THE PRODUCTION OF POTENTIALLY PREBIOTIC OLIGOSACCHARIDES BY *LEUCOSPORIDIUM SCOTTII* Y- 1450

By

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This dissertation is dedicated to my parents. They sacrificed everything so that their children could have opportunities they did not have.

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

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1. Introduction

Consumers are becoming more conscious about their health and lifestyle leading to an increase in the consumption of functional foods (Devlina *et al.,* 2009). The microbiota in humans plays a vital role in the health and well-being of humans with its population changing from the stomach (10^{1} to 10^{3} bacteria per gram of contents) to the small intestines (10^{4} to 10^{7}), and finally to the colon (10^{11} to 10^{12} bacteria per gram of contents), (O'Hara & Shanahan, 2006). During the early stages of life, the microbiota is relatively stable. As time goes on, the microbial population decreases. A plethora of factors account for a decrease in the microbial load, such as age, diet, environmental factors, antimicrobial therapy, susceptibility to infections, immunologic status, transit time and the presence and availability of fermentable material in the gut (Collins & Gibson, 1999; Inna *et al.,* 2010). In the presence of dysbiosis, pathogens may interrupt some of the normal functions of the gastrointestinal tract (GIT) leading to conditions such as diarrhoea, inflammatory bowel disease (IBD) and irritable bowel syndrome (IBS), (Sartor, 2008; Lee & Bak, 2011). In order to maintain a stable microbiota it's important that the diets of humans are systematically supplemented with probiotics, prebiotics and synbiotics (Bielecka *et al.*, 2002).

Probiotics are defined as 'live microorganisms which when administered in adequate amount confer health benefits to the host' (FAO/WHO, 2002). Probiotics are known to exhibit certain benefits to the host like the stimulation and development of the immune system, reducing the risk of lactose intolerance, cholesterol normalisation, and the inhibition of the growth of pathogens (Yadav *et al.*, 2006; Amdekar & Singh, 2012). *Bifidobacterium* and *Lactobacillus* species are the most common probiotics and have been used as functional food ingredients and in combination with prebiotics (synbiotics) (Prasad *et al.,* 1998).

A prebiotic is defined as 'a nondigestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon and thus improves host health' (Gibson & Roberfroid, 1995). Galactooligosaccharides (GOS) and fructooligosaccharides (FOS) are the most common and best-studied prebiotic oligosaccharides. These sugars escape digestion in

the small intestine and reach the large intestine intact where they are fermented by the beneficial microbes to produce organic acids which decrease the pH of the gut and help to eliminate pathogenic microbes (Gibson *et al*., 2004; Brownawell *et al*., 2012). Other products such as succinate, lactate and pyruvate are also produced which act as energy sources to the host. Prebiotics act as a food source to the probiotics, hence promoting the proliferation of these beneficial microbes in the gut (Sekhon & Jairath, 2010). Probiotics have specificity for particular prebiotics (Hachem *et al*., 2013), hence there's a quest for new prebiotics. With an increase in the use of prebiotic oligosaccharide in the food, animal, pharmaceutical and cosmetic industries, there's the search for "new" microorganisms and enzymes that produce oligosaccharides.

1.1 Objective of this study

The aims of this study were to investigate the production and purification of oligosaccharides from the yeast *Leucosporidium scottii* Y-1450 to identify the purified oligosaccharides and to optimise oligosaccharide production from *Leucosporidium scottii* Y-1450 using experimental design.

2. Literature review

2.1 The gut microbiota

The human microbiota is considered as an organ on its own with a huge population of different microbes. Its diversity changes from the stomach to the colon (Fig 1.1). The stomach and duodenum contain very low numbers (10^{1} to 10^{3} bacteria per gram of contents) of microbes, with *Lactobacillus* and *Streptococcus* being the predominant ones. These numbers increase (10^{4} to 10^{7} bacteria per gram of contents) in the jejunum and ileum with the large intestine (the proximal, transverse and distal colons) being the most heavily populated (10^{11} to 10^{12} bacteria per gram of contents) (Vyas & Ranganathan, 2012). It's important to note that the microbes present in the gut microbiota are 10 times greater than those present in the rest of the entire human body. The change in the composition of the microbiota which occurs from the stomach to the colon is as a result of different microbial activities occurring there.

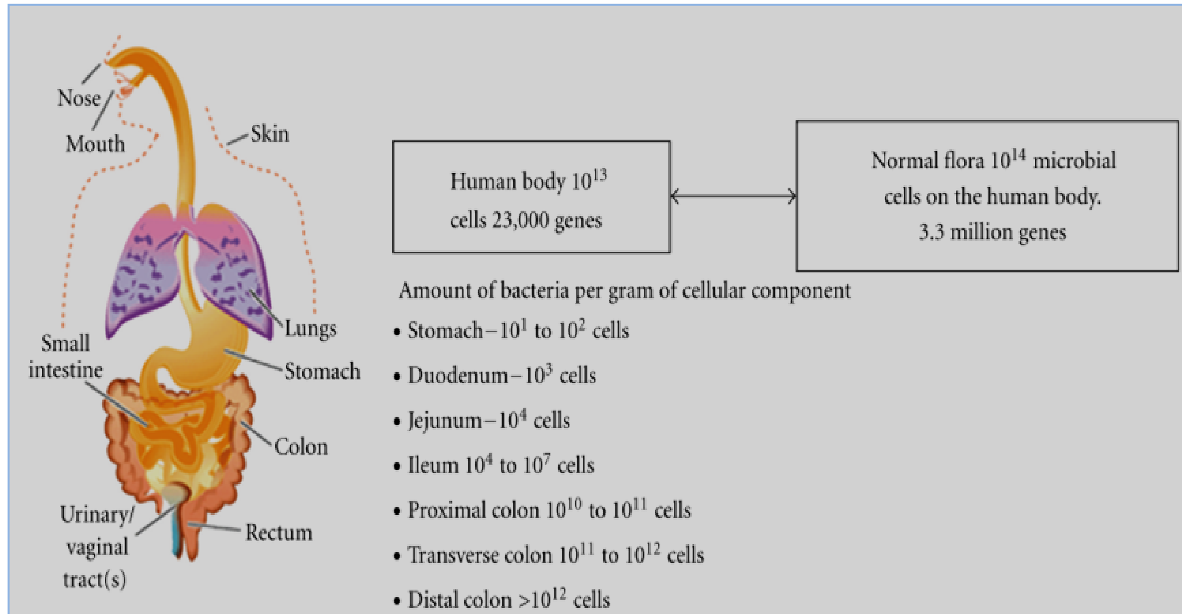

Figure 1.1 The Human body and its microbial population (Vyas & Ranganathan, 2012).

Since a large amount of carbohydrates enters the proximal colon, the microbes there have a good dietary nutrient supply and thus a high growth rate. Short chain fatty acids (SCFA) like acetate, butyrate, and propionate are produced as products of fermentation. These decrease the pH of the proximal colon thus rendering it acidic with the pH ranging between 5.5 - 6.0. The acidic environment prevents the growth of pathogens and the SCFA are absorbed in the colon where they stimulate the absorption of salt and water. SCFAs also act as energy sources to the host. Acetate is metabolised in the kidneys, heart and human muscles. It also serves as a substrate for the biosynthesis of cholesterol. Butyrate which is metabolised by the colonic epithelium serves as an energy substrate. It also helps with cell differentiation and growth, and causes the induction of mucin secretion and antimicrobial peptide secretion which reinforce the defence barrier in the colon. Propionate regulates adipose tissue deposition. Other end products of fermentation include lactate, pyruvate, succinate, ethanol, and gases like H_2, CO_2, CH_4, and H_2S. (Cummings *et al.*, 1987; Gibson & Rastall, 2006; Hamer *et al.*, 2008; Siong *et al.*, 2004). Fermentation is carried out mainly by members of the genera

Clostridium, Lactobacillus, Bifidobacterium, Bacteroides, Ruminococcus and *Eubacterium* (Roberfroid *et al*., 2010).

In the transverse colon there is reduced substrate availability, a slower fermentation rate and a reduced concentration of end products from fermentation. The pH here is slightly greater than that in the proximal colon. Carbohydrate availability decreases in the distal colon, and this account for the characteristic slow growth rate of the bacteria present, the neutral pH and the high rate of proteolysis in this part of the digestive tract. Amino acids and proteins produced from proteolysis are used as energy sources by bacteria (Gibson & Rastall, 2006; Macfarlane *et al*., 1992).The neutral pH of the distal colon makes it a highly favourable environment for bacterial colonisation.

Before birth the guts of neonates are considered sterile. During and after birth, microbial colonisation occurs which is acquired from the mother during the birth process, or from the surrounding environment. In healthy adults, the microbiota stays relatively stable overtime, a state called “normobiosis”. Normobiosis occurs when the beneficial microorganisms predominate over the harmful microorganisms in the gut. However, the microbiota of the gut may be affected by age, diet, environmental exposure, antimicrobial therapies, nutrient availability, pH, immunologic status and transit time (Collins & Gibson, 1999; Costello *et al*., 2009; Inna *et al.,* 2010; Lin *et al*., 2014; Vanhouette *et al*., 2004). These factors can cause an imbalance or dysregulation of the microbiota (dysbiosis) resulting in diseases such as allergy, irritable bowel syndrome (IBS), Inflammatory bowel disease (IBD), Crohn’s disease, Ulcerative colitis, autoimmune disease, obesity, type 2 diabetes, cardiovascular disorders and colorectal cancer (Blottiere *et al*., 2013; Chan *et al*., 2013).

Based on the knowledge of the gut microbiota, it is evident that it is important in maintaining human health. Among the health benefits which these probiotics confer on the host are the alleviation of diseases like allergy, irritable bowel syndrome (IBS), Inflammatory bowel disease (IBD), Crohn’s disease, ulcerative colitis, autoimmune disease, obesity, type 2 diabetes, cardiovascular disorders and colorectal cancer, most of which are caused by dysbiosis (Blottiere *et al*., 2013; Chan *et al*., 2013).It is therefore vital that the gut microbiota be kept stable. To achieve this, probiotics, prebiotics,

synbiotics and antibiotics have been used to reduce the risk of dysbiosis in the colon (Gareau *et al.*, 2010; Preidis & Versalovic, 2009).

2.1.1 Probiotics

Probiotics are live microorganisms which when administered in adequate amounts confer a health benefit on the host (FAO/WHO, 2001). Probiotics are used to restore a perturbed microbiota, and possess certain antagonistic properties which makes them beneficial to the host. These microorganisms produce antioxidants and vitamins which are beneficial to the host, and also protect the host against pathogens (Wallace *et al.*, 2011). *Lactobacillus* spp and *Bifidobacterium* spp are the most common probiotics which are widely used. *Escherichia coli* strain Nissle 1917, *Saccharomyces boulardii, Roseburia*, *Akkermansia muciniphila* and other bacteria species under the genera *Streptococcus*, *Bacteroides*, *Enterococcus* and eubacteria have also been used as probiotics (Czerucka *et al.*, 2007; Duncan *et al.*, 2006; Everard *et al.*, 2013; Meleti *et al.*, 2009.

The following criteria have been outlined as a guideline in the selection of microorganisms as probiotics: resistance to gastric acidity and bile toxicity, ability to persist within the gastrointestinal tract, human origin, ability to modulate immune responses, non-pathogenic behaviour, production of antimicrobial substances, and adhesion to gut epithelial tissue (Brassart *et al.*, 1998; Guarner & Schaafsma, 1998; Huis in't Veld & Shortt, 1996; Marteau & Rambaud, 1993; Salminen *et al.*, 1996; Tannock, 1997).

Probiotics act in different ways to confer health benefits to the host. Their mechanism of action is either direct or indirect and it differs among the different species and strains. Five different ways have been identified by which these microorganisms can act to confer health benefits.

1. Competitive exclusion along the epithelium

2. Modification of the local microenvironment

3. Enhancement of the epithelial barrier function

4. Suppression of intestinal inflammation

5. Modulation of host immune response

Any of the above mechanisms can be used by the microorganism to confer health benefits to the host (O'Hara & Shanahan, 2007).

Due to the specificity of probiotics for particular prebiotics, there is a search for novel prebiotics that may stimulate their proliferation in the intestinal tract.

2.1.2 Prebiotics

Prebiotics are non-digestible food ingredients which beneficially affect the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon and thus improve host health (Gibson & Roberfroid, 1995). The most commonly used prebiotics are fructooligosaccharides (FOS), inulin, galactooligosaccharides (GOS), xylooligosaccharides (XOS), maltooligosaccharides and lactulose (Martinez, 2014; Panesar *et al.,* 2013)). Prebiotics are associated with certain health benefits which include prevention of specific allergies, improved calcium absorption, reduction in the duration, incidence, and symptoms of traveller's diarrhoea, alleviation of irritable bowel syndrome (IBS) symptoms, increased satiety and reduced appetite (Cani *et al.*, 2006; Cani *et al.*, 2009; Cummings *et al.*, 2001; Drakoularakou *et al.*, 2010; Osborn & Sinn, 2013; Whelan, 2011; Whisner *et al.*, 2013). They are made up of sugar molecules which are connected to each other by glycosidic bonds. They vary in chain length, from 3 to 10 sugar molecules. It is important to note that not all oligosaccharides have prebiotic potential. For an oligosaccharide to be called a prebiotic, it must meet the following criteria: The food ingredient must not be hydrolyzed or absorbed in the stomach or small intestine, it must be selective for beneficial commensal bacteria in the colon by encouraging the growth/metabolism of the organisms; and it alters the microbiota to a healthy composition by inducing beneficial luminal/systemic effects within the host (Bandyopadhyay & Mandal, 2014).

Prebiotics have a high specificity for particular beneficial commensal bacteria in the colon. Most prebiotics which have been tested promote the growth of *Lactobacillus* and

Bifidobacterium, hence there's the quest for novel prebiotics which can be utilised by the wide range of other beneficial microorganisms.

2.1.3 Synbiotics

Synbiotics are "mixtures of probiotics and prebiotics that beneficially affect the host by improving the survival and implantation of live microbial dietary supplements in the gastrointestinal tract, by selectively stimulating the growth and/or by activating the metabolism of one or a limited number of health-promoting bacteria, thus improving host welfare" (Gibson & Roberfroid 1995). A mixture of *Bifidobacterium* spp and FOS has been used as synbiotics. In a study carried out by Puccio and co-workers on infants, a reduced risk of respiratory tract infections and a lower risk of constipation was observed in infants who were fed with a formula containing a mixture of *Bifidobacterium longum BL999* and fructo- and galactooligosaccharides as compared to the control group which did not receive synbiotics in the formula (Puccio *et al*., 2007). In the production of synbiotics, care has to be taken on the selection of strain and sugar as the activity, growth, and viability of certain probiotics can only be achieved in the presence of specific prebiotics. (Dellaglio, *et al*., 2002; Nagpal & Kaur, 2011).

2.2 Properties of oligosaccharides

Most oligosaccharides share some common properties, which include their water solubility and mild sweetness (Crittenden & Playne, 1996; Chung & Day, 2002; Voragen, 1998). As the oligosaccharide chain length increases, the sweetness decreases (Roberfroid & Slavin, 2000). The degree of polymerization, the chemical structure and the levels of monosaccharide and disaccharide determine the sweetness of the oligosaccharide. As a result of this sweetness, oligosaccharides are used in the food industry as bulking agents. They are also used to replace artificial sweeteners which have an unpleasant aftertaste. Their mild sweetness has also made them applicable in the health sector where they are used by diabetic patients. The high molecular weight of oligosaccharides leads to an increase in viscosity which improves body and mouth feel (Crittenden & Playne, 1996; Mussatto & Mancilha, 2007). Other properties of oligosaccharides include alteration of freezing temperature of frozen foods,

controlling the intensity of browning in heat-processed food caused by Maillard reactions, and the provision of high moisture-retaining capacity. The high moisture-retaining property of oligosaccharides is very important in the control of microbial contamination of food products as it lowers the water activity (Crittenden & Playne, 1996).

Most prebiotics investigated or used commercially are oligosaccharides. Oligosaccharides are carbohydrates with a low degree of polymerisation (DP) and consequently low molecular weight (Yun, 1996). The IUB-IUPAC nomenclature defines them as saccharides containing between 3 and 10 sugar moieties whereas other authorities extend this range from 3 to 19 monosaccharide units (voragen, 1998). Oligosaccharides are widely used in the food industry as they modify food flavour as well as possessing certain physiological and physicochemical properties which promote the health of humans (Crittenden & Playne, 1996). The type of glycosidic bond and the degree of polymerisation play a very important role in the selective fermentation by the beneficial bacteria (Rowland & Tanaka, 1993; Sanz *et al*., 2005; Sanz *et al*., 2006). The stability of these sugars also depends on the type of glycosidic bonds between the molecules, the anomeric configuration, their ring form, as well as the sugar residues present. Commercially produced oligosaccharides include lactulose, inulin, lactosucrose, glycosyl sucrose, cyclodextrins, palatinose, galacto-, fructo-, isomalto-, malto-, xylo-, gentio-, gluco-, mannano- and soybean-oligosaccharides, (Chen *et al*., 2000; Reis *et al*., 2004; Muzzarelli, 2009). It is important to note that not all oligosaccharides are digestible.

2.2.1 Types of oligosaccharides

Oligosaccharides can either be classified as digestible or non-digestible based on their physiological properties.

2.2.1.1 Non-digestible oligosaccharides

The concept of non-digestible oligosaccharides (NDOs) results from the configuration of the glycosidic bond between monomeric sugar molecules like glucose, fructose, galactose and xylose, or from the substrate selectivity of gastrointestinal digestive enzymes (Conway, 2001; Roberfroid, 1997). The NDOs consist of α or β glycosidic bonds. The α glycosidic bonds are easily hydrolysed by the gastrointestinal digestive enzymes. On the other hand, most NDOs consist of beta glycosidic bonds which cannot be hydrolysed by the gastrointestinal digestive enzymes (Kaur & Gupta, 2002; Priebe *et al.,* 2002; Sako *et al.*, 1999; Tungland, 2003). These NDOs escape digestion by enzymes present in the mouth and small intestine, and arrive the colon intact where they are then cleaved by hydrolytic enzymes. The resulting monomers are fermented to products including short-chain fatty acids (propionate, lactate, acetate and butyrate), which act as energy sources and gases like CO_2, H_2, and CH_4 (Delzenne & Roberfroid, 1994). The fermentation of NDOs is influenced by a number of factors like their structure, degree of polymerization, identity of the monomeric sugar units, complexity of the molecule (branched or linear) and the linkage to non-carbohydrates (Van Laere, 2000). Many NDOs possess prebiotic potential (Table 1.1), for example GOS, FOS, MOS, lactulose and glucooligosaccharides.

Table 1.1 Non-digestible oligosaccharides with bifidogenic functions commercially available (Sako et al., 1999; Teruo, 2003).

Compound	**Molecular structure**[a]
Cyclodextrins	$(Gu)_n$
Fructooligosaccharides	$(Fr)_n$-Gu
Galactooligoaccharides	$(Ga)_n$-Gu
Gentiooligosaccharides	$(Gu)_n$
Glycosylsucrose	$(Gu)_n$-Fr
Isomaltooligosaccharides	$(Gu)_n$
Isomaltulose (or palatinose)	$(Gu\text{-}Fr)_n$
Lactosucrose	Ga-Gu-Fr

Lactulose	Ga-Fr
Maltooligosaccharides	$(Gu)_n$
Raffinose	Ga-Gu-Fr
Soybean oligosaccharides	$(Ga)_n$-Gu-Fr
Xylooligosaccharides	$(Xy)_n$

[a]Ga, galactose; Gu, glucose; Fr, fructose; Xy, Xylose.

Several prebiotics have been investigated and their beneficial effects have been established.

2.2.1.1.1 Fructooligosaccharides

Fructooligosaccharides (FOS) occur naturally in tomatoes, onions, asparagus and artichokes. They are produced enzymatically from sucrose which is obtained from raw materials like sugar cane and sugar beet molasses (Bornet, 1994; Crittenden & Playne, 1996). When ingested, they selectively promote the growth of beneficial bacteria like *Lactobacillus* spp and *Bifidobacterium* spp, and also have a positive effect on the host by lowering cholesterol levels, enhancing mineral absorption and preventing carcinogenic tumours (Gibson & Roberfroid, 1995). They fall under the class of non-digestible oligosaccharides because they escape digestion in the small intestine and arrive in the colon where they are utilised by the beneficial microbes.FOS have a chemical structure which is made up of a single glucose molecule attached to either two, three or four fructose molecules to produce kestose, nystose and fructosyl-nystose respectively (Rivero-Urgell & Santamaria-Orleans, 2001). Their low-cariogenic property makes them useful against tooth decay. They are considered as low energy ingredients, hence are used in alleviating obesity. Osteoporosis has also been treated by the ingestion of FOS, where FOS helped in increasing calcium absorption (Cashman, 2003; Crittenden & Playne, 1996; Qiang *et al*., 2009).They decrease the population of putrefactive bacteria in the colon, alleviating colorectal cancer. A mixture of GOS and long chain FOS has been shown to reduce the incidence of atopic dermatitis in infants (Moro *et al*., 2006). Due to their numerous benefits, they have been incorporated into products like biscuits, energy bars, dairy products, tooth paste and confectioneries.

2.2.1.1.2 Galactooligosaccharides

Galactooligosacchrides (GOS) are produced enzymatically from lactose which is obtained from whey. Like FOS, they are utilised by *Lactobacillus* spp and *Bifidobacterium* spp, and also account for numerous beneficial effects in humans. In a recent study carried out by Sierra and co-workers on healthy infants fed with a formula containing GOS for a year, changes in faecal composition, consistency, frequency of defaecation and changes in the microbiota population were observed (Sierra *et al.*, 2015).They are similar to FOS in their properties. Their low caloric values make them suitable for inclusion in food. GOS are produced enzymatically by transgalactosylation when the galactosyl moiety of lactose is transferred by β-galactosidase to the galactose molecule of another lactose molecule (Kim *et al.,* 1997).Between one to three galactosyl units can be transferred to produce di-, tri-, tetra-, and pentasaccharides. They are sold under trade names like Oligomate 55, Cup-Oligo P, TOS-Syrup and Vivinal-GOS (Panesar *et al.*, 2013).

2.2.1.1.3 Maltooligosaccharides

Maltooligosaccharides (MOS) are produced from starch by the action of three different enzymes. Alpha and beta amylases which hydrolyse starch into maltose, and α-glucosidase which is responsible for the transglucosylation of maltose (Mótyán *et al.*, 2011; Ota *et al.*, 2009). MOS consist of only glucose molecules linked by α- 1- 4 bonds (Crittenden & Playne, 1996). They have a high water-holding capacity, low sweetness and an anti-staling effect, making them useful in the food industry (Park, 1992). Just like other prebiotics, they promote the proliferation of probiotics.

2.2.1.1.4 Xylooligosaccharides

Xylooligosaccharides (XOS) are produced from xylan by the action of xylanase (Sato *et al.*, 2010; Dilokpimol *et al.*, 2011). These prebiotics can be obtained from raw materials like bagasses, hardwood, corn cobs, hulls, straws and malt cakes by either enzymatic treatment, chemical fractionation or hydrolytic degradation. XOS consist of xylose molecules linked by β -1, 4 bonds. This prebiotic occurs in chains consisting of

xylobiose, xylotriose and xylo-tetraose (Vazquez *et al.*, 2000). Xylobiose, xylotriose and xylotetraose consist of 2, 3 and 4 xylose molecules respectively. They have a wide range of application. In the food industry they are used as gelling agents and antioxidants. In the health sector they are used in the treatment of colon cancer, diabetes and arteriosclerosis. XOS are also included in cosmetics, pharmaceuticals and agricultural products, and are known to promote the growth of *Bifidobacterium* spp (Alonso *et al.*, 2003; Katapodis & Christakopoulos, 2008; Madhukumar & Muralikrishna, 2010; Moure *et al.*, 2006).

2.2.1.1.5 Lactulose

Lactulose is a disaccharide which is produced from lactose by either alkali isomerisation or transgalactosylation reactions. In this process, the glucose moiety of lactose is converted to a fructose molecule. Alkalis such as NaOH and MgO have been used in the production of lactulose. This prebiotic is relatively costly to produce due to its low product yield and high purification cost since other by-products are produced during its production (Villamiel *et al.*, 2002). Lactulose is selectively utilised by *Bifidobacterium* in the human gut. It is enzymatically produced by beta- galactosidase via transgalactosylation. This enzyme can be used as whole cells or in the free or immobilized form (Panesar *et al.*, 2013). Lactulose is used as a laxative, infant formula, and a low calorie sweetener. It is also used in the treatment of portosystemic encephalopathy and hyperammonemia (Kim *et al.*, 2006; Goulas *et al.*, 2007).

2.2.1.1.6 Glucooligosaccharides

Glucooligosaccharides are produced chemically by microwave induced acid hydrolysis of glucans (Majumder *et al.*, 2009). They can also be produced enzymatically by β-glucosidase (β-glucooligosaccharides) or α-glucosidase (α-glucooligosaccharides), and are known to result in the proliferation of *Bifidobacterium* spp and *Bacteroides* spp in the human gut (Laere, 2000). Onishi & Tanaka produced glucooligosaccharides from cellobiose by transglucosylation using β- glycosidase (Onishi & Tanaka, 1996). These prebiotics promote beneficial cutaneous flora, and are used in the dermocosmetic industry (Iliev *et al.*, 2008).

2.2.1.2 Digestible oligosaccharides

Unlike the NDOs, which are not hydrolysed by the gastrointestinal digestive enzymes, the digestible oligosaccharides are hydrolysed by enzymes in the small intestine. Some of these digestible oligosaccharides are partially digested in the small intestine, and the portion which reaches the colon exhibits a bifidogenic effect there. An example of a digestible oligosaccharide is maltotriose (Tanabe *et al*., 2014).

2.3 Production of oligosaccharides

The commercial production of oligosaccharides has increased throughout the years due to the health benefits rendered by these sugars. Oligosaccharides have been widely used in the food, cosmetic, feed, pharmaceutical and agricultural industries. Thus, there’s an increasing interest in their production on a larger scale. They can be extracted from natural sources, and can also be produced chemically, physically or enzymatically (Courtois, 2009) (Fig 1.2). Oligosaccharides originate from fungi, bacteria, algae and higher plants (Patel & Goyal, 2011), and have been found in soyabean, milk, lentils, honey, sugarcane juice, mustard, fruits and vegetables like chicory, asparagus, onions, leek, banana, tomato, wheat, artichoke, rye, barley, bamboo shoots and yacon. Others have been produced from almond shells, gram husk, corn cob, wheat bran, brewery spent grains and barley hulls (Katapodis & Christakopoulos, 2008; Mussatto & Mancilha, 2007). Plant cell wall polysaccharides have also been a source of oligosaccharides. They have been produced from polysaccharides like wheat flour arabinoxylan, soy arabinogalactan and sugar beet arabinan (Van Laere *et al*., 2000). Oligosaccharides are commonly produced chemically or enzymatically.

2.3.1 Chemical production

Oligosaccharides can be produced chemically by either polysaccharide hydrolysis, alkali isomerisation or from disaccharide substrates (Fig 1.2). Majumder and co-workers produced glucooligosaccharides chemically by microwave induced acid hydrolysis of glucans (Majumder *et al*., 2009). Raffinose oligosaccharides were also produced from plant material by extraction using water or aqueous methanol or ethanol solutions. The

disaccharide lactulose is produced by alkali isomerisation. In this process, the glucose moiety of lactose is isomerised to fructose with the aid of an alkali catalyst like NaOH (Mussatto & Mancilha, 2007). Chemical hydrolysis is seldom used for oligosaccharide production due to the low yields which arise from this method. This method also requires a number of complex glycosylation steps, and this complexity makes chemical synthesis problematic for large scale production. There's the quest for a single-step glycosylation reaction for oligosaccharide production. During chemical synthesis, the glycosidic bonds are formed when the leaving group of a glycosyl donor reacts with the hydroxyl group of a glycosyl acceptor. The left-over hydroxyl groups of both the glycosyl donor and acceptor are then masked by protecting groups (Fig. 1.3) (Kaeothip & Demchenko, 2011). To overcome this problem, enzymatic methods are usually used in the large scale production of oligosaccharides.

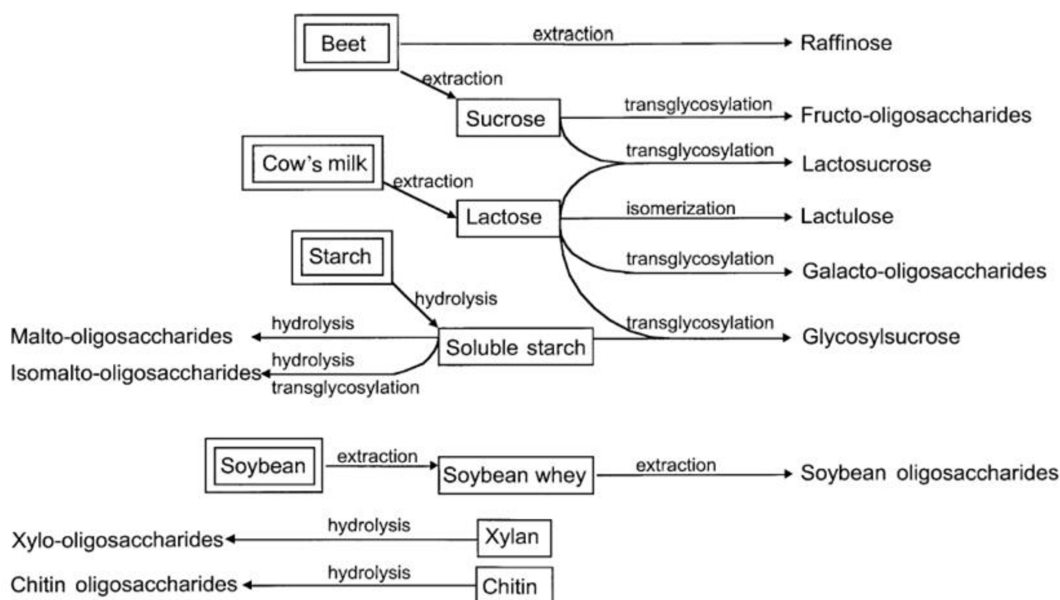

Figure 1.2 Schematic representation of production processes of nondigestible oligosaccharides (Sako *et al.*, 1999).

2.3.2 Enzymatic production

The enzymatic production of oligosaccharides is carried out by the transferases (glycosyl transferases: EC 2.4.) and hydrolases (glycosidases: EC 3.2.) (Boler & Fahey,

2012; Monsan & Paul, 1995). Enzymatic synthesis has advantages over chemical synthesis due to its regio- and stereo-selectivity that can be achieved without the need for protecting functional groups (Perugino *et al.,* 2004). The large scale production of oligosaccharides is hindered by the limited availability of glycosyl transferases, the high cost of their substrates, and the poor yields of the synthetic reactions performed by the glycosidases (Perugino *et al.,* 2004). Pocedicova and co-workers reported the production of galactooligosaccharides from lactose by a transgalactosylation reaction using β- galactosidase (Pocedicova *et al*., 2010). In some cases, both the enzymatic and chemical methods are employed in production. For example Mazzaferro and co-workers produced xylooligosaccharides from agricultural by-products (white poplar, giant cane, apple pomace and grape stalk) by first treating them enzymatically with a cocktail of enzymes (xylanase Buzyme 2511®). This was later followed by a thermal-alkaline treatment. Xylooligosaccharides of up to 96 % w/v was obtained from the grape stalk (Mazzaferro *et al*., 2011). Whole cell biocatalysis has also been reported for the production of oligosaccharides. Tzortzis and co-workers used this approach to produce galactooligosaccharides from whole cells of *Bifidobacterium bifidum* NCIMB 41171 (Tzortzis *et al*., 2005). Fructooligosaccharides have been produced from *Aspergillus sp.* N74 by this same process (Fernando-Sanchez *et al*., 2010).

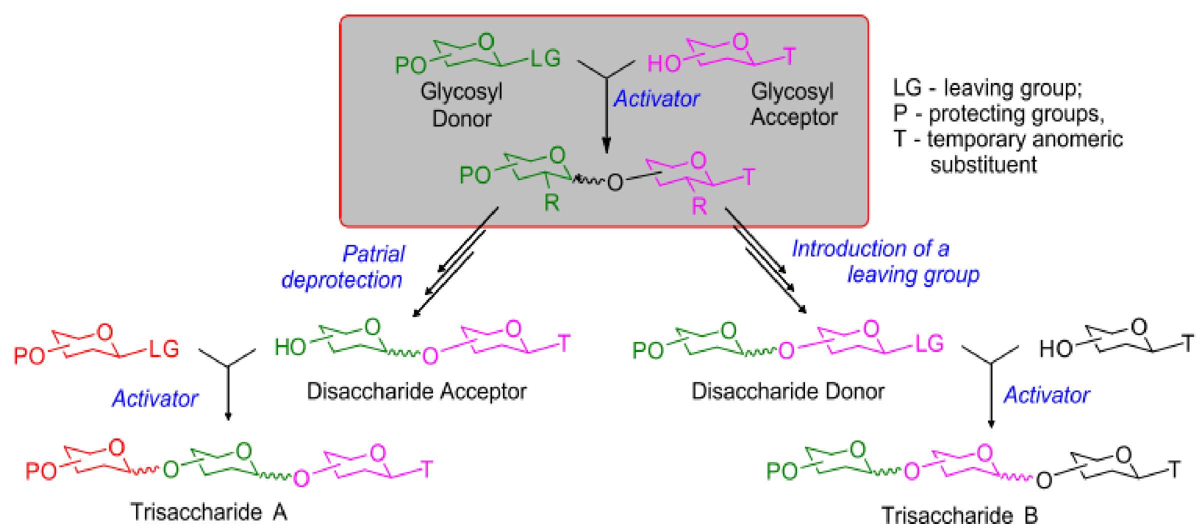

Figure 1.3 Traditional linear oligosaccharide synthesis (Kaeothip & Demchenko, 2011).

2.3.2.1 Glycosidases

Glycosidases are hydrolytic enzymes that are able to catalyse either the direct coupling of glycosyl moieties by simple reversion of the hydrolysis reaction, or the transfer of a glycosyl residue from an activated donor onto an acceptor bearing an OH-group. In reverse hydrolysis, the glycosidases can catalyse the hydrolysis of osidic bonds or their synthesis (Monsan & Paul, 1995). Hydrolysis is then a special type of transfer in which the acceptor is water. Glycosidases are preferably used for oligosaccharide production because they are less expensive; they do not require expensive sugar nucleotide donors and are also more available as compared to the glycosyl transferases. Apart from hydrolysing glycosidic bonds glycosidases are also responsible for glycoside formation (Fujimoto *et al*., 2009). However, they are limited by low yields and poor regioselectivity (Thiem, 1995). Galactooligosaccharides have been produced from lactose by β-glycosidases from the hyperthermophilic archaea, *Sulfolobus solfataricus* and *Pyrococcus furiosus*. Yields of 37 % (w/w) and 44 % (w/w) were obtained respectively (Hansson & Adlercreutz, 2001). Fujimoto and co-workers also reported the production of gentiooligosaccharides by transglycosylation with β-glycosidases from *Penicillium multicolor* using a high concentration of gentiobiose as substrate (Fujimoto *et al*., 2009).

2.3.2.2 Glycosyltransferases

The transferase enzymes are responsible for catalysing group-transfer reactions (Monsan & Paul, 1995). In the presence of a glycosyl donor and an acceptor, these enzymes catalyse the transfer of the glycosyl residue to an acceptor. The enzyme (which can be a hexosyltransferase, pentosyltransferase or those transferring other glycosyl groups) involved in the transfer reaction depends on the nature of the sugar residue being transferred. Moreso, the nature of the donor molecule determines the type of glycosyltransferase enzyme (Leloir-type glycosyltransferases, non-Leloir glycosyltransferases or transglycosidases) to be used (Patel, 2007). Glycosyltransferases and glycosidases belong to the same group based on their reaction mechanism and have both been used in the synthesis of oligosaccharides. These are preferred (because they do not require special activated substrates) over the

Leloir and non-Leloir enzymes which have some shortcomings. Firstly, they require sugar nucleotides or sugar phosphates as substrates whose synthesis is expensive and difficult. Secondly, the nucleotide phosphates which are released have an inhibitory effect. Lastly, these enzymes have a limited availability (Patel, 2007). Despite the limitations of this enzyme; a need for a complex glycosyl donor and the relative inaccessibility of the enzyme, it has a high efficiency and selectivity (Crout & Vic, 1998). Apart from intermolecular transfer, there exist intramolecular transfers where the glycosyl donor acts as an acceptor (Monsan & Paul, 1995). Dextransucrase, a glycosyl transferase has been used to produce the trisaccharide panose with maltose/sucrose as substrates (Fig 1.4) (Rabelo *et al.,* 2006). Yun reported the production of fructooligosaccharides (1-kestose, 1-nystose and 1-fructofuranosyl nystose) by fructosyl transferases from sucrose (Yun, 1996). Fig. 1.4 shows the production of panose by dextransucrase with sucrose acting as the nucleotide donor and maltose as the acceptor.

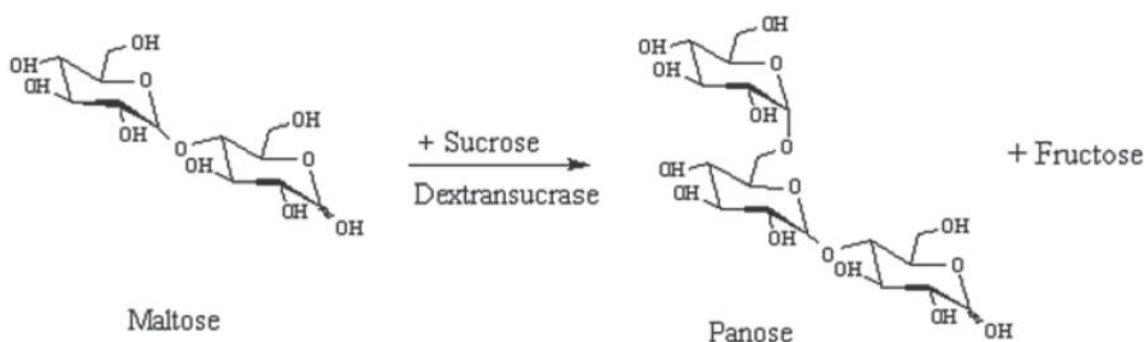

Figure 1.4 Dextransucrase acceptor reaction for panose production (Rabelo *et al.*, 2006).

2.4 Purification of Oligosaccharides

The chemical and enzymatic methods produce oligosaccharides which are not homogeneous. These oligosaccharide mixtures are often made up of oligosaccharides of different molecular weight and a smaller amount of monosaccharides and disaccharides (Fig 1.5). It is important to remove the unreacted substrates and monosaccharides after oligosaccharide formation so as to increase the purity of the

sugars. Purity is vital because it increases the viscosity of the oligosaccharide mixture thus improving body and mouthfeel, it decreases the sweetness and hydroscopicity of the sugar, and also decreases the occurrence of Maillard reactions during heat processing. The absence of simple sugars in the mixtures also lowers cariogenicity and reduces the calorific value of the sugar, thus making them suitable for consumption by diabetic patients (Crittenden & Playne, 1996; Crittenden & Playne, 2002).

A number of chromatographic processes have been used to remove these by-products. Often not all the by-products are successfully removed. About 5-10 % is retained in the purified sugars. Gravity column chromatography using carbon celite columns (Morales *et al.*, 2006), ion exchange columns (Vinjamoori *et al.*, 2004) as well as silica gel columns (Reichardt & Martin-Lomas, 2005) have been used for oligosaccharide purification. Other purification processes include preparative TLC, preparative HPLC and flash chromatography (Ojha *et al.*, 2015; Shimoda & Hamada, 2010; Somiari & Bielecki, 1999).

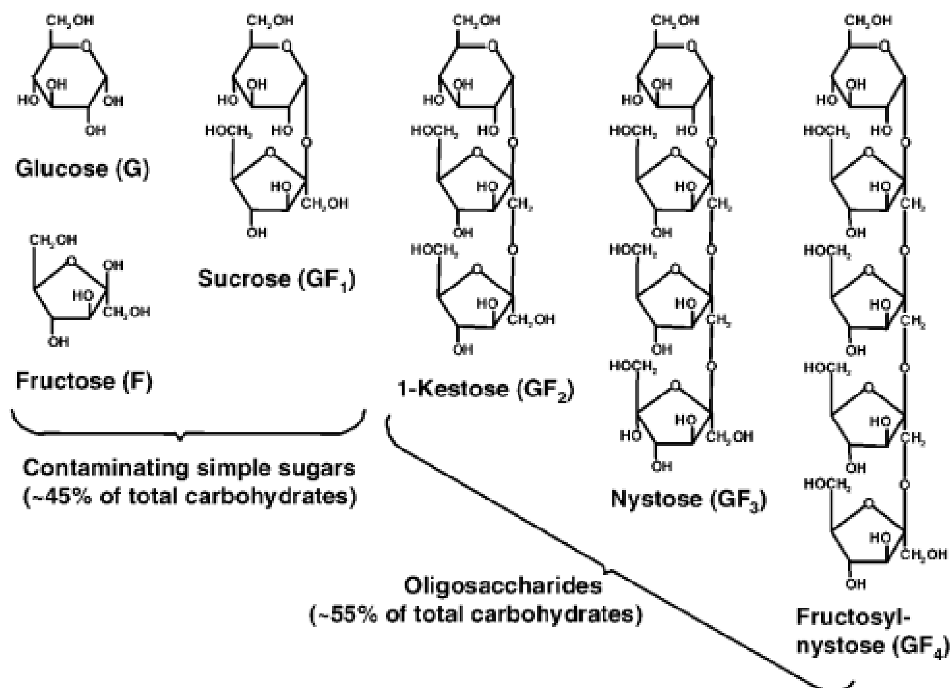

Figure 1.5 Carbohydrate composition of an unpurified fructooligosaccharide product formed from sucrose using transfructosylases (Crittenden & Playne, 2002).

2.4.1 Activated charcoal fixed bed column

There has been an extensive use of activated charcoal fixed bed columns in the purification of fructooligosaccharides (Bali *et al.,* 2015; Kuhn & Filho, 2010a; Kuhn & Filho, 2010b; Kuhn *et al*., 2014; Nobre *et al*., 2012). The principle of this process is based on the adsorption capacity of the activated carbon which is determined by the internal porous structure, the functional groups present on the pore surface and the total surface area. The pore size of the activated carbon is vital because if it is too large, it will not retain small adsorbate molecules, and if it is very small, it will trap the large adsorbate molecules. The functional groups have electrical charges which enhance or hinder the adsorption of target molecules. Like charges (on the functional group and target molecule) will hinder adsorption, while opposite charges will enhance separation (Ahmedna *et al*., 2000). Kuhn & Filho purified fructooligosaccharides from a mixture of sugars using an activated charcoal fixed bed column. A degree of purification of 80 % and a 97.8 % recovery of fructooligosaccharides was obtained. Methanol was found to be better for extraction than ethanol. Ethanol (15% (v/v)) was used as the eluent at 50 °C and gave the best separation (Kuhn & Filho, 2010a). Morales and co-workers reported the separation of oligosaccharides in honey on an activated charcoal column. Monosaccharides were removed with a water-ethanol ratio of 90:10 (v/v). The oligosaccharides were recovered with a 50:50 ratio of water-ethanol (Morales *et al.,* 2006).

The cost effectiveness and ease of operation of this process makes it advantageous. However, only small quantities of oligosaccharides can be purified using this method (Hameed *et al*., 2009; Kuhn & Filho, 2010).

2.4.2 Flash chromatography

Flash chromatography was first described by Still and co-workers in 1978. It has now been widely used in laboratories for the separation of both organic and inorganic compounds (Still *et al*., 1978). This method uses medium pressure (5 – 20bars) and is characterised by short columns (Strum *et al*., 2012; Still *et al*., 1978). Several factors must be in place for flash chromatography to be successful; increasing quantities of

analytes result in poorer resolution (Cox & Snyder, 1989; Still *et al.*, 1978) columns have an optimal flow rate determined by their geometry and silica quality (McGuffin, 2004; Snyder, 1977), more homogenous stationary phases pack better and provide better resolution and more reproducible results (Wellings, 2006) and stationary phases with a larger surface area generally afford better resolution (McGuffin, 2004; Snyder, 1977; Wellings, 2006). C-18 silica gel (230-400 mesh) and pure silica gel are usually used as reversed and normal stationary phases respectively. Typical mobile phases are butanol/acetic acid/water. In the flash chromatography set-up, a column is packed with silica and an air pump applies air pressure which drives the mobile phase or sample through the column (Stevens Jr & Hill, 2009). In modern equipment a piston pump is used instead of an air pump to move the mobile phase (Strum *et al.*, 2012). A fraction collector can be used to collect fractions for further analysis (Fig 1.6). This method has been used for the purification of kestose (Somiari & Bielecki, 1999).

This method is advantageous in that it is rapid, relatively cheap, and easy to setup, operate and manage (Jørgensen *et al.*, 2005; Somiari & Bielecki, 1999).

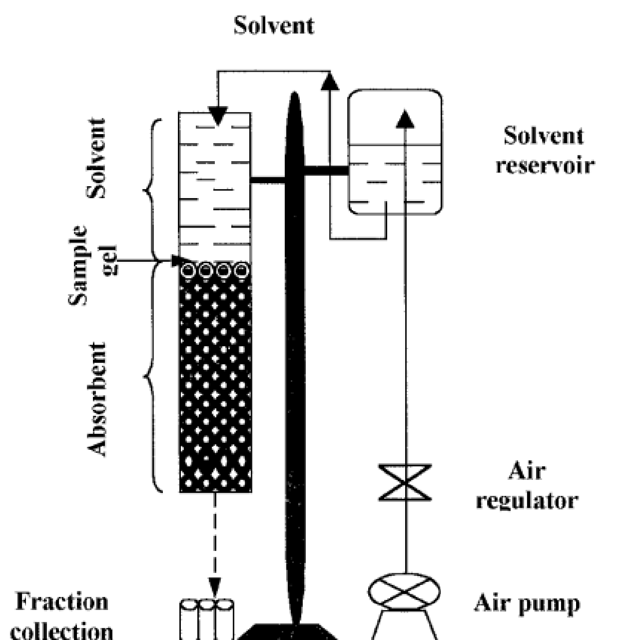

Figure 1.6 Set-up for flash-chromatographic separation of fructooligosaccharides (Somiari & Bielecki, 1999).

2.4.3 Preparative HPLC

The first preparative HPLC system was developed in the 1970's to increase throughput and separation power of valuable products (Unger, 1994). This method makes use of large columns, large amounts of samples applied to the stationary phase and high flow rates. It is an easy-to-use purification method, and purifies large numbers of compounds. Unlike analytical HPLC whose goal is to quantify and/or identify compounds, preparative HPLC is aimed at isolating and/or purifying compounds. The result of this process is usually judged based on the purity of the products, throughput and yield (Huber & Majors, 2007). Preparative HPLC has been used in the purification of oligosaccharides. Typical stationary and mobile phases include aminopropyl silica gel columns and acetonitrile/water respectively (Hicks *et al.*, 1994). The principle is similar to that of analytical HPLC where the carbohydrates elute the column in order of increasing monosaccharide chain length. The elute is collected with a fraction collector, after which the samples are evaporated *in vacuo* and lyophilized. Sadeh and co-workers reported the purification of oligosaccharides using this method (Sadeh *et al*, 1983).

The disadvantage of this method is that it is expensive as compared to other traditional purification processes like extraction, crystallisation and distillation (Huber & Majors, 2007).

2.4.4 Ion Exchange

Among the other methods used for purification of oligosaccharides is ion exchange chromatography. The stationary phase is a resin which has either anions or cations that are covalently bound to the resin. On the surface of this resin are found oppositely charged ions that are electrostatically bound to the resin. As the liquid mobile phase passes through the resin bound stationary phase the electrostatically bound surface ions are released and other ions are preferentially bound to its surface (Faust, 1997). The samples to be separated contain a charge opposite to that present on the resin surface. The rate at which the different compounds move is dependent on the density of the net charge on the sample. Therefore samples with a lower net charge density will elute first. Enzymatically produced FOS have been purified by high pH anion exchange

chromatography (HPAEC). A concentration of 56 gl^{-1} of FOS was obtained (Somiari & Bielecki, 1999). Xylooligosaccharides have also been refined by this method. Prior to ion exchange chromatography, membrane processing and hydrolysis was carried out (Gullon *et al.*, 2008).

2.4.5 Size Exclusion Chromatography

Size exclusion chromatography (SEC) is a technique which has been widely used for oligosaccharide purification. This technique makes use of the degree of polymerisation of the sugars (Hernández *et al.*, 2009), the molecular masses of the particles to be separated, and separation is based on particle size. The sample is injected into an injection valve and is carried with the aid of a pump by the mobile phase into a column packed with porous gel. The pore size has been designed such that it allows the large particles to pass through unimpeded. The smaller particles will be trapped by the gel particles and will only move through the column at a later stage. Therefore the smaller the particle, the longer it takes to pass through the column and vice versa. As the particles elute the column, the elution volume to molar mass is detected by a concentration detector (Faust, 1997; Tayyab *et al.*, 1991; Trathnigg, 2000). Typical gels used for SEC include cross-linked agarose (Sepharose), cross-linked dextrans (Sephadex, cross-linked polyacrylamide (Biogel) controlled pore glass beads and cross-linked allyldextran (Sephacryl) (Tayyab *et al.*, 1991). Yoshida and co-workers purified xylooligosaccharides using SEC with deionized water as the eluent at a flowrate of 0.3 ml/min. Galactooligosaccharides were purified by SEC using a 98.5 x 3 cm column containing Sephadex, after which the samples were run on silica gel TLC plates. Samples with an Rf value of 0.30-0.67 were pooled and freeze-dried (Huebner *et al.*, 2007).

2.4.6 Membrane Filtration

Membrane filtration is a cost effective non-chromatographic method which has also been employed to purify oligosaccharides (Goulas *et al.*, 2002). The size of the compounds to be separated and the membrane characteristics are responsible for the kind of separation techniques to be used. The techniques are classified into

nanofiltration, ultrafiltration, microfiltration, pervaporation, gas permeation, reverse osmosis, dialysis, electrodialysis and membrane distillation (Li *et al*., 2010). The first three techniques have been widely studied and most commonly used. They make use of size exclusion of the unwanted compound. Membrane filtration is based on the selective permeability of the target substance to penetrate through the membrane, while the unwanted substances are retained or rejected by the membrane (Li *et al*., 2010). The molecular weight of the membrane, the pressure used the temperature and the pH play very important roles in choosing a membrane (Michelon *et al*., 2014). Two membrane techniques can be coupled to obtain purity. Nanofiltration and reverse osmosis were used to purify soyabean oligosaccharides (Matsubara *et al*., 1996). Fructooligosaccharides (Li *et al*., 2005) and galactooligosaccharides (Michelon *et al*., 2014) were purified by nanofiltration and pectate oligosaccharides by ultrafiltration (Iwasaki & Matsubara, 2000). Nanofiltration is the most widely used purification method and it is advantageous because of its energy savings, low cost of implementation and maintenance of plants, simplicity of operation and ease of scale up (Michelon *et al*., 2014).

2.4.7 Microbial Treatment

In addition to membrane filtration, microbial treatment is also a non-chromatographic process for the purification of oligosaccharide mixtures. Several microorganisms have been employed to purify oligosaccharide mixtures because they do not possess the carbohydrases which degrade the oligosaccharides. In this way, the mono- and disaccharides are metabolised by the microbes leaving the oligosaccharides intact (Crittenden & Playne, 2002). However, this process is limited in that in some cases, metabolic products (for example CO_2, sorbitol and ethanol) and the biomass produced during fermentation must be removed to obtain highly purified oligosaccharides (Crittenden & Playne, 2002; Goulas *et al*., 2007; Nobre *et al*., 2015; Yoon *et al*., 2003). In addition, Sanz and co-workers found that this process resulted in the modification of the oligosaccharide composition (Sanz *et al*., 2005b). Yeasts (*Saccharomyces carlsbergensis*, *Saccharomyces cerevisiae* L1, *Pichia pastoris, Wickerhamomyces anomala* XL1) and bacterial cells (*Zymomonas mobilis*) have been used (Crittenden &

Playne, 2002; Lu *et al*., 2013; Pan & Lee, 2005; Yang *et al*., 2008). Fructooligosaccharides were purified with a *Wickerhamomyces anomala* strain by Lu and co-workers. In this study 93.6% of monosaccharides in the initial oligosaccharide mixture was metabolised and an improvement in FOS purity from 54.4% to 80.1% (w/w) was obtained (Lu *et al*., 2013). Pan and Lee also purified isomaltooligosaccharides from a mixture containing glucose, maltose and maltotriose using *Saccharomyces carlsbergensis* (Pan & Lee, 2005). Glucose, fructose and sucrose have also been removed by *Zymomonas mobilis* from a food-grade oligosaccharide mixture (consisting of unpurified inulin-, fructo-, malto-, isomalto- and gentio-oligosaccharides). Glucose, fructose and sucrose were fermented to ethanol and CO_2, except in the case of inulinoligosaccharides where a small quantity (2.5 g l^{-1}) of sorbitol was produced (Crittenden & Playne, 2002).

This process is advantageous in that it can be used industrially to produce high purity oligosaccharides at a low cost (Lu *et al*., 2013).

2.5 Identification and Quantification of Oligosaccharides

A number of techniques have been employed to identify or quantify oligosaccharides, with the two most common being thin layer chromatography (TLC) and high pressure liquid chromatography (HPLC). Oligosaccharides are usually separated by HPLC using polar-bonded phase and resin-based HPLC columns with refractive index detectors (RID). Thin layer chromatography (TLC), gas liquid chromatography (GC), nuclear magnetic resonance (NMR), gas chromatography-mass spectrometry (GC-MS) as well as liquid chromatography-mass spectrometry (LC-MS) are other techniques which have been used to structurally identify these sugars (Sangeetha *et al.,* 2005). Some techniques can be used to quantify and also identify the sugars (Liu *et al*., 2014). Accurate results can be obtained by combining a number of the aforementioned identification and quantification processes. However, identification techniques sometimes require special knowledge and large amounts of sample thus reducing its sensitivity, simplicity and rapidity (Kameyama *et al*., 2005).

2.5.1 High Performance Liquid Chromatography (HPLC)

HPLC is one of the most widely used quantitative methods for the identification of oligosaccharides.The principle of this method is that carbohydrates elute from the column in order of increasing monosaccharide chain length (Sangeetha *et al.,* 2005). Polar-bonded and resin-based columns like NH_2 columns, Aminex®HPX-87 C, Sugar-Pak ™ I columns and Shodex Asahipak NH2P-50 4E columns have been used. Common mobile phases include acetonitrile/water, Na_2SO_4 and distilled water which has been degassed and deionised and with flow rates between 0.3 and 1.5ml min^{-1}. Refractive index detectors (RID) are widely used to detect the sugars (Ojha *et al*., 2015; Peng et *al*., 2010; Rabelo *et al*., 2006; Rabelo *et al*., 2009; Sangeetha *et al*, 2005; Trujillo *et al*., 2001). The sugars are quantified by peak area using standards. Isomaltooligosaccharides have been quantified by HPLC using an Aminex HPX- 87C column, ultrapure water as mobile phase at a flow rate of 0.3 mL min^{-1}, and detected using a RID (Rabelo *et al*., 2009).

Although an HPLC is expensive to run, it has a high resolution (Wilson & Walker, 2010) and has led to rapid and accurate analysis of oligosaccharides (Sangeetha *et al.,* 2005).

2.5.2 Thin Layer Chromatography (TLC)

TLC is been used as a semi quantitative and qualitative method in oligosaccharide identification. It is a rapid method used to detect the presence of oligosaccharides after which HPLC is done to quantify the products. The degree of polymerisation can be obtained from this method (Patel & Goyal, 2011). Samples are applied as small spots or streaks to the origin of thin sorbent layers supported on glass, plastic, or metal plates. The mobile phase moves through the stationary phase by capillary action, sometimes assisted by gravity or pressure. Each component of the sample has the same total migration time but different migration distance.

The mobile phase usually consists of a single solvent or a mixture of organic and/or aqueous solvents like butanol-isopropanol-water-acetic acid (7:5:2:1, v/v), isopropyl alcohol-ethyl acetate-water (2:2:1, v/v), butanol-formic acid-water (4:6:1, v/v), ethyl

acetate-methanol-water-acetic acid (12:3:2:3, v/v) and butanol-methanol-chloroform-acetic acid-water (12.5:5:4.5:1.5:1.5:1.5, v/v) (Park *et al.*, 2001; Zhou *et al.*, 2014). The stationary phases are usually made of polar or non polar materials like silica gel, cellulose, alumina, manganese and activated zinc, which are coated onto a suitable support (Fried & Sherma, 1999; Tuzimski, 2011). If the products are colourless, they can be visualised with the aid of dyes which are sprayed on the plates after development. Aniline-diphenylamine-phosphoric acid (4:4:20, w/v/v) and Naphtho-resorcinol reagent (0.2 %) in 5:95 v/v, H2SO4 and ethanol have been used to visualise oligosaccharides (Ojha *et al.*, 2015; Park *et al.*, 2001; Zhou *et al.*, 2014). Park and co-workers reported the identification of FOS by TLC with isopropyl alcohol: ethyl acetate: water (2:2:1, v/v) as the mobile phase. Phenol sulfuric acid was used to spray the plates after development, and the products were visualised by heating the plates in an oven (Park *et al.*, 2001). The sugars can also be quantified by using a flatbed scanner or a densitometer (Halkina & Sherma, 2006).

Although TLC has not been considered to be highly efficient or quantitative, it has been traditionally regarded as a simple, rapid, and inexpensive method for the separation, tentative identification, and visual semi quantification of a variety of substances (Fried & Sherma, 1999).

2.5.3 Gas Chromatography-Mass Spectrometry (GC-MS)

Gas chromatography (GC) is an identification method which uses gas as the mobile phase and either a solid or a non-volatile liquid as the stationary phase. The mobile phase consists of an inert gas such as nitrogen for packed columns or helium or argon for capillary columns. It is a very powerful analytical technique when coupled to mass spectrometry. With mass spectrometry, information about the monosaccharide sequence, branching pattern and the presence of modifying chemical groups on the oligosaccharides can be obtained (Fernández *et al.*, 2004). In addition to this, it produces precise result, it is analytically versatile and has a very high sensitivity (Patel & Goyal, 2011). Gas chromatography exploits differences in the partition coefficients between a liquid stationary phase and a gaseous mobile phase of the volatilised

thermally stable analytes as they are carried through the column by the gaseous mobile phase. The partition coefficients are inversely proportional to the volatility of the analytes so that the most volatile analyte elutes first from the column (Wilson & Walker, 2010). Instruments like the Hitachi M-2000 AM and Agilent 190915-433 GC-MS have been used to identify oligosaccharides. These instruments are usually equipped with a flame ionisation detector in the case of oligosaccharide identification. The HP-5MS and the OB 225 fused silicone columns have been used with Helium as the carrier gas (Peng *et al.*, 2010; Sangeetha *et al.*, 2005; Wilson & Walker, 2010). Samples are usually methylated prior to analysis to make them more volatile and stable (Carlsson *et al.*, 1992). Hayashi and co-workers reported the identification of FOS with GC-MS where the samples were methylated, hydrolysed and reduced prior to analysis (Hayashi *et al.*, 2000).

A drawback of this technique is that samples have to be derivatised before analysis, but it has a high resolution, high sensitivity and high reproducibility (Wilson & Walker, 2010).

2.5.4 Nuclear Magnetic Resonance (NMR)

Nuclear Magnetic Resonance (NMR) has been used for the structural analysis of oligosaccharides (Okada *et al.*, 2010). It is a very powerful method as it provides complete information about the covalent structure of sugars (Agrawal & Pathak, 1996). The principle of NMR is based on atomic nuclei which is situated in a strong magnetic field and absorb radiation at characteristic frequencies. The atomic nuclei of the same element when placed in a different environment will produce different spectra lines, thus making it possible to observe signals from individual atoms in complex biological macromolecules in solution. From the spectra lines produced, some parameters can be obtained, which can then be interpreted in terms of conformation, molecular structure and dynamics (Roberts, 1993). The presence of exchangeable protons (OH and NH) poses a problem in NMR by causing a decrease in resolution. This is overcome by treating the oligosaccharides with deuterium oxide (D_2O) prior to analysis (Agrawal & Pathak, 1996). Isomaltooligosaccharides, FOS, fructopyranose and fucoidan

oligosaccharides have been structurally identified by NMR (Ojha *et al.*, 2015; Okada *et al.*, 2010; Yu *et al.*, 2013; Zambelli *et al.*, 2014)

One of the shortcomings of this method is that it requires samples to have a high degree of purity. A high level of expertise is also required. Nevertheless, it is the most powerful method for the structural characterisation of carbohydrates (Duus *et al.*, 2000; Fernández *et al.*, 2004). It is sensitive, rapid, quantitative, and non-destructive (Prestegard *et al.*, 1982).

2.5.5 Liquid Chromatography-Mass Spectrometry (LC-MS)

Liquid Chromatography-Mass Spectrometry (LC-MS) has been used for some time for the identification and quantification of oligosaccharides. Some structural information about the sugars can also be deduced from this method (Liu *et al.*, 2014). Due to the high sensitivity and specificity of MS, it's usually coupled to LC. The ion source of the mass spectrometer converts analyte molecules to a charged state, and the ions generated are either in the positive or negative ion mode, depending on the nature of the sample. The resulting charged ions or fragment ions are then analysed by a mass analyser based on their mass to charge ratio (m/z).Samples may sometimes require methylation prior to analysis. This makes the sugars less polar thus resulting in a good separation (Kailemia *et al.*, 2013; Pitt, 2009). Electrospray ionisation source (ESI) and Matrix assisted laser desorption ionisation (MALDI) methods have been widely used as ion sources in oligosaccharide identification (Fenn *et al.*, 1989; Hillenkamp *et al.*, 1991; Kailemia *et al.*, 2013). Typical mass analysers used include Matrix assisted laser desorption ionisation- time-of-flight (MALDI-TOF), Quadrupole ion trap (QIT) and Matrix assisted laser desorption ionisation-quadrupole-orthogonal time-of-flight (MALDI QoTOF) (Zaia, 2004). Fucoidan oligosaccharides, isomaltooligosacharides, fructooligosaccharides, N-linked oligosaccharides and human milk oligosaccharides have been identified using LC-MS (Jovanović *et al.*, 2014; Ojha, 2015; Zambelli *et al.*, 2014).

2.5.6 Derivatisation

Derivatisation among other techniques also has been used to identify oligosaccharides. Oligosaccharides are usually derivatised prior to analysis to increase the detection sensitivity (Lattova & Perreault, 2013). In this method the compound to be identified is chemically modified to produce a new compound with properties that are suitable for specific analytical procedures. The active hydrogens on the compound to be identified is usually substituted with a wide range of functional groups which give the compound its desired characteristics, while eliminating the adverse effects of the polar active hydrogens (Pierce, 2006). Three different derivatisation techniques have been widely used. Silylation, acylation and alkylation. Silyl groups, acyl moieties, or alkyl moieties, respectively are introduced into the unidentified compound by the substitution of its active hydrogens (Pierce, 2006). The nature of the group depends on the chromatographic technique. Since sugars are non-volatile, they must first be converted into volatile derivatives before analysis by gas chromatography. Derivatives like Trimethylsilyl (TMS) oximes have been widely used for the GC analysis of many oligosaccharides (Moreno *et al.*, 2014). Hernández-Hernández and coworkers carried out a study where they determined the structure of oligosaccharides derived from lactulose (Hernández-Hernández *et al.*, 2011). The sugars were initially purified by activated charcoal and analyzed by GC-MS as oxime TMS derivatives on a fused silica column coated with cross-linked methyl siloxane. Trisaccharides, galactosyl-galactoses and galactosyl-fructoses were identified. Coulier and coworkers used alditol acetate derivatives to identify the glycosidic linkages present in a GOS mixture (Coulier, 2009).

The short-coming with this process is that many reagents like salts, derivatisation reagents and solvents are used in very large quantities during this process. These reagents have to be removed in a clean-up process to enhance detection (Ruhaak *et al.*, 2010).

2.6 Experimental Design

An experimental design is the collection of predetermined settings of the process variables with each process variable called an experimental factor. Each combination of

settings for the process variables is called a run. A response variable is a measure of process performance (Haaland, 1989). In experimental design the main applications include factor screening, response surface examination, system optimization, and system robustness. In order to make use of the aforementioned applications, the following steps are considered:

1. Determine the overall goal and objectives of the experiment
2. Define the overall outcomes (response) of the experiment
3. Define the factors (and their levels) that will influence the response
4. Choose a design that is compatible with the overall objectives, number of factors considered and required precision of measurements (Hanrahan *et al.*, 2008).

Factor screening is usually the initial step in an experimental design. Here many factors are involved with imprecise knowledge about the factors. The main objective is to identify the important factors and to find out more about their best settings. Factorial designs are examples of screening designs and they are important in determining the initial factor significance for subsequent optimization (Haaland, 1989). A full factorial or fractional factorial design can be used. Fractional factorial design is a good alternative to a full factorial design since they represent a subset of a full factorial design (Otto, 1999). Once the important factors are identified through the screening design, optimization experiments are used to find out the best process performance. The main objective of the optimization experiment is to build a mathematical model which can be used to predict the behavior of the process being investigated. Fewer factors are involved and a lot of information about each factor is required. The optimization experiment produces specific optimal values for the experimental factors (Haaland, 1989). The last stage in the experimental process is verification. The verification experiment verifies that the optimum process performance has been achieved. It confirms the results of the experiments carried out at the predicted best settings, and ensures that the predicted optimal process performance can be reproduced in a second experiment (Haaland, 1989). Manera and coworkers produced galactooligosaccharides using permeabilised cells of *Kluyveromyces marxianus.* A fractional factorial design was

used to study the effects of lactose concentration, enzyme concentration, temperature and pH. The fractional design was later followed by an optimisation experiment using a central composite rotatable design (Manera *et al*., 2010).

2.7 CONCLUSIONS

There is an increase in the consumption of functional foods by consumers due to its numerous health benefits. These benefits extend to the animal and food industries, thus increasing the interest of these sectors in the production of functional foods. Some oligosaccharides are functional food ingredients and have been widely used in the aforementioned sector. Some of these sugars have potential as prebiotics. Prebiotics are associated with certain health benefits like prevention of specific allergies, improved calcium absorption, reduced duration, incidence, and symptoms of traveller's diarrhea, alleviation of irritable bowel syndrome (IBS) symptoms, decrease in cholesterol levels and increased satiety and reduced appetite. These have prompted the food and animal industry to invest more in the production of these ingredients.

Prebiotics are either produced chemically or enzymatically with enzymatic production being the preferred method due to its high yield. Pure and immobilised enzymes as well as whole cells are used as catalyst for prebiotic production. However, the production cost is high when using pure enzymes. Large scale production using whole cells may be cost effective since the whole cells can be reused and therefore preferred. The specificity of these prebiotics for particular probiotics has also increased the search for novel prebiotics which can be utilised by a wide range of the already existing probiotics.

There is no report on the production of oligosaccharides from *Leucosporidium scottii.* Producing these sugars using whole cells may be a cost effective method which can eventually be used in large scale production.

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CHAPTER 2

THE PRODUCTION OF OLIGOSACCHARIDES BY *LEUCOSPORIDIUM SCOTTII* Y-1450

CONTENTS

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1. Abstract

There is an increase in the commercial production of prebiotic oligosaccharides for inclusion in food and animal feed. Only a few yeasts have been investigated for this purpose. This prompted us to investigate the utilisation of sucrose by the yeast *Leucosporidium scottii* and the consequent production of oligosaccharides. This was done aerobically with 100 g l^{-1} sucrose being used as a substrate. The sugars produced were measured by Thin Layer Chromatography (TLC) and High Performance Liquid Chromatography (HPLC). TLC and HPLC analysis showed that *Leucosporidium scottii* was able to hydrolyse sucrose to glucose and fructose. In addition the enzymatic activity of *L. scottii* resulted in the production of three oligosaccharides, two trisaccharides and a tetrasaccharide, with maximum concentrations of 19.8 g l^{-1}, 6.3 g l^{-1}, and 7.8 g l^{-1} respectively, though hydrolysis of all three oligosaccharides was observed after 22 h of cultivation. A maximum oligosaccharide yield coefficient of 0.56 (g oligosaccharide g $sucrose^{-1}$) and a specific growth rate of 0.28 was obtained.

2. Introduction

The interest in the consumption of prebiotic oligosaccharides has intensified over the years due to the beneficial effects they possess. Prebiotic oligosaccharides escape digestion by the hydrolytic enzymes present in the mouth, stomach and small intestine, and reach the large intestine intact where they exhibit numerous benefits in the large intestine and are utilised by probiotic bacteria, thus contributing to their proliferation (Bandyopadhyay & Mandal, 2014). They exert other indirect effects in the gut like the production of lactic acid, short chain fatty acids (SCFAs), hydrogen, methane and carbon dioxide which all have positive effects on the gut. Lactic acid decreases the pH of the gut thus making it unfavourable for the growth of pathogens. SCFAs like acetate, butyrate and propionate act as energy sources for colonocytes (Slavin, 2013). Prebiotic oligosaccharides lower cholesterol levels, contribute to the alleviation of cardiovascular diseases, type II diabetes, helps in the control of glycaemia, weight gain, bioavailability and uptake of calcium, prevents colon cancer, colitis and immune-potentiation (Patel &

Goyal, 2012). Due to these interesting beneficial features of prebiotic oligosaccharides, their production has become important.

The aim of this study was to investigate the production of oligosaccharides by the yeast *Leucosporidium scottii* Y-1450 using sucrose as the carbon source. From preliminary experiments done with *L.scottii* and eight other yeasts, *L. scottii* produced the highest amount of oligosaccharides thus the reason why it was used for this study.

3. Materials and methods

3.1 Microorganism

Leucosporidium scottii Y - 1450 was used in this study. It was obtained from the University of the Free State MIRCEN yeast culture collection. Pure cultures of this yeast were maintained on Yeast Malt (YM) agar slants containing (per litre): 10 g sucrose, 5 g peptone, 3 g yeast extract, 3 g malt extract and 17 g agar.

3.2. Yeast inoculum preparation

Pre-cultures of *L. scottii* were prepared in a sterile rich medium containing (per litre) 10 g sucrose, 1 g yeast extract, 0.5 g citrate, 1.8 g NH_4Cl, 2 g KH_2PO_4, 0.5 g $MgSO_4.7H_2O$, 0.1 g $CaCl_2.2H_2O$, 0.035 g $FeSO_4.7H_2O$, 0.007 g $MnSO_4.7H_2O$, 0.011 g $ZnSO_4.5H_2O$, 0.0005 g $Al_2(SO_4)_3$, 0.00035 g KI, 0.002 g $CoCl_2.5H_2O$, 0.0013 g $Na_2MoO_4.2H_2O$ and 0.002 g H_3BO_3 in 500 ml Erlenmeyer side-arm shake flask containing 100 ml of medium. Sucrose was sterilized separately and added at a final concentration of 100 gl^{-1}.The pH of the medium was adjusted to 5 by the addition of 3M KOH prior to autoclaving. A loopfull of cells from a 24 h slant was inoculated into the flask and this was closed with a cotton plug and aerobically incubated at 25 °C on an orbital shaker at 180 r.p.m. until late exponential phase (30 h) as determined by a previous cultivation. This was used to inoculate the cultivation medium.

3.3 Shake-flask cultivation

Cultivations were carried out in 500 ml Erlenmeyer side-arm flasks with cotton plugs, each with a 100ml working volume, on an orbital shaker at a shaking speed of 180 rpm at 25 °C. A rich medium was prepared with the same components as those used for the yeasts inoculum preparation. An inoculum volume of 10 ml was added to 90 ml of rich medium and the flask was incubated for 50 h. Samples were collected at regular intervals for analyses. After every collection, the samples were immediately kept on ice and later centrifuged at 10 000 *x g* and 4 °C and stored at -20 °C. This experiment was done in duplicates.

3.4 Analytical procedures

Cell concentrations were monitored by measuring culture turbidity against a blank medium with a Photolab S6 spectrophotometer (WTW, Weilheim, Germany) at 690 nm. The dry cell weight was determined using triplicate 10 ml samples which were obtained at the end of the cultivation. These were centrifuged, washed with distilled water and dried to constant weight at 105 °C. Samples collected for the quantification of sucrose and product were immediately cooled on ice before centrifugation at 10 000 *x g* and 4 °C using an Eppendorf 5430 R centrifuge (Eppendorf AG, Hamburg, Germany). Prior to chromatographic analyses the supernatants were again centrifuged for 10 min at 10 000 *x g* and 4 °C. This was done as a precaution measure to remove any cells which might have been transferred during the separation of the supernatant from the cells from the previous centrifugation step. Supernatants not immediately analysed were stored at -20 °C.

Thin layer chromatography (TLC) was done as a rapid analytical method to monitor the levels of sugars in the supernatant. A 1:4 dilution of the samples was done to prevent smearing of the sugars on the TLC plates due to their high concentration and 2 µl was spotted on aluminium 20*20 cm silica gel 60 F_{254} plates (MERCK), dried and run for 5 hours using butanol (water saturated) - ethanol- acetone (50: 20: 2) as mobile phase. The plates were sprayed with aniline diphenylamine phosphate (20 g diphenylamine, 20

ml aniline and 100 ml phosphoric acid dissolved in 1 L acetone) and then baked at 100 °C for sugar detection.

Supernatants were also analysed for the presence of sugars by HPLC using a Phenomex Luna NH 4.6 mm x 250 mm column at 85 °C with 85 % acetonitrile at a flow rate of 2 ml/min as eluent. A refraction index (RI) detector was used to detect the presence of sugars and 25 µl of each sample was injected automatically into a Waters HPLC system. Glucose, fructose and sucrose were used as standards for the quantification of products. Sucrose was used as a standard for quantification of oligosaccharides.

4. Results

4.1. Detection of Oligosaccharides by TLC

Glucose, fructose and sucrose were used as standards for TLC. All compounds with an Rf value lower than that of sucrose were considered to have a degree of polymerisation greater than that of sucrose. Three spots corresponded to oligosaccharides. A retention factor (R_f) of 0.40, 0.33 and 0.26 were obtained for the three spots respectively (Fig. 1). Little sucrose was utilised during the first 10 h of cultivation. After 16 h of cultivation, sucrose was utilised with the concomitant production of glucose and fructose as well as oligosaccharides. The Rf values of fructose and glucose are very similar thus making it difficult to differentiate them on the TLC plates. However, the spraying agent resulted in different colours; brown for fructose and dark green for glucose. The dye or spraying agent stains the monosaccharides differently, thus aiding identification. Based on the intensity of the spots, the oligosaccharide levels were highest between 22 and 34 hours of cultivation after which their levels decreased.

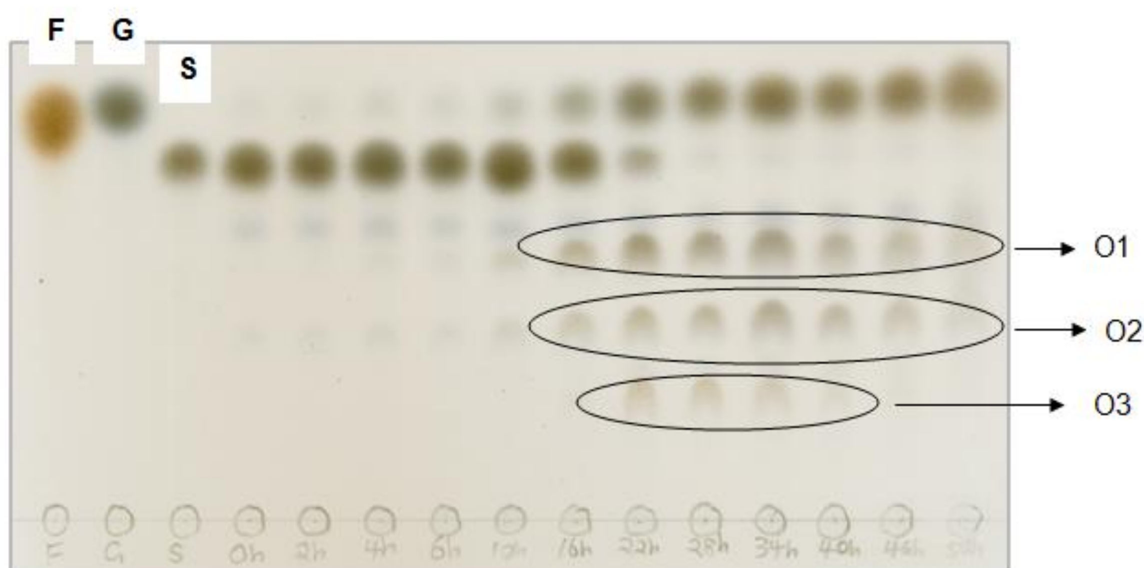

Figure 2.1 TLC plate of oligosaccharide production from 100 g l-1 of sucrose by L. scottii. Glucose (G), fructose (F), sucrose (S) and oligosaccharides (O1, O2 & O3). 0 h to 50 h indicates time of incubation.

4.2 HPLC analysis of sucrose utilisation and oligosaccharide production

TLC was done to confirm the presence of oligosaccharides in the supernatant after which HPLC was done to confirm these observations and to quantify the sugars present. The monosaccharides, glucose and fructose, were first eluted from the column. This was followed by the disaccharide, sucrose, and subsequently by the oligosaccharides, O1, O2 and O3 respectively (Fig 2.2). At the beginning of the cultivation only about 5 g l^{-1} of sucrose was utilised for the first 10 h of cultivation. A similar pattern was also observed on the TLC plates (Fig 2.1). The small amount of sucrose utilised was accompanied by the production of low concentrations of glucose, fructose and the three oligosaccharides (O1, O2 and O3) at concentrations of 2.0 g l^{-1}, 2.5 g l^{-1}, 1.4 g l^{-1}, 0.2 g l^{-1} and 0.2 g l^{-1} respectively. After 10 h of cultivation, there was a rapid utilisation of sucrose accompanied by a rapid increase in the levels of glucose, fructose, O1, O2 and O3. This increase continued to 22 h where the highest

concentrations of O1 (19.8 g l^{-1}) and O3 (7.8 g l^{-1}) were obtained. A concentration of 6.3 g l^{-1} was obtained for O2 at 28 h. After 28 h of cultivation, the sucrose was depleted and this was followed by a decrease in all three oligosaccharides (Fig 2.2). From the 28 h the colour of the monosaccharide spot on the TLC plate shifted to yellow-orange. This seems to match to HPLC results.

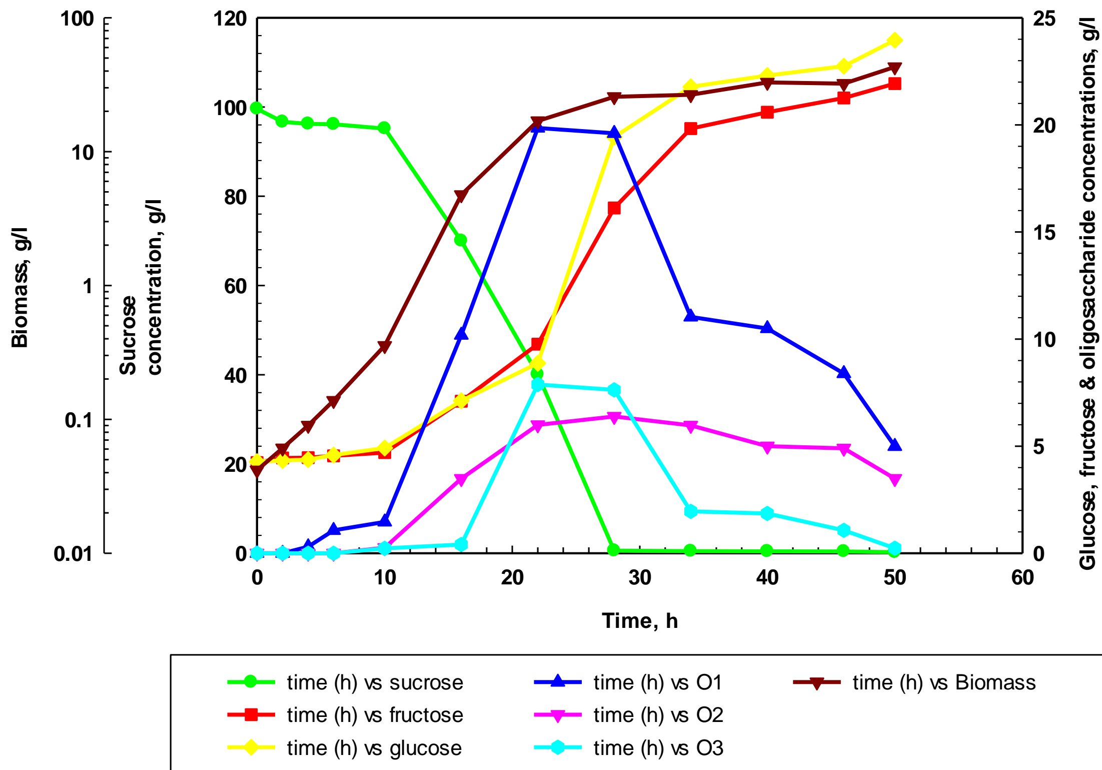

Figure 2.2 Cultivation profile for the production of oligosaccharides from L. scottii grown aerobically on sucrose at 25 °C in a rich medium using sucrose as carbon source.

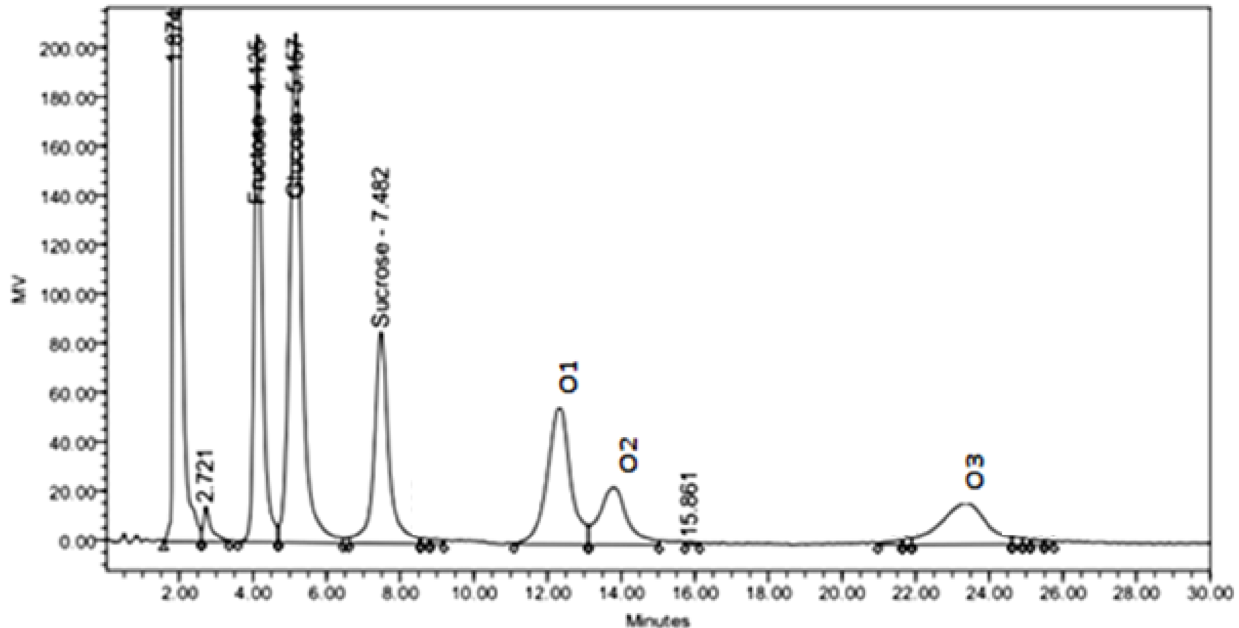

Figure 2.3 Typical HPLC chromatogram showing the elution peaks and retention time of individual sugars; glucose, fructose, sucrose and oligosaccharides (O1, O2 & O3).

The cultivation profile shows an increase in biomass concentration with a concomitant decrease in sucrose concentration and increase in all three oligosaccharides (Fig 2.2). There is a slow increase in the biomass concentration during the first 10 h of cultivation. During this time, the sucrose concentration also decreases slowly. An exponential increase in the biomass concentration is seen after 10 h of cultivation. This happens at the same time with the sucrose concentration declining exponentially. After 28 h of growth, the biomass concentration levels off as the substrate is depleted. When the biomass concentration levelled off after 28 h of growth, decreases in the levels of all three oligosaccharides were observed.

Table 2.1 Growth parameters of *L. scottii* Y- 1450 in aerobic shake flasks in a rich medium containing sucrose at 100 g l^{-1}.

Parameter	*Leucosporidium scottii*
Biomass (g l^{-1})	8.3
Biomass yield coefficient (g biomass g substrate^{-1})	0.28
Maximum specific growth rate (h^{-1})	0.28
Maximum volumetric rate of oligosaccharide production g (l h)$^{-1}$	1.53
Maximum yield coefficient for the production of oligosaccharides (oligosaccharides produced/sucrose assimilated)	0.58

5. Discussion

Oligosaccharides like glucooligosaccharides and fructooligosaccharides have been widely produced from sucrose using yeasts (Chung & Day, 2002; Kilian *et al*., 2002; Kritzinger *et al*., 2003; Kothari *et al*., 2012; Nobre *et al*., 2016; Vega & Zúniga-Hansen, 2011). TLC confirmed the presence of oligosaccharides paving the way for HPLC to be done. In this study, the yeast *L. scottii* produced a total of 33.9 g l^{-1} of oligosaccharides from 100 g l^{-1} sucrose. A maximum oligosaccharide yield coefficient of 0.56 (g oligosaccharide g sucrose^{-1}) and a maximum volumetric rate of oligosaccharide production of 1.53 g (l h)$^{-1}$ was obtained. Prata and co-workers reported a yield coefficient and maximum volumetric rate of 0.68 g g^{-1} and 3.25 g (l h)$^{-1}$ respectively while Mussatto and co-workers reported 0.64 g g^{-1} and 5.36 g (l h)$^{-1}$ for yields and volumetric rates of fructooligosaccharides respectively, obtained from 200 gl^{-1} of sucrose. The fructooligosaccharides were produced by *Penicillium expansum* and *Aspergillus japonicus* respectively. Spore suspensions were used as the inoculate for

the former while whole cells immobilised in different lignocellulosic materials were used for the latter. (Mussatto *et al*., 2009; Prata *et al*., 2010). Taking into consideration the aforementioned studies, one can conclude that the values obtained for the yields and productivity are encouraging, although some other factors like the type of microorganism (yeasts versus molds) may play a part in the outcome. A slight disappearance of sucrose during the lag phase was observed (Fig 2.4A). This can be explained by the fact that the enzyme activity is low during this stage because of the low biomass level; hence sucrose hydrolysis is minimal at this stage. During the exponential phase a rapid increase in oligosaccharide production was observed (Fig.2.4B) because enzyme activity at this stage is high due to a high biomass level, thus the conversion of sucrose to oligosaccharides. This was later followed by sucrose depletion and a decrease in oligosaccharide concentration during the stationary phase. *L. scottii* started consuming the produced oligosaccharide when sucrose was depleted thus accounting for its decrease in concentration. Santos and Maugeri attributed this to an inhibitory effect on the transfructosylation activity caused by the gradual increase of glucose and fructose concentrations (Santos & Maugeri, 2007). Moreover, Ning and co-workers also observed this when they produced neo-fructooligosaccharides from free whole cell biotransformation *by Xanthophyllomyces dendrorhous.* They linked this to the fact that in the presence of a high sucrose concentration, transfructosylation is enhanced while hydrolysis is depressed and vice versa (Ning *et al*., 2010). According to Nobre and co-workers the decrease in oligosaccharide concentration may be due to *L. scottii*'s competition for sucrose, thus reducing the amount of sucrose available to produce the oligosaccharides (Nobre *et al*., 2016). The same trend was observed by Chung and Day for the production of glucooligosaccharides from sucrose using *Leuconostoc mesenteroides* (Chung & Day, 2002) and also by Prata and co-workers for the production of fructooligosaccharides from sucrose using *Penicillium expansum* (Prata *et al*., 2010). With the depletion of sucrose the concentration of fructose and glucose kept increasing. This may arise from oligosaccharide hydrolysis thus leading to an increase in glucose and fructose in the culture broth. In a study carried out by Vera and co-workers (2012), they observed that the presence of a high substrate concentration favours the production of oligosaccharides since the transferring activity is higher at this

stage and minimal in the presence of low substrate concentrations. This may explain why there is oligosaccharide production in the presence of sucrose and its decrease after sucrose depletion. The cultivation profile showed an increase in the rate of oligosaccharide production between 16 h and 28 h. At this same period the concentration of glucose was slightly greater than that of fructose. This suggests that most of the fructose may be used for the production of the oligosaccharides. Belghith and co-workers reported that during transfructosylation the β (1, 2) bond of sucrose is cleaved and there is the transfer of the fructosyl group to another molecule such as sucrose, releasing glucose (Belghith *et al.*, 2012). This might explain why a higher glucose concentration was observed. It had been reported that the presence of glucose had an inhibitory effect on the production of oligosaccharides. It acts as an inhibitor of the enzymes, thus reducing the reaction efficiency (Antošová & Polakovič, 2001; Duan *et al.*, 1994; Jung *et al.*, 1989). The accumulation of glucose might have therefore contributed to a decrease in oligosaccharide production. To remedy this situation, glucose oxidase and glucose isomerase have been used to remove glucose via its transformation to gluconic acid and to fructose respectively (Sheu *et al.*, 2001; Yoshikawa *et al.*, 2008). Yun (1996) reported that fructooligosaccharide yields from *Aureobasidium pullulans* were low due to the hydrolytic activity which gave rise to glucose and fructose as reaction by-products and/or the fact that glucose acts as an inhibitor of the enzymes. Antošová & Polakovič (2001) reported that high substrate concentrations are needed for the production of high amounts of fructoligosaccharides because they increase the ratio of transfructosylating and hydrolysing activities of the β-fructofuranosidase enzymes. They further added that by increasing the substrate concentration the water activity decreases thus leading to an increase in the final FOS yield. Sucrose concentrations as high as 850 g l^{-1} was used. This suggests that the lower yields and productivity obtained from this study when compared to other studies may be attributed to the low sucrose concentration of 100 g l^{-1} which was used. To improve the yield and productivity, higher sucrose concentrations are recommended. Ganaie and co-workers also demonstrated that by using microbial strains with a high transfructosylating activity, high yields of oligosaccharides and low yields of monomeric sugars can be obtained (Ganaie *et al.*, 2013). It was observed that the concentrations of

the oligosaccharides (O1, O2 and O3) decrease as the degree of polymerisation increases. A total concentration of 19.8 g l^{-1}, 6.3 g l^{-1}, and 7.8 g l^{-1} were obtained for O1, O2 and O3 respectively. Mussatto and co-workers reported 46.83 %, 16.31% and 2.75 % for GF2, GF3 and GF4 respectively (Mussatto *et al.*, 2009). Along similar lines, Cruz and co-workers pointed out that FOS synthesis always occurs in the sequence GF → GF 2 → GF 3 → GF 4, as a consequence of the increasing K_m values for the oligosaccharides presented by the transfructosylase. Thus, high concentrations of the preceding oligosaccharide are necessary for the synthesis of the homolog with one more fructose unit (Cruz *et al.*, 1998).

This is the first study done on the production of oligosaccharides from *Leucosporidium scottii*. This study opens up possibilities to develop an efficient process for producing oligosaccharides from this yeast. The results obtained can be improved by establishing the optimal conditions for the process in order to obtain higher yields and productivity. With the search for novel prebiotics which can be utilised by probiotics, further studies need to be done to determine the prebiotic potential of these oligosaccharides.

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CHAPTER 3

PURIFICATION AND IDENTIFICATION OF OLIGOSACCHARIDES PRODUCED BY *LEUCOSPORIDIUM SCOTTII* Y-1450

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1. Abstract

Leucosporidium scottii Y-1450 was cultivated in a complex medium containing 100 g l^{-1} of sucrose. Chromatographic analysis showed the presence of three oligosaccharides (O1, O2 and O3) with a total concentration of 33.9 g l^{-1}. The highest concentration of total oligosaccharides was obtained after 22 h of cultivation and this sample was used for purification of the oligosaccharides. Preparative HPLC was performed on the culture supernatant and fractions were collected. The fractions were analysed by TLC and HPLC. A total concentration of 2.9 g l^{-1} was obtained after purification from 7.8 ml of supernatant. LC-MS peaks confirmed the presence of two trisaccharides (O1 and O2) and a tetrasaccharide (O3). O3 was also analysed using NMR for structural identification. The sample was found to contain mostly two fructooligosaccharides, namely the neonystose, β-Fru*f*-(2→6)-α-Glc*p*-(1→2)-β-Fru*f*-(1→2)-β-Fru*f*, and 1-kestose (α-Glc*p*-(1→2)-β-Fru*f*-(1→2)-β-Fru*f*) which may have originated from the breakdown of neonystose.

2. Introduction

The purity of oligosaccharides is vital to its functionality since the presence of monosaccharides and disaccharide like glucose, fructose and sucrose decreases the prebiotic activity of the mixture (Nobre *et al.*, 2016). Purity is also vital because it increases the viscosity of the oligosaccharide mixture thus improving body and mouthfeel, decreases the sweetness and hydroscopicity of the sugar and decreases the occurrence of Maillard reactions during heat processing. The absence of simple sugars also lowers cariogenicity and reduces the calorific value of the sugar, thus making it suitable for consumption by diabetic patients. In addition, the purity of oligosaccharides are important for characterisation (Crittenden & Playne, 1996; Crittenden & Playne, 2002). Several methods have been employed to improve the purity of oligosaccharides like gravity column chromatography using carbon celite columns (Morales *et al.*, 2006), ion exchange columns (Vinjamoori *et al.*, 2004) as well as silica gel columns (Reichardt & Martin-Lomas, 2005). Other purification processes include preparative TLC,

preparative HPLC and flash chromatography (Ojha *et al*., 2015; Shimoda & Hamada, 2010; Somiari & Bielecki, 1999). Microbial treatment is another process which is gaining popularity. In this method, microorganisms which lack carbohydrases which may degrade oligosaccharide are used to remove monosaccharides and disaccharides, leaving the oligosaccharide intact. Lu and co-workers removed 93.6 % of monosaccharides present in the final fermentation broth after cultivation by *Wickerhamomyces anomala* (Lu *et al*., 2013). This increased oligosaccharide purity from 54.4% to 80.1% (w/w). In this study, preparative HPLC was used to purify the oligosaccharides since it is an easy-to-use method and is also able to purify large quantities of samples.

The α glycosidic bonds present in some food products are easily hydrolysed by the gastrointestinal digestive enzymes. Non-digestible oligosaccharides with prebiotic potential contain β-glycosidic bonds, hence it is important to understand the structure of oligosaccharides because the bonds present between the sugar molecules play a vital role in determining whether the oligosaccharide has potential as a prebiotic (Kaur & Gupta, 2002; Priebe *et al.,* 2002; Sako *et al*., 1999; Tungland, 2003). Thin layer chromatography (TLC), gas liquid chromatography (GC), nuclear magnetic resonance (NMR), gas chromatography-mass spectrometry (GC-MS) as well as liquid chromatography-mass spectrometry (LC-MS) have been used for the structural identification of oligosaccharides (Sangeetha *et al.,* 2005). Linkage analysis is another powerful method which can be used for oligosaccharide identification (Jovanović *et al*., 2014).

The aim of this chapter was to purify oligosaccharides produced by the yeast *Leucosporidium scottii* by preparative HPLC and to structurally identify these oligosaccharides by MS and NMR.

3. Materials and Methods

3.1 Microorganisms and cultivation

Leucosporidium scottii Y - 1450 was used in this study. It was obtained from the University of the Free State MIRCEN yeast culture collection. Pure cultures of this yeast were maintained on Yeast Malt (YM) agar slants containing (per litre): 10 g sucrose, 5 g peptone, 3 g yeast extract, 3 g malt extract and 17 g agar. Pre-cultures and inocula used for fermentation were prepared as described in chapter 2. Shake-flask cultivations were also done as described in chapter 2.

3.2 Analytical Procedures

3.3 Preparative HPLC

The highest concentration of oligosaccharides was obtained after 22 h of cultivation. This sample was used for purification by preparative HPLC. A Phenomex Luna NH 20 cm x 250 cm column at 40 °C with 70 % acetonitrile as effluent at a flow rate of 4 ml/min eluent was used. 75 µl of supernatant was injected after which the different fractions of the three oligosaccharides were manually collected. The sugars eluted in order of increasing molecular masses, with fructose eluting first followed by glucose, sucrose, and finally O1, O2 and O3. O1 and O2 eluted very close to one another, which made their separation difficult, hence four fractions were collected instead of three. Fraction 1 contained the first half of O1 to prevent mixing with O2. Fraction 2 was collected at the end of the O1 peak, and the beginning of the O2 peak because the sample takes time to elute from the column and also to prevent mixing with fraction 2. Fraction 3 was obtained from the start of peak O2 to the end of peak O2. Since the peak for O3 was well separated from O2, it was collected as fraction 4 (Fig 3.1). A total of 105 injections were done with the four different fractions collected every time. A volume of approximately 5 ml was collected per injection for fraction 1, 3.5 ml for fraction 2, 4 ml for fraction 3 and 5.5 ml for fraction 4. Each set of the same fractions were pooled into new test tubes such that each tube contained 10 ml to ease acetonitrile evaporation. After fraction collection and pooling, the acetonitrile was evaporated under vacuum with an Eppendorff speedvac at a temperature of 60 ˚C until a volume of approximately 3 to

4 ml was obtained. Once acetonitrile was removed, as could be determined by the absence of its ether-like odour from the fractions, they were frozen in a -80 ˚C freezer for 24 h. They were then freeze-dried in an FTS Systems Flexi-Dry MP Freeze Dryer for 36 h. Upon collection of the dried oligosaccharides selected fractions were dissolved in distilled water (200 µl) and analysed by TLC and analytical HPLC.

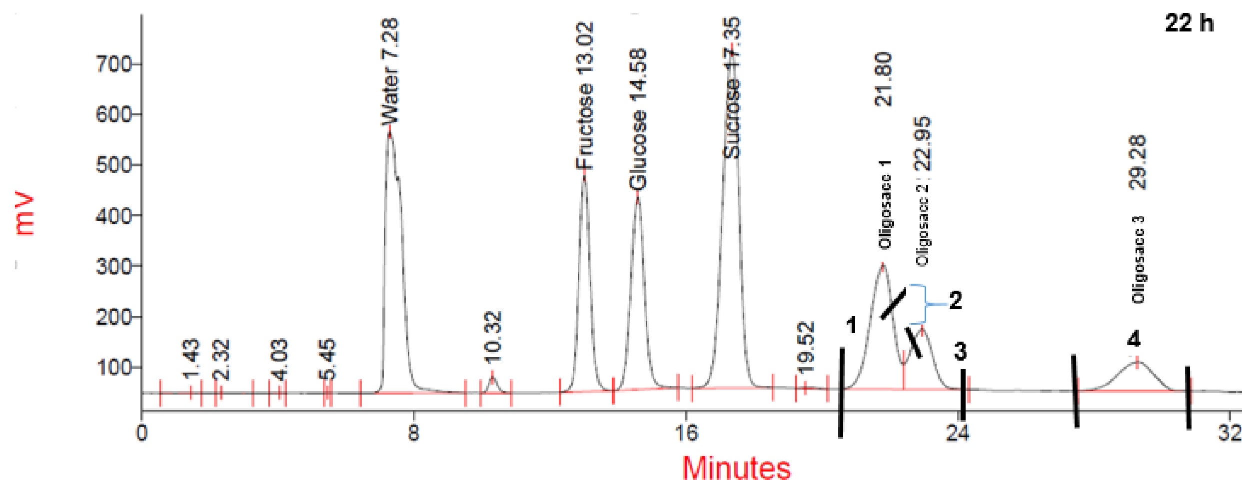

Figure 3.1 Chromatograms for the purification of oligosaccharides by Preparative HPLC. Fractions collected at different time intervals (1, 2, 3 & 4). Bold black strokes indicate the beginning and the end of fraction collection.

3.4 TLC

TLC was done as described in chapter 2. The trisaccharide neokestose which was previously isolated in our lab (unpublished data) was used as a standard together with fructose, glucose and sucrose.

3.5 Analytical HPLC

HPLC was done as described in chapter 2.

3.6 Liquid Chromatography-Mass Spectrometry (LC-MS)

LCMS (Liquid Chromatography Mass Spectrometry) was carried out to determine the composition of the oligosaccharides. A 10 fold dilution of the sample in 10 mM ammonium formate solution was done and it was infused at 10 µl/min into the Turbov electrospray ion source of an AB Sciex 3200 QTRAP mass spectrophotometer. Spectra were acquired in negative Q1 scan mode with a declustering potential of -83 V. Ion spray voltage was at -4500 V, heater temperature at 400 ˚C, a nebulizer gas of 20 psi, heater gas of 30 psi and a curtain gas setting of 20 psi was used.

3.7 Carbohydrate analysis

Carbohydrate analysis was performed at the Complex Carbohydrate Research Center and was supported by the Chemical Sciences, Geosciences and Biosciences Division, Office of Basic Energy Sciences, U.S.

3.8 MALDI-TOF mass spectrometry

MALDI-TOF analysis was performed on the sample using an Applied Biosystems 5800 instrument run in the positive ion reflector mode with DHB as the matrix.

3.9 Glycosyl linkage analysis

Linkage analysis was performed by the Complex Carbohydrate Research Center (CCRC). CCRC specializes in analyzing complex carbohydrates from plants, microbes and animals to determine the role these carbohydrates have in the growth and development, host-pathogen interactions and disease processes. They develop and use advanced analytical techniques like mass spectrometry, NMR spectroscopy, chemical and enzymatic synthesis, computer modeling, cell and molecular biology and immunocyto chemistry (Scientist solutions events, 2016).

The samples were permethylated, depolymerized, reduced, and acetylated; and the resultant partially methylated alditol acetates (PMAAs) analyzed by gas

chromatography-mass spectrometry (GC-MS), essentially as described by Heiss *et al* (2009), except for using a milder hydrolysis method to avoid isomerization of fructose to glucose.

About 1 mg sample was used for linkage analysis. The samples were suspended in 200 µL of dimethyl sulfoxide and left to stir for 1 day. Permethylation was effected by two rounds of treatment with sodium hydroxide (15 min) and methyl iodide (45 min). Following sample workup, the permethylated material was hydrolyzed using 0.1M TFA (0.5 h in a sealed tube at 100 °C), reduced with $NaBD_4$, and acetylated using acetic anhydride/pyridine. The resulting PMAAs were analyzed on an Agilent 7890A GC interfaced to a 5975C MSD (mass selective detector, electron impact ionization mode); separation was performed on a 30 m SP2330 bonded phase fused silica capillary column.

3.10 NMR Spectroscopy

NMR requires a minimum amount of 10 mg for analysis. The samples too must be pure before they can be analysed. However only fraction 4 (O3) qualified for NMR analysis as it was pure and I had a sample of 1200 mg. O4 was sent to the Complex Carbohydrate Research Center in Georgia, U.S.A for NMR analysis.

Total sample was deuterium-exchanged by lyophilization from D_2O, re-dissolved in 200µl D_2O, and transferred to an NMR tube with 3 mm OD. Proton-proton and proton-carbon correlated spectra were acquired on a Varian Inova-600 MHz spectrometer, equipped with a 3-mm cryoprobe. All spectra were acquired at 25 °C. Chemical shifts were referenced to internal acetone [$\delta(^1H)$ = 2.218 ppm, $\delta(^{13}C)$ = 33 ppm]. All experiments (1D proton, 2D gCOSY, TOCSY, gHSQC, HMBC, NOESY) were acquired with standard Varian pulse sequences.

4. Results

4.1 TLC analysis of preparative HPLC fractions

Acetonitrile was removed from the fractions collected by preparative HPLC. The fractions were then analysed by TLC as a rapid method to determine the purity of the oligosaccharides prior to quantitative analysis by HPLC. O1 was not successfully purified as shown by the two spots in fraction 1. It was concluded that fraction 1 was a mixture of O1 and O2 (Fig 3.2).

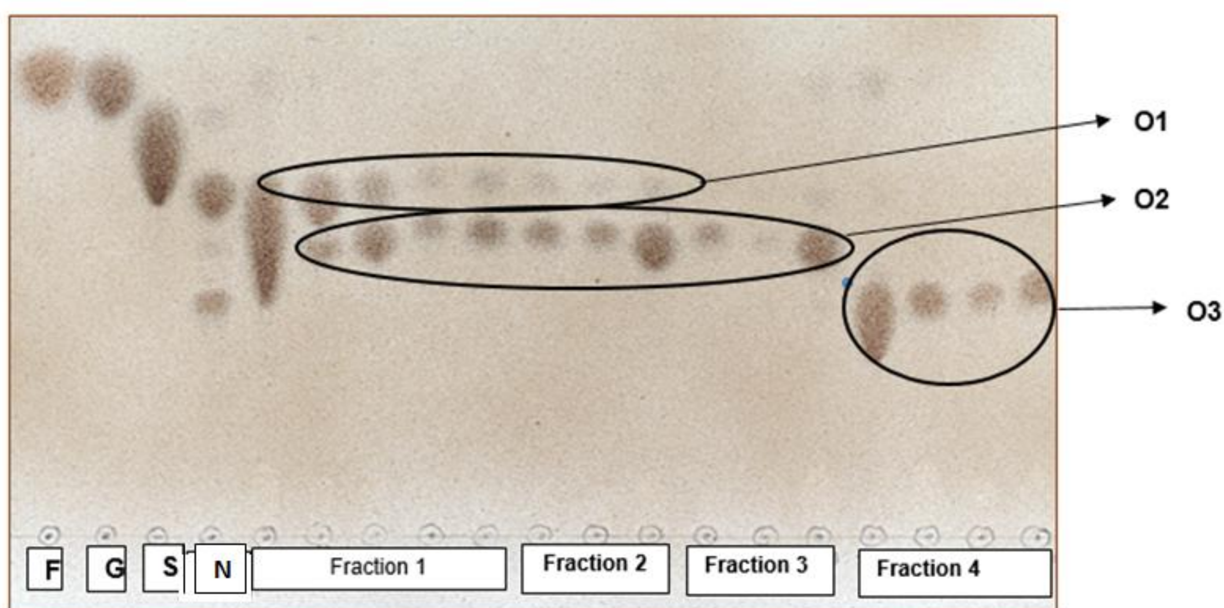

Figure 3.2 TLC analysis of the fractions collected by preparative HPLC. Glucose (G), fructose (F), sucrose (S), Neokestose, 1-kestose & neonystose (N) and oligosaccharides (O1, O2 & O3). Fraction 1 was obtained from the five different test tubes pooled for O1, fraction 2 was from the three test tubes containing the mixed fractions (O1 & O2), fraction 3 was obtained from the three test tubes containing O2 and fraction 4 was obtained from the four test tubes containing O3.

Fraction 2 was not pure due to the presence of both O1 and O2. Fraction 3 contained pure O2 as could be seen from the single spots on the TLC plate. All test tubes containing fraction 4 contained pure O3 since single spots were observed for fraction 4 on the TLC plates. Retention factors of 0.46, 0.39 and 0.32 were obtained for O1, O2 and O3 respectively.

4.2 HPLC analysis of preparative HPLC fractions

HPLC analysis of the four fractions (Fig 3.3 to 3.6) showed that only fractions 3 and 4 were pure. These fractions contained pure O2 and O3 respectively (Figs 3.4 and 3.5). Fractions 1 and 2 were impure and contained mixtures of both O1 and O2 (Figs 3.3 and 3.4).

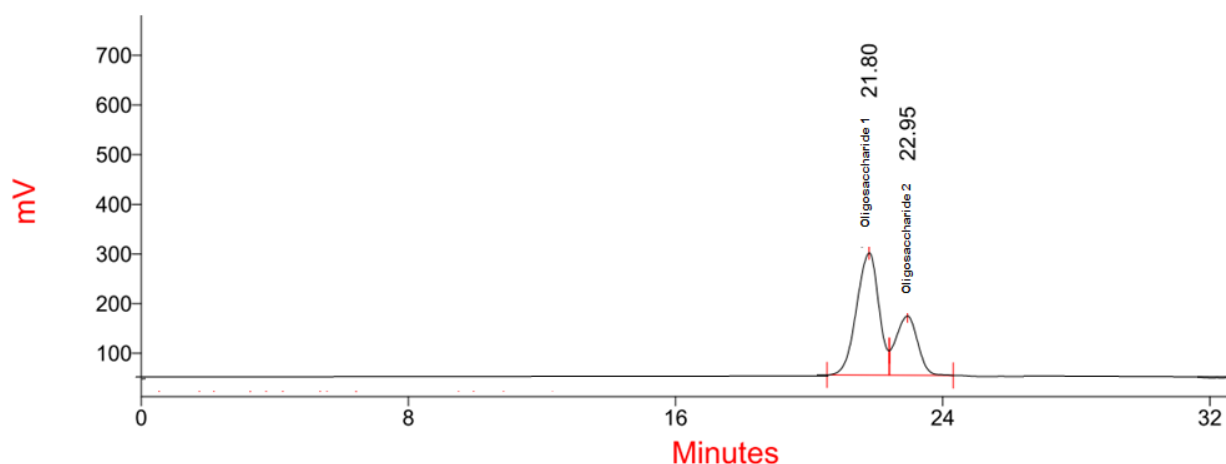

Figure 3.3 A typical HPLC chromatogram obtained from fraction 1 for the purification of oligosaccharides produced by *L. scottii.*

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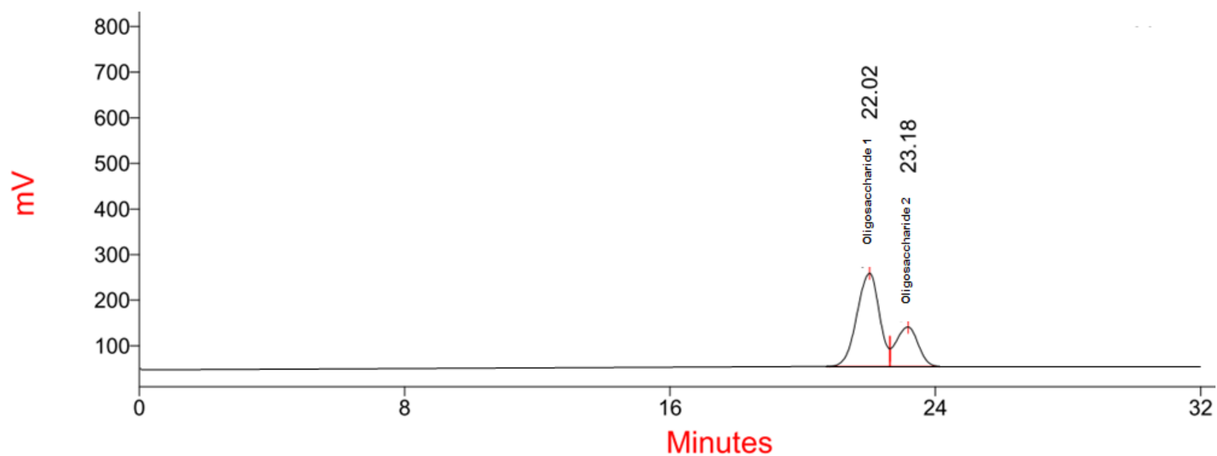

Figure 3.4 A typical HPLC chromatogram obtained from fraction 2 for the purification of oligosaccharides produced by *L. scottii.*

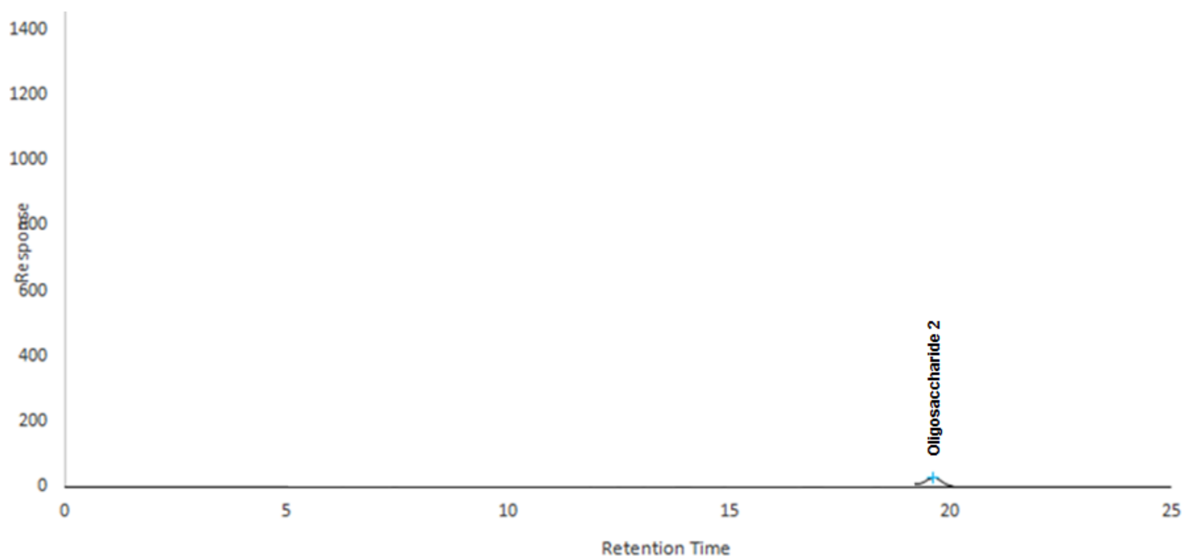

Figure 3.5 HPLC chromatogram obtained from fraction 3 for the purification of oligosaccharides produced by *L. scottii.*

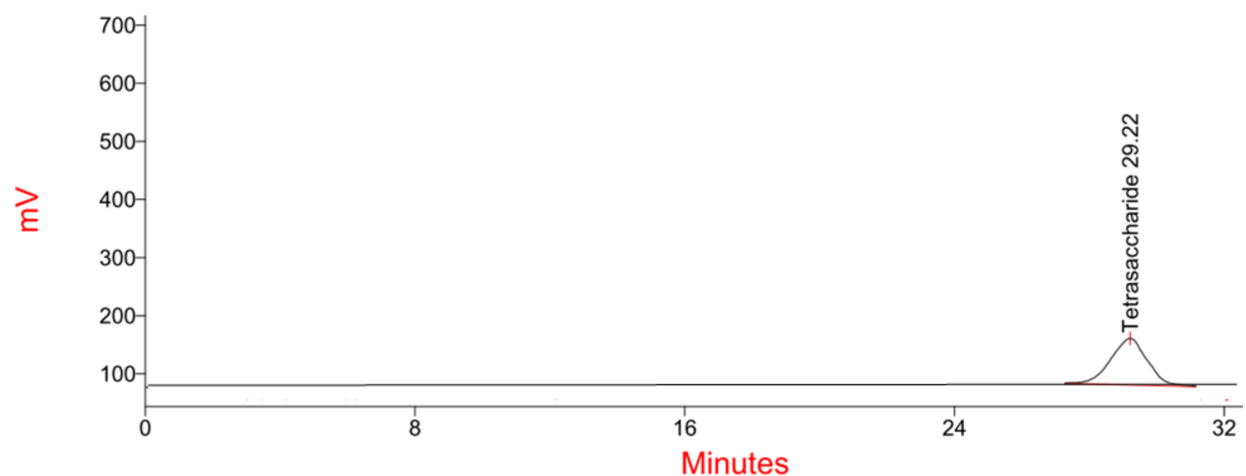

Figure 3.6 HPLC chromatogram obtained from fraction 4 for the purification of oligosaccharides produced by *L. scottii.*

The areas for the chromatograms obtained for fraction 3 were very small indicating the presence of low concentrations of O2. Fructose, glucose and sucrose were completely absent after purification (Figs 3.3 to 3.6). HPLC analysis showed concentrations of 0.99 g l^{-1}, 0.07 g l^{-1}, 0.0074 g l^{-1} and 1.21 g l^{-1} for fractions 1 to 4 respectively after the pooling of fractions (table 3.1).

Table 3.1 Concentration of products obtained by HPLC analysis.

Fractions	**Concentration obtained after HPLC (g l^{-1})**	**Oligosaccharide present in the fraction**
Fraction 1	0.99	O1 and O2
Fraction 2	0.70	O1 and O2
Fraction 3	0.0074	O2
Fraction 4	1.21	O3

4.3 Liquid Chromatography-Mass Spectrometry (LC-MS) analysis

LCMS was initially done on the supernatant and indicated the presence of three oligosaccharides, two trisaccharides and a tetrasaccharide (Fig 3.7).The fractions analysed by LCMS showed the presence of oligosaccharides as is depicted by Fig 3.8. However, the presence of fructose, glucose and sucrose with molecular masses of 179.2 g mol^{-1}, 179 g mol^{-1} and 341.3 g mol^{-1} respectively was also observed.

O1 may be a trisaccharide comprising a sucrose molecule linked to either a fructose or glucose molecule thus accounting for its molecular mass of 504.14 g mol^{-1}. O2 had a molecular mass of 539.3 g mol^{-1}. This may be a trisaccharide linked to an unknown molecule. The third oligosaccharide, O3 had a molecular mass of 665.5 g mol^{-1} indicating that it is a tetrasaccharide comprising of a sucrose molecule linked to two hexose molecules.

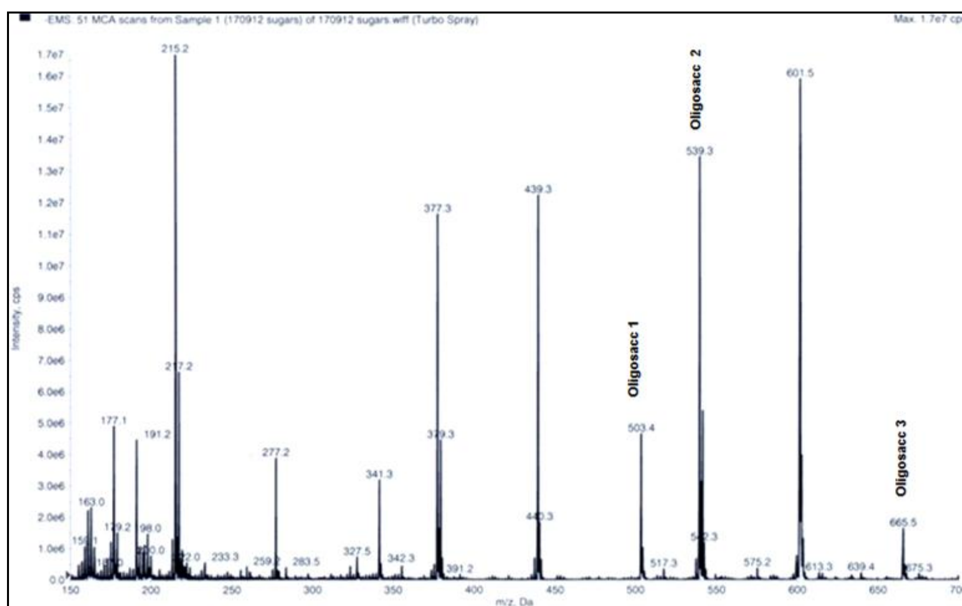

Figure 3.7 ESMS spectrum of the culture supernatant of *L. scottii.*

The fraction containing both O1 and O2 produced the spectrum shown in Fig 3.8. An unknown peak with a high intensity and a molecular mass of 601.5 g mol^{-1} occurs alongside O1 and O2. This may be a trisaccharide linked to an unknown compound.

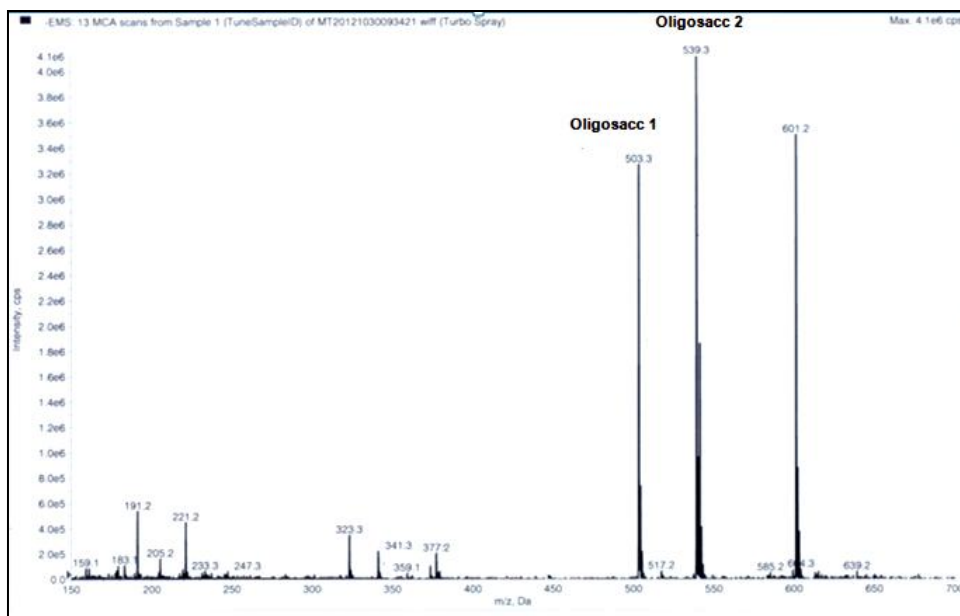

Figure 3.8 ESMS spectrum of fraction 2 containing both O1 and O2 obtained from the purification of oligosaccharides produced by *L. scottii.*

The analysis of fraction 4 produced a peak with a molecular mass of 665.3 g mol^{-1} which is compatible with that of tetrasaccharide (Fig 3.9). Other unidentified fragments were also present.

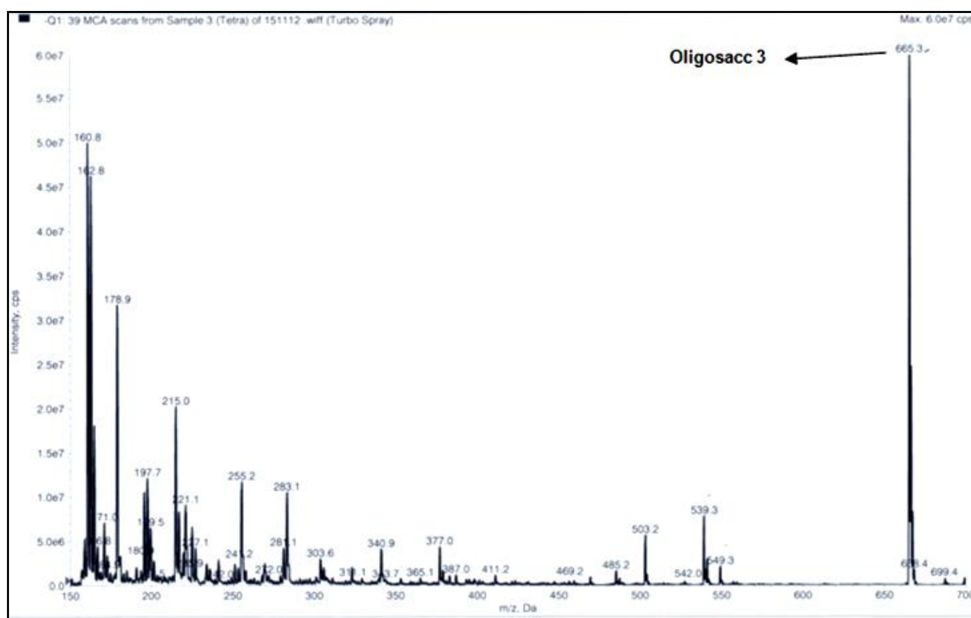

Figure 3.9 ESMS spectrum of fractions containing O3 obtained from the purification of oligosaccharides produced by *L. scottii.*

4.4 MALDI-TOF analysis

The MALDI-TOF spectrum showed the presence of two major oligosaccharides, each of which produced two peaks, one of the sodium and one of the potassium adduct. The more abundant of the two oligosaccharides was from a hexose tetrasaccharide, while the less abundant corresponded to a hexose trisaccharide.

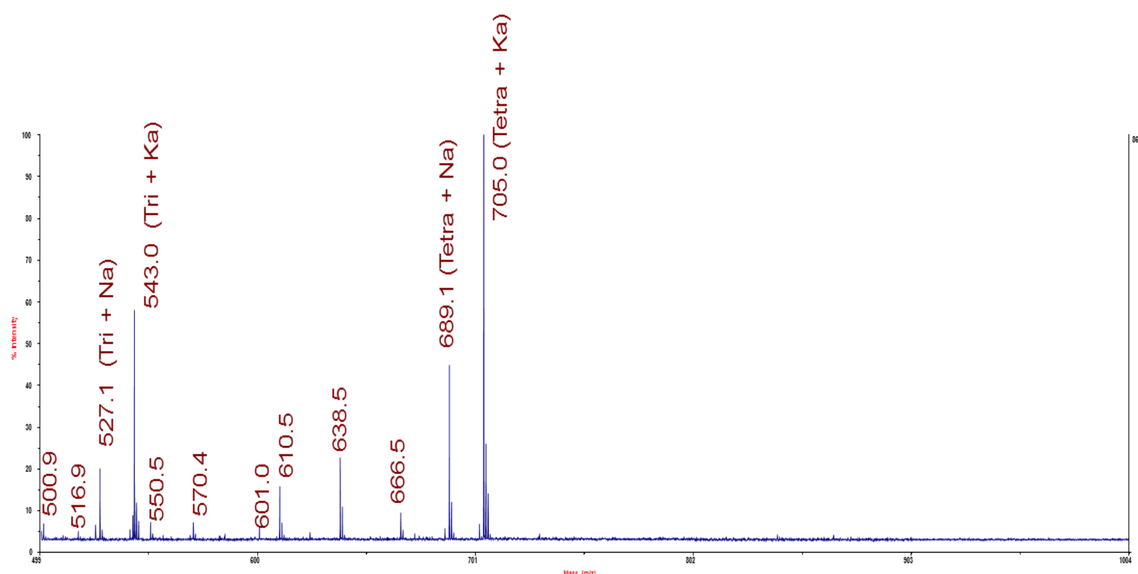

Figure 3.10 MALDI-TOF mass spectrum of the oligosaccharide sample (O3).

4.5 Linkage analysis

Fructose linkage analysis was performed on the sample, as well as on two model compounds, namely 1-kestose and nystose. These were shown to contain terminal fructofuranose (t-Fru*f*), 1-linked fructofuranose (1-Fru*f*), and terminal glucopyranose (t-Glc*p*) in 1:1:1 and 1:2:1 ratios, respectively, as expected from their structure (Figs 3.12 & 3.13). The sample O3 was shown to contain t-Fru*f*, 1-Fru*f*, 6-Glc*p* in a 2:1:1 ratio, consistent with a neonystose structure, but also a significant quantity of t-Glc*p* (Fig 3.11, Table 3.2). The presence of t-Glc*p* indicated heterogeneity in the sample, as also demonstrated by the MALDI-TOF mass spectrum.

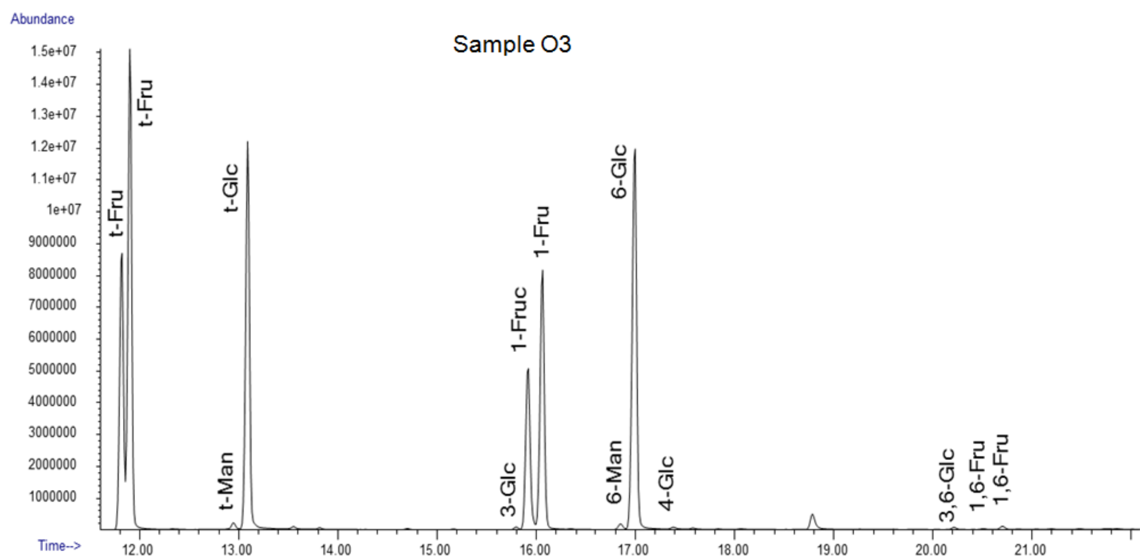

Figure 3.11 The TIC (Total Ion Chromatogram) of sample O3.

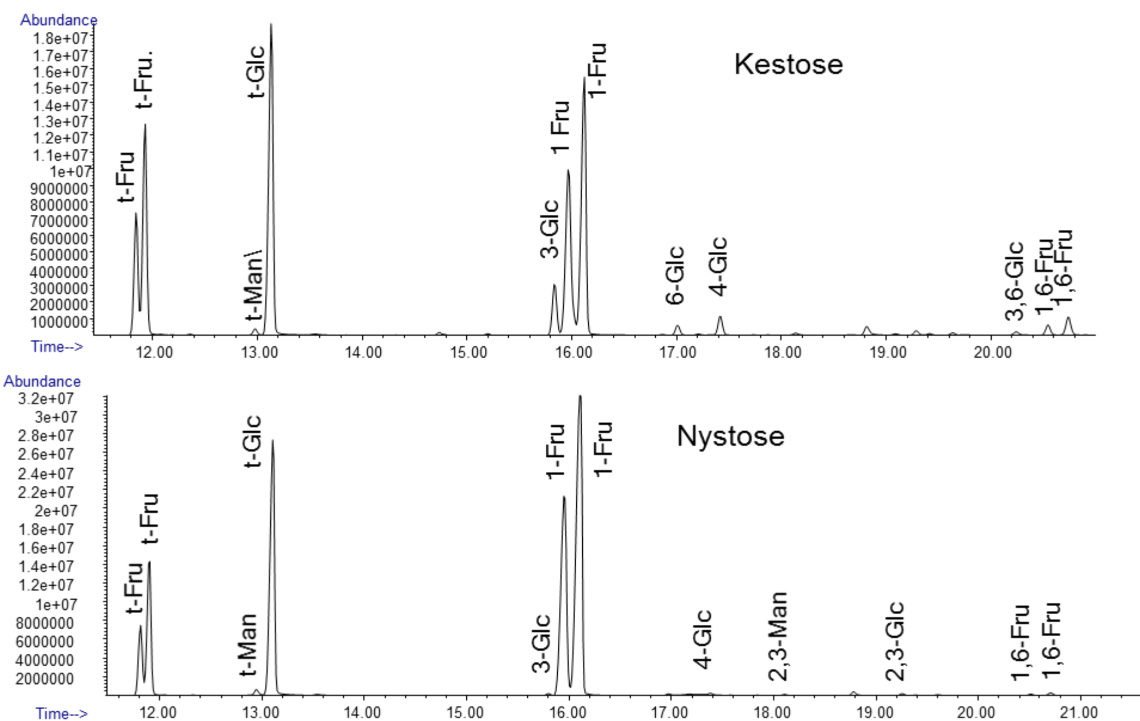

Figure 3.12 The TIC chromatograms of sample 1-kestose and nystose standards run alongside the sample.

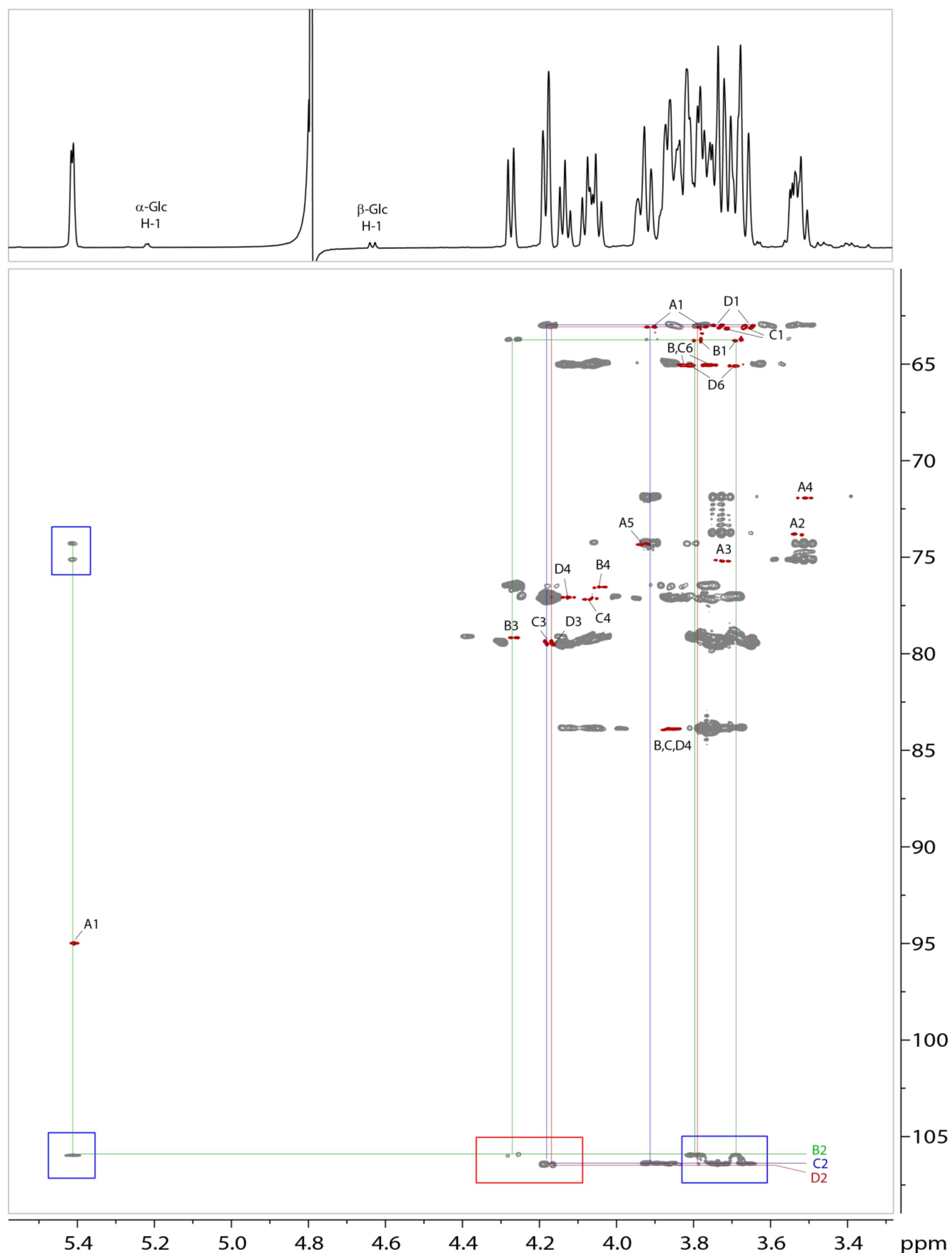

Figure 3.13 1D-Proton (top), 2D-HSQC (bottom, red) and HMBC (bottom, grey) spectra of the fructooligosaccharide. The scale in the z-dimension within red boxes has been zoomed in 10 times; the z-scale in the blue boxes has been zoomed out 5 times to maintain clarity of the HMBC spectrum.

4.6 NMR spectroscopy

A major signal of an α-glucosyl residue at 5.41 ppm was detected in the anomeric region of the 1D proton spectrum (Fig 3.14). Minor α- and β- anomeric signals of reducing glucose were detected at 5.22 and 4.64 ppm, respectively, at low intensity. The 2D NMR analysis showed these minor peaks to be from monomeric glucose.

Table 3.2 The relative percentage of each linkage residue in sample O3.

Residue	% Area
Terminal Fructose residue peak #1 (t-Fru)	14.1
Terminal Fructose residue peak #2 (t-Fru)	21.9
Terminal Mannopyranosyl residue (t-Man)	0.3
Terminal Glucopyranosyl residue (t-Glc)	18.7
3-linked Glucopyranosyl residue (3-Glc)	0.1
1-linked Fructose residue peak #1 (1-Fru)	9.0
1-linked Fructose residue peak #2 (1-Fru)	13.7
6-linked Mannopyranosyl residue (6-Man)	0.4
6-linked Glucopyranosyl residue (6-Glc)	21.3
4-linked Glucopyranosyl residue (4-Glc)	0.1
3,6-linked Glucopyranosyl residue (3,6-Glc)	0.1
1,6-linked Fructose residue peak #1 (1,6-Fru)	0.1
1,6- linked Fructose residue peak #2 (1,6-Fru)	0.2

In HMBC (Fig 3.13), cross-peaks of protons with tertiary carbon indicated the presence of keto-sugars in the sample. Sets of correlations with three different quaternary anomeric carbon signals near 106 ppm indicated presence of at least 3 different ketose residues. The proton chemical shifts of ketoses were assigned from the COSY, TOCSY, HSQC, and HMBC data (Fig 3.14 - 3.19). The α-glucose residue was 6-linked as indicated by slight downfield shift of H5 and H6 protons; in addition to which ring proton signals were not shifted (Table 3.3). In HMBC, a cross-peak at 5.415/105.96 ppm

confirmed the linkage between C2 of β-Fructose B and H1 of α-glucose A. Another HMBC cross-peak at 3.91/106.36 ppm demonstrated glycosidic linkage between H-6 of A and C-2 of C. This led to the partial sequence C-A-B. An additional HMBC cross peak was found at 3.79/106.41 ppm, showing that H1 of B was linked to C2 of D. The identity of Residue B as the 1-linked fructofuranosyl residue was confirmed by the downfield chemical shift of C1. The data taken together indicated the sequence C-A-B-D for the tetrasaccharide. Thus the structure of the tetrasaccharide, which was the major component of the sample, was β-Fru*f*-(2→6)-α-Glc*p*-(1→2)-β-Fru*f*-(1→2)-β-Fru*f*. In addition, looking at the structure of neonystose, hydrolysis of this compound may result in the formation of 1-kestose. (Fig 3.20). This may account for the presence of 1-kestose in the sample.

Table 3.3 Chemical shift assignment of the NMR signals.

No.	**Residue**	**Chemical shift (ppm)**						**NOE**
		1	**2**	**3**	**4**	**5**	**6**	**HMBC**
A	6-α-Glc*p*	5.415	3.53	3.73	3.51	3.93	3.91/3.79	A1-B1
		94.97	73.76	75.14	71.85	74.24	63.01	A1-B2
B	1-β-Fru*f*	3.79/3.69	-	4.274	4.052	3.86	3.83/3.77	B1-A1
		63.66	105.96	79.12	76.47	83.85	64.92	B2-A1
C	β-Fru*f*	3.74/3.66	-	4.183	4.074	3.86	3.83/3.77	
		62.92	106.36	79.32	77.13	83.85	64.95	C2-A6
D	β-Fru*f*	3.73/3.65	-	4.179	4.132	3.86	3.81/3.70	
		63.04	106.41	79.45	77.02	83.85	65.02	D2-B1

NOE: Nuclear Overhauser Effect

HMBC: Heteronuclear Multiple Bond Correlation

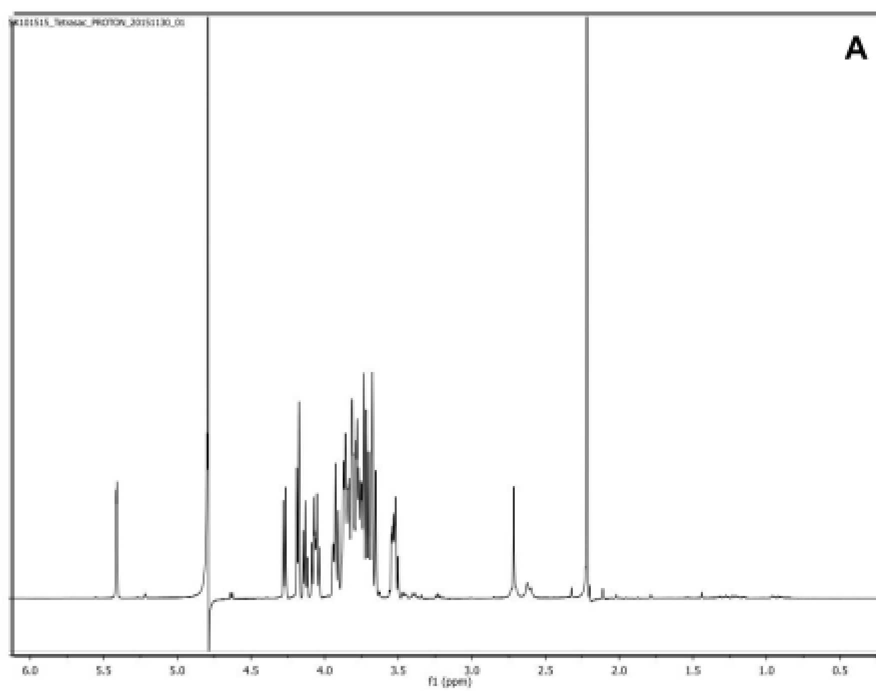

Figure 3.14 The PROTON spectrum of sample O3

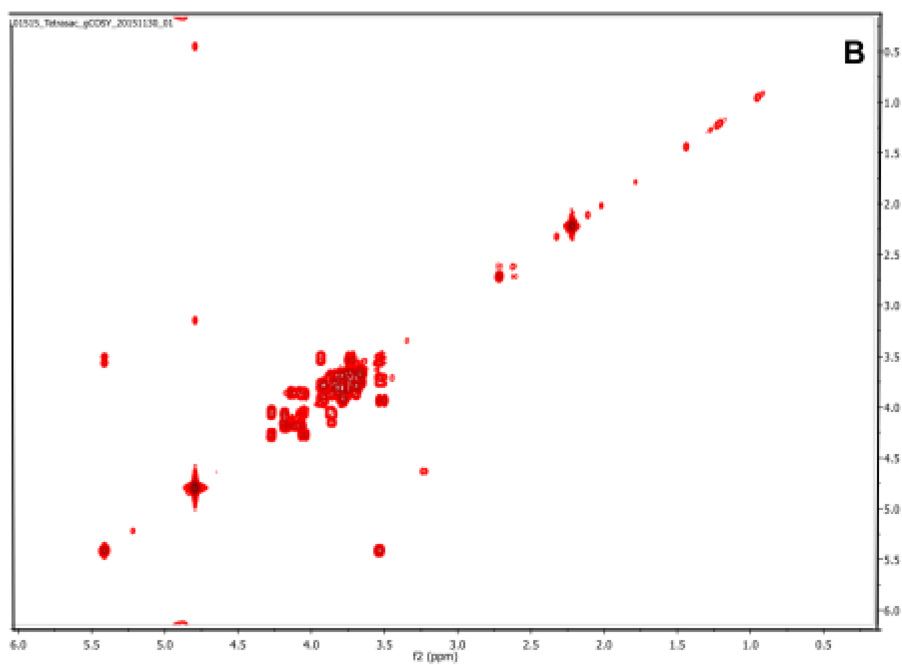

Figure 3.15 gCOSY spectrum of sample O3.

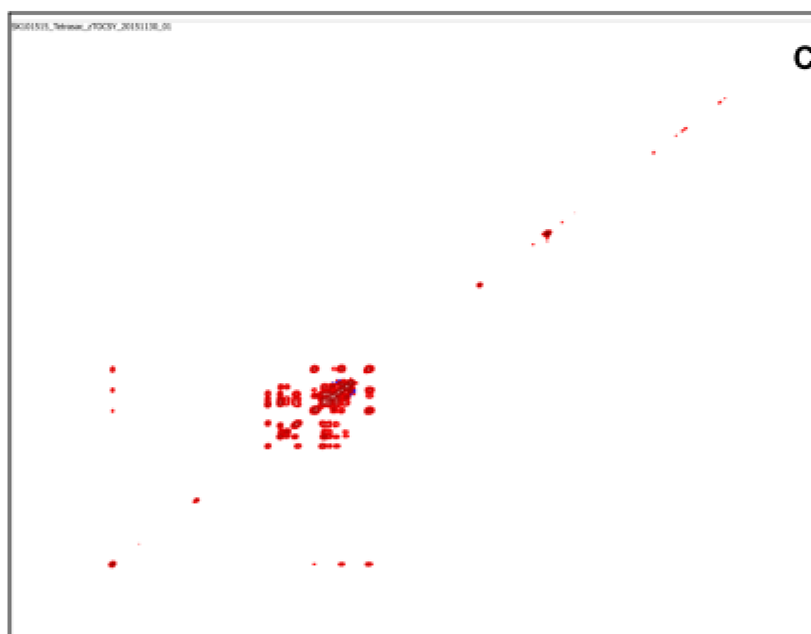

Figure 3.16 zTOCSY spectrum of sample O3.

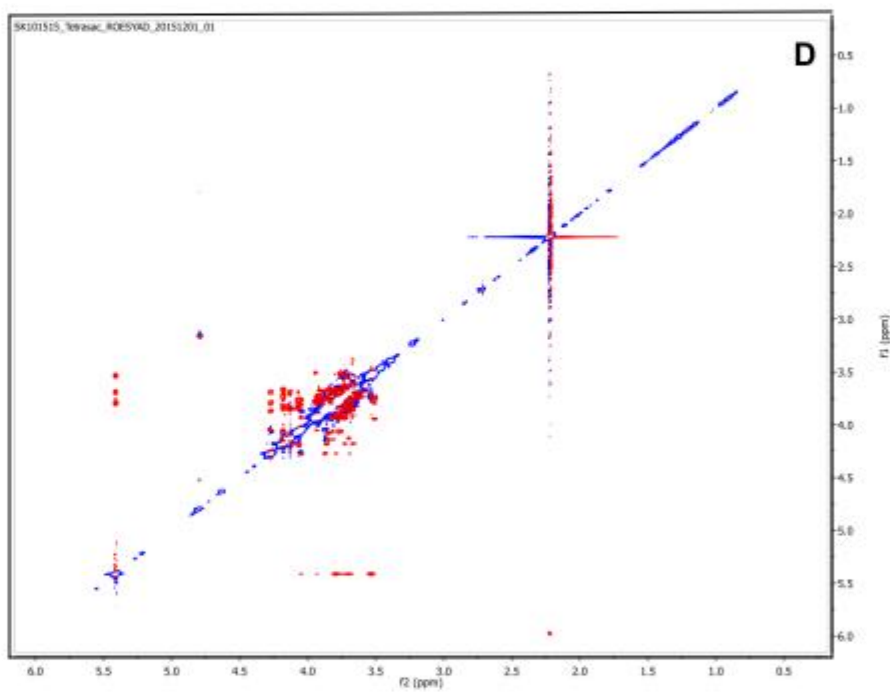

Figure 3.17 ROESYAD spectrum of sample O3.

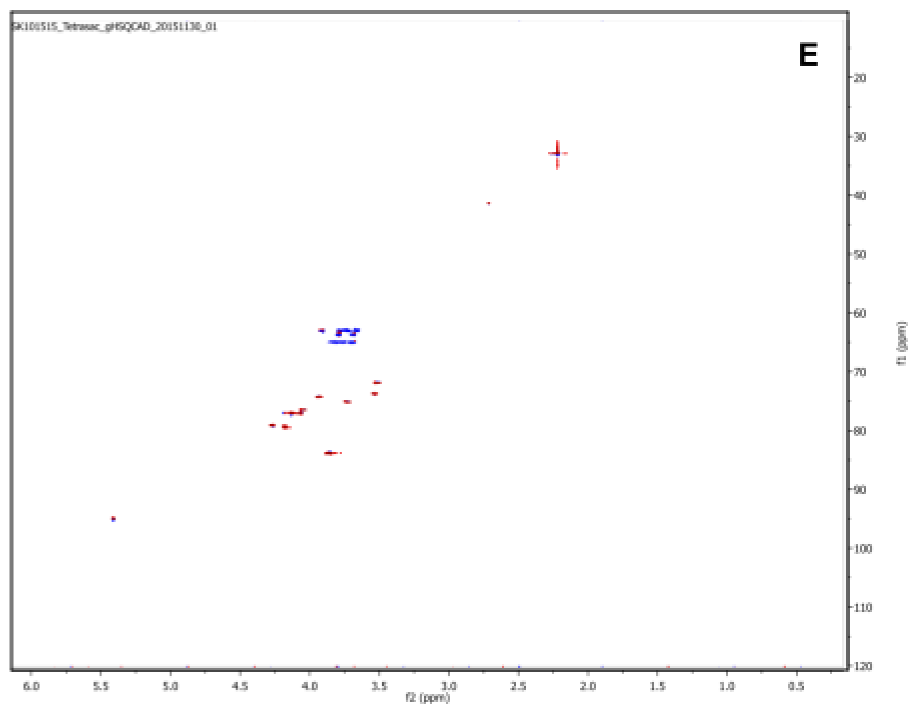

Figure 3.18 gHSQCAD spectrum of sample O3.

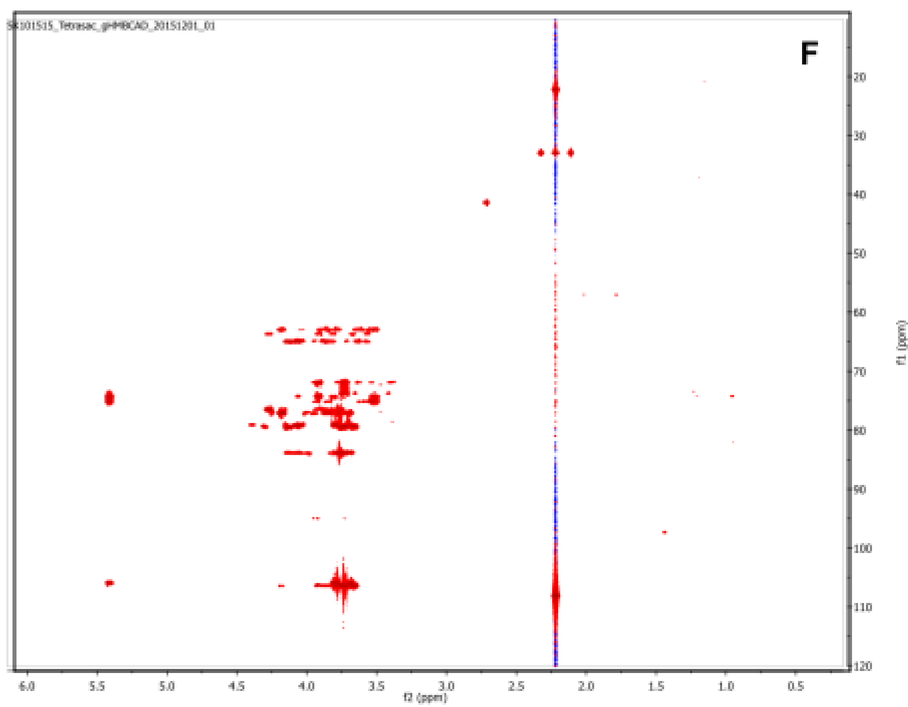

Figure 3.19 gHMBCAD spectrum of sample O3.

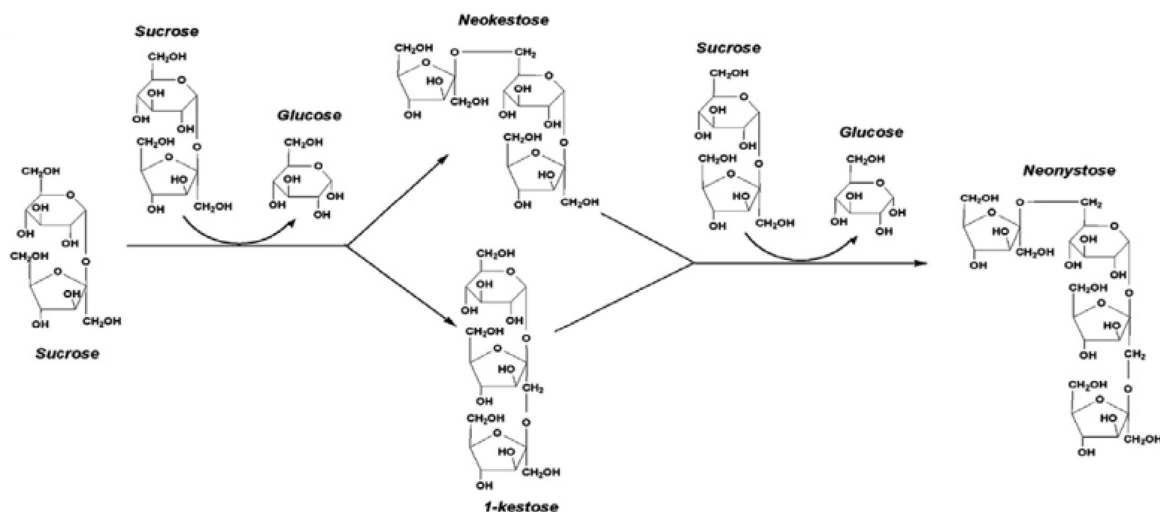

Figure 3. 20 The formation of neofructooligosaccharides (Linde *et al.*, 2012).

5. Discussion

In this study oligosaccharides were isolated from a culture supernatant of *Leucosporidium scottii* growing on sucrose. Purification was followed by analytical TLC and HPLC. The TLC results showed the presence of O2 and O3 in fractions 3 and 4 respectively (Fig 3.2). O1 was not successfully purified by this method. However, fructose, glucose and sucrose were completely removed by preparative HPLC. Given that O1 and O2 elute very close to each other, a pure fraction of neither oligosaccharide was obtained. HPLC showed purity at a low concentration for O2. Rf values obtained for the oligosaccharides (O1= 0.46, O2= 0.39 and O3= 0.32) are similar to the values obtained by Praznik and co-workers and also Kritzinger and coworkers for neokestose, 1-kestose and nystose, respectively, from a fructan containing plant (Praznik *et al.*, 2006). This suggests that the oligosaccharides are two trisaccharides and a tetrasaccharide.

The results obtained for the HPLC analysis of four different fractions were in accordance with what was observed on TLC plates. Purification successfully eliminated monosaccharides and sucrose (Fig 3.3 to Fig 3.6). Oligosaccharide 1 and 2 eluted very

close to one another and this resulted in failure to obtain pure fractions of O1 (Fig 3.3 and Fig 3.4). However, the purification of O2 (Fig 3.5) and O3 (Fig 3.6) was successful. Hotchkiss and Irwin purified cellodextrins which were produced from cellulose by preparative HPLC. In the first purification stage, impure fractions containing significant amounts of glucose and cellobiose were obtained. To overcome this, a second purification step (with Preparative HPLC) was carried out to obtain pure cellodextrins using an Aminex Q-15S, Ca^{2+} -form cation exchange resin unlike in the first where a column packed with AG-50W - X4 Ag^{+} -form was used (Hotchkiss and Irwin, 1994). In order to separate O1 from O2 another purification step can be carried out. Unfortunately, this could not be done due to the small amount of oligosaccharide obtained from the first purification step. Moreover, some of the oligosaccharide may be lost in the second purification step due to transfer between storage containers. Somiari and Bielecki (1999) used five semi preparative HPLC columns to purify the trisaccharide kestose from a mixture of glucose, fructose and sucrose produced enzymatically from sucrose. The isolated kestose still contained carbohydrate impurities. Pure fractions of O3 could easily be obtained as it was well separated from O2. Larger quantities as compared to O1 and O2 were obtained as a result of this. A percentage recovery of 0.12 % and 15.3 % was obtained for oligosaccharide 2 and oligosaccharide 3 respectively. This was calculated as follows: the amount of pure oligosaccharides recovered (g) / amount of oligosaccharides in the sample (g) X 100. The use of preparative HPLC to purify oligosaccharides is uncommon but has been used in some studies (Hicks *et al.*, 1994; Sadeh *et al.*, 1983; Somiari and Bielecki, 1999; Smouter & Simpson, 1993). Purification with carbon-celite column chromatography has been widely used to isolate oligosaccharides produced from sucrose. Kuhn and co-workers obtained a purification efficiency of 94 % using this method (Kuhn *et al.*, 2014). Other researchers used activated charcoal since it has a higher affinity for oligosaccharides as compared to monosaccharides and disaccharides (Kuhn & Filho, 2010; Nobre *et al.*, 2012; Boon *et al.*, 2000). This explains why Kuhn and co-workers obtained a high purification efficiency. Both methods are sometimes employed in oligosaccharide purification (Okada *et al.*, 2006; Okada *et al.*, 2011; Swallow & Low, 1993). In order to obtain larger quantities of pure product, more of the oligosaccharide-containing

supernatant will have to be purified. However, a sufficient amount of tetrasaccharide was obtained for further NMR analysis.

LCMS has been widely used by researchers to aid in the structural identification of oligosaccharides (Harrison *et al*., 2009; Liu & Rochfort, 2015; Moreno *et al*., 2014; Yu *et al*., 2013). Electrospray Mass Spectrometry (ESMS) spectrum of the supernatant showed the presence of three oligosaccharides (Fig 3.8) with m/z of 503.4, 539.3 and 665.5 Da, respectively. In addition to the products of interest, sucrose (341.3 Da) and fructose and / or glucose were also detected (179.2 Da). The ESMS spectrum of the sample containing both O1 and O2 showed peaks for both oligosaccharides, but also showed a peak with a m/z similar to that of O3 which was unexpected since O3 elutes long after O1 and O2 (Fig 3.9). This was not detected by TLC or HPLC. This could be explained by the fact that the sample was too diluted to be detected by TLC and HPLC. The ESMS spectrum of the pure fraction containing O3 showed a noticeable peak of O3 with an m/z of 665.3 Da (Fig 3.10). Ota and co-workers (2009) obtained similar m/z ratios and degree of polymerisation for trisaccharides and tetrasaccharides obtained from a mixture of maltoheptaose and [U-^{13}C]maltose. In their study it was postulated that transglucosidase from *A. niger* transferred a [U- ^{13}C]glycosyl residue to the non-reducing end of maltoheptaose, while the enzyme degraded maltoheptaose into maltooligosaccharides with DP 1-6. A total of 2.9 g l^{-1} of oligosaccharide was obtained after purification. In my study however, only O3 was obtained in a sufficient quantity (greater than 10 mg) for NMR studies (Otte *et al*., 2014).

MALDI-TOF analysis confirmed the presence of two oligosaccharides, a trisaccharide and a tetrasaccharide (Fig 3.10). Furthermore, linkage analysis showed the tetrasaccharide to be neonystose (β-Fru*f*-2-6-α-Glc*p*-1-2-β-Fru*f*-1-2-β-Fru*f*) with a ratio of 2:1:1 for t-Fruf, 1-Fruf and 6-Glcp respectively (Fig 3.11). The presence of a terminal glucose was also detected in about equal proportion to the other linkages. This was partly from free glucose and the trisaccharide 1-kestose, which were both present in addition to neonystose. NMR showed the presence of free glucose a contaminant. However, this was too low to account for the relatively large amount of terminal glucose found in the linkage analysis, but this may just be due to the semi-quantitative nature of

the linkage analysis. The structure of the trisaccharide could not directly be determined by NMR due to signal overlap and the small chemical shift displacements caused by substitution with fructofuranosyl residues that is typical for fructooligosaccharides. However, judging by the linkage results, which showed a high amount of terminal glucose, it is likely that the trisaccharide in the sample is 1-kestose (α-Glc*p*-(1→2)-β-Fru*f*-(1→2)-β-Fru*f*). Moeover, hydrolysis of the glucose moiety of neonystose can give rise to either 1-kestose or neokestose (Fig 3.20). Information on the chemical shifts of neonystose is scarce. Okada and coworkers identified FOS from fermented beverage of plant extract using methylation analysis, MALDI-TOF-MS and 2D NMR measurements. The fructose residues present were in a pyranose form and were identified as p-1-kestose or pyrano-isokestose (Okada *et al.*, 2010). Zambelli and coworkers also identified FOS produced by mycelium-bound transfructosylation activity present in *Cladosporium cladosporioides* and *Penicillium sizovae* using similar methods (Zambelli *et al.*, 2014). NeoFOSs have superior *Bifido*-stimulating effect and chemical and thermal stability compared to other FOSs (Wang, 2015). A study done by Kilian and coworkers showed that neokestose improved the population of *Bifidobacteria* and *Lactobacilli* to a greater extent than currently available FOSs and to inhibit the growth of *Clostridia* (Kilian *et al.*, 2002). Enzymes produced by *Xanthophyllomyces dendrorhous* have been widely used in the production of NeoFOSs. In most cases, sucrose was used as substrate (Kritzinger *et al.*, 2003; Linde *et al.*, 2009; Linde *et al.*, 2012; Ning *et al.*, 2010; Sheu *et al.*, 2013). *Penicillium oxalicum* (Itoh & Shimura, 1987) and *Penicillium citrinum* (Hayashi *et al.*, 2000; Park *et al.*, 2005; Lim *et al.*, 2007) as well as *Aspergillus awamori* (Grizard & Barthomeuf, 1999) have all been used to produce NeoFOSs from sucrose.

This is the first report on the purification and identification of oligosaccharides produced by *Leucosporidium scottii*. Preparative HPLC, methylation analysis, MALDI-TOF-MS and 2D NMR measurements have again proofed to be successful in oligosaccharide purification and identification. The analysis reported did not include the absolute configuration (D or L) of monosaccharides. Further work needs to be done to determine the absolute configurations. Moreover, 1-kestose and neonystose can further be explored for their prebiotic potential.

6. References

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CHAPTER 4

OPTIMISATION OF OLIGOSACCHARIDE PRODUCTION FROM *LEUCOSPORIDIUM SCOTTII* Y-1450 USING DESIGN OF EXPERIMENTS (DOE)

CONTENTS

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1. Abstract

The confirmation of oligosaccharide production by *Leucosporidium scottii* Y-1450 prompted its optimisation using an experimental design. This study focussed on the optimisation of oligosaccharides produced by *L scottii* using a screening design. An irregular fractional factorial design with 12 runs was used. The effects of four variables (cell concentration, sucrose concentration, pH and temperature) on the conversion of sucrose into oligosaccharides by whole cells obtained from the exponential phase and suspended in buffer were investigated. TLC and HPLC were used to quantify the oligosaccharides produced. Three responses were measured: maximum concentration of oligosaccharide produced, maximum yield coefficient (oligosaccharide produced/sucrose utilised) and maximum productivity of oligosaccharides. Pareto charts and interaction plots were used to identify the main effects and the interactions involved in the observed responses while statistical analysis was done using Analysis of variance (ANOVA). The optimum conditions for oligosaccharide production were found to be: sucrose concentration 200 g l^{-1}, cell concentration 8 g l^{-1}, 20 °C and pH 7. These parameters yielded a maximum oligosaccharide concentration, maximum yield coefficient and maximum productivity of 114 g l^{-1}, 0.81 g g^{-1}, and 10.84 g $(l\ h)^{-1}$ respectively. Sucrose was identified as the most significant main effect and had a positive effect on the maximum concentration and yield of fructooligosaccharides (FOS). Strong positive interactions involving cell concentration and sucrose concentration were identified. Temperature, on the other hand, had a negative effect while the pH had no significant effect on the responses.

2. Introduction

Ever since oligosaccharides were accorded prebiotic status, there has been an increase in interest in their production worldwide. Owing to their physiological benefits and to consumers' preference for healthier food, they have received interest from the food and pharmaceutical sectors. In the food industry they have been used as sweeteners,

weight controlling agents and humectants in confectioneries, bakeries and breweries (Park & Oh, 2010). In the health sector they have been found effective in the proliferation of gastrointestinal normal microbiota and suppression of pathogens, prevention of dental caries, enhancement of immunity and facilitation of mineral absorption. They are also used as sources of antioxidants, antibiotic alternatives, as well as regulators of blood glucose in diabetics and serum lipids in hyperlipidemics (Mussato *et al*., 2009). Production of oligosaccharides has been done using free whole cells, extracellular enzymes and immobilised whole cells (Kritzinger *et al*., 2003; Linde *et al*., 2009; Park *et al*., 2005). The use of free whole cells provides a cost effective method. Moreover, the cells can be reused and it also makes use of a single-step process for oligosaccharide production (Ning *et al*., 2010). Ning and co-workers (2010) observed that free cells exhibited a higher productivity of neo-fructooligosaccharides than immobilised cells.

In experimental design, three different approaches can be used: the matrix method, the one-at-a-time method and the statistical design approach. The matrix method involves the layout of a matrix of all the interesting combinations of the variables. All these combinations are investigated until the solution is found. Although this method requires many measurements, it is advantageous because it thoroughly explores the experimental space (Haaland, 1989). In the one-at-a-time method, only one factor or variable is varied at a time while keeping the others fixed. For each variable the best value is found and the process is repeated for the remaining variables until all variables have been considered. The disadvantage of this method is that it takes too many experiments and interactions between the variables may be missed (Czitrom, 1999; Haaland, 1989). The statistical problem-solving approach uses a series of small, carefully designed experiments. Each experiment carefully explores the experimental space while studying many variables using a small number of observations. In addition, the estimates of the effects of each factor are more precise. Using more observations to estimate an effect results in higher precision (reduced variability). Moreover, the interaction between factors can also be estimated systematically with a statistical design (Czitrom, 1999; Haaland, 1989).

The aim of this study was to optimise the conditions for the production of oligosaccharides from *Leucosporidium scottii Y-1450* using a screening statistical design. An irregular fractional factorial design including 12 runs was used. Temperature (20 °C and 60 °C), cell concentration (0.8 g l^{-1} and 8 g l^{-1}), sugar concentration (50 g l^{-1} and 200 g l^{-1}) and pH (4 and 7) were the factors investigated, each at a high and low level. Three responses were measured: maximum concentration of oligosaccharide produced, maximum yield coefficient and maximum productivity of oligosaccharides. With the statistical problem-solving approach, the variables which had main effects on oligosaccharide production, as well as several interactions between the variables, were determined.

3. Materials and methods

3.1 Microorganism

Leucosporidium scottii Y - 1450 was used in this study. It was obtained from the University of the Free State MIRCEN yeast culture collection. Pure cultures of this yeast were maintained on Yeast Malt (YM) agar slants containing (per litre): 10 g sucrose, 5 g peptone, 3 g yeast extract, 3 g malt extract and 17 g agar.

3.2 Yeast inoculum preparation and cultivation

A loopfull of cells from a fresh slant were inoculated into a 500 ml Erlenmeyer shake flask containing 100 ml YM medium, which contained (per litre): 10 g sucrose, 5 g peptone, 3 g yeast extract and 3 g malt extract to a final volume of 100 ml. This was grown till late exponential phase (48 h) after which 10 ml of culture was transferred into each of ten 500 ml Erlenmeyer shake flasks containing 90 ml of YM media with the same composition as described above. These were again grown to late exponential phase (30 h) after which the cells were harvested aseptically. The cells were washed with citrate-phosphate buffer with a pH of 4 or 7, depending on the run the cells were to be used for. Dry cell mass was determined gravimetrically prior to inoculation. Cell concentration, sucrose concentration, pH and temperature were assigned according to

table 4.1. The 12 Erlenmeyer flasks (500 ml) with all combinations were incubated on two orbital shakers (20 °C or 60 °C) at a speed of 180 rpm for 60 h.

Table 4.1 Two-level fractional factorial design showing the 12 different factor combinations.

Factors				
Run	**Sucrose concentration (g l^{-1})**	**Cell concentration (g l^{-1})**	**pH**	**Temperature (°C)**
1	50	0.8	4	20
2	50	8	4	20
3	200	0.8	4	20
4	200	8	4	20
5	50	0.8	7	20
6	50	8	7	20
7	200	0.8	7	20
8	200	8	7	20
9	50	8	4	60
10	200	0.8	4	60
11	50	0.8	7	60
12	200	8	7	60

3.3 Experimental design

Experiments were set up as a two-level fractional factorial design (Resolution V) (Haaland, 1989) in 12 different combinations with sucrose concentration, cell concentration, pH and temperature as main experimental effects (Table 4.1) (Haaland,1989). With this design, all main effects and two-factor interactions can be

estimated. Interaction plots were used to determine which interactions were involved in an observed response while Pareto charts were used to identify the main effects of the different factors on the responses. The results obtained were analysed by ANOVA.

3.4 Statistical Analysis

3.4.1 Model selection

Each dependent variable was analysed by ANOVA (Analysis of Variance) using the SAS procedure GLMSELECT (SAS, 2013), initially fitting all four main effects (ph, cell concentration, temperature and sugar concentration) and the six two-factor interactions. Backward model selection was then applied whereby, at each step, the term that was least significantly associated with the dependent variable was dropped from the model,provided the P-value was larger than (or equal to) the “significance level to stay” (SLS) of 0.15. Thus the backward elimination process stopped when the least significant term in the model had a P-value of less than 0.15.

The backward elimination process obeyed the principle of “marginality”, meaning that a main effect could be eliminated from the model only after all interaction terms involving the main effect in question had been eliminated from the model.

3.4.2 ANOVA of Final Model

Each dependent variable was analyzed using ANOVA, fitting the model identified through the selection process described above. F-statistics and associated P-values for all model effects are reported, as well as least squares mean values (SAS “LS means”) associated with all model effects. Furthermore, the expected responses for various settings of the factor levels were estimated to determine those factor settings that yield the maximal response (SAS, 2013).

3.5 Analytical procedures

Cell concentration was monitored by measuring culture turbidity against a blank medium with a Photolab S6 spectrophotometer (WTW, Weilheim, Germany) at 690 nm. The dry cell mass was determined using triplicate 10 ml samples which were obtained at the

end of the cultivation. These were centrifuged, washed with distilled water and dried to constant mass at 105 °C. Samples collected for the quantification of sugars were immediately cooled on ice before centrifugation at 10 000 *x g* and 4 °C using an Eppendorf 5430 R centrifuge (Eppendorf AG, Hamburg, Germany). Prior to chromatographic analyses the supernatants were again centrifuged for 10 min at 10 000 *x g* and 4 °C to remove any cells which might have been transferred during the separation of the supernatant from the cell pellet in the previous centrifugation step. Supernatants not immediately analysed were stored at -20 °C.

Thin layer chromatography (TLC) was done as a rapid analytical method to monitor the levels of sugars in the supernatant. 2 µl was spotted on aluminium 20*20 cm silica gel 60 F_{254} plates (MERCK), dried and run for 5 hours using butanol (water saturated) - ethanol- acetone (50: 20: 2) as mobile phase. Samples containing 200 g l^{-1} of sucrose were diluted 1:4 to prevent smearing of the sugars on the TLC plates due to their high concentration. The plates were sprayed with aniline diphenylamine phosphate (20 g diphenylamine, 20 ml aniline and 100 ml phosphoric acid dissolved in 1 L acetone) and then baked at 100 °C for sugar detection.

Supernatants were also analysed for the presence of sugars by HPLC using a Phenomex Luna NH 4.6 mm x 250 mm column at 85 °C with 85 % acetonitrile at a flow rate of 2 ml/min as eluent. A refraction index (RI) detector was used to detect the presence of sugars and 25 µl of each sample was injected automatically into a Waters HPLC system. Glucose, fructose and sucrose were used as standards for the quantification of sugars. Sucrose was used as a standard for quantification of oligosaccharides.

4. Results

4.1 Sugar analysis by TLC

Oligosaccharide production was observed in Runs 1, 2, 3, 4, 5, 7, 8, 10, 11 and 12 (Fig 4.1 to 4.4). Minimal to no production was observed for runs 6 and 9 (Fig 4.2F & Fig 4.3I). A general trend was observed for most of the runs where sucrose utilisation was accompanied by a concomitant production in fructose, glucose and oligosaccharides. Three spots on the TLC plate indicated the presence of the three different oligosaccharides as reported in Chapters 2 and 3 (O1, O2 and O3). Sucrose utilisation was minimal in runs 1, 3, 5, 7 and 10. In runs 2, 4, 6, 8, 9, 11, and 12 most or all of the sucrose was converted. Runs 6 and 9 showed a rapid hydrolysis of sucrose to fructose and glucose with minimal production of oligosaccharides (Figs 4.2F & 4.3I, 4.5F & 4.6I). After 4 h of cultivation, the presence of all three oligosaccharides could be observed for runs 4, 8, 10 and 11. (Fig 4.2D, 4.3H, 4.4J & 4.4K). Oligosaccharides were observed at 0 h for runs 2, 5, 6, 7, 8, 9, 11 and 12 (Fig 4.1B, 4.2E, 4.2F, 4.3G, H, I, 4.4K & L) while the disappearance of oligosaccharides after production was observed for some runs (Fig 4.1B, 4.2F, 4.3I & 4.4L).

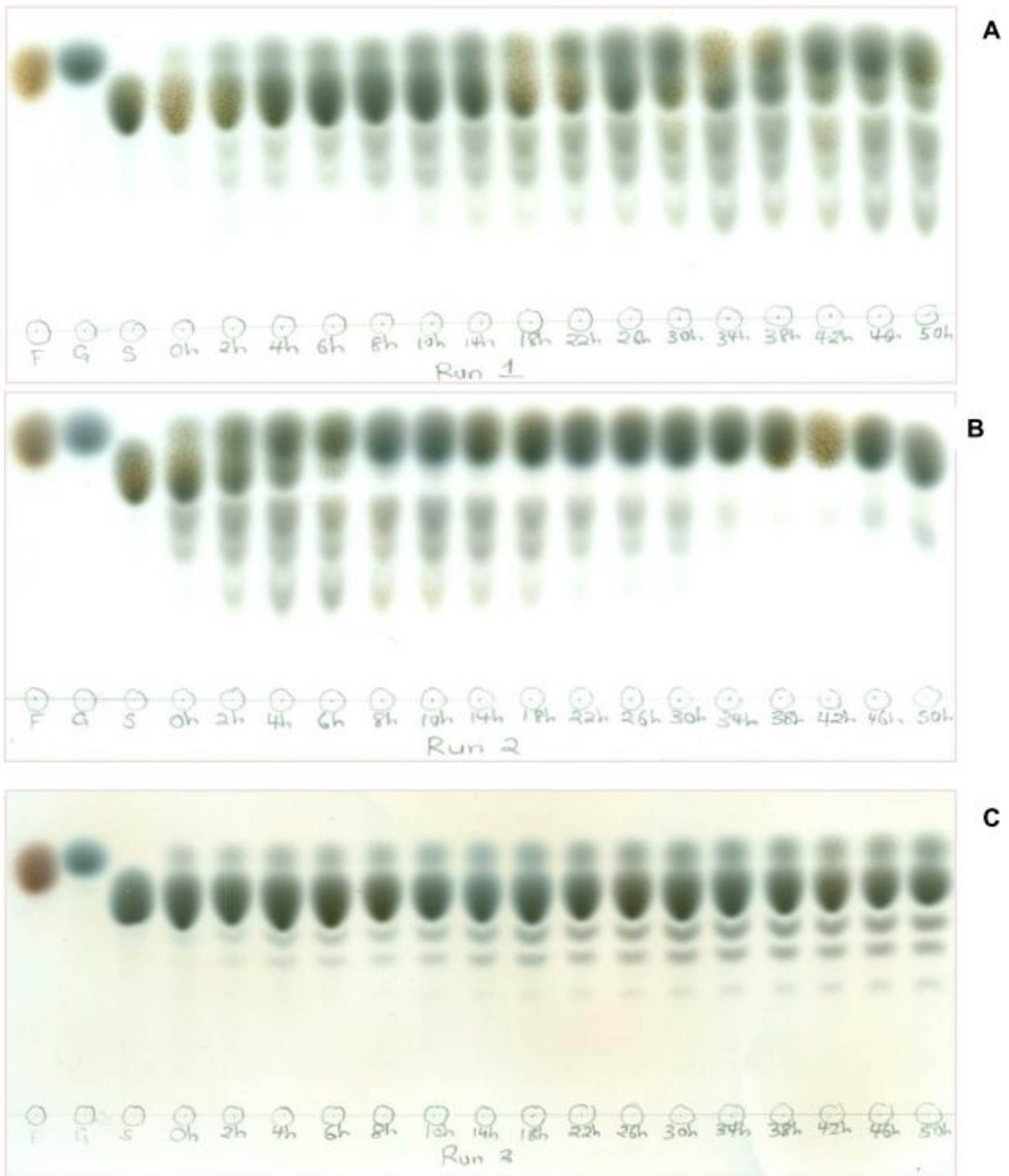


Figure 4.1 TLC plate showing the production of oligosaccharides in Run 1–3 (A-C). Fructose (F), Glucose (G) and Sucrose (S).

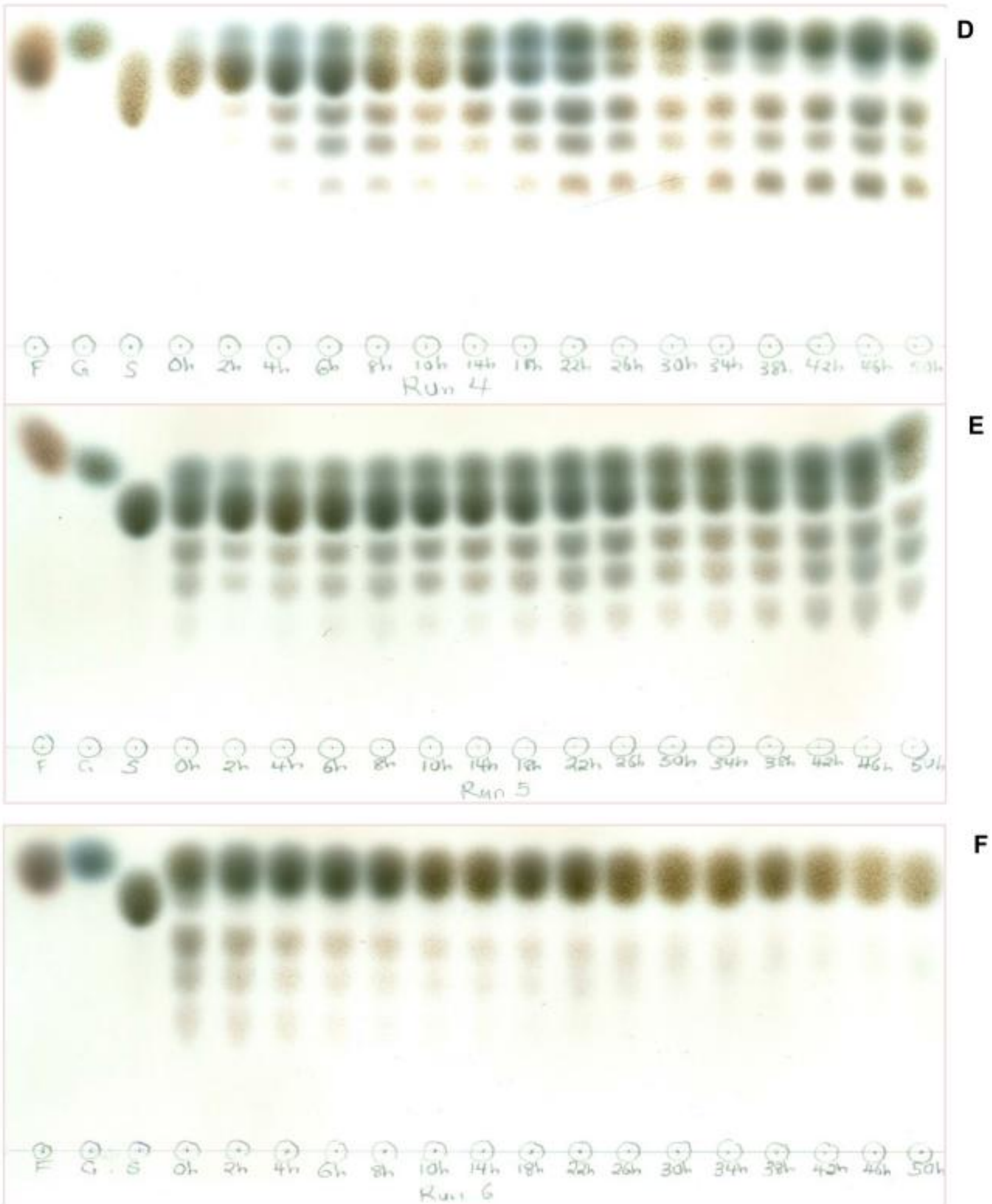


Figure 4.2 TLC plate showing the production of oligosaccharides in Run 4–6 (D-F). Fructose (F), Glucose (G) and Sucrose (S).

Figure 4.3 TLC plate showing the production of oligosaccharides in Run 7–9 (G-I). Fructose (F), Glucose (G) and Sucrose (S).

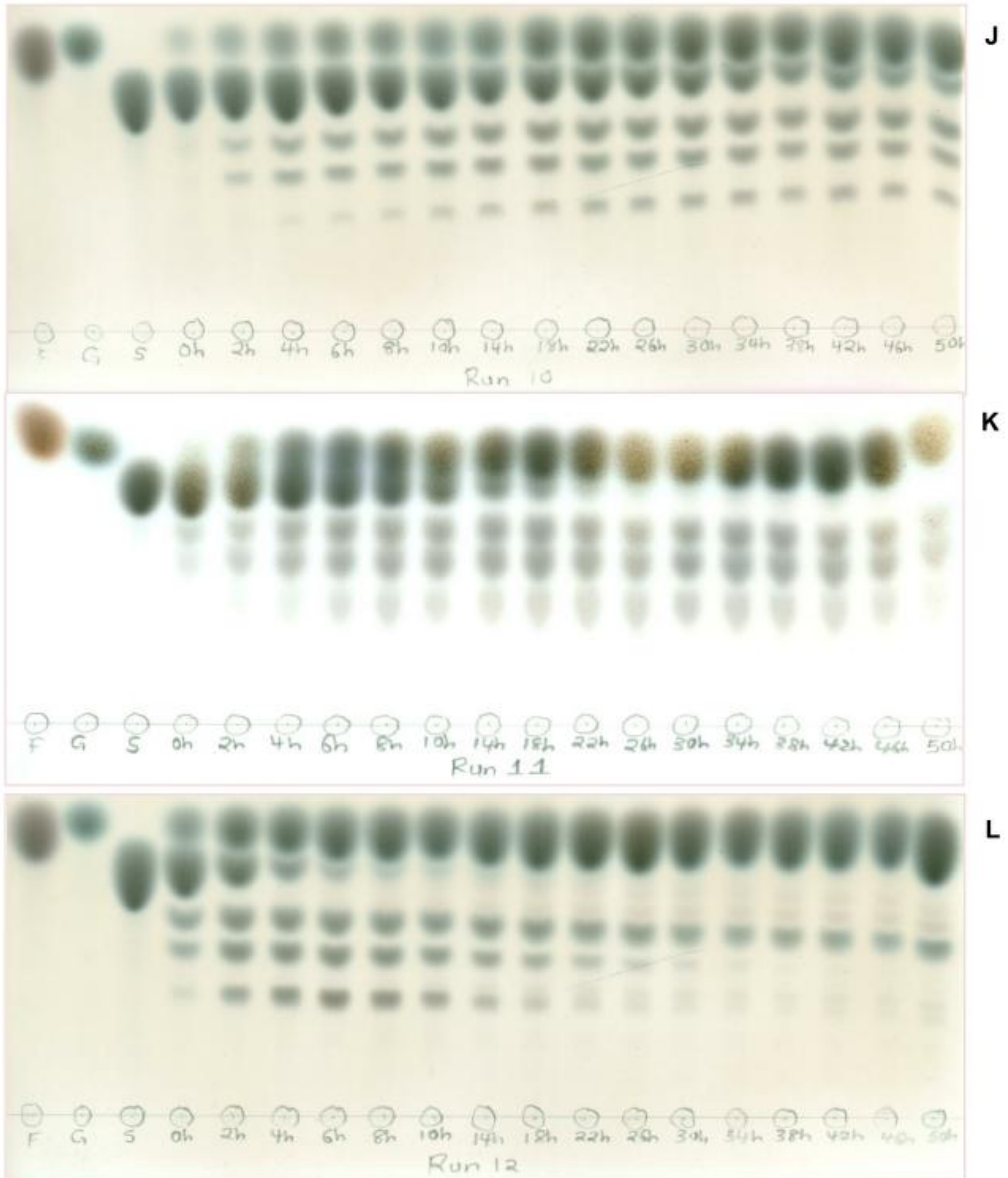


Figure 4.4 TLC plate showing the production of oligosaccharides in Run 10–12 (J-L). Fructose (F), Glucose (G) and Sucrose (S).

4.2 Sugar analysis by HPLC

There was a general trend observed for all 12 runs that sucrose was already utilised at 0 h accompanied by the presence of oligosaccharides. However, the extend of utilisation varied from one run to the other. This may be an indication that the enzymes responsible for the production of oligosaccharides have a very high hydrolytic activity. Moreover, the rapid conversion of sucrose to oligosaccharides probably happens during sample preparation. The cultivation profile for the runs generally showed the utilisation of sucrose with a concomitant increase in the concentrations of glucose, fructose and oligosaccharides (Fig 4.5 & 4.6). This result is in line with what was observed on the TLC plates. Sucrose hydrolysis was very rapid in some runs as could be seen by its consumption at 0 h (Fig. 4.5A, B, D, E, F, 4.6G, H, I & L).The highest concentration of oligosaccharides was obtained at 6 h with a concentration of 114.1 g l^{-1}. 59.4 g l^{-1}, 34.6 g l^{-1} and 23.6 g l^{-1} was obtained for O1, O2 and O3 respectively. This run also had the highest rate of sucrose hydrolysis (Table 4.2).

In run 3 only about half the starting concentration of sucrose was utilised and a continuous increase in all the other sugars was observed (Fig 4.5C). Sucrose was depleted at 34 h and 4 h for runs 6 and 9, with a concomitant production of glucose, fructose and minimal amounts of the oligosaccharides. Due to rapid hydrolysis, an initial sucrose concentration of 2.2 g l^{-1} and 26.6 g l^{-1} was observed for runs 6 and 9 respectively (Fig 4.5F & 4.6I). This clearly demonstrates that hydrolysis outpaced transfer reactions by far in these runs. The lowest amount of oligosaccharides for the entire screening process was obtained for these runs.

Oligosaccharides were observed at 0 h for run 12, indicating the presence of enzymatic activity soon after inoculation and possibly during sample processing. This indicates that the actual peak of oligosaccharide production is therefore missed and remains unknown, but it is almost certainly higher than the measured value. O1 was the predominant oligosaccharide produced throughout the experimental period. This was followed by O2 and then O3.

Table 4.2 Production of oligosaccharides by *Leucosporidium scottii*.

Run	Rate of sucrose hydrolysis g (l h)$^{-1}$	Maximum oligosaccharide concentration (g l^{-1})			Residual sucrose (g l^{-1})	Time of sucrose depletion (h)
		O1	**O2**	**O3**		
1	0.9	7.9	3.6	3.2	1.1	-
2	5.9	7.1	2.6	2.1	0.0	30
3	1.7	28.9	12.7	0.1	101.7	-
4	7.4	54.1	17.9	13.2	8.4	-
5	0.5	5.6	1.9	0.1	2.2	-
6	--	0.1	0.1	0.1	0.0	34
7	1.3	25.2	16.0	2.3	113.9	-
8	18.2	59.4	34.6	23.6	5.0	-
9	13.1	4.2	1.7	0.1	0	4
10	3.5	26.8	17.6	11.6	26.9	-
11	5.6	4.3	3.0	3.1	0.4	-
12	13.0	40.6	28.6	24.7	1.8	-

-: Sucrose was not depleted

--: Not determined due to rapid sucrose hydrolysis.

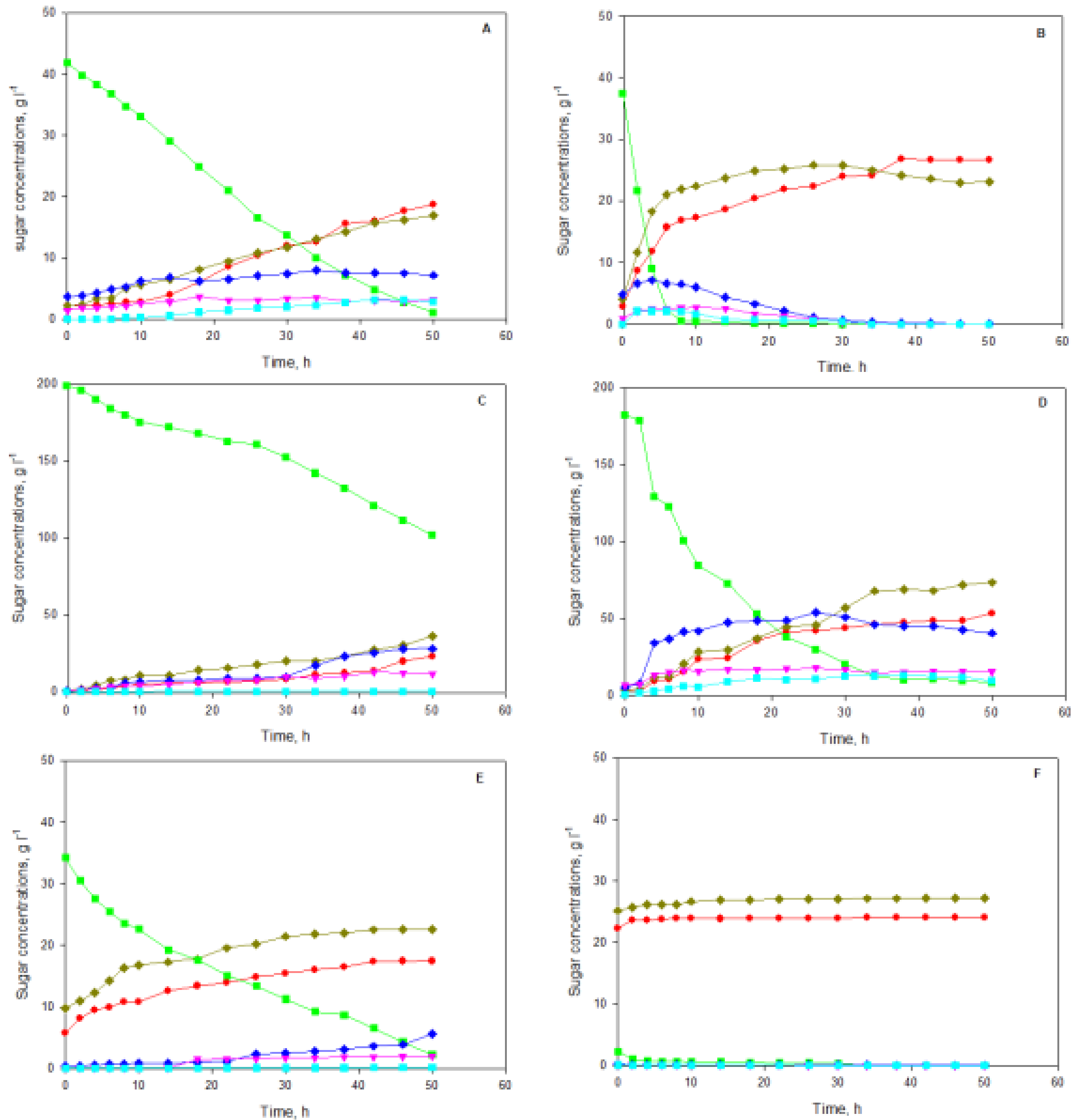


Figure 4.5 The production of oligosaccharides from sucrose by L. scottii suspended in citrate-phosphate buffer in shake flasks. (●) Fructose; (♦) Glucose; (■) Sucrose; (♦) O1; (▼) O2; (■) O3. (A) 50 g l^{-1} sucrose, 0.8 g l^{-1} cells, pH 4, 20 °C. (B) 50 g l^{-1} sucrose, 8 g l^{-1} cells, pH 4, 20 °C. (C) 200 g l^{-1} sucrose, 0.8 g l^{-1} cells, pH 4, 20 °C. (D) 200 g l^{-1} sucrose, 8 g l^{-1} cells, pH 4, 20 °C. (E) 50 g l^{-1} sucrose, 0.8 g l^{-1} cells, pH 7, 20 °C. (F) 50 g l^{-1} sucrose, 8 g l^{-1} cells, pH 7, 20 °C.

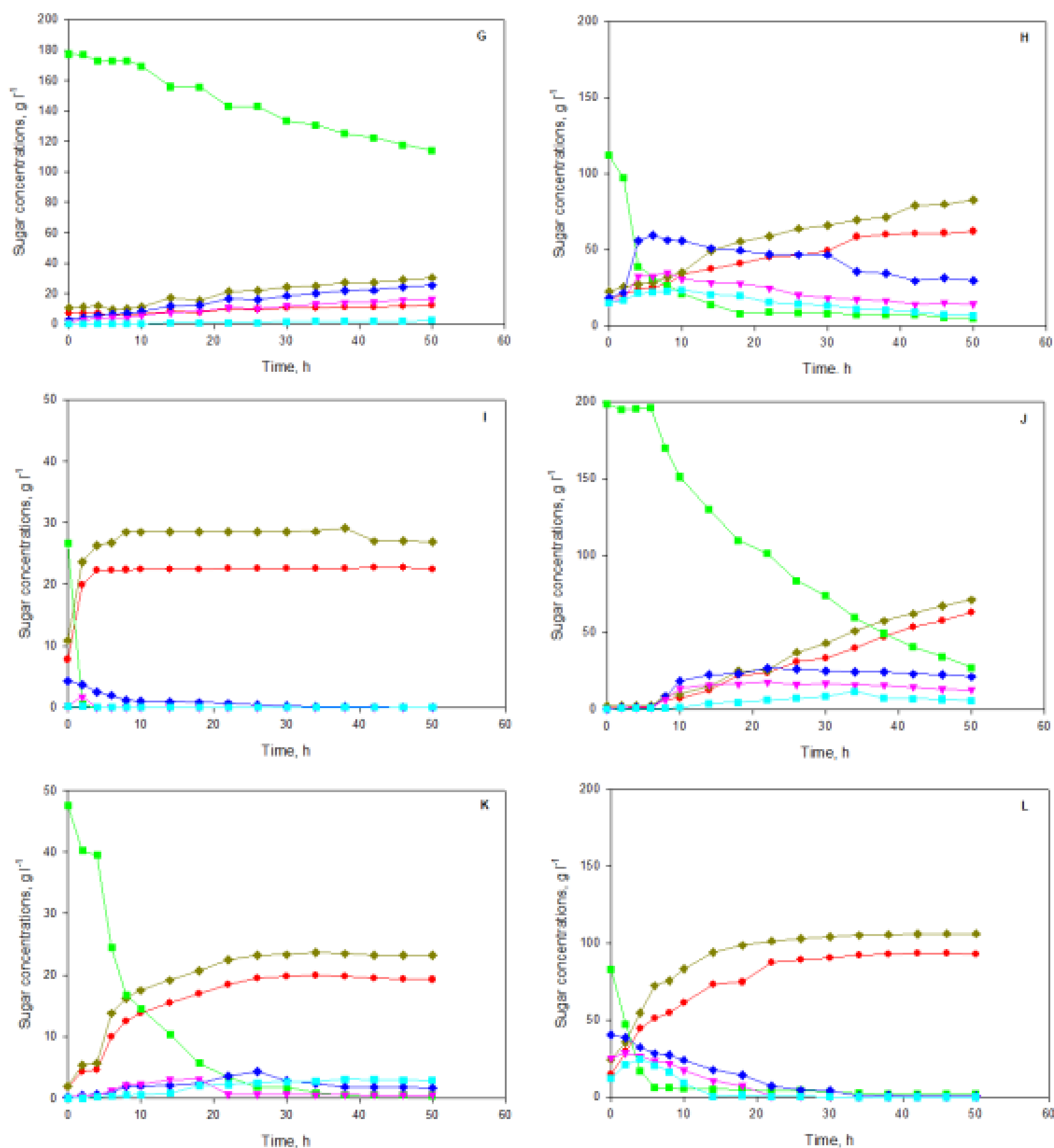


Figure 4.6 The production of oligosaccharides from sucrose by L. scottii suspended in citrate-phosphate buffer in shake flasks. (●) Fructose; (♦) Glucose; (■) Sucrose; (♦) O1; (▼) O2; (■) O3. (G) 200 g l^{-1} sucrose, 0.8 g l^{-1} cells, pH 7, 20 °C. (H) 200 g l^{-1} sucrose, 8 g l^{-1} cells, pH 7, 20 °C. (I) 50 g l^{-1} sucrose, 8 g l^{-1} cells, pH 4, 60 °C. (J) 200 g l^{-1} sucrose, 0.8 g l^{-1} cells, pH 4, 60 °C. (K) 50 g l^{-1} sucrose, 0.8 g l^{-1} cells, pH 7, 60 °C.(L) 200 g l^{-1} sucrose, 8 g l^{-1} cells, pH 7, 60 °C.

Table 4.3 Fractional factorial design for the determination of the effects of the variables sucrose concentration, cell concentration, pH and temperature on the production of oligosaccharides from sucrose by *L. scottii.*

Run	Sucrose concentration (g l^{-1})	Cell concentration (g l^{-1})	pH	Temperature (°C)	Maximum concentration produced (g l^{-1})	Maximum yield coefficient for production (g g^{-1})	Maximum productivity (g (l h^{-1})
1	50	0.8	4	20	13.8	0.23	0.2
2	50	8	4	20	11.7	0.2	1.49
3	200	0.8	4	20	39.9	0.44	0.84
4	200	8	4	20	82.9	0.45	2.68
5	50	0.8	7	20	7.7	0.22	0.14
6	50	8	7	20	0.4	0	0
7	200	0.8	7	20	43.7	0.62	0.79
8	200	8	7	20	114	0.81	10.84
9	50	8	4	60	5.6	ND	ND
10	200	0.8	4	60	51.8	0.37	1.52
11	50	0.8	7	60	7.5	0.17	0.4
12	200	8	7	60	88	ND	ND

ND = Data not suitable for calculation

The highest maximum total oligosaccharide concentration (114 g l^{-1}), yield coefficient (0.81 g g^{-1}) and productivity (10.84 g (l $h)^{-1}$) were all obtained at 200 g l^{-1} sucrose, 8 g l^{-1} cell concentration, pH7 and 20 °C (Table 4.3, run 8). Runs 6 and 9 had the lowest values for all three responses.

4.3 Analysis of the factorial design

Pareto charts indicated that higher sucrose concentrations increased the levels of all three responses (Fig 4.7). However, ANOVA analysis only confirmed that for maximum concentration and the maximum yield coefficient by showing a *P*-value of <0.05 for sucrose (Tables 4.4 and 4.5). *P-values* smaller than 0.05 demonstrated strong main effects or interactions. The maximum productivity was not significantly affected by an

increase in sucrose concentration. High cell concentrations increased the levels of all three responses according to the Pareto charts. However, ANOVA analysis showed that only the maximum concentration was significantly affected by an increase in cell concentration with p-values >0.05 for maximum yield coefficient and maximum productivity. Pareto charts indicated that increased pH positively affected all three responses. However, the effect was not significant as demonstrated by ANOVA analysis. Temperature had a negative effect for all responses on the Pareto charts (Fig 4.7). This was confirmed by the absence of temperature on the ANOVA analysis table which showed that it was eliminated by the model selection process, meaning the negative effect was significantly large.

Table 4.4 Analysis of variance on the maximum concentration of oligosaccharide production.

Source	DF[a]	Sum of Squares	Mean Square	F Value	Pr[b] > F
Model	3	15377.73667	5125.91222	56.96	<.0001
Error	8	719.92000	89.99000		
Corrected total	11	16097.65667			

R-Square	C.V[c]	Root MSE[d]	Maximum concentration mean
0.955278	24.37595	9.486306	38.91667

Source	DF[a]	Type III SS	Mean Square	F Value	Pr[b] > F
Cell	1	1591.60333	1591.60333	17.69	0.0030
Sucrose	1	11631.41333	11631.41333	129.25	<.0001
Cell*Sucrose	1	2154.72000	2154.72000	23.94	0.0012

Table 4.5 Analysis of variance on the maximum yield coefficient of oligosaccharide production.

Source	DF[a]	Sum of Squares	Mean Square	F Value	Pr[b] > F
Model	5	0.49420429	0.09884086	22.61	0.0049
Error	4	0.01748571	0.00437143		
Corrected total	9	0.51169000			

R-Square	C.V[c]	Root MSE[d]	Maximum Yield Coefficient mean
0.965828	18.83669	0.066117	0.351000

Source	DF[a]	Type III SS	Mean Square	F Value	Pr[b] > F
pH	1	0.01714286	0.01714286	3.92	0.1188
Cell	1	0.00017143	0.00017143	0.04	0.8527
Sucrose	1	0.40047619	0.40047619	91.61	0.0007
pH*Sucrose	1	0.08550476	0.08550476	19.56	0.0115
Cell*Sucrose	1	0.03124286	0.03124286	7.15	0.0556

Table 4.6 Analysis of variance on the maximum productivity of oligosaccharide production.

Source	DF[a]	Sum of Squares	Mean Square	F Value	Pr[b] > F
Model	3	60.27428333	20.09142778	3.47	0.0911
Error	6	34.77251667	5.79541944		
Corrected total	9	95.04680000			

R-Square	C.V[c]	Root MSE[d]	Maximum productivity mean
0.634154	127.3740	2.407368	1.890000

Source	DF[a]	Type III SS	Mean Square	F Value	Pr[b] > F
Cell	1	23.12604167	23.12604167	3.99	0.0927
Sucrose	1	27.89380167	27.89380167	4.81	0.0707
Cell*Sucrose	1	16.29688167	16.29688167	2.81	0.1446

[a] = Degrees of freedom

[b] = Probability

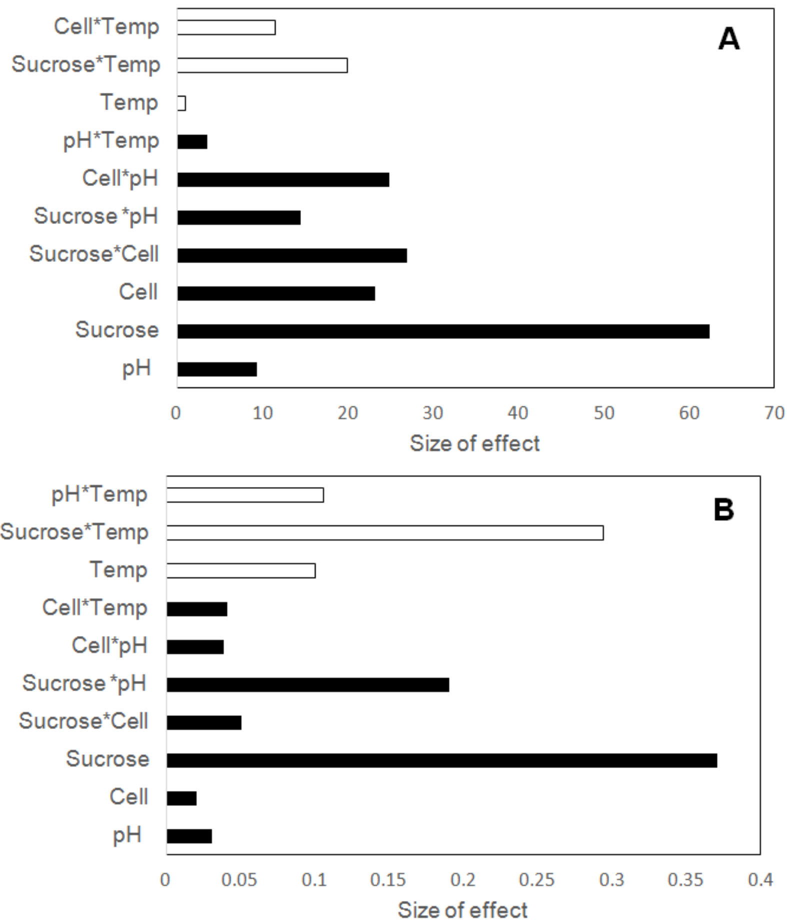
[c] = Coefficient of variance

[d] = Mean square error

F= F statistic (variance of the group means)

Some interactions were observed. Pareto charts showed that sucrose concentration*cell concentration had a positive effect on all three responses. However, ANOVA analysis only confirmed that for maximum concentration by showing a *P*-value of <0.05.The interaction sucrose concentration*cell concentration did not positively affect the maximum yield coefficient and the maximum productivity. Other interactions which positively affected all three responses on the Pareto chart were sucrose concentration*pH and cell concentration*pH. These interactions were not significant according to ANOVA analysis. pH*sucrose concentration had a positive effect on the

maximum yield coefficient and this was confirmed by ANOVA analysis with a *P-value* of 0.01. An increase in sucrose concentration does not necessarily affect the yield coefficient because no matter the sucrose concentration, the same amount of oligosaccharides will be produced per gram of sucrose utilised. The highest yield coefficient was obtained when the sucrose concentration, cell concentration and pH were high (Table 4.3). Sucrose concentration*temperature negatively affected all three responses according to the Pareto charts but these effects were not significant according to ANOVA analysis (Tables 4.4, 4.5 & 4.6). Cell concentration*temperature negatively affected both the maximum concentration and the maximum productivity while pH*temperature negatively affected the maximum yield coefficient and maximum productivity.



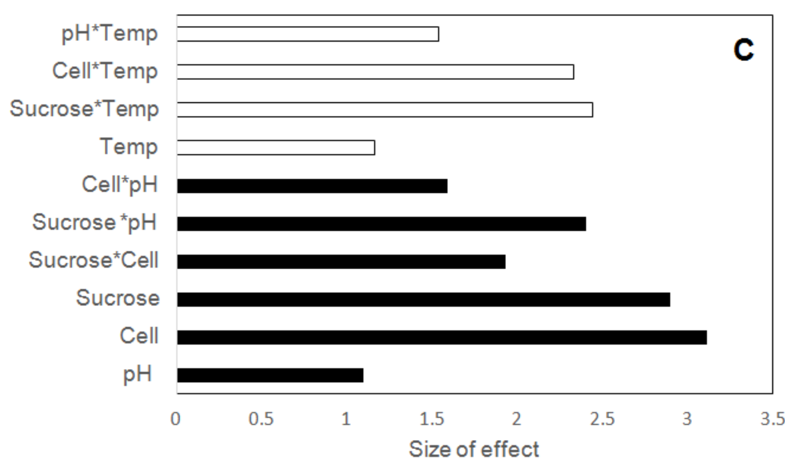


Figure 4.7 Pareto charts showing the influence of cell concentration (g l-1), sucrose concentration (g l-1), temperature (°C), pH and possible interactions on the maximum concentration of oligosaccharides produced (g l-1) (A),the maximum yield coefficient for oligosaccharide production (g g^{-1}) (B) and maximum oligosaccharide productivity g $(l\ h)^{-1}$ (C) from sucrose by *Leucosporidium scottii* cell suspensions incubated in shake flasks in citrate-phosphate buffer containing sucrose. Temp: temperature; cell: cell concentration; sucrose: sucrose concentration; (*): interaction between variables. Shaded columns represent positive effects, unshaded columns represent negative effects.

Interaction plots were used to identify the interactions which had an effect on the responses. Interaction plots with crossed lines were taken as strong interactions (Fig 4.8A). Parallel lines indicated no interactions. The more the lines deviate from parallel, the stronger the interaction. Cell concentration*sucrose concentration showed strong interactions for all three responses (Fig 4.8 D, 4.9J & 4.10P) Sucrose concentration*temperature plot showed strong interactions for only the maximum concentration and maximum oligosaccharide productivity. This interaction was strong especially for the maximum concentration. In addition to these was cell

concentration*temperature showed very strong interactions for the maximum yield coefficient and maximum oligosaccharide productivity.

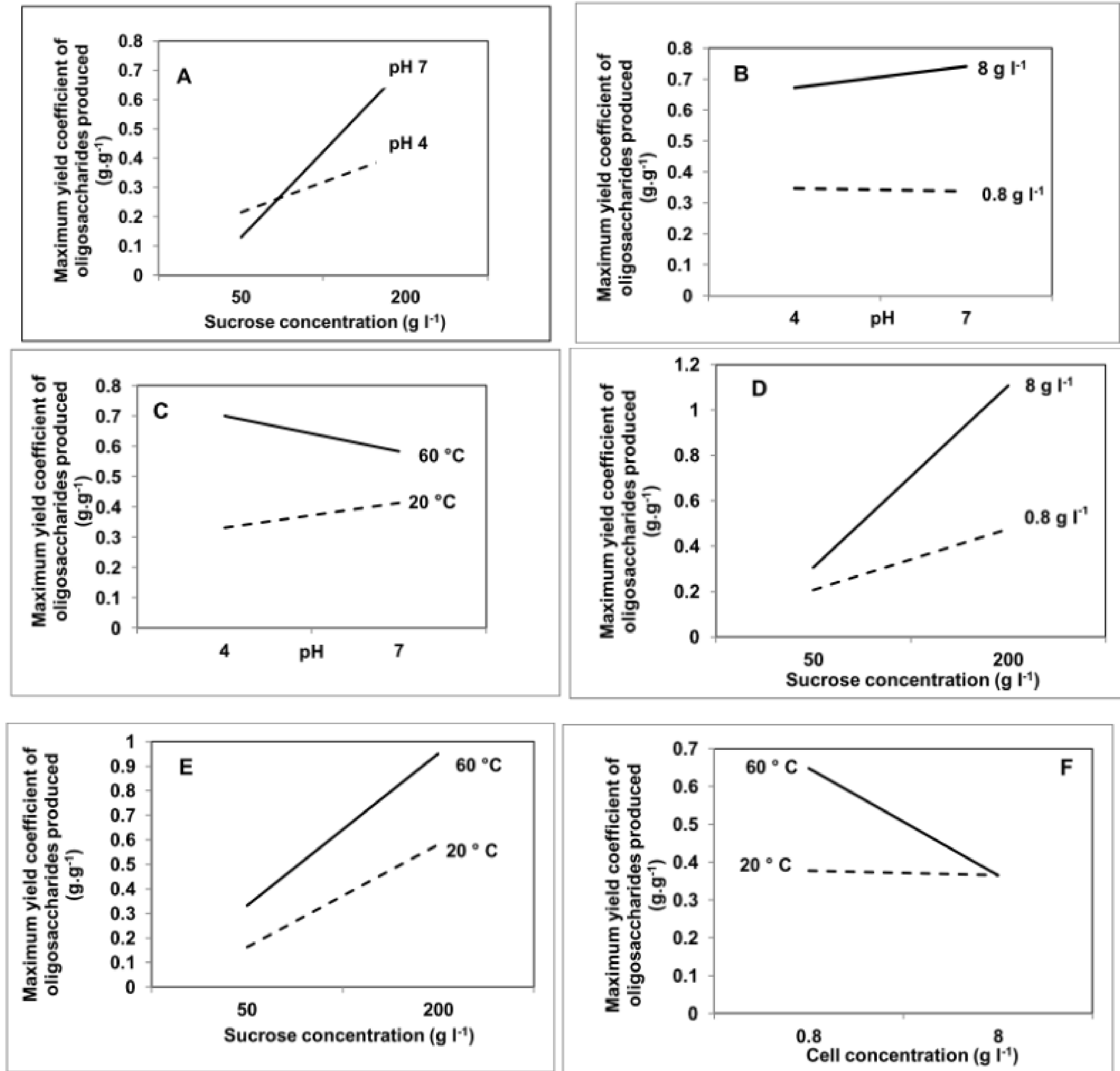


Figure 4.8 The effects of two-factor interactions on the maximum yield coefficient of oligosaccharide production.

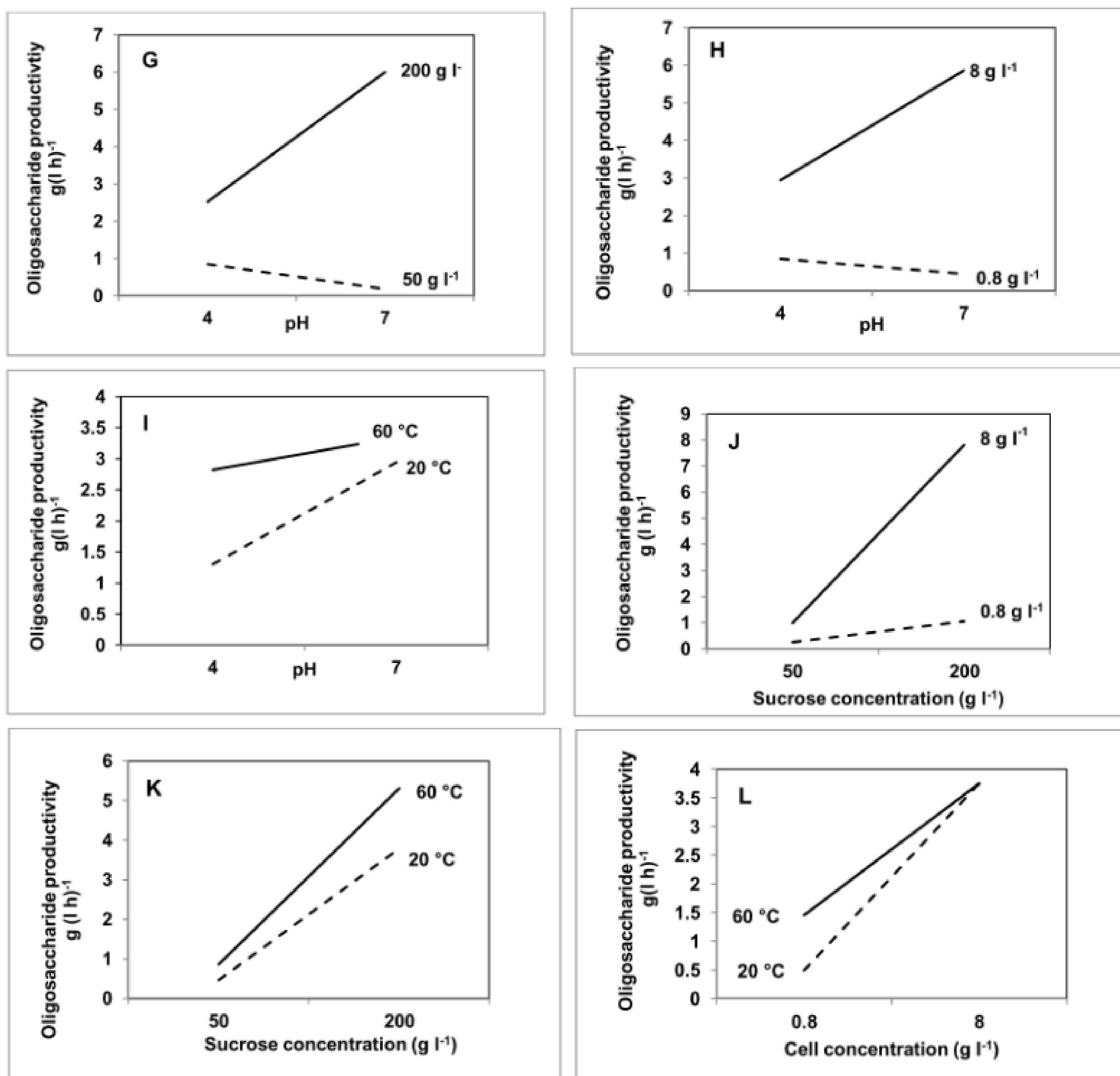


Figure 4.9 The effects of two-factor interactions on the maximum oligosaccharide productivity.

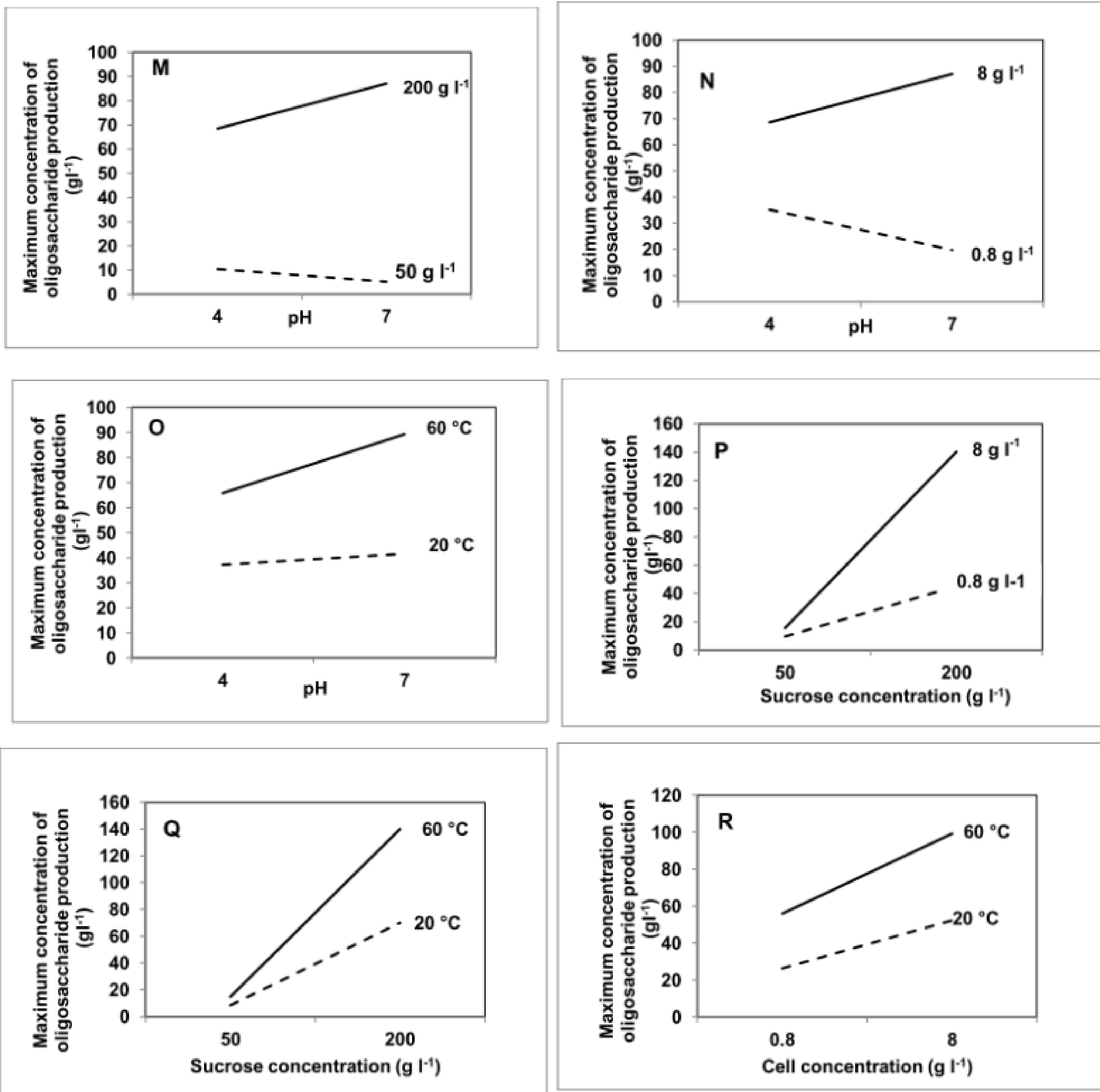


Figure 4.10 The effects of two-factor interactions on the maximum concentration of oligosaccharides.

The maximum concentration showed weak interactions for pH*sucrose concentration, pH*cell concentration, pH*temperature and cell concentration*temperature. Weak interactions were seen for the maximum yield coefficient for pH*cell concentration, pH*temperature and sucrose concentration*temperature. The pairs pH*sucrose

concentration, pH*cell concentration and pH*temperature showed weak interactions for maximum oligosaccharide productivity.

Table 4.7 The different interactions identified by Pareto charts, p-values and interaction plots for the three responses.

Response	Pareto chart	P-value	Interaction plot
Maximum concentration of production (g l^{-1})	pH*Temp Cell*pH Sucrose*pH Sucrose*Cell	Cell*Sucrose	Sucrose*Cell Sucrose*Temp
Maximum yield coefficient for production (g g^{-1})	Cell*Temp Cell*pH Sucrose*pH Sucrose*Cell	pH*Sucrose Cell*Sucrose	Sucrose*pH Cell*Temp Sucrose*Cell
Oligosaccharide productivity (g (l h)$^{-1}$	Cell*pH Sucrose*pH Sucrose*Cell	Cell*Sucrose	Cell*Temp Sucrose*Cell Sucrose*Temp

Cell: Cell concentration

Sucrose: Sucrose concentration

Temp: Temperature

Table 4.8 Significant main effects and interactions as determined by interaction plots and variance analysis.

Response	Factor	Interaction plots	Variance analysis
Maximum concentration of production (g l^{-1})	Cell	NA	+
	Sucrose	NA	+
	Cell*Sucrose	+	+
	Sucrose*Temp	+	NS
Maximum yield coefficient for production (g g^{-1})	Sucrose	NA	+
	pH*Sucrose	+	+
	Cell*Sucrose	+	+
	Cell*Temp	+	NS
Oligosaccharide productivity (g (l h$)^{-1}$	Cell*Temp	+	NS

Cell: Cell concentration

Sucrose: Sucrose concentration

Temp: Temperature

+: Positive effect

NS: Not significant

NA: Not applicable

5. Discussion

HPLC analysis showed that sucrose was consumed in all the runs with the production of fructose, glucose and oligosaccharides (Fig 4.5 & 4.6). In most cases, the oligosaccharides produced were later hydrolysed (Fig 4.5 A, B, D & 4.6H, J, K, L). This phenomenon was reported by other researchers (Kritzinger *et al.*, 2003; Ning *et al.*, 2010). Ning and coworkers attributed this to the fact that at high sucrose concentrations transfructosylation increases while hydrolysis decreased and vice versa (Ning *et al.*,

2010). This then signifies that high sucrose concentrations favour oligosaccharide production. Contrary to the aforementioned studies, this study showed that a decrease in sucrose concentration instead favoured oligosaccharide production as was observed in some runs (Runs 1, 3, 5, 7, 10 & 11) Hang and co-workers also observed this when they produced FOS from sucrose using extracellular fructosyltransferease (Hang *et al.*, 1995). Invertase (β-D-fructofuranoside fructohydrolase, β-fructofuranosidase, sucrase, invertase, saccharase; EC 3.2.1.26) is responsible for the hydrolysis of sucrose and related glycosides (Kotwal & Shankar, 2009). Rapid sucrose hydrolysis observed at the beginning of some runs (Fig 4.5 A, B, D, E, F& 4.6G, H, I, K, L) may be an indication that the enzyme responsible for the production of oligosaccharides has a very high hydrolytic activity. Complete disappearance of sucrose and the production of glucose and fructose in runs 6 and 9 may have been due to very high enzyme activity such that the oligosaccharides were being hydrolysed as they were produced or little oligosaccharides were formed. The best results were obtained in run 8 with pH 7, 200 g l^{-1} sucrose, 20 °C and 8 g l^{-1} cells.

A high cell concentration yielded the highest concentration, yield coefficient and productivity of oligosaccharides (Fig 4.6 H). ANOVA analysis indicated that a high cell concentration had a significant effect for only the maximum concentration of oligosaccharides produced ($p < 0.05$) (Table 4.4 and 4.8). This suggests that as the concentration of cells increases, the amount of enzyme also increases resulting in an increase in the rate of oligosaccharides produced. Manera and co-workers observed an increase in oligosaccharide concentration when high cell concentrations were used (Manera *et al.*, 2010). Contrarily, Ning and co-workers as well as Kritzinger and co-workers found that an increase in cell concentration decreased the concentration of oligosaccharide produced (Kritzinger *et al.*, 2003; Ning *et al.*, 2010). It is possible that an increase in the amount of enzyme may not necessarily result in an increase in enzyme activity, which would explain the discrepancy in these results. It will be beneficial if low cell concentrations can be used to produce high concentrations of oligosaccharides in large scale production.

A high sugar concentration produced the highest concentration, yield coefficient and productivity of oligosaccharides (Fig 4.6, Run 8). A high sugar concentration had a significant effect on only the maximum concentration and the maximum yield of oligosaccharides produced. This was somewhat surprising as one would expect an increase in sucrose concentration to lead to an increase in the rate of oligosaccharide production (Hang *et al.*, 1995; Kaenpanao *et al.*, 2016; Ning *et al.*, 2010). This result is in accordance with that of Kritzinger and co-workers and Kaenpanao and co-workers who also reported a significant effect for sucrose on oligosaccharide concentration and yield coefficient (Kritzinger *et al.*, 2003; Kaenpanao *et al.*, 2016). Sheu and co-workers reported that neofructooligosaccharide (neoFOS) production was faster when the enzyme activity was high as compared to when it was low (Sheu *et al.*, 2013). Pareto charts indicated a positive effect for the interaction cell concentration*sucrose concentration for maximum oligosaccharide productivity. This indicates that a high sucrose concentration may not be sufficient to cause an increase in the productivity of oligosaccharides, but its combination with cell concentration. Moreover, da Silva and co-workers (2014) also highlighted that a high sugar concentration was needed to produce a high concentration of fructooligosaccharide (FOS) production. Hang and co-workers (1995) observed the same with the production of kestose. This is not in accordance with what was observed in some runs (Runs 1, 3, 5, 7, 10 & 11) in this study.

A high pH produced the highest concentration, yield coefficient and productivity of oligosaccharides (Fig 4.6, Run 8). However, ANOVA analysis did not show pH as being a significant factor for any response (Tables 4.4, 4.5 & 4.6). This work agrees with that of Kritzinger and co-workers (2003) who pointed out pH as an insignificant factor. β-fructofuranosidase is responsible for the production of FOS and it is pH dependant (Fernandez *et al.*, 2004).The highest transfructosylating activity occurs at pH 7 (Ning *et al.*, 2010). This could explain why run 8 had the highest concentration of oligosaccharides. Contrarily, Itoh and Shimura (1987) observed an increase in oligosaccharide production with a decrease in pH when they used enzymes from *Penicillium oxalicum.* To explain this, Bali and co-workers (2015) stated that the optimum conditions and the effect of process parameters like pH on FOS production differ from one microorganism to the next. The interaction pH*sucrose concentration

showed a positive effect for the maximum yield coefficient (Table 4.5). Kritzinger and co-workers (2003) observed a positive effect for pH*sucrose concentration on the maximum concentration of neokestose.

A high temperature produced the lowest concentration, yield coefficient and productivity of oligosaccharides (Fig 4.6, Run 8). This result was in line with what was observed on the Pareto charts and the ANOVA analysis, which indicated that temperature was insignificant for all three responses (Fig. 4.7, Tables 4.4, 4.5 & 4.6). An increase in temperature to values higher than the optimum results in enzyme deactivation. This result therefore suggests that the enzyme responsible for oligosaccharide production has its optimum activity between 20 °C and 60 °C. In contrast, Bali and co-workers reported that FOS production was optimum at pH 5.5 and 60 °C. They also stated that the effect of temperature on FOS production differ from one microorganism to the next (Bali *et al.*, 2015). This could be the reason why their results differ from mine.

Cell concentration*sucrose concentration was identified as the most important interaction for all responses according to the Pareto charts, with only the maximum concentration and maximum yield coefficient being statistically significant (Fig 4.7 and Tables 4.4 & 4.5). This agrees with the results from run 8 (Table 4.3) which produced the highest responses obtained at 8 g l^{-1} cell concentration and 200 g l^{-1} sucrose. Setting both factors at high levels should improve oligosaccharide production and yield. pH*sucrose concentration was identified as another important interaction for the maximum yield. This was evident on the interaction plots as indicated by the crossed lines. In addition, this agrees with Table 4.3, run 8 which showed the highest yield at pH 7 and 200 g l^{-1}. In order to improve the yield, a high pH and sucrose concentration is recommended.

The use of high cell concentrations increased process efficiency and productivity. In addition, the use of free whole cells is advantageous as they can be recycled without the process of immobilisation and this is cost-effective. The method used in this study is therefore cost-effective and time saving as it does not require isolation of enzymes from cells. To improve production the combination of factors which produced the highest values for all responses will have to be taken to account pH 7, 8 g l^{-1}, 200 g l^{-1} and 20

°C. Sucrose concentration and cell concentration, which showed significant interactions, should be considered when carrying out optimisation studies. This study demonstrates for the first time efficient oligosaccharide production from *Leucosporidium scottii.* This neoFOS can further be investigated for large scale production and benefits. Enzymatic studies can also be done to characterise the enzyme responsible for oligosaccharide production.

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CHAPTER 5

GENERAL DISCUSSION AND CONCLUSIONS

Fructooligosaccharides ([1]F-FOS) have gained interest in the food, pharmaceutical and animal industries because of the numerous benefits they posses. They are used as prebiotics which stimulate the proliferation of *Bifidobacterium* spp in the intestine and thus improve human health (Steed & Macfarlane, 2009; Nobre *et al*., 2015; Callaway *et al*., 2012). Some of these compounds are made up of one to three fructosyl units bound to the β-2, 1 position of sucrose (GF) such as 1-kestose (GF_2), nystose (GF_3) and ^{1}F-β-fructofuranosyl nystose (GF_4) (Kaenpanao *et al.*, 2016). Another group of FOS which has recently been gaining interest is the neofructooligosaccharides (NeoFOS). These compounds have a superior bifidogenic effect and chemical and thermal stability compared to ^{1}F-FOS and consist of neokestose and neonystose (Kilian *et al*., 2002; Lim *et al*., 2007). FOS production is mostly done enzymatically by transfructosylating activities of enzymes obtained from microbes. Chemical production is avoided due to the stereo- and regioselectivity of enzymes which need complex protection and deprotection steps for the preparation of structurally well-defined oligosaccharides (Wang, 2015). The purification of oligosaccharides is important because it eases identification, which in turn helps in identifying the applications of the oligosaccharides. Moreover, the presence of monosaccharides and disaccharides usually present in the cultivation media after production interferes with identification. Hence, FOS purification is important to eliminate these unwanted sugars. The purpose of this study was therefore to produce, purify and identify oligosaccharides produced *by Leucosporidium scottii* Y-1450.

During the growth of *Leucosporidium scottii* on sucrose, neonystose is produced as the main product, with 1-kestose also produced as a result of neonystose hydrolysis. Neonystose hydrolysis was due to sample degradation and did not occur during production. This is the first report of the production of neofructooligosaccharides from *Leucosporidium scottii.* A maximum yield of 58.0 % was obtained from 100 g l^{-1} of sucrose. Sheu and co-workers (2013) produced neoFOS from 250 g l^{-1} of sucrose using *Xanthophyllomyces dendrorhous* BCRC 21346 with high ^{6}G-FFase activity and *Xanthophyllomyces dendrorhous* BCRC 22367 with low enzyme activity. A yield of 46.0

% and 49.6 % were obtained for these yeasts respectively. This study appears to suggest a better method for NeoFOS production with improved yields. The use of whole cells in this study also lowers production costs compared with established commercial processes of FOS production which employ immobilized enzymes. Novel microorganisms producing potential transfructosylating enzymes need to be explored for their application in NeoFOS production and also in scale-up studies for industrial applications.

Preparative HPLC was used for NeoFOS purification. A total of 2.9 g l^{-1} of oligosaccharide was purified from 7.8 ml of culture supernatant with a percentage recovery of 15.3 % for neonystose. This method was not successful in purifying O1 and O2 since they eluted too close to each other. This study adds to the few which may have been done on the purification of neonystose by preparative HPLC. Information on the purification of by preparative HPLC is scarce. However, Linde and co-workers (2012) used semi-preparative HPLC to purify neonystose and obtained a total of 110 mg of neoFOS. Although activated charcoal columns are frequently used for FOS purification, preparative HPLC seems to be a simpler and less cumbersome method since it eliminates the several washing steps which result in the loss of some FOS.

LCMS (Liquid Chromatography Mass Spectrometry) and NMR (Nuclear Magnetic Resonance) were employed for the identification of oligosaccharides. The molecular masses for the trisaccharide (503.4 Da) and tetrasaccharide (665.5 Da) were in accordance with that observed by Ota and co-workers (Ota *et al.*, 2009). Further identification was carried out on the tetrasaccharide using NMR. MALDI-TOF analysis confirmed the presence of trisaccharides and tetrasaccharide in the sample while linkage analysis showed the tetrasaccharide neonystose with a ratio of 2:1:1 for t-Fru*f*, 1-Fru*f* and 6-Glc*p* respectively. Reports on the structural identification of neonystose are uncommon. The structure of neonystose was identified by Linde and co-workers (2012) by using a combination of 1H, 13C and 2D NMR (COSY, TOCSY, NOESY, HSQC, HSQC-TOCSY, HMBC) techniques. However, the chemical shift assignment was not published. Trisaccharides could not be structurally identified due to signal overlap and the small chemical shift displacements caused by substitution with fructofuranosyl

residues. However the 1-kestose (α-Glc*p*-(1→2)-β-Fru*f*-(1→2)-β-Fru*f*) detected could be a product of the breakdown of neonystose since the addition of a new fructosyl moiety to neokestose through a β-(2-1)-link or to 1-kestose through a β-(2-6)-link with glucose results in the formation of neonystose.

NeoFOS can be produced from non-growing cells of *Leucosporidium scottii* in citrate phosphate buffer with sucrose as carbon source, as demonstrated by the screening experiment. This will reduce production cost. Although cell recycling could reduce the cost of production, it can also reduce production parameters to prohibitively low levels (Kritzinger *et al*., 2003). Pareto charts, interaction plots and ANOVA analysis were used to identify either the main effects or the effects of two-factor interactions on the responses. Sucrose concentration had a high positive effect for all three responses while temperature showed a negative effect for the responses. Interaction plots indicated interactions between sucrose concentration and cell concentration for all three responses. Strong interactions between pH and sucrose concentration as well as temperature and cell concentration were observed for the maximum yield coefficient of oligosaccharides. Temperature and cell concentrations also showed strong interactions for the maximum productivity while strong interactions between temperature and sucrose concentrations were observed for the maximum concentration of oligosaccharides produced. Linde and co-workers (2012) reported that reactions of transfructosylation and hydrolysis mediated by the fructofuranosidases from microorganisms are highly dependent on substrate concentration. This may explain why sucrose concentration showed a significant interaction. The interaction temperature*cell concentration for maximum productivity was unexpected as one would expect an increase in temperature to result in the denaturing of enzymes. ANOVA analysis showed sucrose concentration*cell concentration as a significant interaction for all responses and sucrose concentration as a significant main effect for the maximum concentration and yield coefficient of oligosaccharide production. Kilian and co-workers (2003) reported that sucrose concentration had a significant effect on the maximum concentration, specific rate and yield coefficient for neokestose. Therefore the main factor affecting neoFOS production is sucrose concentration with sucrose concentration*cell concentration as the most important factor combination.

Much research has been done in the area of prebiotics. However, has not been greatly explored. There is need for the discovery of more enzymes from different microorganisms that are capable of producing neoFOS with prebiotic potential. This study is, to my knowledge, the first report on the production, purification and identification of from *Leucosporidium scottii* Y-1450. NeoFOS production could be further optimised since this optimisation was only a screening process to identify the main effects and interactions affecting neoFOS production. In order to evaluate this neoFOS for its prebiotic potential, further animal and human test can be done. Success in this can then be followed by the establishment of a commercially viable process for large scale production.

The results obtained for this study demonstrate the potential of a novel FOS from *L scottii* which can be explored for its prebiotic benefits in the food, animal and pharmaceutical industries.

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Summary

Prebiotic oligosaccharides are gaining interest in the food, poultry and pharmaceutical industries due to their health benefits. These include prevention of specific allergies, improved calcium absorption, reduction in the duration, incidence, and symptoms of traveller's diarrhoea, alleviation of irritable bowel syndrome (IBS) symptoms, increased satiety and reduced appetite. In the food industry they are responsible for modifying food flavour, they are considered as low energy ingredients alleviating obesity and have a high water-holding capacity, low sweetness and an anti-staling effect. The main objectives of this study were to produce, purify and identify oligosaccharides from *Leucosporidium scottii* Y-1450.

Leucosporidium scottii Y-1450 produced neofructooligosaccharides during growth on sucrose. Two trisaccharides and a tetrasaccharide were produced. The highest oligosaccharide concentration obtained was 33.7 g l^{-1} after 22 h of cultivation. A maximum specific growth rate, maximum volumetric rate of oligosaccharide production, and maximum yield coefficient for the production of oligosaccharides (oligosaccharides produced/sucrose assimilated) of 0.28 h^{-1}, 1.53 g $(l\ h)^{-1}$ and 0.58 were obtained respectively. In addition to the products of interest, glucose, fructose and sucrose were also present in the supernatant.

Purification of the supernatant containing mono-, di and oligosaccharides was done by preparative HPLC. A total oligosaccharide concentration of 2.9 g l^{-1} was obtained after purification from 7.8 ml of supernatant. The first unknown trisaccharide was not successfully purified as it overlapped with the second trisaccharide. The second trisaccharide and the tetrasaccharide were successfully purified with percentage recoveries of 0.12 % and 15.3 % respectively.

LCMS identification using Electrospray Mass Spectrometry (ESMS) of the supernatant showed the presence of three oligosaccharides with m/z of 503.4, 539.3 and 665.5 Da, respectively. This was later followed by structural identification of the tetrasaccharide by NMR.

Results from MALDI-TOF and NMR analysis confirmed the presence of two oligosaccharides present in the tetrasaccharide fraction, a trisaccharide (from the breakdown of the tetrasaccharide) identified as 1-kestose (α-Glc*p*-1-2-β-Fru*f*-1-2-β-Fru*f*) and a tetrasaccharide identified as neonystose (β-Fru*f*-2-6-α-Glc*p*-1-2-β-Fru*f*-1-2-β-Fru*f*).

A two level fractional factorial screening design was used to investigate oligosaccharide production, with sucrose concentration, cell concentration, pH and temperature as the factors and maximum oligosaccharide concentration, yield coefficient and productivity as the responses. The highest concentration, yield coefficient and productivity of oligosaccharides were obtained at 200 g l^{-1} sucrose, 8 g l^{-1} cell concentration, pH 7 and 20 °C. ANOVA analysis indicated sucrose concentration as a highly significant main effect for all three responses while the interaction sucrose concentration*cell concentration was identified as a significant interaction for the responses. Temperature was an insignificant factor for all three responses.

This study highlighted the feasibility of the production of neoFOS with possible prebiotic potential from sucrose by *Leucosporidium scottii*.

Keywords: Oligosaccharides, prebiotics, *Leucosporidium scottii*, production, purification, identification

Opsomming

Prebiotiese oligosakkariede wek belangstelling in die kos, pluimvee en farmaseutiese nywerhede as gevolg van hulle gesondheidsvoordele. Dit sluit voorkoming van spesifieke allergieë, verbeterde kalsiumabsorbsie, vermindering in die duur, voorkoms, en simptome van reisigersdiarree, verligting van die simptome van prikkelbare derm-sindroom ("irritable bowel syndrome", IBS), verhoogde versadiging en verminderde eetlus in. In die voedselindustrie is hulle verantwoordelik vir die modifikasie van die smaak van kos, word hulle beskou as 'n lae-energie bestanddeel wat vetsug verlig, het 'n hoë waterhouvermoë, lae soetheid en werk die veroudering van voedsel teë. Die hoofdoelwitte van hierdie studie was om oligosakkariede met *Leucosporidium scottii* Y-1450 te produseer, te suiwer en te identifiseer.

Leucosporidium scottii Y-1450 het neofrukto-oligosakkariedes geproduseer gedurende groei op sukrose. Twee trisakkariedes en 'n tetrasakkariede is geproduseer. Die hoogste oligosakkariedkonsentrasie verkry was 33.7 g l^{-1} na 22 h van kweking. 'n Maksimum spesifieke groeitempo, maksimum volumetriese tempo van oligosakkariedproduksie en maksimum opbrengskoëffisiënt vir die produksie van oligosakkariede (oligosakkariede geproduseer / sukrose geassimileer) van 0,28 h^{-1}, 1.53 g $(l\ h)^{-1}$ en 0.58 is onderskeidelik verkry. Benewens die produkte van belang was glukose, fruktose en sukrose ook teenwoordig in die bostand.

Suiwering van die bostand met mono-, di en oligosakkariede is gedoen deur preparatiewe hoëverrigtingsvloeistofchromatografie. 'n Totale oligosakkariedkonsentrasie van 2,9 g l^{-1} is verkry na suiwering van 7,8 ml bostand. Die eerste onbekende trisakkariede is nie suksesvol gesuiwer nie, aangesien dit naby die tweede trisakkariede geëlueer het. Die tweede trisakkariede en die tetrasakkariede is suksesvol gesuiwer met persentasie herwinnings van 0,12% en 15,3% onderskeidelik.

LCMS identifikasie met behulp van elektrosproei massaspektrometrie (ESMS) van die supernatant het die teenwoordigheid van drie oligosakkariede met m / z van 503,4, 539,3 en 665,5 Da, onderskeidelik, opgelewer. Dit is later gevolg deur strukturele identifikasie van die tetrasakkariede deur KMR.

MALDI-TOF ontleding het die teenwoordigheid van twee oligosakkariede in die tetrasakkariedfraksie, 'n trisakkariede (uit die afbreek van die tetrasakkariede) en 'n tetrasakkariede bevestig. Verdere koppelinganalise het getoon dat die tetrasakkaried neonistose (β-Fru*f*-2-6-α-Glc*p*-1-2-β-Fru*f*-1-2-β-Fru*f*) is en die trisakkariede 1-kestose (α-Glc*p*-1-2-β-Fru*f*-1-2-β-Fru*f*).

'n Twee-vlak fraksionele faktoriaalontwerp is gebruik om oligosakkariedproduksie te ondersoek, met sukrosekonsentrasie, selkonsentrasie, pH en temperatuur as die faktore en maksimum oligosakkariedkonsentrasie, opbrengskoëffisiënt en produktiwiteit as die response. Die hoogste konsentrasie, opbrengskoëffisiënt en produktiwiteit van oligosakkariede is by 200 g l^{-1} sukrose, 8 g l-1 sel konsentrasie, pH 7 en 20 ° C verkry. ANOVA ontleding het sukrosekonsentrasie as 'n hoogs beduidende hoofeffek vir al drie response uitgewys terwyl die interaksie sukrosekonsentrasie * selkonsentrasie geïdentifiseer is as 'n beduidende interaksie vir die response. Temperatuur was 'n onbeduidende faktor vir al drie response.

Hierdie studie beklemtoon die haalbaarheid van die vervaardiging van neoFOS met moontlike prebiotiese potensiaal vanaf sukrose deur *Leucosporidium scottii*.

Sleutelwoorde: Oligosakkariede, prebiotika, *Leucosporidium scotti*, produksie, suiwering, identifikasie