LEVANSUCRASE-CATALYZED TRANSFRUCTOSYLATION REACTION: NOVEL BIOCATALYTIC APPROACH FOR THE PRODUCTION OF FRUCTOOLIGOSACCHARIDES, OLIGOLEVANS AND LEVANS USING MAPLE SYRUPS AS REACTION MEDIA

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ABSTRACT

Maple syrups with selected Brix (Bx) values (15 º, 30 º, 60 º) were investigated as reaction systems for levansucrase from *Bacillus amyloliquefaciens* at 8 ℃ and 30 ℃. The enzymatic conversion of sucrose present in the maple syrups and the production of the transfructosylation products were assessed over a time course of 48 h using levansucrase units of 2 U/ml and 6 U/ml. At 30 ℃, maple syrup 30 ºBx led to the highest total activity (initial conversion rate of sucrose), while maple syrup 66 ºBx led to the highest converted sucrose concentration. The results revealed the absence of substrate inhibition by the excess of sucrose concentration. The sucrose conversion by levansucrase from *B. amyloliquefaciens* at 8 ℃ proceeded with a very low rate as compared to 30 ℃. The highest total activity at 8 ℃ was obtained upon the use of maple syrup 15 ºBx. Transfructosylation reaction was predominantly catalysed at lower temperature and higher sucrose concentration. In the maple syrup 15 ºBx-based reaction system, levansucrase units had more effect on the thermodynamic equilibrium of the reaction and the maximum yield of the transfructosylation products than the reaction temperature. In maple syrup 30 ºBx-based reaction system, oligolevans were synthesized as the major products (more than 80%), and this profile was not affected by the levansucrase unit (2 to 6 U/ml) nor by the temperature (8 to 30 ℃). No significant increase in the yield of the transfructosylation products was achieved at 8 ℃ within the investigated reaction time upon the use of maple syrup 66 ºBx. In addition, the most abundant products were oligolevans at 30 ℃ and levans at 8 ℃ in the maple syrup 66 ºBx-based reaction system. Acceptor specificities were investigated with selected monosaccharides and disaccharides. Galactose with a pyranose ring was a better acceptor for *B. amyloliquefaciens* levansucrase in maple syrups-based reaction systems than xylose with a furanose ring. On the other hand, disaccharides were more favorable fructosyl acceptors than monosaccharides, with lactose being the most preferred acceptor.

Among the three maple syrups, the use of maple syrup 30 ºBx resulted in the highest production of oligolevans/FOSs (83.16%-99.17%); while maple syrup 66 ºBx was identified as the best reaction media for the synthesis of oligolevans/levans (26.81%). The effects of reaction time, pH, and levansucrase enzyme unit on the production of

oligolevans/FOSs and that of oligolevans/levans in the maple syrups 30 °Bx- and 66 °Bx-reaction systems, respectively, were investigated using a response surface methodology (RSM). Reaction time was identified as the most influential factor affecting both oligolevans/FOSs and oligolevans/levans productions. No significant interactive effect between the variables was detected in the oligolevans/FOSs production predictive model of maple syrup 30 °Bx. However, in oligolevans/levans production predictive model of maple syrup 60 °Bx, the interactive effect between levansucrase unit and reaction time was significant.

RÉSUMÉ

Les sirops d'érable avec des valeurs Brix (Bx) sélectionnés (15 °, 30 °, 60 °) ont été étudiés comme des systèmes réactionnels pour la levansucrase de *Bacillus amyloliquefaciens*, à 8 °C et 30 °C. La conversion enzymatique du saccharose présent dans le sirop d'érable et la production des produits de transfructosylation ont été déterminées pendant un temps réactionnel de 48 h en présence d'unités enzymatiques de levansucrase de 2 U/ml et 6 U/ml. À 30 °C, le sirop d'érable 30 °Bx a conduit à une activité enzymatique totale la plus élevée (taux de conversion initial de saccharose), tandis que le sirop d'érable 66 °Bx a conduit à la concentration de saccharose convertie la plus élevée. Ces résultats ont révélé l'absence d'inhibition enzymatique de levansucrase par un excès de substrat saccharose. La conversion du saccharose par levansucrase de *B. amyloliquefaciens* à 8 °C s'est avéré être à un taux plus faible par rapport à 30 °C. L'activité totale la plus élevée à 8 °C a été obtenue suite à l'utilisation de sirop d'érable 15 °Bx. La réaction de transfructosylation a été principalement catalysée à une faible température et en présence d'une concentration élevée du saccharose. Dans le système réactionnel à base de sirop d'érable 15 °Bx, le nombre d'unité enzymatique de levansucrase a plus d'effet sur l'équilibre thermodynamique de la réaction et le rendement maximal en produits de transfructosylation que la température de réaction. Dans le système réactionnel à base de sirop d'érable 30 °Bx, oligolevans ont été synthétisés comme les principaux produits (plus de 80%), et ce profil n'a pas été affecté par l'unité de levansucrase (2 à 6 U/ml) ni par la température (de 8 à 30 °C). Aucune augmentation significative de la production de produits de transfructosylation n'a été obtenue à 8 °C pendant le temps réactionnel étudié pour le sirop d'érable 66 °Bx. En outre, les produits les plus abondants étaient des oligolevans à 30 °C et levans à 8 °C dans le système réactionnel à base de sirop d'érable 66 °Bx. La spécificité vis-a-vis d'accepteurs a été étudiée en utilisant des monosaccharides et des disaccharides sélectionnés comme substrats. Le galactose étant de structure chimique pyranosique était un meilleur accepteur pour la levansucrase de *B. amyloliquefaciens* dans les systèmes réactionnels à base de sirops d'érable que le xylose avec un cycle furanosique. Disaccharides étaient des

accepteurs fructosylés plus favorables que les monosaccharides, le lactose étant l'accepteur préféré.

Parmi les trois sirops d'érable, l'utilisation de sirop d'érable de 30 °Bx a conduit à la plus forte production d'oligolevans/FOSs (83.16% -99.17%); tandis que le sirop d'érable 66 °Bx a été identifié comme étant le meilleur milieu réactionnel pour la synthèse de oligolevans/levans (26.81%). Les effets du temps de réaction, le pH et l'unité enzymatique de levansucrase sur la production d'oligolevans/FOSs et oligolevans/levans dans le système réactionnel à base du sirop d'érable de 30 °Bx et 66 °Bx, respectivement, ont été étudiés en utilisant une méthodologie de surface de réponse (RSM). Le temps de réaction a été identifié comme le facteur le plus influent affectant à la fois la production d'oligolevans/FOSs et oligolevans/levans. Aucun effet interactif significatif entre les variables a été détecté dans le model prédictive de sirop d'érable 30 °Bx pour la production d'oligolevans/FOSs. Cependant, dans le modèle prédictif du sirop d'érable 60 °Bx pour la production de oligolevans/Levans, l'effet interactif entre l'unité de levansucrase et le temps de réaction a été important.

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LIST OF ABBREVIATIONS

ANOVA: Analyses of variance

ATCC: American type culture collection

Arg: Arginine

Asp: Aspartic acid

CCRD: Central composite rotatable design

DNS: 3, 5-Dinitrosalicyclic acid

DP: Degree of polymerization

EC number: Enzyme Classification number

FOS: Fructooligosaccharides

Fru: Fructose

Gal: Galactose

GH: Glycoside hydrolase

Glu: Glutamic acid

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

HPLC: High performance liquid chromatography

NMR: Nuclear magnetic resonance spectroscopy

PEG: Polyethylene glycol

PI: Prediction interval

RSM: Response surface methodology

SCFA: Short chain fatty acid

scFOS: Short chain fructooligosaccharides

$V_{maxapp.}$ Apparent maximum velocity

Xyl: Xylose

CHAPTER I INTRODUCTION

A prebiotic is a selectively fermented ingredient that leads to specific changes, both in the composition and/or the activity of the gastrointestinal microflora, conferring benefits to the host well-being and health (Gibson et al., 2004). Fructooligosaccharides (FOSs) constitute one of the prebiotic classes, whose potential health benefits in terms of supporting intestinal health are increasingly being recognized. Indeed, the β-(2-1)/β-(2-6) configurations of the fructosyl-fructose linkages of FOSs make them resistant to digestion in the upper gastrointestinal tract and help them to reach the colon where they are selectively fermented by beneficial microflora such as *bifidobacteria* and *lactobacilli* (Roberfroid, 2005). Other health benefits of FOSs are the enhancement of mineral absorption, the modulation of lipid metabolism (Azorin-Ortuno et al., 2009), the improvement of body defense mechanism (Lecerf et al., 2012) and the reduction the risk of colonic cancer (Cherbut, 2002; Nyman, 2002). Contributions to the development of such health-promoting ingredients, which have the potential to improve overall public health, have increased during the last years.

In particular, progress in the understanding of the relationship between the structures of FOSs and their health attributes has given rise to a need for efficient synthetic routes to structurally well-defined FOSs. β-(2-6)-FOSs exhibit more bifidogenic effects than their β-(2-1) counterparts (Kilian et al., 2002). β-(2-6)-FOSs can be produced from sucrose through levansucrase-catalyzed transfructosylation reaction. Levansucrase (E.C.2.4.1.10) belongs to the glycoside hydrolase (GH) family 68 (Meng and Futterer, 2003) and catalyzes the formation of β-(2-6)-linked levan with high regio- and stereo-specificity (Mussatto et al., 2012). Interestingly, the formation of β-(2-6)-linked levan can be quantitatively replaced with the formation of FOSs in the presence of various mono- and oligosaccharides as acceptors. Besides sucrose and raffinose, sucrose analogues have recently been successfully used as fructosyl-donors and fructosyl-acceptors by levansucrase (Andersone et al., 2004; Kim et al., 2001; Seibel et al., 2006). Due to its broader acceptor specificity and product spectrum, levansucrases have been identified as promising biocatalysts for the synthesis of β-(2-6)-linked-fructose-based carbohydrates targeting specific structures and functional properties. However, in spite of the progress

that has been made, research efforts are still needed to understand and modulate levansucrase-catalyzed transfructosylation reaction for specific applications.

Maple syrup is one of the most abundant and sucrose-rich renewable feedstock in Canada, which consists primarily of sucrose (68%, w/w) with small amounts of glucose (0.43%, w/w) and fructose (0.34%, w/w) (Stuckel and Low, 1996). It also contains nitrogenous and phenolic compounds (0-0.1 ppm) (Ball, 2007; Kermasha et al., 1995; Underwood et al., 1961), organic acids (0.174%, w/w) (Mollica and Morselli, 1984) and minerals (3638.62 ppm) (Perkins and van den Berg, 2009). Because of its composition, maple syrup seems to be ideal reaction media for levansucrase-catalyzed reaction. The yearly worldwide production of maple syrup is around 8-9 million gal, of which 85% is produced in Canada. Québec is the most important producer of maple syrup, accounting for 90% of the total Canadian production (Perkins and van den Berg, 2009). However, 5-20% of the total production is classified as low-grade and is not marketable because of flavor, dark color, flaring sugar taste or appearance defects (Aider et al., 2007a). The current practice for the use of the low-grade sap is to blend it with the standard quality one (1:60, v/v). However, this practice lowers the quality of maple products and, on a long-term basis it may jeopardize the maple industry. As far as we are aware, little work has been dedicated to the exploration of low grade maple syrups as a renewable substrate for the production of health-promoting products. Such initiative is not only expected to increase the value of low grade maple syrups, but also to diversify their products.

The overall objective of this study was to develop a biocatalytic approach based on levansucrase-catalyzed transfructosylation reaction, for the production of FOSs, oligolevans and levans using maple syrups as reaction media and sucrose source.

The specific objectives were:

1. Investigation of the catalytic efficiency of *Bacillus amyloliquefaciens* levansucrase-catalyzed transfructosylation reaction in selected maple syrups-based reaction systems.
2. Study the acceptor specificity of *B. amyloliquefaciens* levansucrase using selected maple syrups-based reaction systems.

3. Determination of effects of reaction parameters and the optimal conditions for the synthesis of FOSs, oligolevans and levans by *B. amyloliquefaciens* levansucrase in selected maple syrups-based reaction systems.

CHAPTER II LITERATURE REVIEW

2.1. Introduction

FOSs are fructans consisting of a sucrose molecule that is linked by a chain of 3-10 fructosyl units connected through β-(2-1) or β-(2-6) linkages. FOSs belong to one of the prebiotic classes, whose potential health benefits in terms of selective stimulation of the growth of health promoting bacteria (Roberfroid, 2005), enhancement of mineral absorption, modulation of lipid metabolism (Azorin-Ortuno et al., 2009), improvement of body defense mechanism (Lecerf et al., 2012) and reduction the risk of colonic cancer (Cherbut, 2002; Nyman, 2002) are increasingly being recognized. FOSs can be produced by controlled enzymatic hydrolysis of inulin or by chemical synthesis. However, the production is limited by seasonal conditions through fructans hydrolysis and the low product yield through chemical synthesis. On the other hand, FOSs can be produced from sucrose through enzymatic approach on an industrial scale, which provides a cost effective and convenient alternative to other methods. Maple syrup with sucrose as the main component along with trace amounts of glucose and fructose (Stuckel and Low, 1996) makes it an interesting medium for bacterial growth and an interesting base material to produce other novel ingredients such as FOSs (Yezza et al., 2007). This review focuses on the description of health benefits and production of FOSs. Levansucrase catalyzed- reaction for the production of FOSs is discussed in details. This review also covers the production of maple syrups and the challenges related to the production in Québec, Canada. A brief overview about the analysis of FOSs is also provided.

2.2. Probiotics

The intestinal microflora plays a role of prime importance for health. Therefore, it is of great interest to maintain the balance of the gut microflora through increasing the levels of beneficial bacteria (Flynn et al., 2002). Live microorganisms that confer health benefits to the host when administered in adequate amount are defined as probiotics (FAO/WHO, 2002). Probiotic microorganisms include the genera *lactobacilli*, *bifidobacteria*, *Streptococcus* and non-pathogenic yeasts (Fuller, 1989; Morrow et al., 2012).

The mechanisms of health-promoting activity of probiotics often depend on interactions with the specific microflora of the host or with the immune-competent cells of the intestinal mucosa (de Vrese and Schrezenmeir, 2008). It has been demonstrated that probiotics modulate the intestinal microflora. For example, the administration of various *lactobacilli* can temporarily change the number or the composition of the indigenous *lactobacilli* in weaning piglets (Takahashi et al., 2007). In addition, competition between the probiotic microorganisms and pathogenic bacteria for the adhesion to the receptors of the intestinal epithelial cells was demonstrated (Fuller, 1991). Moreover, probiotics can directly inhibit or kill pathogens by the production of metabolites. For instance, *Lactobacillus salivarius* has been reported to produce ABP-118, which is able to inhibit a wide variety of bacteria, including *Bacillus*, *Listeria*, *Enterococcus*, and *Staphylococcus* species (Flynn et al., 2002). Short chain fatty acid (SCFA) production is another essential factor for the beneficial effects of probiotics. It is thought that short-chain fatty acid can increase the cell permeability by disrupting the outer membranes of gram-negative pathogens such as *enterohemorrhagic Escherichia coli* (Alakomi et al., 2000). Furthermore, the accumulation of short chain fatty acid leads to a decrease in intestinal pH, creating an unfavorable environment for pathogenic organisms to grow (Fooks and Gibson, 2002).

2.3. Prebiotics

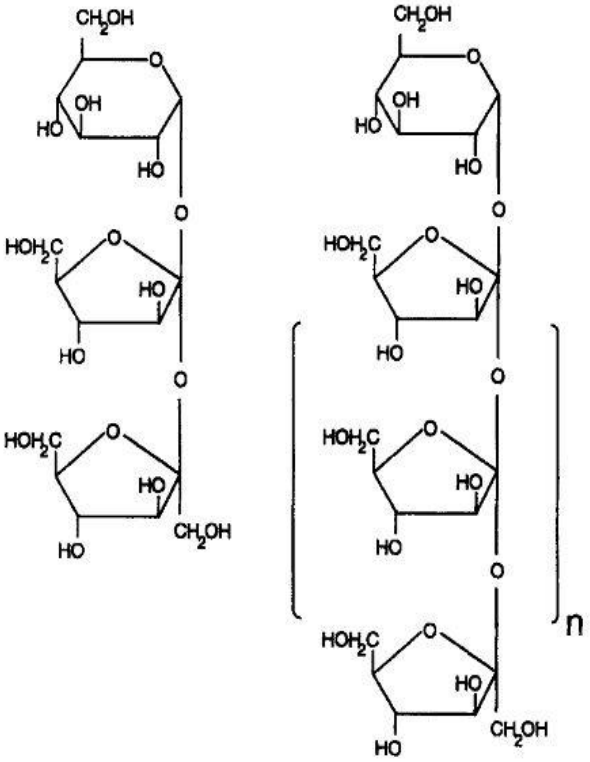
Based upon the success of the use of probiotics as an approach to manage the gut microflora, the concept of a prebiotic was introduced in 1995 (Gibson and Roberfroid, 1995). A prebiotic is a selectively fermented ingredient that allows specific changes, both in the composition and/or activity of the gastrointestinal microflora that confers benefits upon a host well-being and health (Manning and Gibson, 2004). It should be a growth substrate for a limited number of the indigenous intestinal flora of humans, or improve the survival of the indigenous bacteria. Unlike probiotics, which need to establish in the colon and compete with other bacteria for nutrients, which would comprise their chances of survival and effectiveness, prebiotics are not viable entities but rather are growth substrates targeted at bacteria already in the colon, thus overcoming some limitations of the probiotic approach (Gibson and Roberfroid, 1995). Currently, the popular bacterial species targeted for selective stimulation through the use of prebiotics are the indigenous

bifidobacteria and *lactobacilli* (Teitelbaum and Walker, 2002). However, prebiotic success has primarily been achieved with *bifidobacteria*. This may be due to the fact that *bifidobacteria* are a numerically significant bacterial population in the colonic microflora, and they exhibit a preference for oligosaccharides (Brownawell et al., 2012). In addition to their selectivity towards the promotion of gut microbial growth in favor of a healthier composition, prebiotics are resistant to human digestive enzymes; thus, they provide a high bulking effect, increasing feces weight and water content of stools, reliving constipation and reducing the risk of colonic cancer (Cherbut, 2002; Nyman, 2002).

2.4. Fructooligosaccharides

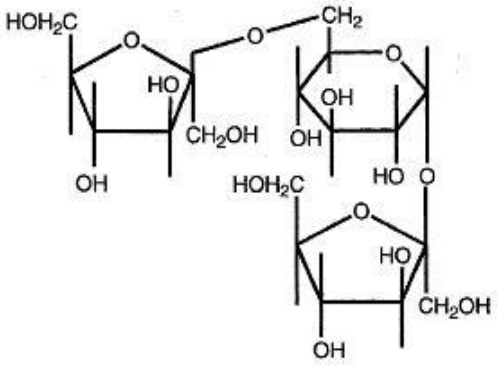
2.4.1. Definitions and Chemical Structures

FOSs are oligomeric linear fructans with β-(2-1) or β-(2-6) fructosyl-fructose linkages. The first monomer of the chain is either a α-D-glucopyranosyl or a β-D-fructopyranosyl residue. The fructosyl–glucose linkage is always β-(2-1) as in sucrose. The degree of polymerization (DP) of FOSs is between 2 and 10 (Roberfroid, 2005). Depending on the different fructosyl-fructose linkages, FOSs can be further subdivided into inulin-type, neoinulin-type, levan-type, neolevan-type and mixed levan-type (Vijn and Smeekens, 1999). Inulin-type FOSs consist of only β-(2-1)-linked β-D-fructosyl units and can be seen as a lower molecular weight version of inulin. Inulin-type FOSs mainly include 1-kestose (GF_2), nystose (GF_3), and 1^F-fructosylnystose (GF_4) as shown in Fig. 2.1 (Damien et al., 2009). Fructosyl units in levan-type FOSs are attached by β-(2-6) fructosyl-fructose linkages and the trisaccharide 6-kestose is the smallest of this type. If a fructosyl unit is β-(2-6) bound to the glucose moiety of 1-kestose or sucrose molecule, respectively, levan-type neo-kestose and neo-nystose are formed (Plou et al., 2007). In the neo-inulin-type FOSs, β-D-fructosyl units are linked to both the C_1 and C_6 of the glucose moiety of sucrose with neo-kestose being the smallest one in this category (Shiomi, 1989). The mixed levan-type FOSs contain both β-(2-1) and β-(2-6) linkages with bifurcose being the smallest one (Sprenger et al., 1995).

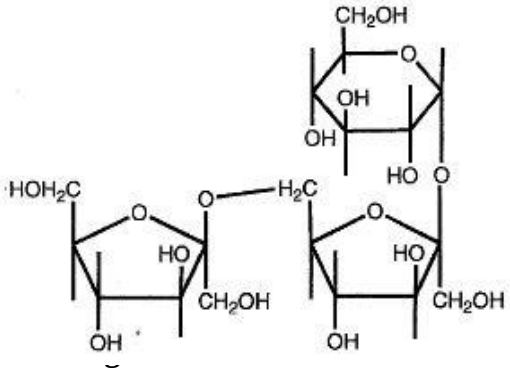


n=1, Nystose

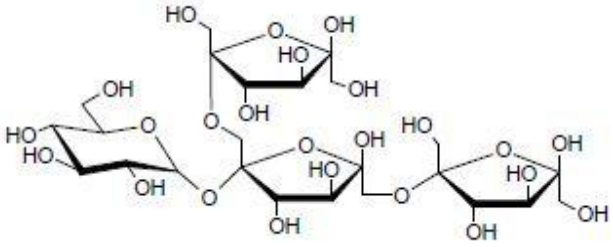
n=2, 1^{F}-β-fructofuranosyl nystose



Neokestose



6-Kestose



Bifurcose

Figure 2.1. Chemical structures of FOSs

2.4.2. Prebiotic Activity of Fructooligosaccharides

FOSs are one of the prebiotics that has been most studied. The β-(2-1) and β-(2-6) configurations of the fructosyl-fructose linkages make FOSs resistant to digestion in the upper gastrointestinal tract. They then enter the colon where they are fermented by specific microflora (Roberfroid, 2005). Both *in vivo* and *in vitro* studies have demonstrated that FOSs can selectively stimulate the growth of *bifidobacteria* and *lactobacilli* (Gibson and Wang, 1994a; Sghir et al., 1998). *In vitro* studies using batch culture systems or continuous culture systems showed that *bifidobacteria* preferred fructans as a source of carbon and energy and that FOSs could significantly increase the numbers of *bifidobacteria* compared with other larger molecular weight carbohydrates (Gibson and Wang, 1994a, b). This may be due to the fact that *bifidobacteria* have relatively high amounts of intracellular β-fructofuranosidases, which are capable of hydrolyzing β-(2-1) glycosidic bonds present in FOSs (Bouhnik et al., 1996). Moreover, Sghir et al. (1998) tested the abilities of *bifidobacteria* and *lactobacilli* from human fecal samples to utilize FOSs and demonstrated that *lactobacilli* were also able to utilize FOSs and could outcompete with *bifidobacteria* in continuous culture under controlled conditions. Recently, levan-type FOSs have been demonstrated to be more bifidogenic and effective than commercial FOSs (Kilian et al., 2002). The authors also reported that neo-kestose, which belongs to neo-levan-type FOSs, was able to stimulate the growth of *bifidobacteria* and *lactobacilli* significantly while inhibiting the growth of potentially detrimental *Coliforms*, *Clostridia* and *Bacteroides*. This has been confirmed by Losada and Olleros (2002) who have reported that FOSs could only stimulate the growth of beneficial bacteria, while harmful bacteria including *E. Coli*, *Clostridia* and *Salmonella* were not capable to grow on FOSs.

In vivo human studies with FOSs included a controlled diet with varying doses, durations and volunteers. The results showed that as prebiotics, FOSs were able to demonstrate bifidogenic effect at a low dose (2.5g/day) (Bouhnik et al., 1996). However, the effect of FOSs remained only with continued FOSs administration. Once the administration ceased, a decrease in *bifidobacteria* was observed (Bouhnik et al., 2006).

2.4.3. Safety Toxicity of Fructooligosaccharides

The safety and toxicity of FOSs have been extensively evaluated in a series of standard toxicological tests in animals and humans. No specific safety issues were raised in these studies. When FOSs were administered in the diet at high levels, they did not result in mortality, morbidity or target organ toxicity and had no genotoxic and cancerigenous effects (Carabin and Flamm, 1999). The only detected limitation for FOSs use in human diet was the gastrointestinal tolerance. In a study with healthy volunteers, excessive flatus occurred at 30 g FOSs/day and borborygmi and bloating appeared at higher levels (> 40g FOSs/day). When the intake reached 50 g FOSs/day, abdominal cramps and diarrhea occurred. Up to 20 g FOSs/day was well tolerated (Briet et al., 1995). An average daily FOSs consumption of approximately 13.7 mg/kg body weight/day or 806 mg/day was recommended (Voragen, 1998).

2.4.4. Limitations of Current Fructooligosaccharides

FOSs have great potential as functional ingredients because of their prebiotic activity, low caloric value and non-toxicity. Commercially, the β-(2-1)-FOSs are produced by the hydrolysis of fructans or by the enzymatic synthesis from sucrose by microbial enzymes with transfructosylating activity. The FOSs mixtures obtained from these two processes are similar, except that fructans hydrolysis usually generates FOSs of longer chains (Dominguez et al., 2014). However, the limitations associated with current industrial production of FOSs are the low product yield through fructans hydrolysis and low DP through the transfructosylation of sucrose. Because of their low DP, the current β-(2-1)-FOSs are quickly fermented in the proximal colonic region into short-chain fatty acids by the gut bacteria, and are not able to reach the distal colonic region, which is more susceptible to chronic diseases (Rastall and Maitin, 2002). As a result, the β-(2-1)-FOSs are limited by their low colonic persistence. In contrast, the β-(2-6)-FOSs have been reported to show more bifidogenic effects than their β-(2-1) counterparts (Kilian et al., 2002). Therefore, the production of structurally defined β-(2-6) FOSs is expected to result in an increase in the colonic persistence of FOSs and prebiotic effects.

2.5. Fructan

2.5.1. Inulin

Inulin consists of fructose units, linked by β-(2-1) fructosyl-fructose linkages and usually terminates with only one glucose unit. In some cases, inulin can be highly branched through β-(2-6) linkages (Waterhouse and Chatterton, 1993). Inulin is present as a reserve carbohydrate in more than 30,000 vegetable products, including Jerusalem artichoke, dahlia and chicory. The latter is the most commonly used source for industrially extraction of inulin (Baumgardner and Praznik, 1995).

Inulin can improve the rheological and sensory properties of food products as sugar or fat replacers. It was observed that inulin can provide spread-ability to chocolates (Aidoo et al., 2014), optimize the texture in biscuits (Laguna et al., 2014) and increase viscosity in low-fat dairy products (Guggisberg et al., 2009). Due to the β-(2-1) fructosyl-fructose linkages, inulin cannot be hydrolyzed by the digestive enzymes in the human small intestine. However, inulin can be quantitatively fermented in the large intestine by the colonic microflora. Therefore, inulin is considered as a nondigestible carbohydrate and a dietary fiber (Roberfroid, 2005), contributing to the improvement of the gastrointestinal health. In fact, it was observed that inulin could selectively stimulate the growth of *bifidobacteria* (Harmsen et al., 2002) and *lactobacilli* (de Souza Oliveira et al., 2012), which are the most well-known probiotic microorganisms. Moreover, inulin can also exhibit physiological and immunological effects such as the enhancement of mineral absorption, modulation of lipid metabolism (Azorin-Ortuno et al., 2009), improvement of body defense mechanism (Lecerf et al., 2012), and reduction of colon cancer risk (Pool-Zobel and Sauer, 2007). Due to these properties, inulin has wide variety of applications in food and pharmaceutical industries for the production of functional foods, nutritional composites and drugs.

2.5.2. Levan

Levan consists of D-fructofuranosyl residues linked by β-(2-6) linkages as a main chain with some β-(2-1) linked branched chains (Han and Clarke, 1990). Levan is naturally present in plants (Van den Ende et al., 2011), and can be produced by yeasts (Franken et al., 2013) and bacteria (Dahech et al., 2012). Levan from plants were reported to have lower molecular weight than levan from microbial sources (Jathore et al., 2012).

Microbial levans are produced by microorganisms grown on sucrose. The most common levan-producing microorganisms include *B. polymyxa* (Han and Clarke, 1990), *Zymomonas mobilis* (Jang et al., 2001), *Pseudomonas phaseolicola* (Gross and Rudolph, 1987) and *B. licheniformis* (Ramsay et al., 1989). New strains that can produce levan are continuously being sought. Poli et al. (2009) have reported for the first time that *Halomonas species* can produce high levels of levan.

Levan is non-toxic, non-mutagenic and tasteless. Levan has an unusually low intrinsic viscosity and is readily soluble in water at room temperature. In contrast to the low solubility of β-(2-1) linked inulin, the high solubility of levan may be attributed to the β-(2-6) linkages (Han and Clarke, 1990). In the food industry, levan can be used as an encapsulating agent, thickener, surface-finishing agent, emulsifier as well as a carrier for colors and flavors (Jathore et al., 2012). In the pharmaceutical industry, levan has been shown to have antioxidant and antitumor activities (Abdel-Fattah et al., 2012). However, the antitumor activity of levan likely depends on the branching degree and the microbial source. Yoon et al. (2004) have reported that as the branching degree of levan reduced, levan-induced antitumor activity on SNU-1 tumor cell lines linearly decreased. Yoon et al. (2004) have also tested the antitumor activities of levans synthesized by several selected microorganisms, and the antitumor activities were found to differ significant among the levans. Recently, levan has been found to exhibit antibacterial activity against major foodborne pathogenic bacteria and therefore has the potential to be used as an alternative preservative (Byun et al., 2014).

Levan may have a wide range of applications in pharmaceuticals, foods and cosmetics; however, the main factor limiting the application of levan is its availability. Optimization of levan production is another interesting research area.

2.6. Production of Fructooligosaccharides and Fructans

2.6.1. Extraction of Fructooligosaccharides from Plants

FOSs can be found as natural components in plants (Flamm et al., 2001), especially in monocotyledonous and dicotyledonous families, such as *Liliaceae*, *Amaryllidaceae*, and *Compositae*. FOSs are usually stored in bulbs, tubers and tuberous roots, which can be easily extracted and processed to purified products (Fuchs, 1991). Jerusalem artichoke,

asparagus and chicory roots are well-known sources of FOSs. For chicory roots, FOSs concentrations range between 5 and 10%, while in Jerusalem artichoke, they can go up to 20% (Voragen, 1998). In addition, wheat, honey, tomato, banana, onion and garlic are special sources of FOSs. Table 2.1 illustrates the natural sources of FOSs (Sangeetha et al., 2005b). For most of these sources, the concentration of FOSs is extremely low and mass production is limited by seasonal conditions (Sangeetha et al., 2005a).

2.6.2. Chemical Synthesis of Fructooligosaccharides

FOSs may be produced by chemical synthesis from disaccharide substrates. However, the chemical synthesis of oligosaccharides is very laborious. It often requires very elaborate synthetic steps involving extensive protecting and/or leaving group manipulations between each glycosylation step. Then, the desired compound must be separated chromatographically and deprotected, thereby decreasing both the efficiency and yield (Halcomb and Wong, 1993; Palcic, 1999; Premathilake and Demchenko, 2011). In addition, because of the involvement of toxic reagents, food safety is another issue associated with chemical synthesis.

2.6.3. Hydrolysis of Fructans to Fructooligosaccharides

FOSs can be produced either by chemical hydrolysis or by controlled enzymatic hydrolysis of inulin or levan. Chemical hydrolysis of inulin can be carried out using organic or mineral acids or throughout heterogeneous catalysis using solid acidic catalysts (Rocha et al., 2006). However, acid hydrolysis requires catalyst removal and expensive anion exchange resins. In addition, the formation of undesirable colored compounds lowers the product yields (Fleming and Grootwassink, 1979).

Enzymatic approach provides a cost effective and convenient alternative to the chemical hydrolysis. The enzyme that hydrolyzes β-(2-1)-fructan linkages in inulin is commonly known as inulinase (EC 3.2.1.7). Depending on its mode of action, inulinase can be categorized into exo-inulinase (EC 3.2.1.80) and endo-inulinase (EC 3.2.1.7) (Barman, 1969). Exo-inulinase acts on every β-(2-1)-fructan linkage and produces fructose as the main end product, while endo-inulinase yields FOSs of varying sizes through partial and random hydrolysis of inulin (Kang et al., 1998; Ricca et al., 2007).

Table 2.1. Concentration of FOSs in natural foods

Source	FOSs (%, w/w)
Barley	0.15
Tomato	0.15
Onion	0.23
Banana	0.30
Brown sugar	0.30
Rye	0.50
Garlic	0.60
Honey	0.75

Similarly, levanases (EC 3.2.1.65) can hydrolyze β-(2-6)-fructan linkages in levan to produce levan-type FOSs. Most levanases are characterized as fructose- or levanbiose-producing exo-levanases, some of which are also able to hydrolyze inulin, raffinose and sucrose in addition to levan (Menéndez et al., 2002), whilst endo-levanases degrade levan resulting in the production of various FOSs as the final hydrolysis products (Miasnikov, 1997).

2.6.4. Enzymatic Synthesis of Fructooligosaccharides and Fructans

FOSs can be produced from substrate sucrose or sucrose analogues through the enzymatic transfructosylation reactions catalyzed by β-fructofuranosidase (β-FFase, EC 3.2.1.26) or β-fructosyl-transferase (β-FTase, EC 2.4.1) (Hidaka et al., 1988). The enzymes are mainly derived from plants, such as asparagus, sugar beet, onion, etc. or from bacteria and fungi such as *Aspergillus* sp., *Aureobasidium* sp., *Fusarium* sp., etc. (Yun, 1996). FOSs formed by enzymes usually contain two to four fructosyl units with small amount of glucose and fructose as by-products, as well as unused sucrose (Crittenden and Playne, 1996). The transfructosylating process is considered to have a greater potential compared to the FOSs production through the hydrolysis of fructans, since the intrinsic selectivity of transfructosylating enzymes can lead to the synthesis of FOSs of defined chain length. In addition, it can lead to the production of desired composition mixtures by controlling the reaction time and to FOSs products with higher purity (Mussatto et al., 2012). In addition, not all of the FOSs produced by hydrolysis of fructans are headed with a glucose moiety (Crittenden and Playne, 1996).

Transfructosylation activity cleaves the β-(2-1) linkage of sucrose and transfers the fructosyl group to an acceptor such as sucrose, resulting in the formation of FOSs, while the hydrolytic activity catalyzes the hydrolysis of sucrose to glucose and fructose (Belghith et al., 2012). There is still some ambiguity in that whether FOS-producing enzymes should be categorized as β-FFase or β-FTase. Both β-FFases and β-FTase possess transfructosylation and hydrolytic activity. However the ratio between transfructosylation and hydrolysis is different. The latter denomination is probably due to the fact that β-FTase is able to exert sufficient transfructosylating activity when acting on a low concentration of sucrose to synthesize FOSs (L'Hocine et al., 2000; Straathof et al., 1986).

2.6.4.1. β-Fructofuranosidases-catalyzed Synthesis

β-FFase is an efficient biocatalyst for the production of FOSs. Generally β-FFase catalyzes naturally the hydrolysis of sucrose, but its synthetic reaction may be favored over the hydrolytic one through the use of high substrate concentration, elevated temperatures, organic co-solvents and/or highly selective fructosyl- acceptor than water (Fernández et al., 2004; Plou et al., 2007). (Fernández et al., 2004; Plou et al., 2007)Generally speaking, the ratio of transfructosylation over hydrolysis of β-FFase depends on the microbial sources and the reaction conditions. β-FFase from *Aureobasidium pullulans* was used for FOSs production and the maximum FOSs yield reached 69% (Yoshikawa et al., 2008). Maximal FOS production for any particular enzyme depends on the relative rates of the transfructosylation and hydrolysis reactions (Nguyen et al., 2005). Despite the wide availability of β-FFase, only a few have a transfructosylation activity significant enough for industrial FOSs production. In addition, the production of FOSs is limited by low to modest yields (Ashokkumar et al., 2001).

2.6.4.2. β-Fructosyl-transferase-catalyzed Synthesis

β-FTase comprises of levansucrase (EC 2.4.1.10) and inulosucrase (EC 2.4.1.9), which is able to catalyze the transfer of the fructosyl unit from sucrose to growing chain of fructose to synthesize β-(2-6) levan (Gross et al., 1992) and β-(1-2) inulin (Olivares-Illana et al., 2002), respectively. In addition to the difference in product specificities (levan and inulin), levansucrase and inulosucrase also differ in the transfructosylation over hydrolysis ratio (Ozimek et al., 2006b). Moreover, levansucrase is able to use various acceptors *in vitro*, such as water, glucose, sucrose or fructan, while there are no reports on the use of water or glucose as acceptor by inulosucrase (Velazquez-Hernandez et al., 2009). Due to the broader range of acceptors and product spectrum, levansucrase has been identified of great potential as fructosyl-transferase.

2.7. Levansucrases

2.7.1. Microbial Sources of Levansucrases

Levansucrase can be expressed by gram-negative bacteria, such as *A. diazotrophicus* (Tambara et al., 1999), *Gluconacetobacter* diazotrophicus (Martinez-Fleites et al., 2005), *Z. mobilis* (Bekers et al., 2002) and *Erwinia amylovora* (Gross et al., 1992) or gram-positive bacteria including *B. subtilis* (Chambert and Gonzytreboul, 1976a), *B. circulans*

(Oseguera et al., 1996), *B. megaterium* (Homann et al., 2007), *B. amyloliquefaciens* (Rairakhwada et al., 2010), *S. salivarius* (Garszczy.Sm and Edwards, 1973), *L. reuteri* (van Hijum et al., 2004), *L. panis* (Waldherr et al., 2008) and *Actinomyces viscosus* (Pabst, 1977). Although levansucrases share some similarity with respect to their amino acids sequence, they differ in molecular weight, degree of polymerization of end-products synthesized, chemical susceptibility and substrate specificity (van Hijum et al., 2006).

For instance, *B. subtilis* levansucrase tends to synthesize high-molecular-mass levan. In contrast, levansucrases from *A. diazotrophicus, G. diazotrophicus* and *Z. mobilis* have been found to produce a large amount of FOSs, especially scFOSs like 1-kestose from sucrose (Doelle et al., 1993; Hernandez et al., 1995; Martinez-Fleites et al., 2005). Moreover, most levansucrases are monomeric enzymes with a molecular weight ranging from 46 to 73 kDa. However, levansucrases from *A. viscous* can form dimers and therefore have a molecular weight as high as 220 kDa (Velazquez-Hernandez et al., 2009). The optimum pH of levansucrases is slightly acidic, ranging from 5.0 to 6.2 (Hettwer et al., 1995; Yanase et al., 1992). The optimum temperatures of levansucrases have a very broad range. For most levansucrases, such as the ones from *B. megaterium* (Homann et al., 2007), *Z. mobilis* (Yanase et al., 1992) and *L. reuteri* (van Hijum et al., 2004), the optimum temperature is between 45 ℃ and 50 ℃. Levansucrase from *B. subtilis* has been reported to show the highest activity at a lower temperature of 30 ℃ (van Hijum et al., 2004). However, *R. aquatilis* levansucrase was the most active at temperatures as high as 60 ℃ (Ohtsuka et al., 1992).

2.7.2. Levansucrase-catalyzed Reactions

Bacterial levansucrases catalyze the transfructosylation reaction by transferring the β-fructofuranosyl group of sucrose to a variety of acceptors including water, glucose, fructan and sucrose with β-(2-6) linkages (Hernandez et al., 1995). Levansucrase can catalyze the hydrolysis of sucrose when water is used as an acceptor. In the presence of glucose, levansucrase has a low hydrolytic activity and catalyzes an exchange reaction. In the presence of the growing fructan chain, levansucrase is able to catalyze polymerization, leading to levan formation. With sucrose alone as the substrate, levansucrase may catalyze transfructosylation, synthesizing FOSs (Feingold et al., 1956; Hernandez et al.,

1995; Meng and Futterer, 2003). In addition, with other acceptors, levansucrases are also able to catalyze other transfructosylation reactions, such as the formation of methyl fructoside in the presence of methanol (Gon Kim et al., 2000), the formation of fructosyl glycerol in the presence of glycerol (Gonzalez-Munoz et al., 1999)and lactosucrose in the presence of lactose (Han et al., 2009).

2.7.3. Mechanism of Action of Levansucrases

2.7.3.1. Active Site of Levansucrases

According to the database of carbohydrate-active enzymes, levansucrase belongs to the glycoside hydrolase (GH) family 68 (Meng and Futterer, 2003). The active site of the GH family 68 enzymes is composed of three conserved amino acids: a catalytic nucleophile, a general acid/base catalyst, and a transition state stabilizer. They are key catalytic acidic residues at the active site and critical for the catalytic activity (Ozimek et al., 2004). It is assumed that the reaction catalyzed by levansucrase follows a ping-pong mechanism involving the formation of a covalent fructosyl-enzyme intermediate (Chambert et al., 1974). The three-dimensional structures of levansucrase from gram-positive bacteria *B. subtilis* and levansucrase from gram-negative *G. diazotrophicus* were revealed by Meng and Futterer (2003) and Martinez-Fleites et al. (2005), respectively. Both levansucrases displayed a five-bladed-propeller fold with a deep, negatively charged central cavity. The three acidic residues in the active site of *B. subtilis* levansucrase have been identified as Asp86, Glu342 and Asp247. Asp86 residue acts as a nucleophile, which usually attacks the glucopyranosyl residue of the sucrose and forms an enzyme-fructosyl intermediate with the inversion of the glycosidic bond in the intermediate state (Chambert and Gonzytreboul, 1976b). Asp247 is able to form strong covalent bond with the C-3’ and C-4’ hydroxyls of the fructosyl unit, therefore, Asp247 is considered as a transition state stabilizer. The sucrose-bound complex structure suggests that Asp86 and Glu342 are the essential catalytic residues that are conserved in most glycoside hydrolases (Meng and Futterer, 2003). The catalytic triad of *G. diazotrophicus* levansucrase has been determined as Asp135, Asp309 and Glu401, which act as the catalytic nucleophile, the transition-state stabilizer and the general acid-base catalyst, respectively (Martinez-Fleites et al., 2005).

2.7.3.2. Reaction Selectivity: Hydrolysis vs. Transfructosylation

Levansucrases possess both transfructosylation and hydrolytic activities. Euzenat et al. (1997) investigated the ability of levansucrase from *B. subtilis* to synthesize levan. At the beginning, hydrolysis of sucrose was the main reaction. Later, levan appeared to be synthesized faster. This may be due to the fact that low molecular weight levans are better acceptors of fructosyl residues than water or glucose, inducing an increase in the activity of levansucrase, together with a shift in enzymatic action from hydrolysis to levan synthesis.

The ratio of transfructosylation over hydrolysis is likely affected by reaction conditions, such as temperature, pH and substrate concentration. Although, levansucrases differ widely from each other in terms of their optimum temperatures for transfructosylation and hydrolysis, in general, transfructosylation is favored at lower temperatures. For instance, levansucrase from *P. syringae* pv. Phaseolicola preferentially catalyzed the formation of levan at low temperature (18 ℃), but when the temperature increased to 60 ℃, hydrolysis reaction was preferred over the levan formation reaction (Hettwer et al., 1995). Similarly, *Bacillus* sp. levansucrases showed the optimum activity for levan production at 50 ℃. However, the maximum hydrolytic activity was at 60 ℃ (Ben Ammar et al., 2002). Tambara et al. (1999) have studied the effect of the substrate concentration on FOSs production by *A. diazotrophicus* levansucrase with a sucrose concentration ranging from 292 mM (100 g/l) to 2047 mM (700 g/l). Transfructosylation reaction increased as the sucrose concentration was higher, indicating that high sucrose concentration favored the transfructosylation activity of the enzyme.

Site-directed mutations of amino acid residues have improved our understanding of the important residues involved in the transfructosylation activity over those involved in the hydrolytic one. Chambert and Petitglatron (1991) have demonstrated that the transfructosylation activity of *B. subtilis* levansucrase significantly increased when Arg331 in the active site of the enzyme was substituted. In addition, Senthikumar et al. (2003) have reported that serine substitutions of cysteine at positions 121, 151 or 244 abolished levan forming activity of levansucrase from *Z. mobilis* whilst only halving its activity in sucrose hydrolysis.

2.7.3.3. Reaction Selectivity: Oligomerization vs. Polymerization

Levansucrases are able to synthesize levan as well as FOSs. However, there are great differences in the ratio of polymer versus FOSs synthesized depending on the enzyme and the reaction conditions (Ozimek et al., 2006a). The three-dimensional structure of *B. subtilis* levansucrase revealed that a total of eight amino acid residues are involved in the substrate recognition, binding and catalysis at the -1 (W85, D86, R246, D247 and W163) and +1 subsites (R360, E340 and R246) (Meng and Futterer, 2003). Ozimek et al. (2006b) proposed a "processive vs. non-processive" model for the organization of the levansucrase sugar-binding subsites to explain the reaction selectivity towards polymerization and oligomerization (Fig. 2.2). The -1 subsite has high affinity for fructose units; whereas the +1 subsite is able accommodate both glucose (binding of sucrose and raffinose) and fructose (binding sucrose as an acceptor substrate during transfructosylation). When sucrose enters the active site, it occupies the -1 and +1 subsites and a covalent fructosyl-enzyme intermediate is formed at the -1 subsite. Then another (sucrose) acceptor substrate enters the active site, binds to the +1 and +2 subsites, and reacts with the fructosyl-enzyme intermediate forming FOS (DP2). If the subsites +2, +3 and/or further have a relatively low affinity for binding the growing fructan polymer chain, the fructan polymer chain will be released after virtually every chain elongation with a fructosyl unit from sucrose, which may result in a non-processive reaction (disproportionation, oligomerization). On the contrary, high affinity of the fructan polymer chain at subsites +2, +3 and/or further leads to the production of high molecular weight polymers (proportionation, polymerization).

Levansucrases from different bacteria synthesize different types and DP of fructans. For instances, *B. subtilis* and *B. amyloliquefaciens* levansucrases have been reported to mainly produce high molecular mass levan without or with low FOSs accumulation (Hernandez et al., 1995; Tian and Karboune, 2012). This indicates that the enzyme may employ a processive type of reaction for fructan-chain elongation. In contrast, *Z. mobilis* levansucrase (Doelle et al., 1993) and *L. sanfranciscensis* levansucrase (Korakli et al., 2001) synthesized FOSs and low amounts of levan, which were produced in a non-processive manner.

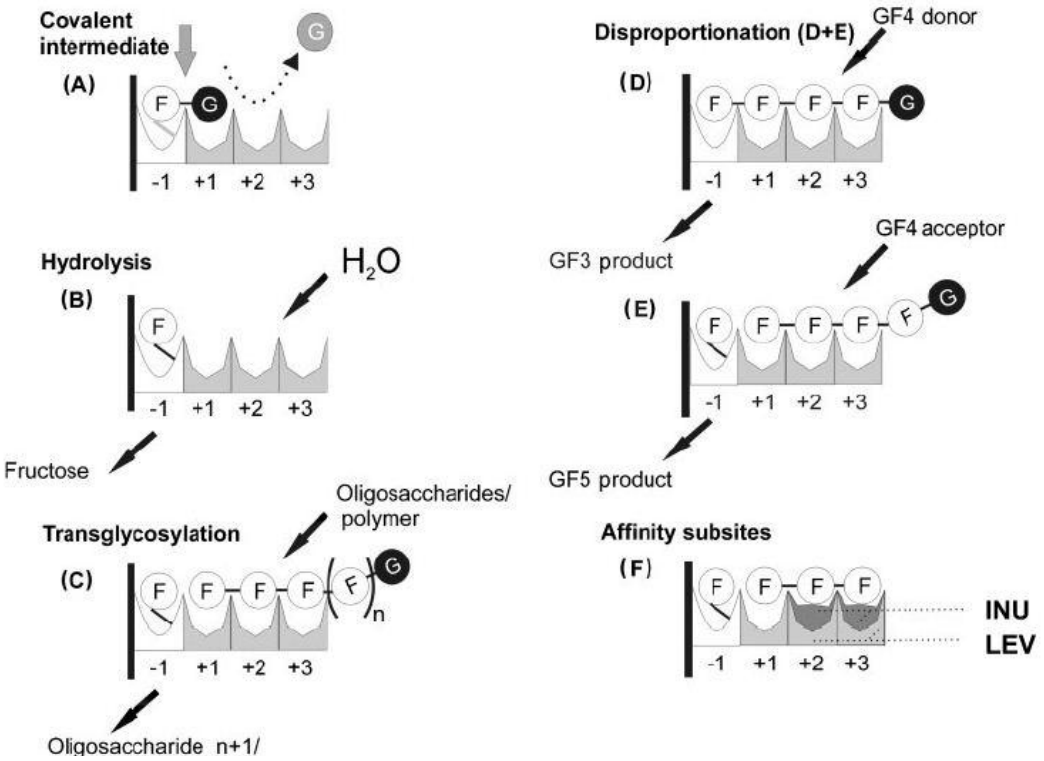


Figure 2.2. Model of the substrate-binding at the active site and levansucrase-catalyzedproportionate and disproportionate reactions (Ozimek et al., 2006b) G (Glucose), F (Fructose) and LEV (Levansucrase)

2.7.3.4. Donor and Acceptor Specificities of Levansucrases

The -1 subsite of levansucrase binds fructose residue of the donor sucrose molecule with a high affinity. The +1 subsite that participates in both donor and acceptor binding, has a lower substrate specificity. Accordingly, levansucrases are able to use different saccharides as fructosyl acceptors to produce oligosaccharides (Park et al., 2005). Monosaccharides like galactose, xylose and fructose (Bekers et al., 2002; Tian and Karboune, 2012) and disaccharides such as maltose, cellobiose, lactose (Kim et al., 2001; Tian and Karboune, 2012) and melibiose (Seibel et al., 2006) have been identified as fructosyl acceptors of levansucrases with sucrose as fructosyl donor. Moreover, it has been reported that disaccharides were more favorable fructosyl acceptors than monosaccharides (Ohtsuka et al., 1992). The saccharides having a pyranose ring in their chemical structure, such as galactose, maltose, lactose, melibiose, and cellobiose were better acceptors than the saccharides having a furanose ring, such as arabinose, xylose and raffinose (Park et al., 2003). Interestingly, Mena-Arizmendi et al. (2011) reported that *B. subtilis* levansucrase was also able to utilize non-sugars, such as hydroquinone, butanol and benzyl alcohol as acceptors for the fructosylation of aromatic or aliphatic alcohols in organic solvent.

In addition, levansucrase can synthesize levan and FOSs with sucrose, stachyose (Park et al., 2003) as well as raffinose (Meng and Futterer, 2008; Tian and Karboune, 2012) as fructosyl donor. Levansucrase from *Z. mobilis* showed a higher affinity towards raffinose than sucrose at low concentrations (Andersone et al., 2004). However, because of the low cost, large availability, purity, and easier access to the active site of levansucrase, sucrose has more advantages as a fructosyl donor substrate (Monchois et al., 1999; Ozimek et al., 2006b).

2.8. Maple Syrup as a Source of Fructooligosaccharides

Maple syrup is indigenous to North America, and more specifically to Québec. It is a natural product, free of any colorings or additives, made from the sap of three maple *(Acer)* species, including sugar maple (*Acer saccharum*), red maple (*Acer rubrum*) and silver maple (*Acer saccharinum*). The yearly worldwide production of maple syrup is roughly 8–9 million gal, of which 85% is produced in Canada. Québec is the most important Canadian producer of maple syrup, accounting for 90% of the total Canadian

production (Perkins and van den Berg, 2009). The fact that the main component of maple syrup is sucrose along with trace amounts of glucose and fructose (Stuckel and Low, 1996) makes it an interesting medium for bacterial growth and an interesting base material to produce other novel ingredients such as FOSs (Yezza et al., 2007).

2.8.1. Chemical Composition

Maple syrup consists primarily of sucrose (68%) with small amounts of glucose (0.43%, w/w) and fructose (0.34%, w/w) (Stuckel and Low, 1996). Maple syrup also contains nitrogenous and phenolic compounds (0-0.1ppm) (Ball, 2007; Kermasha et al., 1995; Underwood et al., 1961), organic acids (0.174%, w/w) (Mollica and Morselli, 1984), and minerals (3638.62 ppm) (Perkins and van den Berg, 2009). It is the phenolic compounds in maple syrup that contribute to the distinctly unique flavor and provide antioxidant, antiradical and antimutagenic potential of maple products (Theriault et al., 2006). A one-fourth cup serving of maple syrup holds an antioxidant capacity comparable to that of a portion of broccoli or a banana. In addition, the portion also provides 100% of manganese, 34% of riboflavin (vitamin B2) and 11% of zinc of the Canadian and US FDA daily recommended value and lesser amounts of magnesium, calcium, and potassium (Perkins and van den Berg, 2009).

2.8.2. Standards and Quality Determinants of Maple Syrup

In Canada, the Canadian Food Inspection Agency (CFIA) regulates the safety and quality of maple syrup and ensures that producers meet federal standards. Pure maple syrup must generally meet strict standards for soluble solids, density, clarity, color, flavor and microbial counts (Aider et al., 2007a).

2.8.2.1. Physicochemical Quality of Maple Syrup

Physicochemical quality of maple syrup includes soluble solids, pH, color, flavor, etc. Canadian standards stipulate that maple syrup must at least 66% total solids at 20℃ (Dumont et al., 1993). Since the major solid constituent of syrup is mainly sucrose, % total solids and °Brix values can be used interchangeably. The pH of maple syrup is near neutral, with mean values around pH 6.3-6.8 (Stuckel and Low, 1996). Maple syrup varies greatly in opacity and color, which ranges from a very light yellow-amber to near black. Thus, maple syrup color (% light transmittance at 560 nm) is a key variable used in

current classification systems (Perkins and van den Berg, 2009). The standards in Canada include three grades of maple syrup with five different classes of color: Canada No. 1 extra light (AA), Canada No. 1 light (A), Canada No. 1 medium (B), Canada No. 2 amber (B) and Canada No. 3 dark (C) (Aider et al., 2007a). In addition, maple syrup is also graded based on its flavor. The lightest syrups (Canada No. 1 extra light) are generally sweet, with only a slight hint of maple flavor. In general, lighter-color (higher grade) syrups tend to contain lower levels of flavor compounds than darker (lower grade) syrups (Perkins and van den Berg, 2009). In some syrup-producing areas, greater economic benefits can be obtained if the syrup is of a light amber color, with a very special "maple bouquet" flavor (Morselli and Whalen, 1991).

2.8.2.2. Microbiological Quality of Maple Syrup

Maple sap is sterile in the tree (Morselli and Whalen, 1991), but during collection, storage and production, it becomes contaminated by a wide variety of microorganisms, including bacteria, fungi and yeasts (Dumont et al., 1993). Microbial contamination may cause defects in color, flavor, viscosity and safety concerns in maple syrup (Fabian and Buskirk, 1935; Frank and Willits, 1961). One of the most common defects reported by the maple syrup industry is the production of ropy and unmarketable maple syrup resulting from *Enterobacter agglomerans* contamination of maple sap (Britten and Morin, 1995; Fabian and Buskirk, 1935). In addition, microorganism activity converts sucrose to invert (reducing) sugars, which is mainly responsible for the color darkening of maple syrup during processing. The reduction of microorganism in maple sap can result in syrup one grade lighter than the syrup from untreated sap (Perkins and van den Berg, 2009). Maple syrup with more than 3% of invert sugar cannot be used to produce value-added maple products such as maple candy, maple cream and granulated maple sugar (Aider et al., 2007b). Moreover, microorganisms can cause fermentation of maple syrup and result in a moldy off-flavor (Perkins and van den Berg, 2009).

2.8.3. Production of Maple Syrup

2.8.3.1. Production

In early spring when the temperature fluctuates around the freezing point at night, maple sap is collected by drilling small holes into the stem of sugar maple trees. An elaborate

tubing system connects each tree and sends sap directly to the sugar shack. In general, each taphole will produce about 10-20 gal of sap during the season. The sap itself is thin, slightly sweet and as clear as water, which contains all the precursors required for the development of flavor and color which are characteristic of maple syrup. Once the sap is collected, it is partially concentrated by reverse osmosis followed by heating in an evaporator to reach the minimum value of 66 °Bx. The unique flavors of maple syrup are developed during this evaporation process (Deslauriers, 2000). However, the production of maple syrup from maple sap is time-consuming and uses large amounts of energy, since 40 gal of sap are required to produce 1 gal of maple syrup (Khalf et al., 2010).

2.8.3.2. Challenges Associated with Maple Syrup Production

Every year, most of the concentrated maple sap that is produced is consumed as syrup, however, 5-20% of the total production is classified as low-grade because of flavor, color, taste or appearance defects (Morin et al., 1995). This kind of syrup is not marketable because of its dark color and very strong taste similar to that of the flaring sugar (Aider et al., 2007a). In the food industry, the current way of reusing this low-grade sap is to blend 1 liter of it into each 60 liters of concentrated maple sap of standard quality. However, this practice lowers the quality of maple products and it may jeopardize the maple product industry in the long term. Finding alternative usage of the low-grade maple syrup without having to decrease the quality of pure maple syrup could be of great importance to the maple syrup industry (Morin et al., 1995).

2.8.4. New Applications and Diversification of Maple Products

In addition to being a natural sweetener, maple syrup also represents a raw material from which other maple products can be produced. The common maple products include maple sugar, granulated maple sugar, maple spread and maple candy (Perkins and van den Berg, 2009). Maple syrup has also gone beyond these traditional uses, and is being used as a natural flavoring ingredient in processed, value-added products. According to Mintel's Global New Products Database (GNPD), which documents new food and drink launches in Europe from January 2011 to August 2012, maple syrup was being used in a variety of food from dairy-based frozen products to cold cereals, snacks and bakery

products. Many of these products focus on the natural, healthy, and quality attributes of maple syrup (Mintel, 2012).

In addition, since the dominant component of maple syrup is sucrose along with trace amounts of glucose and fructose (Stuckel and Low, 1996), it may be an ideal medium for bacterial growth and demonstrates the potential biotransformation of maple syrup into other novel value-added products (Yezza et al., 2007). Many authors have investigated the potential of maple syrup as functional food. Cochu et al. (2008) observed that maple sap was able to stimulate the growth of *lactobacilli* and enhance the production of lactic acid. Later, Khalf et al. (2010) developed a symbiotic maple sap product containing the probiotic strains *Bifidobacterium lactis* Bb12 and *Lb. rhamnosus* GG and the prebiotic inulin. This product has shown potential as a vehicle for the delivery of viable *B. lactis* Bb12 and *Lb. rhamnosus* GG in large numbers in the gastrointestinal tract.

2.9. Product Analysis

2.9.1. Thin-layer Chromatography (TLC)

Thin-layer chromatography (TLC) has been widely used in the rapid detection and the characterization of sugars in complex mixtures due to its high sensitivity and low cost (Sherma, 2003). FOSs can be characterized by TLC analysis using a developing phase of butanol/acetic acid/deionized water (5:4:1, v/v/v) followed by spraying of resorcinol solution in acetic acid (0.1% w/v resorcinol and 0.25% w/v thiourea acid) and of sulfuric acid in methanol (2%, v/v) for detection. The plate is then heated at 100 ℃ for 2h. The FOS-containing spots are identified as yellowish to brown spots (Park et al., 2003; Tian et al., 2011). TLC analysis is most efficient when many samples must be analyzed and only qualitative data are required (Folkes and Jordan, 2006). However, TLC analysis has been mostly replaced by quantitative instrumental chromatographic methods which have higher separation efficiencies, low detection limits and higher speed of analysis (Folkes and Jordan, 2006).

2.9.2. Gas Chromatography

Gas chromatography (GC) can provide both quantitative and qualitative determination of FOSs. However, the prerequisite for analysis by GC is the derivatization to render the sugars volatile. Barez et al. (1999) described the use of O-trimethylsilyl (O-TMS) ether

derivatives of the sugars to quantify the oligosaccharides in honey. Hayashi et al. (2000) treated samples with methyl iodide followed by hydrolysis with sulfuric acid and reduction with $NaBD_4$ before subjecting the samples to GC analysis. Due to the complexity in the preparation of volatile derivatives, high-performance liquid chromatography which requires fewer steps in sample preparation has received much attention in the analysis of FOSs.

2.9.3. High-performance Liquid Chromatography

High-performance liquid chromatography (HPLC) is the most employed method for FOSs analysis. The separation efficiency and sensitivity depend on the columns and detector. The most commonly used columns are amino-bonded phase columns and resin-based columns equipped with a Refractive Index Detector (RID) (Sangeetha et al., 2005b). L'Hocine et al. (2000) reported the use of Merck Lichrosorb-NH_2 column at a temperature of 30 ℃ with acetonitrile-water (80:20, v/v) as the mobile phase at a flow rate of 1.2 ml min^{-1}. By changing the proportion of water and acetonitrile, the resolution and the speed of elution can be controlled. The amino-bonded phase columns are efficient, and carbohydrates elute in the order of increasing molecular weight. However, the drawback of amino-bonded phase columns is the gradual loss of the amino groups caused by the reaction between reducing sugars in the sample and the stationary phase (Cataldi et al., 2000). In addition, acetonitrile is a relatively expensive solvent which adds to the costs of using amino-bonded phase columns. In contrast, water is commonly used as the mobile phase in resin-based columns, which is more affordable. As early as 1980s, Slavin and Marlett (1983) has used Aminex HPX-85 heavy metal cation-exchange carbohydrate column to determine saccharides content. Other resin-based columns examples include Aminex HPX-42C column (Kim et al., 2001), Aminex HPX 87 N column (Trujillo et al., 2001) and Aminex HPX 87C (Crittenden and Playne, 2002). In resin-based size exclusion columns, oligosaccharides elute first, followed by disaccharides and then monosaccharides. However, this method has poor resolution of structurally similar carbohydrates (Barez et al., 1999).

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

Anion-exchange chromatography has grown in importance as it is able to resolve a complex sugar mixtures separation that cannot be achieved using amino-bonded phase columns or resin-based columns in HPLC (Folkes and Jordan, 2006). In basic solutions (pH >11), carbohydrates become weakly anionic, therefore, by ionizing the sugar hydroxyl groups with NaOH as mobile phase, separation can occur by differential retention of the ionized sugars on the anion-exchange resin surface (Cataldi et al., 2000). Sugar resolution can be further improved by the addition of competitor ions, usually acetate ions, which compete with sugar molecules for the resin-binding sites, allowing the separation of closely related monosaccharides and disaccharides (Folkes and Jordan, 2006). The gold electrode surface is able to catalyze the oxidation of carbohydrates in alkaline media; therefore, HPAEC-PAD provides highly specific detection and sensitivity without derivatization of samples (Folkes and Jordan, 2006). Borromei et al. (2009) have used a Carbopac PA 100 analytical anion exchange column using a 20 min linear gradient from 5% to 22% of 500 mM sodium acetate with 16% of 600 mM NaOH solution to detect FOSs (DP 3-10) and inulin. The main drawback of HPAEC-PAD is the interference of carbonate ions, which have higher affinity than acetate ions to the resin-binding sites, in the sodium hydroxide solution affecting the separation and reproductivity. It is important to keep the eluent solution saturated with nitrogen, thus hindering the CO_2 intake (Cataldi et al., 1999).

2.9.5. Nuclear Magnetic Resonance Spectroscopy

Nuclear magnetic resonance (NMR) spectroscopy is a reliable and powerful method for the analysis of carbohydrates (Lerner, 1996). It can provide a complete assignment of all the structural features of carbohydrates, including the stereochemistry of the monosaccharide units and the type of linkages between the units without destructing or degrading the carbohydrate samples. NMR methods are often applied for the structural determination of polysaccharides isolated from bacteria and plants, which exhibit large structural diversity in terms of unusual linkages (Lieth, 2009).

The analysis of the polysaccharides is often offered by ^{13}C-NMR and ^{1}H-NMR spectroscopy. According to the spectral patterns, all of the fructosyl units in sucrose, FOSs, levan and inulin are shown to be β-D-linked. In particular, levan is generally formed by a repeated unit of β-fructose residues linked by β-(2-6) bonds, while inulin is composed of linear chains of (2-1)-linked β-D-fructofuranosyl residues (Dahech et al., 2013; Fischer and Geyer, 2007; Matulová et al., 2011; Tian and Karboune, 2012).

2.10. Conclusion

This review presented the potential for low grade maple syrups as a renewable substrate to be transformed into value-added health-promoting products through *B. amyloliquefaciens* levansucrase-catalyzed reactions. As was reviewed, levansucrase is able to catalyze the formation of β-(2-6)-linked levan and FOSs with high regio- and stereo-specificity in the presence of sucrose as fructosyl-donor (Mussatto et al., 2012). Furthermore, *B. amyloliquefaciens* levansucrases have been reported to mainly produce high molecular mass levan without or with low FOSs accumulation (Hernandez et al., 1995; Tian and Karboune, 2012). Maple syrup consisting of mainly sucrose seems to be ideal reaction media for *B. amyloliquefaciens* levansucrase-catalyzed reaction. The exploration of low grade maple syrups as a renewable substrate is not only expected to increase the value of low grade maple syrups, but also to diversify their products.

CHAPTER III MATERIALS AND METHODS

3.1. Chemicals and Materials

D-(+)-galactose, D-(+)-xylose, α-lactose, D-(+)-cellobiose, D-(+)-melibiose and sucrose were all purchased from Sigma Chemical Co. (St-Louis, MO). Chemical reagents, including 3, 5-dinitrosalicylic acid (DNS), K_2HPO_4, KH_2PO_4, NaOH, polyethylene glycol (PEG) 200 and Triton X-100 were also obtained from Sigma Chemical Co (St-Louis, MO). $CaHPO_4$, $FeSO_4 \cdot 7H_2O$, $MgSO_4 \cdot 7H_2O$, $MnSO_4$, Na_2HPO_4, $Na_2MoO_4 \cdot 2H2O$, $(NH_4)_2SO_4$, and yeast extract were purchased from Fisher Scientific (Fair Lawn, NJ). Carbohydrate standards 1-kestose, nystose, and 1^F-fructosylnystose were purchased from Wako Pure Chemical (Japan). Maple syrups 15 °Bx, 30 °Bx and 66 °Bx were kindly provided by Dr. Luc Lagacé from the Centre de recherche, de développement et de transfert technologique acéricole inc. (Centre ACER).

3.2. Microbial Strain and Growth Conditions

B. amyloliquefaciens (ATCC 23350) was obtained from American type culture collection (Manassas, USA) and used as a microbial source for the production of levansucrase. The strain was maintained on a potato dextrose agar (39 g/l).

3.3. Production and Purification of Levansucrase

A loopful of *B. amyloliquefaciens* was inoculated in a nutrient broth preculture (8 g/l) and incubated at 35°C for 24 h with agitation of 150 rpm (New Brunswick Scientific co., inc.-Shaker). 4 ml of preculture was withdrawn and transferred into a 1-L baffled Erlenmeyer flask containing 400 ml of the culture medium. The modified mineral salt medium (Tian et al., 2011) was comprised of (in g/100ml): KH_2PO4 (0.136), $Na_2HPO_4 \cdot 2H_2O$ (0.267), $(NH_4)_2SO_4$ (0.05), $MgSO_4 \cdot 7H_2O$ (0.02), $CaPO_4 \cdot 2H_2O$ (0.001), $FeSO_4 \cdot 7H_2O$ (0.0005), $MnSO_4 \cdot H_2O$ (0.00018) and $Na_2MoO_4 \cdot 2H_2O$ (0.00025). This medium was supplemented with yeast extract (10 g/l) as organic source of nitrogen and sucrose (10 g/l) as inducer. *B. amyloliquefaciens* was grown in the medium at 35°C for 11 h with agitation at 150 rpm. The medium was then centrifuged at 8000 rpm for 20 min to recover the bacterial cells. To recover the intracellular levansucrase, the recovered cells were re-suspended in 50 mM potassium phosphate buffer (pH 6.0) containing 1% Triton X-100 and disrupted by

ultrasonication at 2 kHz with a cycle 25/50 s (550 Sonic Dismembrator, Fisher Scientific). The resulted suspension was centrifuged at 8000 rpm for 15 min to recover intracellular levansucrase. Levansucrase was then purified by PEG 200 fractionation at a concentration of 30% (v/v). The mixture was gently stirred for 24 h at 4 ℃. The pellet containing precipitated levansucrase was recovered by centrifugation at 8000 rpm for 45 min and re-suspended in 50 mM potassium phosphate buffer (pH 6.0). The resulted suspension was dialyzed against 5 mM potassium phosphate buffer (pH 6.0) using a membrane with a 5-6 kDa cut-off for 48 h at 4 ºC and then freeze dried. The activity and the protein content of the intracellular levansucrase extract were assessed.

3.4. Total Protein Content

The protein content was measured using the Standard BioRad Protein Assay. 20 µl of the sample was added into 1 ml of a 25% (v/v) diluted Bradford Dye. The absorbance was measured at 595 nm spectrophotometrically. The concentration of protein was then estimated from a standard curve using bovine serum albumin (0.0625 to 1 mg/ml).

3.5. Enzymatic Assays of Levansucrase Activity

The total levansucrase activity was assayed using sucrose as substrate. One unit of total levansucrase is defined as the amount of the biocatalyst that produces 1 µmol of reducing sugars (glucose and fructose) per min. The total released reducing sugars were measured as glucose equivalents by dinitrosalicylic acid (DNS) method (Miller, 1959). The enzymatic reactions were initiated by adding a series of appropriately diluted levansucrase extract suspension (from 10 to 80) to 250 µl of 1.8 M sucrose solution prepared in potassium phosphate buffer (50 mM, pH 6.0). Two blank assays, without the substrate or without the enzyme, were also conducted. The reaction mixtures were incubated at 30ºC for 20 min. 750 µl of DNS solution, consisting of a 1% (w/v) 3, 5-dinitrosalicylic acid in 1.6% (w/v) NaOH was added into each reaction mixture and then the mixtures were boiled in water for 5 min. To reduce the tendency of the solution to dissolve oxygen, 250 µl of a 50% (w/v) potassium sodium tartrate solution was added. The absorbance was measured at 540 nm (DU800 spectrophotometer, Beckman Coulter) against the blanks. The amount of released reducing sugars was then determined from a standard curve using glucose (from 0.5mM to 10mM). The specific activity was

expressed as the μmol of released glucose or fructose per minute of reaction and per mg of protein.

3.6. Reaction Time Courses for the Enzymatic Conversion of Sucrose from Selected Maple Syrups

The catalytic efficiency of levansucrase from *B. amyloliquefaciens* in maple-based reaction systems was assayed. Three maple syrups 15 °Bx, 30 °Bx and 66 °Bx were prepared from the same maple sap from centre ACER and these syrups were used as reaction media and sucrose source. The reaction time courses in these maple-based systems were investigated at two temperatures of 8 °C and 30 °C. The reactions were initiated by adding levansucrase enzyme (72 U/ml) in 0.1 M potassium phosphate buffer (pH 6.0) to yield 2 and 6 U/ml maple syrup. Aliquots of enzymatic reactions were withdrawn at selected time intervals within a 48h time period. To stop the reaction, the aliquots were boiled for 5 min. The protein and levan polymers were precipitated with methanol (1:1, v/v). The analyses of the reaction mixture components were carried out by HPAEC-PAD (Dionex).

3.7. Identification and Characterization of the End-Product Spectrum

To determine the total sucrose conversion yield and the concentration of transfructosylation products, sucrose, glucose and fructose were determined by HPAEC on Dionex (ICS-3000) chromatography system equipped with pulsed amperometric detector and CarboPac PA 20 (3×150 mm) column. Subtracting the total amount of fructose from that of glucose provides the amount of glucose resulting from transferring fructose. The elution of the reaction components was carried out at 0.4 mL/min using 10 mM NaOH for 30 min. Integration was carried out with the Chromeleon Software (Version 7).

On the other hand, the concentrations of fructooligosaccharides, oligolevans and levans were determined by high performance size exclusion chromatography (HPSEC) using waters HPLC system equipped with refractometer 2489 detector and three size exclusion columns (7.8 mm x 30cm) sequentially, TSKgel G3000PWXL-CP, TSKgel G4000PWXL-CP and TSKgel G5000PWXL-CP. The elution of the reaction components was carried out at 0.4 ml/min using 0.1 M NaCl for 90 min. The integration was carried

out with the Breeze^{TM}2 software. Dextrans (50 to 670 kDa) were also used as standards for calibration.

3.8. Substrate Acceptor Specificity Study

To study the acceptor specificity of levansucrase in maple syrup 15 °Bx and 30 °Bx-based reaction systems, selected saccharides (galactose, xylose, lactose, cellobiose and melibiose) were used as acceptors. The maple syrup 15 °Bx and maple syrup 30 °Bx were enriched with selected acceptors and used as reaction media and as sucrose fructosyl donor source. The enzymatic reactions were performed with a fructosyl donor to acceptor molar ratio of 2:1 with an enzyme amount of 2 U/ml over a time course of 48 h at 30 °C. Samples were taken at different time intervals and boiled for 5 min to stop the reaction. The protein and levan polymers were precipitated with methanol (1:1, v/v). The analysis of the reaction mixtures was carried out by HPAEC on Dionex (ICS-3000) chromatography system equipped with pulsed amperometric detector and Chromeleon Software (Version 7) (Tian and Karboune, 2012). CarboPac PA 200 (3×250 mm) column was used with a linear gradient of sodium acetate from 0 to 0.2 M in 0.1 M NaOH at 0.5 ml/min for 20 min. Standard curves of selected saccharides (fructose, glucose, sucrose, xylose, galactose, lactose, cellobiose, melibiose, 1-kestose, 6-kestose, nystose, and 1^{F}-fructosylnystose) were constructed. The levan was also quantified by HPSEC as described above.

3.9. Optimization of Fructooligosaccharides, Oligolevans and Levans Production by Response Surface Methodology

3.9.1. Experimental Design

Optimization of the synthesis of oligolevans/FOSs and oligolevans/levans in maple syrup 30 °Bx and 66 °Bx-based reaction systems, respectively, was achieved using response surface methodology (RSM). A five-level, three factor central composite rotatable design (CCRD) was employed. The variables included enzyme unit (X_1, 2-6 U/ml), reaction time (X_2, 6-36 h) and pH (X_3, 5.7-7). The full design consisted of 8 factorial points, 6 axial points (2 axial points on the axis of each design variable at a distance of 1.68 from the center) and 6 center points, leading to 20 sets of experiments (Table 3.1).

Table 3.1. Variables and their coded levels used in a central composite rotatable design for optimization of oligolevan/FOSs and oligolevans/levans productions in the maple syrups 30 ℃Bx- and 66 ℃Bx-reaction systems, respectively.

	Coded values		
Variables	-1	0	1
X_1: enzyme unit	2	3.5	5
X_2: reaction time	12.08	21	29.92
X_3: pH	6.04	6.55	7.06

The response variables were the oligolevans/FOSs yield and oligolevans/levans yield (%, w/w; g/l) as compared to the initial sucrose concentration. The analysis of the reaction components was carried out by HPSEC.

3.9.2. Statistical Analysis

Experiment data was obtained based on the design. Regression was performed and fitted to quadratic equations using the software Design-Expert 8.0.7 (Stat-Ease, Inc. Minneapolis, MN, USA).

$$Y= \beta_0 + \sum_{i=1}^{3} \beta_i X_i + \sum_{i=1}^{3} \beta_{ii} X_i^2 + \sum_{i<j=1}^{3} \beta_{ij} X_i X_j \quad (1)$$

Where Y are the predicted responses for oligolevans/FOSs and oligolevans/levans yields, β_0, β_i, β_{ii} and β_{ij} are constant, linear, quadratic and cross-product terms, respectively, $X_{i\ (i=3)}$ are the coded independent variables. The variability of the fit of the polynomial model equation was expressed by the coefficient of the determination R^2 and its statistical significance was checked using *F*-test.

CHAPTER IV RESULTS AND DISCUSSION

4.1. Time Course for Levansucrase-catalyzed Reactions in Maple Syrups

Levansucrase uses the free energy of cleavage of non-activated sucrose to transfer the fructosyl group to a variety of acceptors and hence catalyzes four types of reactions: exchange (the acceptor is a monosaccharide), hydrolysis (the acceptor is water), transfructosylation (the acceptors could be sucrose, sucrose analogues or FOSs), and polymerization (the acceptor is levan). Maple syrups being made of mainly sucrose (68 w/w) (Ball, 2007) can be used as reaction media for levansucrase. To assess the efficiency of the maple syrup as reaction medium and as source of sucrose, the time courses for levansucrase from *B. amyloliquefaciens*-catalyzed transfructosylation and hydrolysis reactions were investigated using maple syrups 15 °Bx (0.44 M), 30 °Bx (0.88 M) and 66 °Bx (1.93 M). It has been also reported that decreasing the reaction temperature can lead to the change in the reaction selectivity (transfructosylation/hydrolysis) and to the alteration of the end-product profile (FOSs/levans) (Tian and Karboune, 2012). Therefore, the conversion of sucrose present in the maple syrups by levansucrase from *B. amyloliquefaciens* was assessed at 8 °C and 30 °C using an enzyme concentration of 6 U/ml of syrup.

The results show that at 30 °C, the sucrose concentration present in maple syrups 30 °Bx (Fig. 4.1B) and 15 °Bx (Fig. 4.1C) decreased linearly during the first 3h of the reaction time course to reach 0.51 M and 0.26 M, respectively; beyond this reaction time, it decreased with a much lower extent to reach a concentration of 0.37 M and 0.20 M at 7 h, respectively, corresponding to 57.6% and 52.4% of the total conversion yield. A further increase in reaction time to 24h resulted in a more or less similar total conversion yield of sucrose present in maple syrups 30 °Bx and 15 °Bx; however, with maple syrup 30 °Bx, a significant increase in the converted sucrose concentration was, thereafter, observed to reach a maximum yield of 74.28% upon 48 h of reaction. These results may be due to the fact that the reaction thermodynamic equilibrium may have been reached faster in maple syrup 15 °Bx.

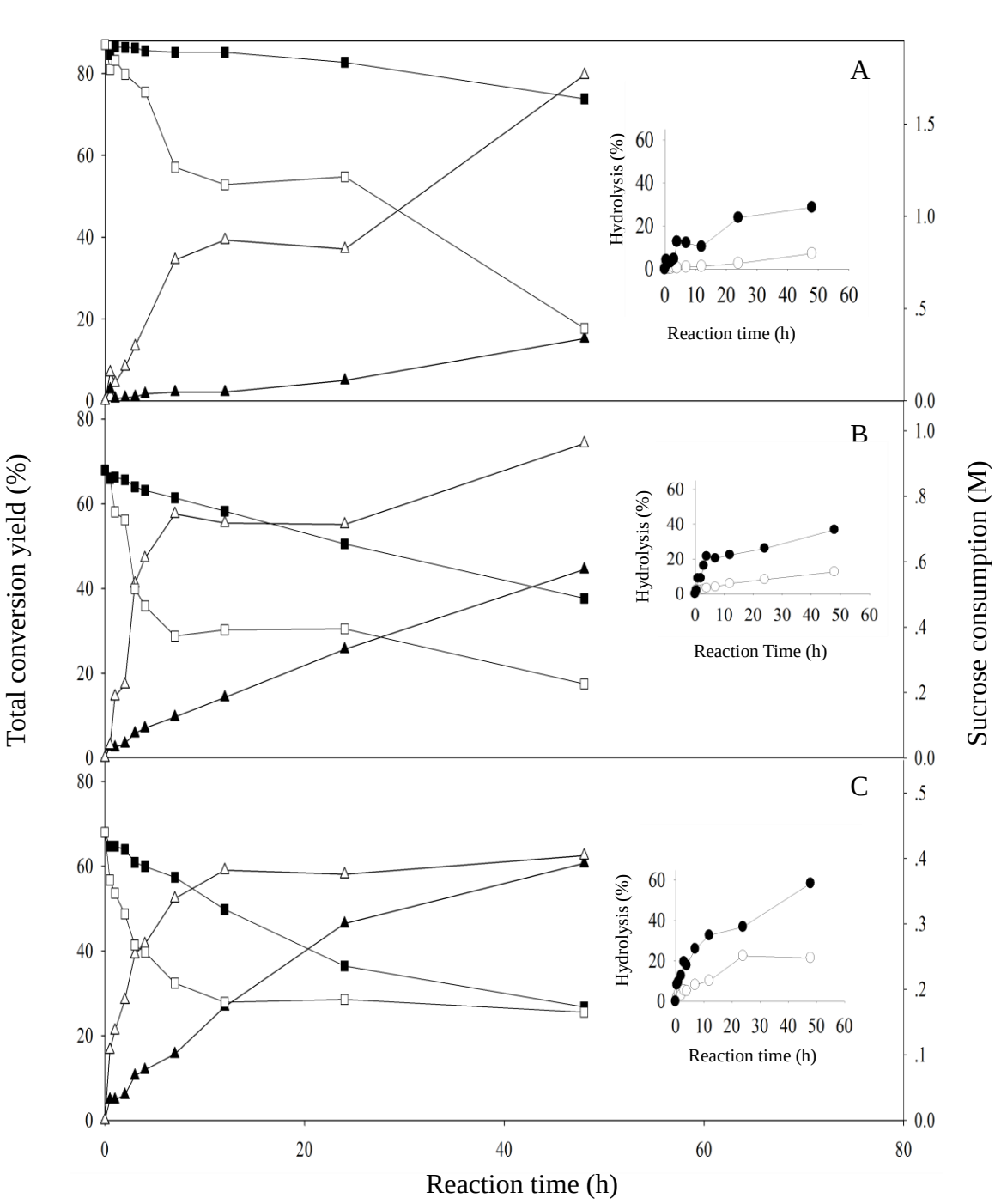


Figure 4.1. Reaction time courses for levansucrase-catalysed reaction in maple syrup 66 ℃Bx (A), maple syrup 30 ℃Bx (B) and maple syrup 15 ℃Bx (C)-based reaction systems: sucrose consumption (right scale) at 30 ℃ (□) and 8 ℃ (■), the total conversion yield at 30 ℃ (△) and 8 ℃ (▲), and hydrolysis at 30 ℃ (●) and 8 ℃ (○).

Using maple syrup 66 °Bx and 30 °C, different reaction time course was obtained in which the rate of conversion of sucrose was high at both the early and the last stages of the reaction with an intermediate saturation stage to reach 0.39 M upon 48 h (conversion yield of 80.0%) (Fig. 4.1A). These results may be explained by the fact that as the sucrose was converted, the viscosity of maple syrup 66 °Bx as reaction medium may have changed, affecting the substrate/product diffusion and hence the rate of conversion. However, maple syrup 30 °Bx led to the highest total activity (initial conversion rate of sucrose) at 30 °C (Table 4.1). As compared to maple syrup 30 °Bx, the low total activity obtained in maple syrup 15 °Bx is expected because of its low sucrose content (0.44 M). However, the low total activity of levansucrase when maple syrup 66 °Bx was used as reaction medium may be attributed to the combined effects of enzyme saturation and low substrate/enzyme interactions due to the high viscosity of the syrup. The V_{maxapp} value reported for *B. amyloliquefaciens* levansucrase total activity was 388.6 µmol/min of reaction/mg proteins with sucrose as substrate (Tian et al., 2011). The use of maple syrup 30 °Bx as reaction medium resulted in a comparable total activity of 427.53 µmol/min of reaction/mg proteins at 30 °C.

Although the sucrose present in maple syrup 66 °Bx was converted with a low rate at the initial stage at 30 °C, it led to the highest converted sucrose concentration of 1.54 M (79.66%). These results reveal the absence of substrate inhibition by the excess of sucrose concentration. However, the levan-forming activity of levansucrase from *P. syringae* and *B. circulans* has been reported to decrease due to the substrate inhibition (Hettwer et al., 1995; Oseguera et al., 1996). Table 4.1 also shows that 0.27 M (62.49%) and 0.65 M (74.28%) of sucrose concentration in maple syrup 15 °Bx and 30 °Bx, respectively, were maximally converted into hydrolytic and transfructosylation products at 30 °C. The high conversion yields of sucrose (more than 60%) reveal that *B. amyloliquefaciens* levansucrase was not inhibited by all the three maple syrups. In contrast, α-amylase and α-glucosidase inhibitions (IC_{50} of 68.85-135.0 µg phenolics) by phenolic-enriched extracts of Canadian maple syrup have been reported (Apostolidis et al., 2011). Similarly, Thériault et al. (2006) reported that maple syrup showed antimutagenicity properties by inhibiting the activity of β-galactosidase.

Table 4.1. Reaction kinetics of levansucrase-catalyzed transfructosylation reaction in selected maple syrups-based reaction systems

	Maple syrup 15 °Bx		Maple syrup 30 °Bx		Maple syrup 66 °Bx	
	6U/30 °C	6U/8 °C	6U/30 °C	6U/8 °C	6U/30 °C	6U/8 °C
Total activity(μmol/mg protein.min) [a]	328.86	47.54	427.53	46.05	377.35	29.17
Hydrolytic activity(μmol/mg protein.min) [b]	114.57	17.85	198.35	14.99	161.95	13.22
Transfructosylation activity(μmol/mg protein.min) [c]	214.29	29.7	229.18	31.05	215.4	15.95
Total converted sucrose concentration(M) [d]	0.274	0.267	0.653	0.391	1.537	0.294
Transfructosylation products concentration (M) [e]	0.122	0.172	0.330	0.281	0.983	0.155
Productivity(g/l.h) [f]	0.80	1.13	2.23	1.89	8.43	1.17

[a] Total activity was calculated as the μmol of released glucose per mg proteins per min of reaction.

[b] Hydrolytic activity was calculated as the μmol of released fructose per mg proteins per min of reaction.

[c] Transfructosylation activity was calculated as the μmol of fructose resulting from subtracting the concentration of free fructose from that of glucose per mg proteins per min of reaction.

[d] The maximum total converted sucrose concentration was achieved at 48 h for all the reactions.

[e] The transfructosylation products concentration was achieved at the corresponding time for the maximum total converted sucrose concentration.

[f] Productivity was calculated at the corresponding time for the maximum total converted sucrose concentration.

As expected, the sucrose conversion by levansucrase from *B. amyloliquefaciens* at 8 ℃ proceeded with a very low rate as compared to 30 ℃ (Fig. 4.1). Compared to 30 ℃, the highest total activity (initial reaction rate) at 8 ℃ was obtained upon the use of maple syrup 15 °Bx (Table 4.1), in which a maximum bioconversion yield of 60.7% was achieved upon 48 h of reaction (Fig 4.1). On the other hand, the limited mass diffusional transfers at low temperature in the highly viscous maple syrup 66 °Bx resulted in low total levansucrase activity. Within the investigated reaction time course, the sucrose concentration linearly decreased at a steady rate to reach 0.48 and 1.63 M in maple syrups 30 °Bx and 66 °Bx, respectively, corresponding to a conversion yield of 44.53 and 15.25%. As a result, the maximum total converted sucrose concentration present in maple syrups 30 °Bx and 66 °Bx at 8 ℃ was 1.67 and 5.2 times lower, respectively, than that at 30 ℃ (Table 4.1). However, in maple syrup 15 °Bx, similar maximum total converted sucrose concentration (0.27-0.27 M) was obtained at 8 ℃ and 30 ℃.

The sucrose conversion by levansucrase releases glucose and fructose, which can be transferred to the fructosyl 6'-hydroxyl (assuming β-(2-6)-linkage) of the acceptor products (levan, FOSs). Since the free fructose cannot act as donor or acceptor substrate, its concentration reflects the extent of the hydrolysis reaction of sucrose. Moreover, subtracting the concentration of free fructose from that of glucose gives the extent of the transfructosylation reaction. The results show that at 30 ℃, the extend of the hydrolysis of sucrose in maple syrup 15 °Bx (Fig. 4.1C) increased linearly during the first 7h of reaction to reach 25.98%; however, during this first 7h-reaction period, the transfructosylation reaction was slightly more favored than the hydrolytic reaction-a transfructosylation to water. Indeed, the transfructosylation activity (initial reaction rate) of levansucrase was 2 times higher than the hydrolytic activity, taking up to 65% of the total activity (Table 4.1). As the reaction was proceeded to 48 h, the reaction was shifted in favor of the hydrolytic reaction to reach a maximum hydrolysis and transfructosylation reaction extents of 34.71 and 27.77%, respectively. These results may be due to the high affinity of levansucrase toward the acceptor glucose as compared to other fructosyl acceptors.

In the maple syrup 30 °Bx-based reaction system at 30 ℃, the extent of the hydrolysis reaction and that of the transfructosylation one were more or less similar during the first

2h-reaction period (Fig. 4.1B); contrary to maple syrup 15 °Bx, the transfructosylation reaction (37.25-33.19%, 7-12h) was more favored than the hydrolytic one (20.38-22.29%, 7-12h) as the reaction time was proceeded to 12h. A further increase in reaction time to 48h resulted in a more significant increase in the hydrolysis reaction of sucrose (36.76%) than in the transfructosylation one (37.52%). As a result, 0.33 M of sucrose present in maple syrup 30 °Bx was converted, at the end stage of the reaction, into the transfructosylation products at 30 °C as compared to only 0.122 M with maple syrup 15 °Bx (Table 4.1). These results reveal that the accumulation of the fructosyl-acceptors/or donors in the reaction medium induced an increase in the apparent transfructosylation activity of levansucrase as well as a shift towards the production of transfructosylation products. The same effect has been reported by Chambert et al. (1974) for *B. subtilis* levansucrase and by Oseguera et al. (1996) for *B. circulans* in sucrose-based media.

Different reaction selectivity profile (transfructosylation/hydrolysis) was obtained over the investigated time course with maple syrup 66 °Bx in which the extent of the hydrolysis reaction and that of the transfructosylation was low and more or less constant during the initial stage (2-4h) of the reaction (Fig. 4.1A). As the reaction was extended, the transfructosylation reaction was preferentially catalyzed rather than the hydrolytic reaction of sucrose, revealing the high reaction selectivity of levansucrase in maple syrup 66 °Bx. Indeed, the hydrolytic reaction began to increase but proceeded with a much lower rate as compared to maple syrup 15 °Bx and maple syrup 30 °Bx, reaching a maximum hydrolysis yield of 28.71% upon 48h of reaction. Upon 48 h of reaction, 0.983 M of transfructosylation products were formed from sucrose (Table 4.1), which was 54.6 and 2.9 times higher than those with maple syrup 15 °Bx and maple syrup 30 °Bx, respectively. These results reveal a very important characteristic of *B. amyloliquefaciens* levansucrase with its high transfructosylation-to-hydrolysis ratio at high sucrose concentration. Tian and Karboune (2012) reported that at low sucrose concentration (0.1 M), transfructosylation activity and hydrolytic activity of *B. amyloliquefaciens* levansucrase were comparable, but the transfructosylation activity increased to 85% of the total activity when sucrose concentration increased to 1.2 M. This behavior has also been reported for *B. subtilis* levansucrase by Chambert et al. (1974) and *L.*

sanfranciscensis levansucrase by Tieking et al. (2005). In contrast, levansucrases from *B. megaterium* and *Rhodotorula* sp. displayed higher hydrolysis activity than transfructosylation activity at high sucrose concentration (Alvarado-Huallanco and Maugeri Filho, 2011; Homann et al., 2007)

As expected, the sucrose hydrolysis by levansucrase from *B. amyloliquefaciens* at 8 ℃ proceeded with a very low rate as compared to 30 ℃ (Fig. 4.1). The extent of the hydrolysis reaction and that of the transfructosylation one were comparable at the initial stage of the reaction in maple syrup 15 ℃Bx-based reaction system (0-7h) at 8 ℃ (Fig 4.1 C); however, contrary to 30 ℃, the transfructosylation reaction became dominant at the late stage of the time course (12-48h). Similarly, Rairakhwada et al. (2010) have reported that the optimum transfructosylation temperature of *B. amyloliquefaciens* levansucrase was around 4 ℃. *P. syringae* pv levansucrase displayed the highest transfructosylation activity at 18 ℃ (Hettwer et al., 1995). On the other hand, the experimental results show a similar trend of the reaction selectivity for *B. amyloliquefaciens* levansucrase at 8 ℃ and 30 ℃ in the maple syrup 30 ℃Bx-based reaction system with the transfructosylation reaction being preferentially catalyzed at the advanced stage of the reaction. These results may be attributed to the synergistic effects of the temperature on the levansucrase tridimensional structure and of the high substrate concentration on the thermodynamic shift of the reaction towards the transfructosylation. Contrary to maple syrup 15 ℃Bx and maple syrup 30 ℃Bx, low conversion of sucrose through the hydrolysis reaction and the transfructosylation one was obtained at 8 ℃ reaching 7.20% and 8.05% at 48h, respectively (Fig. 4.1A). As a result, the transfructosylation-over-hydrolysis ratio fluctuated during the investigated time course with no obvious dominant reaction observed. These results may due to the limited mass diffusional transfer in the highly viscous maple syrup 66 ℃Bx at 8 ℃ as well as to the low levansucrase activity at low temperature.

4.2. Production of Fructooligosaccharides, Oligolevans and Levans in Selected Maple Syrups-Based Reaction Systems

The effects of selected reaction conditions (temperature, levansucrase units, reaction time) on the production of the transfructosylation products and on the modulation of their

spectrum profile were investigated using maple syrups with three selected Bx values (15 °, 30 °, 66 °). Figure 4.2 shows the concentration of the total transfructosylation products and the relative proportion of FOSs (2< DP ≤10), oligolevans (10< DP ≤30) and levans (DP≥30). Using maple syrup 15 °Bx as a reaction medium, 6 U levansucrase/ml at 30 °C, more or less ~23.85 % (33.39 g/l) of its sucrose content was converted into transfructosylation products at 12 and 24 h of reaction (Fig. 4.2C); increasing the reaction time to 48 h resulted in a slight decrease in the yield to 19.5% (27.31 g/l). With low levansucrase unit of 2 U/ml, an increase in the yield of the transfructosylation products from 13.80 to 31.59% (44.23 g/l) was obtained over the investigated time course of 48 h. Lower reaction temperature of 8 °C resulted in a similar maximum yield of 23.92% (33.50 g/l) as that achieved at 30 °C. In the maple syrup 15 °Bx-based reaction system, levansucrase units had more effect on the thermodynamic equilibrium of the reaction and the maximum yield of the transfructosylation products than the reaction temperature. Such results may be due to the activation of the enzyme by the levan formed and/or to the accumulation of the fructosyl-acceptors/or donors in the reaction medium (Homann et al., 2007; Ozimek et al., 2006b; Tian et al., 2014). The profile of the end-products synthesized using maple syrup 15 °Bx reveals that sucrose was mainly converted into oligolevans (70.97-89.93%) at the early stage of the reaction (12 h). Although there was no significant changes in the yield of the transfructosylation products at 12 and 24 h, the relative proportion of levan increased from 22.57%, 61.48% and 38.09% to 79.72%, 64.20% and 69.71%, at the following conditions of 2 U/ml/ 30 °C, 6 U/ml/30 °C and 6 U/ml/8 °C, respectively. These results may be due to the use of the oligolevans as fructosyl-acceptors by levansucrase to form levan. However, the relative proportions of levan formed dropped significantly to 0.34-14% at 48 h; the main end-product was FOSs (70.65-99.65%) at 30 °C and oligolevans (93.52%) at 8 °C. The apparent decrease of levan may be attributed to its non-covalent cross linking, which may have led to its loss upon centrifugation before size exclusion chromatography analysis. Indeed, increasing the reaction time from 12 to 48 h led to an increase in molecular weight of levan from 3,413 kDa to 28,094 kDa (data not shown). El-Refai et al. (2009) have also reported an increase in the molecular weight of levan synthesized by *B. circulans* levansucrase with the extension of the reaction time from 6 to 24 h.

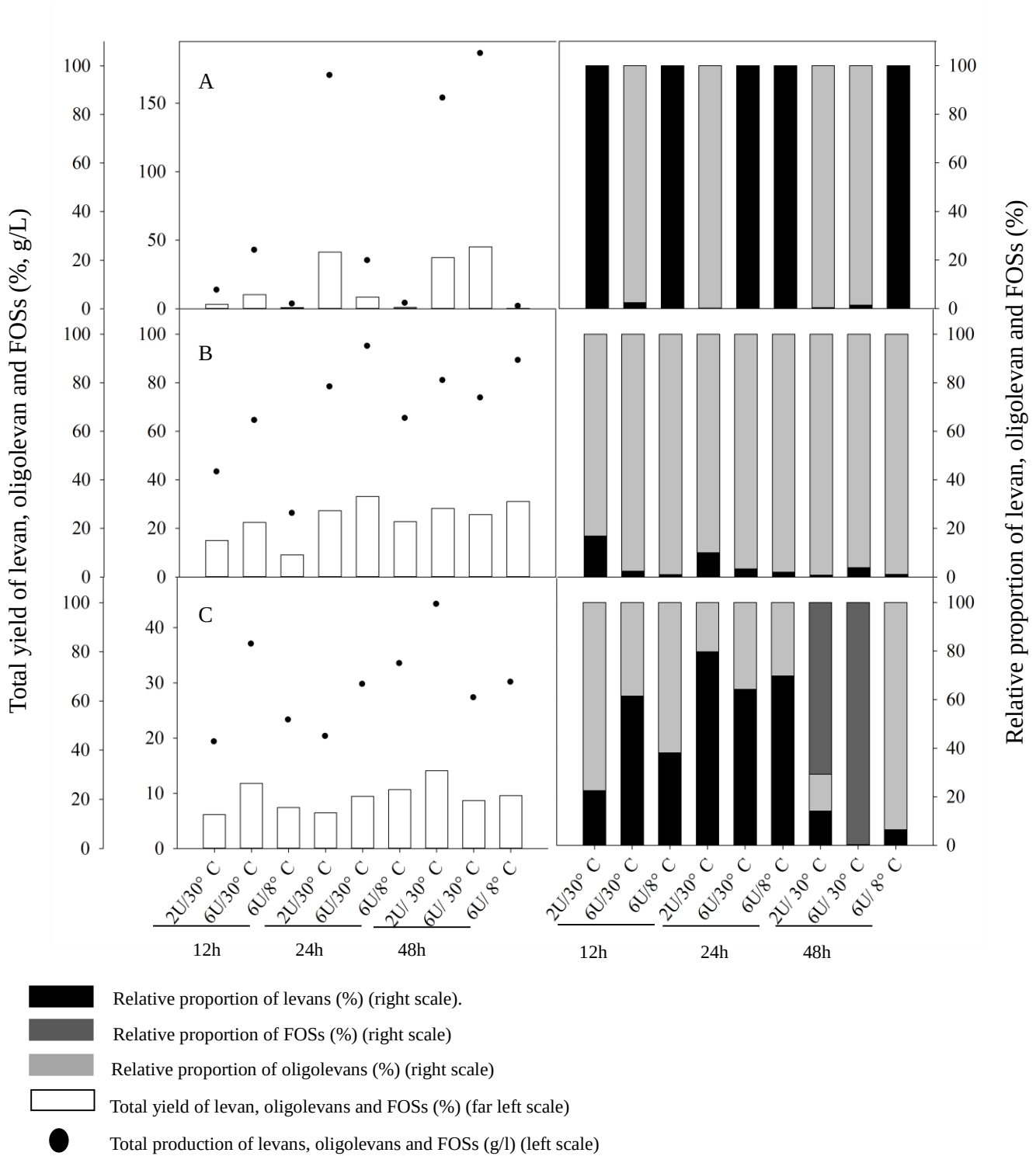


Figure 4.2. End-product profiles of transfructosylation products obtained upon levansucrase-catalyzed transfructosylation of sucrose present in maple syrup 66 °Bx (A), maple syrup 30 °Bx (B) and maple syrup 15 °Bx (C).

On the other hand, the use of low FOSs and oligolevans as fructosyl donor and acceptor through disproportionation type of reaction can explain their accumulation at the late stage of reaction. Ozimek et al. (2006b) have reported that levansucrase from *L. reuteri* 121 was able to catalyze a disproportionation type of reaction converting GF_n substrates into mainly $GF_{(n\pm1)}$, and small amounts of $GF_{(n\pm2)}$.

Using maple syrup 30 °Bx as reaction medium, the highest yield of the transfructosylation products of 33.10% (94.98 g/l) and 31.02% (89.17 g/l) was achieved upon 24 and 48 h of reaction at 30 and 8 °C, respectively, using 6 levansucrase units/ml. Contrary to maple syrup 15 °Bx, the use of lower levansucrase units (2 U/ml) led to a lower maximum yield of the transfructosylation products of 28.20% (80.90 g/ml) in the maple syrup 30 °Bx-based reaction system. At 30 °C, the limited increase of the yield of the transfructosylation products beyond 24 h was less important, which may be due to the enzyme deactivation and/or to the achievement of the reaction equilibrium. The results (Fig. 4.2B) also show that oligolevans were the major products (83-98%), and this end-product profile was not significantly affected by the levansucrase unit (2 to 6U/ml) nor by the reaction temperature (8 to 30 °C).

As compared to maple syrups 15 °Bx and 30 °Bx, the effects of reaction time, temperature and levansucrase unit were more significant in the maple syrup 66 °Bx-based reaction system. At the initial stage of the reaction (12 h), the transfructosylation products yields were only 0.47-5.85% (3.48-42.72 g/l) with the lowest values obtained at low temperature of 8 °C and low levansucrase unit of 2 U/ml. As the reaction was proceeded to 24 h, a significant increase in the yield to 23.33 % (170.26 g/l) was obtained at 30 °C and remained more or less constant thereafter upon 48 h. While no significant increase in the yield was achieved at 8 °C within the investigated reaction time. In addition to the low catalytic activity at low temperature, these results may be attributed to the high viscosity of maple syrup 66 °Bx at 8 °C leading to mass transfer limitations and hence to low substrate binding at the levansucrase active site. Regarding the end-product profile, the most abundant ones were oligolevans at 30 °C and levans at 8 °C. Similarly, with an increase in temperature, the formation of FOSs by levansucrase from *R. aquatilis* was preferred, while at lower temperatures, levan was formed (Kim et al., 1998). Chambert

and Petit-Glatron (1993) found levansucrase from *B. subtilis* had an optimum levan production temperature at 5 ℃; when the temperature was increased to 60 ℃, there was a significant drop in levan production and an increase in the hydrolysis. Jang et al. (2001) have reported that the DP and the yield of levan formed by levansucrase from *Z. mobilis* were lower when the incubation temperature was increased. These results reveal that temperature could be considered as an effective factor for controlling the molecular weight of the synthesized levan and the diversity of transfructosylation products.

A previous study (Tian and Karboune, 2012) in our laboratory has shown that *B. amyloliquefaciens* levansucrase synthesized mainly levan (47%, w/w) and low amount of FOSs (16 mM; 3%, w/w) in the presence of 0.8 M sucrose concentration. The use of maple syrup 30 °Bx as reaction medium with a comparable sucrose concentration (0.88 M) resulted in mainly oligolevans and FOSs with low amounts of levan. In addition to sucrose, maple syrup contains water, minerals (Ball, 2007), amino acids (Morselli and Whalen, 1986), organic acids (Mollica and Morselli, 1984) and phenolic compounds (Kermasha et al., 1995; Underwood et al., 1961). The other components of maple syrup seems to limit the ability of levansucrase to retain the accepting molecule at the subsites +1, +2 and +3, resulting in the release of products with short chain length. A change in the end-product profile was observed upon the use of maple syrups 30 °Bx and 60 °Bx in which more than 98% oligolevans were synthesized. Such results may be due to the presence of higher sucrose concentrations and other components. Abdel-Fattah et al. (2005) have reported that the degree of polymerization of transfructosylation products decreased inversely with sucrose concentration from *B. subtilis* levansucrase. The conversion of sucrose to levan reached its maximum using 5% sucrose in the reaction mixture while the molecular weight of levan decreased with increased sucrose concentration up to 40%. It has been assumed that high sucrose concentration enhances its relative effectiveness as a fructosyl acceptor, thus increasing the synthesis of lower molecular products. Kim et al. (1998) reported that oligosaccharides synthesized by *R. aquatilis* levansucrase at 100, 300, 600 g sucrose/l were 15, 62 and 86%, respectively. At high sucrose concentration (3 M), *Leuconostoc mesenteroides* levansucrase synthesized 59.5% of FOSs and only 2.7% of levan (Seo et al., 2004). Sucrose concentration has been identified as the most effective factor controlling the molecular weight of the synthesized

transfructosylation products, this leads to tailor made levans of different molecular sizes for applications of different purposes (Wu et al., 2013).

4.3. Acceptor Substrate Specificity of Levansucrase from *B. amyloliquefaciens*

4.3.1. Monosaccharide Acceptor Specificity Study

The acceptor specificity of *B. amyloliquefaciens* levansucrase towards monosaccharide acceptors, including galactose and xylose, were investigated in maple syrup 15 °Bx and maple syrup 30 °Bx-based reaction systems in which sucrose was as a fructosyl donor. A fructosyl donor to acceptor molar ratio of 2:1 and a reaction temperature of 30 °C were used. The product spectrum profiles were identified by comparing their retention times with those of external inulin-type FOS standards, including 1-kestose, nystose and 1^F-fructosylnystose, and with the product spectrum of sucrose/monosaccharide acceptor reactions (Tian and Karboune, 2012).

Figure 4.3 shows the time courses for the investigated acceptor reactions over a time course of 48h. These results show that *B. amyloliquefaciens* levansucrase was able to transfer the fructosyl-unit from sucrose of maple syrup 15 °Bx and maple syrup 30 °Bx to both investigated monosaccharide acceptors, but the spectrum profiles and yields of the end-products were acceptor dependent. In addition, for a given acceptor, the product spectrum profiles obtained in maple syrup 15 °Bx and maple syrup 30 °Bx were different. Indeed, the results (Figs 4.3A, B) indicate that in the presence of galactose in maple syrup 15 °Bx-based reaction system, the total transfructosylation product yield increased linearly during the investigated reaction time course, reaching a maximum yield of 29.65% (11.48g/l) upon 48 h of reaction. Maple syrup 30 °Bx led to different galactose acceptor reaction time course, in which the yield of total transfructosylation products increased with a high extent within the initial 4 h of the reaction to reach a yield of 13.54%, but decreased to 8.82% at 12h before reaching the maximum yield of 18.51% (14.34g/l) at 48 h. The galactose-acceptor reactions in maple syrups 15 °Bx and 30 °Bx resulted in the same transfructosylation products including D-Gal-2Fru, D-Gal-3Fru, D-Gal-4Fru and nystose; however; their concentrations were dependent on the type of maple syrups.

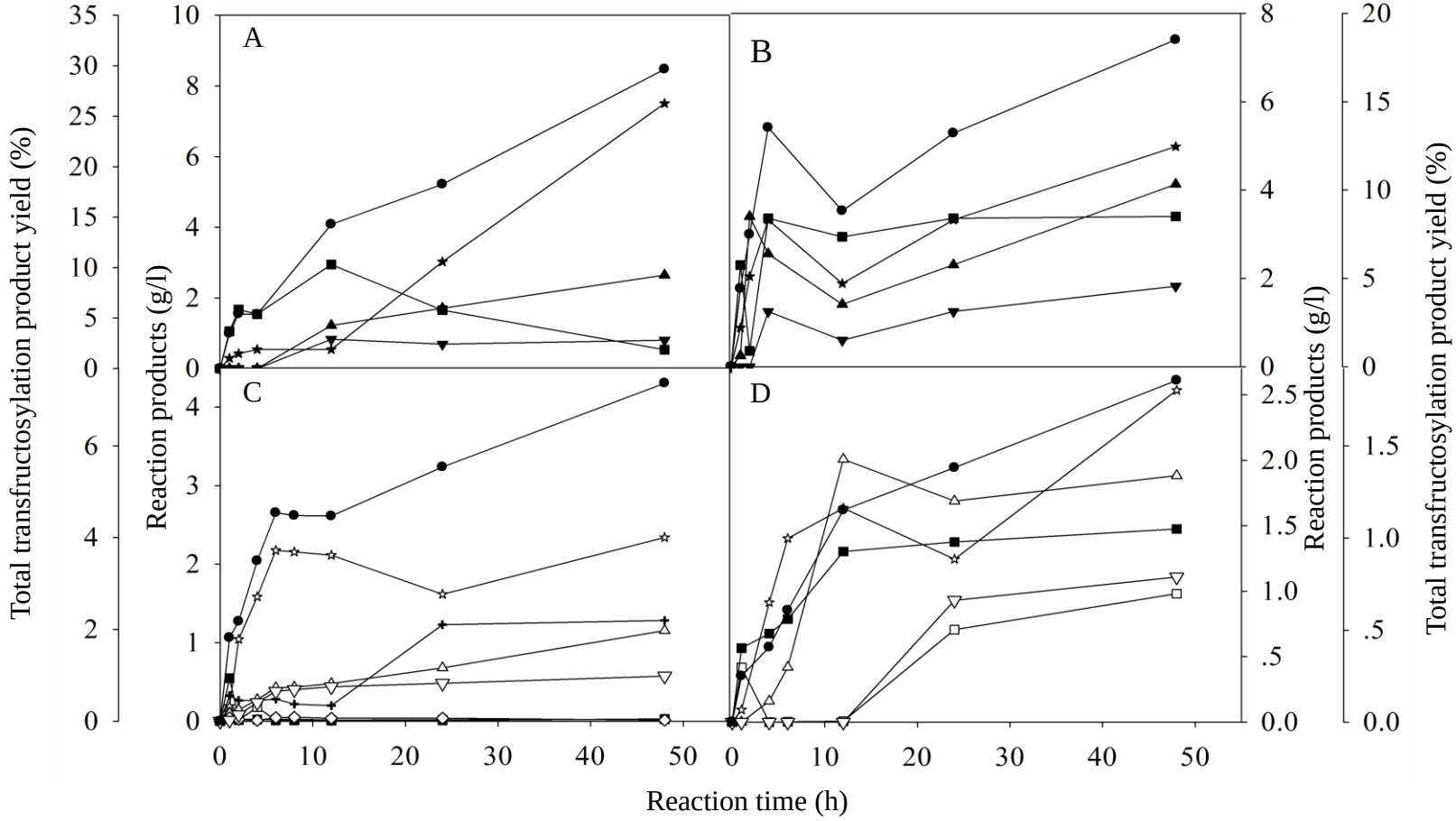


Figure 4.3. Reaction time courses for *B. amyloliquefaciens* levansucrase–catalyzed transfructosylation acceptor reactions using galactose (A : maple syrup 15 ℃Bx, B : maple syrup 30 ℃Bx), and xylose as a fructosyl acceptor (C: maple syrup 15 ℃Bx, D: maple syrup 30 ℃Bx): ★galactose-2fructose; ■ nystose; ▲ galactose-3fructose; ▼galactose-4fructose; ● total transfructosylation product yield (far left scale and far right scale); ┼ 1-kestose; □ xylose-fructose,☆ xylose-2fructose; ∆ xylose-3fructose;◇ 1^F-fructosylnystose;▽xylose-4fructose.

Indeed, the main product of galactose-acceptor reaction in maple syrup 15 °Bx was D-Gal-2Fru (α-D-galactopyranosyl-(1→2)-O-β-D-fructofuranosyl-(1→2)-O-β-D-fructofura

noside), which increased significantly within the time course of reaction to reach 7.50 g/l at 48 h, whereas in maple syrup 30 °Bx, D-Gal-2Fru (4.98 g/l), D-Gal-3Fru (4.13 g/l) and nystose (3.40 g/l) were the main products over the 48 h-time course.

The maximum concentration of D-Gal-4Fru was 2.3 times lower in maple syrup 15 °Bx (0.80 g/l) than maple syrup 30 °Bx (1.82 g/l). These experimental findings confirm the use of D-Gal-nFru sucrose analogues by *B. amyloliquefaciens* levansucrase as fructosyl acceptor substrates through disproportionation type of reaction. The brix of maple syrup, its viscosity and its enrichment with other components (phenols, flavonoids) seem to affect the length of transfructosylation products and the binding of D-Gal-nFru in the subsites. In particular, the other components of maple syrup may have decrease the affinity of levansucrase to retain the accepting molecule at the subsites +1, +2 and +3, inhibiting mainly the transfructosylation process and resulting in the release of products with short chain length. No detection of D-Gal-Fru may be due to their use as fructosyl donor substrates. It has been reported that both sucrose and sucrose analogues (D-Gal-Fru) are competing donor substrates because they contain highly energetic α-glycosidic bonds (van Hijum et al., 2006). Beine et al. (2008) have reported that fructan synthesis with D-Gal-Fru by levansucrase from *B. subtilis* followed the same manner like sucrose.

The results (Figs 4.3 C, D) also indicate that *B. amyloliquefaciens* levansucrase was able to use xylose as acceptor substrate. Xylose was not a good acceptor substrate as galactose for *B. amyloliquefaciens* levansucrase in maple syrup-based reaction systems. In both maple syrups, the conversion rate of xylose-acceptor reactions was higher within the first 6 h-reaction period and decrease thereafter to lead to a maximum yield of 7.36% (5.42g/l) and 1.86% (7.98g/l) in the maple syrup 15 °Bx and 30 °Bx-based reaction systems, respectively. Similarly to galactose-acceptor reactions, lower yield and higher proportion of transfructosylation products with high chain length were obtained in the maple syrup 30 °Bx-based reaction system. In addition to kestose, nystose and 1^F-fructosylnystose, D-Xyl-Fru, D-Xyl-2Fru, D-Xyl-3Fru and D-Xyl-4Fru were formed. The most abundant transfructosylation product was identified to be D-Xyl-2Fru (α-D-xylopyranosyl-(1→2)-β-

D-fructofuranosyl-(1→2)-O-β-D-fructofuranoside) (2.34-2.53 g/l) in both systems. The concentration of D-Xyl-2Fru increased more rapidly at the initial stage of the reaction (0h-6h) to 2.17g/l in the 15 °Bx-based reaction system than in the 30 °Bx-based one (1.4 g/l). These results may be attributed to the high viscosity of maple syrup 30 °Bx leading to mass transfer limitations and hence to low substrate binding at the levansucrase active site. Upon slight decrease of D-Xyl-2Fru concentration at the intermediate stage (12-24 h), it thereafter increased with higher rate in the 30 °Bx-based system. The results also show that in the 30 °Bx-based system, the decrease in D-Xyl-2Fru and D-Xyl-3Fru concentrations at the intermediate stage correlated with the increase of D-Xyl-4Fru to 1.1 g/l. However, in the 15 °Bx-based system, the decrease in D-Xyl-2Fru was accompanied with an increase in D-Xyl-3Fru concentration to 1.15 g/l. The maximum concentration of D-Xyl-4Fru was 2.0 times lower in maple syrup 15 °Bx-based reaction system than maple syrup 30 °Bx. In addition, kestose (1.28 g/l) was only produced in maple syrup 15 °Bx-based reaction system in the presence of xylose, whereas nystose (1.47 g/l) was mainly produced in maple syrup 30 °Bx-based reaction system. Such results confirm the effect of the type of syrup and its components on the binding affinity of growing chain acceptors in the subsite +1 and +2. The product profiles (Figs 4.3 C, D) also indicate that xylose-fructose was accumulated in detectable amounts, which was not observed when galactose was used as an acceptor. This might be explained by the fact that D-Xyl-Fru was used as donor/acceptor with low affinity. Indeed, xylose has no 6-hydroxyl group and may show less affinity to Asn242 at the subsite+2 as compared to D-Gal-Fru (Beine et al., 2008; Meng and Futterer, 2008).

Previously, Tian and Karboune (2012) have reported that the use of galactose and xylose as acceptors by *B. amyloliquefaciens* levansucrase led to the formation of D-Gal-Fru and D-Xyl-Fru with a maximum yield of 46 and 55%, respectively. Similarly, *B. subtilis* levansucrase yielded 61% D-Gal-Fru and 56% D-Xyl-Fru in the acceptor reactions (Seibel et al., 2006). However, the experimental results showed that in maple syrup-based reaction systems, the product yields were much lower in the presence of xylose and galactose. These results may be attributed to the limited access of substrates to the active site the enzyme due to the presence of other components in maple syrups. In addition, with xylose as acceptor, lower product yields were obtained as compared with galactose.

It has been reported that saccharides having a pyranose ring in their chemical structure, such as galactose were better acceptors for *M. laevaniformans* levansucrase than the saccharides having a furanose ring, such as xylose (Park et al., 2003).

4.3.2. Disaccharide Acceptor Specificity Study

To investigate the disaccharide acceptor specificity of *B. amyloliquefaciens* levansucrase in maple syrup-based reaction systems, maple syrups 15 °Bx and 30 °Bx were enriched with lactose, cellobiose and melibiose as acceptors and used as reaction media and sucrose source. A fructosyl donor to acceptor molar ratio of 2:1 and a reaction temperature of 30 °C were used. The results (Figs 4.4-4.6) show that *B. amyloliquefaciens* levansucrase was able to transfer the fructosyl-unit from sucrose to all the investigated disaccharide acceptors with high yield of conversion. These results demonstrate the broad acceptor specificity of *B. amyloliquefaciens* levansucrase, with lactose as the most preferred fructosyl acceptor.

The enrichment of maple syrups 15 °Bx and 30 °Bx with lactose led to the highest total transfructosylation product yields of 79.25 (61.39g/l) and 91.71% (142.09 g/l), respectively (Fig 4.4), the major transfructosylation product was identified to be lactose-fructose(O-β-D-galactopyranosyl-(1→4)-α-D-glucopyranosyl-(1→2)-β-D-fructo-furanoside) produced with a maximum yield of 76.90 % (60.73g/l) and 87.97% (136.30g/l), respectively (Table 4.2), upon 24 h reaction time. The time course profile for the formation of lactose-fructose was dependent on the type of maple syrups, the lactose-fructose trisaccharide decreased significantly to reach 37.13 g/l (47.94%) at 12 h after a significant increase during the first initial 4h-period of time course; this decrease was followed by an increase up to 60.73 g/l (76.90%) at 24 h. This time course profile of lactose-fructose production in the maple syrup 15 °Bx-based reaction system may reveal two phenomena: (a) lactose-fructose may have been used as acceptor to form the growing chain of levan. Indeed, as indicated in Figure 4.4, levan yield was only 5.42% (4.20g/l) at 12h and it increased significantly to 55.31% (42.85g/l) at 48 h in the maple syrup 15 °Bx-based reaction system; (b) the shift of lactose-acceptor reaction equilibrium may be highly dependent on the availabilities of the transfructosylation products in the maple syrup 15 °Bx-based system.

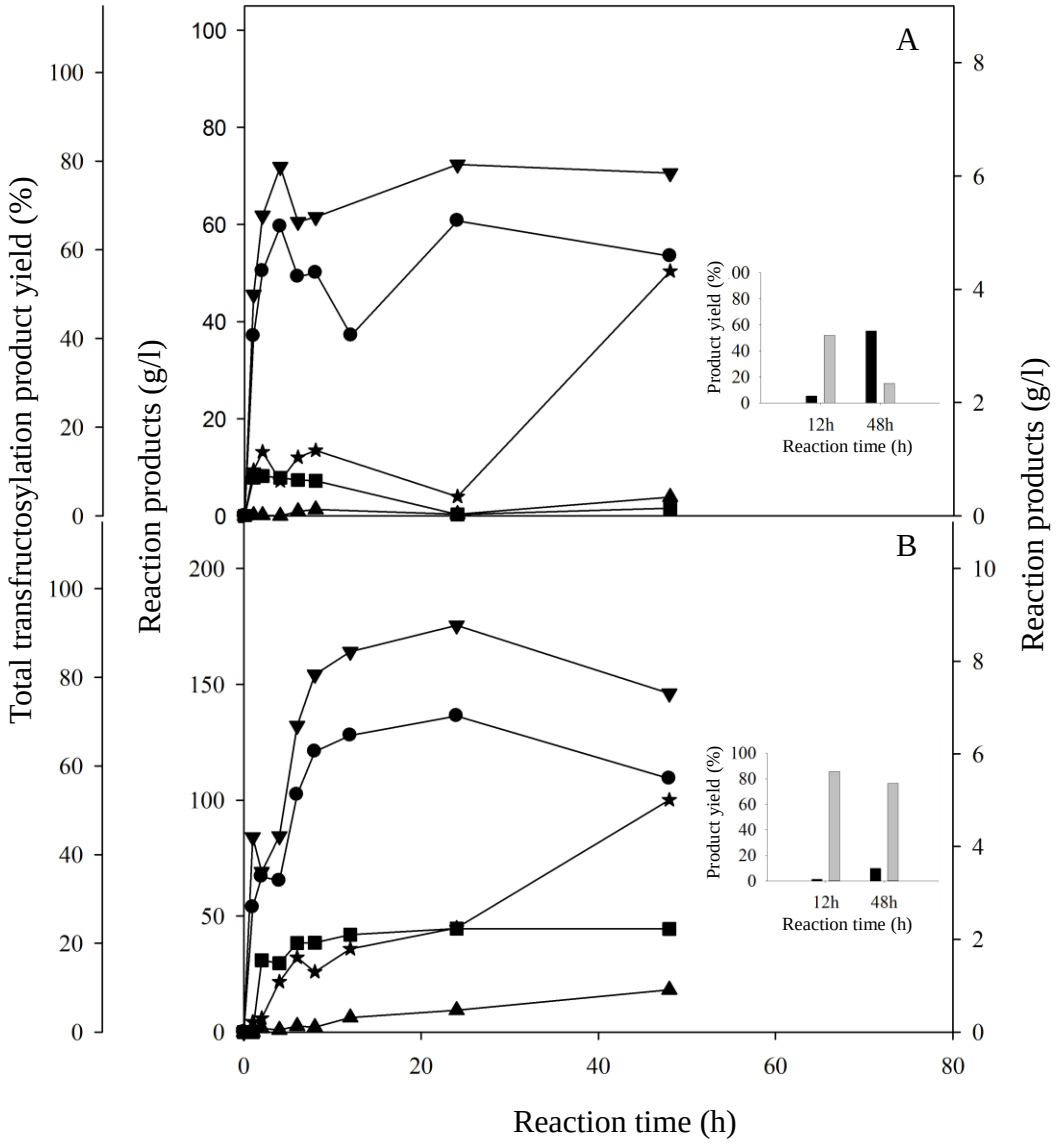
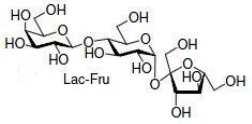
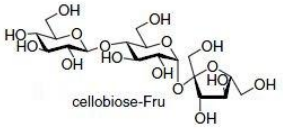
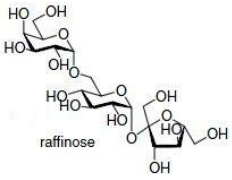


Figure 4.4. Reaction time courses for *B. amyloliquefaciens* levansucrase–catalyzed transfructosylation acceptor reaction using lactose as afructosyl acceptor (A: maple syrup 15 ℃Bx, B: maple syrup 30 ℃Bx): ● lactose-fructose (left scale); ★nystose (right scale); ■ lactosucrose/lactose-2fructose (right scale); ▲1^F-fructosylnystose (right scale); ▼total transfructosylation products (far left scale); ▬ levan; ▬ FOS.

Table 4.2. Acceptor specificity of levansucrase from *B. amyloliquefaciens*-catalyzed transfructosylation reactions in selected maple syrups-based reaction systems.

Acceptor	Product	Yield (%)[a]	
		maple syrup 15 °Bx	maple syrup 30 °Bx
Lactose	 Lac-Fru	76.90 (60.73)[b, e]	87.97 (136.30)[b]
Cellobiose	 cellobiose-Fru	62.39 (48.33)[c]	51.91(80.42)[d]
Melibiose	 raffinose	48.86 (37.85) [c]	36.25(56.17) [d]

[a] The yield was determined as the concentration of the product over that of the acceptor, multiplied by 100; [b] The maximum yield was achieved at 24h; [c] The maximum yield was achieved at 6h; [d] The maximum yield was achieved at 12h; [e]concentration of the products in g/l.

Indeed, a shift of reaction equilibrium towards the hydrolysis of lactose-fructose may have occurred within the investigated reaction time course. The results also show that in the maple syrup 15 °Bx-based reaction system, the time course profile for the lactosucrose/lactose-2fructose production was more or less similar to that of lactose-fructose; however, its concentration was much lower with a maximum value of 0.7 g/l obtained upon 2 h reaction.

In contrast, higher concentration of lactosucrose/lactose-2fructose 2.23 g/l was accumulated in the maple syrup 30 °Bx-based reaction system upon 48 h. Lactose-fructose concentration being the major transfructosylation product in the maple syrup 30 °Bx-based reaction system increased with a high extent at the initial stage of the reaction (up to 6h) and with very lower rate thereafter to reach a maximum concentration of 136.30 g/l (87.97%) at 24 h before slightly decreasing to 109.34g/l (70.57%) at 48h. The high accumulation of the lactose-fructose without significant decrease and the low accumulation of levan in the maple syrup 30 °Bx-based reaction system (9.89 % at 48 h) reveal that (a) the formation of lactose-fructose was more favored in the presence of high sucrose concentration and (b) the binding affinity of lactose-fructose as an acceptor was low in a highly-rich maple syrup 30 °Bx with other components. Minor FOSs such as, nystose and fructosyl-nystose, together with some other unidentified tetra- and pentasaccharides were also observed over the reaction time course at very low extents.

It has also been reported that *B. amyloliquefaciens* levansucrase in the lactose-acceptor reaction synthesized lactose-fructose as the major product with a maximum yield of 60% (Tian and Karboune (2012). In addition, the authors also reported that levan was produced in the lactose acceptor reaction. Similarly, *B. subtilis* levansucrase showed a high acceptor specificity toward lactose and yielded lactose-fructose (34%) (Seibel et al., 2006). However, Park et al. (2005) have identified the main product of the levansucrase from *B. subtilis* using lactose as the acceptor to be lactosucrose with high amount (181.0 g/l).

With cellobiose as acceptor, the overall yield of the transfructosylation products increased significantly during the first 6 h of reaction, in the maple syrup 15 °Bx-based reaction system, to reach a maximum value of 63.66% (49.31 g/l) (Fig 4.5); beyond this

reaction time, it decreased to reach 15.73 % (12.18g/l) upon 48h of reaction. On the other hand, the time course of cellobiose-acceptor reaction in the maple syrup 30 °Bx-based system showed no significant conversion during the first 4 h and increased significantly thereafter to achieve a maximum value of 55.26 % (85.62 g/l) at 12 h. This low initial conversion rate may be attributed to the limited solubility and availability of cellobiose in maple syrup 30 °Bx; as the reaction was proceeded, the high viscosity and the polarity of the reaction medium may have changed. Contrary to maple syrup 15 °Bx-based reaction system, a lower decrease in the yield (44.70%; 69.26g/l; 48 h) was observed upon reaching the maximum value in the maple syrup 30 °Bx. In both systems, the major transfructosylation product was identified to be cellobiose-fructose (β-D-glucopyranosyl-(1→4)-α-D-glucopyranosyl-(1→2)-β-D-fructofuranoside), with a maximum yield of 62.39% (48.33g/l) and 51.91% (80.42g/l) in maple syrup 15 °Bx and 30 °Bx-based systems, respectively (Table 4.2). The results also show that the decrease in the concentration of the cellobiose-fructose was in parallel correlated with an increase in the concentration of cellobiose-sucrose that achieved a maximum concentration of 3.05% (2.36g/l) and 2.13% (3.31g/l) at 12 and 48 h, respectively, in maple syrup 15 °Bx and 30 °Bx-based systems. An increase in the levan yield as the reaction proceeded from 12 h to 48 h, was also obtained reaching a yield of 12.42% (9.62 g/l) and 7.39% (11.45 g/l) at 48 h in maple syrup 15 °Bx and 30 °Bx-based systems, respectively. These results may reveal the use of cellobiose-fructose as acceptor and explained partially the decrease of its concentration at the late stage of reaction. The shift of reaction equilibrium towards the hydrolysis may also explain the decrease of cellobiose-fructose concentration. Minor unidentified FOSs such as pentasaccharides were also observed in the reaction mixture. However, the concentration of the pentasaccharides was higher in maple syrup 30 °Bx-based system as compared to those in maple syrup 15 °Bx-based system. Seibel et al. (2006) reported that the β-D-glucopyranoside moiety at C-4'in cellobiose has lowered its affinity towards the active site of *B. subtilis* levansucrase, leading to the formation of cellobiose-fructose in yield of only 30%. Similarly, Kim et al. (2001) reported that levansucrase from *Bacillus* sp. produced acceptor products ranging from DP2 to DP7 with a maximum yield of 30% when cellobiose was utilized as acceptor.

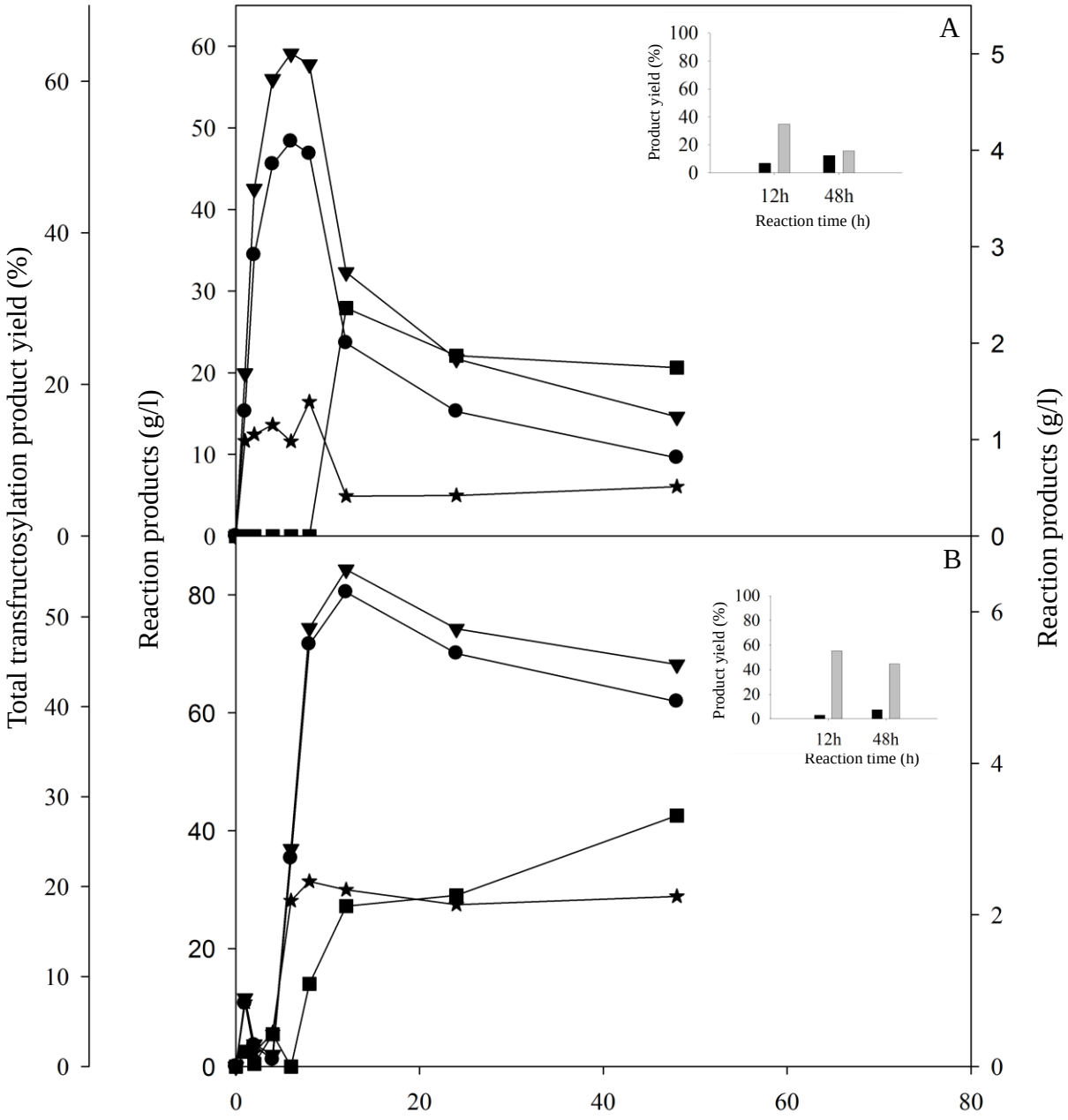


Figure 4.5. Reaction time courses for *B. amyloliquefaciens* levansucrase–catalyzed transfructosylation acceptor reactions using cellobiose as a fructosyl acceptor (A: maple syrup 15 ℃Bx, B: maple syrup 30 ℃Bx). ● cellobiose-fructose (left scale); ★ nystose(right scale); ■ cellobiose-sucrose (right scale); ▼ total transfructosylation products (far left scale); ▬ levan ; ▬ FOS.

The results show that in the presence of melibiose, the overall yields of the transfructosylation products increased significantly during the initial stage of the reaction, to reach a maximum value of 55.40% (42.91g/l) and 41.95% (64.95g/l) upon 6h and 12 h of reaction in the maple syrup 15 °Bx- (Fig. 4.6A) and the maple syrup 30 °Bx- based reaction systems (Fig. 4.6B), respectively. The major transfructosylation product was identified to be raffinose produced with a maximum yield of 48.86% (37.85 g/l) and 36.25% (56.17 g/l) upon 6 h and 12 h reaction time in maple syrup 15 °Bx- and maple syrup 30 °Bx based reaction systems, respectively; a further increase in reaction time to 48h resulted in a decrease in raffinose yield to 10.80% (8.37g/l) and 24.60% (38.11g/l), respectively. The decrease in raffinose concentration could be due to the hydrolysis of raffinose to melibiose or the use of raffinose as acceptor to form longer-chain products. In fact, the results show that in both maple syrup 15 °Bx- and maple syrup 30 °Bx-based systems, the decrease in raffinose concentrations at the late stage correlated with an increase of melibiose-3fructose concentration to 1.99 and 1.49 g/l, respectively. In addition, the formation of levan increased from 12h to 48h, reaching a yield of 28.94% (22.41g/l) and 10.81% (16.74g/l) in maple syrup 15 °Bx and maple syrup 30 °Bx, respectively. Indeed, it has been reported that levansucrases from *B. amyloliquefaciens* and *Z. mobilis* have affinity towards raffinose as fructosyl donor leading to the formation of fructan polymer (Andersone et al., 2004; Tian and Karboune, 2012). In addition, in the presence of melibiose as acceptors, 1^{F}-fructosylnystose (0.26 g/l) was mainly produced in maple syrup 15 °Bx-based reaction system, whereas nystose (2.30 g/l) was mainly produced in maple syrup 30 °Bx-based reaction system. Contrary to *B. amyloliquefaciens* levansucrase, levansucrase from *B. subtilis* showed a moderate acceptor affinity towards melibiose and yielded raffinose (18.2%) (Seibel et al., 2006). The experimental findings also show that in maple syrup 30 °Bx-based reaction system, compared with lactose and cellobiose, the maximum total transfructosylation product yields were lower in the presence of melibiose. These results could be attributed to the fact that the acceptor melibiose whose α (1→6) linkage between glucose and galactose could come into conflicts with binding site of *B. amyloliquefaciens* levansucrase and interfere its uses as an acceptor (Tian and Karboune, 2012).

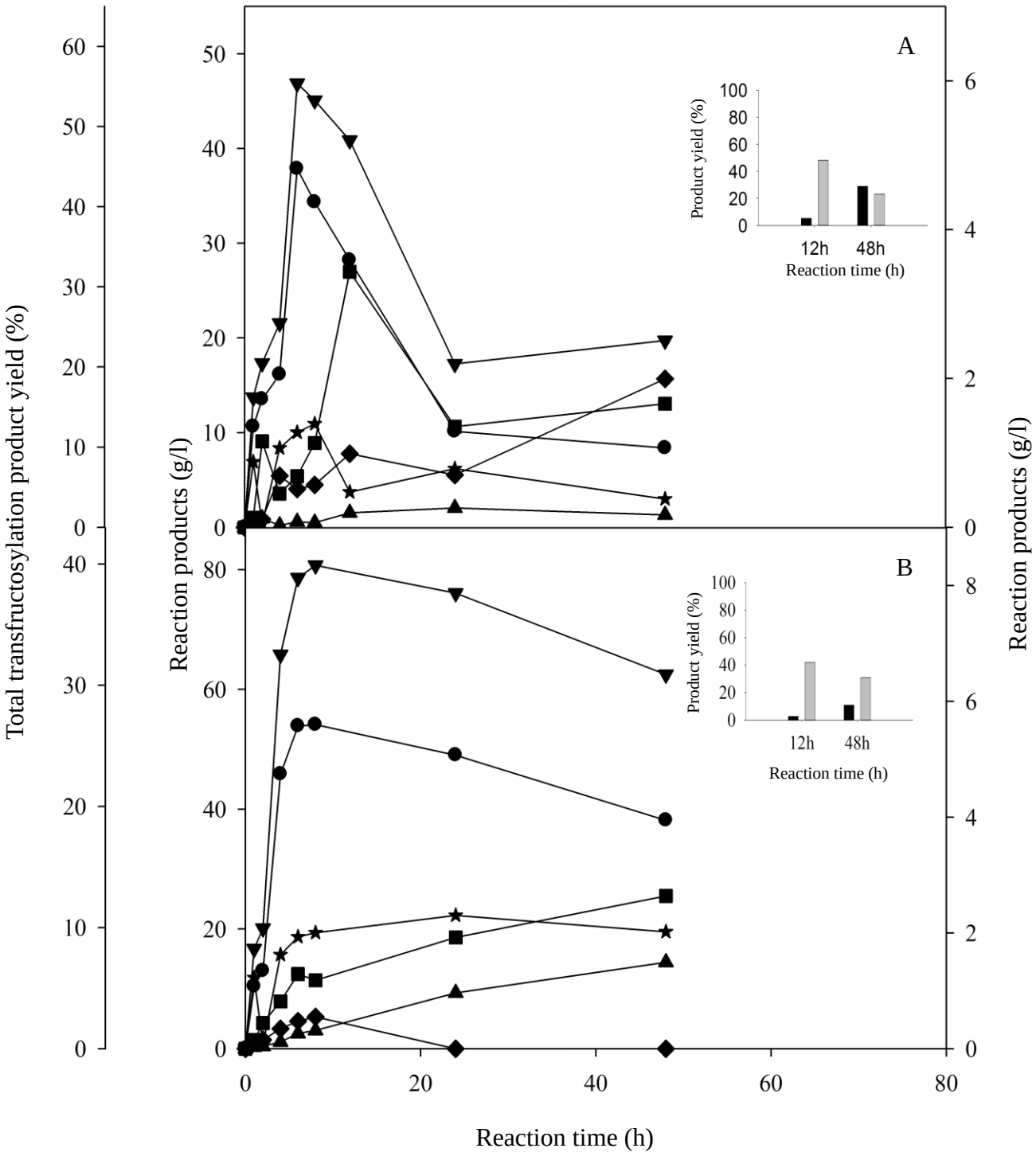


Figure 4.6. Reaction time courses for *B. amyloliquefaciens* levansucrase–catalyzed transfructosylation acceptor reactions using melibiose as a fructosyl acceptor (A: maple syrup 15 ºBx, B: maple syrup 30 ºBx): ● raffinose (left scale);★nystose (right scale); ■ melibiose-2fructose (right scale); ◆ 1^F-fructosylnystose (right scale); ▲ melibiose-3fructose (right scale);▼total transfructosylation products (far left scale); ▬ levan; ▬ FOS.

4.4. Optimization of Levansucrase-Catalyzed Reaction for the Production of Fructooligosaccharides, Oligolevans and Levans in Selected Maple Syrups

4.4.1. Model Fitting and Statistical Analysis

Maple syrups 30 °Bx and 66 °Bx were identified as the best reaction media for the synthesis of oligolevans/FOSs and oligolevans/levans, respectively. In order to better understand the effects of reaction parameters on oligolevans/FOSs and oligolevans/levans production, and to optimize theses parameters, RSM has been used. CCRD was selected for the optimization of the production of these bioactive molecules using five-level and three factors, including levansucrase unit (X_1, 2-6 U/ml), reaction time (X_2, 6-30 h) as well as pH (X_3, 5.7-7). The design consisted of 8 factorial points, 6 axial points (2 axial points on the axis of each design variable at a distance of 1.68 from each design center), and 6 center points, leading to 20 runs

Table 4.3 shows the experimental conditions and their corresponding quantified responses of oligolevans/FOSs and oligolevans/levans yields (%, w/w; g/l). Among the various treatments of maple syrup 30 °Bx, the highest bioconversion yield of oligolevans/FOSs (40.35-48.62%; 115.75-139.47 g/l) was obtained at Runs (#6, 10, 14, 15, 17) corresponding to the center point, where 3.5 U/ml levansucrase unit, pH 6.6 and 21 h of reaction were used. At the same conditions (3.5 U/ml, 21 h), changing the pH values to 5.7 (Run#2) or 7.4 (Run#13) resulted in a significant decrease in the yield of oligolevans/FOSs to 14.79% (42.42 g/l) and 12.79% (36.68 g/l), respectively. Tian et al. (2011) have reported that levansucrase from *B. amyloliquefaciens* exhibited the maximum activity in the pH range of 6.0-6.5 and retained only 62% of its maximum activity in the pH range of 5.0–7.5. The lowest yields of oligolevans/FOSs (7.4-9.7 %; 21.26-27.91 g/l) produced in maple syrup 30 °Bx were obtained upon the use of low enzymatic units of 2 U/ml levansucrase unit and pH 6.0 (Run #12 and run #8). The results also show that using maple syrup 66 °Bx, the highest yield of oligolevans/levans (26.81%; 195.56 g/l) was achieved after 36 h of reaction using 3.5 U/ml levansucrase unit and pH 6.6 (Run #16); the use of shorter reaction time of 21 h (Runs#6, 10, 14, 15, 17) resulted in lower yields (2.43-7.48%; 17.72-54.52 g/l).

Table 4.3. Central composite rotatable design arrangement of the actual and coded independent variables and the estimated responses.

Run	Independent variables			Responses	
				Maple syrup 30 °Bx	Maple syrup 66 °Bx
	X_1[a]	X_2[b]	X_3[c]	Oligolevans/FOSs[d]	Oligolevans/levans[e]
1	2.00 (-1)	29.92 (1)	7.00(1)	10.63(30.49)[f]	5.09(37.15)[f]
2	3.50 (0)	21.00 (0)	5.70(-1.68)	14.79(42.42)	5.17(37.69)
3	5.00 (1)	29.92 (1)	6.00 (-1)	16.35(46.90)	18.71(136.47)
4	5.00 (1)	12.08 (-1)	6.00 (-1)	15.31(43.93)	1.71(12.47)
5	0.98 (-1.68)	21.00 (0)	6.60 (0)	27.78(79.69)	2.89(21.06)
6	3.50 (0)	21.00 (0)	6.60 (0)	48.62(139.47)	4.83(35.21)
7	3.50 (0)	21.00 (0)	6.60 (0)	34.96(100.29)	6.61(48.24)
8	2.00 (-1)	29.92 (1)	6.00 (-1)	9.73(27.91)	2.77(20.18)
9	3.50 (0)	6.00 (-1.68)	6.60 (0)	10.48(30.07)	9.03(68.88)
10	3.50 (0)	21.00 (0)	6.60 (0)	46.33(132.90)	4.24(30.92)
11	5.00 (1)	12.08 (-1)	7.00 (1)	13.25(38.00)	7.86(57.36)
12	2.00 (-1)	12.08 (-1)	6.00 (-1)	7.41(21.26)	4.80(35.00)
13	3.50 (0)	21.00 (0)	7.40 (1.68)	12.79(36.68)	4.69(34.23)
14	3.50 (0)	21.00 (0)	6.60 (0)	46.38(133.07)	7.48(54.52)
15	3.50 (0)	21.00 (0)	6.60 (0)	41.66(119.51)	2.43(17.72)
16	3.50 (0)	36.00 (1.68)	6.60 (0)	26.99(77.45)	26.81(195.56)
17	3.50 (0)	21.00 (0)	6.60 (0)	40.35(115.75)	6.39(46.61)
18	6.02 (1.68)	21.00 (0)	6.60 (0)	17.43(49.99)	4.82(35.15)
19	5.00 (1)	29.92 (1)	7.00 (1)	34.96(100.29)	19.00(138.58)
20	2.00 (-1)	12.08 (-1)	7.00 (1)	14.13(40.55)	12.79(93.30)

[a] Levansucrase unit (U/ml); [b] Reaction time (h); [c] pH; [d] oligolevans/FOSs yield (%, w/w) with maple syrup 30 °Bx; [e] oligolevans/levans yield (%, w/w) with maple syrup 66 °Bx; [f] Product concentration in g/l.

The lowest bioconversion yield of levans (1.71%; 12.47g/l) produced in maple syrup 66 °Bx was obtained upon the use of pH 6.0 and shorter reaction time of 12.08 h (Run#4). These results reveal the significance of the reaction time for the enzymatic synthesis of oligolevans/levans in maple syrup 66 °Bx. Although the maximum yield of the oligolevans/FOSs (~ 44.39%) generated in maple syrup 30 °Bx is higher than that (26.81%) of oligolevans/levans produced in maple syrup 66 °Bx, the absolute amount of these compounds is more or less 2 times higher in maple syrup 66 °Bx.

Multiple regression analysis was used to determine the best-fitting model using the software Design-Expert version 8.0.6. Box-Cox plot was used to determine if a power transformation is needed to normalize the response data and to find models. The Lambda value of 1 indicates that no transformation of the data into a normal distribution is required. The best-fitting models were evaluated by the determination of *p*-value, lack of fit, adjusted R-Squared and R-Squared (R^2) values. The analyses of variance (ANOVA) are summarized in Table 4.4.

The results show that the quadratic model was statistically the most suitable for the description of oligolevans/FOSs and oligolevans/levans productions in maple syrup 30 °Bx and 60 °Bx, respectively with *P* value of 0.0018 and 0.0005, and *F* value of 7.76 and 10.38, respectively. Indeed, the higher is the *F* value, the better predictor the model is. The R^2 of the fitted models were 0.8747 and 0.9033 for oligolevans/FOSs and oligolevans/levans production models; these R^2 values indicate that the models can explain the relationship between the responses and the variables significantly. In addition, the "lack of fit" is also a measure of the goodness of fit of the model. If the "lack of fit" is significant, then a more complicated model is needed to fit the experimental data. Here, the non-significant "lack of fit" with *P* value of 0.1402 and 0.0945 for oligolevans/FOSs and oligolevans/levans production models, respectively, indicate that the experimental data fitted well in the quadratic polynomial model. An adequate precision of 6.711 and 11.751 were obtained, which is greater than 4. The overall results show that the models chosen can satisfactorily explain the effects of enzyme amount, reaction time and pH on FOSs/oligolevans/levans production by *B. amyloliquefaciens* levansucrase using maple syrup 30 °Bx and 66 °Bx as reaction media and sucrose substrate source.

Table 4.4. Analyses of variance for response surface quadratic models for the productions of oligolevan/FOSs and oligolevans/levans in the maple syrups 30 °Bx- and 66 °Bx-reaction systems, respectively

	Maple syrup 30 °Bx			Maple syrup 66 °Bx		
	Oligolevans/FOSs yields (%, w/w)			Oligolevans/levans yields (%, w/w)		
Source	Sum of squares	F value	p-value	Sum of squares	F value	p-value
Model	3397.1	7.76	0.0018	735.58	10.38	0.0005
X_1[a]	30.93	0.64	0.4438	46.07	5.85	0.0361
X_2[b]	178.22	3.66	0.0846	170.86	21.71	0.0009
X_3[c]	31.67	0.65	0.4385	18.67	2.37	0.1546
X_1X_2	71.59	1.47	0.253	179.23	22.77	0.0008
X_1X_3	9.95	0.2	0.6607	1.88	0.24	0.6358
X_2X_3	27.57	0.57	0.4689	16.62	2.11	0.1769
X_1^2	827.64	17.01	0.0021	4.01	0.51	0.4918
X_2^2	1152.78	23.7	0.0007	284.91	36.19	0.0001
X_3^2	1648.59	33.89	0.0002	0.31	0.039	0.8467
Lack of Fit	358.97	2.82	0.1402	61.49	3.57	0.0945

[a] Levansucrase unit (U/ml); [b] Reaction time (h); [c] pH

Similarly, Lim et al. (2005) have described the production of FOSs by *Penicillium citrinum* in a quadratic model. Tian and Karboune (2012) have reported that oligolevans production in a levansucrase/endo-inulinase system can be described by a quadratic model. As for the levan production, Silbir et al. (2014) evaluated and optimized levan production by *Z. mobilis* B-14023 using quadratic model. Zhao et al. (2013) have also reported that the production of extracellular polysaccharide by *P. fluorescens* PGM37 was most suitably described with a quadratic model.

According to the established quadratic model of maple syrup 30 °Bx, the linear terms of levansucrase unit (X_1, *F* value of 0.64, *P* value of 0.4438), and pH (X_3, *F* value of 0.65, *P* value of 0.4385) had no significant effect on oligolevans/FOSs synthesis; while the reaction time (X_2, *F* value of 3.66, *P* value of 0.0846) had the most significant effect. Reaction time has also been identified as a key factor affecting FOSs production by an extracellular fructosyltransferase from *Rhodotorula* sp.(Hernalsteens and Maugeri, 2010). In the oligolevans/levans production predictive model of maple syrup 66 °Bx, the linear terms with the largest effects on oligolevans/levan synthesis were levansucrase unit (X_1, *F* value of 5.85, *P* value of 0.0361) and reaction time (X_2, *F* value of 21.71, *P* value of 0.0009). Similarly, reaction time was also identified as a key factor affecting levan production by *B. subtilis* (Dos Santos et al., 2013). In addition, the positive coefficient for this linear term (X_2) indicates a commitment increase in the oligolevans/levans production as the reaction time was extended. The results (Table 4.4) also show that the quadratic terms of the three investigated factors exhibited significant effects on oligolevans/FOSs production in maple syrup 30 °Bx. In contrast, only the quadratic term of reaction time (X_2, *F* value of 36.19, *P* value of 0.0001) was significant in the oligolevans/levans production predictive model of maple syrup 66 °Bx. However, no significant interactive effects between the variables were detected in the oligolevans/FOSs production predictive model of maple syrup 30 °Bx. In oligolevans/levans production predictive model, the interactive effect between levansucrase unit and reaction time (X_1X_2, *F* value of 22.77, *P* value 0.0008) was highly significant. In addition, the positive sign of this interaction term (X_1X_2) indicates the positive synergistic effects of the variables. Neglecting the insignificant terms and considering all the significant terms, oligolevans/FOSs production and oligolevans/levans

production can be described by the following predictive equations obtained in terms of coded variables:

$$Y_1=+38.01-6.52X_1^2-7.32X_2^2-8.76X_3^2 \quad (2)$$

$$Y_2=+5.33+1.84\ X_1+3.54X_2+4.73\ X_1X_2+4.45X_2^2 \quad (3)$$

Where X_1 is the coded value of enzyme amount, X_2 is the coded value of reaction time, X_3 is the coded value of pH, Y_1 is the Oligolevans/FOSs yield (%) and Y_2 is the levan yield (%).

4.4.2. Effect of Reaction Parameters and Optimum Conditions

The relationships between reaction parameters and yields of oligolevans/FOSs and oligolevans/levans can be better understood by studying the planned series of two dimensional (2D) contour plots of fitted models. The 2D contour plots provide a method to predict response for different values of the parameters and to help in the identification of the type of interactions between the parameters. If there is no significant interation between two parameters, the contour plots are straight and circular, otherwise the contour plots are inclined at different angles depending on the effect of parameters.

The interactive effects of the investigated factors on the oligolevans/FOSs production in maple syrup30 °Bx are illustrated in the 2D contour plots presented in Figure 4.7 (A, B and C). All the three contour plots display similar circular trends; these results confirm the non-siginficance of the interactions occuring between the factors in the oligolevans/FOSs production predictive model of maple syrup 30 °Bx. However, the yield of oligolevans/FOSs produced in maple syrup 30 °Bx increased with a commitment increase of the reaction time to 23.12 h, levansucrase units to 3.72 U/ml and pH to 6.6 up to a maximum value of 43.72% and decreased thereafter. The decrease in the oligolevans/FOSs yield with the increase of the reaction time may be due to their uses as acceptors/donors by levansucrase. Figure 4.7A also shows that the effect of levansucrase units was more significant at the initial stage of the reaction. These results may be due to the saturation of the levansucrase at the late stage of the reaction time course. As expected, the effect of pH is not reaction time- nor levansucrase unit-dependent (Figs 4.7 B, C).

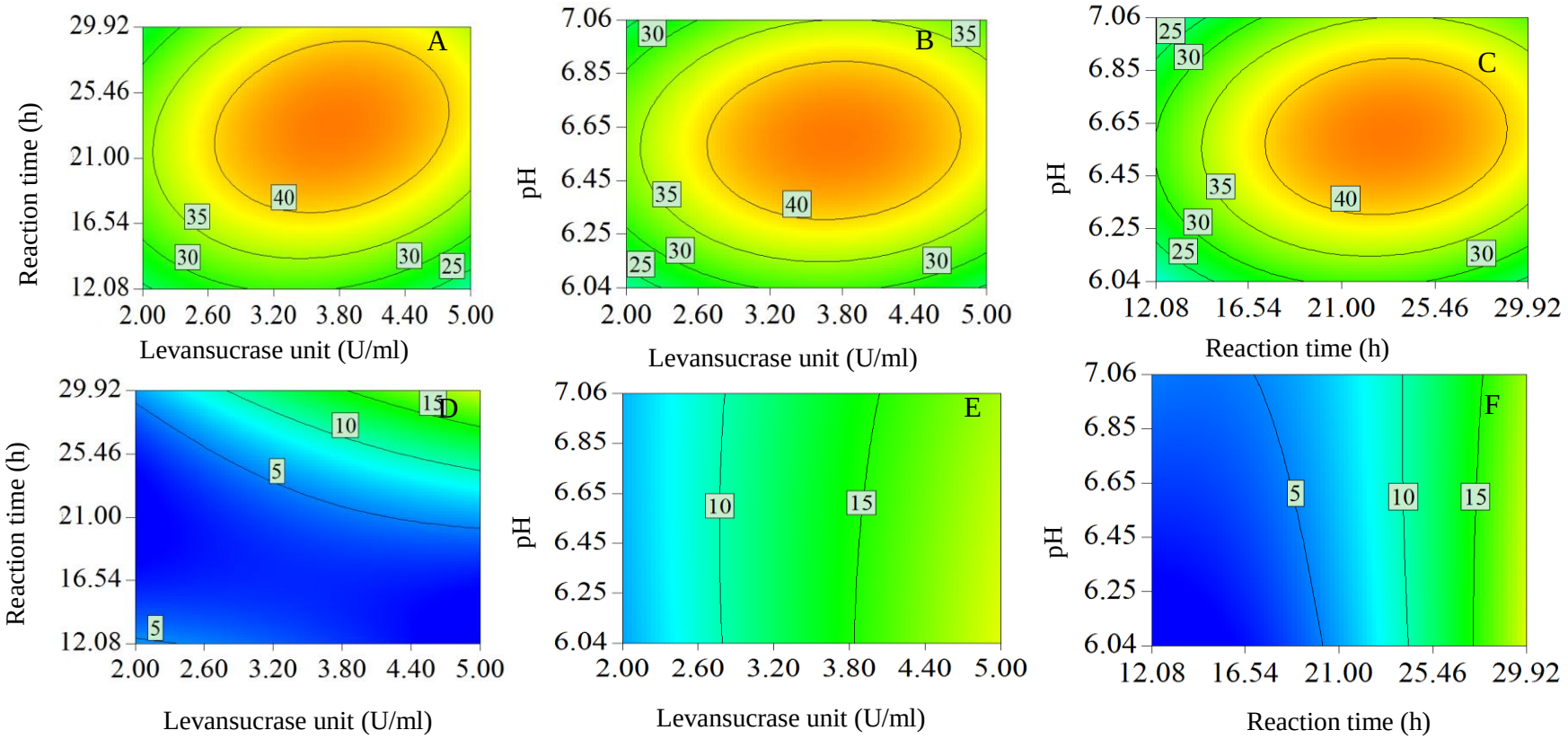


Figure 4.7. Contour plots of predicted models for the yields of oligolevan/FOSs (A, B, C) and oligolevans/levans (D, E, F) generated in the in the maple syrups 30 ℬx- and 66 ℬx-reaction systems, respectively. The numbers inside the contour plots indicate conversion yields (%, w/w) under given reaction conditions.

The 2D contour plots (Figs 4.7 D, E, F) generated from the oligolevans/levans production predictive model of maple syrup 66 °Bx show the interactive effects of levansucrase unit/reaction time (pH 6.04), levansucrase unit/pH (reaction time of 29.92h) and reaction time/pH (levansucrase unit of 5 U/ml). Among all interactions, only that of levansucrase unit/reaction time with an elliptic trend of its 2D contour plot was significant;however, the effect of this interaction on the oligolevans/levans production was more pronounced at the late stage of the reaction time course (more than 21 h). These results may be due to the reduced viscosity of maple syrup 66 °Bx at this stage, favoring the substrate/enzyme interaction. The vertical lines obtained in the contour plots (Figs 4.7 E, F) reveal the negligible effect of pH on oligolevans/levans production as compared to levansucrase unit and reaction time. The oligolevans/levans yield increased as levansucrase unit increased or as the reaction was prolonged within the investigated design space.

4.4.3. Model Verification

The optimal conditions for the synthesis of FOSs, oligolevans and levans were estimated via the numerial optimization of the Design Expert 8.0.6 software. The uncoded optimal conditions and the predicted and the experimental maximum bioconversion yields are summaried in Table 4.5. The un-coded optimal conditions for the oligolevans/FOSs production in maple syrup 30 °Bx-based reaction system were: levansucrase unit of 3.73 U/ml, pH 6.60 and reaction time of 23.12 h. The un-coded optimal conditions for the oligolevans/levans production in maple syrup 66 °Bx-based reaction system were: levansucrase unit of 5 U/ml; pH 6.04 and reaction time of 29.92 h. Under the optimum conditions, the predicted conversion yields of oligolevans/FOSs and oligolevans/levans were 38.81% (109.20 g/l) and 20.16% (147.09 g/l), respectively. Both the maximum conversion yields are within the statistically significant range of the estimated optimum values with 95% prediction intervals (PIs). The good correlation between these results confirmed that the response models were adequate for reflecting the expected optimization. As an overall, validation of the RSM models were confirmed.

Table 4.5. Model verification and optimization of yields of oligolevans/FOSs and oligolevans/levans.

Optimum conditions	Oligolevans/FOSs yield (%)[a]				Oligolevans/levans yield (%)[a]			
	Uncoded	95% PI	Experimental	Predicted	Uncoded	95% PI	Experimental	Predicted
Levansucrase unit (U/ml)	3.73				5			
Reaction time (h)	23.12	26.31-59.88	38.51	43.10	29.92	11.89-28.04	20.16	19.96
pH	6.6				6.04			

[a]Yield (%) is expressed in the weight percentage (w/w) of the end-products as compared to the initial sucrose concentration.

CHAPTER V CONCLUSION

An innovative biocatalytic approach based on *B. amyloliquefaciens* levansucrase-catalyzed transfructosylation reaction approach was developed for the production of levans, oligolevans and FOSs using selected maple syrups (15 °Bx, 30 °Bx and 66 °Bx) as reaction media and source of sucrose substrate. The biotransformation of moderate to low-grade maple products into high value-added enriched products is expected to overcome their defects and broaden their applications.

In the three maple syrups-based reaction systems, altering reaction conditions resulted in the changes in the reaction selectivity (transfructosylation/hydrolysis) and in the product spectrum (levans/oligolevans/FOSs). At 30 °C, the use of maple syrup 30 °Bx led to the highest total levansucrase activity (initial conversion rate of sucrose), while the highest concentration of converted sucrose was reached in maple syrup 66 °Bx. The absence of substrate inhibition by the excess of sucrose concentration was observed. The sucrose conversion by levansucrase from *B. amyloliquefaciens* at 8 °C proceeded with a very low rate as compared to 30 °C. The highest total activity of levansucrase at 8 °C was obtained upon the use of maple syrup 15 °Bx as reaction medium. Transfructosylation reaction was predominantly catalyzed at lower temperature and higher sucrose concentration. These results reveal a very important characteristic of *B. amyloliquefaciens* levansucrase with its high transfructosylation-to-hydrolysis ratio. In the maple syrup 15 °Bx-based reaction system, low levansucrase unit of 2 U/ml at 30 °C yielded more transfructosylation products as compared to high levansucrase unit of 6 U/ml. Lower reaction temperature of 8 °C resulted in a similar maximum yield as that achieved at 30 °C. In maple syrup 30 °Bx-based reaction system, oligolevans were synthesized as the major products (more than 80%) and this profile was not affected by the levansucrase unit (2 to 6 U/ml) nor by the temperature (8 to 30 °C). As compared to maple syrups 15 °Bx and 30 °Bx, the effects of reaction time, temperature and levansucrase unit were more significant with maple syrup 66 °Bx. A significant increase was obtained at 30 °C from 0-12 h and remained more or less constant thereafter upon 48 h. While no significant increase in the yield was achieved at 8 °C within the investigated reaction time. In addition, in maple syrup 66 °Bx, the most abundant products were oligolevans at 30 °C and levans at 8 °C. This study is the

first to highlight the potential of *B. amyloliquefaciens* levansucrase to produce FOSs, oligolevans and levans with maple syrups as substrate.

Acceptor specificities of *B. amyloliquefaciens* levansucrase were investigated with selected monosaccharides and disaccharides. Galactose having a pyranose ring in its chemical structure was a better acceptor than xylose who has a furanose ring. Disaccharides were more favorable fructosyl acceptors than monosaccharides, with lactose being the most preferred acceptor. Furthermore, maple syrups 30 ° and 66 °Bx were identified as the best reaction media for the synthesis of oligolevans/FOSs and oligolevans/levans, respectively. According to the RSM optimization studies, reaction time, levansucrase unit and pH showed significant negative quadratic effects on oligolevans/FOSs yield, whilst the linear terms of levansucrase unit and reaction time were the most important variables for the yield of oligolevans/levans. The experimental values of the oligolevans/FOSs yield and oligolevans/levans yield were within the statistically significant range of the estimated optimum values with 95% prediction intervals (PIs). The good correlation confirmed the effective ability of the established models to describe the relationship between the reaction variables and the yield of oligolevans/FOSs and the yield of oligolevans/levans, respectively.

As overall, the production of FOSs, oligolevans and levans using maple syrups as reaction systems through *B. amyloliquefaciens* levansucrase-catalyzed transfructosylation reaction is a promising approach for the valorization of low-grade maple syrups.

Recommendations for future work:

- Immobilization of levansucrase for efficient biotransformation of maple syrups at the industrial level.
- Investigation of the prebiotic activity of the produced novel value-added maple products for better understanding of their functional properties and health attributes as they are related to their chemical compositions.

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