that regular milk usually contains around 4-5% (w/w) of lactose (Ohlsson et al., 2017). Hence, reducing/increasing the lactose content in milk will not be necessary before the biotransformation reaction. As for the sucrose content, 5.49% (w/w) is approximately what is usually used in chocolate milk processing (Data provided by a local dairy cooperative). The predictive model suggested a sucrose concentration of 7.53 %(w/w), which will eventually be reduced after the transfructosylation of lactose and sucrose by LS. Finally, the predictive model suggested a LS concentration of 5 U/mL.

The experimental responses for the lactosucrose produced, conversion of lactose to lactosucrose, conversion of sucrose to lactosucrose, conversion of sucrose to oligolevan/levan, relative transfructosylation and apparent viscosity at 15 and 60 °C all fell within the predictive models' confidence intervals (See Table 5.6). The only exception was the apparent viscosity at 37 °C. The apparent viscosities of a commercial chocolate milk at 50 s^{-1} are shown in Table 5.4 (6.29 mPa.s at 15 °C; 3.37 mPa.s at 37 °C; 2.39 mPa.s at 60 °C). The experimental responses resulted in slightly lower viscosities, indicating that the chocolate milk might need to be supplemented with

Table 5.6. Responses of the optimal conditions for lactosucrose production with *V. natriegens* LS in chocolate milk

	Predicted confidence interval	Experimental responses
Selected parameters		
Lactose % (w/w)	4.00	
Sucrose % (w/w)	7.53	
LS U/mL	5	
Responses		
Lactosucrose (g/L)	18.22 - 50.46	21.22 ± 3.94
Conversion of lactose to lactosucrose (%)	34.65 - 50.54	36.04 ± 6.70
Conversion of sucrose to lactosucrose (%)	16.44 - 49.99	19.14 ± 3.56
Conversion of sucrose to oligolevan/levan (%)	10.77 - 33.36	11.96 ± 2.51
Relative transfructosylation (%)	53.48 - 89.55	76.16 ± 1.26
η50 (mPa.s) at 15 °C	5.71 - 13.3	6.05 ± 0.96
η50 (mPa.s) at 37 °C	4.02 - 11.55	2.63 ± 0.07
η50 (mPa.s) at 60 °C	1.15 - 3.00	1.95 ± 0.08
Final sugars content		
Final lactose % (w/w)		2.56
Final sucrose % (w/w)		4.45
Final total sugars % (w/w)		7.01
Total sugar reduction %		39.17

a stabilizer like carrageenan or the predictive model could as well be altered to also favor the production of both lactosucrose and levan, which could increase the viscosity of the chocolate milk (Sahyoun et al., 2024). The final sugar content was then calculated from the selected parameters and experimental responses. The biotransformation reaction successfully reduced the final sugar content, achieving a total reduction of 39.17%. The final lactose and sucrose content was valued at 2.56 and 4.45% (w/w), respectively. Hence, the application of LS could result in a sugar-reduced, lactosucrose-enriched (21.22 to 35.56 g/L) chocolate milk. Furthermore, with epidemiological survey data showing that 70% of the world's population has some degree of lactase deficiency (Li et al., 2023), this study provides an interesting alternative to produce low-lactose/lactose-free beverages, necessary to cater for the needs of lactose intolerant consumers.

5.4. Conclusion

This study demonstrated the high potential of *V. natriegens* LS to produce a low-sugar, lactosucrose-enriched chocolate milk. Analysis of variance helped identify the critical parameters of each response. Lactose concentration was the critical parameter for the conversion of lactose to lactosucrose, relative transfructosylation extent, color difference and apparent viscosities at 50 s^{-1}. Sucrose concentration dictated the sucrose conversion to lactosucrose, sucrose conversion to oligolevan/levan, and LS concentration was the most important parameter for the lactosucrose production. The contour plots generated helped visualize the predictive models. Finally, the selected biotransformation parameters to maximize the selectivity of *V. natriegens* LS towards lactosucrose synthesis were determined for a chocolate concentrate. The sucrose, lactose and LS concentrations were valued at 7.53% (w/w), 4% (w/w) and 5 U/mL, respectively. The resulting chocolate milk could be fortified with 21.22 to 35.56 g/L lactosucrose.

Supplementary Table 5.1. Experimental design parameters

Run	Parameter 1	Parameter 2	Parameter 3
	Lactose %(w/w)	Sucrose %(w/w)	LS unit (U/mL)
1	11.8717	19.8447	1.81079
2	8.75	3.5	3
3	11.8717	7.65531	4.18921
4	8.75	13.75	3
5	8.75	24	3
6	5.62833	7.65531	1.81079
7	11.8717	7.65531	1.81079
8	5.62833	19.8447	1.81079
9	14	13.75	3
10	3.5	13.75	3
11	8.75	13.75	1.5
12	8.75	13.75	1.81079
13	8.75	13.75	5
14	5.62833	19.8447	4.18921
15	5.62833	7.65531	4.18921
16	11.8717	19.8447	4.18921
17	8.75	13.75	1
18	8.75	13.75	2

CHAPTER VI. GENERAL CONCLUSION, FUTURE WORK & CONTRIBUTIONS TO KNOWLEDGE

This study was focused on developing a biocatalytic process for the endogenous production of prebiotic functional ingredients in dairy products using levansucrases (LS, EC 2.4.1.10). LS strains from *Gluconobacter oxydans* (strain 621H) (LS1), *Vibrio natriegens* NBRC 15636 (LS2), *Novosphingobium aromaticivorans* (LS3), and *Paraburkholderia graminis* C4D1M (LS4) were selected.

First, the acceptor specificity of selected LSs was characterized. Phenolic compounds, including catechol, catechin, epicatechin, coniferyl alcohol, gallic acid, caffeic acid, chlorogenic acid, and rosmarinic acid, except vanillic acid, were successfully fructosylated by *V. natriegens* LS2. *N. aromaticivorans* LS3 and *P. graminis* LS4 also proved to be efficient biocatalysts for the transfructosylation of phenolic compounds. *N. aromaticivorans* LS3 successfully transfructosylated catechol, catechin and epicatechin while *P. graminis* LS4 catalyzed the transfructosylation of catechol and catechin. Interestingly, it was also found that more than one fructosyl unit could be attached to the glycosylated phenolic compounds. Furthermore, the presence of phenolic compounds prevented the formation of other LS-catalyzed end-products such as fructooligosaccharides (FOSs). As for when carbohydrates were used as acceptor substrates, *V. natriegens* LS2 and *P. graminis* LS4 led to high yields of fructosylated trisaccharides with maltose, cellobiose and lactose. LS2 also demarcated itself as being the most selective towards the transfructosylation of disaccharides, not simultaneously producing FOSs, unlike the other selected LSs. No transfructosylation activity was reported with sorbitol.

With lactose and phenolic compounds proving to be suitable acceptor substrates, dairy products with particular attention to chocolate milk were selected as food systems for the LS-catalyzed biocatalytic process in the second part of this study. The effect of pH and temperature on the transfructosylation of lactose was first evaluated. *V. natriegens* LS2 showed the highest potential, with high lactosucrose production even at the pH of milk (pH 6.6) and at a low temperature of 10 °C. However, *P. graminis* LS4 and *N. aromaticivorans* LS3 were also found to be promising with high FOS production, especially with LS3. At pH 6.6, very low catalytic activities were recorded for *G. oxydans* LS1 compared to reactions carried out at pH 4, indicating that the application of *G. oxydans* LS1 might be instead more suitable for moderately acidic food systems, like fruit juices, with a pH range of about 3.0-5.0. During the 24-hour time courses, a shift in the

thermodynamic equilibrium of the transfructosylation of lactose was observed for the four selected LSs suggesting that the reaction might have shifted towards lactosucrose hydrolysis. It could also be concluded that sucrose was used to transfructosylate other end-products such as FOSs and levan since the overall transfructosylation extent did not always follow the same trend as the lactosucrose production. Then, LS was used in reconstituted sweetened milk and chocolate milk formulations. An increase in the lactose content from 4.9% to 7.84% (w/v), and an increase in the sucrose content from 3.38% to 10.30% (w/v) did not significantly affect the reaction selectivity at 1h or 24h. No significant changes were observed in the reaction selectivity, sucrose conversion, lactosucrose and FOS production with additional cocoa powder. Finally, levan produced from *G. oxydans* LS1 was added to chocolate milk to evaluate its potential as a stabilizer. Less than 1% (w/w) of high molecular weight (HMW) levan or less than 0.5% (w/w) of mixed low and high molecular weight (MIX) levan was sufficient to bring the viscosity of the fortified chocolate milk equivalent to that of commercial chocolate milk.

Consequently, *V. natriegens* LS2 was selected for the optimization of the biogeneration of lactosucrose in chocolate milk. The effects of the concentrations of substrates (sucrose and lactose) and LS units were investigated using response surface methodology (RSM). Predictive models were developed to deduce the significance of the biotransformation reaction parameters towards the relative transfructosylation extent, the lactosucrose production, the lactose conversion to lactosucrose, the sucrose conversion to lactosucrose, the sucrose conversion to oligolevan/levan, the colour difference relative to commercial chocolate milk and the apparent viscosities at 50 s^{-1}. Finally, the selected biotransformation parameters to maximize the selectivity of *V. natriegens* LS towards lactosucrose synthesis were determined for a chocolate milk concentrate.. A chocolate milk, prepared from a bio-transformed chocolate concentrate, could contain 21.22 to 35.56 g/L lactosucrose.

Future works

Future works may include further characterization of the phenolic fructosides produced in the first part of this study, determining their aqueous solubility, stability, and functional properties. The effects on levan production during the biotransformation reactions of the different acceptor substrates could also be investigated since only FOS production was analyzed. As for the effect of temperature on lactose transfructosylation, the biotransformation reactions could be assessed at the refrigeration temperature of milk (4 °C). Lastly, RSM methodology could be applied for the optimization of the biogeneration of levan using *G. oxydans* LS1, FOSs using *N. aromaticivorans* LS3, or a mix of lactosucrose and FOSs using *P.graminis* LS4, in chocolate milk or any other dairy products such as yoghurt. Alternatively, a bi-enzymatic process could be adopted to develop dairy products rich in diverse prebiotic functional ingredients.

Contributions to Knowledge

The major contributions to knowledge of this study are:

1. This is the first study to fully characterize the acceptor specificity of LS from *G. oxydans* (LS1), *V. natriegens* (LS2), *N. aromaticivorans* (LS3), and *P. graminis* (LS4) towards the selected phenolic compounds and carbohydrates.
2. Phenolic compounds catechol, epicatechin, chlorogenic acid and vanillic acid were not previously tested as potential acceptor substrates for LS-catalyzed reactions. This is also the first study that suggests the potential of phenolic compounds as inhibitors, preventing the formation of other LS-catalyzed end-products.
3. For the first time, the effect of pH and temperature on the transfructosylation of lactose by selected strains was evaluated.
4. This is the first study that assessed the endogenous biogeneration of functional ingredients by LS in dairy beverages. The biocatalytic reactions were first carried out in reconstituted sweetened milk and chocolate milk formulations. Levan produced from *G. oxydans* (LS1) was evaluated as a potential stabilizer in chocolate milk. Finally, the optimization of the biogeneration of lactosucrose in chocolate milk by *V. natriegens* (LS2) was performed.

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