Enzymatic Synthesis of Fructooligosaccharides from Sucrose by Endo-Inulinase-Catalyzed Transfructosylation Reaction in a Non-Conventional Media

By

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August, 2016

A thesis submitted to the Graduate and Post-Doctoral Studies Office in partial fulfillment of the requirements of the degree of Master of Science

ABSTRACT

A biocatalytic approach based on endo-inulinase-catalyzed transfructosylation reaction in non-conventional media using sucrose as substrate was investigated for the synthesis of well-defined fructooligosaccharides. Endo-Inulinase from *Aspergillus niger* was purified by anion exchange chromatography. The transfructosylation reactions were carried out in selected organic/buffer biphasic systems (3:1, v/v) at 35/40°C and 150 rpm. Heptane, heptanone, cyclohexane, butyl acetate and ethyl acetate were the investigated organic solvents. The reaction components were analyzed by high performance anion exchange chromatography equipped with pulsed amperometric detector. The highest initial velocity of 1,500.0 μmol/ml.min was observed in the heptane/buffer biphasic system followed by the butyl acetate /buffer biphasic system with an initial velocity of 1,000.7 μmol/ml.min. However, the lowest initial velocity of 69.6 μmol/ml.min was obtained in the hexane/buffer biphasic system. Although the butyl acetate/buffer system resulted in the highest sucrose conversion of 93.94%, the relative proportion of the transfructosylation reaction was only 43.33%. The highest transfructosylation relative proportions of 79.34% was reported in the heptane/buffer biphasic systems. The highest fructooligosaccharides bioconversion yield of 60.24% was obtained in the butyl acetate/buffer biphasic system after 48 h of reaction, with nystose as the main end-product. 1-kestose was the most abundant fructooligosaccharides produced followed by nystose and fructosyl-nystose.

The effects of enzyme unit, proportion of butyl acetate solvent and reaction time on the kestose, nystose and fructosyl-nystose concentrations as well as the bioconversion yield of fructooligosaccharides were studied, using a response surface methodology (RSM). The RSM analysis showed that the most significant variable was different for the various responses. The linear term of the enzyme unit exhibited the most significant effect on the kestose concentration, whereas for the nystose concentration and fructooligosaccharides bioconversion yield responses, the reaction time and the proportion of solvent were the most significant variables, respectively. However, for the fructosyl-nystose concentration response, all linear terms were found to be significant. Among the interactive effects, the one between the reaction time and the proportion of solvent had the most significant effect on kestose and nystose concentrations and on the bioconversion yield of fructooligosaccharides.

An investigation in the production of fructooligosaccharides by endo-inulinase catalysed transfructosylation reaction in maple syrup-based biphasic media was carried out using selected

maple syrups with different Brix (Bx) values (15º, 30º, 60º). The synthesis was performed over a time course of 16 to 80 h to assess the transfructosylation products obtained by endo-inulinase in a butyl acetate/maple syrup based biphasic system. Among the three maple syrups, the one with 66°Bx at 80 h of reaction resulted in the highest fructooligosaccharide bioconversion yield of 52.53%. As for the end product profile, the highest production of kestose was obtained with the use of maple syrup15°Bx, whereas the maple syrup 66°Bx resulted in the highest productions of nystose and fructosyl nystose. The biocatalytic approach of the study showed a great potential in synthesizing well defined fructooligosaccharides as functional ingredients in food industry and the development of new applications with maple syrup.

RÉSUMÉ

Une approche biocatalytique basée sur la réaction de transfructosylation de saccharose catalysé par endo-inulinase dans un milieu non-conventionnel a été étudiée pour la synthèse de fructooligosaccharides bien définies. Endo-inulinase d'*Aspergillus niger* a été purifiée par une chromatographie d'échange d'anion. Les réactions de transfructosylation ont été effectuées dans un milieu biphasique composé d'une phase organique/tampon (3: 1, v: v) à 35/40°C sous une agitation de 150 trs par minute. L'heptane, l'heptanone, le cyclohexane, l'acétate de butyle et l'acétate d'éthyle ont été utilisés comme solvants organique dans le milieu biphasique. Les composantes de la réaction ont été analysées par la chromatographie d'échange d'anions à haute performance équipée d'un détecteur ampérométrique pulsée. La vitesse initiale la plus élevée de 1500.00 µmol/ml.min a été obtenue dans le milieu biphasique composé d'heptane/tampon, suivie d'une vitesse initiale de 1000.67 µmol/ml.min qui a été observée dans le milieu biphasique composé d'acétate de butyle/tampon. Tandis que l'utilisation d'un milieu biphasique composé d'hexane/tampon a conduit à une vitesse initiale la plus faible de 69.62 µmol/ml.min. Bien que le milieu biphasique composé d'acétate de butyle/tampon a conduit à la bioconversion la plus élevée de 93.94%, la réaction de transfructosylation dans ce milieu ne représentait que 43.33% par rapport à celle d'hydrolyse. La proportion relative de la réaction du transfructosylation la plus élevée de 79.34% a été estimée dans le milieu biphasique contenant l'heptane. Le milieu biphasique composé de l'acétate de butyle/tampon a permis l'obtention d'un rendement de bioconversion des fructooligosaccharides le plus élevé de 60.24%. 1-kestose était le plus abondant fructooligosaccharides suivi par nystose et fructosyl-nystose.

Les effets de l'unité enzymatique, la proportion du solvant acétate de butyle et le temps réactionnel sur la synthèse de fructooligosaccharides (réponses : concentrations de kestose, nystose et fructosyl nystose et le rendement de bioconversion) ont été évalués en utilisant la méthode de surface de réponse (RSM). L'analyse RSM a démontré que la variable la plus significative dépendait des réponses. L'unité enzymatique a montré un effet linéaire très significatif dans le model prédictive de la concentration kestose, tandis que dans les autres modèles prédictifs de la concentration de nystose et du rendement de bioconversion, le temps de réaction et la proportion de solvant étaient les variables les plus déterminantes, respectivement. Toutefois, pour la réponse de concentration de fructosyl nystose, tous les termes linéaires des variables étaient significatifs dans son model.

La production de fructooligosaccharides par l'endo-inulinase dans un milieu biphasique contenant des sirops d'érable ayant des Brix sélectionnée*s* (15º, 30º, 60º) a été investiguée sur une période de réaction de 16 à 80h. Parmi ces trois sirops d'érable, celui ayant 66°Bx a conduit au rendement de bioconversion le plus élevé de 52,53% après 80h de réaction. La production de kestose la plus élevée a été obtenue dans le sirop d'érable de 15º Bx tandis que le taux le plus élevé de nystose et fructosyl nystose ont été obtenus avec le sirop d'érable de 66 ° Bx. L'étude avec l'approche biocatalytique a démontré un grand potentiel pour la synthèse de fructooligosaccharides comme ingrédients fonctionnels et aussi dans le développement de nouvelles applications pour le sirop d'érable.

CONTRIBUTION OF AUTHORS

The present thesis consists of four chapters.

Chapter I presents an assessment of the literature of prebiotics, their benefits and their synthesis. A brief description of common techniques employed to produce the fructooligosaccharides is also reported. The properties of the enzyme, inulinase, and the non-conventional media of high interest in the study are also presented. The maple syrup, which is one of the substrate in this study, is also described. The chapter ends by demonstrating some of the analytical techniques used for the quantitative and the qualitative analyses of fructooligosaccharides.

Chapter II covers the different materials and methods that have been used throughout this study. In particular, the enzyme purification, the enzymatic assays, the transfructosylation reaction catalyzed by the endo-inulinase enzyme in biphasic media, the characterization of end product profile as well as the transfructosylation reaction catalyzed in a maple syrup biphasic medium were described. This chapter also covers the optimization of the production of fructooligosaccharides and the various effects of parameters involved. The connecting statements in between the chapters provide the rational linking the different chapters presented in this study.

Chapter III outlines all the results obtained in the form of figures and tables as well as the discussion supporting the results that were obtained through out the study.

Chapter IV provides a general conclusion to the thesis with a summary of major findings.

Neeyal Appanah, the author, was responsible for the experimental work and the preparation of the thesis.

Dr. Salwa Karboune, the supervisor of the author's Master’s work, guided all the research and critically revised the thesis prior to submission.

ACKNOWLEDGEMENTS

First, I would like to convey my gratitude to my supervisor Dr. Salwa Karboune, for her continuous support, guidance and patience in allowing me to complete my M.Sc. research work with success. Her ongoing constructive remarks, advices and knowledge in the food science field have helped me to become more knowledgeable in this research field.

A special thanks is dedicated to my ex colleague, Dr. Feng Tian for introducing me to various laboratory techniques. I am also grateful to all my previous and present laboratory mates: Afshan, Andrea, Dr. Amanda Waglay, Erin, Eugenio, Jin, Juan Tamayo, Dr. Juan Pablo, Marika, Dr Marya Aziz, Mengxi, Mikki, Dr. Nastaran Khodaie, Parsley, Shi, Dr. Soo Seo and Yi for their friendship, kindness and moral support throughout my graduate studies.

I would also like to extend my appreciation to few special friends who motivated me and cheered me up throughout this journey.

Last but not least, I am indebted towards the almighty, my mum Radha, my dad Balaram, my husband Kevin, and my daughter Elliana for their unconditional love and sacrifices. Today, due to their ongoing support, my graduate studies have become a dream come true.

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LIST OF ABBREVIATIONS

ANOVA: Analysis of variance

Arg: Arginine

Asp: Aspartate

Bx: Brix

CCRD: Central composite rotatable design

CV: Column volume

DNS: 3,5-Dinitrosalicyclic acid

EC number: Enzyme Classification number

FOSs: Fructooligosaccharides

Fr: Fructose

Ga: Galactose

GF_4 : Fructosyl nystose

GH: Glycoside hydrolase

Gu: Glucose

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

HPLC: High performance liquid chromatography

HPSEC: High performance size exclusion chromatography

RSM: Response surface methodology

SCFA: Short chain fatty acid

Sp; Species

TLC: Thin liquid chromatography

Xy: Xylose

INTRODUCTION

According to Gibson and Roberfroid (2008), a prebiotic can be defined as "a selectively fermented ingredient that allows specific changes, both in the composition and/or activity in the gastrointestinal microbiota that confers health benefits". Besides stimulating the growth of *bifidobacteria* in the intestinal system, prebiotics can activate the immune system, increase the production of short chain fatty acids and increase the absorption of minerals, such as calcium (Dominguez et al., 2014). The gut microbiota ferments a wide range of substances, such as oligosaccharides, non-starch polysaccharides and resistant starch, which cannot be digested (Dominguez et al., 2014).

Fructooligosaccharides consist of fructose oligomers containing one glucose unit and 2 to 10 fructose units bound together by β- (2→1) glycosidic linkages (Silva et al., 2013b). The growing interest in fructooligosaccharides by the food industry is due to their beneficial functional properties, such as low calorie, low sweetness intensity, non-cariogenic, and their ability to promote the growth of probiotic bacteria, which produce short chain fatty acids (Silva et al., 2013b, Dominguez et al., 2014). Due to the complexity of the carbohydrate structures and their multiple attachment sites, the production of fructooligosaccharides with high purity is very challenging. Indeed, fructooligosaccharides can be produced by extraction from natural sources, chemical synthesis or through biocatalytic approaches. Advantages associated with the use of biocatalytic approaches include the use of milder reaction conditions, the high reaction selectivity that could lead to the synthesis of structurally well-defined fructooligosaccharides in regio- and stereo-specific manner and the ability to market natural products to consumers.

Fructooligosaccharides can be obtained through the enzymatic hydrolysis of inulin or by the enzymatic transfructosylation of sucrose (Dominguez et al., 2014). The current transfructosylation reaction is catalyzed by fructofuranosidases (EC 3.2.1.26) at high sucrose concentrations. Although fructofuranosidases have proven to be valuable in fructooligosaccharide synthesis, their application is subject to many key limitations, including (*i*) the challenge of driving the reaction in a thermodynamically disfavored direction; (*ii*) their severe inhibition by glucose by-product; (*iii*) the narrow acceptor specificity; (*v*) the poor regioselectivity. On the other hand, the use of fructosyltransferases (EC 2.4.1) is limited by their low availability and their low thermal stability. Inulinases (EC 3.2.1.7), which belong to the glycoside hydrolase family, has been little explored as a biocatalyst for the catalysis of the transfructosylation of sucrose (Chi et al., 2009). Indeed, inulinase is currently used for the production of high fructose syrup and/or inulooligosaccharides

by the controlled hydrolysis of inulin (Silva et al., 2013b). While the exo-inulinase cleaves fructose from the non-reducing β-(2,1) end of inulin, the endo-inulinase hydrolyses the internal inulin linkages to yield inulooligosaccharides. The synthetic reaction catalyzed by inulinase may be favored over the hydrolytic one by the use of high sucrose concentration, elevated temperatures and the use of organic co-solvents.

Canada accounts for 85 % of yearly worldwide production of maple syrup with Quebec contributing about 90 % of total Canadian maple syrup production (Perkins and van den Berg, 2009; Li, 2015). Since maple syrup consists mainly of sucrose (68%), glucose (0.43%, w/w) and fructose (0.34%, w/w) (Stuckel and Low, 1996), therefore it is an interesting reaction medium for favoring transfructosylation reaction.

The overall objective of this study was to develop a biocatalytic approach, based on the endo-inulinase-catalyzed transfructosylation reaction, for the production of fructooligosaccharides with great potential health benefits. The specific objectives were:

1) Assessment of the catalytic efficiency of endo-inulinase in selected non-conventional reaction media.
2) Investigation of the effects of the reaction parameters on the transfructosylation reaction of sucrose catalyzed by endo-inulinase.
3) Application of the biocatalytic approach to enrich Maple products with fructooligosaccharides using the defined optimal conditions.

CONNECTING STATEMENT 1

This previous part provided a general introduction and outlined the objectives of the present study. Chapter I provides a comprehensive literature review on the prebiotics, in particular the non-digestible oligosaccharides and the fructooligosaccharides, and their health benefits. This chapter also describes the common techniques employed to produce the fructooligosaccharides, in particular the enzymatic approaches and the different types of non-conventional media. The different analytical methods for the analysis of fructooligosaccharides are also reported.

CHAPTER I

LITERATURE REVIEW

1. Prebiotics

1.1. Definition

The concept of prebiotics was first introduced by Gibson and Roberfroid in 1995 (Charalampopoulos and Rastall, 2012); these authors defined a prebiotic as a "non-digestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon, potentially improving the host health" (Gibson and Roberfroid, 1995). The definition of prebiotic was afterwards refined to "*a selectively fermented ingredient that allows specific changes, both in the composition and/or activity in the gastrointestinal microbiota that confers benefits*" (Gibson and Roberfroid, 2008). Moreover, the concept of prebiotic has gained scientific and industrial research interest throughout the years (Roberfroid, 2007). In simple words prebiotic can be defined as a non-digestible oligosaccharide that helps to increase the growth of beneficial bacteria present in the gastrointestinal tract.

1.2. Health Benefits

Nowadays, due to their potential health benefits, the consumption of prebiotics has become more prevalent among consumers and prebiotics have found different applications in various food products ((Mussatto and Mancilha, 2007; O'Neill, 2011). An intake of prebiotics can have the following health benefits:

1) Enhance and increase the bioavailability of certain minerals, such as calcium and magnesium (Brownawell et al., 2012).
2) Lower the risk for colon cancer (Charalampopoulos and Rastall, 2012).
3) Reduce the risk factors associated with coronary heart disease (Brownawell et al., 2012).
4) Prevent the onset of various gastrointestinal infections, such as traveller's diarrhea and acute diarrhea as well as reduce their duration (Charalampopoulos and Rastall, 2012).
5) Decrease inflammatory conditions, such as irritable bowel syndrome and inflammatory bowel disease (Brownawell et al., 2012).
6) Promote weight loss and prevent obesity by regulating metabolic disorders.

1.3. Techno-Functional Properties of Prebiotics

Besides their beneficial health effects, prebiotics can be useful in various food applications. Certain prebiotics, such as inulin and oligofructose can influence the textural and organoleptic properties of food products (Charalampopoulos and Rastall, 2012). Due to its good binding and moisture

retention properties, oligofructose can help to prevent the hardening of a product and therefore can contribute to an increase in its shelf life (O'Neill, 2011). Owing to their high acid stability, galactooligosaccharides are being used along with inulin in juices as well as in infant formula (Gibson and Roberfroid, 2008). Due to its gelling characteristic, inulin is also used in the making of low fat food, such as cream cheese to help maintain their taste and texture (Gibson and Roberfroid, 2008). Since inulin also contributes to fewer calories (8.63KJ/g) compared to sugar (16.74KJ/g), the cereal market has started formulating their bakery and cereal products using prebiotics, which can eventually help people to manage and decrease their calorie intake (O'Neill, 2011).

2. Non-Digestible Oligosaccharides

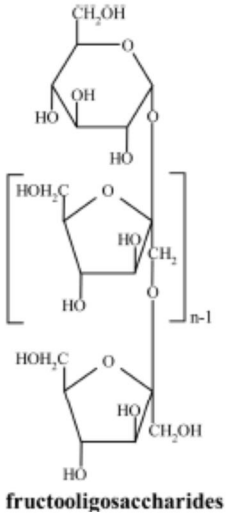
2.1. Classification and Chemical Structures

Oligosaccharides can be categorized as non-digestible oligosaccharides (NDOs) when they possess β-glycosidic bonds between monosaccharide units (Mussatto and Mancilha, 2007). According to Roberfroid and Slavin (2000), "the concept of non-digestible oligosaccharides originates from the observation that the anomeric C atom (C1 or C2) of the monosaccharide units of some dietary oligosaccharides has a configuration that makes their osidic bounds non-digestible to the hydrolytic activity of the human digestive enzymes".

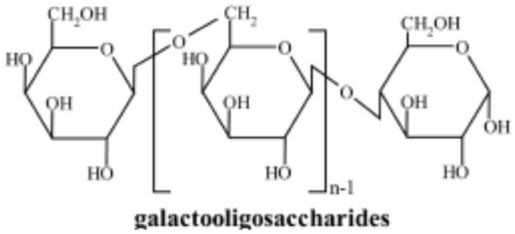
Table 1. Commercially available non-digestible oligosaccharides, their molecular structure (Mussatto and Mancilha, 2007) and their osidic bond (Patel and Goyal, 2010).

Compound	Molecular structure	Osidic bond
Fructooligosaccharides	$(Fr)_n$-Gu	ß -(2 →1) and α -(1→2) linked
Galactooligosaccharides	$(Ga)_n$ –Gu	α -(1→4) and ß -(1→6)-linked
Isomaltooligosaccharides	$(Gu)_n$	α -(1→6)-linked
Maltooligosaccharides	$(Gu)_n$	α -(1→4)-linked
Soybean oligosaccharides	$(Ga)_n$ -Gu-Fr	α -(1 →6)- α -(1→2)- ß -fructose linked
Xylooligosaccharides	$(Xy)_n$	ß -(1→4)-linked

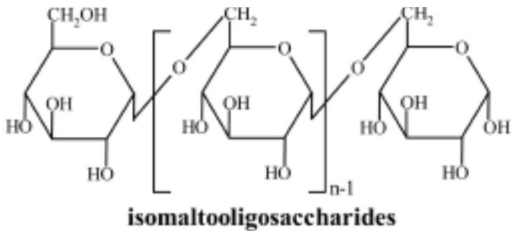
Ga: Galactose, Gu: Glucose, Fr: Fructose, Xy: Xylose



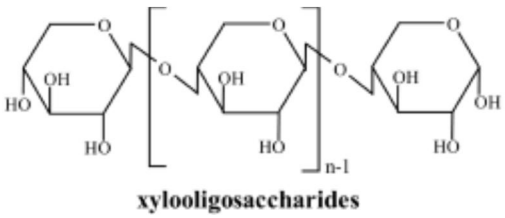
Scheme 1: Chemical structure of fructooligosaccharide (Swennen et al., 2006)



Scheme 2: Chemical structure of galactooligosaccharides



Scheme 3: Chemical structure of isomaltooligosaccharides

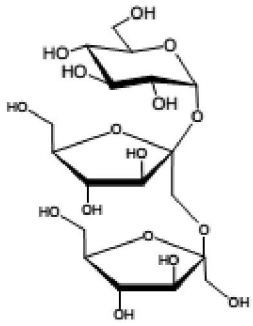


Scheme 4: Chemical structure of xylooligosaccharides

2.2. Fructooligosaccharides

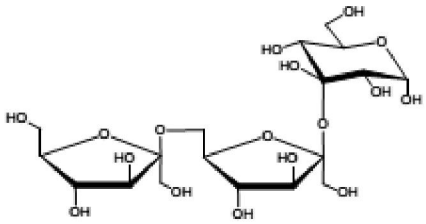
Fructooligosaccharides are composed of 3-10 monomeric sugar residues with fructose as the major one and are linked by β (2→1) or β (2→6) glycosidic linkages with or without a terminal D-glucose group (Roberfroid, 1996; Tian, 2013). According to their structures and the type of linkages between the monosaccharide residues, they can be classified into four major classes: inulin-type, levan-type, mixed levan type and neoseries type (Monsan and Ouarne, 2009).

Inulin type fructooligosaccharides are composed of β-D-fructofuranosyl units which are attached by β (2→1) linear linkages (Roberfroid and Slavin 2000). The commercially available inulin-type fructooligosaccharides consist of 1-kestose, nystose and fructofuranosylnystose (Plou et al., 2007).



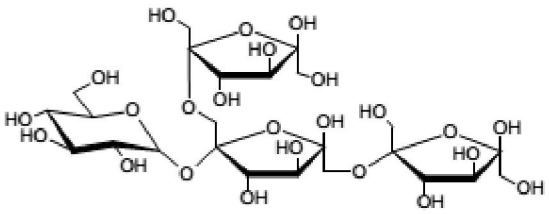
Scheme 5: Chemical structure of 1-kestose

Levan type fructooligosaccharides are composed of β- D-fructofuranosyl units which are attached by β (2→6) linear linkages. The fructofuranosyl units are β- linked to the 6 position of sucrose (Monsan and Ouarne, 2009). An example of levan type fructooligosaccharides is 6-kestose.



Scheme 6: Chemical structure of 6-kestose

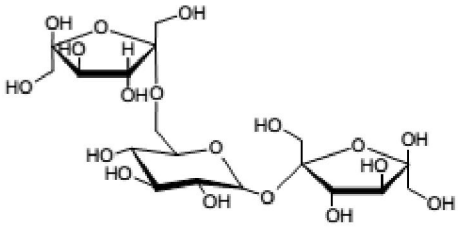
Mixed levan type fructooligosaccharides are composed of β- D-fructofuranosyl units which are attached to sucrose by both β (2→6) and β (2→1) linkages (Monsan and Ouarne, 2009). An example of mixed levan type fructooligosaccharides is the bifurcose, which is the smallest tetrasaccharide.



Scheme 7: Chemical structure of bifurcose

Neoseries type

Neoseries fructooligosaccharides are composed of β- D-fructofuranosyl units which are attached by linear β (2→1) linkages. The fructofuranosyl are attached to C6 of the glucose moiety of sucrose. Examples of neoseries fructooligosaccharides are neokestose and neocystose (Linde et al., 2012).



Scheme 8: Chemical structure of neokestose

3. Production of Fructooligosaccharides

Fructooligosaccharides can be obtained by various methods, such as extraction, chemical synthesis and enzymatic synthesis.

3.1. Extraction of Fructooligosaccharides

Fructooligosaccharides can be extracted from natural sources, such as the flowering plants, which are found in temperate and arid climates (Banguela and Hernandez, 2006). They are usually found in storage organs, such as bulbs and tuberous roots which can be easily processed to purified products (Fuchs, 1991). However, they can also be extracted from some edible parts of plants, such as banana, tomato and asparagus (Voragen, 1998; Flamm et al., 2001). Jerusalem artichoke with upto 20 % of fructooligosaccharides concentration and chicory ranging from 5-10% are considered as the major plant sources for commercial production of GF-type fructooligosaccharides (Kurtoglu and Yildiz, 2011; Voragen 1998).

3.2. Chemical Synthesis

Fructooligosaccharides can be obtained by chemical synthesis. However, this method of synthesis of fructooligosaccharides is elaborate and laborious. Due to the presence of different functional groups and chiral centers in monosaccharides, sequential selective protection-deprotection steps of the functional groups are required to control the stereochemical and regio-chemical specificity of the glycosidic bond that is formed (Inthanavong, 2011). Chemical synthesis also requires the use of toxic reagents, which may not comply with the food safety conditions (Inthanavong, 2011).

3.3. Enzymatic Synthesis

Fructooligosaccharides are produced commercially from sucrose by the microbial enzymes β-fructosyltransferases or β-fructofuranosidase with a high transfructosylating activity (Prata et al., 2010). In comparison to chemical synthesis, the enzymatic synthesis has the advantage of providing regio-specificity and stereo-specificity to the glycosidic linkage and therefore can be a more suitable method for the production of commercial oligosaccharides (Plou et al., 2007).

3.3.1. Hydrolysis Reaction

Fructooligosaccharides can be obtained by the hydrolysis process of oligo- or polymeric fructans by the enzyme levanase or inulinase. The enzyme inulinase was discovered in 1888 by J. Reynolds Green while he was experimenting on Jerusalem artichoke (Dean, 1903). He carried out various tests on germinating Jerusalem artichoke and concluded that inulinase was able to hydrolyze inulin (Dean, 1903). Inulinases are fructofuranosyl hydrolases which are obtained from various sources, such as plants and microorganisms. Inulinase reaction generally involves the action of exo-inulinase and endo-inulinase (Kango and Jain, 2011). Exo-inulinase breaks down the terminal fructose units from inulin, whereas endo-inulinase breaks down inulin into inulooligosaccharides (Kango and Jain, 2011). The hydrolysis process is a single bioconversion reaction and an immobilized inulase can be used for continuous production of fructooligosaccharides (Parekh and Margaritis, 1986).

3.3.2. Transfructosylation Reactions

3.3.2.1 Fructooligosaccharides Production by Fructosyl-tansferase Enzymes

Fructosyltransferase enzymes belong to the glycoside hydrolase family (GH68) and are β-retaining enzymes, which have a double displacement mechanism (Ozimek et al., 2006). They can catalyze two types of reactions: the transglycosylation reaction and the hydrolysis reaction. The most common bacterial fructosyltransferase enzymes are levansucrases and inulosucrases. The levansucrases reaction is more specific to β (2→6) glyosidic bond whereas the inulosucrases reaction is more specific to the β (2→1) linked polymers (Ozimek et al., 2006).

Fructosyltransferase only possesses a transfructosylating activity whereas β-D fructofuranosidase possesses hydrolytic and transfructosylating activities (Maiorano et al., 2008). The properties of fructosyltransferase are dependent on the type of microorganism and on the composition of the culture medium used e.g. the carbon source can have an inducer role (Maiorano et al., 2008). Fructosyltransferase can undergo a disproportionation reaction where it can catalyze the transfer of a fructosyl moiety of sucrose to another sucrose or fructooligosaccharides to generate higher yield of fructooligosaccharides (Maiorano et al., 2008).

3.3.2.2. Fructooligosaccharides Production by β-Fructofuranosidases

β-Fructofuranosidases are used in the production of fructooligosaccharides and they naturally catalyze the hydrolysis reaction of sucrose. They can be obtained from microbial sources, such as *Aspergillus niger and Aerobasidium* pulluans species. Moreover, β-Fructofuranosidases obtained from these microbial species show high transfructosylation activity and are used in industrial fructooligosaccharides production (Hidaka et al., 1988; Yun, 1996; Tian, 2013). Despite being widely available, only few of them exhibit significant transfructosylation activity. Their use in the production of fructooligosaccharides is also limited due to their low yield (< 20%) and low regio-selectivity (Plou et al., 2007; Inthanavong, 2011; Li, 2015). However, the synthesis of fructooligosaccharides through synthetic reaction can be favored by the use of high substrate concentration, high temperature and non-conventional media in order to reduce the water activity through an equilibrium controlled reaction (Fernández et al., 2004; Plou et al., 2007). The yield of fructooligosaccharides produced by thermodynamic controlled reaction is dependent on various parameters, such as the initial substrate concentration, pH, temperature, ionic strength and solvent composition (Plou et al, 2007; Inthanavong, 2011).

3.3.2.3. Fructooligosaccharide Production by Inulinase

The use of inulinase in the food industry has become important as it is used in the production of fructooligosaccharides (Risso et al., 2012a). Since the enzyme possesses a transfructosylating activity in high sucrose concentration, it can therefore synthesize frutooligosaccharides by the transfructosylation reaction (Risso et al.,2012b). Moreover, the synthesis of fructooligosaccharides through the reverse of hydrolysis reaction can also be achieved by the use of organic solvent which shifts the equilibrium of the reaction to favor the transfructosylation reaction (Risso et al., 2012b). Santos and Maugeri (2007) reported that temperature and sucrose concentration were found to be the two most vital parameters in the production of fructooligosaccharides from sucrose using inulinase from *Kluyveromyces marxianus* var. bulgaricus. Kuhn et al. (2013) reported a higher yield of fructooligosaccharides, when an immobilized commercial inulinase from *Aspergillus niger,* was treated in pressurized fluid and sucrose was used as substrate. However, a lower yield of fructooligosaccharides was reported with inulin as a substrate.

4. Inulinase

Inulinase (EC 3.2.1.7) belongs to the glycoside hydrolases family (Chi et al., 2009) and catalyzes the hydrolysis of inulin to produce high fructose syrup or inulo-oligosaccharides (Kango and Jain, 2011). Inulinase can be obtained from various sources, such as the inulin storage of plants and microorganisms, like bacteria, yeasts and molds (R.S.Singh and R.P.Singh,2010). Microorganisms are the best sources for the commercial production of inulinase since they are easily cultivated and can produce high amounts of enzyme (Chi et al., 2009).

Inulinase hydrolyses the β (2→1) linkage of inulin into fructose and glucose (Chi et al., 2009). There are two types of inulinase: the exo-inulinases and the endo-inulinases (Chi et al., 2009). The exo-inulinase splits off the terminal fructose unit from the non-reducing end of the inulin, whereas the endoinulinase hydrolyses internal linkages of inulin into inulo-oligosaccharides, such as inulotriose, inulotetaose and inulopentaose (Chi et al., 2009; Kang et al., 1998). The exo and endo acting property depends on the microbial origin of the enzyme (R.S.Singh and R.P.Singh, 2010). Inulinases type 3 of *Aspergilus niger* were found to selectively hydrolyze inulin and according to Zittan (1981), inulinases from *Aspergillu*s species were highly specific to inulin (Kango and Jain, 2011).

The multiple sequence alignment of inulinase revealed the presence of Arg-Asp-Pro (RDP) motif, which has a functional role in substrate binding and is responsible for the specificity of the enzyme towards fructopyranosyl residues (Kango and Jain, 2011). A study on three dimensional models of exo-inulinase from *Bacillus stearothermophilus* and endo-inulinase from *Aspergilus niger* revealed that these inulinases had a high degree of similarity in the structural domain opposite to the active site region; however, main differences were found in the catalytic site of the enzymes (Basso et al., 2010). The catalytic site of the endo-inulinase was wide with about 90 of 516 residues and the active site of the exo-inulinase was smaller with 42 of 493 amino acids due to a large site of natural substrates present (Basso et al., 2010). The molecular weight of inulinase is said to be strictly related to the producing microorganism (Ricca et al., 2007).

4.1. Catalytic Properties of Inulinase

4.1.1. Effect of pH

The effect of pH on inulinase activity depends on the microbial source (Ricca et al., 2007). Fungal inulinase has an optimal pH range of 4.5-7.0, whereas the pH of yeast inulinase is between 4.4 and 6.5 and that of bacteria inulinase ranges from 4.8 to 7.0 (Ricca et al., 2007). The optimum pH of purified inulinase from fungi and yeast strains was reported to be in the range of 4.5-6.0 (Chi et al., 2009). Although the exo-inulinase from bacteria had an optimum pH of 6.0, the optimum pH of exo-inulinase from *Bacillus polymyxa* was 7 (Chi et al., 2009). The endo-inulinase activity from *Penicillium* sp TN-88 and *Arthrobacter* sp S37 was optimum at pH 5.2 and 7.5, respectively; these findings show that the optimal pH of bacteria is higher than that of fungi (Chi et al., 2009).

4.1.2. Effect of Temperature

Temperature is considered as an important parameter in the production of fructooligosaccharides. It has been shown to have a positive effect on the fructooligosaccharides synthesis when there is an increase from level -1 (40^{o}C) to +1 (50^{o}C) (Santos and Maugeri, 2007). Ricca et al. (2007) reported that the optimal temperatures for inulinases from bacteria and yeasts were usually higher than those from fungi. There is a need for a high thermal stable enzyme to favor inulin solubility (Ricca et al., 2007). The optimal temperature for the production of fructooligosaccharides from inulin using a recombinant endo-inulinase was found to be 55 and 50^{o}C in a batch and continuous system, respectively (R.S Singh and R.P Singh, 2010).

Table1.2. Effect of substrate type and enzyme source on the yield and type of fructooligosaccharides produced.

Article	**Enzyme**	**Substrate**	**Yield of FOS % /product obtained**
Zhengyu et al.,2005	*Aspergillus ficcum*	Inulin 5 %	70.37
Kim et al., 1997	*Pseudomonas* sp	Inulin 5%	75.60
Yokota et al., 1995	*Streptomyces* sp	Inulin 5%	71.00
Naidoo et al., 2009	*Xanthomanas campestris*	Inulin 3 %	60.00
Cho et al., 2001	*Xanthomonas oryzae*	Inulin 5%	74.90
Park et al., 1999	*Xanthomonas* sp	Inulin 5%	86.00
Chi et al., 2009	Recombinant yeast from *A.ficuum* and *S.Cerevisiae*	Inulin	1-kestose
Chi et al., 2009	*Penicillium* sp TN-88	Inulin	Inulotriose
Silva et al., 2013b	*K.marxianus* NRRL Y-7571	Inulin	Kestose, nystose and fructosyl nystose
Silva et al., 2013b	*K.marxianus* NRRL Y-7571	Sucrose	Kestose
Silva et al., 2013b	*Aspergillus niger*	Inulin	Kestose,nystose and fructosyl nystose
Silva et al., 2013b	*Aspergillus niger*	Sucrose	Kestose,nystose and Fructosyl nystose

5. Biocatalysis in Non-Conventional Media

The non-conventional media can favor the transfructosylation reaction over the hydrolysis reaction through an equilibrium controlled reaction approach. Due to simple preparation and milder reaction conditions, the production of fructooligosaccharides using enzymes in non-conventional media is more viable than that by chemical synthesis (Silva et al., 2013b).

The catalysis of enzymes in organic solvent systems has many advantages over that in aqueous systems, which include: (a) Increased solubility of hydrophobic substrates/products; (b) Elimination of microbial contamination; (c) Thermodynamic equilibrium favoring synthesis over hydrolysis; and (d) Suppression of water-dependent side reactions. Despite these advantages, the use of enzymes in organic media is still limited due to the denaturation and/or the inhibition of the biocatalyst by the organic solvent (Doukyu and Ogino, 2010). However, enzyme activity and stability in organic solvents can be improved by protein engineering as well as by physical and chemical methods, such as immobilization, entrapment and modification (Doukyu and Ogino, 2010).

Organic solvents are hydrocarbons of organic compounds containing mostly carbon atoms. The type of organic solvents can affect the enzyme catalytic activity. Various parameters, such as dielectric constant, dipole moment, hydrogen binding, polarizability and logarithm of partition coefficient (logP), can be used to correlate the enzyme activity with nature of organic solvent. Log P is used to demonstrate the solvent effect on the activity as well as the stability of the enzyme. Solvents with log P values greater than 4 (hydrophobic solvents) tend to have a lower inactivation effect on the biocatalyst (Choi and Yoo, 2012). Two main types of organic solvent systems exist based on the miscibility of an organic acid with water and the relative proportion of the solvent and water in the medium: (1) organic monophasic system; and (2) organic biphasic system (Doukyu and Ogino, 2010).

5.1. Organic Monophasic System

The organic monophasic system can be obtained upon the addition of water-miscible co solvents to the medium to promote the solubility of compounds that are insoluble in aqueous systems (Doukyu and Ogino, 2010). This system can favor more rapid reaction rates for hydrophobic compounds by decreasing the mass transfer limitations (Doukyu and Ogino, 2010). However, an increase in the concentration of organic co-solvent will always result in a decrease in the enzymatic

activity since the enzyme will be in direct contact with the organic solvent (Doukyu and Ogino, 2010).

5.2. Organic Biphasic System

The organic biphasic system consists of an aqueous phase containing a dissolved enzyme and a phase of an immiscible organic solvent (Doukyu and Ogino, 2010). The aqueous phase forms a separate layer in contact with the layer of the organic solvent through the interfacial area and the enzymatic reaction occurs in the aqueous phase where the enzyme is present (Doukyu and Ogino, 2010). This system is used mostly to shift the reaction equilibrium towards synthesis. The main advantages of the biphasic system include the simple preparation, the easy regeneration of enzyme and the easy separation of products. In addition, the enzyme is quite stable in this system due to its limited direct contact with the organic solvent (Doukyu and Ogino, 2010).

5.3. Characteristics of Organic Solvents

The deep eutectic solvents are mixtures of an ammonium salt and a hydrogen bond donor, like chloride. They possess various characteristics, such as a melting point below room temperature, high thermal stability and low volatility (Gorke et al., 2008). Ionic liquids consist of ions, large quaternary ammonium cations (R_4N^+) and large anions, such as PF_{6-} and BF_{4-} (Gorke et al., 2008). These solvents are liquid below 100°C. They are used as solvents in some applications, such as biocatalysis. As compared to conventional organic solvents, ionic liquids have an extremely low vapor pressure, a wide liquid range, possess low flammability and have high ionic and thermal conductivities.

6. Maple Syrup

6.1. Maple Syrup Production and its Challenges

Canada and United States are the main producers of maple syrup worldwide. In Canada, the province of Quebec is the largest maple syrup producer and is considered to be contributing to 75% of the worldwide production. The central and eastern parts of Quebec are the regions where most of the production is concentrated. According to Agriculture and Agri-Food Canada statistics of 2014, Quebec has produced 8584 (thousands of gallons) of maple syrup with a gross value of

321,700 (thousands of Canadian dollars) of maple products. It is also the largest exporter of maple products, with 95.28% of the total Canadian export sales.[1]

However, there are certain challenges that are associated with maple production, which have major impacts on the maple industry. These challenges are climatic change, fluctuation in oil pricing, change in dietary trend, imbalance between the demand and supply chain, and competitiveness of the market. In Canada, due to the climate change (higher temperatures and warmer weather), maple producers are faced with earlier tapping dates and a shorter season leading to drastic changes in regional production. On the other hand, in warmer regions, maple plants are prone to pest and diseases. The fluctuation in oil pricing also affects the maple industry. An increase in oil price will result in an increase in the operating cost of maple farms, which is considered as an important challenge to the farmers. Maple producers also face a very competitive market with various cheaper types of sweeteners, such as honey, sugarcane and corn syrup. In addition, in the past decade, there has been an imbalance between the demand and supply of maple products due to the high production rate compared to the domestic sales and exports (Agriculture and Agri -Food Canada[2]

6.2. Composition and Quality of Maple Syrup

Maple syrup is composed primarily of sucrose (68%) and small amounts of glucose (0.43%, w/w) and fructose (0.34%, w/w) (Stuckel and Low, 1996). Maple syrup also contains certain vitamins and minerals, such as thiamin, niacin, riboflavin, folic acid, biotin, pyridoxine, calcium, manganese, magnesium, zinc and potassium (Perkins and Van den Berg, 2009). In addition, nitrogenous and phenolic compounds (0-0.0001kg/m^3) are found in maple syrup (Underwood et al., 1961; Kermasha et al., 1995; Ball, 2007; Li, 2015); these phenolic compounds are responsible for the distinct unique flavor of maple syrup and for their antioxidant and antimutagenic properties in maple products (Theriault et al., 2006; Li, 2015).

"According to the Canada Agricultural act, the maple products must be obtained exclusively by the concentration of maple sap or maple syrup, excluding substitutes and maple syrup must be

[1] http://www.agr.gc.ca/eng/industry-markets-and-trade/statistics-and-market-information/by-product-sector/horticulture/horticulture-canadian-industry/sector-reports/statistical-overview-of-the-canadian-maple-industry-2014/?id=1447784793831

[2] http://publications.gc.ca/collections/collection_2014/aac-aafc/A71-35-2007-eng.pdf

obtained exclusively by the concentration of maple sap or by the dilution or solution of maple product in potable water". The Canadian food inspection agency is the board that controls and regulates the safety and quality of maple syrup throughout Canada (Li, 2015). Their role is to ensure that the maple producers are following the federal standards. The standards in Canada comprised of 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., 2007; Li, 2015). Moreover, pure maple syrup must follow the strict standards for soluble solids, density, clarity, color, flavor and microbial counts (Aider et al., 2007; Li, 2015). Besides its standards on color grading, maple syrup is also graded based on its flavor components. The lightest syrup (Canada No. 1 extra light, AA) has a generally sweet flavor but with only a slight hint of maple flavor whereas the lighter-color syrups tend to contain lower levels of flavor compounds than darker syrups (Perkins and van den Berg, 2009; Li, 2015).

6.3. New Applications and Diversification of Maple Products

In addition to its traditional use as a natural sweetener various studies have shown that maple syrup can have many potential new applications. In 2008 and 2009, the Center Acer Inc. in Quebec, had undergone various research studies on maple. The impact of air injection on the properties of color development and taste characteristics of maple syrup was investigated. In addition, a study was also carried out in collaboration with Laval University in Quebec on the use of probiotic bacteria for the development of a new maple sap drink, which can promote health. Maple sap has been used as a base in a study for the synthesis of a natural biodegradable polymer; using the bacteria *Alcaligenes latus,* the sucrose in sap was converted through a fermentation process into a biologically inert polyester, which could be used in medical applications, such as surgical sutures (Yezza et al., 2007). Another study by Cochu et al. (2008) showed that maple sap was used as a substrate and a good carbon source to promote the growth of *Lactobacilli* to produce lactic acid for the development of nondairy probiotic drink. Zeng et al. (2011) investigated the production of bacterial cellulose by optimizing the culture conditions using maple syrup as an alternative carbon source to traditional glucose, fructose and sucrose. These authors concluded that maple syrup was a more suitable carbon source for this experiment than traditional glucose, fructose and sucrose. Li and Sreeram (2011), isolated Quebecol, a novel phenolic compound, from the Canadian maple syrup.

7. Structural Characterization of End-Products

7.1. Thin Layer Chromatography (TLC)

TLC is a type of liquid chromatography, in which the mobile phase is liquid and the stationary phase is a thin layer of material on top of a flat plat (Santiago et al., 2012). It is used in many research and industrial applications, including clinical analysis, industrial chemistry and food chemistry (Santiago et al., 2012). TLC possesses many advantages, which include quick development time, simplicity, high sensitivity, good reproducibility and low cost. It can be used to separate one compound as well as to detect and characterize sugars in complex mixtures. (Santiago et al., 2012; Sherma, 2003; Li, 2015). Sucrose and fructans can be characterized using TLC and a reflectance densitometry method, where glass-backed TLC plates are developed in a solution of propanol–ethyl acetate–water (45:35:20, v/v) at 30°C for 3.5 h. For the detection of fructans, the plates are sprayed with urea and phosphoric acid and then heated at 110°C for 6 min (Reiffov´a et al., 2006). For the characterization of fructooligosaccharides, TLC plates were developed in a mobile phase of butanol/acetic acid/deionized water (5:4:1, v/v/v). After their spraying with a resorcinol solution in acetic acid (0.1% w/v resorcinol and 0.25% w/v thiourea acid) or a sulfuric acid solution in methanol (2%, v/v), the plates are heated at 100°C for 2h in order to identify the fructooligosaccharides corresponding to yellowish and brown spots (Park et al., 2003; Tian et al., 2011; Li, 2015). A modern instrumental TLC can be used for a more rapid method to quantify fructooligosaccharides. The instrumental TLC involved the use of acetonitrile and acetone as solvents and diol high performance TLC plates. However, derivatization, using 4-aminobenzoic acid reagent, glacial acetic acid, water, 5% phosphoric acid and acetone, was necessary. Plates are then heated at 115^{o}C for 15 min in order to identify the fructooligosaccharides corresponding to yellowish and brown spots (Vaccari et al., 2001; Inthanavong, 2011).

7.2. High-Performance Anionic Exchange Chromatography with Pulsed Amperometric Detection (HPAEC-PAD)

Carbohydrates can be analyzed using a high performance anion-exchange chromatography with a pulsed amperometric detector (HPAEC-PAD). HPAEC-PAD is a simple and rapid quantifying method requiring small sample volumes and minimal sample preparation (Hogarth et al., 2000; Inthanavong, 2011). It is considered as an effective and reliable method to separate carbohydrates with different complex matrices due to the combination of the liquid chromatography with the electrochemical detection (Cataldi et al; 2000). CarboPac PA 100 is the analytical anion exchange

column commonly used for the analysis of fructooligosaccharides by HPAEC-PAD. It is packed with macroporous resin composed of co-polymerized ethylvynil- and divylbenzene making it more suitable for the isolation of both mono and oligosaccharides than the divylbenzene-packed CarboPac PA10 column (Dionex Corp., 1995; Inthanavong, 2011). The CarboPac PA200 can also be used to isolate fructooligosaccharides by using high concentration of saturated sodium hydroxide and sodium acetate (Inthanavong, 2011). Borromei et al. (2009) used a CarboPac PA 100 analytical anion exchange column to detect fructooligosaccharides with degree of polymerization of 3 to 10 and inulin. The separation is carried out by a gradient solvent system, using water as solvent (A), 600 mM aqueous sodium hydroxide solution as solvent (B) and 500 mM aqueous sodium acetate solution as solvent (C) (Li, 2015). However, an important drawback is the formation of sodium carbonate from the reaction between carbon dioxide and sodium hydroxide. To prevent this reaction from occurring, the eluent solution must be kept saturated with nitrogen (Cataldi et al., 1999; Li, 2015).

CONNECTING STATEMENT 2

A comprehensive literature review on the prebiotics, the enzymatic synthesis of fructooligosaccharides, the effects of different reaction parameters was discussed in chapter I. Chapter II describes the different materials and methods that were used to carry out the study. This includes the purification of the enzyme, the enzymatic assays, the reactions catalyzed in the biphasic media, the characterization of product spectrum and the optimization of the enzymatic reaction of endo-inulinase in butyl acetate/buffer biphasic media. It also describes the synthesis of fructooligosaccharides in a maple syrup based biphasic media and the characterization of the end product profile.

CHAPTER II

MATERIALS AND METHODS

2.1. Sources of Chemicals

The chemical reagents purchased from Sigma Chemical Co. (St Louis, Missouri) were Inulin, 3,5-dinitrosalicylic acid (DNS), D-fructose, D-glucose, D-maltose, α-lactose, D-raffinose, sucrose, 2-heptanone, dextran standards (50-670 kDa), acetic acid and sodium acetate. Cyclohexane, ethyl acetate, heptane, sodium chloride and potassium phosphate dibasic (K_2HPO_4) were obtained from Fisher Scientific (Fair Lawn, New Jersey). Carbohydrate standards, including 1-kestose, nystose, and 1^F-fructosylnystose, were purchased from Wako Pure Chemical (Osaka, Japan). The organic solvents N-butyl acetate and 2-Octanone were obtained from Acros Organics (New Jersey, USA). Potassium phosphate monobasic (KH_2PO_4) was purchased from MP Biomedicals (Fair Lawn, New Jersey) and sodium hydroxide (NaOH) from Fluka Chemicals (Fair Lawn, New Jersey). Maple syrups 15°Bx, 30°Bx and 66°Bx were kindly provided by the Centre de recherche, de développement et de transfert technologique acéricole inc. (Centre ACER, St Hyacinthe, Quebec). The commercial endo-inulinase produced from *Aspergillus niger* was purchased from Sigma Chemical Co. (St-Louis, Missouri).

2.2. Purification of Endo-Inulinase

The commercial endo-inulinase from *A. niger* may contain exo-inulinase enzyme. To purify the endo-inulinase, the enzyme preparation was subjected to an anion exchange chromatography on a MonoQ column at a flow rate of 0.6 mL/min. The column was conditioned using potassium phosphate buffer (20 mM, pH 6.0) as the running buffer. After injection of the samples, the column was first washed with the running buffer (2 column volumes, cv), and a NaCl elution gradient from 0 to 0.5 M (4 cv) was applied thereafter. The purified enzyme was dialyzed against 5 mM of potassium phosphate buffer (pH 6.0) using a membrane with a 5-6 kDa cut-off for 48 h at 4°C. The dialyzed enzyme was freeze dried and stored at -80°C. The endo-inulinase activity and the total protein content of the purified endo-inulinase were assessed as indicated here below.

2.3. Determination of Protein Content

The total protein content of the purified endo-inulinase was measured using the Standard BioRad Protein Assay. A volume of 20 µl of the sample was added into 1 mL of a 25% (v/v) diluted Bradford Dye. The absorbance was read at 595 nm, using a spectrophotometer. The concentration

of protein was then estimated, using a standard curve of bovine serum albumin (0.0625 to 1 mg/mL).

2.4. Enzymatic Assays of Inulinase Activity

The endo-inulinase activity assays were carried out, using sucrose and inulin as substrates. One unit of total endo-inulinase activity was defined as the amount of biocatalyst to liberate 1 μmol of the reducing sugars (glucose and fructose) from sucrose per min at the standard assay conditions. One hydrolytic unit of endo-inulinase is defined as the amount of the biocatalyst to produce 1 μmol of fructose per min, whereas one transfructosylation unit of endo-inulinase is defined as the amount of the biocatalyst to release 1 μmol of glucose per min as a result of transferring fructose. The subtraction of the total amount of fructose from that of glucose will provide the amount of glucose resulting from the transferring fructose.

To initiate the enzymatic reaction, the enzyme suspension (0.0025- 0.05g/μl) was added to the substrate solution at a ratio of 1:1 (v/v). The final concentration of substrates was set at 2% (w/v). Blanks containing all components except substrate or enzyme were conducted in tandem with the enzymatic reactions. The reaction mixtures were incubated at 45ºC for 20 min, and thereafter, the dinitrosalicylic acid (DNS) solution, consisting of a 1% (w/v) DNS in 1.6% (w/v) NaOH, was added at a ratio of 1:1 (v/v) in order to quantify the amount of total released reducing sugars. The mixture was then boiled in water for 5 min, and a 50% (w/v) of potassium sodium tartrate solution, a color stabilizer, was added at a ratio of 1:2 (v/v). The absorbance of the resulting mixture was measured at 540 nm (DU800 spectrophotometer, Beckman Coulter). A standard curve was constructed, using fructose as a standard (0.5 to 10 mM). The specific activity was expressed as the enzymatic unit of endo-inulinase per mg of protein.

The hydrolytic and transfructosylation activities of endo-inulinase were measured by quantifying glucose and fructose, respectively using a Dionex ICS-3000 high pressure anionic exchange chromatography system equipped with pulsed amperometric detector (HPAEC-PAD), a CarboPac PA20 column (3 × 150 mm) and a Chromeleon Software version 7.0. An isocratic elution of the reaction components was carried out using a 10 mM NaOH solution as the mobile phase at a flow rate of 0.5 mL/min for a total elution time of 30 min. The concentration of each product was determined by constructing standard curves of glucose and fructose. All assays were run in duplicates.

2.5. Transfructosylation Reaction-Catalyzed by Endo-Inulinase in Biphasic Systems

The transfructosylation reaction of sucrose was carried out according to a modification of the method described by Li et al. (2014). Prior to each enzymatic reaction, a stock solution of sucrose (1.2 M) was prepared in a sodium acetate buffer solution (0.1 M, pH 5). An organic solvent was added to the reaction mixture to achieve a solvent: buffer ratio of 1:3 (v/v). The transfructosylation reaction was initiated by the addition of purified endo-inulinase (4-10 U/ml) to the reaction mixture. The reactions were carried under vacuum at 40^{o}C, with continuous shaking at 150 rpm for up to 120 h of reaction. However, the temperature was 35^{o}C when heptanone was used as the organic solvent. Aliquots of 1 ml reaction mixture were collected and boiled for 5 min to stop the enzymatic reaction. Ethanol was then added to the recovered aliquot at a ratio of 1:1 (v/v) to precipitate the enzymes. Control reactions, containing all components except endo-inulinase, were carried out in tandem with the enzymatic reactions. The sugar profile was analyzed using the Dionex HPAEC-PAD system as described previously.

2.6. Effect of Type of Biphasic System on the Transfructosylation Reaction Time Course

The effect of biphasic systems on the transfructosylation reaction-catalyzed by endo-inulinase was investigated over a time course of 120 h. The organic solvents studied were butyl acetate, cyclohexane, ethyl acetate, heptane, heptanone octanone and hexane, with a logarithm of the 1-octanol/water partition coefficient (log *P*) of 1.25, 2.67, 0.28, 3.58, 2.14, 2.4 and 3.6, respectively. The transfructosylation enzymatic reactions were carried as described previously.

2.7. Identification and Characterization of End Product Spectrum

The oligosaccharide end products obtained upon the transfructosylation of sucrose by endo-inulinase in selected biphasic systems were determined using a Dionex ICS-3000 high performance anion exchange chromatography (HPAEC) system equipped with a pulsed amperometric detector (PAD) and a CarboPac PA 200 column (3 × 250 mm). The integration was carried out using the Chromeleon Software version 7. The elution of the reaction components was carried out using a linear gradient of sodium acetate from 0 to 0.2 M in 0.1 M NaOH at a flow rate of 0.5 ml/min for 20 min. Standard curves were constructed, using selected saccharides (D-fructose,

D-glucose, sucrose, maltose, raffinose, lactose, 1-kestose, nystose, and 1^F-fructosylnystose). In order to identify the peaks, 1-kestose, nystose and 1^F-fructosylnystose were used as internal standards.

On the other hand, a high performance size exclusion chromatography (HPSEC) was applied to characterize the profile of the polysaccharide end products obtained from the transfructosylation of sucrose in selected biphasic systems. Aliquots of 20 µl of reaction mixtures were recovered after 48-120 h of transfructosylation reactions. Ethanol was then added to the recovered aliquot at a ratio of 1:1 (v/v) to precipitate the enzyme. The samples were centrifuged at 14,000 rpm for 4 min and then subjected to an HPLC system (Waters 1525) equipped with a refractometer 2489 detector and three size exclusion columns sequentially, TSKgel G3000PWXL-CP, TSKgel G4000PWXL-CP and TSKgel G5000PWXL-CP. The elution of the reaction components was carried out using a buffer solution of 0.1 M NaCl at a flow rate of 0.4 ml/min for 90 min. Dextrans (50, 150, 270, 470 and 670 kDa), 100 mM sucrose and 100 mM raffinose were used as standards. The standard curve was constructed by plotting the log of molecular weight versus the elution time.

2.8. Enrichment of Maple Syrups with Fructooligosaccharides using Endo-Inulinase as Biocatalyst

The efficiency of endo-inulinase from *A. niger* in maple-based reaction systems was assayed. Three maple syrups 15°Bx, 30°Bx and 66ºBx were prepared from the same maple sap by ACER center, and these syrups were used as reaction media and sucrose source. The reaction time courses in these maple-based systems were investigated at 40ºC and 150 rpm. The reactions were initiated by adding endo-inulinase enzyme (6 U/ml) in the medium comprised of a mixture of butyl acetate and maple syrup at a ratio of 1:2 (v:v). Aliquots of enzymatic reactions were withdrawn at selected time intervals within 80 h time period. To stop the reaction, the aliquots were boiled for 5 min and then kept at -80ºC. To determine the total sucrose conversion yield and the concentration of transfructosylation products, the HPAEC on Dionex (ICS-3000) chromatography system with a pulsed amperometric detector and a CarboPac PA 200 column (3 × 250 mm) was used. The integration was carried out using the Chromeleon Software version 7. The elution of the reaction components was carried out using a linear gradient of sodium acetate from 0 to 0.2 M in 0.1 M NaOH at a flow rate of 0.5 mL/min for 20 min. Standard curves were constructed using the selected

saccharides (fructose, glucose, sucrose, maltose, raffinose, lactose, 1-kestose, nystose, and 1^F-fructosylnystose).

2.9. Optimization of Fructooligosaccharides Production by Response Surface Methodology

2.9.1. Experimental Design

Optimization of the synthesis of fructooligosaccharides using sucrose and butyl acetate as solvent 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, 2.5-9.5 U/ml), reaction time (X_2, 10-120 h) and solvent proportion (X_3, 10-40 %). The full design consisted of 10 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 22 sets of experiments (Table 2.0). The response variables were kestose, nystose, fructosyl nystose and bioconversion yield. The analysis of the reaction components was carried out by HPAEC-PAD (Dionex).

2.9.2. Statistical Analysis

The experimental data was obtained based on the CCRD and regression was carried out and fitted to quadratic equations using the software Design-Expert 8.0.7 (Stat-Ease, Inc. Minneapolis, MN, USA).

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

Where Y are the predicted responses for kestose, nystose, fructosyl nystose and bioconversion yield;

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.

Table 2. Variables and their coded levels used in a central composite rotatable design for optimization of fructooligosaccharides production in butyl acetate/buffer system, using sucrose as substrate.

	Coded values				
Variables	-α	-1	0	+1	+α
X_1:enzyme unit	2.5	3.92	6	8.08	9.5
X_2:reaction time	10	32.3	65	97.7	120
X_3:proportion of solvent	10	16.08	25	33.92	40

CONNECTING STATEMENT 3

The different materials and methods that were used throughout this study was described in chapter II. The endo-inulinase catalyzed transfructosylation reaction was investigated in the biphasic medium and the optimization of this reaction was studied. Maple syrup with different brix values were evaluated as sucrose source and reaction media. In chapter III, the results of the bioconversion yield and the end-product profiles in selected biphasic media were presented and discussed.

CHAPTER III

RESULTS AND DISCUSSION

3.1. Purification of Inulinase

The endo-inulinase enzyme from *A. niger* was purified and its substrate specificity was assessed using inulin and sucrose as substrates. Figure 1 shows the elution profile of an anionic exchange chromatogram of endo-inulinase from *A. niger* using a MonoQ column. Using NaCl as the elution gradient, the big peak eluted in the beginning of the chromatogram (Figure 1) can be considered as other protein of bigger molecular weight and the two distinct peaks (labelled as Peak #1 and #2) are the ones expressing inulinase activity at 0.35 - 0.37 M of NaCl concentration, respectively.

The enzymatic activity of the recovered peak using inulin and sucrose as substrates are summarized in Table 3.1. When sucrose was used as a substrate, the enzymatic activity expressed in Peak#1 and Peak#2 was 100.3 and 2.4 μmol/ml.min respectively. However, when inulin was used as the substrate, the enzymatic activity towards inulin was higher in Peak#2 (416.4 μmol/ml enzyme.min) than in Peak# 1 (335.8 μmol/ml enzyme.min). These results show the high specificity of the enzyme activity expressed in Peak#2 towards the inulin than sucrose. The low reducing ends generated upon the hydrolysis of inulin by endo-inulinase present in Peak#2 also reveal its endo-mode action. While the enzyme activity expressed in Peak#1 found to be more of exo-action mode, corresponding to the exo-inulinase. These experimental findings are in agreement with those reported by Fawzi (2011) who showed that endo-inulinases are specific for inulin, whereas exo-inulinases can split off terminal fructose units in sucrose, raffinose and inulin to release fructose.

The specific activity of the crude commercial inulinase enzyme was estimated to be 1,259.6 μmol/mg protein.min, while that of the pure endo-inulinase enzyme was found to be 10,372.0 μmol/mg protein.min (Table 3.1). This reveals that a purification factor of 8.2 was achieved upon the purification of endo-inulinase enzyme from *A. niger*. using an anionic exchange chromatography. A study carried out by Kang et al. (1998) showed that an endo-inulinase from the *Arthrobacter sp. S* 37 was purified by a factor of 16 when it was applied on DEAE-cellulose column. On the other hand, Skowronek et al. (2006) carried out the purification of endo-inulinase from *A. niger* 20 OSM on DEAE Sepharose column and obtained a purification factor of 14.

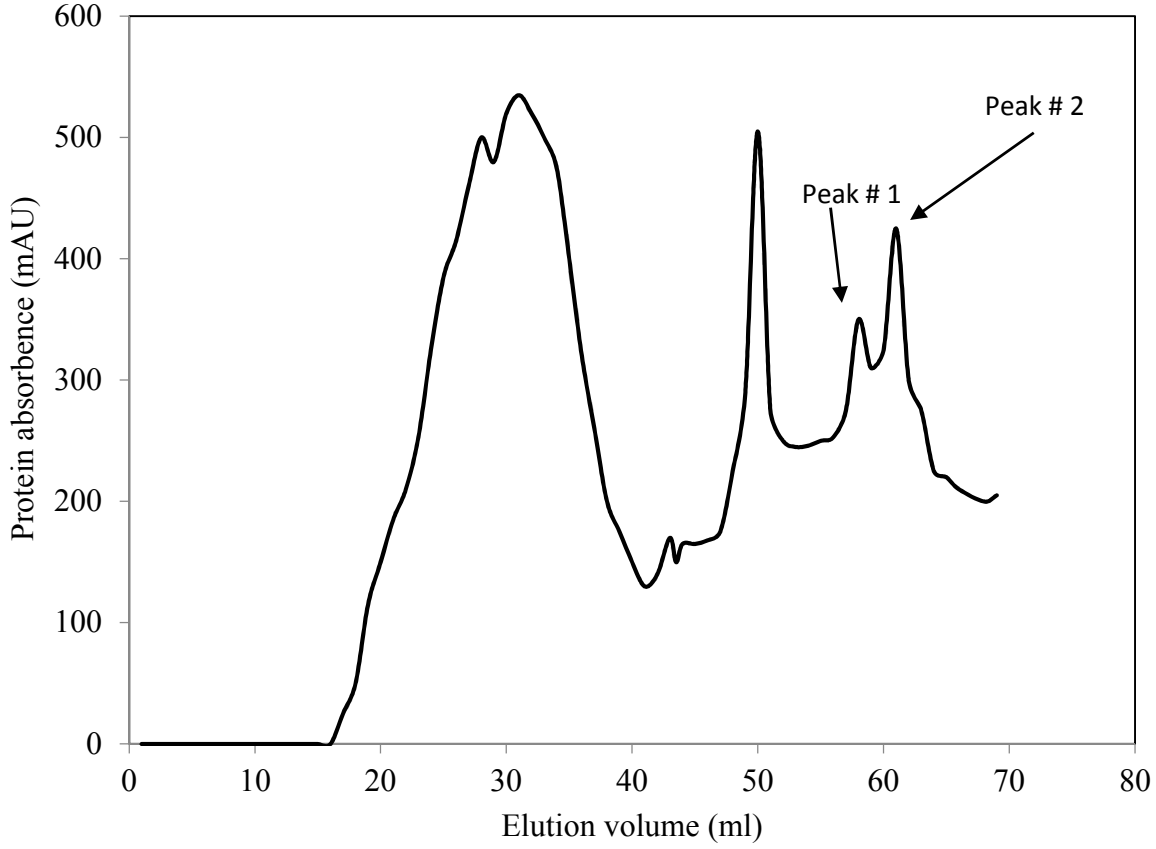


Figure 1. Anionic exchange chromatogram of the extract of endo-inulinase from *Aspergillus niger* on a MonoQ column. Peaks#1 & 2 were identified as exo- and endo-inulinase, respectively.

Table 3.1. Enzymatic activity of the recovered peaks and of the purified endo-inulinase

	Inulinase activity (μmol/mg protein.min)	
Substrate	Sucrose	Inulin
Peak #1	100.3	353.8
Peak #2	2.4	416.4
Specific activity of Enzyme (μmol/mg protein.min)		
Crude Inulinase	Purified Endo-inulinase	
1,259.6	10,372.0	

3.2. Time Courses for Endo-Inulinase-Catalyzed Sucrose Conversion in Biphasic Media

An organic solvent/buffer biphasic system and a high sucrose concentration (1.2 M) were used in order to favor the transfructosylation activity of endo-inulinase. However, the type of organic solvent can affect the enzymatic activity and lead to low conversion of sucrose. The time course for the total conversion of sucrose (hydrolysis & transfructosylation) by the purified enzyme, endo-inulinase from *A. niger* which was recovered from peak # 2(Figure 1) was catalyzed in selected biphasic media was investigated (Figure 2). Hexane (Log P 3.6), heptane (Log P 3.58), butyl acetate (Log P 1.25), cyclohexane (Log P 2.67), octanone (Log P 2.4) and heptanone (Log P 2.14) were used to construct the biphasic media at an organic solvent to buffer ratio of 1:3 (v:v). In addition to shifting the reaction equilibrium, the use of organic solvent-based systems may offer other advantages such as the decrease in the substrate inhibition and/or the easy recovery of the end-products (Illanes and Barberis, 1994).

The time course of 24 h for the conversion reaction of sucrose using purified enzyme, endo-inulinase, measuring the release of the reducing-ends as an indicator the conversion are illustrated in Figure 2. The results show the time course profile of the conversion of sucrose by endo-inulinase was dependent on the type of the biphasic media. In all selected biphasic media with the exception of hexane, the conversion reaction of the sucrose by endo-inulinase proceeded in two phases, in which the initial period of high rate showed a burst phase and was followed by a steady state phase with a slower linear reaction rate. The burst phase amplitude, in terms of rate and saturation, was dependent on the type of solvent, while the rate of steady state phase varied within a narrow range with the type of solvent. The two phase-reaction behavior may be attributed to the catalysis of two simultaneous reactions (hydrolysis & transfructosylation) by endo-inulinase. The presence of burst phase may also reflect the elapsing time needed for the enzyme to become saturated with substrate before reaching the linear reaction velocity. Similarly, exocellulase which hydrolyzes cellulose by sequential cleaving of soluble sugars from one end of a cellulose strand, undergo an initial burst effect, which is followed by a pronounced slowdown in the presence of a large amount of substrate (Praestgarrd et al., 2011). Another study carried out by Kipper et al. (2005) showed that the hydrolysis of cellulose by cellobiohydrolase Cel7A from *Trichoderma reesei* had a burst behavior when the first cellobiose molecule was released from each cellulose strand.

Contrary to other biphasic systems, the reaction of sucrose conversion in hexane and cyclohexane based-ones reached a plateau at the end of the reaction stage. This limited release of reducing ends may be due to the enzyme denaturation by specific interactions with the solvent (Amorim and Halling, 2002) and/or to the reaction having reached its equilibrium (Hadzir et al., 2001). The highest amount of reducing ends (621.92 mM) released was observed with butyl acetate with the lowest Log P of 1.25, whereas the lowest one (69.62 mM) was obtained with hexane with a Log P of 3.6. Cyclohexane, octanone and heptanone with relatively similar Log P (2.1 to 2.67) exhibited more or less similar initial reaction velocity (680 to 857.50 µmol/ml.min) and reached a maximum reducing end concentration of 307.54 to 444.86 mM (Table 3.2). These results may reveal the non-significant effect of solvent-enzyme interactions on the catalytic efficiency of endo-inulinase in the presence of cyclohexane, octanone and heptanone. The highest initial velocity of (1500 µmol/ml.min) was observed in the heptane (Log P of 3.58)/buffer biphasic system, followed by the butyl acetate (Log P of 1.25)/buffer biphasic system with an initial velocity of (1006.67 µmol/ml.min). Contrary to the initial velocity, the highest release of reducing end concentration was achieved in the butyl acetate/buffer biphasic system (621.92 mM) than in the heptane-based one (444.86mM). Although hexane has a high Log P value of 3.6, it resulted in the lowest initial velocity (69.62 µmol/ml.min) and reducing end concentration (18.07mM).

The experimental findings (Table 3.2) are not in agreement with those reported by Laane et al. (1987) who indicated that the most suitable organic solvents for the enzymatic synthesis are those with relatively high Log *P* values (Log $P > 2.5$). Generally, a concomitant increase in solvent hydrophobicity, as denoted by higher Log P values, is expected to lead to higher catalytic efficiency. This trend was attributed to the fact that the hydrophilic solvents with lower Log P values have higher tendency to strip off the water from the surface of the enzyme, which is essential for its catalytic activity (Halling, 2002). However, the overall findings (Figure 2 and Table 3.2) indicate clearly that there was no correlation between the catalytic efficiency of endo-inulinase and the Log P value of the organic solvent in the biphasic system. This may be due to (a) the use of organic solvent/buffer mixture media in which the enzyme-bound water interaction may have not been affected by the presence of solvent and to (b) the presence of the direct effect of the organic solvent on the enzyme itself by binding in or near its active site, affecting its catalytic efficiency.

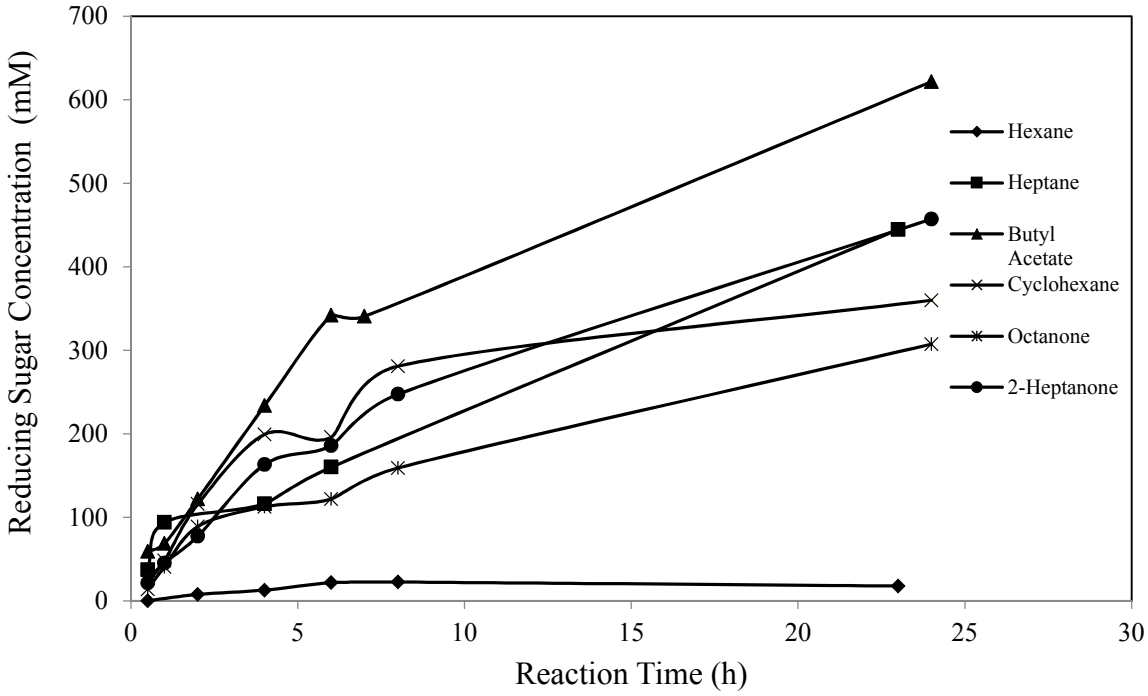


Figure 2. Time courses for the conversion reaction of sucrose catalyzed by purified endo-inulinase from *Aspergillus niger* in biphasic media composed of buffer and hexane (♦), heptane (■), butyl acetate (▲), cyclohexane (×), octanone (x) or heptanone (•) as the organic solvent.

Table 3.2. Reaction Selectivity (Transfructosylation vs. hydrolysis) of endo-inulinase from *Aspergillus niger* in selected biphasic media.

Organic Solvent	Log P	Initial Velocity (μmol/ml.min)	Reducing-End Concentrations (mM)	Converted Sucrose (%)[a]	Transfructosylation Relative Proportion (%)[b]	Hydrolysis Relative Proportion (%)[c]
Heptane	3.58	1500.00	444.86	73.53	79.34	20.69
Butyl acetate	1.25	1006.67	621.92	93.94	43.33	56.66
Cyclohexane	2.67	857.50	360.31	55.97	47.42	52.58
Octanone	2.4	828.33	307.54	61.28	21.15	78.85
Heptanone	2.14	680.00	457.60	74.62	42.45	57.55
Ethyl acetate	0.28	-	ND	73.85	61.14	38.86
Hexane	3.6	69.62	18.07	-	-	-

[a]The total converted sucrose concentration was determined after 24 h of reaction.

[b]Transfructosylation proportion was calculated from the amount of biocatalyst that releases glucose as a result of transferring fructose. The subtraction of the total amount of fructose from that of glucose provides the amount of glucose resulting from transferring fructose.

[c] Hydrolysis relative proportion is the amount of biocatalyst that produces fructose.

It is known that organic solvent has an impact on the binding of substrates at the active site of the enzyme by changing the actual K_m values (Dordick, 1989). The presence of solvent may have also affected the substrate partition between the micro/microenvironment of enzyme and hence the availability of substrate and products (Adlercreutz, 2000). Similar results have been reported for the porcine pancreatic lipase-catalyzed esterification of tributyrin with hexanol, using mixtures of hexane and a wide range of co-solvents (Van Tol et al., 1995) and for the chorophyllase-catalyzed the hydrolysis of chlorophyll in neat organic solvent (Arriagada et al., 2007).

3.3. Reaction Selectivity of Endo-Inulinase in Selected Biphasic Systems

The endo-inulinase can simultaneously catalyze the transfructosylation and the hydrolysis reactions by transferring the fructosyl unit from sucrose into water and/or a fructosyl-based acceptor, respectively. The reaction selectivity (transfructosylation vs. hydrolysis) of endo-inulinase can be likely affected by the reaction conditions, such as substrate concentration (e.g. sucrose), reaction temperature, and type of organic solvent. To assess the effect of selected biphasic systems on the reaction selectivity of endo-inulinase, the total yield of converted sucrose and the extent of the hydrolysis vs transfructosylation reactions were investigated. Indeed, the conversion of sucrose by endo-inulinase can result in glucose and fructose, which can be incorporated into the acceptor products fructooligosaccharides. Therefore, the concentration of glucose reflects the total amount of sucrose converted. Because the free fructose cannot act as donor or acceptor substrates, its concentration can be used for the estimation of the extent of the hydrolysis of sucrose. By subtracting the concentration of free fructose from that of glucose, the extent of the transfructosylation reaction catalyzed by endo-inulinase can be determined.

The yield of converted sucrose as well as the relative proportion of the hydrolysis and the transfructosylation reactions are summarized in Table 3.2. The overall yields of converted sucrose are in a good agreement with those of reducing ends concentration. The results also show that the endo-inulinase catalyzed both the hydrolysis and transfructosylation reactions in all the investigated biphasic systems but at different extents. Endo-inulinase in the butyl acetate/buffer biphasic system led to the highest converted sucrose of 93.94%; however, the extent of the hydrolysis reaction (56.66%) in this medium was higher as compared to the transfructosylation one (43.33%). The use of heptane, heptanone and ethyl acetate with different Log P values of 3.58, 2.14 and 0.28, respectively, led to a comparable amount of converted sucrose, which was around

74.0%. The transfructosylation reaction was more predominant in heptane (79.34%) and ethyl acetate/buffer (61.14%) biphasic systems. In the heptanone/buffer biphasic system, only 42.45% of sucrose was converted into the transfructosylation products, the rest (57.55%) was hydrolyzed. Contrary to other biphasic systems, the octanone/buffer system favored the hydrolysis (78.85%) reaction over the transfructosylation reaction (21.15%). These differences may be due to the occurrence of some conformation changes in the presence of octanone and/or to its binding in or near the enzyme active site. In contrast, the use of heptane and ethyl acetate improve the ability of endo-inulinase from *A. niger* to use more efficiently the growing fructosyl chain as an acceptor, shifting the reaction further towards the transfructosylation side. Similarly, a research conducted by Risso et al. (2010) showed that after screening different organic solvents like cyclohexane, n-heptane, ethyl acetate and heptanone, the highest fructooligosaccharides yield of 3.9 % (w/w) was achieved, when butyl acetate was used. The reaction selectivity of purified endo-inulinase was also found to be dependent on the type of organic solvent used in the biphasic system.

Santos and Maugeri (2007) carried out the synthesis of fructooligosaccharides from sucrose in stirred reactors using the enzyme inulinase from *Kluyveromyces*. *marxianus* var. *bulgaricus*. These authors reported that there was an increase in the concentration of kestose, nystose, glucose and fructose over the reaction time course; however, as the reaction was proceeded, the fructooligosaccharides undergo hydrolysis with a gradual increase in glucose and fructose concentration. Indeed, a high concentration of 50g/L fructooligosaccharides was obtained at 3-4h reaction time, and all the fructooligosaccharides were hydrolyzed at 7h. It has been reported that the release of glucose and fructose may inhibit the transfructosylation activity (Santos and Maugeri, 2007). On the other hand, Park and Almeida (1991) have investigated the production of fructooligosaccharides by β fructofuranosidase from A. *niger* and reported that during a time course of 80h, the enzymatic reaction showed an initial rapid (77 -86 %) hydrolysis of sucrose in the reaction mixture as well as the formation of kestose. A study was carried out by Tian et al. (2014) on the synthesis of fructoololigosaccharides and levan with combined use of levansucrase from *Bacillus amyloliquefaciens* and endo-inulinase from *A. niger.* The authors reported that during the early stage of reaction (2h), 97% of sucrose substrate was consumed and 60-66% transfrusctosylation reaction was favored over hydrolytic. However, as the reaction time increased, there was more hydrolytic reaction. During the first 1.5h of reaction time, the transfructosylation

products reached a maximum of 51.6-61.7%, but after 2.5h there was a decrease in transfructosylation products.

3.4. Effect of Biphasic Media on the Enzymatic Synthesis Fructooligosaccharides

The HPAEC-PAD chromatogram of the reaction mixture is illustrated in Figure 3. The end-product profiles of fructooligosaccharides were identified by comparing their retention times with those of inulin-type fructooligosaccharide standards. 1-kestose (peak1, O-β-D-fructofuranosyl-(2→1)-O-β-D-fructofuranosyl-(2→1)-α-D-glucopyranoside),nystose(peak2,O-β-D-fructofuranosyl-(2→1)-O-β-D-fructofuranosyl-(2→1)-O-β-D-fructofuranosyl-(2→1)-α-D-gluco-pyranoside) and 1^F-fructosyl-nystose(peak3,O-β-D-fructofuranosyl-(2→1)-O-β-D-fructofuranosyl-(2→1)-O-β-D-fructo-furanosyl-(2→1)-O-β-D-fructo-furanosyl-(2→1)-α-D-glucopyranoside) were identified as the main end-products.

The effect of biphasic medium type on the bioconversion yield of fructooligosaccharides is shown in Figure 4A. The yield increased as the reaction time increased from 24 to 48 h; beyond this reaction time, it remained more or less constant thereafter (data not shown). The end-product profile of fructooligosaccharides varied depending on the type of biphasic system and the reaction time. As an overall, a shift towards higher molecular-weight fructooligosaccharides was observed as the reactions were proceeded in all biphasic media with the exception of cyclohexane/buffer system. Indeed, the decrease of the relative proportion of kestose over the reaction time course correlated with the increase of that of nystose and 1^F-fructosyl-nystose. This finding reveal that endo-inulinase may have followed a non-processive mechanism in which a release of the fructooligosaccharides from the enzyme active site occurred after each fructosyl unit transfer (Ozimek et al., 2006).

The results show that the highest fructooligosaccharide bioconversion yield of (60.24%) was obtained in the butyl acetate/buffer biphasic system after 48 h of reaction, with nystose being the main end-product (a relative proportion of 56.52 %). In the heptane/buffer biphasic system, there was more obvious shift of the reaction side during the reaction time course as the bioconversion yield of fructooligosaccharides decreased from (52.15 to 43.51%) when the reaction time increased from 24 to 48 h.

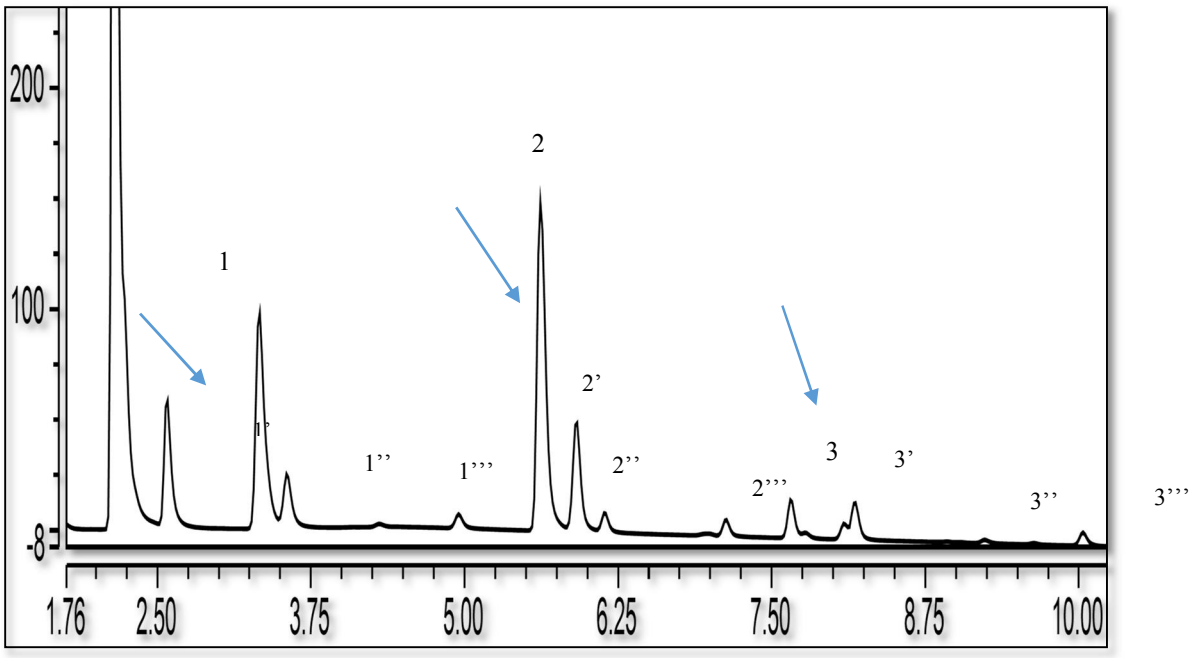


Figure 3. End products profile of purified endo-inulinase from *Aspergillus* niger-catalyzed transfructosylation reaction. Peak numbers were identified as follow: Peak #1 kestose, Peak # 1'-1'''' tri fructosyl units (kestose type), Peak #2 nystose, Peak #2'-2''' tetrafructosyl units (nystose type), Peak #3 fructosyl-nystose # and Peak#3'-3''' pentafructosyl units (fructosyl-nystose type).

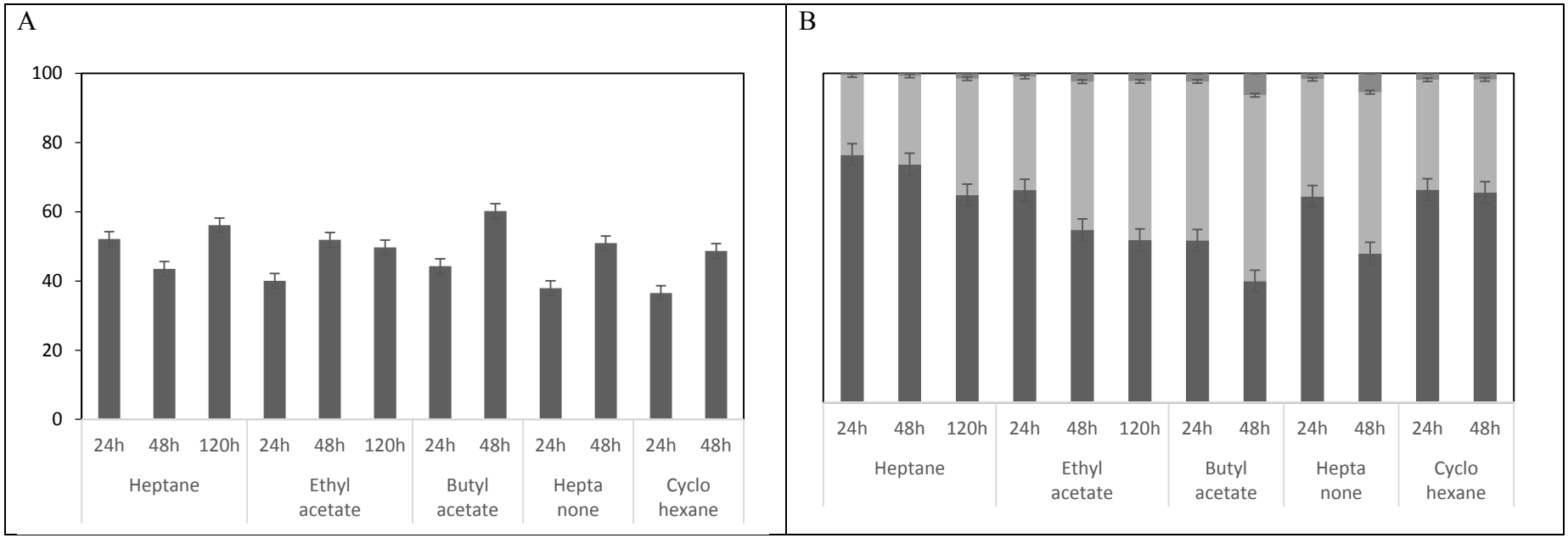


Figure 4. Effect of biphasic media on the bioconversion yield of fructooligosaccharides **(A)** and on the end product profile of endo-inulinase- catalyzed-transfructosylation reaction (**B)**: Kestose ■, Nystose □, Fructosyl nystose ■The standard deviation is represented by the Y error bars.

The highest yield of (56.12%) in heptane /buffer biphasic system was achieved at 120 h, with kestose being the main end product (relative proportion of 63.06 %), followed by nystose (35.34%). The use of ethyl acetate, heptanone and cyclohexane in the biphasic media led to more or less similar bioconversion yield of fructooligosaccharides, with an average of (50.80%), after 48 h reaction. However, the end-product profiles of fructooligosaccharides in these biphasic systems were different. Indeed, the cyclohexane/buffer biphasic system favored the synthesis of kestose (63.80%), while the nystose (48.94%) was the most dominant fructooligosaccharides in the heptanone/buffer biphasic system. On the other hand, similar relative proportion of kestose and nystose were accumulated in the ethyl acetate/buffer biphasic system. The relative proportion of synthesized 1^F-fructosyl-nystose was lower than kestose and nystose in all the studied biphasic systems. This last fructooligosaccharide was detected at higher reaction time, and its relative proportion (5.72- 6.58%) was higher in the biphasic system composed of heptanone and butyl acetate. The results reveal that the most polar solvents, butyl acetate, heptanone and ethyl acetate, as denoted by their lower Log P values (0.28 to 2.14) favored the elongation of the fructooligosaccharides by exhibiting higher affinity toward the binding of the growing fructosyl chain. These results may be due to the high solvation of the substrate in the presence of polar solvents and/or to their direct effects on the enzyme binding affinity. As mentioned by Halling, (1994), enzyme specificity can be influenced by solvent, which can change the partitioning of substrates and products between the bulk organic phase and the active-site micro-aqueous phase (Koskinen and Klibanov, 1996).

Risso et al. (2012a) reported that a maximum fructooligosaccharides yield of 17% was obtained in a butyl acetate/buffer biphasic system with ratio of 25/100, using free inulinase from *K. mariaxianus* NRRL Y-7571. Another study carried out by the same authors (Risso et al., 2012b) using immobilised inulinase from *K.mariaxianus* NRRL Y-7571 reported a yield of 18.20% of frucrooligosaccharides in a butyl acetate/buffer biphasic system with ratio of 25/75. On the other hand, Silva et al. (2013) conducted a research on the synthesis of fructooligosaccharides in an ethyl acetate/buffer biphasic medium with ratio 25/70 using immobilized inulinase from A. *niger* and sucrose as a substrate and using immobilized inulinase from *K. marxianus* NRRL Y-7571 and inulin as a substrate; both enzymes were treated with liquefied petroleum gas. The authors reported a production yield of 26.62% of kestose, 30.62% of nystose and 8.47% of 1^F-fructosyl-nystose with the immobilized inulinase from A. *niger* treated and using sucrose as substrate, while using

the immobilized inulinase from K. *marxianus* NRRL Y-7571 and inulin as a substrate, a yield of 11.89% of kestose and 20.83% of nystose were obtained.

The molecular weights of oligomers and polysaccharides released in biphasic media were estimated using high performance size exclusion chromatography (HPSEC) on three sequential columns. The results obtained in Table 3.3 shows that the concentration of released oligomers/polysaccharides was relatively low (2-19.2 mg/L) in all the investigated biphasic systems except in the ethyl acetate/buffer biphasic system where it was 2,131.0 mg/L. Moreover, the amount of oligomers/polysaccharides produced was found to be significantly low when compared to that of the fructooligosaccharides. Oligomers with the lowest molecular weights (3-10 kDa) which were found in the butyl acetate/buffer and ethyl acetate/buffer biphasic systems could imply that the transfer of fructose to its growing chain was somehow limited.

Table 3.3 Molecular weights and concentrations of oligomers and polysaccharides produced by endo-inulinase from *A. niger* in biphasic media.

Biphasic Reaction Media	Oligomers/polysaccharide concentration (mg/L)[a]	Molecular weight (kDa)[b]
Butyl acetate	2	10
Ethyl acetate	2,131.00	<3
Heptanone	19.2	1,518.00
Cyclohexane	12	1,534.00
Heptane	5	1,960.50

[a]Concentration of oligomers/polysaccharides is expressed in mg/L.
[b]Molecular weights of oligomers/polysaccharides are expressed in kDa.

3.5. Optimization of Fructooligosaccharides Production in Butyl acetate/buffer biphasic Medium by Response Surface Methodology

The synthesis of fructooligosaccharides in butyl acetate/buffer biphasic medium was optimized by response surface methodology (RSM), using a five-level three factor central composite rotatable design (CCRD). RSM is a combination of mathematical and statistical techniques used to optimise a response (output), which is influenced by different independent variables (parameters) (Montgomery, 2008; Seo, 2014). RSM helps to minimize the number of experiments required by making use of mathematical models, which assess the relevance and statistical significance of the variables being studied as well as their interaction effects (Seo, 2014). RSM approach was applied to evaluate the effects of the variables, enzyme unit (X_1, 2.5-9.5 U/ml), reaction time (X_2, 10-120 h) and proportion of solvent (X_3, 10-40%), on the production of well-defined fructooligosaccharides, mainly kestose, nystose and fructosyl nystose. The CCRD consisted of 10 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 22 sets of experiments.

The experimental conditions and their corresponding responses for kestose, nystose and fructosyl nystose concentrations (mM) as well as for the bioconversion yield of fructooligosaccharides (%) are described in Table 3.4. The highest kestose concentration of 263 mM was obtained at Run #4, with 6.0 U/ml enzyme unit, 25% solvent proportion and 10 h of reaction as experimental conditions. The highest nystose concentration of 277 mM and fructooligosaccharides bioconversion yield of 61% were obtained at Runs #19 and 20, where the experimental conditions were 3.92 U/ml enzyme unit and 16.08% solvent proportion with longer reaction time of 97.7 h for (Run #19) or 33.92% solvent proportion with shorter time of 56.3 h (Run #20). The concentration of fructosyl nystose was found, in general, to be lower than those of kestose and nystose, under the investigated conditions. Nevertheless, the highest fructosyl nystose concentration of 84 mM was achieved in the presence of a high proportion of co-solvent (33.92%) in the biphasic system and using 8.08 U/ml enzyme unit and 56.3 h of reaction (Run #15). These experimental findings may be attributed to the high affinity of enzyme to water and/or to the low binding affinity of nystose as acceptor, which may have limited the transfer of fructose to its growing chain. Risso et al. (2012a) reported that the proportion of water in an aqueous-organic medium can determine whether an inulinase enzyme will exhibit a synthetic or a hydrolytic activity.

Table 3.4 Central composite rotatable design arrangement of the actual and coded independent variables and the estimated responses.

	Independent Variables			Responses			
Run	X_1[a]	X_2[b]	X_3[c]	Kestose[d]	Nystose[e]	GF4[f]	Bioconversion[g]
1	3.92(-1)	56.3 (-0.266)	16.08 (-1)	181	177	17	42
2	9.5 (- 1.682)	65 (0)	25 (0)	129	207	49	43
3	6 (0)	65 (0)	25 (0)	159	236	47	49
4	6 (0)	10 (-1.682)	25 (0)	263	148	10	47
5	2.5 (1.682)	65 (0)	25 (0)	194	192	15	45
6	6 (0)	65 (0)	25 (0)	164	233	46	49
7	6 (0)	65 (0)	10 (-1.682)	158	236	45	49
8	8.08 (1)	56.3 9(-0.264)	16.08 (-1)	179	256	49	54
9	8.08 (1)	97.7 (1)	16.08 (-1)	202	269	55	58
10	8.08 (1)	97.7 (1)	33.92 (1)	176	243	46	52
11	6 (0)	65 (0)	25 (0)	166	238	55	51
12	6 (0)	120 (1.682)	25 (0)	187	226	60	53
13	6 (0)	65 (0)	40 (1.682)	178	241	70	54
14	3.92 (-1)	97.7 (1)	33.92 (1)	192	251	60	56
15	8.08 (1)	56.3 (-0.266)	33.92 (1)	187	268	84	60
16	6 (0)	65 (0)	25 (0)	176	256	63	55
17	6 (0)	65 (0)	25 (0)	186	266	65	57
18	6 (0)	65 (0)	25 (0)	189	271	70	59
19	3.92 (-1)	97.7 (1)	16.08 (-1)	218	277	56	61
20	3.92 (-1)	56.3 (-0.266)	33.92 (1)	215	277	61	61
21	3.92 (-0.999)	32.3 (-1)	16.08 (-1)	166	172	30	41
22	8.08 (-0.999)	32.3 (-1)	16.08 (-1)	122	145	44	35

[a] Enzyme unit (U/ml); [b] Reaction time (h); [c] Proportion of solvent (%) ; [d] Kestose (mM); [e] Nystose (mM); [f] Fructosyl nystose (mM) ; [g] Bioconversion yield (%).

Multiple regression analysis was used to determine the best-fitting model using the software Design-Expert version 8.0.7. The best-fitting models were evaluated by the determination of *p*-value, lack of fit and R-Squared (R^2) values. The analysis of variance (ANOVA) for responses of kestose, nystose and fructosyl nystose concentrations as well as for that of bioconversion yield of fructooligosaccharides in butyl acetate/buffer biphasic system is summarized in Table 3.5. The results show that the quadratic model was statistically the most suitable model for the description of productions of kestose, nystose and fructosyl nystose, with *P* value of 0.0301, 0.0102, and 0.0172 and *F* value of 3.26, 4.37 and 4.4, respectively. Indeed, the higher is the *F* value, the better predictor the model is. The R^2 of the fitted models were 0.7096, 0.7661, 0.4233 for kestose, nystose and fructosyl nystose 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 0.0672, 0.0892 and 0.1314 for kestose, nystose and fructosyl nystose production models, respectively indicate that the experimental data fitted well in the quadratic polynomial model. An adequate precision measures the signal to noise ratio. An adequate precision of 7.059 7.094 and 7.453 were obtained, which is greater than 4 and therefore makes it desirable. The overall results show that the models chosen can satisfactorily explain the effects of enzyme unit, reaction time and solvent proportion on fructooligosaccharides production by endo-inulinase in butyl acetate/buffer biphasic system, using sucrose as substrate. Similarly, Tian and Karboune (2012) reported that oligolevans production in levansucrase/endo-inulinase system can be described by a quadratic model. Moreover, Lim et al. (2005) described the use of a quadratic model for the production of fructooligosaccharides using *Penicillium citrinum*. Zhao (2013) also reported the use of a quadratic model to describe the production of extracellular polysaccharides by *Pseudomonas fluorescens* PGM37.

According to the established quadratic model, the linear term of endo-inulinase enzyme unit (X_1, *F* value of 7.12, *P* value of 0.0205) had the most significant effect on the kestose concentration, whereas, the linear terms of reaction time (X_2, *F* value of 12.19, *P* value of 0.0044) and the proportion of solvent (X_3, *F* value of 5.89, *P* value of 0.0282) were the most significant variables, for the nystose concentration and the bioconversion yield of fructooligosaccharides, respectively (Table 3.5).

Table 3.5 Analysis of variance (ANOVA) for response surface quadratic model for the total transfructosylation products and the bioconversion yield of fructooligosaccharides.

	Kestose (mM)		Nystose (mM)		Fructosyl-nystose (mM)		Bioconversion Yield (%)	
Source	*F* value	*p*-value	*F* value	*p*-value	*F* value	*p*-value	*F* value	*p*-value
Model	3.26	0.0301	4.37	0.0102	4.4	0.0172	3.9	0.0151
X_1[a]	7.12	0.0205	0.15	0.7038	3.43	0.0804	0.12	0.7358
X_2[b]	2.14	0.1694	12.19	0.0044	4.22	0.0548	2.97	0.1052
X_3[c]	3.88	0.0724	2.24	0.1604	4.06	0.0591	5.89	0.0282
X_1X_2	0.45	0.5141	2.18E-04	0.9885	-	-	6.61E-03	0.9363
X_1X_3	0.1	0.752	0.39	0.5429	-	-	0.27	0.6087
X_2X_3	11.42	0.0055	5.14	0.0427	-	-	10.16	0.0061
X_1^2	0.031	0.863	3.3	0.0945	-	-	-	-
X_2^2	11.05	0.0061	8.21	0.0142	-	-	-	-
X_3^2	0.044	0.8381	0.037	0.8511	-	-	-	-
Lack of Fit	4.18	0.0672	3.59	0.0892	2.8	0.1314	1.89	0.2499

[a] Enzyme unit (U/ml); [b] Reaction time (h); [c] Proportion of solvent (%).

However, the linear terms of enzyme unit (X_1, *F* value of 3.43, *P* value of 0.0804), reaction time (X_2, *F* value of 4.22, *P* value of 0.0548) and proportion of solvent (X_3, *F* value of 4.06, *P* value of 0.0591) had all significant effects on fructosyl nystose concentration. Among the interactive effects, the one between the reaction time and the proportion of solvent had the most significant effect on the kestose and nystose concentrations as well as on the bioconversion yield of fructooligosaccharides. In addition, the quadratic term, reaction time (X_2^2) had the most significant effect on kestose and nystose concentrations.

Considering all the significant terms, the production of kestose, nystose and fructosyl nystose as well as bioconversion yield of fructooligosaccharides can be described by the following predictive equations obtained in terms of coded variables:

Y_1=+175.14-14.43*X_1-9.46*X_2+10.84*X_3+5.85*X_1X_2-2.25*X_1*X_3-30.50*X_2*X_3-0.98* X_1^2+18.51* X_2^2+1.16* X_3^2

Y_2 =+251.94+2.62* X_1+28.13* X_2+10.25* X_3+0.16* X_1* X_2-5.42* X_1* X_3-25.47* X_2* X_3-12.56* X_1^2-19.85* X_2^2+1.33* X_3^2

Y_3= +50.18+7.14* X_1+9.18* X_2+7.90* X_3

Y_4 =+52.21-0.48* X_1+2.82* X_2+3.40* X_3-0.18* X_1* X_2-0.94* X_1* X_3-7.25* X_2* X_3

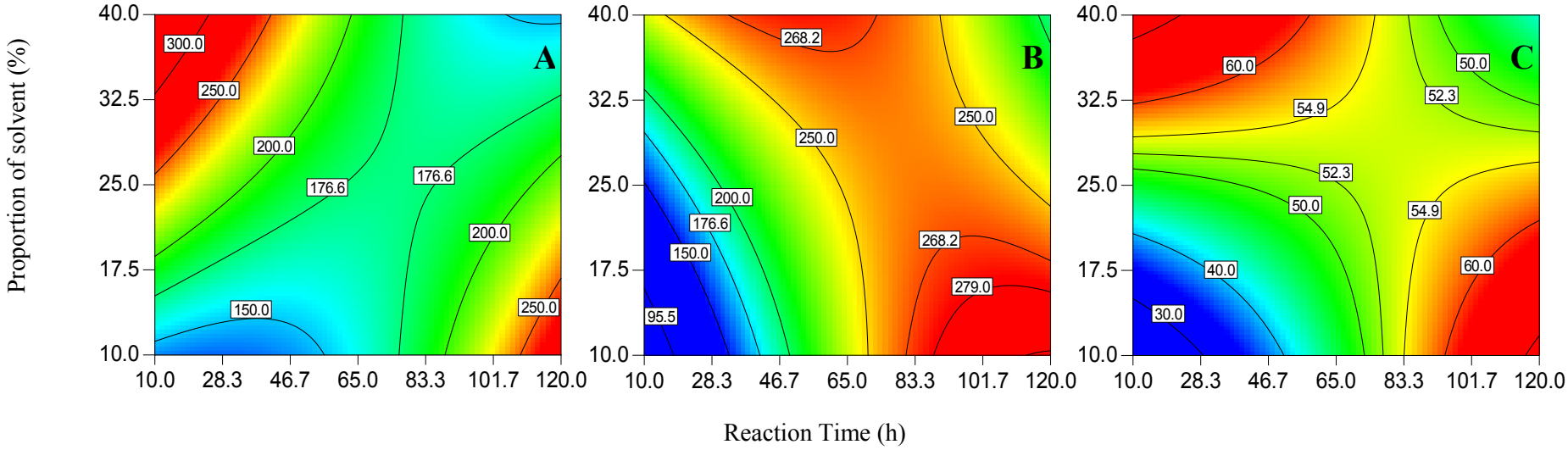
Where X_1 is the coded value of enzyme unit, X_2 is the coded value of reaction time, X_3 is the coded value of proportion of solvent, Y_1 is the kestose concentration (mM), Y_2 is the nystose concentration (mM), Y_3 is the fructosyl nystose concentration (mM) and Y_4 is the bioconversion yield of fructooligosaccharides (%).

3.5.1. Effect of Reaction Parameters on the Production of Fructooligosaccharides by Endo-inulinase

The relationships between the reaction parameters and the concentration of kestose and nystose as well as the bioconversion yield of fructooligosaccharides 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 interaction 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 kestose and nystose concentrations as well as on the bioconversion yield of fructooligosaccharides are illustrated in the 2D contour plots presented in Figure 5 (A, B and C). The contour plot of the kestose predictive model (Figure 5A) showed that in the biphasic medium containing lower solvent proportions (<25%, w/w), the kestose concentration increased when the reaction time was increased; however, higher solvent proportions (>25%, w/w) had a positive effect on the production of kestose at the early stage of the reaction but a negative one at the advanced stage. These results reveal that the presence of higher solvent proportions may have favored the transfructosylation reaction by limiting the water availability. Olvera et al. (2012) reported that the use of organic solvent can limit the availability of water for the hydrolytic activity. Similarly, when organic solvents were used instead of water, the transgalactosylation reaction was favored resulting in a higher yield of galactooligosaccharides (Ganzle, 2012). However, the results (Figure 5A) show that as the reaction was proceeded in the biphasic medium containing high solvent proportions, the concentration of kestose decreased. This decrease in kestose concentration was partially accompanied by the increase in the nystose concentration, revealing the elongation of the chain. In the nystose predictive model (Figure 5B), the effect of reaction time was more pronounced in the biphasic media with lower solvent proportions (<25%, w/w) than higher ones. A shift of the endo-inulinase-catalyzed reaction towards the hydrolysis one was observed at higher solvent proportions and longer reaction time. This may be attributed to the mass action effect.



Figures 5. Contour plots of predictive models for the transfructosylation products: (A): Kestose concentration (mM), (B): Nystose concentration (mM), and (C): Bioconversion yield of fructooligosaccharides (%).

The predictive model of the bioconversion yield of fructooligosaccharides (kestose, nystose, fructosylnystose) (Figure 5C) showed hyperbolic profile with two regions exhibiting maximum yields. Coupling shorter reaction times/higher solvent proportions and longer reaction times/lower solvent proportions can lead to high bioconversion yields of fructooligosaccharides. These results showed that the achievement of a compromise between the effect of solvent on the endo-inulinase catalytic efficiency, the reaction selectivity (hydrolytic/transfructosylation) and the mass action effect is important for efficient production of fructooligosaccharides by endo-inulinase in non-conventional media.

3.5.2 Model Verification

The optimal conditions for the synthesis of fructooligosaccharides using endo-inulinase in a butyl acetate biphasic medium were estimated using the numerical optimization of the Design Expert 8.0.6 software. The non-coded optimal conditions are reported in Table 3.6. The confirmations of these optimal conditions and the liability of the model predictions were investigated through additional runs. The predicted and the experimental maximum concentrations of the products are shown in Table 3.6. The uncoded optimal conditions for the fructooligosaccharides production were (a) endo-inulinase unit of 6 U/ml, reaction time 32.3 h with a proportion of solvent 33.52 % and (b) endo-inulinase unit of 6 U/ml, reaction time 56.3h with proportion of solvent 33.52 %. Under the optimum conditions of (a), the predicted bioconversion yield of 46.28 %, kestose concentration of 250.34 mM, nystose concentration of 241.08 mM and fructosyl nystose concentration of 47.39 mM were obtained. As for the optimum conditions of (b), the predicted bioconversion yield of 42.93 %, kestose concentration of 202.90 mM, nystose concentration of 261.45 mM and fructosyl nystose concentration of 54.13 mM were estimated. The maximum conversion yields are within the statistically significant range of the estimated optimum values with 95% prediction intervals (PIs). Therefore, the good correlation between these results confirmed that the response models were adequate to reflect the expected optimization.

Table 3.6. Model verification and optimisation of yields of fructooligosaccharides

A

Optimum Conditions	Uncoded	95%PI	Fructooligosaccharides	Experimental	Predicted
		190.63-310.06	Kestose (mM)	198.35	250.34
Endo-inulinase (U/ml)	6	166.75-315.41	Nystose (mM)	235.55	241.08
Reaction time (h)	32.3	11.63-83.15	fructosyl nystose (mM)	84.55	47.39
Proportion solvent (%)	33.92	36.86-55.69	Bioconversion Yield (%)	55.34	46.28

B

Optimum Conditions	Uncoded	95%PI	Fructooligosaccharides	Experimental	Predicted
		152.3-253.51	Kestose (mM)	164.48	202.90
Endo-inulinase (U/ml)	6	198.46-324.44	Nystose (mM)	219.07	261.45
Reaction time (h)	56.3	19.9-88.37	fructosyl nystose (mM)	89.02	54.13
Proportion solvent (%)	33.92	34.81-51.05	Bioconversion Yield (%)	51.79	42.93

3.6. The Production of Fructooligosaccharides by Endo-inulinase Catalysed Transfructosylation Reaction in Maple Syrup-based Biphasic Media.

Endo-inulinase can catalyze two types of reactions: the hydrolysis reaction, where the acceptor is water and the transfructosylation reaction, where the acceptors could be sucrose or growing chain of fructose. Since maple syrup consists primarily of sucrose (68%) with small amounts of glucose (0.43%, w/w) and fructose (0.34%, w/w), it can be used as a substrate for the transfructosylation reaction **(**Stuckel and Low, 1996; Li et al., 2015). To assess the effect of maple syrup as a substrate on the bioconversion yield of fructooligosaccharides, the time course for endo-inulinase-catalyzed transfructosylation reaction was investigated, using maple syrups 15°Bx (0.44 M), 30°Bx (0.88 M) and 66°Bx (1.93 M) with the experimental conditions of 6 U/ml enzyme unit and 33.92 % butyl acetate solvent proportion and reaction time of 16h, 48h and 80h (Figure 6).

The results show that when 15°Bx (0.44 M) maple syrup was used as the sucrose source the bioconversion yield of fructooligosaccharides increased with the reaction time reaching a high yield of 37.91% at 80 h of reaction. Using 30°Bx (0.88 M) as substrate, an increase in the bioconversion yield from 23.42 to 50.14 % was obtained, when the reaction time was increased from 16 to 32 h; however, no significant change in the fructooligosaccharides bioconversion yield was observed after an additional 16 h of reaction. With the use of 66°Bx (1.93 M), the bioconversion yield of fructooligosaccharides increased from 39.8 to 54.54 % with the increase in reaction time from 16 to 80 h. Based on these results, the highest fructooligosaccharides bioconversion yield of 52.53 % was obtained using 66°Bx, followed by 30°Bx. A study carried out by Li et al. (2015) using levansucrase from *B. amyloliquefaciens* and 15° and 30°Bx maple syrup systems to synthesize fructooliogsaccharides, oligolevans and levans showed that the transfructosylation reaction was more favored than the hydrolysis one during the first 7 to 12 h. However, as the reaction was proceeded, there was a shift towards the hydrolytic reaction to reach 34.71-36.76 % at 48 h. When the reaction was carried out in a 60°Bx maple syrup system, the transfructosylation reaction was the dominant one over the reaction time course, and its extent was higher than in maple syrup 15°Bx and 30°Bx systems. Li et al. (2015) also reported that when 15°Bx was used, a maximum yield of transfructosylation products of 27.8 % was achieved, whereas with 30°Bx and 66°Bx maple syrup systems, the yield of the transfructosylation products were 37.5% and 54.6 % respectively at 48h.

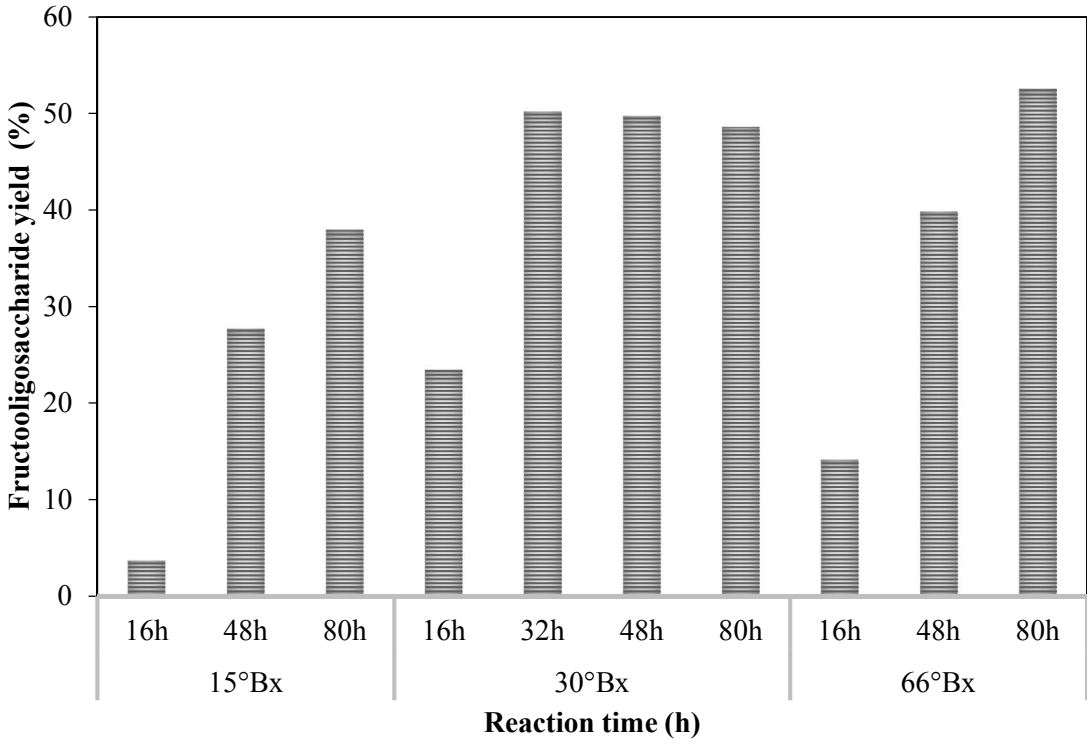


Figure 6. Effect of maple syrup on the bioconversion yield of fructooligosaccharides produced by the endo-inulinase-catalyzed transfructosylation reaction.

The end product profiles of endo-inulinase catalyzed transfructosylation reaction in maple syrup-based biphasic media are shown in Figure 7. The results show that using maple syrup 15°Bx as the sucrose source, kestose was the main end product over the reaction time course; only 10.22% relative proportion of nystose was produced at the advanced stage of the reaction. These results may be due to low substrate concentration and/or to the low affinity of endo-inulinase to kestose as a substrate acceptor. When maple syrup 30°Bx was used, higher relative proportions of nystose and fructosyl nystose were obtained. Indeed, a shift in the end-product profile was observed in which the kestose was the main end-product (76.43%) at 16h of reaction, while nystose and fructosyl nystose (51.28%) were the most dominant ones at 48h of reaction. This may reveal the dominance of the transfructosylation reaction, favoring the transfer of the fructose to kestose. However, at 80h an increase in kestose production from (48.72% to 56.26%) and a decrease in both nystose (43.99 % to 37.72%) and fructosyl nystose (7.29% to 6.02 %) productions were observed. This result may be attributed to the hydrolysis of nystose and fructosyl nystose into kestose as the bioconversion yield remained almost constant.

With the use of maple syrup 66°Bx-based biphasic medium, kestose, nystose and fructosyl nystose were all produced during the first 16h, with a relative proportion of 78.79%, 20.34% and 0.86%, respectively. However, at 48h the relative proportion of kestose was decreased to 54.94%, and there was an increase in the relative proportion of nystose to 38.94% and fructosyl nystose to 6.12%. At 80 h, a similar trend was also observed as that at 48h, the relative proportion of kestose was decreased from 54.94 to 47.24%, the relative proportion of nystose and fructosyl nystose was increased from 38.94 to 41.71% and from 6.12 to 11.04 %, respectively.

Based on the experimental results, the highest production of kestose was obtained with the use of maple syrup 15°Bx, whereas maple syrup 66°Bx resulted in the highest productions of nystose and fructosyl nystose. Similarly, Li et al. (2015) carried out a study on the synthesis of fructooligosaccharides/oligomers using levansucrase in maple syrup based systems. The study showed that when 15°Bx maple syrup system was used, the main products formed at 12h reaction time were oligolevans (70.97-89.93%). However, as the reaction proceeded to 48h, the main products were fructooligosaccharides (70.65 -99.65 %). In both 30°Bx and 66°Bx maple systems, the dominant transfructosylation products formed were oligolevans.

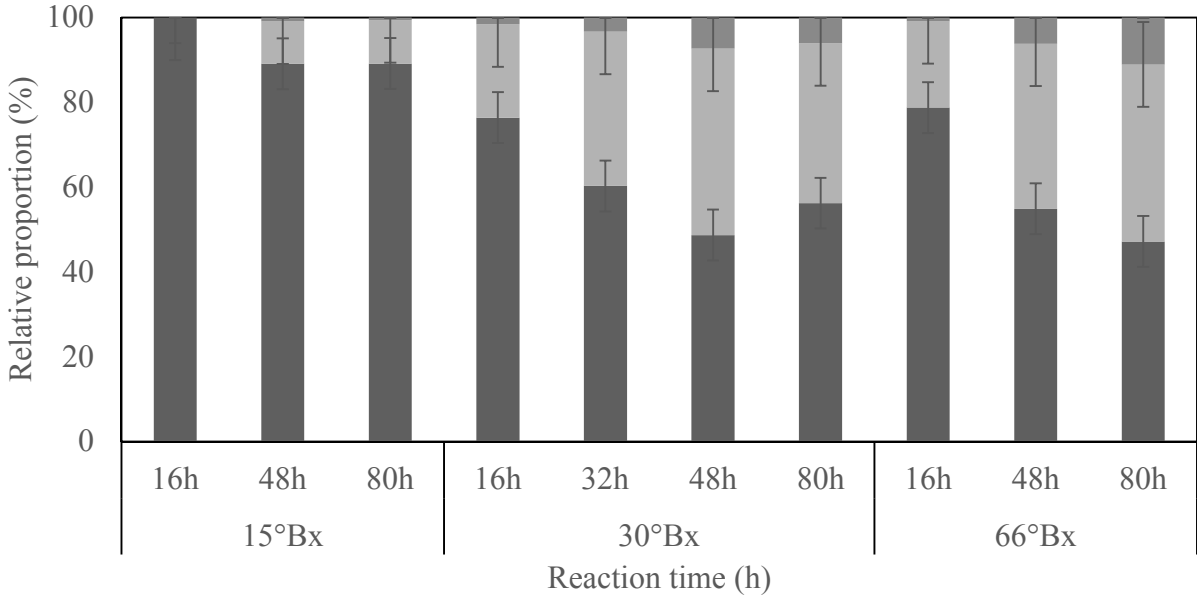


Figure 7. Effect of maple syrup on the end product profile of endo-inulinase-catalyzed transfructosylation reaction. ■ Kestose □ Nystose ■ Fructosyl nystose. Standard deviation is represented by the Y error bars.

CHAPTER IV

CONCLUSION

The objective of this study was to develop a biocatalytic approach based on endo-inulinase-catalyzed transfructosylation reaction for the production of well-defined fructooligosaccharides in non-conventional media, using sucrose as substrate. The enzyme endo-inulinase was purified with a purification factor of 8.2 from the exo inulinase. Among the investigated biphasic systems, the one using butyl acetate as the organic solvent resulted in the highest converted sucrose of 93.94%. The highest proportion of the hydrolysis reaction was obtained in the octanone /buffer biphasic medium with a relative proportion of 78.85 %, while the highest relative proportion of the transfructosylation (79.34%) was achieved in the heptane/buffer biphasic medium. Moreover, the highest fructooligosaccharide bioconversion yield of 60.24% was obtained in the butyl acetate/buffer biphasic system after 48 h of reaction. The fructooligosaccharides end products were identified to be mainly kestose, nystose and fructosyl nystose. However, this end-product profile varied depending on the type of biphasic system and the reaction time. For the butyl acetate/buffer biphasic system, the main end product was nystose with a relative proportion of 56.52 %. The highest kestose concentration of 263 mM was obtained with the use of 6.0 U/ml enzyme unit, 25% solvent proportion and 10 h of reaction. The highest concentration of nystose (277 mM) and bioconversion yield of fructooligosaccharides (61%) were obtained after 97.7h of reaction, using 3.92 U/ml enzyme unit and a butyl acetate solvent proportion of 16.08%.

According to the response surface methodology optimization study, the enzyme unit was found to be the most significant variable for the kestose concentration response, whereas reaction time and solvent proportion were found to be the most significant variables for nystose concentration and bioconversion yield, respectively. The experimental values for the fructooligosaccharides production were within the statistically significant range of the estimated optimum values with 95% prediction Intervals (PIs). Therefore, a good correlation confirmed the effectiveness of the established models to describe the relationship between the reaction variables and the yield fructooligosaccharides.

Furthermore, the effect of maple syrup on the bioconversion yield of fructooligosaccharides produced by endo-inulinase-catalyzed transfructosylation reaction was investigated. Maple syrups 15°Bx (0.44 M), 30°Bx (0.88 M) and 66°Bx (1.93M) were used as sources of sucrose and the aqueous phase of the biphasic media. The highest bioconversion yield of fructooligosaccharides of 52.53% was obtained, using 66°Bx (1.93M). The effect of maple syrup on the end product

profile of endo-inulinase catalyzed transfructosylation reaction was also studied using maple syrup 15°Bx, 30°Bx and 66°Bx. The highest production of kestose (89.15%) was obtained with the use of maple syrup15°Bx, whereas maple syrup 66°Bx resulted in the highest productions of nystose (41.71%) and fructosyl nystose (11.04%).

Overall, endo-inulinase catalyzed transfructosylation reaction in non-conventional media is a promising approach for the synthesis of fructooligosaccharides, using sucrose as substrate. The overall experimental results obtained throughout the present study contribute to the synthesis of fructooligosaccharides with specific composition. Furthermore, using maple syrup as a substrate give rise to a different approach in the use of maple syrup in research field.

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