TR 08 - 100

Purification, Characterisation and Application of Inulinase and Transferase Enzymes in the Production of Fructose and Oligosaccharides

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Abstract

Inulin hydrolysis can occur as a result of the action of exoinulinases and endoinulinases acting alone or synergistically. Exoinulinases cleave the non-reducing β-(2, 1) end of inulin releasing fructose while endoinulinases act on the internal linkages randomly to release inulotrioses (F_3), inulotetraoses (F_4) and inulopentaoses (F_5) as major products. Fructosyltransferases act by cleaving a sucrose molecule and then transferring the liberated fructose molecule to an acceptor molecule such as sucrose or another oligosaccharide to elongate the short chain fructooligosaccharide. The production of high yields of oligosaccharides of specific chain length from simple raw materials such as inulin and sucrose is a challenge. Oligosaccharides of chain length up to degree of polymerisation (DP) 5 and fructose were produced using preparations of three commercial microbial enzymes. Production of these novel oligosaccharides was achieved by employing response surface methodology (RSM) with central composite experimental design (CCD) for optimising product yield. Using a crude Novozyme 960 endoinulinase preparation isolated from *Aspergillus niger*, the following conditions gave a high inulooligosaccharide (IOS) yield, temperature (60 °C), 150 g/L inulin concentration, 48 h incubation, pH 6.0 and enzyme dosage of 60 U/ml. Under these conditions, inulotrioses (70.3 mM), inulotetraoses (38.8 mM), and inulopentaoses, (3.5 mM) were produced. Response surface regression predicted similar product levels under similar conditions. The crude endoinulinase was purified through a three step purification procedure with a yield of 1.11 % and 3.5 fold purification. The molecular weight of this endoinulinase was estimated to be 68.1 kDa by SDS-PAGE and its endoinulinase nature was confirmed by native PAGE. The purified endoinulinase was more efficient in production of IOS than the crude endoinulinase preparation. The purified endoinulinase demonstrated a high affinity for the inulin substrate (K_m 3.53 mM, V_{max} 666.67 µmol/min/ml). Pectinex Ultra SP-L, a commercial crude enzyme preparation isolated from *Aspergillus aculeatus* is a cocktail of several enzymes including a fructosyltransferase. The crude enzyme showed both transfructosylation and hydrolytic activity in 200 to 600 g/L sucrose. The main fructooligosaccharides produced from sucrose were 1-kestose (GF_2), nystose (GF_3) and fructofuranosyl nystose (GF_4). After the first RSM, with the coded independent variables of temperature, incubation time, pH and

sucrose concentration, the highest levels of GF_2, was 68.61 mM, under sucrose concentration 600 g/L, temperature 60 °C, enzyme dosage 20 U/ml, pH 5.6, after 4 h incubation. A sucrose concentration of 400 g/L favoured the synthesis of high levels of GF_3 and GF_4. In the second RSM the maximal yields of GF_2, GF_3 and GF_4 were 152.07 mM, 131.38 mM and 43.99 mM respectively. A purified fructosyltransferase did not synthesise GF_4. Ammonium ions were demonstrated to enhance the yield of FOS. A mixture of glucose and fructose was used as substrate for FOS synthesis and no FOS were formed. Glucose was shown to be an end product inhibitor of the fructosyltransferase and therefore hinders the formation of high FOS yield. Fructozyme, isolated from *Aspergillus ficuum* is a mixture of exo and endoinulinases with the former being predominant was used for fructose production from inulin hydrolysis. The exoinulinase was purified to electrophoretic homogeneity by a three step purification procedure. The molecular weight of the enzyme was estimated to be 53 kDa with a 21 % yield and 4.2-fold. Response surface regression was used to predict the maximum fructose levels achievable under the combinations of temperature, enzyme dosage and incubation time. A reaction time (48 h), enzyme dosage (100 U/ml) and inulin concentration (150 g/l) at pH 5.0 at 50 °C gave higher fructose levels (106.6 mg/ml) using crude exoinulinase as compared to 98.43 mg/ml using the purified exoinulinase. These findings indicate that higher levels of fructose require longer incubation periods and higher inulin substrate concentrations with higher enzyme dosage. The crude exoinulinase preparation gave fairly higher levels of fructose than the purified exoinulinase and this is due to the presence of other hydrolytic enzymes in the crude preparation. The conditions established by RSM and CCD were adequate in producing high yield of oligosaccharides and fructose and can therefore be applied for their industrial production since they are in high demand due to their health benefits as prebiotics.

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List of Abbreviations

1-FEH	Fructan 1-exohydrolase
AOAC	Association of Analytical Chemists
ANOVA	Analysis of Variance
DNS	3, 5-Dinitrosalicylic Acid
CCD	Central composite design
DP_n	Degree of Polymerisation (compound with n units)
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
FDA	Food and Drug Administration
FFD	Fractional factorial design
F	Fructose
F_n	Inulooligosaccharide with n Fructose units
F_3	Inulotriose
F_4	Inulotetraose
F_5	Inulopentaose
FI	Flame Ionisation
FOS	Fructooligosaccharides
Ftase	Fructosyltransferase
$FT-IR$	Fourier Transform coupled with Infrared Spectroscopy
g	gravity
G	Glucose
g/L	Gram per litre
$GC-MS$	Gas Chromatography coupled with Mass Spectrometry
GF_n	Fructooligosaccharide with n fructose units and a terminal glucose
GF	Sucrose
GF_2	1-kestose
GF_3	Nystose
GF_4	Fructofuranosyl nystose
GHF	Glycoside hydrolase family
h	hour
HPAEC-PAD	High Performance Anion Exchange Chromatography with Pulsed Amperometry Detection
HPLC	High Performance Liquid Chromatography
ICPAE	Inductively Coupled Plasma Atomic Emission Spectroscopy
IOS	Inulooligosaccharide
K_m	Affinity constant (Michaelis-Menten)
MALDI-TOF	Matrix Assisted Laser Desorption Ionisation Time of Flight
Mg	Milligram
mg/ml	Milligram per millilitre
min	minute
mM	Millimolar
MS-ESI	Mass Spectrometry with Electrospray Ionisation
NBS	N-bromosuccinimide

NMR	Nuclear Magnetic Resonance spectroscopy
°C	Degree Celsius
p	Probability
PCR	Polymerase chain reaction
ppb	parts per billion
ppm	parts per million
pH	Logarithm of the reciprocal of the hydrogen ion concentration
PEG 2000	Polyethylene glycol 2000
RI	Refractive Index
R^2	Correlation coefficient
RSM	Response Surface Methodology
scFOSs	Short Chain Fructooligosaccharides
SD	Standard Deviation
SDS	Sodium Dodecyl Sulphate
SmF	Submerged fermentation
SSF	Solid state fermentation
t	Time
TLC	Thin Layer Chromatography
μl	Microlitre
USDA	United States Department of Agriculture
v/v	Volume per volume
V_{max}	Maximal velocity
w/v	Weight per volume

Acknowledgements

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List of Outputs

A Publications in Peer-Reviewed Scientific Journals

Mutanda, T., Wilhelmi, B., and Whiteley, CG. Enzymatic synthesis of fructooligosaccharides with an endoinulinase from *Aspergillus niger* (submitted to *Enzyme Microbial Technology*).

Nemukula, A., **Mutanda, T.,** Wilhelmi, B., and Whiteley, C.G. Double response surface methodology: synthesis of short chain Fructooligosaccharides with a fructosyltransferase from *Aspergillus aculeatus*. (submitted to *Biotechnology Progress*).

Mutanda, T., Wilhelmi, B., and Whiteley, C.G. Controlled Synthesis of Fructose and Fructooligosaccharides by an Exoinulinase from *Aspergillus ficuum* (submitted to *Applied Biochemistry and Microbiology*).

B International Conference Proceedings

Mutanda, T., Wilhelmi, B. and Whiteley C. G (2006) *Optimisation for Enzymatic Synthesis of Fructooligosaccharides.* 20th IUBMB International Congress of Biochemistry and Molecular Biology and 11th FAOBMB Congress held in Kyoto, Japan from 18-23 June 2006.

C Local Conference Proceedings

Mutanda, T., Wilhelmi, B. and Whiteley C.G. (2008) Abstract accepted for presentation entitled *'Enzymatic Hydrolysis of Chicory Inulin for the Production of Novel Short Chain Inulooligosaccharides and Fructose'.* This paper will be presented at the forthcoming Bio-08 Conference organised by SASBMB, Biotech SA Conference, SASM Congress to be held at Rhodes University, Grahamstown, South Africa from 21 to 25 January 2008.

Mutanda, T., Wilhelmi, B. and Whiteley C.G. (2006) *Synthesis of Short Chain Fructooligosaccharides Using Response Surface Methodology'* at the South African Society of Biochemistry and Molecular Biology (XXth SASBMB Conference), held at the University of KwaZulu-Natal, Pietermaritzburg, South Africa, from 2 - 5 July 2006.

Mutanda, T., Wilhelmi, B. and Whiteley C.G. (2005) *Enzymatic synthesis of fructooligosaccharides (FOS) of varying chain length from inulin and sucrose'* at the 15th annual Meeting of the Eastern Cape division of South African Society for Biochemistry and Molecular Biology hosted by the Biochemistry, Microbiology and Biotechnology Department, Rhodes University, Grahamstown, South Africa on 25 November 2005.

D Technical Annual Reports to African Products Pty Ltd (Industrial Sponsor)

Mutanda, T., Wilhelmi, B. and Whiteley C.G. (2005) *Synthesis of Short Chain fructooligosaccharides from sucrose using a fructosyltransferase from* Aspergillus niger.

Mutanda, T., Nemukula A., Wilhelmi, B. and Whiteley C.G. (2006) *Synthesis of oligosaccharides from sucrose and inulin using transferases and inulinases with response surface methodology.'*

Mutanda, T., Nemukula, A., Wilhelmi, B. and Whiteley C.G. (2007) *Enzymatic production of fructose and short chain fructooligosaccharides.*

I hereby declare that this thesis submitted at Rhodes University for the award of a PhD degree is original and my own work and has not been submitted by me for the award of a degree at another University or Institution of higher education.

Taurai Mutanda

This _____7th_____ day of__ *December* ___ 2007

Grahamstown
South Africa

Chapter 1

General Introduction and Literature Review

1.1 Background

Fructooligosaccharides (FOS) of the inulin type constitute an important class of carbohydrates that have gained attention recently due to their beneficial health effects (Ghazi *et al.*, 2006; Huebner *et al.*, 2007). Fructooligosaccharides are found naturally in foods such as onion (*Allium cepa* L), banana (*Musa* sp.), Jerusalem artichoke (*Helianthus tuberosus* L), chicory (*Cichorium intybus* L), tomato (*Solanum lycopersicum* L), cereal plants, and some grasses (Jing *et al.*, 2003[a]; Zhengyu *et al.*, 2005; Rocha *et al.*, 2006; Sheng *et al.*, 2007; Lingyun *et al.*, 2007; Xiong *et al.*, 2007). It is estimated that more than 36 000 vegetable plants in the *Compositae* and *Asteraceae* families accumulate large amounts of polyfructans as reserve carbohydrate (Vandamme and Derycke, 1983; Leiti *et al.*, 2004; Sharma *et al.*, 2006; Itaya *et al.*, 2007). Generally, these compounds have a number of biofunctions which have been documented in several publications though some of the claims have not been thoroughly elucidated and consequently require further research for validation (Tanriseven and Aslan, 2005; Villegas and Costell, 2007; Goulas *et al.*, 2007).

Fructooligosaccharides have been classified as prebiotics because of their bifidogenic nature and also their health promoting properties when consumed in sufficient amounts (Modler, 1994; Roberfroid *et al.*, 1998; Van Laere *et al.*, 2000; Huebner *et al.*, 2007). There are essentially three main criteria that should be met before a food ingredient can be classified as a prebiotic: 1) it should not be hydrolysed or absorbed in the upper part of the gastrointestinal tract, 2) it should be a selective substrate for one or a limited number of probiotics, and 3) it should be able to alter the colonic microflora toward a potentially more healthy composition and / or activity (Roberfroid *et al.*, 1998). In general terms, probiotics are potentially beneficial bacteria commensal to the colon, (for example bifidobacteria and lactobacilli), which are stimulated to grow by the prebiotics with concomitant suppression of potentially pathogenic bacteria (Modler, 1994; Alles *et al.*, 1999; Van Laere *et al.*, 2000; Roberfroid *et al.*, 1998).

Recently, a new term, synbiotics, has been introduced, which is the combination of prebiotics and probiotics in food products such as yogurt and other fermented dairy products that have the ability to influence and improve the gastrointestinal health of

humans (Huebner *et al.*, 2007; Villegas and Costell, 2007). Health benefits and applications of FOS in human nutrition are well documented and these include the immune system activation, resistance to some infections, synthesis of B-complex vitamins and enhanced calcium absorption in the gastrointestinal tract (Yun, 1996; Tanriseven and Aslan, 2005).

The biofunctional applications of fructooligosaccharides are due to the following four properties that make them important food ingredients (Yun, 1996; Sangeetha *et al.*, 2005[a]; Villegas and Costell, 2007). First, FOS have a low sweetness intensity, since they are about one third as sweet as sucrose and this property is useful in the various kinds of foods where the use of sucrose is restricted by its high sweetness (Yun, 1996). Second, FOS have low calorie levels and they are rarely hydrolysed by digestive enzymes and are not used as an energy source in the body and consequently are safe for consumption by diabetics (Yun, 1996; Sangeetha *et al.*, 2005[a]; Villegas and Costell, 2007). Third, FOS are noncariogenic, that is, they are not used by *Streptococcus mutans* to form acids and insoluble β-glucan that are implicated in the formation of dental caries (Yun, 1996). Fourth, as prebiotics, FOS encourage the growth of the bifidobacteria and discourage the growth of potentially putrefactive microorganisms that have a tendency of causing diarrhoea (Yun, 1996; Biedrycka and Bielecka, 2004). The suppression of the growth of harmful bacteria in the gut is possibly due to the formation of lactic, acetic and other short chain organic acids that may be antagonistic to the potentially pathogenic intestinal competitors (Huebner *et al.*, 2007). Important physiological roles of FOS in human beings include the reduction of levels of serum cholesterol, phospholipids, and triglycerides (Yun, 1996).

An oligosaccharide is characterised by the degree of polymerisation (DP), type and sequence of its monosaccharide moieties (Prapulla *et al.*, 2000). On average, up to 10 monomeric units are contained in the chain, which can either be linear or branched (Roberfroid *et al.*, 1998; Prapulla, *et al.*, 2000). The degree of polymerisation is defined as the number of fructose units linked to the terminal glucose moiety. In some plants, for instance *Cichorium intybus*, the degree of polymerisation is determined by the type of cultivar, plant's life cycle and the time of harvest (Van Waes *et al.*, 1998; Hebette *et al.*, 1998; Paseephol *et al.*, 2007). It is reported that early harvesting produces inulin with a higher DP as compared to late harvesting,

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before the onset of inulinases which can cleave the long molecules into short chain oligosaccharides (Modler, 1994). FOS are also defined as lower mass linear molecules that are made up of 2 to 20 fructose monomers with a terminal glucose moiety that are joined together by glycosidic linkages (Van Loo *et al.* 1995; Roberfroid, *et al.*, 1998; Wang *et al.*, 1999).

Fructooligosaccharides are generally regarded as safe (GRAS) for human consumption. They are not hydrolysed by digestive enzymes in the upper gastrointestinal tract and due to their non-digestibility, they therefore reach the colon intact (Roberfroid, *et al.*, 1998). Among the non-digestible oligosaccharides are compounds containing fructose, glucose, xylose and galactose (Roberfroid *et al.*, 1998; Niness, 1999). The chain length, presence of the β-bond and branching make these compounds resistant to hydrolysis by the digestive enzymes in the upper gastro intestinal tract and consequently are classified as natural fibre (Niness, 1999).

Fructooligosaccharides derived from inulin are referred to as inulooligosaccharides (IOS). Inulin is the storage carbohydrate in the roots and tubers of plants such as Jerusalem artichoke, chicory, and dahlia with exclusive 2→1 fructosyl-fructose linkages (Kalil *et al.*, 2001; Ohta *et al.*, 2002; Zhengyu *et al.*, 2005). In a broader context, fructans are carbohydrate polymers in which fructosyl-fructose linkages constitute the majority of linkages (French, 1989; Lewis, 1993). Fructans include both small oligomers, polymers with more than 10 000 residues and dimeric inulobiose (Lewis, 1993). Currently the nomenclature of different types of fructans is under intense debate and some fructan names can occasionally be interchanged.

1.2 Classification and chemical structure of fructans

Inulin is a linear compound consisting of β-(2, 1) linked D-fructofuranose units and one terminal α-(1, 2) linked D-glucopyranose unit. There are several types of fructans depending on their chemical structure and organisms producing them (Corradini *et al.*, 2004). Fructans are categorised as follows: inulin, levan, phlein, graminan, kestoses and kesto-*n*-oses. Inulin is a linear plant β-(2, 1)-fructan that is polydisperse with a degree of polymerisation ranging from 2 to 60 fructose units (Van Loo *et al.*, 1995; Sharma *et al.*, 2006; Sharma and Gill, 2007). In most cases, the inulin molecule

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has a terminal glucose moiety (GF_n) but in some instances, it can have only fructose molecules only and lacks the terminal glucose moiety (F_m) (Modler, 1994; Hebette *et al.*, 1998) (Figure 1.1 a and b).

(a) GF_n (b) F_m

Figure 1.1 Chemical structures of two types of inulin molecules. (a) GF_n is a fructan of degree of polymerisation of n + 2 whereby the glucose is the terminal moiety and (b) F_m is a fructan of degree of polymerisation of m + 1 with fructofuranosyl units only without the terminal glucose moiety; G and F represent glucose and fructose respectively (Adapted from Roberfroid *et al.*, 1998).

Levan is primarily found as a microbial exopolysaccharide and is a fructose biopolymer mainly linked by β-(2, 6)-glycosidic bonds, with β-(2, 1)-linked side chains (Yoon *et al.*, 2004; Gonta *et al.*, 2004; Corradini *et al.*, 2004; Nagem *et al.*, 2004). Levan is also a fructan of higher plants that has mostly the (2, 6) fructosyl-fructose linkage and it consists of high DP (DP > 100) polymers such as those found in bacterial systems (French, 1988; Lewis, 1993) (Figure 1.2). Phlein are plant derived compounds which contain mostly the (2, 6) fructosyl-fructose linkage and these polymers are of lower molecular weight (DP <100) (Lewis, 1993).

Grasses possess fructans with a more complicated branched structure containing a preponderance of β-2, 6 linkages of the levan type in addition to the β-2, 1 linkages (Nagem *et al.*, 2004; Simmen *et al.*, 1993). Graminan has both (2, 1) and (2, 6)

fructosyl-fructose linkages in significant amounts with branching (Lewis, 1993). Kestoses are trimeric fructans containing glucose and two fructose units while kesto-*n*-oses are oligomeric fructans of DP $\geq$ 3 that contain a sucrose unit with the DP designated by a Greek root for instance, kestohexaose and kestodecaose (Lewis, 1993).
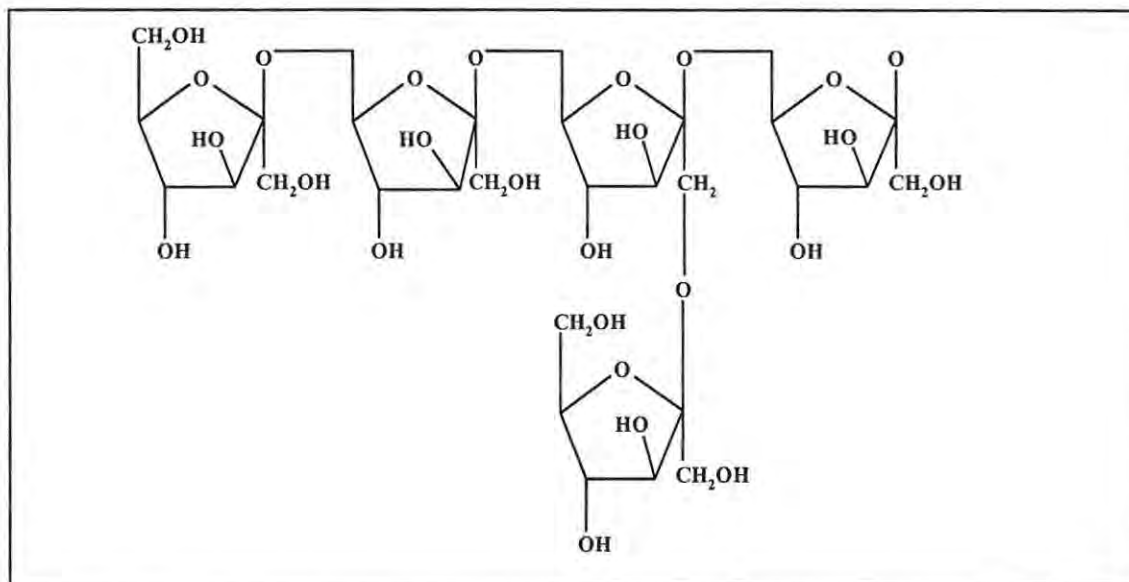
Figure 1.2 Chemical structure of part of a levan molecule showing the side branching pattern (Gonta *et al.*, 2004, French, 1988).

1.3 Sources of inulin and fructooligosaccharides

Fructooligosaccharides are found in trace amounts as natural components in fruits, vegetables and honey (Sangeetha *et al.*, 2005[a]). The major sources of inulin are chicory (*Cichorium intybus*) and Jerusalem artichoke (*Helianthus tuberosus*). Inulin content on fresh weight basis in these plants ranges from 10 to 20 % (Rocha *et al.*, 2006). The distribution of inulin from various plant sources is illustrated in Figure 1.3. However, a major limitation of chicory as a source of inulin is the presence of fructan 1-exohydrolase (1-FEH, EC 3.2.1.153), an inulinase that degrades fructosyl-fructose linkages at low temperatures, reducing the quality of the inulin at the end of the growth period and during storage (Van den Ende, 2004; Modler, 1994).

The inulins derived from plant sources are polydisperse and are mixtures of different carbohydrates (Lopez-Molina *et al.*, 2005). The average chain length of inulin varies

between plants and in different growth conditions. Due to polydispersity, the molecular weight of inulin varies between ± 3500 and 5500 (Vandamme and Derycke, 1983).
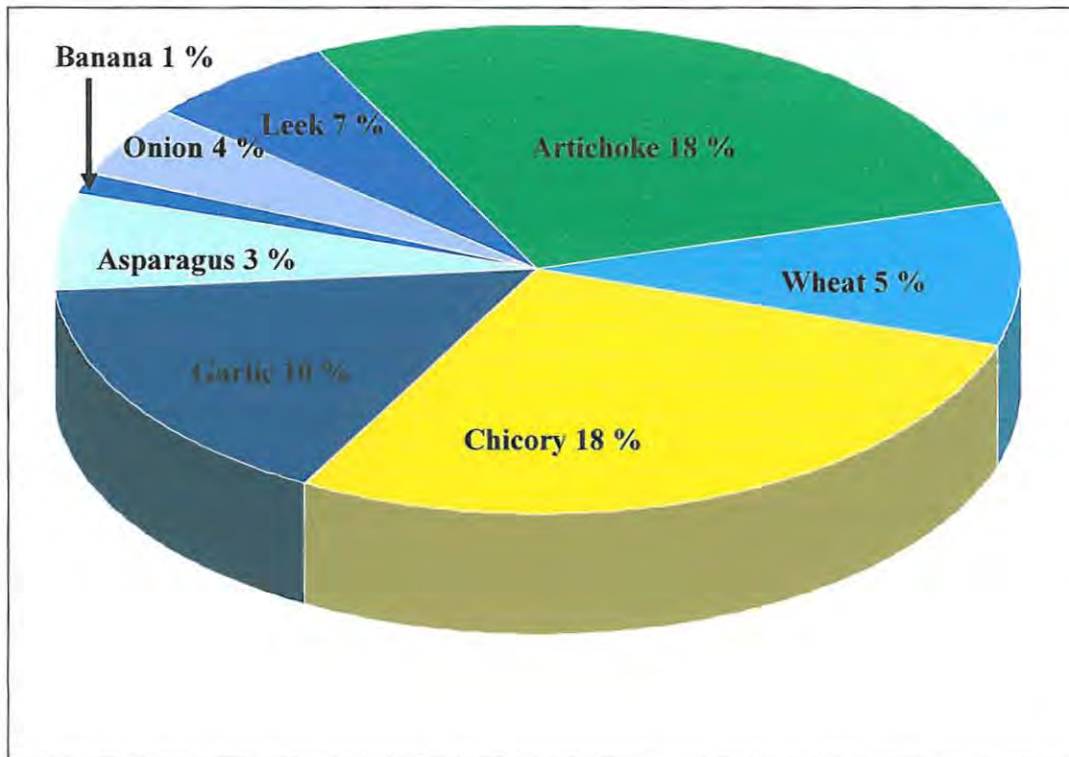
Figure 1.3 A graphical illustration showing the distribution of inulin from various plant sources that are commonly used in human nutrition (Van Loo *et al.*, 1995; Roberfroid *et al.*, 1998).

South Africa is one of the major producers of chicory inulin in the world after European countries such as Belgium and Germany. South Africa produces 63 million tonnes of chicory (beverage) annually. In South Africa, chicory is cultivated in the Alexandria area in the Eastern Cape Province where the climate is mild temperate because the area is coastline, lying near the Indian Ocean, and therefore is ideal for this crop (Figure 1.4). In addition good edaphic factors such as good deep rocky sandstone soils make this area suitable for chicory farming. Growing chicory in this area is more agronomically advantageous because there is increased production of chicory per hectare as compared to Jerusalem artichoke. The availability of modern day state of the art infrastructure such as good road, rail, airports, reliable communication net works, sufficient power supplies, cheap labour and a competitive

banking sector in the Eastern Cape Province also make this area ideal for chicory cultivation.

Figure 1.4 A map of South Africa showing the main chicory growing area of Alexandria in the Eastern Cape Province where 6 000 hectares is under chicory cultivation (Representational Map: not to scale).

1.4 Other bifidogenic factors

Besides fructooligosaccharides and inulooligosaccharides, there are other types of oligosaccharides which are reported to be bifidogenic (Crittenden and Playne, 1996). Bifidogenic factors are defined as carbohydrate bearing materials that survive direct metabolism by the host and are preferentially metabolised by bifidobacteria in the large intestines (Modler, 1994; Vazquez *et al.*, 2000). There are 12 classes of food-grade oligosaccharides currently in commercial production (Crittenden and Playne, 1996). Xylooligosaccharides (XO) are sugar oligomers composed of xylose monomers with only (1, 4) - β-D-xylopyranosyl linkages that are widely found in bamboo shoots, fruits, vegetables and honey (Modler, 1994; Crittenden and Playne, 1996). Xylooligosaccharides are mainly used in pharmaceutical formulations, feed formulations for agricultural purposes and in foods for human consumption (Vazquez *et al.*, 2000). The main advantage of xylooligosaccharides over inulin is that they can resist acid and thermal degradation and consequently have found wide use in low pH fruit juices and carbonated prebiotic drinks (Prapulla *et al.*, 2000; Modler, 1994).

Isomaltooligosaccharides are $[\alpha\text{-D-Glu } (1, 6)]_n$, where n = 2 - 7 and are commercially produced from starch by the action of debranching enzymes such as pullulanase (EC 3.2.1.41) and isoamylase (EC 32.2.1.68) (Crittenden and Playne, 1996; Prapulla et al., 2000). Intake of isomaltooligosaccharides has been found to improve colonic conditions by reducing the levels of intestinal putrefactive bacteria such as *Clostridia perfringens* and members of the family Enterobacteriaceae (Prapulla et al., 2000).

Galactooligosaccharides do not occur naturally but are synthesised from lactose using the galactosyltransferase activity of β-galatosidase and different types are produced commercially, namely, isogalatobiose, galsucrose and lactosucrose (Crittenden and Playne, 1996; Prapulla et al., 2000). Galactooligosaccharides are used as prebiotic food components and they are bifidogenic since they enhance the growth of bifidobacteria in the colon (Prapulla et al., 2000).

1.5 Industrial and pharmaceutical importance of inulin

The name inulin was used after the compound was first isolated from *Inula helenium* (Azis et al., 1999). Many other plants have been shown to make inulins as storage polysaccharides (Azis et al., 1999). Chicory inulins have interesting industrial applications. It is a cheap, renewable and readily available raw material of fructose, which is widely used in the food industry as a sweetener (Yun et al., 2000). Inulins are the only class of polysaccharides based on the furanose structure (Azis et al., 1999). Inulin is used as a special food for diabetics, as diuretics and is also used in kidney clearance tests because it remains intact in the blood for long after injection (Azis et al., 1999).

Inulin solutions have different properties depending on the origin of the inulin (Diaz, et al., 2006). Inulin solubility depends significantly on temperature, degree of polymerisation, distribution of the molecular chains, degree of molecular branching and the processing method (Lopez-Monila et al., 2005; Diaz et al., 2006). It has been reported that native chicory inulin is soluble to about 60 g/L at 10 °C while at 90 °C it is soluble to about 330 g/L (Diaz et al., 2006). Native chicory inulin is dispersible in water under normal conditions and has a tendency to clump during hydration due to its hygroscopic character.

1.6 Chicory inulin composition

Inulin is a polydisperse polymer made of between 2 – 60 fructose units (Lopez-Monila *et al.*, 2005). However, a high sugar content and long inulin chains are preferred by the sugar industry (De Leenheer and Hoebregs, 1994; Van Waes *et al.*, 1998). In Belgium, it has been observed that inulin has the highest DP early in the harvesting season, from end of September to October as shown in Table 1.1 (De Leenheer and Hoebregs, 1994).

Table 1.1 The effect of time of harvest on the chain length (average DP and maximal DP) of native chicory inulin (De Leenheer and Hoebregs, 1994).

Date of Harvest	Average DP	DP_{max}
End of September	11.7	72
Mid November	9.0	60
End of December	6.0	51

Native inulin is form of inulin that has been extracted from the plant source with hot water, minimising any chance of hydrolase activity that may lead to its degradation. Commercial inulin preparations obtained from various sources are not native and they should not be considered as products representing the inulin which is typical for the plants from which they were extracted (De Leenheer and Hoebregs, 1994). The composition of native inulin is GF_n molecules, accompanied by a small percentage of F_m molecules (Figure 1.1 page 5).

Several methods for inulin extraction from various sources have been documented (Lingyun *et al.*, 2007). The hot water extraction process at 85 °C is one of the most popular method followed by evaporation and spray drying (Tofano *et al.*, 2007). Another efficient method for inulin extraction that is widely used at laboratory scale is the precipitation from aqueous solution using ethanol (Lingyun *et al.*, 2007). This method was shown to be uneconomical and not suitable for industrial production of inulin (Lingyun *et al.*, 2007). Lingyun and co-workers developed an extraction strategy using high intensity and high frequency ultrasound waves (sonication). This

disrupts plant cell walls thereby facilitating the release of extractable compounds and enhances mass transport of solvent from the continuous phase into plant cells (Lingyun *et al.*, 2007). In addition the use of pectolytic enzymes allows for the enzymatic disruption of cell walls which leads to release of inulin from plant cells. This enzymatic treatment also reduces the viscosity and clarifies the final inulin solution.

1.7 Enzymes for oligosaccharide production

A wide range of inulinases and fructosyltransferases are involved in the formation of oligosaccharides and are produced by plant and microbial sources (Table 1.2). Principal FOS such as 1-kestose, nystose and fructofuranosylnystose (Figure 1.5) are synthesised by a wide range of enzymes. For industrial application, it is technically difficult to source these enzymes from plants. Traditionally inulinases have been produced by submerged fermentation (SmF) but recently, solid state fermentation (SSF) is gaining importance though there are few reports on the application of this technique (Xiong *et al.*, 2007). SSF is associated with numerous advantages over SmF such as superior productivity, simplicity, low capital investment, low energy requirement, less water output, and better product recovery (Xiong *et al.*, 2007). Due to these advantages, SSF is regarded as the most appropriate process (Xiong *et al.*, 2007). Enzyme properties have been shown to be greatly influenced by the nature of their source.

1.7.1 Microbial exoinulinases

Inulin hydrolysis is achieved by the use of exoinulinase (2, 1-β-D-fructan fructo-hydrolase, EC 3.2.1.80) which successively cleaves fructose from the non-reducing β-2, 1 end of inulin (Pandey *et al.*, 1999; Jing *et al.*, 2003[b]). Microorganisms involved in the production of exoinulnases include *Aspergillus* sp., *Klyveromyces* sp., *Pseudomonas* sp., *Xanthomonas* sp., *Penicillium* sp., *Chrysosporium* sp., *Bacillus* sp., and others as shown in Table 1.2. Generally, inulinases sourced from fungal and bacterial sources are more thermotolerant and this property is important for their industrial application, as elevated temperatures prevent microbial contamination of

the final product. Inulinases from microbial sources work under mild conditions and are stable over a broad pH range.

Table 1.2 Microbial and plant sources of oligosaccharide producing enzymes (Yun, 1996).

Plant Sources	Microorganism Sources
Agave americana (agave)	*Aureobasidium pullulans*
Agave vera cruze (agave)	*Aureobasium* sp.
Asperagus officinalis (asparagus root)	*Arthrobacter* sp.
Allium cepa (onion bulbs)	*Aspergillus japonicus*
Cichorium intybus (chicory)	*Aspergillus niger*
Crinum longifolium (sugar-beet leaves)	*Aspergillus oryzae*
Helianthus tuberosus (Jerusalem artichoke)	*Asperigullus phoenecis*
Lactuca lativa L. (lettuce)	*Aspergillus sydowi*
Lycoris radiate (monocot)	*Claviceps purpurea*
Taraxacum offinale (dandelion)	*Fusarium oxysporium*
	Pecicillium frequentans
	Penicilium spinulosum
	Phytophthora parasitica
	Scopulariopsis sp.
	Saccharomyces cerevisiae

1.7.2 Microbial endoinulinases

Microbial endoinulinases (2, 1-β-D-fructan-fructan hydrolase, EC, 3.2.1.7) hydrolyses the internal linkage of inulin to release intermediates such as inulotriose (F_3), inulotetraose (F_4) and inulopentaose (F_5) (Yun *et al.*, 2000; Jing *et al.*, 2003[b]) (Figure 1.1 b page 5). It has been well documented that the two endoinulinases and exoinulinases act either alone or synergistically to produce fructose, but it is not known whether the two enzymes coexist (Pandey *et al.*, 1996; Yun *et al.*, 2000; Jing *et al.*, 2000[b]; Rocha *et al.*, 2006).
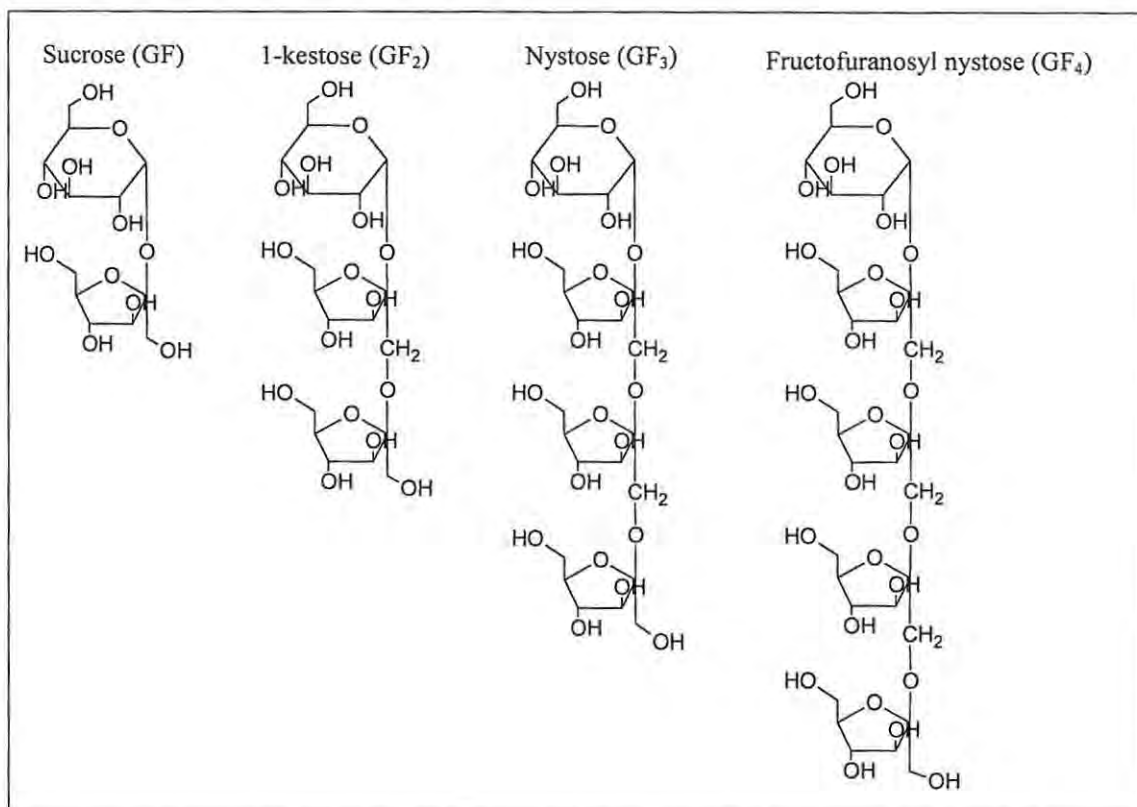
Figure 1.5 Chemical structures of principal FOS synthesised by fructoslytransferases.

1.7.3 Plant fructosyltransferases and mechanism of action

Fructosyltransferases obtained from plants are distinct transferases such as 1-SST and 1-FFT. Plants such as *Cichorium intybus* and *Helianthus tuberosus* produce high levels of fructosyltransferases such as sucrose: sucrose 1-fructosyltransferase (1-SST), (EC. 2.4.1.99) and fructan: fructan 1-fructosyltransferase (1-FFT), (EC. 2.4.1.100). The two enzymes are produced during the fructan accumulation stages and during the cold months.

The established method for fructan biosynthesis in Jerusalem artichoke (*Helianthus tuberosus*) tubers was first demonstrated by Edelman and Jefford (1968). They showed that fructan synthesis occurs through the formation of a trisaccharide intermediate by the concerted action of two distinct fructosyltransferases, 1-SST and 1-FFT (Cairns, 1995; Luscher *et al.*, 1996). In this model, 1-SST is a key enzyme that initiates fructan biosynthesis from sucrose to form a trisaccharide, 1-kestose, with the concomitant stoichiometric release of glucose and that 1-FFT transfers fructose

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moieties between fructan molecules to form long chain fructans. The product 1-kestose from the initial reaction with 1-SST is reported to be an efficient fructose donor for the formation of long chain fructans (Cairns, 1993; Luscher *et al.*, 1996; Van den Ende *et al.*, 2004). The mechanism of this reaction is illustrated in reactions 1.1 and 1.2. The wide array of enzymes involved in fructan enzymology is shown diagrammatically in Figure 1.6.

$$\text{G1, 2F + G1-2F} \xrightarrow{\text{1-SST}} \text{G1, 2F1, 2F + G} \qquad 1.1$$

$$\text{G1, 2F (1, 2F)}_m + \text{G1, 2F (1, 2F)}_{\bar{n}} \xrightarrow{\text{1-FFT}} \text{G1, 2F (1, 2F)}_{m-1} + \text{G1, 2F (1, 2F)}_{n+1} \quad 1.2$$
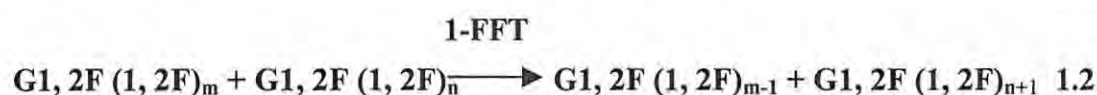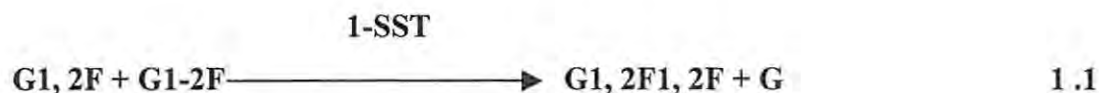
(Where $m > 0$ and $n > 1$, G and F represents glucose and fructose molecules respectively and 1, 2 represents the β (2, 1) glycosidic bond).

The two enzymes have since been purified and characterised and consequently the model proposed by Adelman and Jefford validated (Cairns, 1993; Koops and Jonker 1994; Luscher *et al.*, 1996; Van den Ende and Van Laere, 1996). An important finding of this method is that the enzymatic *de novo* synthesis of fructan with a degree of polymerisation greater than 3 from sucrose by a mixture of 1-SST and 1-FFT is impossible, as a result of enzyme inhibition by sucrose (Cairns, 1995; Koops and Jonker, 1996). According to Cairns (1995), long chain fructan synthesis in some grasses is not inhibited by sucrose and a high enzyme concentration is a requirement to overcome inhibition by sucrose. Enzymes that are involved in fructan synthesis are shown diagrammatically in Figure 1.6.

1.7.4 Microbial fructosyltransferases and mechanism of action

A microbial fructosyltransferase (Ftase; E.C. 2.4.1.9) catalyses the formation of FOS from sucrose (Sangeetha *et al.*, 2004[b]). A wide range of microbial sources for fructosyltransferases (Ftases) have been reported in the literature (Sangeetha *et al.*, 2005[a]) (Table 1.2). These enzymes synthesise FOS with different linkages to form

several kinds of FOS of varying yields as a direct result of the initial concentration of sucrose in the reaction mixture (Sangeetha *et al.*, 2005[a]; Catana *et al.*, 2007). It has
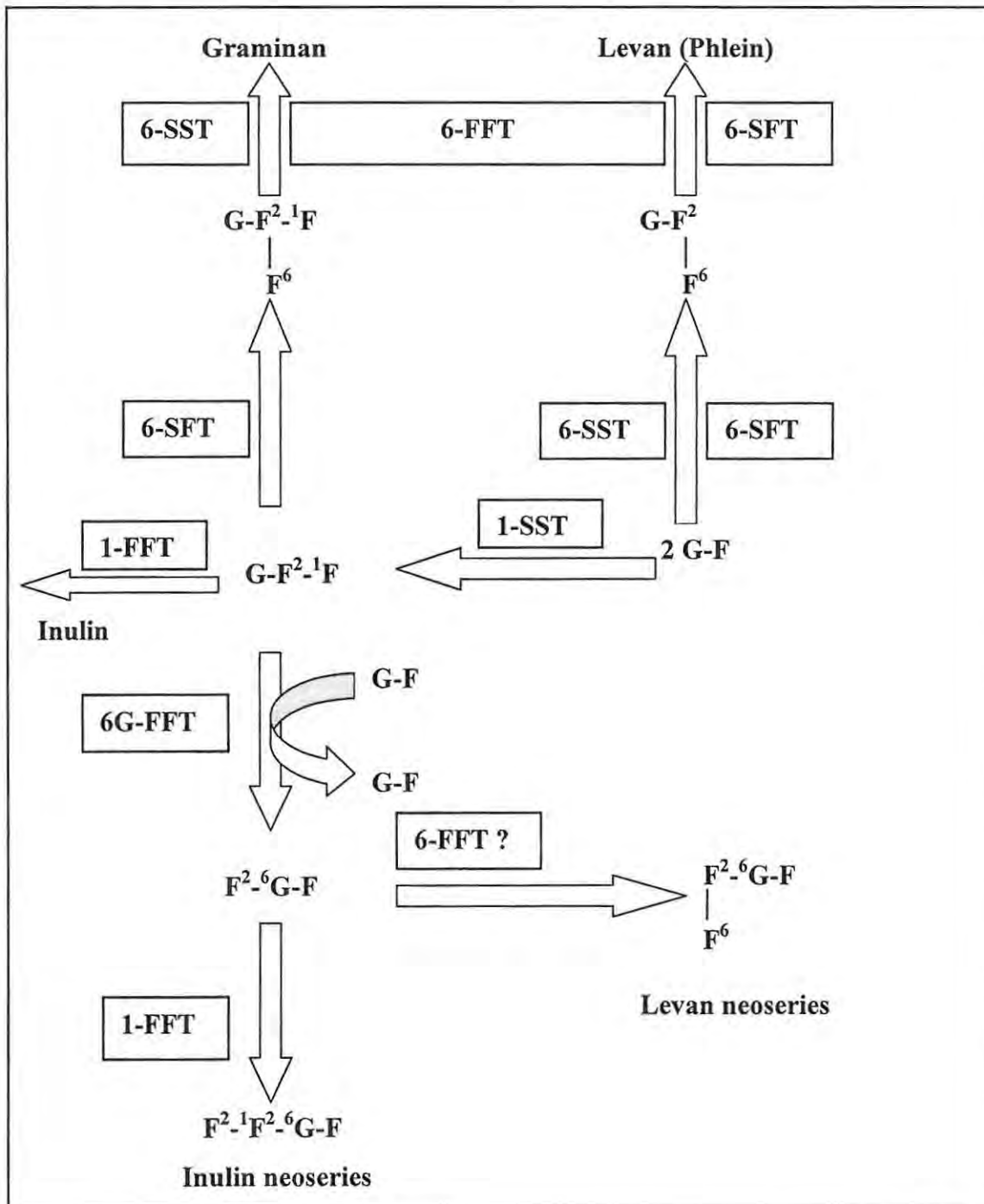
Figure 1.6 Enzymology of fructan biosynthesis in plants (Heyer *et al.*, 1999; Van den Ende *et al.*, 2004). 1-kestose (G-F2-1F) and 6-kestose (G-F2-6F) are both products of sucrose (G-F) from fructosyltransferase action. 1-SST catalyses the synthesis of 1-kestose formation. 1-kestose is a substrate for inulin and inulin neoseries synthesis using 1-FFT for chain elongation. Sucrose: sucrose 6-fructosyltransferase (6-SST) catalyses the synthesis of unbranched levan in barley and has the ability to introduce branches into longer chains. Fructan: fructan 6-fructosyltransferase (6-FFT) has chain elongating activity producing branched levan. Fructan: fructan 6-glucose-fructosyltransferase (6-SFT) is involved in the production of fructan neoseries.

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been established that fructosyltransferases obtained from microorganisms are single enzymes with both transferase and hydrolase activities (Tanriseven and Gokmen, 1999; Sangeetha *et al.*, 2005[a]). Figure 1.7 illustrates the mechanisms of FOS synthesis using a microbial fructosyltransferase.
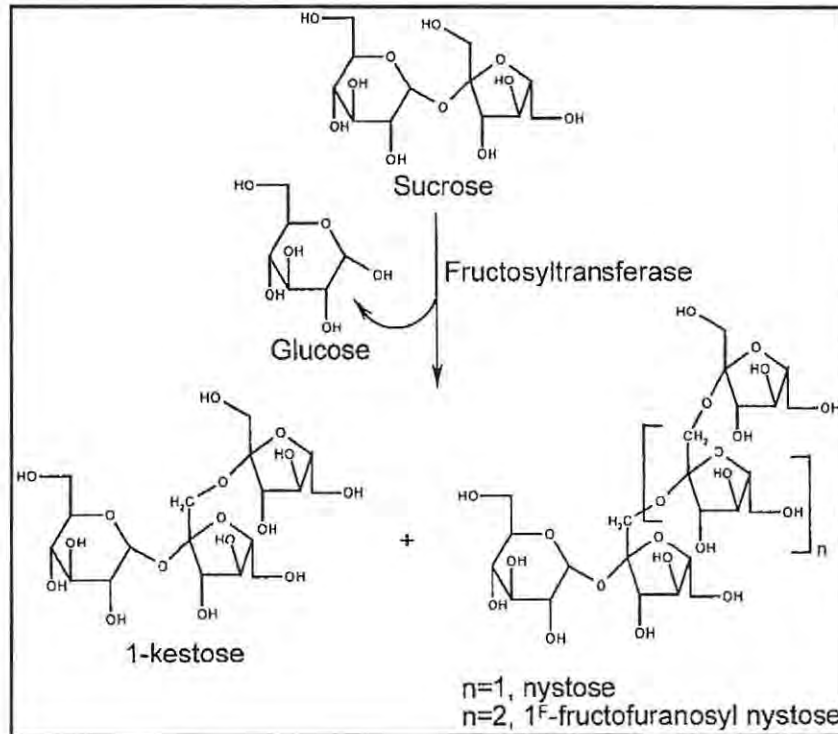
Figure 1.7 A schematic illustration of the microbial fructosyltransferase catalysed synthesis of fructooligosaccharides from sucrose (Tanriseven and Gokmen, 1999).

A mathematical model has been postulated to elucidate the mechanism of FOS synthesis with FTase from a set of disproportionation reactions which is slightly different from the mechanism of plant fructosyltransferases (Jung *et al.*, 1989). The mechanism of FOS synthesis by microbial fructosyltransferases has been elucidated and in this reaction 2 moles of sucrose act as fructose donors and acceptors for the formation of 1 mole of glucose and 1 mole of 1-kestose. In turn, the 1-kestose acts as an acceptor for the formation of a tetrasaccharide as illustrated in Figure 1.8. The reaction mechanism can be exemplified by reactions 1.3 and 1.4. It has been shown that the glucose that is liberated in the reaction mixture acts as an Ftase inhibitor (Jung *et al.*, 1989). Consequently, the batch enzymatic synthesis of polysaccharide from sucrose is disadvantageous due to a large loss of enzyme activity by end product inhibition of the enzyme (Hicke *et al.*, 1999).

Ftase

$$GF + GF \longrightarrow G + GF_2 \qquad\qquad 1.3$$

Ftase

$$GF_n + GF_n \longrightarrow GF_{n-1} + GF_{n+1} \qquad\qquad 1.4$$
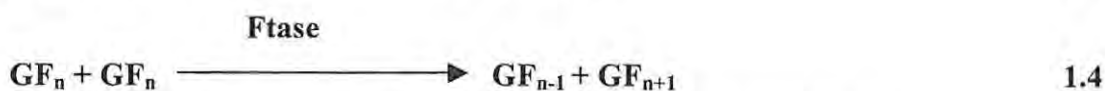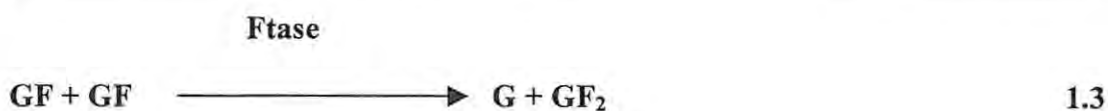
Where $n > 1$ and G and F represent glucose and fructose molecules respectively.

A fructosyltransferase from *Aureobasidium pullulans* has been isolated, characterised and optimum reaction conditions for the synthesis of FOS determined (Jung *et al.*, 1989). Parameters such as optimal pH, optimal temperature, pH stability and thermal stability and kinetic parameters such as K_m and V_{max} were established and the data generated was used to propose the reaction mechanism (Jung *et al.*, 1989).
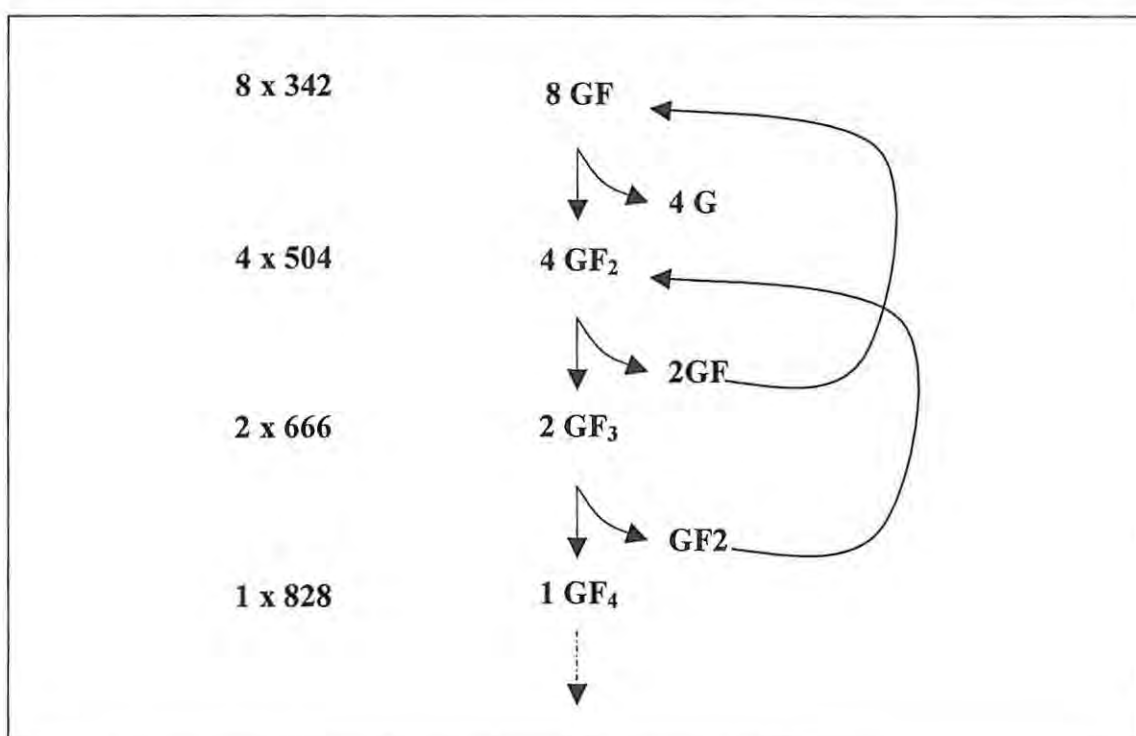
Figure 1.8 Illustration of a mathematical model to explain the disproportionation reaction mechanism for the formation of FOS from sucrose (Jung *et al.*, 1989; Yun, 1996). According to this model, 8 moles of sucrose (GF) are used to form 4 moles of 1-kestose (GF$_2$). Also 1 mole of 1-kestose is formed from 2 moles of nystose (GF$_3$) and simultaneously, 2 moles of nystose and 2 moles of sucrose are formed from 4 moles of 1-kestose. One mole of fructofuranosyl nystose (GF$_4$) is formed by the removal of nystose.

It has been reported that the synthesis of FOS is a kinetically controlled reaction that involves a fructosyl-enzyme intermediate whereby the two nucleophiles; water and sucrose, compete for the fructosyl-enzyme intermediate (Ghazi *et al.*, 2006). When water is the nucleophile, the enzyme acts as a hydrolase to liberate glucose and

fructose, and when sucrose is the nucleophile, the enzyme acts as a transfructosidase synthesising high DP FOS (Ghazi et al., 2006). As a result, the first condensation product, 1-kestose, can also be hydrolysed by the enzyme.

1.8 Isolation and purification of inulinases and fructosyltransferases

In order to produce pure IOS and FOS, it is desirable to purify enzymes used for IOS and FOS to electrophoretic homogeneity so that their properties and mode of action can be studied as well as to find out if the purified enzyme can enhance the production of FOS or IOS. FTases have been purified from various sources as shown in Table 1.3. The purified FTase was found to produce 1-kestose and nystose unlike the crude enzyme which produced GF_5 and GF_6 oligosaccharides (Sangeetha et al., 2005[a]).

Table 1.3 Characteristics of fructosyltransferases from various microbial sources (Sangeetha et al., 2005[a]).

Source of Ftase	Purification Fold	Molecular Weight (kDa)	Optimal pH	Optimal Temperature	Stability pH	Stability Temperature
B. macerans EG-6	63.5	66	5.0	50 °C	5.0-7.0	20-50 °C
A. oxydans J17-21	95.5	54	6.5	45 °C	5.0-11.0	20-40 °C
M. laevaniformans ATCC 15953	45.6	64	6.0	30 °C	5.0-7.0	
A. niger ATCC 20611	51.6	340	5.0-6.0	50-60 °C	4.5-10	Up to 60 °C
Arthrobacter sp. K-1	405.3	52	6.5-6.8	55 °C	5.5-10	Up to 40 °C
S. salivarius ATCC 25975	34.5	125.4	6.0-7.0	37-40 °C	-	-
Microbacterium sp AL-210	98.8	46	7.0	40 °C	7.0-8.0	Up to 40 °C

1.9 Physiological roles of fructans

Fructans are fructose oligomers and polymers synthesised by a wide range of plant and bacterial species, and mainly function as reserve carbohydrates that are hydrolysed by fructan 1-exohydrolase when energy supplies are needed (Simmen et al., 1993; Nakamura et al., 1995; Luscher et al., 1996; Rocha et al., 2006; Sheng et

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al., 2007). There are other physiological roles that have been postulated by other workers but these have not been fully elucidated (Van den Ende *et al.*, 2003; Van den Ende *et al.*, 2004; Cerantola *et al.*, 2004).

Fructans increase the osmotic pressure of plant cells resulting in osmoprotection which protects the plant against abiotic stress (De Leenheer and Hoebregs, 1994; Van den Ende *et al.*, 2004; Guignard *et al.*, 2005). A wide range of carbohydrates are involved and these consist mainly of hexoses (mostly fructose and glucose), disaccharides (sucrose and trehalose), sugar alcohols (for example inositol and mannitol) and complex carbohydrates (for example raffinose and stachyose) (Guignard *et al.*, 2005). The presence of fructans allows for frost tolerance and as a result contributes to membrane stabilisation (Van den Ende *et al.*, 2001; Van den Ende *et al.*, 2004). According to De Leenheer and Hoebregs (1994), the average DP is decreased with growth time and as a result there will be a higher intracellular osmotic pressure, and this leads to cold resistance in the plant. The variation in DP may be induced by external weather conditions (De Leenheer and Hoebregs, 1994). Fructans have been examined for their biological role as an adaptation to low temperature photosynthesis (Wang *et al.*, 1999; Van den Ende *et al.*, 2000).

1.10   Production of inulooligosaccharide and fructose from inulin

Complex polysaccharides are difficult to synthesise and manipulate. Currently, there is no commercial process that can synthesise these compounds in an automated fashion, which poses a major limitation to detailed study of their biological functions (Duus *et al.*, 2000; Seeberger and Werz, 2007). In addition, there is no method available to biologists for the structural assignment of carbohydrates with DP < 10 as compared to other biological molecules such as nucleic acids which can be synthesised *in-vitro* using the polymerase chain reaction (PCR) (Duus *et al.*, 2000).

Despite these constraints, however, there are several methods that can be employed for the synthesis of fructose and fructooligosaccharides with DP < 10 from simple inexpensive raw materials. Fructose can be obtained by acid hydrolysis of inulin at elevated temperatures though the product monosaccharide can be easily degraded at low pH and the process can give rise to colouring of the inulin hydrolysate and

unwanted by-product formation in the form of inulin anhydrides that lower the yield and require extensive downstream processing (Vandamme and Derycke, 1983; Gill *et al.*, 2006; Diaz *et al.*, 2006; Rocha *et al.*, 2006).

Another major drawback of chemical hydrolysis is that the technique requires refluxing for extended periods which can be expensive and there is need to use expensive acid-resistant equipment (Ma and Ooraikul, 1986). Chemical inulin hydrolysis can be carried out by treatment with organic or mineral acids or by heterogeneous catalysis using solid acidic catalysts such as acid-cation resins, zeolites or oxidized activated carbon (Rocha, *et al.*, 2006). The energy economy and low formation of by-products, mainly those resulting from the cyclisation of glucose and fructose at acid pH and high temperature, suggest the advantages of using enzymes over acid hydrolysis (Tomotani and Vitolo, 2007).

Enzyme-based processes operate at lower temperatures, produce less toxic and pollutant wastes, produce fewer emissions and by-products compared to conventional chemical processes (Tomotani and Vitolo, 2007). Enzymatic methods for the production of fructooligosaccharides have demonstrated to be the best option for the industrial production of fructose syrups since they are specific and are not associated with the shortcomings of the chemical approach (Rocha *et al.*, 2006).

Fructose can be produced from starch by enzymatic methods involving α-amylase, amyloglucosidase and glucose isomerase (Reaction 1.5) resulting in the production of a mixture consisting of oligosaccharides (8 %), fructose (45 %) and glucose (50 %) (Gill *et al.* 2006). The major drawback of this method is that downstream processing of the final fructose product is costly (Gill *et al.*, 2006; Diaz *et al.*, 2006; Sharma and Gill, 2007). The use of inulinases is an alternative enzymatic method which can give rise to 95 % pure fructose and the remainder being a mixture of inulooligosaccharides (IOS) (inulobiose, inulotriose, inulotetraose and inulopentaose) and small amounts of glucose.

$$\text{Starch} \xrightarrow[\text{Amylase}]{\text{Amyloglucosidase}} \text{Glucose} \xrightarrow{\text{Glucose isomerase}} \text{Fructose} \qquad 1.5$$
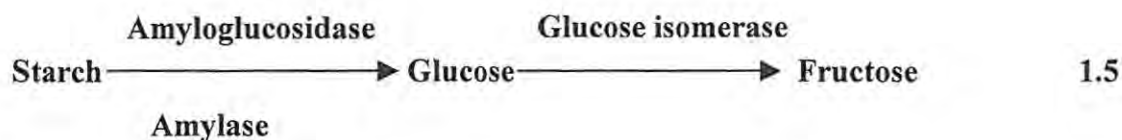
Inulin hydrolysis using enzymes is usually carried out at 60 °C and mild pH conditions (Yun *et al.*, 1997[b]). The temperature is especially critical because it prevents microbial contamination of the final product, it lowers the viscosity, it improves transfer rates and it allows for the use of higher concentrations of inulin due to increased solubility (Gill *et al.*, 2006; Bruins *et al.*, 2003). Under these conditions it is crucial to employ highly thermostable inulinolytic enzymes. For IOS production, the main benefit of using a higher reaction temperature lies in the increase of the substrate concentration that improves the IOS yield. The disadvantages at these elevated temperatures may be the instability of enzymes, substrates or products and the occurrence of side reactions (Bruins *et al.*, 2003).

The production methods for IOS can either be batch or continuous using free or immobilised enzymes. Batch production of IOS has limitations such as requirements for high amounts of enzymes and product purification. Due to the high cost of enzymes, this method has high production costs (Diaz *et al.*, 2006). Continuous methods are desirable because the enzymes are amenable to immobilisation, which allows for continual biocatalyst reuse (Rocha *et al.*, 2006). Continuous mode of operation prevents contamination of the final product (Rocha *et al.*, 2006, Tomotani and Vitolo, 2007). Inulinases or whole microbial cells with inulinase activity can be immobilised on suitable resins such as amberlite and the procedure must be effective, cheap and simple for industrial scale process (Rocha *et al.*, 2006). The yield of target products such as pentamers and hexamers however decreases with progression of hydrolysis. This is because they are intermediates of the reaction and as a result, stricter control of the hydrolysis reaction must be achieved in order to produce these oligosaccharides more efficiently (Kuroiwa, *et al.*, 2002).

1.11 Glucose removal from the reaction mixture

Inhibition of the Ftase by glucose is one important factor limiting maximal FOS yield under batch conditions (Hicke *et al.*, 1999; Sheu *et al.*, 2001; Sangeetha *et al.*, 2005[a]). This is due to the accumulation of by-products such as glucose in the reaction mixture which may in turn inhibit the fructosyltransferase activity (Park *et al.*, 1999; Steinberg *et al.*, 2002; Itaya *et al.*, 2007). Techniques for the removal of the reducing sugars

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involving nanofiltration and microfiltration have been proposed by some workers to eliminate low molecular weight carbohydrates from a mixture of oligosaccharides (Goulas *et al.*, 2002; Diaz *et al.*, 2006; Sjoman *et al.*, 2007; Goulas *et al*, 2007; Sanz and Martinez-Castro, 2007).

The use of immobilised enzymes such as glucose oxidase (GOD) for the bioconversion of D-glucose to D-gluconic acid and hydrogen peroxide has been evaluated and its efficacy looks promising (Tanriseven and Gokmen, 1999; Sheu *et al.*, 2001; Mislovicova *et al.*, 2007) (Reaction 1.6). On the other hand the use of glucose isomerase for glucose removal from the reaction mixture is reported to be ineffective (Yun, 1996; Tanriseven and Gokmen 1999).

Glucose oxidase

Glucose ────────────▶ Gluconic acid + hydrogen peroxide   1.6

Oxidation
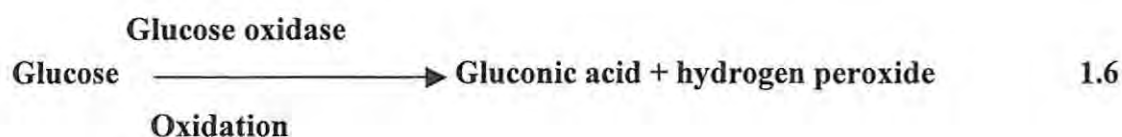
Saccharomyces cerevisiae and *Zymomonas mobilis* were used separately for the removal of glucose from the FOS reaction mixture (Crittenden and Playne, 2002; Yoon *et al.*, 2003). Glucose and fructose were completely fermented to ethanol and carbon dioxide with minor amount of sorbitol as a fermentation by-product (Crittenden and Playne, 2002; Yoon *et al.*, 2003; Goulas *et al.*, 2007). Degradation of oligosaccharides was not observed due to the selective fermentation characteristics of the yeast towards different sugars (van der Heijden *et al.*, 1999; Yoon *et al.*, 2003; Goulas *et al.*, 2007). Strains of *Saccharomyces cerevisiae* and *Zymomonas mobilis* lack carbohydrases that hydrolyse most oligosaccharides (Crittenden and Playne, 2002). High content FOS (98 %) was obtained by eliminating the released glucose and unreacted sucrose from the reaction mixture (Sangeetha *et al.*, 2005[a]).

Removal of glucose confers oligosaccharides with several advantages such as increased viscosity, reduced sweetness and hygroscopic, and causes fewer Maillard reactions during heat processing (Crittenden and Playne, 2002). Furthermore, removal of contaminating simple sugars lowers cariogenicity and caloric value, and allows the oligosaccharides to be included as diabetic foods (Crittenden and Playne, 2002).

1.12 Applications of fructose

High-fructose syrup (HFS) has major applications as a sweetener in food, pharmaceutical industries, and is used as a substitute for sucrose (Rocha *et al.*, 2006; Gill *et al.*, 2006; Tomotani and Vitolo, 2007). Functional attributes of fructose include high osmotic pressure, high solubility, source of energy, twice as sweet as sucrose, prevents crystallisation of sugar in food products, prevents microbial growth, flavour, colour, product stability enhancement and it is organoleptically desirable (Nakamura *et al.*, 1995; Rocha *et al.*, 2006; Diaz *et al.*, 2006).

Fructose is an attractive food additive which can replace glucose since it does not involve the metabolic pathway of glucose which requires insulin and hence complications with diabetes (Giacco, *et al.*, 2004; Diaz *et al.*, 2006). Fructose does not stimulate insulin secretion. Consequently, fructose can be consumed by diabetic patients without compromising their health. Due to the growing need of foods with health benefits, there is a growing need to produce fructose for diabetic people.

The fate of fructose after absorption in the gastrointestinal tract involves its transformation into fructose-1-phosphate and then metabolised into triose phosphate, glyceraldehydes, and dihydroxyacetone (Messier *et al.*, 2007). The events that occur after fructose enters the glycolytic pathway are threefold. A negligible portion of the fructose is converted into glucose and this leads to a small but measurable increase in blood glucose level (Messier *et al.*, 2007). A large part of the fructose is transformed into lactate and this leads to a three fold increase in the levels of blood lactate (Messier, *et al.*, 2007). Therefore fructose bypasses the regulatory processes involved with glucose metabolism in the liver.

1.13 Applications of fructooligosaccharides

Fructooligosaccharides are relatively new functional food constituents that are becoming popular because they have a potential for enhancing the quality of flavour and physicochemical properties of food products (Modler, 1994; Prapulla *et al.*, 2000). The formation of lactic, acetic, propionic and other short chain organic acids

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are thought to be antagonistic to other potentially pathogenic gastrointestinal competitors (Huebner *et al.*, 2007). As a result, FOS are slowly replacing some harmful sugar products such as sucrose that can cause health problems such as diabetes mellitus. FOS are produced from natural compounds and this has led to increased interest from consumers across the world (Prapulla *et al.*, 2000).

A functional food is defined as any food that has a positive impact on an individual's health, physical performance or state of mind in addition to its nutritional content (Loo *et al.*, 1995; Sangeetha *et al.*, 2005[a]). FOS are widely applied in food formulations because of their functional properties. Examples of their use in food include light jam products, ice cream and confectionery (Sangeetha *et al.*, 2005[a]). Application of FOS in food formulations is a new phenomenon.

Fructooligosaccharides have different applications depending on the method of production. Fructooligosaccharides synthesised by transfructosylation from sucrose are used as prebiotic ingredients while the longer chain oligosaccharides derived from controlled enzymatic hydrolysis of inulin are used as fat replacers (Prapulla *et al.*, 2000). The functional properties of fructooligosaccharides are greatly dependent upon their molecular weight with pentamers (DP5) and hexamers (DP6) being especially significant (Kuroiwa, *et al.*, 2002). As a result, products with a high content of pentamers and hexamers are preferred for their effective stimulation of growth of beneficial bacteria in the gastro-intestinal tract (Kuroiwa, *et al.*, 2002).

1.14 Biofunctional properties of fructooligosaccharides

Investigations have been carried out to confirm that FOS have biofunctional properties (Roberfroid *et al.*, 1998; Flamm *et al.*, 2001; Flickinger *et al.*, 2003; Sangeetha, *et al.*, 2005[a]). Published research data indicate that FOS have desirable health effects such as dietary fibre, prebiotics in food formulations, enhancing mineral absorption, role in defence mechanisms, role in lipid metabolism, anticancer effects, control of diabetes among other health claims which still need further research for validation (Lopez-Molina *et al* 2005; Sangeetha, *et al.*, 2005[a]).

1.14.1 Fructooligosaccharides as prebiotics

The bifidogenic activity is reported to be optimum with short chain FOS in which the fructosyl units (n = 2-8) are bound by a β-(2, 1) linkage (Barthomeuf et al., 1997). The proliferation of bifidobacteria is associated with beneficial effects such as improved digestion and absorption, increased vitamin availability and most importantly prevention of gut colonisation by pathogens and putrefactive bacteria (Prapulla et al., 2000). The prebiotic role of FOS has been promulgated from in vivo and in vitro studies of the metabolism of these compounds by intestinal bacteria (Sangeetha et al., 2005[a]). Durieux and co-workers (2001) used two types, Fibruline instant and Fibrulose 97, to demonstrate the prebiotic role of chicory fructooligosaccharides. They found that all the bacterial strains investigated, *Bifidobacterium longum, B. infantis* and *B. angulatum* utilised the fructose oligomers in the commercial chicory FOS and this unequivocally proved that FOS can be used as prebiotics (Durieux et al., 2001).

Furthermore, the prebiotic role of FOS was shown by Rycroft and co-workers (2001) by carrying out a comparative evaluation of the fermentation properties of prebiotic oligosaccharides by the predominant gut bacterial groups. Rycroft et al., (2001), and Roberfroid et al., (1998), found that all prebiotics investigated increased the level of bifidobacteria from 20 to 71 % and clostridia levels dropped from 3 to 0.3 % (Figure 1.9 a and b)

1.14.2 Control of diabetes

It has been demonstrated that a daily intake of 20 g of FOS significantly reduced the basal hepatic glucose production in healthy human subjects with no effect on insulin stimulated metabolism (Luo et al., 2000). It was further investigated that other compounds such as serum triacylglycerol, total and HDL cholesterol, free fatty acids, apolipoproteins A1 and B were not modified by the consumption of FOS (Kaufhold et al., 2000; Luo et al., 2000). During the enzymatic synthesis of FOS glucose and sucrose are some of the side products formed and these sugars are implicated in diabetes. Hence it is imperative to have pure FOS that are free from glucose and sucrose in the mixture to be marketable to diabetic patients (Sangeetha et al., 2005[a]).

(a)

Bacteroides   Bifidobacteria   Fusobacteria

20 %

65 %

12 %

3 %

Clostridia

(b)

Bifidobacteria

Fusobacteria

71 %

3 %

Bacteroides ───────────▶ 25.7 %

0.3 %

Clostridia
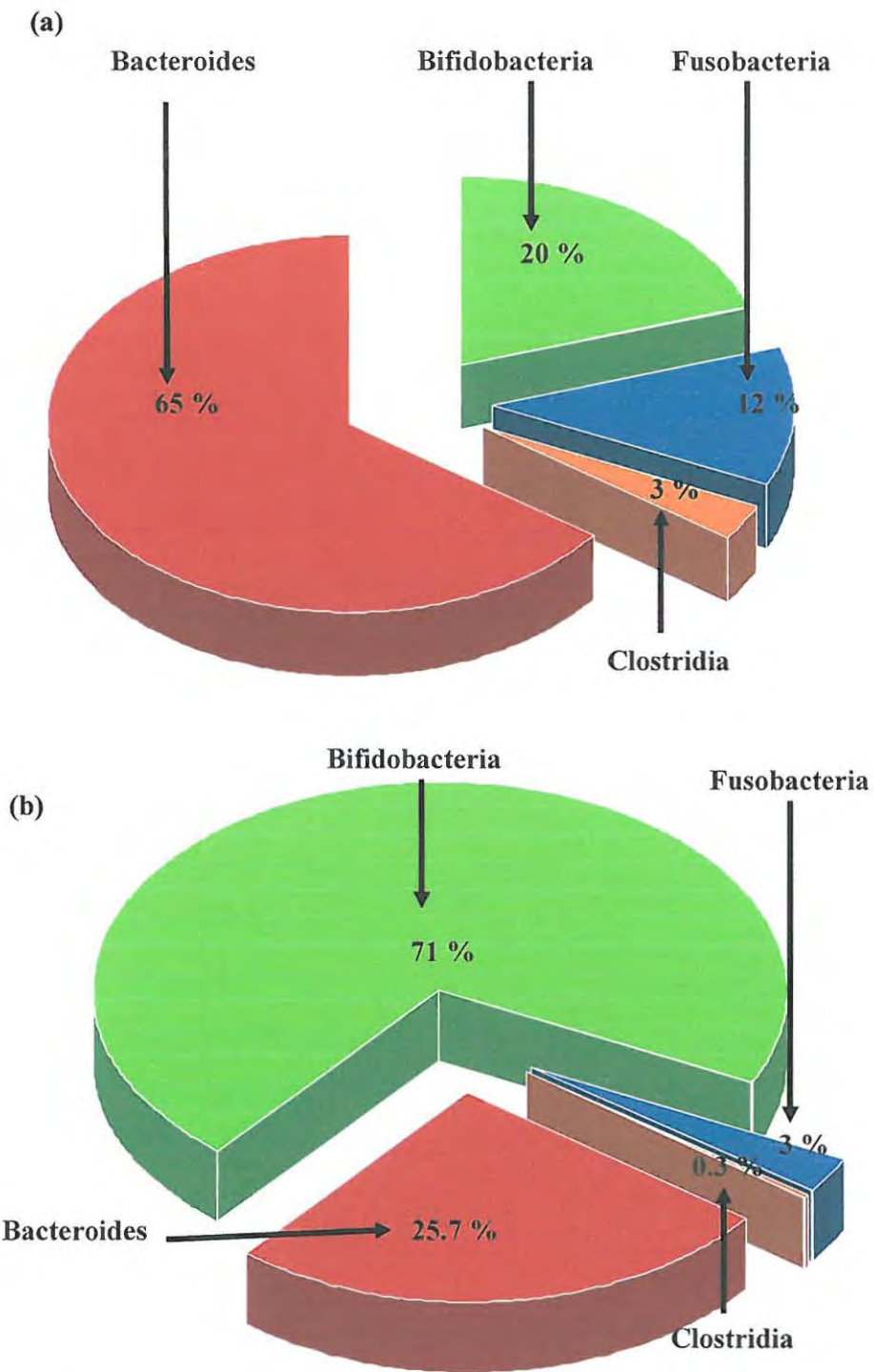
Figure 1.9 Gastrointestinal microbial populations before inulin intake (a) and after inulin intake (b). (Roberfroid, *et al.*, 1998).

1.14.3 Role as anticancer agents

Recent research in experimental animal models has revealed the physiological effects of FOS and inulin in the possible protection against the development of colon cancer and also reduction of chemically induced aberrant crypts (Prapulla *et al.*, 2000; Sangeetha *et al.*, 2005[a]). The anticarcinogenic attributes of FOS are reported to be a result of the proliferation of the bifidobacteria (Pool-Zobel *et al.*, 2002; Pool-Zobel, 2005).

Experiments demonstrated that a 15 % supplementation of basal diets of experimental animals resulted in significant reduction of colon tumours, inhibited the proliferation of transplantable malignant tumours in mice and also lowered the incidence of lung metastases of a malignant tumour implanted intramuscularly in mice (Taper and Roberfroid, 2002). Moreover, Taper and Roberfroid (2002) reported that the dietary treatment with FOS or inulin significantly potentiated the effects of subtherapeutic doses of six different cytotoxic drugs used in cancer treatment in human beings.

1.14.4 Role as dietary fibre

Dietary fibre is defined as those substances that consist of remnants of edible plant cell polysaccharides that are resistant to hydrolysis by human alimentary enzymes (Cherbut, 2002). Fructooligosaccharides are fermented by the bacteria in the large intestines producing short-chain fatty acids mainly acetate, propionate and butyrate which are absorbed efficiently (Luo *et al.*, 2000; Huebner *et al.*, 2007). This has led to FOS being regarded as dietary fibre (Cherbut, 2002). By closely monitoring the levels of ingested FOS in individuals with conventional ileostomy, Cherbut (2002) found that 90 % of the ingested FOS was recovered at the end of the ileum. In the colon the FOS are completely fermented and as a result of these fermentation properties, FOS affect the intestinal epithelium that may strengthen mucosal protection and reduce the risk of gastrointestinal diseases (Cherbut, 2002).

1.14.5 Role in mineral absorption

Research carried out on animal models has shown that non-digestible oligosaccharides (NDO) such as inulin, oligofructose and transgalatooligosaccharide (TOS) stimulate mineral absorption, mainly calcium and magnesium (Scholz-Ahrens and Shrezenmeir, 2002). This role of FOS has been demonstrated by the accumulation of bone mineral content and also the prevention of osteoporosis in ovariectomised rats whereby the addition of 5 % FOS led to a significant increase in bone mineral content (Sangeetha et al., 2005[a]). Increase in bone mineralisation is possibly as a result of the enhancement of passive and active mineral transport across the intestinal epithelium, mediated by an increase in certain metabolites of the intestinal flora and a reduction in pH (Scholz-Ahrens and Schrezenmeir, 2002).

1.14.6 Role in lipid metabolism

Apart from their beneficial effect on the gastrointestinal tract, studies on animal models have shown that inulin and oligofructose modified the hepatic metabolism of lipids (Delzenne et al., 2002). By feeding rats with inulin or oligofructose, it was shown that these carbohydrate rich diets markedly lowered serum triacylglycerols (TAG) and phospholipid concentrations.

1.14.7 Role in defence mechanisms

Fructooligosaccharides are known to stimulate the growth of beneficial bacteria while suppressing the growth of potentially harmful microorganisms. This is attributed to the low pH environment as a result of the production of antibiotic like compounds by the beneficial bacteria during the fermentation of FOS (Sangeetha et al., 2005[a]). Supplementing the diets of chicken, pigs and rats with oligofructose and other NDO resulted in a reduction of the faecal density of Salmonella (Sangeetha et al., 2005[a]). Furthermore, a diet consisting of 100 g/Kg of inulin and oligofructose that was fed to mice infected with virulent strains of Listeria monocytogenes and Salmonella typhimurium was shown to reduce mortality as compared to mice fed with a placebo of cellulose rich diet as a source of fibre (Buddington et al., 2002).

1.15 Quantification, detection and structural determination of FOS

Methods for accurate qualitative and quantitative analysis of oligosaccharides are rare because of the high diversity of carbohydrates occurring in nature (Van Waes, 1998; Guignard *et al.*, 2005). Chromatographic methods, in particular HPLC, have led to rapid and accurate determination of FOS and play a central role for the analysis and characterisation of carbohydrates (Churms, 1996[a]; Churms, 1996[b], Rassi, 1996; Prapulla *et al.*, 2000; Farine *et al.*, 2001; Bruggink *et al.*, 2005; Sangeetha *et al.*, 2005[a]; Sanz and Martinez-Castro, 2007).

Pure authentic compounds are needed for the determination of the exact response factors in several quantitation methods and it is technically difficult to separate and quantitate inulin or oligofructose because no commercial standards are available in sufficient quantities (De Leenheer and Hoebregs, 1994; Van Waes, 1998; Guignard *et al.*, 2005; Sangeetha *et al.*, 2005[a]; Ronkart *et al.*, 2007). Moreover, the wide range of functional groups (hydroxyl, amino, acetamino, phosphate) add to the chemical diversity and consequently complicate the choice of stationary and mobile phases for high resolution with HPLC (Guignard *et al.*, 2005).

Photometric detection of carbohydrates is problematic and insensitive because of the lack of a natural chromophore or fluorophore and therefore requires derivatisation for analysis by fluorescence and gas chromatography (Honda, 1984; Guignard *et al.*, 2005). Modern methods for the analysis of carbohydrates include gas chromatography (GC), high performance liquid chromatography (HPLC), and capillary electrophoresis (CE) or by a combination of techniques which include nuclear magnetic resonance (NMR) and gas chromatography - mass spectrometry with electron impact ionisation (GC-MS-EI), *inter alia* (Duus *et al.*, 2000; Cheng *et al.*, 2006; Campa *et al.*, 2004; Montilla *et al.*, 2006; Matamoros Fernandez, 2007).

Advances in the development of desorption/ionisation techniques, such as matrix assisted laser desorption ionisation (MALDI) and electrospray ionisation (ESI) in conjunction with the development of new instruments in MS has allowed application of multiple MS techniques for the structural characterisation of carbohydrates

(Matamoros Fernandez, 2007). It has been reported that the efficient separation of underivatised sugar alcohols, mono, di- and oligosaccharides up to degree of polymerisation of 60 is possible using high performance anion exchange chromatography coupled with pulsed amperometric detection (HPAEC-PAD), using alkali hydroxide and alkali acetate based eluents (Guignard *et al.*, 2005; Bruggink *et al.*, 2006; Ronkart *et al.*, 2007). This method is efficient and most preferable because of its ease and power of separation, detection, and quantification in comparison to other chromatographic and enzymatic methods (Timmermans *et al.*, 1994; Van Waes *et al.*, 1998; Guignard *et al.*, 2005; Ronkart *et al.*, 2007;).

Pulsed amperometric detection (PAD) is essentially an electrochemical detection method based on the oxidation of carbohydrates on a gold electrode by application of a positive potential. The main advantage of HPAEC-PAD is that it can analyse carbohydrates without derivatisation and is a most powerful tool as its resolution power is able to separate each degree of polymerisation and their isomers (distinction between F_n and GF_{n-1}) (Corradini *et al.*, 2004; Ronkart *et al.*, 2007). The method suffers, however, from unstable baselines, coelution, loss of sensitivity due to excessive noise and use of high pH and high salt concentration (Anumula and Dhume, 1998; Campa *et al.*, 2004; Guignard *et al.*, 2005; Ronkart *et al.*, 2007). The prediction of the elution order of the sugar oligomers is also a problem since, in most instances, no standards are available (Ronkart *et al.*, 2007). Interferences between the matrix components and the target compounds affect the results by hindering peak attribution and integration (Guignard, *et al.*, 2005). Verification of the identity of the individual sugars is achieved by comparing the retention times of the peaks with those obtained from authentic sugar standards if they are available. The peaks of unknown sugars are collected and identified offline by MS, NMR or any other suitable procedure.

1.15.1 HPLC and LC/MS

HPLC is a method of choice for the quantification and identification of sugars from a wide range of sources. This method uses a suitable column for the separation of sugars and the main advantage is that it is convenient and direct (Cheng *et al.*, 2006; Medeiros and Simoneit, 2007). Polar-bonded-phase and resin-based HPLC columns are commonly used for the analysis of FOS of different degree of polymerisation and

different glycosidic linkages (Prapulla *et al.*, 2000; Farine *et al.*, 2001; Sangeetha *et al.*, 2005[a]). The former columns are efficient and carbohydrates elute in order of increasing chain length in contrast to the latter type of columns where the sugar components elute in order of decreasing molecular weight (Prapulla *et al.*, 2000; Sangeetha *et al.*, 2005[a]).

A number of detection methods are available for HPLC analysis of sugars, and these include, refractive index (RI) detection, PAD, evaporative light-scattering detection (ELSD) and mass spectrometry (MS) (Cheng *et al.*, 2006). The concentration detection limits of RI and PAD are 0.1 parts per million (ppm) and 0.01 parts per billion (ppb) respectively but, the major drawback is that these conventional detectors can not determine the chemical structure of the carbohydrates; hence the use of MS (Cheng *et al.*, 2006).

Recently a method has been developed whereby a fluorescence detector has been used to detect carbohydrates at the picomole level, after labelling with 2-aminobenzamide (Wing *et al.*, 2001). Derivatisation for the ionisation of carbohydrates for MS can be done by post column addition of lithium chloride to form sugar lithium adducts for detection in the positive and negative mode. Some carbohydrate derivatives absorb UV light in the range 180 to 220 nm, though detection based on these measurements is reported to be nonselective and requires expensive instrumentation (Honda, 1984).

Carbohydrates are conveniently detected using refractive index. The drawback however is lack of sensitivity, and is affected by column temperature effects and mobile phase composition (Honda, 1984). Carbohydrate detection with RI is only amenable to isocratic elution using reverse phase partition chromatography (Abreu, and Relva, 2002). HPLC determination of long chain compounds such as inulin is problematic because the inulin molecule becomes insoluble in the mobile phase and is deposited in the HPLC column and does not elute (De Leenheer and Hoebregs, 1994).

More recently, liquid chromatography (LC) coupled with electrospray ionisation mass spectrometry (ESI-MS) has been proposed as a useful and sensitive method for analysing trace sugars in complex media (Camp *et al.*, 2004; Cheng *et al.*, 2006). Moreover, a number of spectroscopic methods are available such as Fourier transform

infrared spectroscopy (FT-IR), inductively coupled plasma atomic emission spectroscopy (ICPAE) and laser polarimeter for use in conjunction with HPLC (Cheng *et al.*, 2006). The main disadvantage of the FT-IR and the laser polarimeter detection methods are low sensitivities of 1000 ppm and 500 ppm respectively (Cheng *et al.*, 2006). Despite the attractiveness of the HPLC-ESI-MS method, there is need to use high quality standards and pure analytes. HPLC samples are pre-treated to remove salts and other matrices that can cause high chemical background noise, baseline instability and interference for certain carbohydrates (Cheng *et al.*, 2006).

The LC-MS technique identifies sugars by their mass to charge ratio (*m/z*). The typical alkali acetate and hydroxide eluents are incompatible with atmospheric pressure ionisation (API) due to their non-volatility and high conductance, and consequently, a desalting devise is installed between the LC column and the MS detector (Bruggink *et al.*, 2005). The desalting device converts the alkali hydroxide and acetate into water and acetic acids continuously exchanging the alkali cations by hydronium ions using a selective cation exchange membrane and a regenerant (Bruggink *et al.*, 2005). However, according to Bruggink and co-workers, (2005), the sensitivity of the neutral carbohydrates can be improved by adding 0.5 mM LiCl after the desalter as the LiCl forms charged complexes with carbohydrates, whereby the sugars are detected as Li-adducts $[M + Li]^+$ at $[M + 7]^+$ in the positive mode or as chloride adducts in the negative mode $[M + Cl]^-$. The positive charged complexes are detected with a higher sensitivity (Bruggink *et al.*, 2005).

1.15.2 Gas chromatography with mass spectrometry (GC-MS)

Due to their high polarity, hydrophilicity and low volatility, carbohydrates have to be converted into volatilisable and stable derivatives, i.e., trimethylsilyl (TMS) or acetate derivatives, before GC analysis with flame ionisation detection (FID) or MS detection (Guignard *et al.*, 2005; Mederois and Simoneit, 2007). The advantage of GC is that it is relatively cheap, simple yet powerful analytical technique widely found in academic and industrial laboratories (Montilla *et al.*, 2006). The main disadvantage of this analytical technique is that the compositional information of FOS (DP) may be lost due to heat damage (Montilla *et al.*, 2006). Besides, oligosaccharides are

hydrolysed before analysis and this leads to breakage of the oligosaccharide linkages and hence loss of compositional DP information (Montilla *et al.*, 2006).

The conversion of the sugars to trimethyl derivatives is a laborious procedure and needs careful manipulation during methylation of sugars to avoid incomplete methylation which could lead to incorrect results and damage the column (Prapulla *et al.*, 2000). Moreover, the GC method though particularly accurate for low sugar concentrations, is time-consuming and prone to errors (Agblevor *et al.*, 2004). However due to these reasons, HPLC is often preferred for carbohydrate identification and quantification.

Several detectors coupled with chromatographic methods have been used to quantify sugars and these consist mainly of Flame Ionization Detection (FID) and mass spectrometry (MS). Mass spectrometry has been used widely to give both qualitative and quantitative analysis due to its capability of molecular identification at a high sensitivity level (Mederois and Simoneit, 2007).

1.15.3 Thin layer chromatography

Thin layer chromatography (TLC) is one of the simplest, cheapest and rapid qualitative methods that can be used for sugar separation (Prapulla *et al.*, 2000). This method relies on the use of a polar mobile phase and a non-polar stationary phase normally made of silica on a thin glass plate or aluminium.

The main draw back of this method is that it is not quantitative and that separation of long chain length carbohydrates such as inulin is difficult hence the method does not find wide application as an analytical method for FOS and IOS.

1.15.4 NMR spectroscopy

The main field of application of NMR spectroscopy is that of elucidating the chemical structures of molecules and in this respect, the necessary information is obtained by measuring, analysing, and interpreting high resolution NMR spectra recorded on liquids of low viscosity (Prapulla *et al.*, 2000; Friebolin, 2005). NMR is one of the

most powerful techniques for structural elucidation but the major constraint is the cost of the equipment and also that the protocol for sample preparation is too elaborate (Prapulla *et al.*, 2000). Semipreparative HPLC is recommended before samples are applied to NMR analysis.

1.16 International method for fructan analysis

The Food and Drug Administration (FDA) and the United States Department of Agriculture (USDA) have defined dietary fibre as the material that precipitates in 78% ethanol by the Association of Analytical Chemists (AOAC) international method for dietary fibre analysis for nutritional labelling purposes (Prosky and Hoebregs, 1999). The classical international method was proposed by the AOAC for dietary fibre analysis. This method is not suitable for the determination of inulin and FOS because they are partially precipitated in the alcohol fraction (Korakli *et al.*, 2003).

Direct highly accurate and precise quantitative methods involving liquid chromatography have been thoroughly explored in the previous sections. However indirect quantitative methods for the analysis of inulin involve enzymatic or acid hydrolysis of inulin with subsequent measurement of the liberated reducing sugars (Prosky and Hoebregs, 1999; Korakli *et al.*, 2003).

The AOAC has reported an enzymatic method for the determination of fructans in food products. The method relies on the enzymatic treatment of the sample with an inulinase enzyme (Prosky and Hoebregs, 1999; Sangeetha *et al.*, 2005[a]). The first step involves inulin extraction by the hot water extraction method. One aliquot of the extract is kept untreated as the initial sample and a second aliquot of the extract is hydrolysed using an amyloglucosidase enzyme. A sample of the hydrolysate is kept as the second sample and the rest is hydrolysed using a fructofuranosidase (Prosky and Hoebregs, 1999; Sangeetha *et al.*, 2005[a]).

The reducing sugars released can be measured using 3, 5 -dinitrosalicylic (DNS) method, the glucose oxidase method, or by HPAEC-PAD. Inulin concentration is then calculated by subtracting the sugars in the first and second sample from the third sample. An analytical method for the determination inulin is depicted in Figure 1.10.

Sample

↓

Extraction (± 1g/L inulin and ± 1 g/L (FOS)

Dilution 1: 5

↓

Hydrolysis of inulin and FOS by the addition of β-fructofuranosidase

↓

Inulin and Actilight ®

Hydrolysis of Actilight® by the addition of invertase

↓

Reduction of monosaccharides to sugar alcohol by the addition of sodium borohydride followed by neutralisation

↓

Hydrolysis of inulin by the addition of β-fructofuranosidase

↓

Inulin

Figure 1.10 Flow diagram illustrating the analytical method for determination of inulin and Actilight (Prosky and Hoebregs, 1999; Krokali *et al.*, 2003).

1.17 Response surface methodology

Response surface methodology (RSM) is a statistical method that uses quantitative data from appropriate experimental designs to determine and simultaneously solve multivariate equations which specify the optimum product for a specified set of factors through mathematical models (Giovani, 1983; Anjum *et al.*, 1997; Bas and Boyaci, 2007a; Bas and Boyaci, 2007b; Paseephol *et al.*, 2007, Abdullah *et al.*, 2007). This process involves trial and error, is time consuming and may not eventually generate a right model because the approximating functions are polynomials (Anjum *et al.*, 1997).

RSM is an attractive and efficient optimisation strategy that can be used for product optimisation as compared to an optimisation strategy which tests one variable at a time. The one-factor-at-a-time approach is inefficient for three main reasons. First, a

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large number of experiments are required which can be expensive and time consuming for an industrial process or product development strategy. Second, the effects of interactions among the variables are not known and, the optimum response factor may not be determined by this classical approach (Bas and Boyaci, 2007a). Third, there is no equation that best describes the relationship between the variables and responses to these variables (Giovani, 1983). The equations generated are represented graphically as response surfaces which are used to describe how the test variables affect the response, to determine the interrelationships among the test variables and to describe the combined effect of all test variables on the response (Giovani, 1983). RSM involves four important steps: (1) identification of critical factors for the product or process, (2) determination of the range of factor levels, (3) selection of specific test samples by the experimental design, (4) analysis of the data by RSM and data interpretation. Generally, the RSM is basically an approximation procedure (Box and Wilson, 1951).

RSM relies on 5 assumptions whereby the factors critical to the product are known, the region of interest where the factor levels affecting the product are known, the factors vary continuously throughout the experimental range tested, there is a mathematical function which relates the factors to the measured response and finally, the response which is defined by this function is a smooth surface (Giovani, 1983).

The main limitations of the RSM approach to product optimisation is that there is large variation in the factors which could lead to misleading conclusions and this variation is a result of experimental error or bias. This variation can be decreased by controlling all sources of variation possible and to increase the number of replications. The critical factors may not be specified or adequately defined. The optimum response factor may not be determined because the range of factor levels is too narrow or too broad to specify the optimum (Giovani, 1983). Biased results can be obtained with RSM if sound statistical principles such as randomisation and blocking are not followed. Finally, in order to generate reproducible and accurate data, it is prudent to meet all requirements of the assumptions and also to control all the limitations.

1.18 Project justification

There is no satisfactory and cost effective protocol for the production of pure FOS of specific chain length. New technologies are required for synthesis of novel healthy food products for today's consumer since sucrose has problems associated with cariogenicity, corpulence, atherosclerosis and diabetes (Gill *et al.*, 2006). FOS with higher DP ($\geq$ 4) are expected to have greater prebiotic potential than the smaller oligosaccharides (Montilla, *et al*, 2006).

1.19 Hypothesis

Under controlled reactions, novel IOS, FOS and fructose can be produced from simple raw materials such as inulin and sucrose using crude and purified inulinases and fructosyltransferases.

1.20 Research objectives

The principal objective of this work was to use response surface methodology (RSM) for the optimisation of enzymatic production of IOS, FOS, and fructose under batch conditions using inulin and sucrose as substrates.

The specific objectives of this research were:

1. By using response surface methodology optimise for IOS production using a crude and purified endoinulinase for inulin hydrolysis,

2. By using response surface methodology optimise for fructose production using a crude and purified exoinulinase preparation for inulin hydrolysis,

3. By using response surface methodology optimise for the synthesis of FOS from sucrose *in-vitro* using a crude and purified fructosyltransferase from *Aspergillus* sp.,

4. To isolate, purify and characterise inulinases i.e. to separate exoinulinase and endoinulinase in Fructozyme and Novozyme preparations respectively.

1.21 Research approach and organisation

This research work is divided into 6 chapters and each chapter is subdivided into 4 sections, Introduction, Materials and Methods, Results, Discussion, and Conclusions. Chapter 1 gave a detailed background of this research and literature review. Chapter 2 investigates the production of IOS using a crude endoinulinase preparation using RSM, Chapter 3 explores the purification and characterisation of an endoinulinase and production of IOS using a purified endoinulinase using RSM, Chapter 4 studies the synthesis of FOS using a crude and purified fructosyltransferase using RSM, Chapter 5 investigates the production of fructose using a crude and purified exoinulinase preparation using RSM, and Chapter 6 is the general discussion and conclusions.

Chapter   2

Production of Inulooligosaccharides by a Crude Endoinulinase from _Aspergillus niger_ optimised using Response Surface Methodology

2.1 Introduction

Inulin hydrolysis occurs as a result of the action of fructofuranosyl hydrolases, namely exoinulinases (EC. 3.2.1.80) and endoinulinases (EC. 3.2.1.7) acting solely or synergistically (Yun *et al.*, 2000; Jing *et al.*, 2002[b]; Zhang *et al.*, 2004; Zhengyu *et al.*, 2005; Rocha *et al.*, 2006; Catana *et al.*, 2007). Exoinulinases cleave the non-reducing β-(2, 1) end of the inulin molecule releasing fructose while endoinulinases act randomly on the internal linkages to release inulotrioses, inulotetraoses and inulopentaoses as major products (Ohta *et al.*, 2002; Zhengyu *et al.*, 2005; Santos *et al.*, 2007).

The products resulting from total and partial inulin hydrolysis have commercial value (Catana *et al.*, 2007). Inulooligosaccharides (IOS) are reported to act as prebiotics and research is ongoing to establish and validate this claim (Roberfroid *et al.*, 1998; Kaplan and Hutkins, 2003). The principal objective of inulin hydrolysis is to obtain high levels oligosaccharides with a degree of polymerization (DP) of between 4 and 8 since it has been demonstrated that these have greater prebiotic potential (Montilla *et al.*, 2006).

Optimal conditions for the production of inulooligosaccharides by endoinulinases can be established using response surface methodology (RSM) as compared to the traditional one-factor-at-a-time approach which is tedious and time consuming (Anjum *et al.*, 1997). The method was developed by Box and Wilson (1951) and the main idea behind this method is to use a set of designed experiments to obtain an optimal response. Response surface methodology is a modeling procedure whereby important multivariate operational parameters such as temperature, pH, enzyme dosage and reaction time are simultaneously varied to achieve optimal conditions giving a high product yield.

The modeling procedure involves the use of experimental designs such as the central composite design (CCD) to establish suitable combinations of the parameters. CCD is one of the most useful approaches in determining optimum conditions of many processes (Abdullah *et al.*, 2007). In addition, it is normal practice to perform preliminary experiments where important factors for the experimental procedure can

be chosen (Zhengyu *et al.*, 2005). Work from previous studies using other microbial enzyme systems have demonstrated that temperature is a crucial factor if an optimisation strategy is to succeed (Diaz *et al.*, 2006).

Batchwise production of inulooligosaccharides is an attractive procedure since process control is easy. Product yield may be affected by end product inhibition of the enzyme. In addition, inhibition by other accumulating by products in the reaction mixture may occur. The major advantage of this production method is that industrial scale up is easy and that the production parameters can be efficiently controlled for optimal yield of products. Optimal conditions for the generation of pure and high quality inulooligosaccharides from cheap raw-materials such as inulin still remain a challenge (Yun *et al.*, 2000). Therefore it is important to investigate the optimal parameters for the production of these biofunctional products using enzymes from microorganisms (Diaz *et al.*, 2006).

The main objective of the work in this chapter was to develop a set of optimum conditions for controlled partial inulin hydrolysis with the aim of producing maximal yield of IOS using a commercial crude endoinulinase preparation from *Aspergillus niger*. The specific objective were:

1   to produce inulooligosaccharides from pure chicory inulin using a commercial crude endoinulinase preparation (Novozyme® 960)

2   to optimise reaction parameters for maximal yield of inulooligosaccharide using response surface methodology (RSM) in which multiple factors such as temperature, incubation time, enzyme dosage and inulin concentration were varied simultaneously with the CCD experimental design.

2.2 Materials and Methods

2.2.1 Materials

Pure non-hydrolysed commercial chicory inulin from chicory tubers (*Cichorium intybus*, L) was used as a model substrate for inulooligosaccharide production, 3, 5-dinitrosalicylic acid reagent and anisaldehyde spray reagent, 50 µl Hamilton syringe

for injecting samples into the HPLC system, were obtained from Sigma-Aldrich, (St Louis, USA). Authentic pure FOS standards, 1-kestose (GF_2), 1-nystose (GF_3) and 1-fructofuranosyl nystose (GF_4) were purchased from Wako Pure Chemical Industries, Osaka, Japan. Novozyme® 960 (Batch KNN00107) a liquid commercial enzymatic preparation isolated from *Aspergillus niger* was purchased from Novozymes, South Africa. Acetonitrile (HPLC grade) (BDH), lithium chloride, 20 cm x 20 cm silica coated aluminium TLC plates, soda glass capillary tubes (75 mm) for spotting samples onto the TLC plate, Techne Dri-Block DB-2A heating block and Mini Spin Eppendorf microcentrifuge, safe lock Eppendorf tubes, and the Aquamate Thermo Spectronic spectrophotometer were purchased from Merck, South Africa.

An analytical Prevail Carbohydrate ES column (250 x 4.6 mm) and a guard column (7.5 x 4.6 mm) packed with 5 μm spherical polymer beads coated with proprietary material were purchased from Alltech Associates Inc, Deerfields, IL, USA. All HPLC analysis was done using an HPLC system with a Beckman System Organiser 20 μl injector loop and a Beckman 110B Solvent Delivery Mobule pump coupled to a Knauer RI detector. A Millivac Maxi Diaphragm vacuum pump was used for filtration and degassing of the mobile phase for HPLC. Nylon membranes (0.45 μm) were purchased from Millipore, South Africa.

The Snake-Skin™ Pleated dialysis tubing with a MWCO of 10 kDa was purchased from Pierce Chemical Company, Rockford, USA. A Finigan LCQ mass spectrometer system equipped with an ElectroSpray Ionisation source at atmospheric pressure was obtained from Finnigan MAT, Oaks Parkway, USA. All other chemicals and reagents were acquired from reputable commercial sources and were of analytical grade unless otherwise stated.

2.2.2 Determination of fructose concentration

Fructose concentration was determined using the 3, 5-dinitrosalicylic acid (DNS) method (Miller, 1959). The DNS reagent was prepared as described in Appendix A2. Standard fructose solutions of 0 to 2 mg/ml were analysed simultaneously with enzyme reaction product samples using the DNS assay as described in Appendix A3.

2.2.3 Analytical Procedures

2.2.3.1 High performance liquid chromatography

The HPLC system was optimized for the accurate, reliable and reproducible separation of oligosaccharides by varying the mobile phase composition, flow rate and pump pressure. Inulooligosaccharides produced were identified and quantified using high performance liquid chromatography (HPLC) coupled to a refractive index (RI) detector. The optimal mobile phase was prepared using 73 % (v/v) HPLC grade acetonitrile in Milli-Q distilled water. The mobile phase was filtered using a 0.45 μm Millipore nylon membrane using a Millipore filtering unit and then degassed for 15 minutes using a Millipore vacuum pump. After HPLC, 32 Karat peak integration software was used for data acquisition and analysis. In order to obtain a stable base line the HPLC system was pre-equilibrated at a flow rate of 1 ml/min at a constant pressure of 1 000 psi at an ambient temperature using acetonitrile : distilled water (73 : 27) (v/v) as the mobile phase. After each HPLC run, the system was equilibrated for 10 to 15 minutes with 73 % acetonitrile in distilled water (v/v) to prevent carry over of adsorbed analytes on the column. Peak areas were converted to quantities with appropriate standards using a calibration curve of authentic standards (Appendix A4). The HPLC method used was validated for reliability, accuracy and precision by comparing the retention times of the known external standards to those of the IOS sample products after each HPLC run.

2.2.3.2 Thin layer chromatography

Qualitative determination of inulooligosaccharides produced in the reaction mixture was performed by using thin layer chromatography (TLC). An aliquot (2 - 4 μl) of the reaction mixture was spotted on the TLC plate and then developed with a solvent system of butanol / acetic acid / water (5 : 4 : 1) (v / v / v) in a TLC developing tank. In order to get good separation of the reaction products, ascending development was repeated twice at room temperature. The plate was allowed to dry in a fume hood and then developed by spraying with anisaldehyde spray reagent using an Aldrich® flask-type sprayer. The plate was then dried in an oven at 110 °C for 15 minutes to visualise the reaction spots.

2.2.3.3 Mass Spectrometry

Electrospray ionisation (ESI) with a quadrupole ion trap mass analyser was used to ascertain the degree of polymerisation of inuloogosaccharides produced in the reaction mixture. The IOS samples were prepared by ionising the sugars with 1 mM LiCl to form IOS lithium adducts. The IOS samples were directly infused into the ion source using the syringe pump and mass spectra were recorded. The response was monitored in the positive ion mode $[M + Li]^+$ (in the range 100 to 1 000 m/z) which gave enhanced response factor as compared to the negative ion mode $[M + Cl]^-$ where the IOS ions were poorly observable. The IOS were identified using the mass to charge ratios of available authentic IOS standards. Bruker Daltonics data analysis 3.2 software was used for data acquisition and analysis.

2.2.4 Preparation of inulin and crude endoinulinase

The substrate inulin (5 % w/v), suspended in citrate-phosphate buffer (0.1 M, pH 6.0) was prepared by heating the solution at 80 °C for 10 minutes to facilitate dissolution in an aqueous solution. The commercial crude endoinulinase preparation (20 ml) was dialysed overnight against citrate-phosphate buffer (0.1 M, pH 6.0) with gentle shaking at 4 °C to remove traces of salts and reducing sugars.

2.2.5 Determination of endoinulinase activity

Endoinulinase activity was measured by the modified procedure of Yun *et al.*, (2000) by determining the increase in reducing power liberated from random hydrolysis of inulin chains (5 %) as the substrate. The substrate solution was allowed to equilibrate at 60 °C for 3 minutes before adding the enzyme solution. The enzyme assays were carried out in triplicate and the mean was reported as the enzyme activity. The assay mixture for endoinulinase contained the enzyme aliquot (300 µl) and 5 % (w/v) inulin (1 200 µl) dissolved in 0.1 M citrate - phosphate buffer (pH 6.0) in a safe lock Eppendorf tube.

The control tube was prepared by replacing the enzyme solution with the buffer solution and incubating under the same conditions as the experimental tubes. The

reaction mixture was incubated at 60 °C in a heating block under static conditions for 1 h after which the reaction in all tubes was stopped by boiling for 10 minutes. The reaction products were centrifuged (4 000 x *g*, 10 min, 4 °C) and then syringe filtered using a 0.45 μm Millipore nylon membrane.

Reducing power was estimated by the DNS method described in section 2.2.2. The reducing sugars already present in the enzyme samples were estimated and an appropriate correction made while calculating the actual amount of sugar liberated due to endoinulinase activity. The reducing sugars liberated were estimated from the calibration curve of an authentic fructose standard (Appendix A3). Reducing sugar contribution from thermal hydrolysis of inulin was ruled out as no reducing sugars were observed after incubating the inulin mixture at 60 °C for 1 h. One endoinulinase unit was defined as the amount of enzyme catalysing the liberation of 1 μmole fructose per minute under the experimental conditions.

2.2.6 Preliminary inulin hydrolysis using crude endoinulinase

Approximate conditions for inulooligosaccharide (IOS) production by the hydrolysis of inulin by a crude endoinulinase were chosen from a preliminary RSM with CCD experimental runs. The critical parameters chosen for IOS production in the first RSM were pH (5.6 to 6.4), temperature (40 to 60 °C), and enzyme concentration (20 to 100 U/ml (Table 2.1). The experimental run treatments are presented in Table 2.3.

Table 2.1 Generation of coded levels using central composite design for preliminary IOS production using Response Surface Methodology by a commercial crude endoinulinase preparation.

Independent Variable	Symbol	Coded Levels		
		-1	0	+1
pH	X_1	5.6	6.0	6.4
Temperature (°C)	X_2	40	50	60
[Enzyme] (U/ml)	X_3	20	60	100

Incubation time was 8 h and initial inulin concentration was 50 g/L in all experimental runs. An aliquot (1 ml) of the crude enzyme of appropriate concentration was added to pure inulin substrate (4 ml). The reaction mixture in all the experimental runs was incubated at the appropriate temperature for 8 h. The control tube containing the boiled enzyme with the inulin substrate was incubated under same conditions with experimental tubes. Reaction products were boiled for 10 min to stop the enzyme activity and IOS concentration was determined by the HPLC with a RI detector as previously described in section 2.2.3.1. Calibration curves were generated from pure fructooligosaccharide (FOS) standard (Appendix A4) and these were used to extrapolate the concentration of IOS produced in the experimental runs.

2.2.7 Experimental design for IOS production by crude endoinulinase

Inulooligosaccharide production was optimised by RSM with CCD after the preliminary experimental runs to establish the important multivariate factors for inulin hydrolysis. In the second RSM experimental runs, the most important independent variables were simultaneously varied, incubation time (24 to 72 h) X_1, crude enzyme dosage (20 to 100 U/ml) X_2, and inulin concentration (50 to 150 g/L) X_3 as shown in Table 2.2.

The coded levels in Table 2.2 were calculated using equation 2.1:

$$x_i = (X_i-X_0)/\Delta x_i \qquad\qquad\qquad\qquad 2.1$$

Where x_i is the coded value of an independent variable, X_i is the real value of an independent variable, X_0 is the real value of an independent variable at the centre point and Δx_i is the step change value. In the second RSM experiment, 15 experimental runs (Table 2.3) were carried out with runs 1 to 8 being the classic factorial design and runs 9 to 14 being the axial points and run 15 being the experimental run at the centre point. The results generated were fitted into a response surface regression analysis using statistics software, Statistica 7, (Statsoft, Inc.).

Table 2.2 Generation of coded levels using central composite design for IOS production using Response Surface Methodology by a commercial crude endoinulinase preparation.

Independent Variable	Symbol	Coded Levels		
		-1	0	+1
Time (h)	X_1	24	48	72
[Endoinulinase] (U/ml)	X_2	20	60	100
[Inulin] (g/L)	X_3	50	100	150

2.2.8 Optimisation of IOS production by the crude endoinulinase

The reaction mixtures were subjected to RSM with CCD to find out optimal conditions for the production of inulooligosaccharides. In all the 15 experimental runs, fixed initial parameters were pH, 6.0 and temperature, 60 °C. All the experimental runs were carried out in a heating block under static conditions. For each experimental run, an aliquot of inulin solution of appropriate concentration, (50 to 150 g/L, 1 200 μl) suspended in 0.1 M citrate-phosphate buffer (pH 6.0) was allowed to equilibrate at 60 °C before adding an appropriate concentration of dialysed crude endoinulinase (20 to 100 U/ml, 300 μl). After the necessary incubation time (24 to 72 h), the reactions were stopped by boiling the tubes for 10 minutes. Any effects of thermal and acid hydrolysis were ruled out after reducing sugars and IOS intermediates were not detected after incubating the inulin substrate at 60 °C, at pH 6.0 for the duration of the experiment

The samples were briefly centrifuged, syringe filtered (0.45 μm nylon membranes) and a 20 μl aliquot was injected into the HPLC system and the relative concentrations of the IOS produced were calculated from the calibration curve using authentic FOS standards DP 3 to DP 5 (Appendix A4). In this study, only fructose linked IOS i.e. inulotriose (F_3), inulotetraose (F_4) and inulopentaose (F_5) concentrations were estimated and all F_2 and other IOS with a terminal glucose moiety were not considered since the synthesis of FOS with a terminal glucose moiety was evaluated in a separate study.

Table 2.3 Coded combinations of independent variables at different levels using RSM with CCD. Experimental runs 1 to 8 are classic factorial design, runs 9 to 14 are the axial points and 15 is the experimental run at the centre point. The coded levels were generated as described in equation 2.1.

Experimental Run	Coded Levels		
	X_1	X_2	X_3
1	-1	-1	-1
2	+1	-1	-1
3	-1	+1	-1
4	+1	+1	-1
5	-1	-1	+1
6	+1	-1	+1
7	-1	+1	+1
8	+1	+1	+1
9	-1	0	0
10	+1	0	0
11	0	-1	0
12	0	+1	0
13	0	0	-1
14	0	0	+1
15	0	0	0

In order to promote the formation of short chain inulooligosaccharides such as those with $DP \geq 3$, a third set of experimental runs was needed where the incubation time was changed. Long incubation time (24 to 72 h) was observed to prolong inulin hydrolysis hence low yield of IOS intermediates so the incubation time was changed to 1 to 3 h. The third set of experimental runs was achieved by simultaneously varying reaction time (1 to 3 h), temperature (40 to 60 °C), and inulin substrate dosage (50 to 150 g/L). The coded levels were calculated as before (equation 1) and the independent variables were incubation time, X_1 (1 - 3 h), temperature, X_2 (40 - 60 °C) and inulin concentration, X_3 (50 to 150 g/L) (Table 2.4). In this set of experimental runs, initial pH and enzyme dosage were kept constant at 6.0 and 60 U/ml respectively.

In order to fully describe the response surface around the optimum region, a CCD was used with 3 coded levels as in Tables 2.2 and 2.3. The quadratic model for predicting the optimal yield was calculated according to equation 2.2; provided that a measurable response is obtained.

$$y = b_o + \sum b_r x_i + \sum b_{ir} x_i^2 + \sum b_{ij} x_i x_j \qquad 2.2$$

Where y is the response variable, b_o is the constant, b_r is the coefficient for the linear effect, b_{ir} is the coefficient for the quadratic effect, b_{ij} is the coefficient for the interaction effect and x is the coded level of the respective independent variable. The data generated was statistically analysed using response surface regression using Statistica 7.

Table 2.4 Response Surface Methodology and CCD for inulooligosaccharide production using 60 U/ml dialysed crude endoinulinase preparation. The coded levels were generated using equation 2. 1.

Independent Variable	Symbol	Coded Levels		
		-1	0	+1
Time (h)	X_1	1	2	3
Temperature (°C)	X_2	40	50	60
[Inulin] (g/L)	X_3	50	100	150

2.2.9 Statistical and Data Analyses

Empirical data generated throughout this study was statistically analysed using Statistica 7 software for first, second order, and response surface regression models to predict the yield as the response factor. All RSM experimental runs in all chapters were performed in duplicate and the average product yield was taken as the response. Mean and standard deviation calculations and comparison of data sets using analysis of variance (ANOVA) was performed with Microsoft Excel 2003 statistical tools. The ANOVA was performed to 5 % level of significance.

2.3 Results

2.3.1 Endoinulinase Activity

Endoinulinase activity in the dialysed crude enzyme preparation was found to be 929 U/ml using the DNS assay for reducing sugars.

2.3.2 Preliminary inulin hydrolysis using crude endoinulinase

In order to choose important parameters that are crucial for inulooligosaccharide production from inulin hydrolysis by a crude endoinulinase, 3 factors were chosen, pH (5.6 to 6.4), temperature (40 to 60 °C), and enzyme dosage (20 to 100 g/L). The highest inulotriose (F_3), inulotetraose (F_4) and inulopentaose (F_5) concentrations achieved were 32.51 mM, 17.39 mM and 12.42 mM respectively after 8 h incubation at pH 6.0, temperature 60 °C and 100 g/L inulin concentration in experimental run 12 (Figure 2.1). Response surface regression predicted F_3 (30.27 mM, R^2 of 99.23), F_4 (18.45, R^2 of 97.45) and F_5 (10.23 mM, R^2 of 97.45) under similar experimental conditions. Using the response surface regression model, the predicted IOS concentrations were statistically significant ($p < 0.05$).
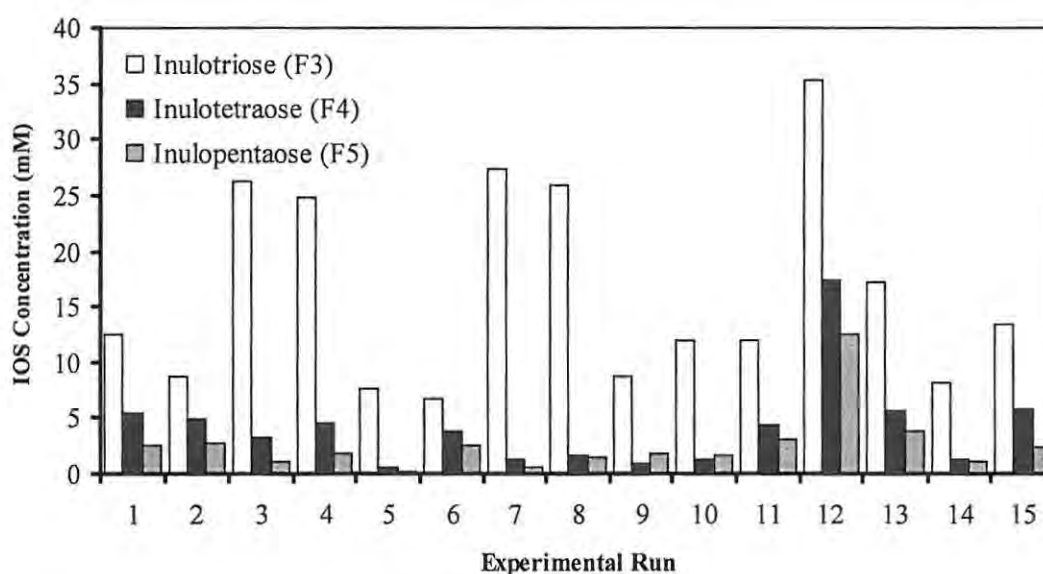
Figure 2.1 Production of inulooligosaccharides from preliminary RSM experimental runs to find important parameters for inulin hydrolysis using RSM for 8 h and crude exoinulinase (100 U/ml). The experimental conditions are shown in Table 2.1 and Table 2.3.

2.3.3 Optimisation of IOS production by the crude endoinulinase

By varying incubation time (24 to 72 h), enzyme dosage (20 to 100 U/ml) and inulin concentration (50 to 150 g/L), inulotrioses (F_3), inulotetraoses (F_4) were the dominant products with trace amounts of inulopentaoses (F_5) also produced. After 48 h of incubation with 60 U/ml enzyme dosage and 150 g/L inulin concentration, the highest levels of F_3 (70.3 mM), F_4 (38.8 mM) and F_5, (3.5 mM) were produced (Figure 2.2). After analysis of all the reaction products, no IOS were detected in experimental runs 4 at (72 h, 100 U/ml, 50 g/L) and 13 at (48 h, 60 U/ml, 50 g/L) incubation conditions. Under experimental run 3 at (24 h, 100 U/ml, 50 g/L), 8 at (72 h, 100 U/ml, 150 g/L), 12 at (48 h, 100 U/ml, 100 g/L), and 15 at (48 h, 60 U/ml, 100 g/L) experimental conditions, 2.63 mM, 12.3 mM, 1.6 mM and 2.22 mM of inulotrioses were produced respectively (Figure 2.2). The IOS levels produced were subjected to response surface regression to adequately describe the interactive effects of the 3 independent variables tested for IOS production. A summary of the actual and predicted maximum concentrations of the IOS from the second RSM experimental runs is shown in Table 2.5.

Table 2.5 Maximum actual and predicted IOS concentrations produced in the second RSM runs.

IOS	Actual maximum concentration (mM)	Predicted maximum concentration (mM)	R^2	p
F_3	70.3	62.3	0.75	0.012
F_4	38.8	38.9	0.84	0.011
F_5	6.9	6.9	0.92	0.016
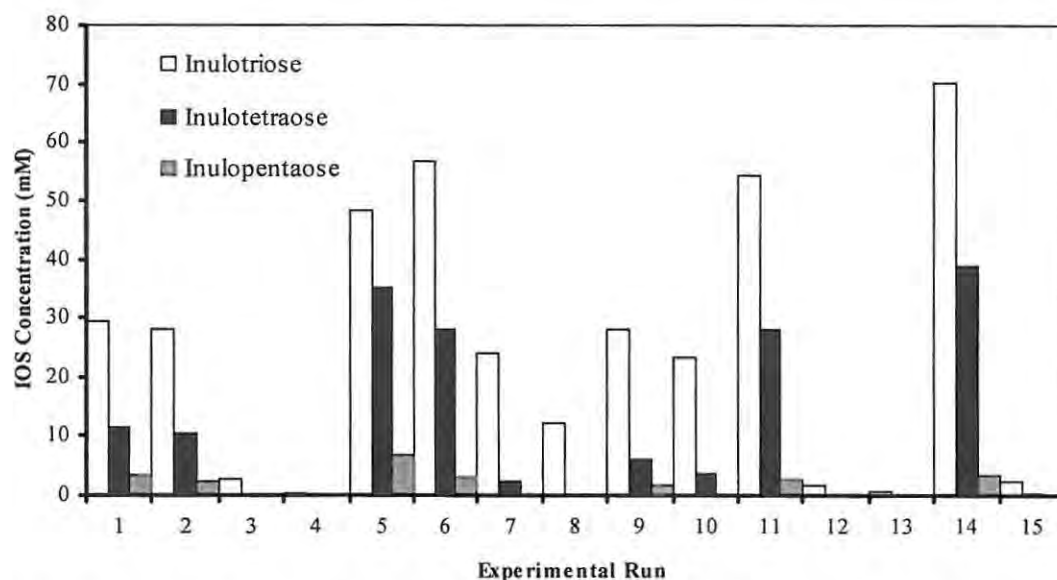
Figure 2.2 Actual IOS concentrations produced by the second RSM experimental runs with crude dialysed endoinulinase preparation with 3 coded levels and 3 independent variables with temperature and pH fixed at 60 °C and 6.0 respectively.

In the third set of RSM experiments, incubation time was varied from 1 to 3 h and temperature was simultaneously varied from 40 to 60 °C and inulin concentration was varied from 50 to 150 g/L as in the first experimental runs and enzyme dosage and initial pH were kept at 60 U/ml and 6.0 respectively. The data obtained show slightly lower levels of IOS as compared to the first set of RSM experimental runs (Figure 2.3). The maximum IOS were produced after 3 h incubation at 60 °C with 150 g/L inulin concentration and response surface regression model was used to predicted IOS concentrations under similar conditions (Table 2.6).

Table 2.6 Maximum actual and predicted IOS concentrations after the third RSM.

IOS	Actual maximum concentration (mM)	Predicted maximum concentration (mM)	R^2	p
F_3	50.9	42.1	0.85	0.0156
F_4	8.85	8.35	0.75	0.0189
F_5	6.6	12.3	0.86	0.7163
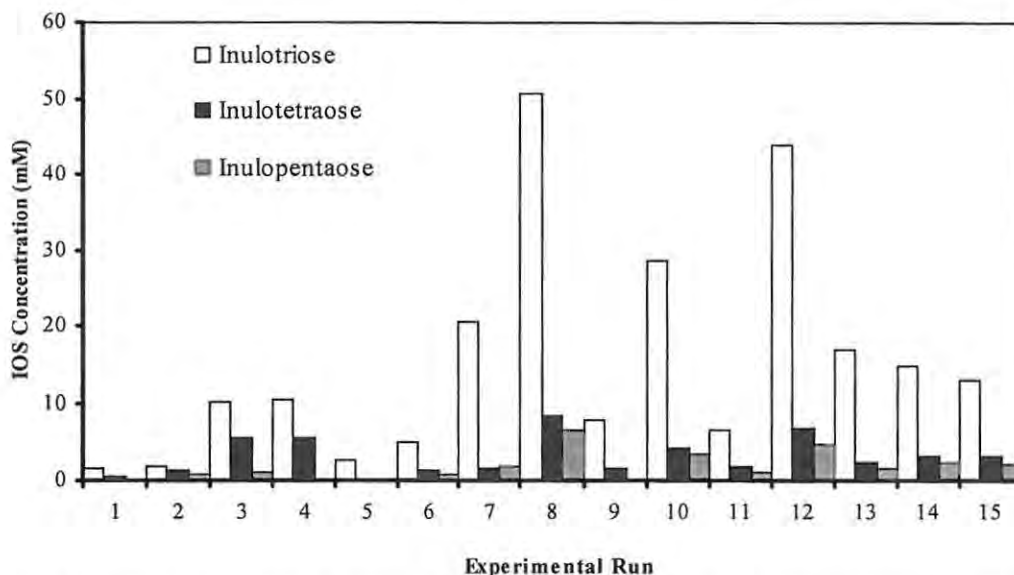
Figure 2.3 Actual IOS concentrations produced by the third RSM with crude dialysed endoinulinase with 3 coded levels and 3 independent variables with enzyme dosage at 60 U/ml and pH kept at 6.0.

The reaction products were analysed by HPLC and the concentrations were calculated from peak areas using suitable FOS standards. The HPLC trace of run 8 of the second RSM showing high levels of inulotrioses is shown (Figure 2.4).
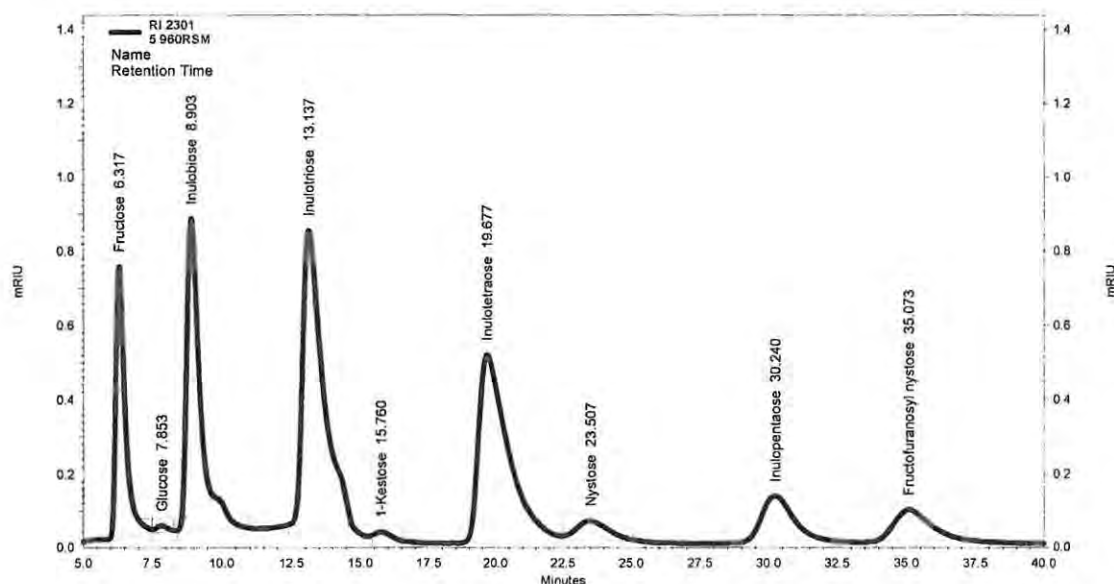
Figure 2.4 Chromatogram of IOS reaction products under optimal conditions from the third RSM (run 8) after 3 h incubation period at 60 °C with 150 g/L inulin concentration, with 60 U/ml crude endoinulinase and pH kept at 6.0. The sample was analysed by HPLC with RI detection at room temperature and eluted with 73 % acetonitrile in water at a flow rate of 1 ml/min. Peak integration was done with 32 Karat Software.

The reaction products of all runs of the third RSM were subjected to TLC to identify the products (Figure 2.5). Lanes 1 to 15 have different spot intensities corresponding

to their different sugar concentration (Figure 2.5). No inulooligosaccharides of DP6 and above were detected in all the experimental run samples. It was noticed that the migration of the higher molecular weight IOS was zero.

The reaction products of run 8 of the third RSM were also subjected to mass spectrometry with electrospray ionisation in order to confirm the findings of other analytical methods for time course monitoring of the reaction products. At the start of the reaction only free ionised inulobiose (F_2) $[M + Li]^+$ (m/z, 349) was the dominant product detected in the reaction mixture (Figure 2.6). After 3 h of reaction, other intermediates appeared in the reaction mixture such as ionised F_3 $[M + Li]^+$ being the dominant product (m/z, 511), F_4 (m/z, 673), F_5 (m/z, 835), with a small trace corresponding to F_6 (m/z, 997) were detected (Figure 2.7). The main drawback of the MS-ESI method is that it cannot distinguish IOS with or without a terminal glucose moiety. The presence of DP6 IOS shows that the MS-ESI is more sensitive in detecting IOS than the HPLC and TLC methods.
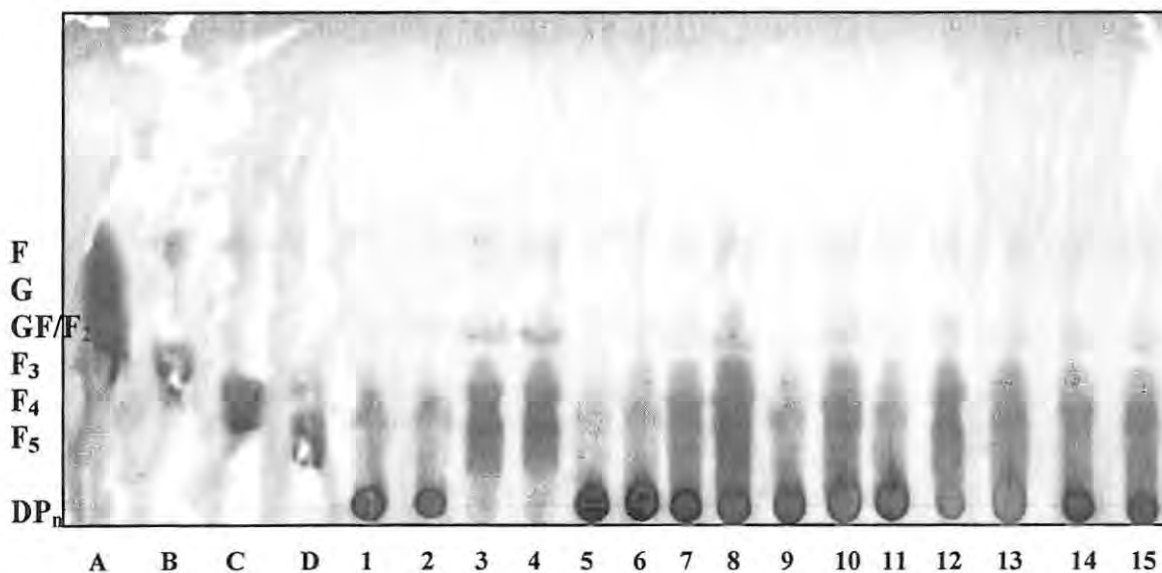
Figure 2.5 Thin layer chromatogram of inulin hydrolysis products .with 60 U/ml dialysed crude endoinulinase using RSM. Lane A: standard mix (5 % F, G, GF); lane B: DP3 standard; lane C DP4 standard; lane D DP5 standard. Lanes 1 to 15 correspond to hydrolysis products of experimental runs 1 to 15 respectively of the third RSM. The end products of inulin hydrolysis were analysed by using TLC plate (Silica gel 60, Merck, Germany). The TLC plate was irrigated with butanol: acetic acid: water (5:4:1) (v/v/v) and then visualised by spraying with anisaldehyde spray reagent before heating at 110 °C for 15 minutes in an oven.
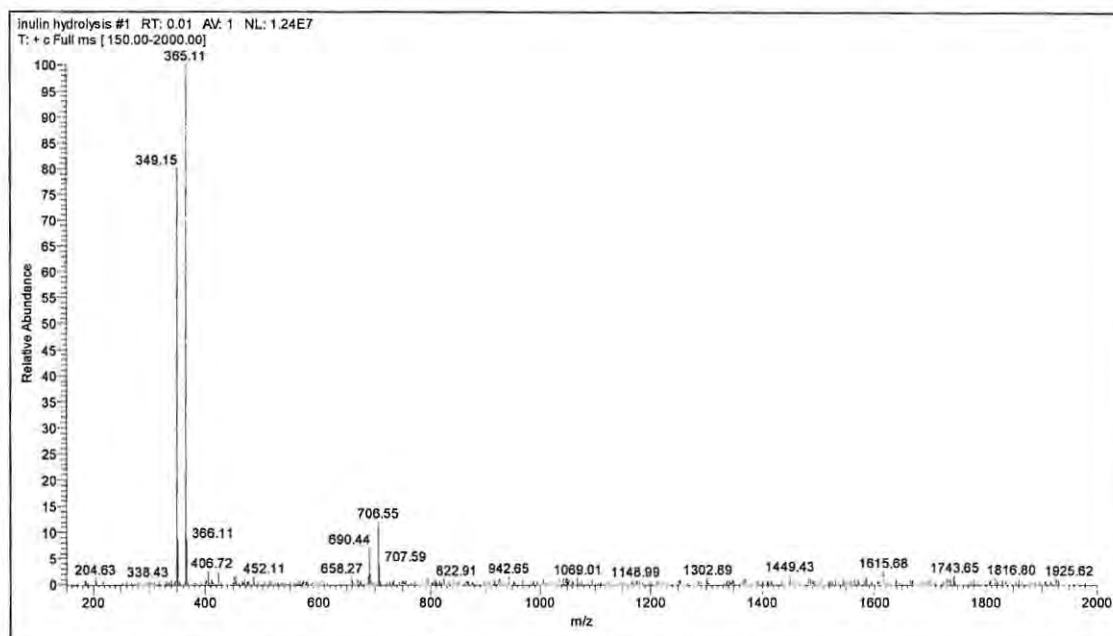
Figure 2.6 MS-ESI of the reaction mixture at the start of the reaction in experimental run 8 of the third RSM. The reaction mixture consisted of 150 g/L inulin, 60 U/ml dialysed crude endoinulinase, and the reaction was carried out at 60 °C. An aliquot was withdrawn from the reaction mixture and boiled for 10 minutes to stop the reaction. The reaction products were analysed with MS-ESI in the positive mode after ionising the sugar sample with 1 mM LiCl. The m/z ratio of 349.15 corresponds to inulobiose $[M + Li]^+$.
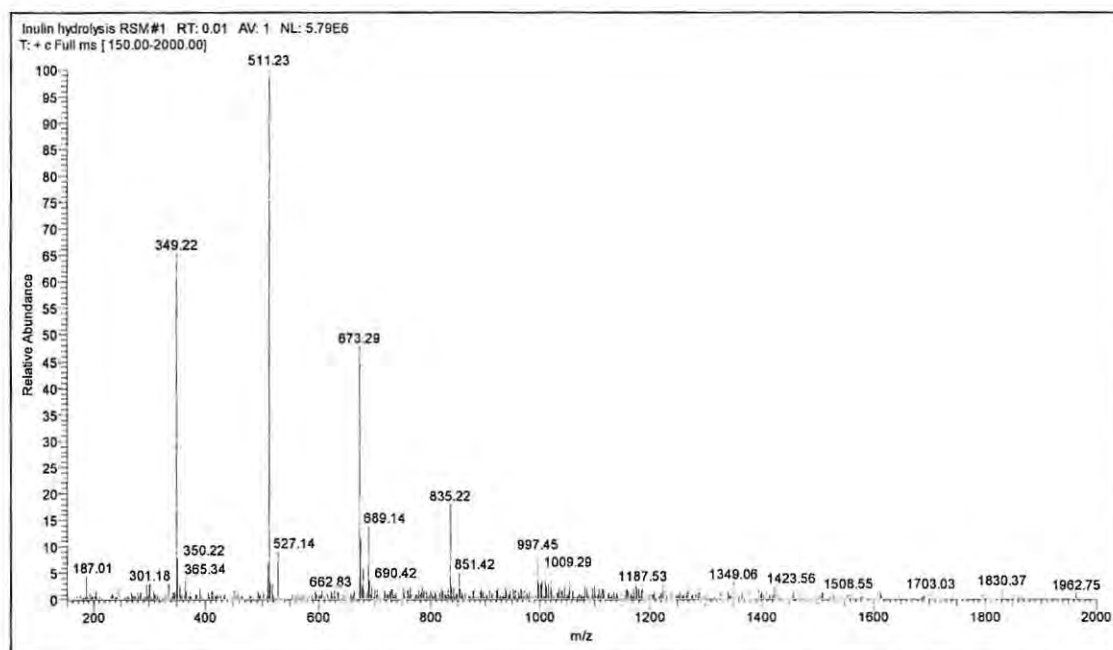
Figure 2.7 Analysis of inulin hydrolysis products using MS-ESI. Inulin was hydrolysed using 60 U/ml crude dialysed Novozym 960 at 60 °C with 150 g/L inulin and the reaction was stopped after 3 h incubation of experimental run 8 of the third RSM. The reaction products were analysed with MS-ESI in the positive mode after ionising the sugar sample with 1 mM LiCl. The m/z ratio of 187.01, 349.22, 511.23, 673.29, and 835.22 correspond to monosaccharides, disaccharides, DP 3, DP 4, and DP5 respectively $[M + Li]^+$.

2.4 Discussion

Endoinulinase (2, 1-β-D-fructan fructanohydrolase; EC 3.2.1.7) is specific for the random hydrolysis of internal β-(2-1) fructofuranosidic bonds of inulin into shorter chain intermediates. Maximum amounts of inulotriose (70.3 mM), inulotetraose (38.8 mM), and inulopentaose (3.5 mM) were obtained with optimum conditions of 60 U/ml enzyme concentration, 48 h incubation period and 150 g/L inulin concentration. This indicates that high substrate concentration is not saturating to this crude endoinulinase preparation which is an attractive property for industrial application of this enzyme. This was also observed by Zhengyu and co-workers (2005) when they used a fungal endoinulinase preparation for IOS production.

The actual concentrations of IOS produced from RSM experiments in this study can possibly indicate the synergism that exists for inulin hydrolysis. The crude endoinulinase preparation has some traces of exoinulinase in the enzyme cocktail. There was a preponderance of short chain intermediates such as inulotrioses, inulotetraoses and inulopentaoses when the reaction was prolonged to 48 h. By increasing the reaction time to 72 h, no inulotrioses, inulotetraoses and inulopentaoses were detected in the reaction mixture in experimental runs 4, 8, 12 and 13 (Figure 2.2) indicating that inulin was completely degraded to monosaccharides and disaccharides. Incubation time was critical for the production of IOS using RSM with CCD. The high temperature (60 $^{\circ}$C) used could have led to a reduction in enzyme activity with extended incubation time at this temperature, hence the need to shorten the incubation time. The data generated suggest that high inulin concentrations contributed to diffusional problems in the reaction mixture under batch conditions and also that high inulin concentration (>10 %) has a tendency of precipitating at lower temperatures (Yun and Kim, 1999).

Furthermore, Yun and Wim (1999) in their work reported that using a continuous system of immobilised endoinulinase from *Pseudomonas* sp., obtained a maximum yield of IOS with 50 g/L inulin after 25 h of reaction. The high concentrations of short chain intermediates released from inulin clearly suggest a probable reaction mechanism of this endoinulinase under optimal conditions. It is postulated that high yield of F_3 might indicate that early products of GF_4 or F_5 are hydrolysed to F_3 and F,

GF_4 is hydrolysed to GF and F_3 and F_5 in cleaved to F_3 and F_2. It is possible that this enzyme can only cleave a maximal size of DP5 from the inulin molecule hence the absence of IOS of DP $\geq$ 6. Moreover work done by other workers (Cho *et al.*, 2001) demonstrated that IOS production of DP 3 to 5 was maximal at 50 g/L. They observed substrate inhibition causing a lower product yield where high inulin concentration is used.

In this study, pH was maintained at pH 6.0 in all the experiments because it was found to be the optimal pH from the preliminary experimental runs. The active centre of inulinases is reported to contain imidazole and sulfhydryl groups and the hydrolysis of inulin is catalysed by the imidazole group by a nucleophile attack on carbon in the 2-position, yielding a tetrahedral intermediate complex (Zherebtsov *et al.*, 2003; Diaz *et al.*, 2006). The sulfhydryl group may act as hydrogen acceptor or donor so the reaction medium pH plays a key role in the enzyme activity, as is often found in biocatalysis (Zherebtsov *et al.*, 2003). Inulinase from *Aspergillus niger* is stable within a pH range of 3.5 – 8.5 and the lower pH optima are advantageous for industrial fructose syrup preparations as it prevents undesired colour formation (Gill *et al.*, 2006; Diaz *et al.*, 2006).

The analytical techniques used to quantify the IOS were adequate but it should be noted that peak areas do not supply full information about the concentrations of the oligosaccharides, since the response factors of the RI detector vary significantly with the various oligomers, with lowest responses occurring with oligosaccharides with a high DP. Regression analysis was performed to fit the response function with the experimental data. The fit value, termed R^2, of the response surface regression model was calculated to be 0.84 for F_4 and 0.92 for F_5, indicating that 84 % and 92 % of the variability in the response (F_4 and F_5 yield) could be adequately explained by the response surface regression prediction equation. Response surface regression results showed that this model is appropriate for predicting IOS concentrations after inulin hydrolysis.

2.5 Conclusions

Using RSM, it was possible to establish the optimal conditions to obtain high IOS concentrations in the reaction mixture using the crude endoinulinase preparation. The following conclusions can be drawn from this study:

1   The optimised parameters for inulin hydrolysis were pH, incubation time, temperature, inulin concentration and enzyme dosage. By applying the RSM system, the maximum IOS productivity was achieved.

2   Major products of inulin hydrolysis were F_3 and F_4 and small traces of F_5 with F_3 IOS dominant indicating the possible mechanism of action of endoinulinase.

3   Response Surface Methodology with central composite design was adequate as a modelling tool for predicting IOS production under various conditions.

4   There is a need to purify the endoinulinase to establish the levels of IOS produced using RSM after removing other contaminating enzymes.

The next chapter focuses on the purification, characterisation of the endoinulinase, and its application for IOS using RSM.

Chapter 3

Purification and Characterisation of Endoinulinase and Production of Inulooligosaccharides with a Purified Endoinulinase Optimised Using Response Surface Methodology

3.1 Introduction

Inulinolytic enzymes are used for inulin hydrolysis to produce short chain inulooligosaccharides (IOS) and fructose at elevated temperatures around 60 °C (Sharma and Gill, 2007). At these high temperatures, most microbial inulinases gradually lose their activity and therefore require further replenishment, which adds to the cost of production (Vandamme and Derycke, 1983; Sharma and Gill, 2007). There is a strong demand to isolate, purify and characterise these inulinases due to their attractive and desirable biotechnological applications. Most microbial inulinases are amenable to immobilisation on suitable supports which allows for continuous production of IOS (Sharma and Gill, 2007).

Inulooligosaccharides can be produced using crude endoinulinase preparations where there is synergism between the endoinulinases and exoinulinases for the hydrolysis of inulin. The isolation and purification of the endoinulinase enzymes may enhance the yield of IOS after inulin hydrolysis. It is expected that with the use of pure endoinulinase, under controlled conditions, there will be a prevalence of short chain IOS in the reaction mixture and low concentrations of fructose due to the absence of exoinulinase and invertase in the enzyme preparation (Yun et al., 1997[b]). The reaction products of the purified endoinulinase were analysed and compared with those obtained using a crude preparation to determine if there were any significant difference in product composition and quantity. However from an industrial point of view, extensive purification of inulinase is not necessary since the crude enzyme preparation usually displays high activity (Vandamme and Derycke, 1983; Yun and Kim, 1999).

Inulinases have been isolated and characterised from a number of sources such as fungi, bacteria and plants. Separation of endoinulinase and exoinulinase using standard conventional methods is technically difficulty because of the similarities in properties of the two enzymes (Yun et al., 1997[b]; Jing et al., 2003[b]). Some workers have successfully developed a new and convenient method for separating and identifying a mixture of inulinases obtained from *Aspergillus ficuum* by analysing their reaction products after native polyacrylamide gel electrophoresis (PAGE) (Gabriel and Wang, 1969; Jing et al., 2003[a]). In order to elucidate the important

characteristics of inulinases from different microbial sources, several purification procedures have been used and these include 65 % ammonium sulphate precipitation, dialysis, lyophilisation, acetone precipitation, chromatographic fractionation with Sephadex G-1000, DEAE-cellulose ion exchange and gel filtration with a final recovery in the region of 43.3 % (Vandamme and Derycke, 1983).

Characterisation of inulinases has revealed an average optimum temperature between 50 °C and 60 °C. This temperature regime is recommended for industrial application to avoid microbial contamination of reactors and final product and also to increase solubility of the inulin substrate (5% maximal solubility at 50 °C) with the main disadvantage of a reduction in enzyme stability at these elevated temperatures (Vandamme and Derycke, 1983). The optimal pH of most inulinases ranges from 4.5 to 6.0.

The principal aim of this study was to purify and characterise endoinulinase isolated from a commercial enzyme cocktail, Novozyme 960 that has been obtained from *Aspergillus niger*. The specific objectives were:

1   to determine temperature and pH profiles as well as temperature and pH stability of the purified endoinulinase.

2   to investigate the kinetic parameters (K_m, and V_{max}), substrate specificity and the effect of metals and compounds on endoinulinase activity.

3   to evaluate the purified endoinulinase for production of IOS using RSM.

3.2 Materials and Methods

3.2.1 Materials

Sephacryl S-200 for molecular exclusion chromatography and the Bradford reagent for protein concentration determination, Bis-acrylamide, polyacrylamide, and the Belly Dancer™ undulating orbital shaker AC input 115 V, 60 Hz, for staining and destaining gels were purchased from Sigma (South Africa). The Mini Protean II for gel electrophoresis, power pack and the fraction collector were purchased from Bio-Rad (South Africa). Polyethylene glycol (PEG) 2000 was purchased from Merck

(South Africa). Ultracentrifugation was done using the Avanti® J-E centrifuge purchased from Beckman Coulter, USA. The Power Wave$_x$ microtitre plate reader and the KC Junior software were purchased from Bio-Tek Instruments, South Africa. The freeze drier was purchased from Modulyo, Edwards, USA. All other chemicals and reagents were of analytical grade unless otherwise stated.

3.2.2 Endoinulinase Assay

The endoinulinase activity was determined as previously described in Chapter 2 section 2.2.5.

3.2.3 Protein concentration assay

Protein concentration was determined using the Bradford assay (Bradford, 1976). A standard calibration curve was done using 0 to 2 mg BSA (Appendix A1). A 5 µl protein sample was mixed with 245 µl sample of the Bradford's reagent in a microtitre plate and allowed to react for 5 minutes. Absorbance values were determined at 595 nm using a Power Wave$_x$ microtitre plate reader with KC Junior software (Bio-Tek Instruments). Protein concentration in the samples was then calculated by interpolation from the protein standard curve (Appendix A1)

3.2.4 Purification of endoinulinase

Endoinulinase from the crude commercial preparation was purified to electrophoretic homogeneity through a 3 step purification procedure which involved ammonium sulphate precipitation, dialysis and Sephacryl S-200 size exclusion chromatography. All the purification steps were done in a cold room at 4 °C to avoid enzyme denaturation.

3.2.5 Ammonium sulphate precipitation

Endoinulinase activity and protein concentration were determined as previously described on the crude endoinulinase extract. A pilot scale ammonium sulphate fractionation procedure was done from 10 to 80 % ammonium sulphate saturation to

establish the concentration of ammonium sulphate required for salting out a large fraction of the target endoinulinase protein. Based on the findings from the pilot scale fractionation, the crude endoinulinase fraction (500 ml) was subjected to 80 % saturation ammonium sulphate precipitation by adding the salt slowly at 4 °C with gentle stirring for 4 h to avoid foaming and enzyme denaturation. The precipitate was centrifuged (10 000 x g, 20 min, 4 °C). The pellet was washed 3 times with distilled water and then reconstituted in 10 ml distilled water and then subjected to dialysis.

3.2.6 Dialysis, PEG 2000 concentration and lyophilisation

The reconstituted pellet was subjected to dialysis using a Snake skin dialysis tubing (Pierce) with a MWCO point of 10 000 Da. Dialysis was done overnight against distilled water and the dialysed enzyme solution was concentrated by 12.5 % (w/v) PEG 2000 and 0.1 % glycine (w/v). The concentration step was done by immersing the dialysis tubing into the PEG 2000 solution for 3 h and enzyme activity and protein concentration was determined as before. A 5 ml aliquot in a freeze drying round bottomed flask was frozen by swirling in liquid nitrogen. The sample was freeze dried in a freeze drier overnight, and the dried powder was kept at -20 °C till required for further purification.

3.2.7 Size exclusion chromatography

Sephacryl S-200 was packed into a 2.5 cm x 45 cm column with a bed volume of 176 cm^3 and equilibrated with 0.1 M citrate-phosphate buffer (pH 6.0). The freeze dried sample (53 mg) was reconstituted in 5 ml distilled water and then applied at the top of the column using a Pasteur pipette. The sample was eluted with a 0 to 3 M NaCl gradient elution in 0.1 M citrate-phosphate buffer (pH 6.0) and 5 ml fractions were collected. Protein concentration in the fractions was monitored by the Bradford method as previously described in section 3.2.3 and endoinulinase activity was determined as previously described (Chapter 2 section 2.2.5). Fractions displaying endoinulinase activity were pooled, freeze dried and reconstituted when required.

3.2.8 SDS-PAGE

Protein purity was monitored by SDS-PAGE (Laemmli, 1970) using 8 % resolving gel and a 5 % stacking gel (Appendix B1). Protein samples for SDS-PAGE were prepared using a standard protocol as described by Bollag *et al.*, (1996) (Appendix B1). Peqgold Protein Marker II was used as the molecular weight marker. Electrophoresis was run for 45 minutes at 150 V. The gel was stained with Coomassie Brilliant Blue R-250 stain for 30 minutes with gentle shaking and then destained overnight with glacial acetic acid.

3.2.9 Native PAGE

A discontinuous native PAGE was prepared to confirm the endoinulinase nature of the purified enzyme using 8 % resolving gel and a 5 % stacking gel. Protein samples for activity gels were prepared using a standard procedure as described by Bollag *et al.*, (1996) (Appendix B2). Electrophoresis was run for 45 minutes and the gel was stained with Coomassie Brilliant R250 stain for 30 minutes with gentle shaking and then destained overnight with glacial acetic acid/ methanol/water (1:1:8) (v/v/v). The single band of the pure endoinulinase was cut off from the gel and macerated into small pieces in a minimal volume of distilled water. The macerated band was tested for enzyme activity with 5 % inulin under assay conditions as before and the products were analysed by HPLC and TLC as previously described in Chapter 2 sections 2.2.3.1 and 2.2.3.2 respectively.

3.2.10 Physico-chemical characterisation of purified endoinulinase

3.2.10.1 Effect of temperature on endoinulinase activity

The effect of temperature on the purified endoinulinase activity was determined by incubating the purified enzyme at 30, 40, 50, 60, 70, and 80 °C. Enzyme activity was determined as previously described in Chapter 2 section 2.2.5. The reaction was allowed to proceed for 1 h and then stopped by boiling the tubes for 10 minutes. The reducing sugars liberated were assayed by the DNS assay as previously described in Chapter 2 section 2.2.2.

3.2.10.2 Effect of pH on endoinulinase activity

The effect of pH on the purified endoinulinase was determined by carrying out the endoinulinase activity in the pH range of 3 to 10 using the following buffer systems: pH 3 to 5 (sodium acetate, 0.1 M), pH 6 to 8 (citrate-phosphate, 0.1 M), and pH 9 to 10 (glycine-NaOH, 0.1 M). This was done by preparing 5 % inulin at the appropriate pH. Enzyme activity was determined as previously described in Chapter 2 section 2.2.5. The reaction was allowed to proceed for 1 h and then stopped by boiling the tubes for 10 minutes. The reducing sugars liberated were assayed by the DNS method as previously described in Chapter 2 section 2.2.2.

3.2.10.3 Thermal stability of endoinulinase

Thermal stability of the purified endoinulinase was determined by incubating the enzyme at the optimum temperature (60 °C) for 72 h and aliquots of the reaction mixture were taken at 6 h intervals to test for residual activity under assay conditions. Enzyme activity was determined as previously described in Chapter 2 section 2.2.5. The reaction was allowed to proceed for 1 h and then stopped by boiling the tubes for 10 minutes. The reducing sugars liberated were assayed by the DNS assay as previously described in Chapter 2 section 2.2.2.

3.2.10.4 pH stability of endoinulinase

The pH stability of the purified endoinulinase was determined by incubating the enzyme at pH 6.0 at room temperature for 72 h and samples were taken at 6 h interval to test for residual endoinulinase activity. Enzyme activity was determined as previously described in Chapter 2 section 2.2.5. The reaction was allowed to proceed for 1 h and then stopped by boiling the tubes for 10 minutes. The reducing sugars liberated were assayed by the DNS assay as previously described in Chapter 2 section 2.2.2.

3.2.10.5 Substrates hydrolysed by the purified endoinulinase

A wide range of carbohydrate substrates such as cellobiose, lactose, maltose, melezitose, mellibiose, palatinose, raffinose, stachyose, sucrose, and trehalose were assessed for endoinulinase specificity. A 5 % of each carbohydrate was prepared in 0.1 M citrate-phosphate buffer (pH 6.0). Endoinulinase activity was done under assay conditions for 1 h and the reaction products were analysed by the DNS method and HPLC as previously described in sections 2.2.2 and 2.2.3.1 respectively.

3.2.10.6 Effect of metals and reagents on endoinulinase activity

The effects of various metal salts and reagents were investigated at a concentration of 1 mM. This was done by incubating different metal ions with enzyme solution in 0.1 M citrate-phosphate buffer (pH 6.0) at room temperature for 1 h. The following compounds were investigated, $AgNO_3$, $NaNO_3$, EDTA, $CuSO_4$, Hg_2Cl_2, urea, and $CoCl_2$. The control tube had no metal ions. The residual endoinulinase activity was performed in triplicate under assay conditions.

3.2.10.7 Determination of kinetic parameters (K_m, V_{max})

In order to get an insight of the affinity of the purified endoinulinase for inulin, kinetic parameters of the purified endoinulinase were assumed to follow the Michaelis-Menten kinetics (Cornish-Bowden, 2004) (equation 3.1). Apparent Michaelis-Menten constant (K_m) and maximum reaction velocity (V_{max}), for the substrate inulin were determined from a simple linear regression using the Lineweaver–Burk double reciprocal, which is a result of the linearisation of the Michaelis-Menten equation as follows (equation 3.2):

$$v = \frac{V_{max} \cdot [S]}{[S] + K_m} \qquad \qquad 3.1$$

$$\frac{1}{v} = \frac{1}{V_{max}} + \frac{K_m}{V_{max}} \cdot \frac{1}{[S]} \qquad \qquad 3.2$$

Different inulin concentrations were chosen to give measurable reaction rates and the reactions were performed in triplicate. The substrate concentration was carefully chosen as described by Cornish-Bowden (2004), since a low substrate concentration range relative to the K_m will result in the plot being nearly horizontal while a high substrate concentration range relative to the K_m will result in the plot intersecting the axes close to the origin. Average relative molecular weight for inulin was estimated to be 5500 from the average degree of polymerisation of 34 due to the polydisperse nature of the compound (Vandamme and Derycke, 1983; Moriyama and Ohta, 2007). Endoinulinase activity was determined at 5 to 20 mM inulin in 0.1 M citrate-phosphate buffer (pH 6.0) at 10 minute interval for 1 h at 60 °C under standard assay conditions as previously described (section 2.2.5) and the reactions were performed in triplicate. Initial hydrolysis rate at each concentration was estimated from the linear part of the progress curves.

3.2.12 Optimisation of IOS production by the purified endoinulinase

The purified endoinulinase was evaluated for the production of IOS using RSM under the same conditions used as previously described for the crude endoinulinase in section 2.2.6 and 2.2.7. In the first RSM, time was varied from 1 to 3 h (X_1), temperature (40 to 60 °C) (X_2), and inulin concentration (50 to 150 g/L) (X_3). In the second RSM, time was varied from 24 to 72 h (X_1), enzyme dosage (20 to 100) (X_2), and inulin concentration (50 to 150 g/L) (X_3). The IOS produced were identified and quantified using HPLC as described in Chapter 2 section 2.2.3.1

3.2.13 Statistical Analysis

Statistical analysis was performed as stated in Chapter 2 section 2.2.9.

3.3 Results

3.3.1 Purification of Endoinulinase

A 500 ml portion of Novozyme 960 crude endoinulinase (929 U/ml) was precipitated with 80 % saturation ammonium sulphate at 4 °C and the reconstituted pellet was

further purified using Sephacryl S-200 molecular exclusion chromatography. Gradient elution was done using 0 to 3 M NaCl and 5 ml fractions were collected using a fraction collector and protein concentration was determined by the Bradfords method. The elution profile is shown in Figure 3.1 and fractions 6 to 16 with highest endoinulinase activity were pooled, concentrated with PEG 2000 (12.5 %, w/v) with glycine (0.1 %, w/v) and the aliquot was subjected to SDS-PAGE. A second protein peak (fractions 17 to 21) appeared in the chromatogram which had little endoinulinase activity but was identified as an exoinulinase.
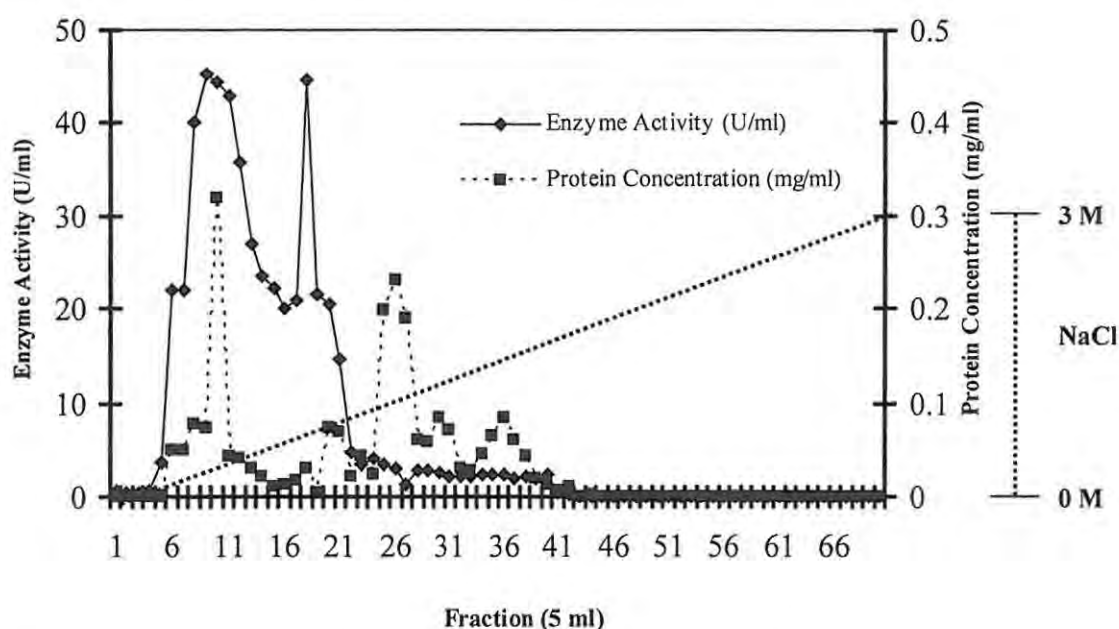
Figure 3.1 Gradient elution (0 to 3 M NaCl) profile of Novozyme 960 using Sephacryl S-200 molecular exclusion chromatography. Fractions (5 ml) were collected and protein concentration was monitored by the Bradford method and endoinulinase activity was determined by incubating the enzyme fraction with 5 % inulin at 60 °C for 1 h and reducing sugars were assayed by the DNS method.

3.2.2 SDS-PAGE

The purity of the pooled fractions after Sephacryl S-200 size exclusion chromatography was checked by 8 % discontinuous SDS-PAGE with a 5 % stacking gel as shown in Figure 3.2. The homogenous monomeric endoinulinase band was estimated to be 68.1 kDa by SDS-PAGE using the molecular weight calibration curve (Appendix C1). A purification table was constructed for each step of the purification process. The final endoinulinase activity after Sephacryl S-200 size exclusion chromatography was 325 U/ml with a specific activity of 565.60 U/mg. After the size

exclusion purification step, the endoinulinase was purified 3.50 fold with a final yield of 1.1 % (Table 3.1).
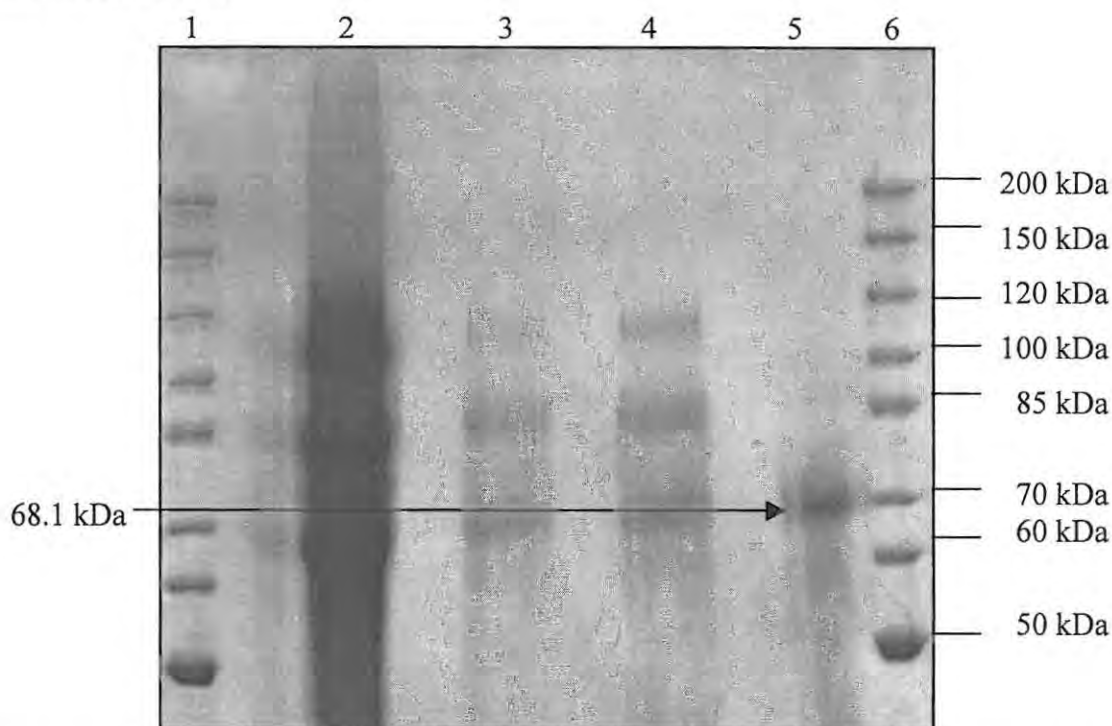
Figure 3.2 SDS-PAGE of the purified Novozyme 960 Endoinulinase using 8 % separating gel and a 5 % stacking gel. Lane 1 and 6, MWM, lane 2, dialysed crude Novozyme 960, lane 3, reconstituted pellet after 80 % ammonium sulphate precipitation, lane 4, dialysed pellet after 80 % ammonium sulphate precipitation, and lane 5 pooled fractions 6 to 16 after Sephacryl S-200 molecular exclusion chromatography.

Table 3.1 Purification of Novozyme 960 endoinulinase.

Fraction	Volume (ml)	[Protein] (mg/ml)	Total Protein (mg)	Activity (U/ml)	Total Activity (U)	Specific Activity U/mg	Yield (%)	Fold Purification
Crude	500	5.75	2875	929	464500	161.57	100	1
AS[a]	20	3.82	76.4	890	17800	232.98	3.83	1.4
SS-200[b]	15	0.63	9.15	345	5175	565.6	1.11	3.50

[a]Dialysed pellet after 80 % ammonium sulphate precipitation, freeze dried and reconstituted.
[b]Sephacryl S-200 with NaCl linear gradient elution (0 – 3 M).

3.2.3 Native PAGE

Discontinuous nondenaturing gels were prepared and the purified endoinulinase was resolved in a single band (Figure 3.3). The pure band was cut out and macerated for further analysis to confirm if it was an authentic endoinulinase.
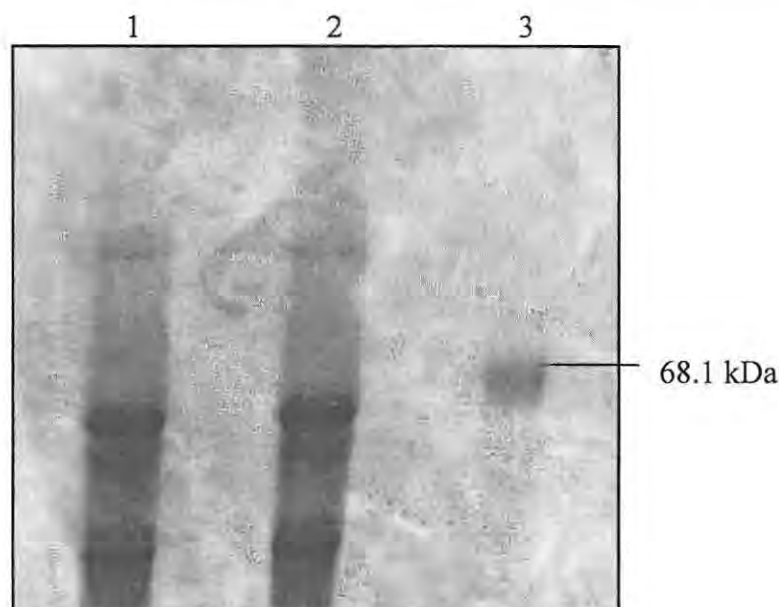
Figure 3.3 Activity PAGE of the pure endoinulinase after Sephacryl S-200 molecular exclusion chromatography using 8 % separating gel and a 5 % stacking gel. Lane 1, dialysed crude Novozyme 960, lane 2, freeze dried reconstituted dialysed pellet after 80 % ammonium sulphate precipitation, and lane 3 pooled fractions 6 to 16 after Sephacryl S-200 molecular exclusion chromatography. The gel was stained with Coomassie Brilliant Blue R-250 for 30 minutes and destained with glacial acetic acid destaining solution overnight with gentle shaking.

3.2.4 Determination of endoinulinase activity after native PAGE

The pure single band obtained after native PAGE was cut from the gel and macerated in a minimal volume of 0.1 M citrate-phosphate buffer (pH 6.0). This enzyme solution was used for inulin hydrolysis and the reaction products were analysed by TLC (Figure 3.4). There were no reaction products in the control tube with boiled endoinulinase and 5 % inulin because the enzyme was denatured; suggesting that the products observed in the experimental tubes were due to endoinulinase activity. The pooled fractions 6 to 16 gave major F_3 and F_4 reaction products (lane 4, Figure 3.4). The macerated pure band after native PAGE also gave similar products confirming that the enzyme is endo acting (lane 5, Figure 3.4). The reaction products of the macerated pure band after native PAGE were further analysed using HPLC with RI detection showing absence of monosaccharides from the reaction mixture (Figure 3.5). The main products identified were F_2, F_3, F_4, and F_5.
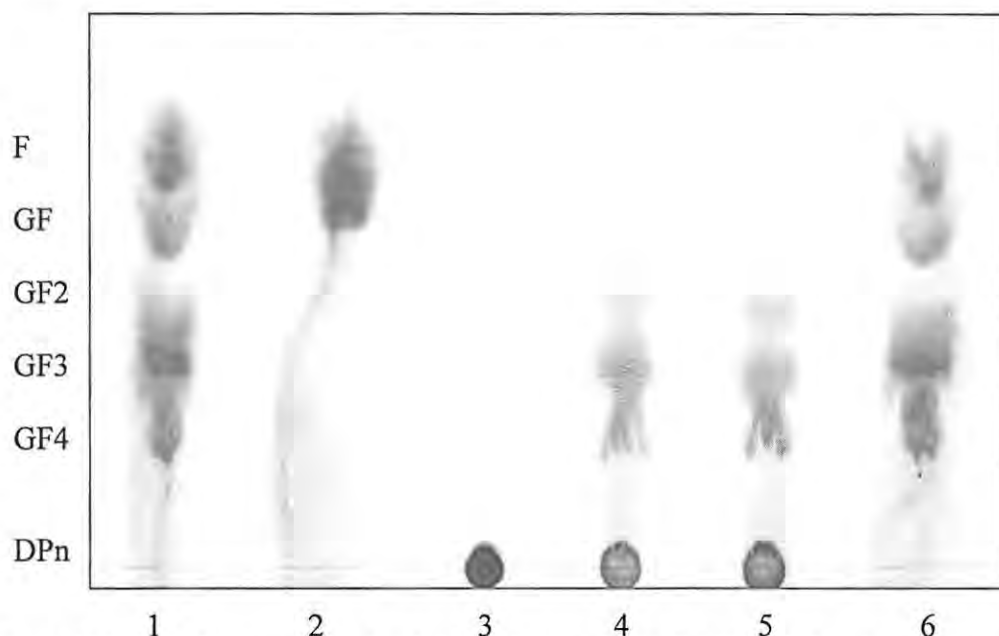
Figure 3.4 TLC chromatogram showing reaction products of the purified and native endoinulinase with 5 % inulin (pH 6.0), at 60 °C for 1 h. The solvent for irrigation was butanol: acetic acid: water (5: 4: 1) (v/v/v) and the spots were visualised by heating the plate at 110 °C for 15 minutes after spraying with anisaldehyde spray reagent. Lane 1 and 6, standard FOS mixture of 5 % fructose (F), sucrose (GF), 1-kestose (GF_2), nystose (GF_3) and fructofuranosyl nystose (GF_4), lane 2, 5 % Fructose, lane 3, control with boiled Novozyme 960 endoinulinase and 5 % inulin, lane 4, Sephacryl S-200 pooled fractions 6 to 16 with 5 % inulin reaction products, and lane 5, reaction products of the native PAGE pure band plus 5 % inulin. Main products of the purified enzyme are F_3 and F_4 and no fructose was liberated indicating that the purified enzyme is an endoinulinase.
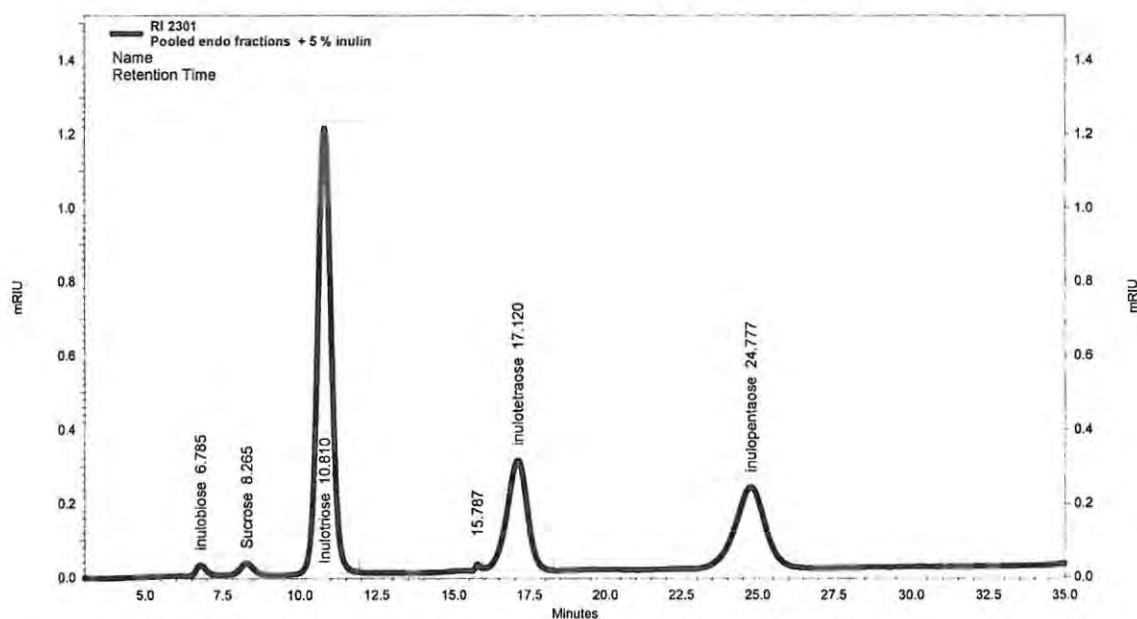
Figure 3.5 HPLC chromatogram of the reaction products of the pure endoinulinase band after native PAGE and 5 % inulin at 60 °C for 1 h. Diluted 20 µl of the reaction products were injected into the sample port and eluted with acetonitrile / water (73: 27) mobile phase at a flow rate of 1 ml/min. The products were detected with a refractive index detector at room temperature.

3.2.5 Effect of temperature on endoinulinase activity

The optimal temperature of the purified endoinulinase was determined to be 60 °C (Figure 3.6). After 1 h of incubation, the relative activity was 25.69 ± 2.13 % at 30 °C, 100 ± 4.31 % at 60 °C and 23.4 ± 1.98 % at 80 °C.
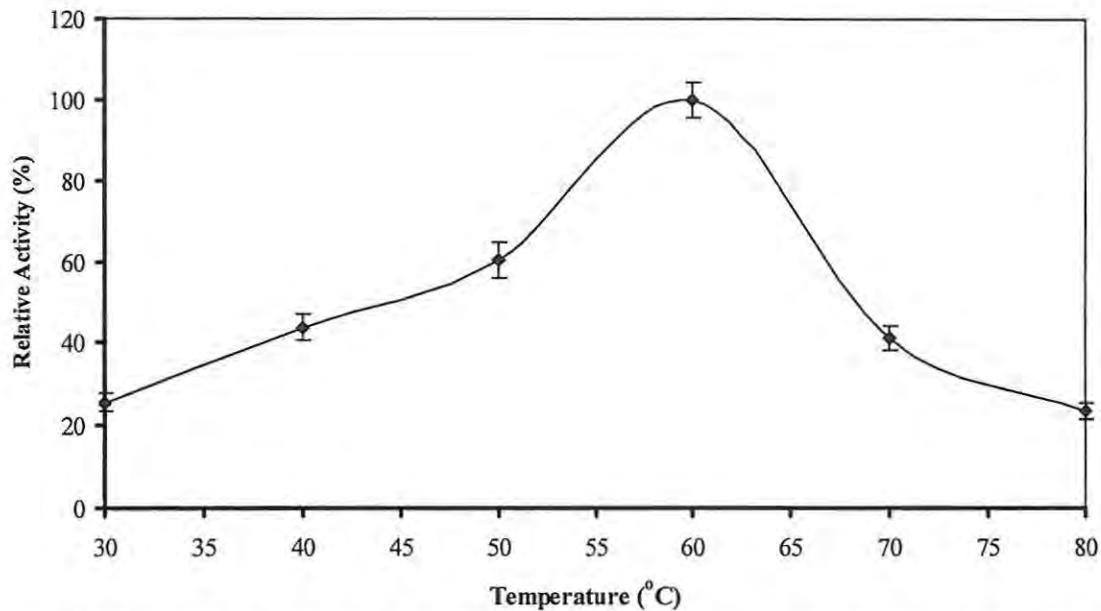
Figure 3.6 Temperature profile of the purified endoinulinase. Data points represent a triplicate determination and error bars on data points represent standard deviations (± SD).

3.2.6 Effect of pH on endoinulinase activity

The effect of pH on the activity of the purified endoinulinase was determined in the pH range of 3 to 10. The residual activity was 25 ± 2.45 % at pH 3 and increased to 56 ± 4.67 % at pH 4. The optimal pH was 6.0 (Figure 3.7). The residual activity decreased to 93 ± 4.54 % at pH 7 and finally dropped to 22 ± 1.89 % at pH 10.
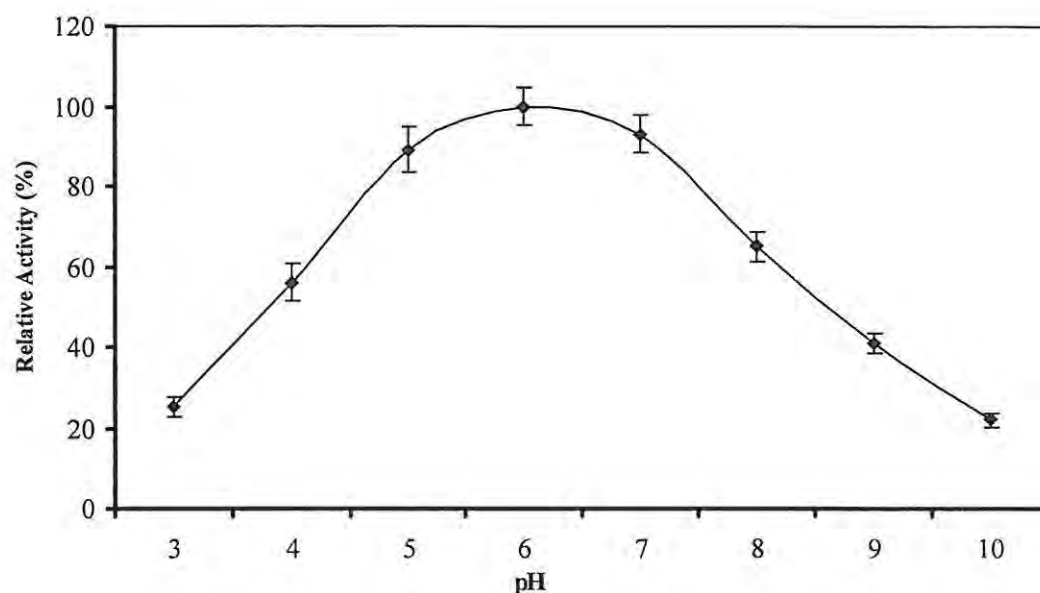
Figure 3.7 Effect of pH on the activity of the purified endoinulinase. Enzyme activity was determined by incubating the purified endoinulinase with 5 % inulin for 3 h in the pH range of 3 to 10 using 0.1 M sodium acetate (pH 3 to 5), citrate-phosphate (pH 6 to 8) and glycine-NaOH (pH 9 to 10). Data points represent the mean of a triplicate determination and error bars on data points represent standard deviations (± SD).

3.2.6 Thermal and pH stability of endoinulinase

Temperature stability of the purified endoinulinase was determined over 72 h of incubation at 60 °C and samples were retrieved for analysis at 6 h interval. Endoinulinase activity was determined as previously described. Figure 3.8 shows the profile generated whereby the enzyme exhibited good thermostability. The endoinulinase was significantly stable at 60 °C after 6 h with 97.93 ± 4.34 % residual activity. After 72 h of incubation, the enzyme had 61.23 ± 4.30 % residual activity.

Stability of the purified endoinulinase was determined at pH 6 for 72 h and samples were collected for analysis at 6 h interval. The purified endoinulinase was highly stable at pH 6 for 36 h without any decrease in activity and thereafter the activity started to decrease (Figure 3.8). The residual activity was 82 ± 4.31 % after 48 h of incubation at pH 6.0. After 72 h incubation at this pH, the residual activity was 62 ± 2.31 %.
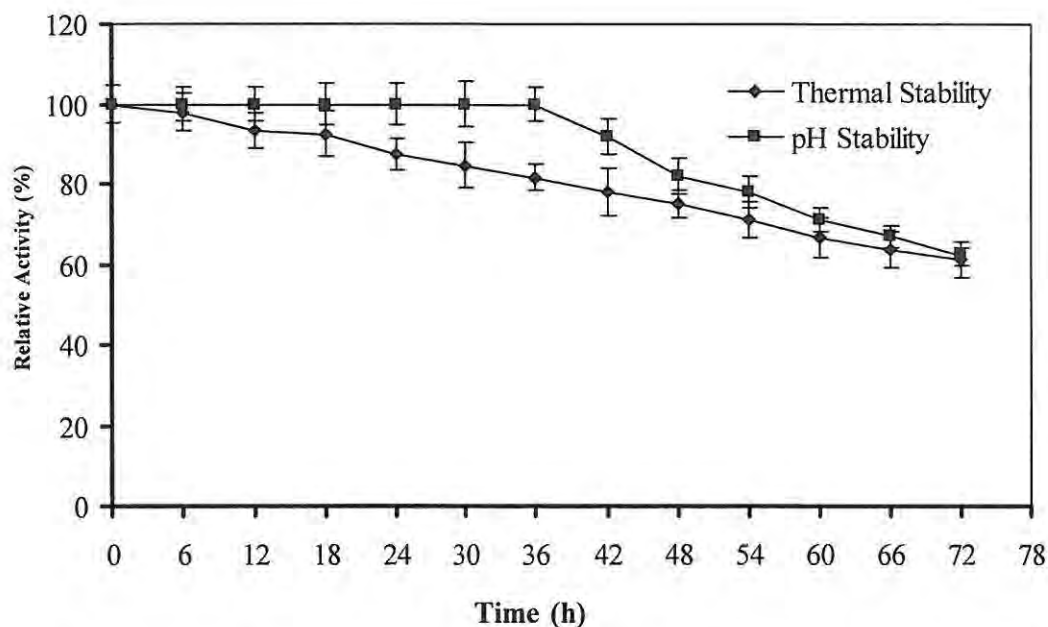
Figure 3.8 Temperature and pH stability of the purified endoinulinase. Data points represent the mean of a triplicate determination and error bars on data points represent standard deviations (± SD).

3.2.8 Effect of metals and reagents on endoinulinase activity

The effect of different metal ions and reagents on endoinulinase activity was investigated at a concentration of 1 mM (Table 3.2). Cobalt chloride increased the residual activity of the endoinulinase by 156 ± 6 %. There was no endoinulinase activity in the reaction mixture with EDTA, $HgCl_2$, and $NaNO_3$.

3.2.9 Substrates hydrolysed by endoinulinase

A wide range of carbohydrates such as cellobiose, lactose, melezitose were evaluated for their applicability as substrates for the purified endoinulinase under assay conditions (Table 3.3). Inulin was the most preferred substrate (345.0 ± 7 U/ml) followed by D-maltose monohydrate (251.6 ± 5 U/ml) and D-(+)-melibiose (243.6 ± 5 U/ml). Melezitose hydrate and trehalose dihydrate were not hydrolysed at all by the purified endoinulinase.

Table 3.2 Effect of metals and reagents on endoinulinase activity[b].

Compound (1 mM)	Relative Activity (%)[a]
Control	100 ± 4
AgNO$_3$	97 ± 3
EDTA	Nil
Urea	83.5 ± 4
CuSO$_4$	64 ± 4
Hg$_2$Cl$_2$	Nil
CoCl$_2$	156 ± 6
NaNO$_3$	Nil

[a] 100 % Relative activity is defined as the activity in the control reaction with no added metal ions. Values represent the means ± SD, n = 3, [b] (100 % endoinulinase activity is 345 U/ml).

Table 3.3 Substrates hydrolysed by the purified endoinulinase.

Substrate (5 %)	Enzyme Activity (U/ml)[a]
Inulin	345.0 ± 7
D (+) Cellobiose	241.3 ± 4
D-Lactose	247.8 ± 3
D-Maltose monohydrate	251.6 ± 5
Melezitose hydrate	Nil
D-(+)-Melibiose	243.6 ± 5
Palatinose hydrate	123.5 ± 3
D-(+)-Raffinose pentahydrate	112.8 ± 6
Stachyose hydrate	67.9 ± 2
Sucrose	154.8 ± 5
D-(+)-Trehalose dihydrate	Nil

[a] The values represent means of enzyme activities ± SD n = 3

3.2.10 Kinetic parameters (K_m, V_{max}) of endoinulinase

The purified endoinulinase did not exhibit saturation behaviour by inulin and the kinetic parameters were determined by the classical initial rate experiments and did fit

the Lineweaver-Burk plot (Figure 3.9). Therefore, it was possible to estimate the apparent Michaelis-Menten constant (K_m) value of 3.53 mM and the maximum velocity (V_{max}) for the purified endoinulinase of 666.67 µmoles/min/ml at 60 °C, pH 6.0.
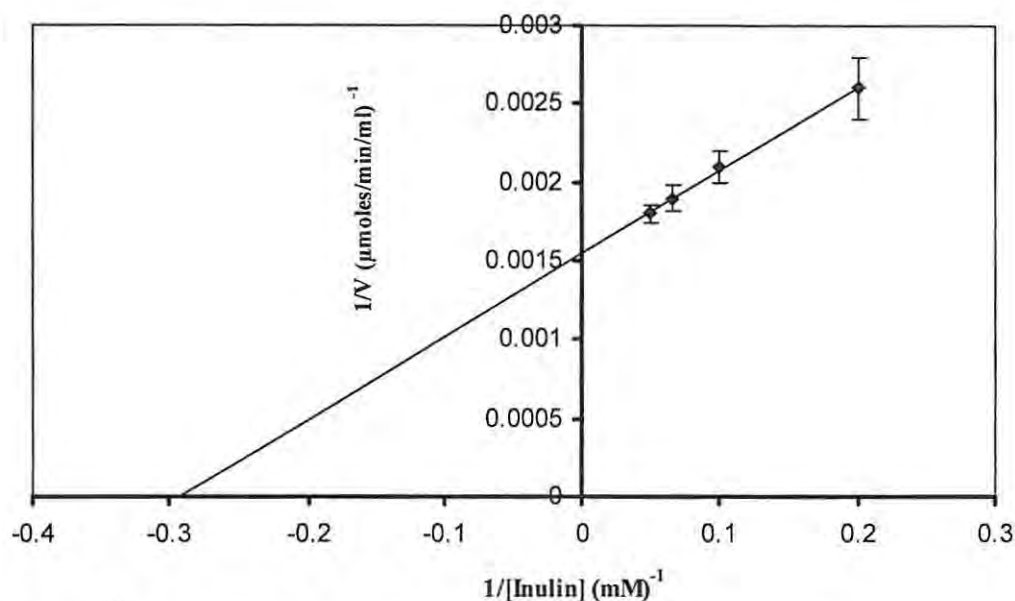
Figure 3.9 Lineweaver-Burk double reciprocal plot of the purified endoinulinase. Each point on the graph represents the mean ± SD of three replicate determinations.

3.2.11 Optimisation of IOS production by the purified endoinulinase

In the first RSM, the following parameters were simultaneously varied, time (1 to 3 h), temperature (40 to 60 °C), inulin concentration (50 to 150 g/L), and enzyme dosage and pH were kept at 20 U/ml and 6.0 respectively. The different experimental runs resulted in different yields of IOS (Figure 3.10). The highest yield of F_3, F_4 and F_5 are summarised in Table 3.4. Incubation time significantly ($p < 0.05$) affected the IOS yield and composition and was therefore varied from 24 to 72 h in a second set of RSM experimental runs. Three independent variables, incubation time (24 to 72 h), enzyme dosage (20 to 100 U/ml) and inulin concentration (50 to 150 g/L) were simultaneously varied in an RSM experiment with 15 experimental runs. Hydrolysis products were periodically withdrawn and analysed by HPLC. Inulotrioses (F_3), inulotetraoses (F_4) were the major products produced with trace amounts of inulopentaoses (F_5) produced and no F_6 and longer chain intermediates were detected in the reaction mixtures (Figure 3.11) (Table 3.5).

Table 3.4 Maximum actual and predicted IOS concentrations produced in the first RSM runs.

IOS	Actual maximum concentration (mM)	Predicted maximum concentration (mM)	R^2	p
F_3	62.9	61.56	0.88	0.046
F_4	17.48	15.22	0.87	0.013
F_5	8.71	12.58	0.78	0.017
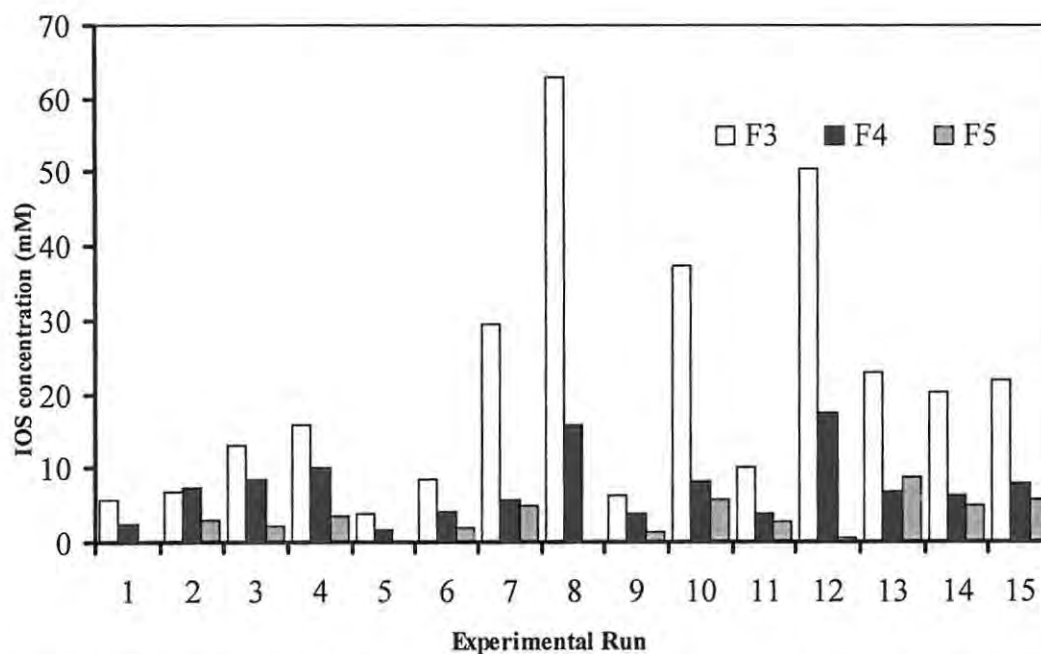
Figure 3.10 Inulooligosaccharide concentrations produced by the purified endoinulinase with 3 coded levels and 3 independent variables with enzyme dosage fixed at 20 U/ml and pH kept at 6.0.

After analysis of all the reaction products, no IOS ≥ F5 were detected in experimental runs 3, 4, 5, 7, 8, 10, 12, 13, and 15. Experimental runs 3, 4, 8, 12, and 15, produced 5.9 mM, 0.8 mM, 16.4 mM, 2.8 mM and 3.2 mM of inulotrioses respectively (Figure 3.11).
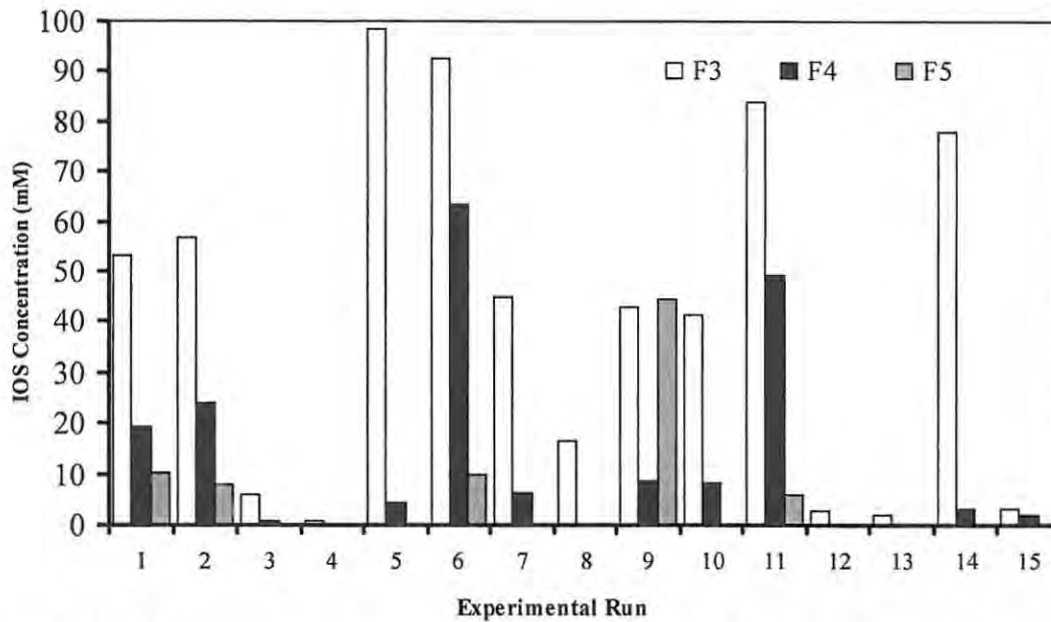
Figure 3.11 Inulooligosaccharide concentrations produced by RSM with the purified endoinulinase with 3 coded levels and 3 independent variables with temperature at 60 °C and pH kept at 6.0.

Table 3.5 Maximum actual and predicted IOS concentrations produced in the second RSM runs.

IOS	Actual maximum concentration (mM)	Predicted maximum concentration (mM)	R^2	p
F_3	98.3	107.45	0.89	0.042
F_4	63.4	56.73	0.85	0.031
F_5	44.4	23.99	0.48	0.036

3.4 Discussion

It is desirable to purify and know the properties of enzymes if they are to be used for large scale industrial and biotechnological processes. The commercial endoinulinase preparation (Novozyme 960) isolated from *Aspergillus niger* was purified to electrophoretic homogeneity as confirmed by SDS-PAGE shown in Figure 3.2 through a 3 step purification procedure. Three major bands were observed in the crude preparation and these could be exoinulinase and other contaminating proteins. The purified endoinulinase gave a single band on SDS-polyacrylamide gel electrophoresis and its molecular weight was estimated to be 68.1 kDa. The size of

the endoinulinase purified in this investigation agrees reasonably well with the findings of other workers (Cho and Yun, 2002).

The molecular mass of endoinulinases isolated from *Pseudomonas* sp. were 210 kDa and 170 kDa, whereas those of many other microbial endoinulinases were 75, 68, and 53 kDa from *Arthrobacter* sp., *Penicillium* sp., and *Aspergillus* sp. respectively (Cho and Yun, 2002; Nakamura *et al.*, 1997). The endoinulinase in the present study was purified 3.5 fold with a 1.11 % suggesting a remarkable loss of enzyme activity and protein during the purification process.

The purified endoinulinase was confirmed to be an authentic endoinulinase after native PAGE. The apparent absence of fructose in the TLC chromatogram lane 4 and 5 (Figure 3.4) indicates that the purified enzyme was endo-acting because of the preponderance of inulotriose and inulotetraose intermediates in the reaction mixture. Moreover, Zhengyu, *et al.*, (2005) observed that it is technically difficulty to get a selective endoinulinase preparation free from exoinulinase and invertase activity. The HPLC trace in Figure 3.5 also further confirms that fructose was not produced in the reaction mixture. The absence of fructose in the reaction mixture with the purified endoinulinase is attributed to the complete removal of invertases and exoinulinases during the purification process. Refractive index detection is attractive because it offers a wide linear range for sugar analysis but it suffers from the disadvantage of not being sensitive to low analyte concentrations (Ronkart *et al.*, 2007; Giannoccaro *et al.*, 2008). The variation of retention times of the reaction products analysed by HPLC with RI detection is attributed to temperature fluctuations which affect RI detection.

The optimal temperature of the purified endoinulinase was found to be 60 °C and highly thermostable with a residual activity of 61.23 % at this temperature after 72 h of incubation (Figure 3.6 and Figure 3.7). This optimal temperature is higher than the range reported for microbial inulinases from 45 to 55 °C (Vandamme and Derycke, 1983). This suggests that the enzyme has attractive and favourable properties for industrial production of IOS production which have been accepted as functional sweeteners, similar to other microbial oligosaccharides (Zhengyu, *et al.*, 2005). In addition, the high optimal temperature allows the industrial production of IOS

intermediates from inulin with concomitant inactivation of contaminating microorganisms in the reaction mixture (Sharma *et al.*, 2006). In a similar study using a purified endoinulinase isolated from *Aspergillus ficuum*, Zhengyu and co-workers (2005) found that the purified endoinulinase produced 70 % IOS yield after 72 h at 45 °C using 5 % inulin pH 5.0 with an enzyme concentration of 10 U/ g substrate.

The optimal pH was established to be 6.0 (Figure 3.8) which indicates that the purified endoinulinase prefers slightly mild acidophilic conditions for maximal production of IOS and this could possibly be due to the ionisation of the catalytic acidic residues. This result is important because inulin is degraded at pH less than 3 to give fructose and other unwanted coloured products such as fructose anhydrides with no sweetening properties (Sharma *et al.*, 2006). There was no change in residual activity at pH 6.0 for 36 h with a residual activity of 62 % after 72 h of incubation, thus indicating that the purified endoinulinase was highly stable at this pH. Zhengyu and co-workers (2005) also found pH 6.0 to be favourable for IOS production where they obtained a maximum yield of 54 % total IOS after 72 h of incubation with 5 % inulin, at 45 °C with an enzyme dosage of 5 U/g substrate.

The purified endoinulinase hydrolysed a wide range of carbohydrate substrates such as cellobiose, maltose, raffinose and sucrose showing that it has wide substrate specificity. Trehalose and melezitose were not hydrolysed by the purified endoinulinase suggesting that inappropriate glycosidic bonding confirmation for endoinulinase attack. In a related investigation Sharma *et al.*, 2006 demonstrated that endoinulinase from *Streptomyces* sp. can hydroylse raffinose, sucrose and inulin.

Various metal ions reagents were investigated for their activity on endoinulinase activity (Table 3.2). Cobalt chloride enhanced endoinulinase activity while AgNO$_3$ had a marginal inhibition on endoinulinase activity. Mercury ions (Hg^{2+}) affect -SH-groups and completely inhibit endoinulinase activity. The complete inhibition by Hg^{2+} ions indicates that some -SH-groups in the protein might be required for endoinulinase activity since Hg^{2+} is known to affect thiol groups (Sharma *et al.*, 2006). Previous studies have also confirmed similar observations for other microbial inulinases (Ettalibi and Baratti, 1987; Sharma, *et al.*, 2006). The purified

endoinulinase was strongly inhibited by metal chelating agent EDTA, indicating that metal cofactors are required for endoinulinase activity. Urea had low residual activity which is normal for all inulinases investigated to date (Mukherjee and Sengupta, 1987).

The purified endoinulinase showed a low K_m value of 3.53 mM and a V_{max} of 666.67 μmoles/min/ml suggesting that it has a high affinity for the substrate inulin. Therefore this enzyme makes a valuable tool as a biocatalyst for industrial application for the production of IOS using inulin as a substrate. This also cuts cost since pure inulin as a substrate is expensive since inulin can be used in low concentrations. The findings of this study are in agreement with the findings of other workers who investigated inulinases from other organisms. Beluche *et al.*, (1980) found the K_m of an inulinase isolated from *Debaryomyces cantarelli* to be 15 mM while that of an inulinase isolated from *Candida salmenticensis* was 17 mM (Guiraud *et al.*, 1980) and in another study, Ettalibi and Baratti, 1987 found the K_m of an inulinase from *Aspegillus ficuum* to have K_m value ranging from 10 to 15 mM.

Response surface regression was adequate in explaining the concentrations of IOS produced under multivariate conditions without IOS with higher chain length being detectable in the reaction mixture. Long chain IOS ($\geq$ DP6) could be present but the sensitivity of the analytical column used may cause the higher chain sugars to get stuck onto the matrix of the column and not eluted. According to Ronkart, *et al.*, (2007), the preparation and quantitative determination of high DP fractions of IOS and accurate separation of GF_n and F_n oligosaccharides still remains a challenge. In addition higher chain sugars do not resolve well on TLC. The IOS produced by the purified endoinulinase are slightly higher than the levels produced by the crude endoinulinase preparation and this is explained by the removal of contaminating exoinulinases and other enzymes in the reaction mixture which cleave the long chain molecules to monosaccharides hence decreasing the concentration of IOS intermediates (Zhengyu *et al.*, 2005).

3.5 Conclusions

Some conclusions can be drawn from this study:

1. The purified endoinulinase was found to have an optimal temperature of 60 °C and a pH of 6.0. The enzyme was highly stable at these optimal conditions for 72 h and these properties for the purified endoinulinase make it a potential candidate for biotechnological application for the production of IOS.

2. The purified endoinulinase was purified to electrophoretic homogeneity through a 3 step purification procedure and it was estimated to be monomeric with a molecular weight of 68.1 kDa after SDS-PAGE. The enzyme was confirmed to be an authentic endoinulinase by native PAGE.

3. The purified endoinulinase had wide substrate specificity and had low K_m of 3.53 mM and a V_{max} of 666.67 µmoles/min/ml. The endoinulinase activity of the purified enzyme was completely inhibited by EDTA, Hg_2Cl_2, $NaNO_3$, and SDS.

4. Response surface methodology was used for optimisation of IOS production using the purified enzyme and the optimal conditions were established. The levels of IOS were higher in comparison to the use of a crude endoinulinase preparation observed in Chapter 2.

Chapter 4 explores the synthesis of frucooligosaccharides from sucrose using a microbial fructosyltransferase.

Chapter 4

Synthesis of Short Chain Fructooligosaccharides from Sucrose by a Fructoslytransferase from *Aspergillus aculeatus* Optimised Using <u>Response Surface Methodology</u>

4.1 Introduction

Fructosyltransferase (Ftase) (EC 2.4.1.9) and β-fructofuranosidase, also called invertase (EC 3.2.1.26), are two classes of enzymes that catalyse the formation of short chain fructooligosaccharides (scFOS) from sucrose (Park *et al.*, 1999; Sangeetha *et al.*, 2004[a]; Ghazi *et al.*, 2006; Ghazi *et al.*, 2007). Microbial fructosyltransferases are important for the industrial production of FOS due to their higher fructose transferring activity as compared to the hydrolase activity (Gorrec *et al.*, 2002; Ghazi *et al.*, 2006; Soro *et al.*, 2007). The search for novel thermophilic microbial fructosyltransferases for biotechnological applications is ongoing (Van den Burg, 2003; Ghazi *et al.*, 2007).

Fructooligosaccharides are non-digestible, nonreducing water-soluble carbohydrates, which are currently gaining a lot of importance because of their excellent biological functional properties (L'Hocine *et al.*, 2000). The development of enzymatic systems for the synthesis of scFOS from cheap, renewable and readily available substrates such as sucrose is gaining importance (L'Hocine *et al.*, 2000; Ghazi *et al.*, 2005; Soro *et al.*, 2007). Fructooligosaccharides are mainly composed of 1-kestose (GF_2), nystose (GF_3) and 1^F-fructofuranosyl-nystose (GF_4) in which fructosyl units are bound at the β-2, 1 position of sucrose (Chen and Liu, 1996; Yun, 1996; Katapodis *et al.*, 2003). In recent years, the production of FOS by reverse hydrolysis and transfructosylation using microbial fructosyltransferases has attracted attention (Ghazi *et al.*, 2005).

Among the various optimisation strategies, response surface methodology (RSM), an experimental strategy for seeking the optimum conditions for a multivariable system, is a much more efficient approach for optimisation in contrast to one-factor-at-a-time approach which is too approximate and time consuming (Box and Wilson, 1951; Ma and Ooraikul, 1986; Chen *et al.*, 2002). Central composite design (CCD) is one of the most useful approaches in determining optimum conditions of many biochemical processes (Abdullah *et al.*, 2007). The CCD explores the response surfaces covered in the experimental design, thus making the optimisation process more efficient and effective and is especially suitable to account for the interactions of the factors (Chen *et al.*, 2002). It is however difficult to find the most important factors and to

optimise them, hence optimisation of reaction parameters can be approached by either empiric or statistical methods (Kalil *et al.*, 2001; Chen *et al.*, 2002).

A major problem encountered in the synthesis of FOS is that the purification of the FOS products is technically difficult. Consequently, available products on the market consist of a mixture of different sugars with some low levels of reducing sugars and sucrose which may not be suitable for diabetics (Crittenden and Playne, 1996; Yun *et al.*, 1997[a]). This problem can be solved by controlled enzymatic synthesis of FOS of specific chain length (Kim *et al.*, 1998). Microbial fructosyltransferases are reported to have both transferase and hydrolase activity and this causes a low yield of long chain FOS as a result of the hydrolase activity (Yun *et al.*, 2000; Ghazi *et al.*, 2007).

The synthesis of FOS of higher DP still remains a challenge and consequently, RSM was adopted in order to optimise the FOS yield with a minimal number of experimental runs. The principal objective of this study was to use RSM with central composite design (CCD) to maximise the production and yield of FOS using a commercial fructosyltransferase. The specific objectives were:

1   to use RSM for the optimisation of the synthesis of FOS.

2   to evaluate the effect of metal compounds and reagents on FOS synthesis.

3   to investigate the effect of initial glucose concentration on FOS synthesis.

4   to establish if monosaccharides act as precursors for FOS synthesis and to evaluate the effect of ammonium compounds on FOS synthesis.

5   to use a mixed enzyme system for glucose removal from the reaction mixture in order to increase FOS yield under optimal conditions.

4.2 Materials and Methods

4.2.1 Materials

Pectinex Ultra® SP-L, a commercial liquid enzyme preparation isolated from *Aspergillus aculeatus* was provided by Novozymes (South Africa). Glucose oxidase (GOD) and glucose (HK) Assay Kit (Product Code GAHK-20), for measuring glucose concentration, were purchased from Sigma-Aldrich (South Africa). A water bath for incubating reaction mixtures and sucrose were obtained from Merck, South

Africa. All other chemicals were obtained from Sigma-Aldrich (South Africa), Merck (South Africa) and other reputable commercial sources and all chemicals were of analytical grade unless otherwise stated.

4.2.2 Analytical Procedures

Identification and quantification of reaction products was done using high performance liquid chromatography with a refractive index detector (HPLC-RI), thin layer chromatography (TLC), and mass spectrometry with electrospray ionisation (MS-ESI) as previously described for the analytical procedures in Chapter 2, section 2.2.3.

4.2.3 Determination of fructosyltransferase activity

The fructosyltransferase activity was measured according to a modified procedure of Ghazi *et al.*, (2005) using sucrose as the substrate. The crude fructosyltransferase preparation (50 ml) was filtered through a Millipore membrane (0.45 µm) and then desalted by dialysing the crude enzyme against citrate-phosphate buffer (0.1 M, pH 5.6) at 4 °C (in the cold room) overnight with gentle stirring. The reaction mixture consisted of the sucrose substrate (4 ml, 10 % w/v) suspended in citrate-phosphate buffer (0.1 M, pH 5.6) and the crude dialysed fructosyltransferase (1 ml). The mixture was incubated (60 °C, 1 h) and the reaction terminated by boiling for 10 min. The crude enzyme preparation was replaced with distilled water in the control reaction mixture.

Sucrose conversion by a fructosyltransferase liberates glucose (and fructose which is partly used for FOS chain elongation) and the amount of glucose released allows determination of the overall enzyme activity. Therefore fructosyltransferase activity was determined based on the amount of glucose liberated after the reaction. Glucose released was measured by the Glucose (HK) Assay Kit (Product Code GAHK-20) as recommended by the manufacturer (Sigma-Aldrich) (Appendix A5. Glucose concentration was extrapolated from a glucose standard curve (Appendix A5, Figure A5). One unit of fructosyltransferase was defined as the amount of enzyme required to release 1 µmole of glucose per minute under the conditions of the experiment.

4.2.4 Preliminary investigation of FOS synthesis using crude fructosyltransferase

Approximate conditions for FOS synthesis were chosen and FOS were synthesised at 60 °C with sucrose (60 %, w/v) suspended in citrate-phosphate buffer (0.1 M, pH 5.6) for 8 h under batch conditions before adopting the RSM strategy. An aliquot of enzyme dosage (1 ml, 50 U/ml) was added to pure sucrose substrate (4 ml, 60 % w/v) and incubated at 60 °C for 8 h. The control tube contained boiled enzyme with the substrate and was incubated under same conditions with experimental tubes. A calibration curve was generated from authentic pure fructooligosaccharide standards using HPLC with a RI detector (Appendix A4, Figure A.3). The reaction products were identified and quantified by HPLC with a refractive index detector as previously described in section 2.2.3.1. The identity of the FOS was further confirmed by TLC and MS-ESI as described in sections 2.2.3.2 and 2.2.3.3 respectively.

4.2.5 Experimental design for FOS synthesis by the crude fructosyltransferase

From the preliminary experiments, temperature, sucrose concentration, pH, enzyme dosage and incubation time were found to be critical for FOS synthesis. Optimisation for the synthesis of FOS using the crude fructosyltransferase preparation was done using the RSM as key function of these critical parameters: temperature, sucrose concentration, enzyme dosage, time and pH. The RSM with CCD is as shown in Table 4.1 to 4.3. The coded levels were calculated using equation 2.1 in Chapter 2 section 2.2.7 and the experimental runs were performed according to Table 2.3 in Chapter 2.

4.2.6 FOS synthesis using crude fructosyltransferase with RSM

In the first RSM trial, FOS synthesis was carried out at sucrose concentrations ranging from 200 g/L to 600 g/L (X_1), temperature of 50 to 70 °C (X_2), and pH 4.8 to 6.4 (X_3), using crude fructosyltransferase (20 U/ml) for 4 h (Table 4.1). In the second RSM trial, FOS synthesis was carried out for 4 to 12 h (X_1), enzyme dosage 50 to 150 U/ml (X_2), sucrose concentration of 200 to 600 g/L (X_3), with temperature and initial pH being kept constant at 60 °C and 5.6 respectively (Table 4.2). In the third RSM trial, FOS synthesis was carried out for 16 to 24 h (X_1), enzyme dosage 50 to 150 U/ml

(X_2), sucrose concentration of 200 to 600 g/L (X_3), with temperature and initial pH being kept constant at 60 °C and 5.6 respectively (Table 4.3).

Table 4. 1 RSM and CC design for FOS synthesis using crude fructosyltransferase (20 U/ml) for 4 h.

Independent Variable	Symbol	Coded Levels		
		-1	0	+1
Sucrose Concentration (g/L)	X_1	200	400	600
Temperature (°C)	X_2	50	60	70
pH	X_3	4.8	5.6	6.4

Table 4. 2 RSM and CC design for FOS synthesis using crude fructosyltransferase at 60 °C, pH 5.6.

Independent Variable	Symbol	Coded Levels		
		-1	0	+1
Time (h)	X_1	4	8	12
Enzyme Dosage (U/ml)	X_2	50	100	150
Sucrose Concentration (g/L)	X_3	200	400	600

Table 4. 3 RSM and CC design for FOS synthesis using crude fructosyltransferase at 60 °C, pH 5.6.

Independent Variable	Symbol	Coded Levels		
		-1	0	+1
Time (h)	X_1	16	20	24
Enzyme Dosage (U/ml)	X_2	50	100	150
Sucrose Concentration (g/L)	X_3	200	400	600

In all experimental runs, the reaction mixture consisted of the crude fructosyltransferase solution (1 ml) and sucrose (4 ml) in citrate-phosphate buffer (0.1

M) at appropriate pH. For long term incubation, 0.02 % (w/v) NaN_3 was added to the reaction mixture to prevent microbial growth. Each experiment was performed in duplicate and the reactions were terminated by boiling for 10 min. The samples were stored in vials at -20 °C pending analysis by HPLC as previously described in Chapter 2 section 2.2.3.1. The average FOS yield from a duplicate determination was taken as the response.

4.2.7 FOS synthesis using purified fructosyltransferase

In order to increase the yield of FOS, the crude fructosyltransferase was purified to electrophoretic homogeneity (Nemukula and Whiteley, *pers. com.*). The purified fructosyltransferase (20 U/ml) was used for the synthesis of FOS using RSM as previously described for the crude fructosyltransferase above. This was done by varying sucrose concentration (200 to 600 g/L) (X_1), temperature, (50 to 70 °C) (X_2), and pH 4.8 to 6.4 (X_3). The experimental runs using the coded levels were as described in Table 2.2 in Chapter 2.

4.2.8 FOS Synthesis using monosaccharides as substrates

This experiment was performed to establish if FOS could be synthesised from a mixture of simple monosaccharides in the absence of sucrose by the dialysed crude fructosyltransferase. This could give an insight into the mechanism of action of this enzyme. A mixture of glucose and fructose (40 %, w/v), prepared in citrate-phosphate buffer (0.1 M, pH 5.6), in equal proportions, was then used as a substrate for FOS synthesis using the crude fructosyltransferase (20 U/ml) at 60 °C for 8 h as previously described in section 4.2.4. The reaction products were taken at 1 h interval and the reaction was stopped by boiling for 10 min. The products were analysed by HPLC as previously described in Chapter 2 section 2.2.3.1 and authentic FOS were used as standards (Appendix A4).

4.2.9 Effect of ammonium compounds on the synthesis of FOS

Ammonium compounds are reported to be activating compounds for fructosyltransferase and to therefore enhance the synthesis of FOS (Park *et al.*, 1999).

FOS were synthesised under optimal conditions in the presence of ammonium ions. A sucrose solution (600 g/L, 1 200 µl) in citrate-phosphate buffer (0.1 M, pH 5.6) was prepared in the presence of ammonium compound (100 mM). The ammonium compounds used were ammonium nitrate, ammonium chloride, ammonium sulphate, ammonium dihydrogen phosphate and urea. The dialysed crude fructosyltransferase (50 U/ml, 300 µl) was used for FOS synthesis at 4 h and 60 °C as previously described in section 4.2.4 and the reactions were performed in triplicate. The effect of the ammonium compounds was tested on the yield of GF_2, GF_3 and GF_4 relative to the control sample which contained no ammonium salt. The reaction mixture was boiled for 10 min to stop the reaction and was analysed by HPLC as previously described in Chapter 2 section 2.2.3.1.

4.2.10 Effect of compounds on FOS synthesis by the crude fructosyltransferase

The influence of different metals and reagents was investigated on the crude fructosyltransferase activity. The following reagents were prepared in the presence of sucrose (600 g/L) suspended in citrate-phosphate buffer (0.1 M, pH 5.6). A 10 mM concentration of N-bromosuccinimide (NBS), barium chloride, silver chloride, calcium chloride, magnesium chloride, ferric chloride hexahydrate, manganese chloride, zinc chloride, copper sulphate heptahydrate, ferrous sulphate heptahydrate, and the chelating agent EDTA were included in the substrate solution. A control sample was set up without added compounds under same conditions with the experimental samples. FOS synthesis was carried out at 60 °C for 3 h under batch conditions as previously described in section 4.2.4 and the reaction products were analysed by HPLC. The FOS synthesised in the presence of metal ions and reagents was calculated relative to the amount of GF_2, GF_3, and GF_4 synthesised in the control sample that had no added metal ions or reagents.

4.2.11 Effect of initial glucose on FOS synthesis using fructosyltransferase

Glucose is produced as a by-product with concomitant synthesis of FOS by a fructosyltransferase. Sucrose (600 g/L, w/v, pH 5.6) with initial glucose concentration ranging from 0 to 40 % (w/v) was used for FOS synthesis at 60 °C for 8 h with the crude fructosyltransferase as previously described in section 4.2.4. The

aliquots of the reaction products were taken at 1 h interval and the reaction was stopped by boiling for 10 min and the reaction mixture was analysed by HPLC.

4.2.12 Mixed enzyme reaction for glucose removal

Glucose was observed to have an inhibitory effect on FOS synthesis, therefore a mixed enzyme system was evaluated for glucose removal from the reaction mixture in order to increase the FOS yield. The method used was a modification of the procedure by Sheu *et al.*, (2001), using a mixture of fructosyltransferase from *Aspergillus japonicus* and a commercial glucose oxidase. Glucose oxidase converts glucose to gluconic acid and hydrogen peroxide by oxidation. The crude fructosyltransferase (50 U/ml, 500 µl) was mixed with glucose oxidase (30 U/ml, 500 µl) and sucrose (60 %, w/v, 4 ml) in citrate-phosphate buffer (0.1 M, pH 5.6). The reaction was initiated by adding 1 ml of the mixed enzyme solution with 4 ml of the sucrose substrate. The incubation temperature was lowered to 40 °C in order to avoid denaturation of glucose oxidase at temperatures above 50 °C. Aliquots were taken at 1 h interval for 8 h and then analysed by HPLC as previously described in chapter 2 section 2.2.3.1. The pH decreased from 5.6 to 4.5 after 8 h of incubation due to production of gluconic acid even though the reaction mixture was buffered in citrate-phosphate buffer.

4.2.13 Statistical Analysis

Statistical analysis was performed as stated in Chapter 2 section 2.2.9.

4.3 Results

4.3.1 Determination of fructosyltransferase activity

The fructosyltransferase activity in the dialysed crude commercial preparation was found to be 177 U/ml.

4.3.2 Preliminary investigation of FOS Synthesis by the crude fructosyltransferase

The pattern of FOS synthesis was investigated from preliminary batch experiments. Fructose composition in the reaction mixture increased gradually from 3.30 % after 1 h reaction to 10.36 % after 8 h incubation (Figure 4.1). Glucose levels increased from 22.13 % after 1 h to 36.95 % after 8 h incubation. Sucrose composition decreased sharply from the initial 100 % to 11.76 % after 8 h reaction. FOS composition in the reaction mixture after 8 h reaction was as follows: 1-kestose (16.15 %), nystose (22.89 %) and fructofuranosyl nystose (1.89 %) (Figure 4.1). In order to confirm the data generated by HPLC, the reaction products were monitored by TLC as shown in Figure 4.2. No FOS were detected in the control experiment indicating that the crude fructosyltransferase was responsible for FOS synthesis.
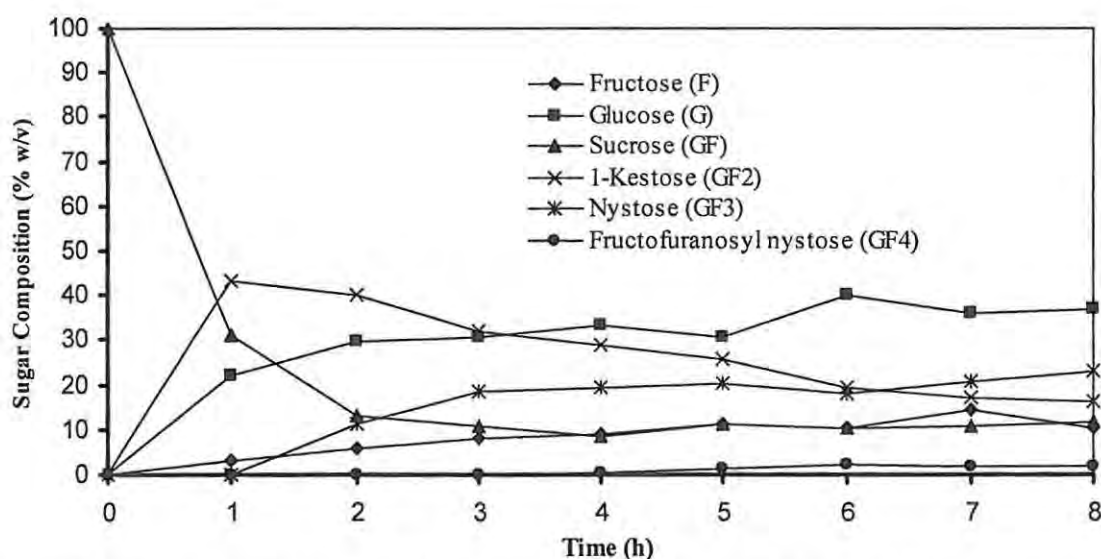
Figure 4.1 Preliminary investigation of FOS synthesis from sucrose (600 g/L, pH 5.6) at 60 °C for 8 h with dialysed 20 U/ml crude fructosyltransferase. The profile of the reaction products were analysed by HPLC with RI detection.

G F GF GF$_2$ GF$_3$ GF$_4$ T$_0$ T$_{0.5h}$ T$_{1h}$ T$_{1.5h}$ T$_{2h}$ T$_{3h}$ T$_{4h}$ T$_{5h}$ T$_{6h}$ T$_{7h}$ T$_{8h}$
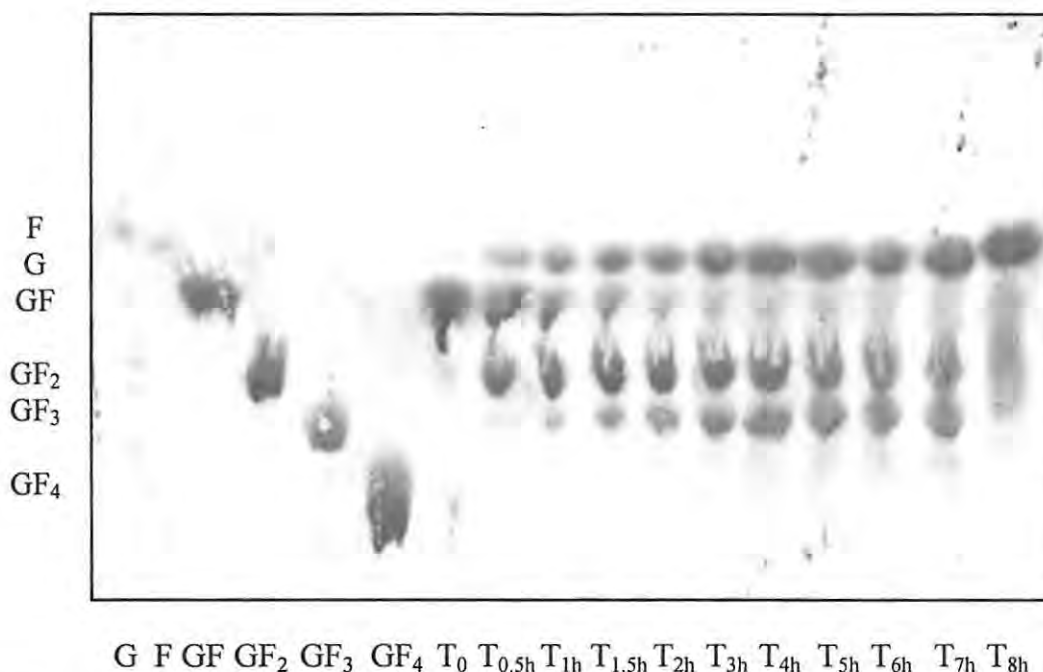
Figure 4.2 TLC chromatogram of FOS synthesised from sucrose (600 g/L) at 60 °C for 8 h in a preliminary batch experiment. The plate was developed with butanol/acetic acid/water (5:4:1) (v/v/v) in a developing tank. Ascending development was repeated twice for good resolution of the FOS spots. The plate was allowed to dry in a fume hood and the spots were visualised by spraying with anisaldehyde spray reagent before heating in an oven at 110 °C for 15 min.

4.3.3 FOS synthesis by RSM using crude fructosyltransferase

The experimental conditions of sucrose concentration 600 g/L, temperature 60 °C, and pH 5.6 promoted the synthesis of high levels of GF$_2$ (68.61 mM) from experimental run 10 (Figure 4.3). Response surface regression predicted GF$_2$ levels to be 66.23 mM (R^2 of 0.90, $p < 0.05$) under same conditions as the experimental run 10 (Table 4.4). On the other hand, sucrose concentration of 400 g/L temperature 60 °C, and pH 5.6 favoured the synthesis of high levels of GF$_3$ and GF$_4$. There was a strong correlation between the actual FOS synthesised and predicted concentrations with the high R^2 values. The actual and predicted maxima of the FOS yield were statistically significant (p < 0.05) (Table 4.4). No GF$_3$ and GF$_4$ were synthesised in the experimental runs 3, 7, 8 and 12 (Figure 4.3).

Table 4.4 Maximum actual and predicted FOS concentrations produced in the first RSM runs.

FOS	Actual maximum concentration (mM)	Predicted maximum concentration (mM)	R^2	p
GF_2	68.61	66.23	0.90	0.046
GF_3	16.31	14.28	0.93	0.022
GF_4	5.88	4.48	0.91	0.034
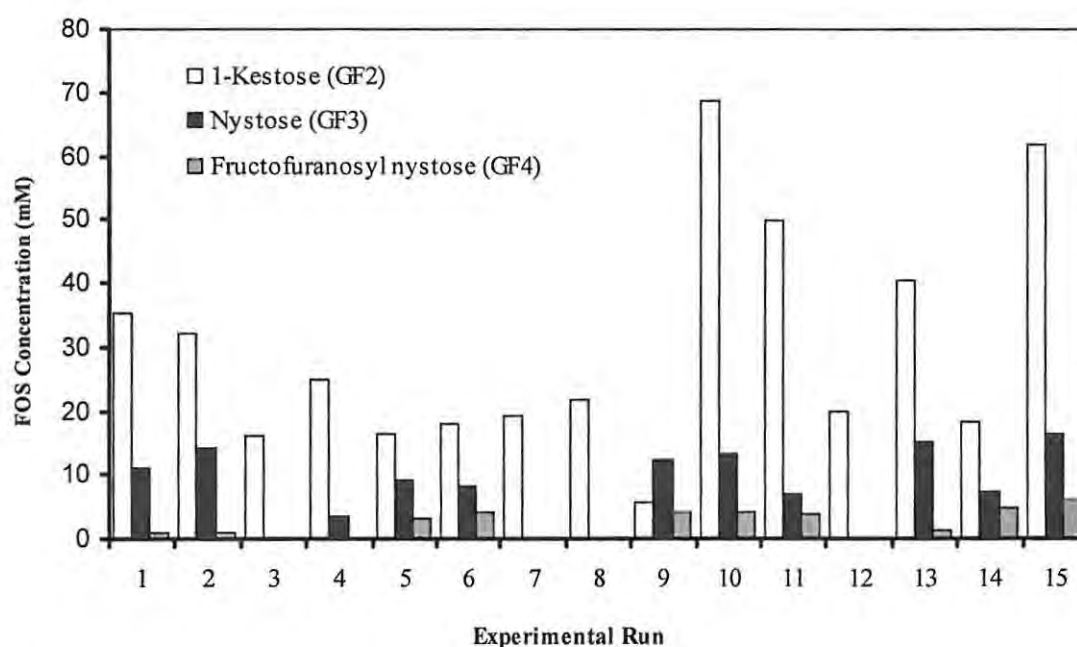
Figure 4.3 FOS synthesis by RSM using dialysed crude fructosyltransferase (20 U/ml) by simultaneously varying sucrose concentration (200 to 600 g/L), temperature (50 to 70 °C) and pH (4.8 to 6.4). The experimental runs were incubated for 4 h and the conditions of each experimental run were described in Chapter 2 Table 2.3.

In the second RSM experimental runs, the maximum yield of GF_2 was 152.07 mM after 4 h incubation, with 50 U/ml enzyme dosage at 600 g/L sucrose concentration pH 5.6 at 60 °C (Figure 4.4). The response surface regression model predicted 148 mM of GF_2 (R^2 of 0.99, $p < 0.05$) under the same experimental conditions. The highest GF_3 yield was 131.38 mM in experimental run 5 and the response surface regression model predicted 145.58 mM (R^2 of 0.95, $p < 0.05$). Maximal GF_4 was obtained in experimental run 14 with a yield of 43.99 mM after 8 h incubation with 100 U/ml at a sucrose concentration of 600 g/L. Response surface regression model

predicted 43.59 mM (R^2 of 0.96, $p < 0.05$) after a 12 h incubation period, 100 U/ml enzyme concentration and 600 g/L sucrose concentration.

Table 4.5 Maximum actual and predicted FOS concentrations produced in the second RSM runs.

FOS	Actual maximum concentration (mM)	Predicted maximum concentration (mM)	R^2	p
GF_2	152.07	148.00	0.99	0.001
GF_3	131.38	145.58	0.95	0.005
GF_4	43.99	44.59	0.96	0.031
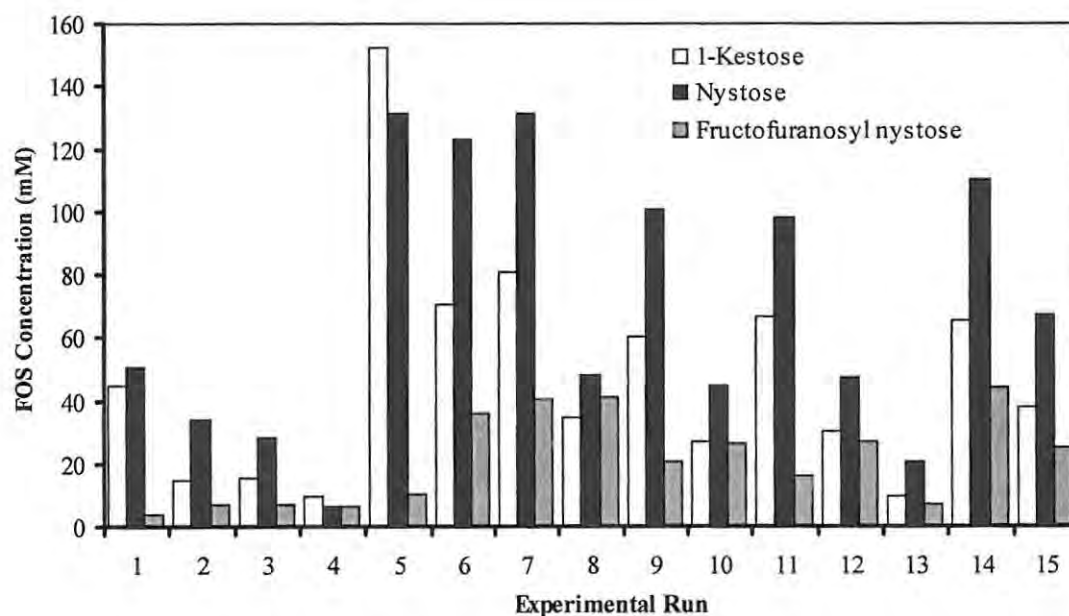
Figure 4.4 FOS synthesis by RSM using dialysed crude fructosyltransferase by simultaneously varying time (4 to 12 h), enzyme concentration (50 to 150 U/ml) and sucrose concentration (200 to 600 g/L). The reaction products were analysed and quantified by HPLC.

Time significantly affected FOS yield and composition, consequently in the third RSM experimental runs, time was varied from 16 to 24 h and temperature and pH were kept constant at 60 °C and 5.6 respectively. The maximal GF_2 yield (211.09 mM) was obtained after 16 h incubation, enzyme dosage (100 U/ml), and sucrose concentration (400 g/L) (Figure 4.5). Using response surface regression model, GF_2 concentration was predicted to be 183.46 mM (R^2 of 0.86, $p > 0.05$) under similar experimental conditions. Maximal GF_3 yield (156.06 mM) was achieved after 16 h incubation, enzyme concentration of 150 U/ml, and with a sucrose concentration 600

95

g/L. Response surface regression predicted a slightly higher yield of GF_3 (159.98 mM) (R^2 of 0.97, $p < 0.05$) after 24 h incubation with 150 U/ml enzyme dosage and sucrose concentration of 600 g/L. The highest yield of GF_4 (22.5 mM) was obtained after 24 h incubation, 150 U/ml enzyme dosage and a 600 g/L sucrose concentration (Figure 4.5). The predicted GF_4 yield was the same (22.5 mM, R^2 of 0.99, $p < 0.05$) to the empirical GF_4 yield under similar experimental conditions.

Table 4.6 Maximum actual and predicted FOS concentrations produced in the third RSM runs.

FOS	Actual maximum concentration (mM)	Predicted maximum concentration (mM)	R^2	P
GF_2	211.09	183.46	0.86	0.09
GF_3	156.06	159.98	0.97	0.002
GF_4	22.5	22.5	0.99	0.012
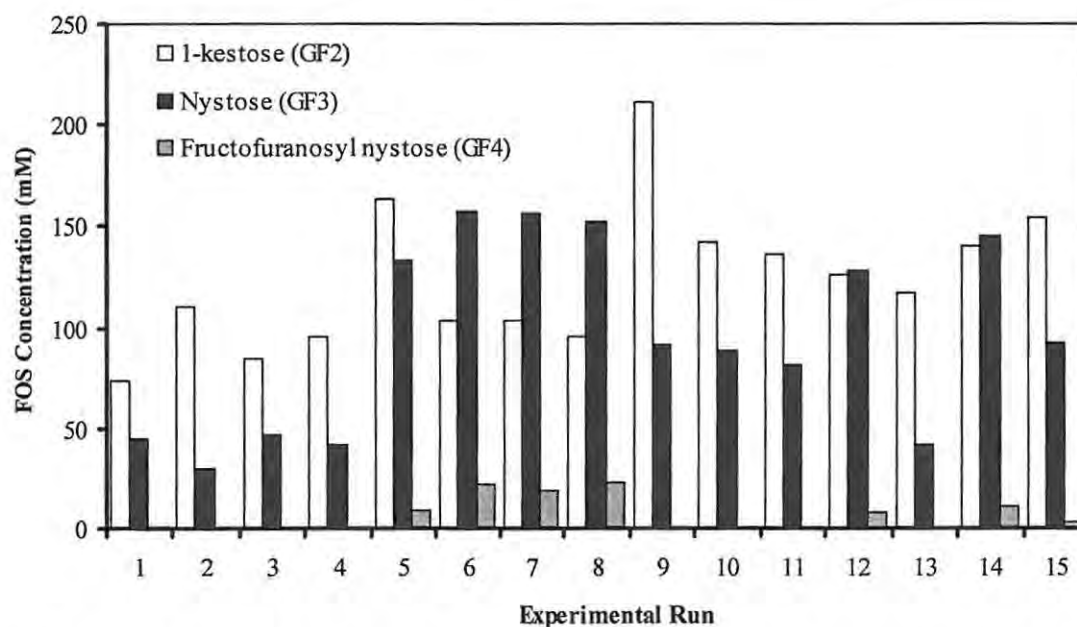
Figure 4.5 FOS synthesis by RSM using dialysed crude fructosyltransferase by simultaneously varying time (16 to 24 h), enzyme concentration (50 to 150 U/ml) and sucrose concentration (200 to 600 g/L). The reaction products were analysed and quantified by HPLC.

In all the experimental runs, no GF_5 and higher were synthesised by the crude fructosyltransferase. To confirm this, the reaction products in the second RSM, experimental run 5 were subjected to MS-ESI and the results are presented in Figures

4.6 to 4.8. The reaction mixture consisted of sucrose (600 g/L), dialysed crude fructosyltransferase (50 U/ml), and the reaction was carried out at 60 °C for 4 h. A mixture of authentic FOS standards (1 mM) was delivered into the ion source by a syringe pump and monitored in the positive mode. The m/z ratio of 187.03, 349.15, 511.24, 673.13, and 835.29 correspond to monosaccharides, sucrose, GF_2 GF_3 and GF_4 respectively (Figure 4.6).
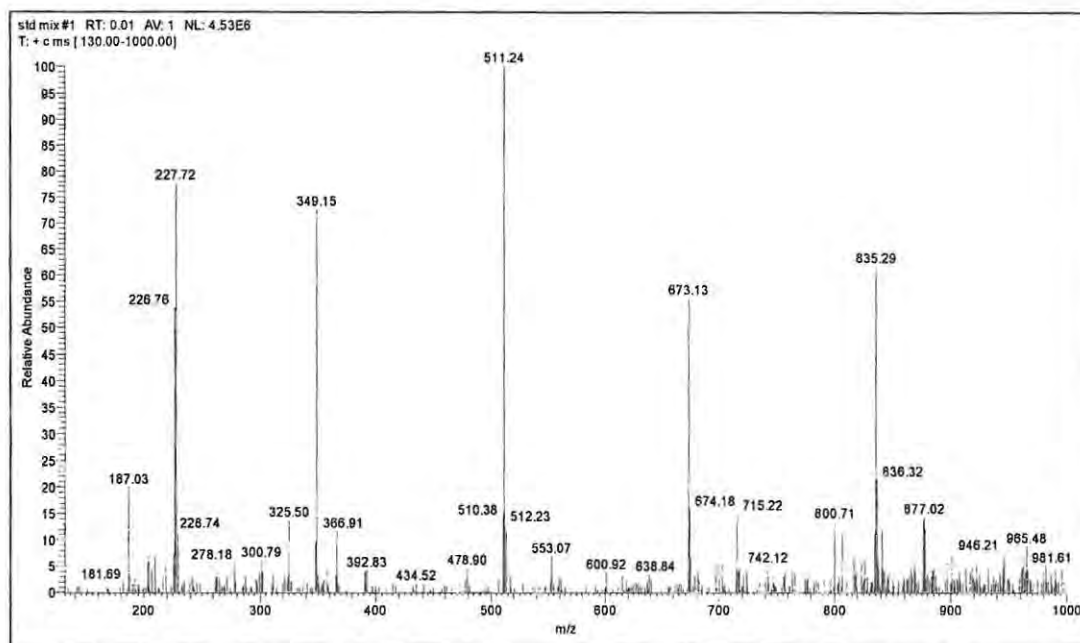
Figure 4.6 MS-ESI trace of 1mM FOS standard mixture ionised with 1 mM LiCl.

The sample was withdrawn from the reaction mixture at the beginning of the reaction (time 0) and boiled for 10 minutes to stop the reaction. The reaction products were analysed with MS-ESI in the positive ion mode after ionising the sugar sample with 1 mM LiCl. The m/z ratio of 349.09 corresponds to sucrose $[M + Li]^+$ (Figure 4.7). At the end of the reaction, the reaction mixture was boiled for 10 minutes to stop the reaction. The reaction products were analysed with MS-ESI in the positive ion mode after ionising the reaction mixture with 1 mM LiCl and the m/z ratio of 187.98, 349.19, 511.25, 673.27, and 835.45 correspond to $[M + Li]^+$ of monosaccharides, disaccharides, GF_2 GF_3 and GF_4 respectively (Figure 4.8). The absence of FOS of higher DP (> 5) in this reaction mixture, confirms the data obtained by HPLC with refractive index detection.
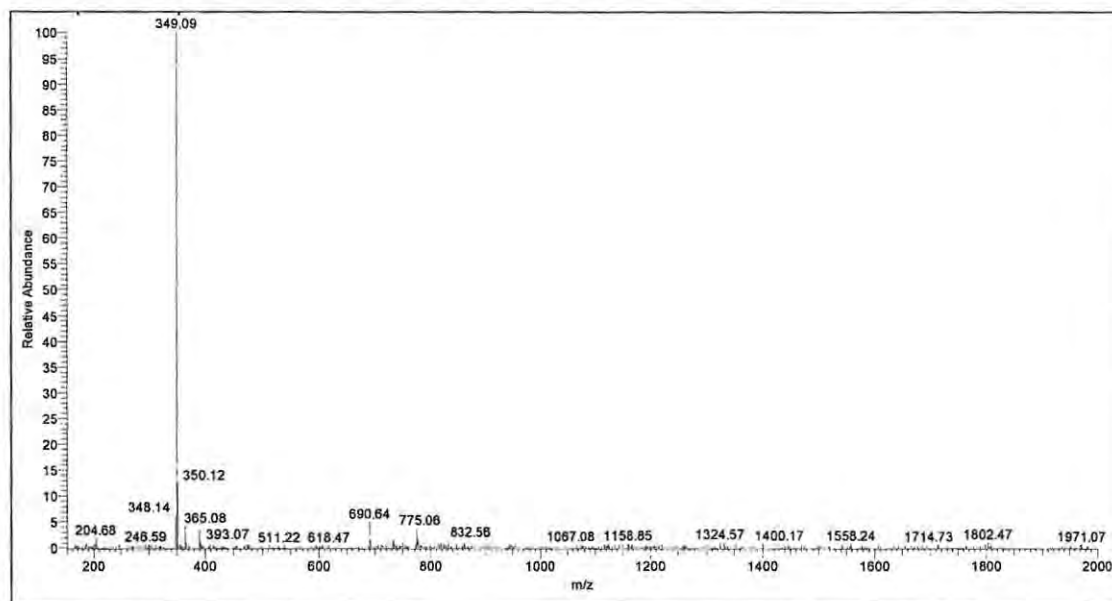
Figure 4.7 MS-ESI of the reaction mixture at the start of the reaction in experimental run 5 of the second RSM.
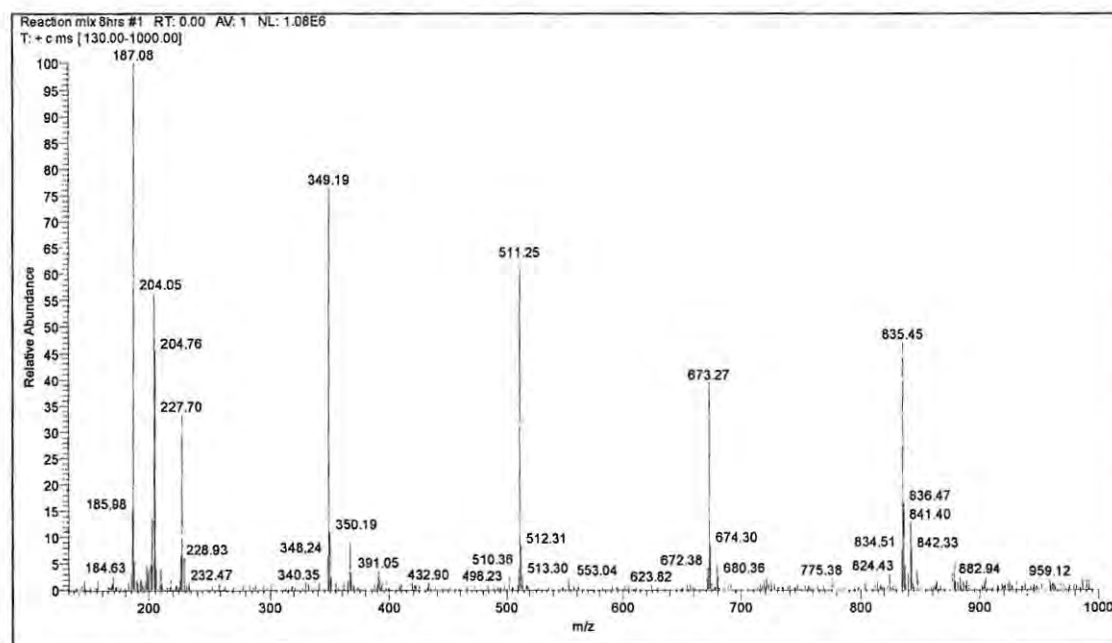
Figure 4.8 MS-ESI of the reaction mixture at the end of 4 h incubation in experimental run 5 of the second RSM.

4.3.4 FOS synthesis using purified fructosyltransferase

In a separate study in our laboratory (Nemukula and Whiteley, *pers. com*), the crude fructosyltransferase was purified to electrophoretic homogeneity. The purified enzyme was used for FOS synthesis using RSM with CCD. All the experimental runs

were performed for 4 h with 20 U/ml enzyme dosage. Three parameters were simultaneously varied, namely sucrose concentration (200 to 600 g/L), temperature (50 to 70 °C) and pH (4.8 to 6.4). The maximum GF_2 synthesised was 199.36 mM in experimental run 6 at 600 g/L sucrose concentration, at a temperature of 50 °C at initial pH 6.4 (Figure 4.9). Response surface regression model predicted 205.17 mM of 1-kestose (R^2 of 0.87, $p > 0.05$) with 600 g/L sucrose concentration at 60 °C at initial pH 5.6. Nystose yield (73.32 mM) was achieved in experimental run 10 at 600 g/L sucrose concentration, 60 °C at initial pH 5.6 (Figure 4.9). The yield of nystose was predicted by response surface regression model and was found to be 57.66 mM (R^2 of 0.81 $p > 0.05$) at 600 g/L, 60 °C at initial pH 5.6. No GF_4 was synthesised using the purified FTase in all the experimental runs.
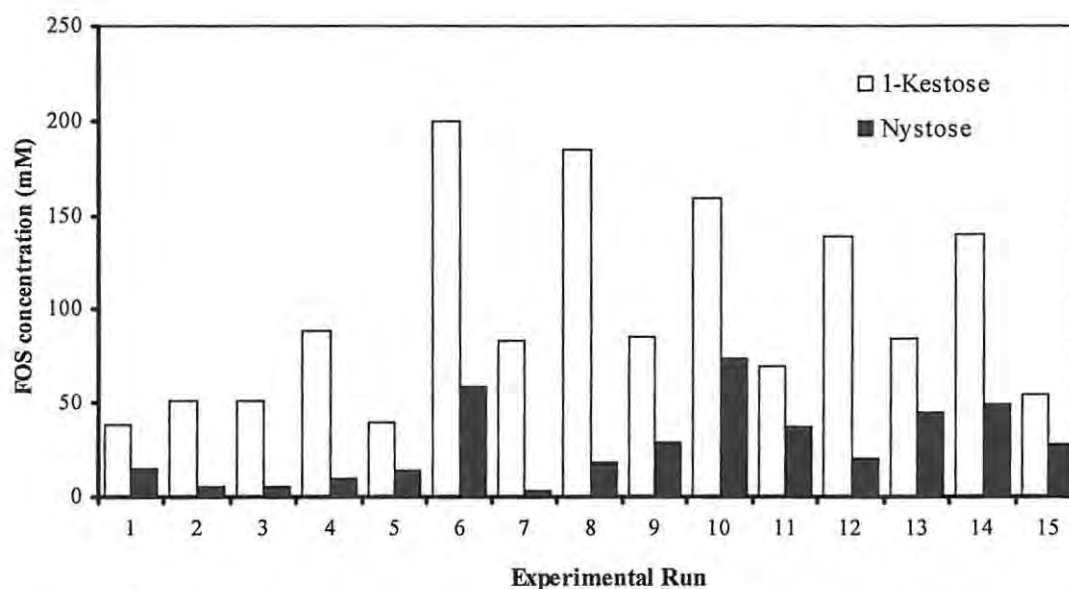
Figure 4.9 Synthesis of FOS by a purified fructosyltransferase using RSM by simultaneously varying sucrose concentration (200 to 600 g/L), temperature (50 to 70 °C) and pH (4.8 to 6.4). Enzyme dosage and incubation time were kept at 20 U/ml and 4 h respectively in all experimental runs. The reaction mixture was analysed and quantified by HPLC with a RI detector.

4.3.5 FOS synthesis using monosaccharides as substrates

The substrate for FOS synthesis was a mixture of glucose and fructose (40 %, w/v) in equal proportions suspended in citrate-phosphate buffer (0.1 M, pH 5.6) with a crude fructosyltransferase (20 U/ml). The crude fructosyltransferase did not synthesise any fructose linked FOS from a mixture of glucose and fructose as revealed by HPLC

(Figure 4.10). This indicates that fructose and glucose are not precursors for FOS synthesis hence neither of the two acts as a donor or acceptor for FOS synthesis.
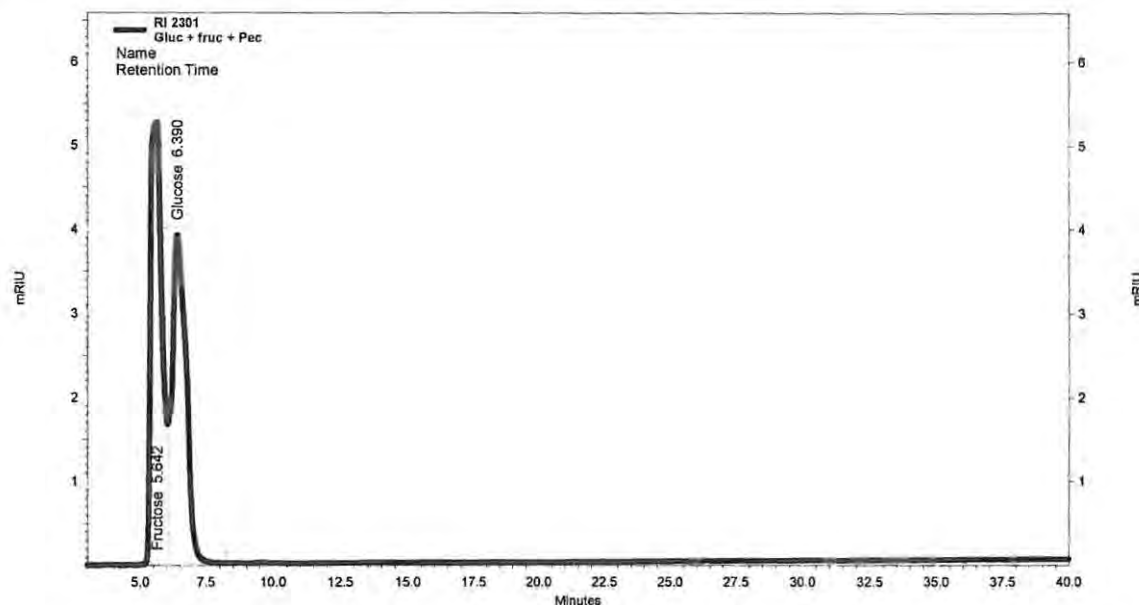
Figure 4.10 HPLC chromatogram of a reaction mixture after 8 h of incubation at 60 °C showing only fructose and glucose in the reaction mixture.

4.3.6 Effect of initial glucose on FOS synthesis

Initial glucose concentration in the reaction mixture was investigated for its effect on FOS synthesis by the crude fructosyltransferase. Glucose concentration was varied from 0 to 40 % (w/v) with sucrose concentration (600 g/L) as the substrate and crude fructosyltransferase (20 U/ml). The yield of GF_2 decreased from 172.32 ± 5.28 mM without any initial glucose to 10.28 ± 1.34 mM with 30 % initial glucose (Figure 4.11). GF_3 yield decreased from 75.28 ± 3.82 mM without added glucose to 2.82 ± 0.04 mM with 30 % initial glucose (Figure 4.11). GF_4 decreased from 4.91 ± 0.92 mM without added glucose to 1.82 ± 0.05 mM with 10 % initial glucose and no GF_4 was synthesised with 30 % and 40 % initial glucose in the reaction mixture (Figure 4.11). There was complete inhibition of fructosyltransferase activity at 40 % initial glucose, with no scFOS detected in the reaction mixture (Figure 4.12). Unreacted sucrose, glucose and traces of fructose were detected in the reaction mixture.
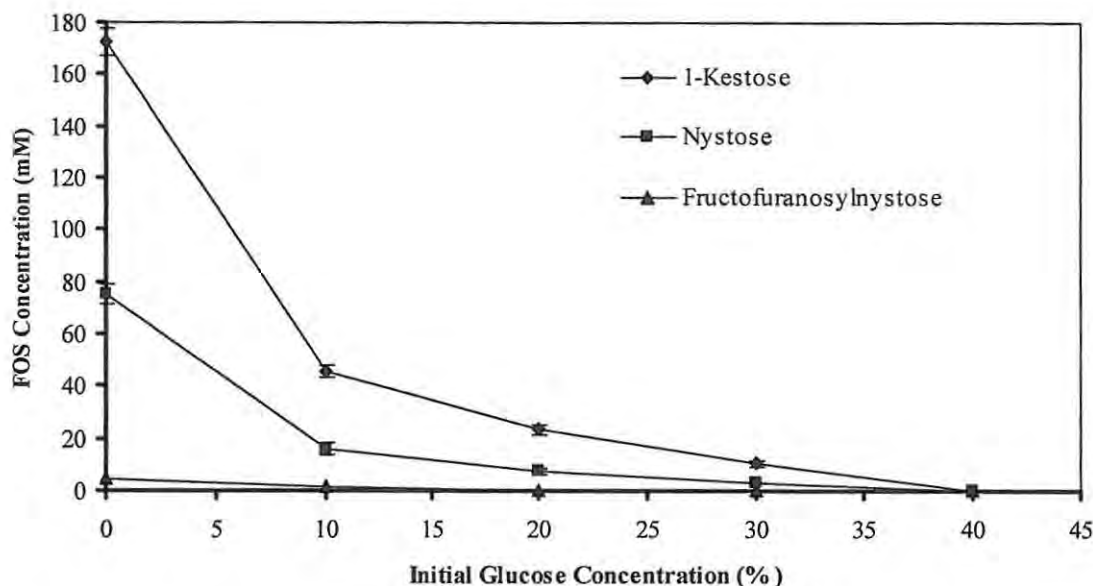
Figure 4.11 Effect of initial glucose concentration (0 to 40 % w/v) on FOS synthesis by the fructosyltransferase (20 U/ml) in the presence of sucrose (60 % w/v, pH 5.6). The reaction mixture was incubated for 8 h at 60 °C. The FOS yield was quantified by HPLC with RI detection. Each point on the graph represents the mean ± SD of three replicate determinations.
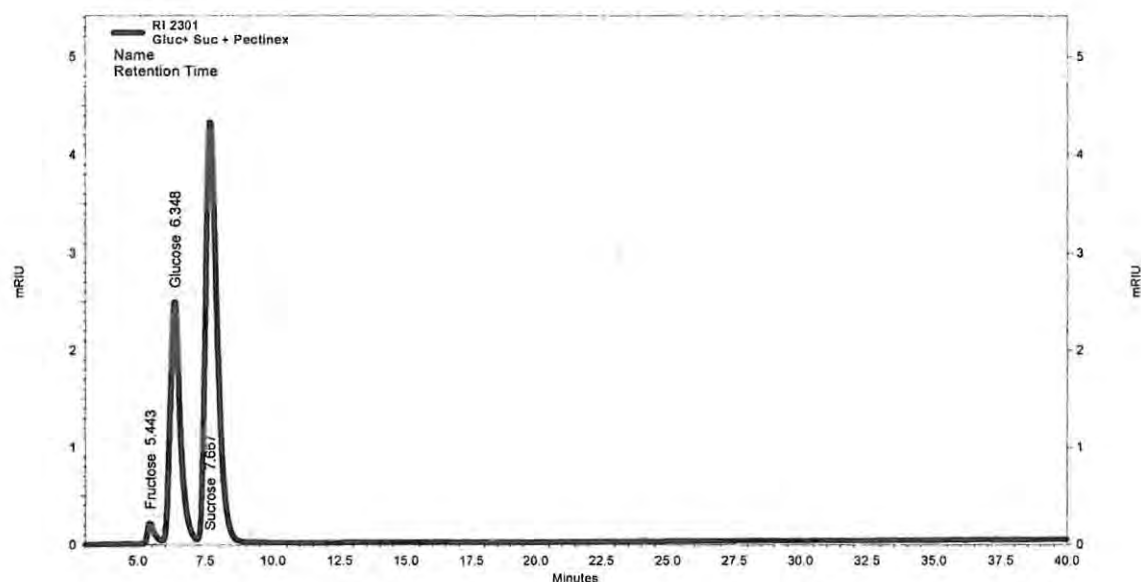
Figure 4.12 HPLC chromatogram showing the effect of initial glucose (40 %, w/v) on FOS synthesis by the fructosyltransferase (20 U/ml). The reaction mixture was incubated with sucrose (60 % w/v, pH 5.6) for 8 h at 60 °C. The reaction mixture was analysed by HPLC with RI detection. The identities of the sugars and retention times are displayed on the peaks.

4.3.7 Effect of ammonium compounds on the synthesis of FOS

Ammonium compounds were investigated for their effect on the synthesis of GF_2, GF_3 and GF_4 under batch conditions using crude fructosyltransferase. Ammonium

nitrate enhanced the yield of GF_4 (142 ± 2.91 %) and ammonium chloride increased the yield of GF_4 (112.3 ± 2.81 %) (Figure 4.13). This shows that ammonium nitrate acts as an activator for transferase activity. Ammonium sulphate increased the yield of GF_4 (119.4 ± 2.77 mM). The effect of different ammonium compounds on the yield of GF_2 ranged from 91.1 to 107.6 %. Generally, all the ammonium ions tested resulted in an increase in FOS yield (Figure 4.14).
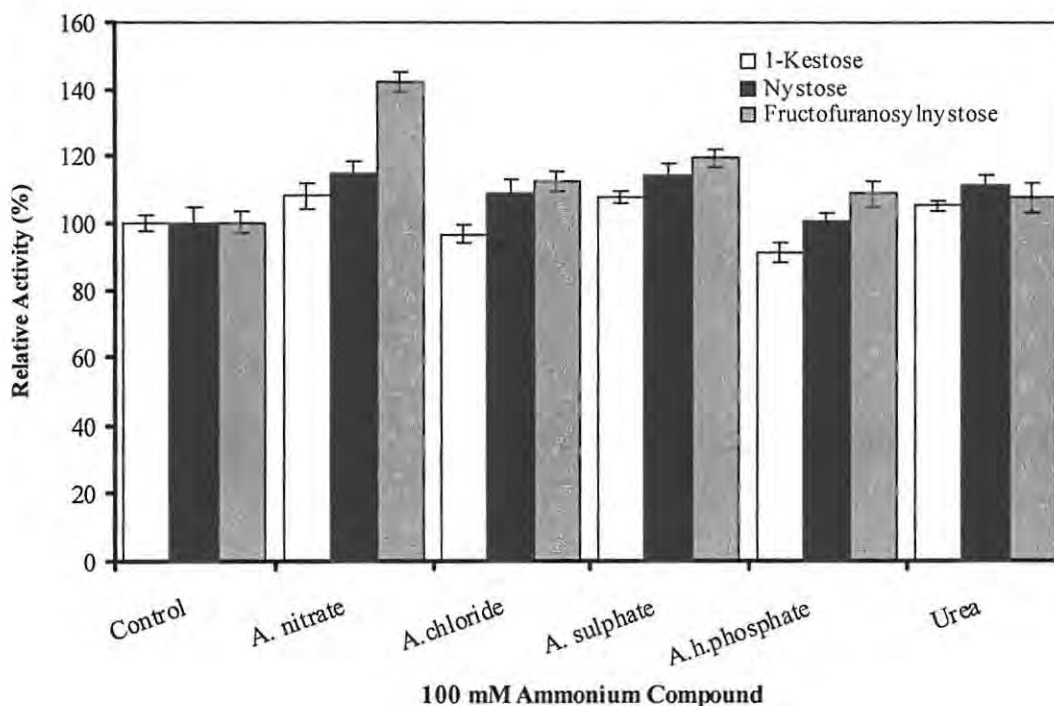
Figure 4.13 Effect of different ammonium compounds (100 mM) on the yield of FOS. The ammonium compounds were mixed with sucrose (60 % w/v, pH 5.6) and incubated at 60 °C fro 4 h. The data points represent the mean of 3 replicates and the error bars represent standard deviations (± SD). The FOS yield from the control reaction mixture was 78.7 mM, 112.3 mM and 35.0 mM of GF_2, GF_3 and GF_4 respectively and the mean data values were calculated relative to concentrations in the control reaction mixture. (A) and (h) represents ammonium and hydrogen respectively in the names of the compounds in the x-axis.

4.3.8 Effect of compounds on FOS synthesis by the crude fructosyltransferase

The effects of different reagents and compounds were evaluated on the yield of FOS synthesised by the crude fructosyltransferase. NBS and barium chloride are reported to be strong inhibitors of hydrolase activity, therefore these compounds were investigated in order to stop hydrolase activity and promote transferase activity. The following metallic monovalent and divalent cations, Mg^{2+}, Ca^{2+}, Mn^{2+}, and Co^{2+} slightly enhanced the levels of GF_2, GF_3, and GF_4 with varying degrees suggesting

that these ions are activators for this enzyme (Table 4.7). FOS yield was reduced by 50 % in the presence of Fe^{2+} and in the presence of Zn^{2+} the relative yield of GF_2, GF_3, and GF_4 was 18 ± 2.1 %, 21 ± 2.3 %, and 41 ± 3.2 % respectively. No FOS were synthesised in the presence of Fe^{3+}, Cu^{2+}, Ag^+, Ba^{2+} and N-bromosuccinimide indicating that they are strong inhibitors of the fructosyltransferase enzyme (Table 4.7). The chelating agent EDTA had no significant effect on FOS synthesis by the fructosyltransferase.
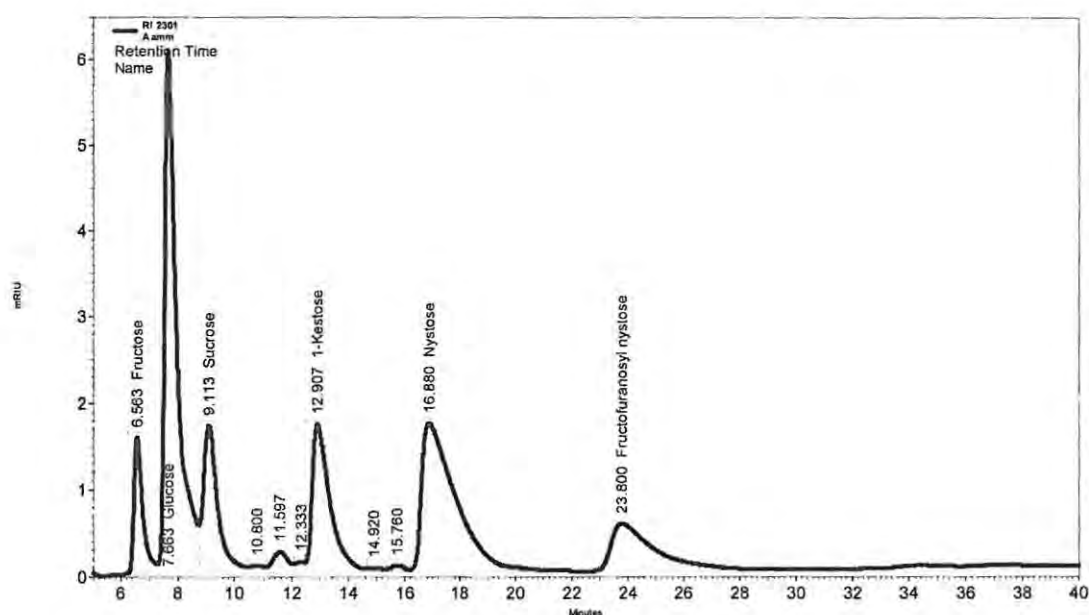
Figure 4.14 HPLC chromatogram showing different FOS synthesised in the presence of ammonium chloride (100 mM). The reaction mixture consisted of sucrose (60 %, pH 5.6) and 100 mM ammonium chloride at 60 °C for 4 h. The reaction products were boiled to stop the enzyme reaction and then analysed by HPLC.

4.3.9 Mixed enzyme reaction for glucose removal

By using a mixed enzyme system for FOS synthesis under batch conditions, there was an improved yield of FOS suggesting that the system is efficient. After 8 h of incubation, the composition of GF_2, GF_3 and GF_4 was 22.87 %, 28.91 and 25.89 % respectively (Figure 4.15). After 8 h reaction, 9.89 % of unreacted sucrose remained in the reaction mixture. Though glucose was not completely eliminated from the reaction mixture, glucose levels were significantly reduced from 14.02 % after 5 h of reaction to 1.86 % after 8 h of reaction.

Table 4.7 Effect of compounds on FOS yield by the crude fructosyltransferase.

Metal Compound	Metal Concentration	Relative FOS Yield (%) [a]		
		GF_2	GF_3	GF_4
Control	-	100	100	100
$MgCl_2.6H_2O$	10 mM	109 ± 6.7	110 ± 5.1	107 ± 5.9
$CaCl_2$	10 mM	103 ± 4.3	106 ± 3.8	98 ± 5.2
$FeCl_3.6H_2O$	10 mM	0	0	0
$MnCl_2$	10 mM	107 ± 5.4	108 ± 3.6	88 ± 4.3
$ZnCl_2$	10 mM	18 ± 2.1	21 ± 2.3	41 ± 3.2
$CuSO_4.7H_2O$	10 mM	0	0	0
$FeSO_4.7H_2O$	10 mM	50 ± 4.5	49 ± 3.2	50 ± 5.3
$CoCl_2.6H_2O$	10 mM	123 ± 4.9	127 ± 6.1	132 ± 4.8
AgCl	10 mM	0	0	0
EDTA	1 %	100 ± 5.2	100 ± 4.7	100 ± 4.5
$BaCl_2.2H_2O$	10 mM	0	0	0
N-bromosuccinimide	10 mM	0	0	0

[a] Data represent the mean of 3 replicates with the standard deviations shown ($\pm$ SD). The data is relative to FOS synthesised in the control experiment with GF_2, GF_3, and GF_4 concentration of 78.7 mM, 112.3 mM, and 35 mM respectively.
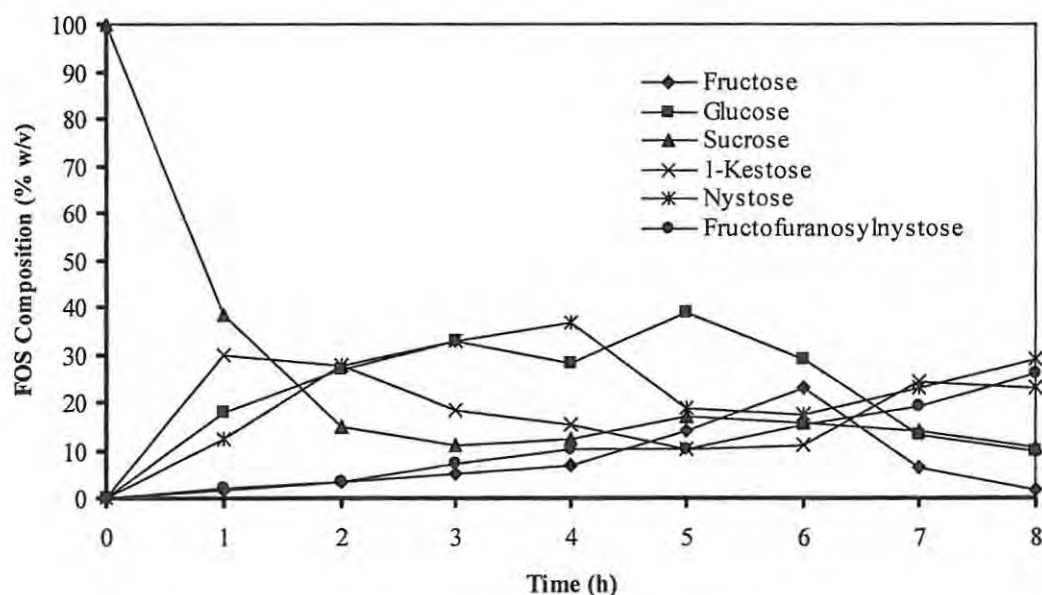
Figure 4.15 Time course monitoring of FOS composition and evaluation of the mixed enzyme system for the removal of glucose from the reaction mixture. The system was set up with crude fructosyltransferase (50 U/ml) with glucose oxidase (30 U/ml) in the presence of sucrose (60 %, w/v) at 40 °C for 8 h. The reaction products were analysed by HPLC.

4.4 Discussion

The preliminary experiment established the critical multivariate parameters to be subjected to RSM for the optimisation of FOS synthesis by a crude fructosyltransferase. The crucial variables chosen from the preliminary investigation were temperature, sucrose concentration, enzyme dosage, reaction time, and pH. The mild pH regime prevented acid hydrolysis of the synthesised FOS which occurs at pH below pH 3. Furthermore, the pH (5.6) promoted transfructosylation instead of the hydrolase activity which is reported to occur at lower pH around 4 (Katapodis, *et al.*, 2003). Ghazi *et al.*, (2007) characterised a fructosyltransferase from *Aspergillus* and found that it had a broad optimal pH of 5.0 to 7.0. The temperature of 60 °C was advantageous because it increases sucrose solubility, prevents microbial contamination, lowers viscosity and improves transfer rates (Bruins *et al.*, 2003; Gill *et al.*, 2006).

The first RSM resulted in high levels of GF_2 and low yield of GF_4 indicating possible interplay of glucose inhibition and the hydrolase activity of the fructosyltransferase. The by-product, glucose, has been reported to be the main factor lowering yield during FOS synthesis (Sheu *et al.*, 2001, Kim *et al.*, 1998). In the three consecutive RSM trials, there were no significant differences between the observed FOS yield and the yield predicted by response surface regression (ANOVA, $p < 0.05$) suggesting that the model predicted conditions suitable for FOS synthesis. The following parameters, pH 5.6, sucrose concentration, 600 g/L and temperature 60 °C were found to be optimal for FOS synthesis. In the first RSM, no FOS above GF_4 were synthesised in experimental runs 3, 7, 8 and 12 (Figure 4.3) and this is explained by the high temperature of 70 °C in all the runs which inactivated the fructosyltransferase enzyme with time (Ghazi, *et al.*, 2007).

High sucrose concentration (600 g/L) did not cause any substrate saturation for this enzyme and in a similar study using a microbial fructosyltransferase, Kim *et al.*, (1998), reported that high sucrose levels (500 g/L) promoted transfructosylation reaction that results in high FOS yield in preference to any hydrolysis reaction. According to Katapodis *et al.*, (2003), the rate of fructose transfer was observed to be decreased at high sucrose concentrations and this is possibly due to a decrease in

water activity. Both hydrolase and transfructosylation rates are high at low sucrose concentration of 100 g/L (Kim *et al.*, 1998; Katapodis *et al.*, 2003). According to Ghazi *et al.*, 2007, the transferase or hydrolase ratio, and therefore the maximum yield of FOSs, depends basically on 2 parameters: the concentration of sucrose and the intrinsic enzyme properties i.e. its ability to bind the nucleophile and to exclude water (Ghazi *et al.*, 2007).

Incubation time had a critical effect on the yield of FOS. In the second RSM using the crude fructosyltransferase, the major product was GF_2 after 4 h of incubation at 60 °C pH 5.6, 600 g/L sucrose concentration, and the maximal GF_4 was achieved after 8 h incubation. The increase in the yield of GF_2 in the second RSM experiment is as a result of the increase in enzyme concentration in the reaction mixture and reduced incubation time that favour accumulation of GF_2. Product composition in the reaction mixture is a result of time since short chain FOS like GF_2 are synthesised in the early stages and are then used as acceptors for fructose for chain elongation. The high yield of GF_4 after 8 h of incubation is attributed to the fact that GF_2 and GF_3 are disproportionately converted to GF_4 over time. The absence of GF_5 in the reaction products in all experimental runs is possibly due to the fact that the active site of the fructosyltransferase enzyme may not be able to accommodate a FOS longer than GF_4.

A prolonged incubation time of 16 h resulted in high yield of GF_2 in the third RSM experimental trial. This is explained by the hydrolysis of GF_4 into GF_3 and GF_2 as a result of self hydrolase activity by the fructosyltransferase. A GF_3 yield of 211.09 mM was synthesised after 16 h of incubation and 22.5 mM of GF_4 was synthesised after 24 h incubation. This pattern of FOS synthesis indicates that sugars with greater degree of polymerisation are formed by incubating the reaction mixture for extended time. Kim and co-workers (1998) found that the fructosyltransferase from *Bacillus macerans* EG-6 synthesised exclusively GF_4 at high sucrose concentration of 500 g/L after 40 h incubation at 37 °C. This observation has been shown by Yun *et al.*, (1993) to be true for all microbial fructosyltransferases disregarding their source. Accordingly, it is therefore ideal to incubate the reaction mixture under optimal conditions for 3 to 4 h for GF_2 sugars. It is reasonable to presuppose that FOS are formed by a self-transfer mechanism, whereby microbial fructosyltransferases are reported to be regiospecific, by selectively transferring the fructofuranosyl moiety of

sucrose to the 1-OH furanoside of the sucrose molecule, forming 1-kestose (Kim *et al.*, 1998).

The purified fructosyltransferase was subjected to RSM whereby parameters such as pH, temperature and sucrose concentration were varied. The purified fructosyltransferase synthesised GF_2, GF_3 and no GF_4 was synthesised in all experimental runs indicating that purification of the enzyme could have disturbed the integrity of the enzyme for the synthesis of higher chain FOS. In addition, a crucial component or cofactor could have been removed during the purification process. Yun *et al.*, 1997[a] also purified a fructosyltransferase and obtained similar observations with no GF_4 being synthesised.

The absence of any FOS after using fructose and glucose as precursors for FOS synthesis further suggests the mechanism of the fructosyltransferase. This shows that this enzyme requires a disaccharide such as sucrose as the fructose donor and acceptor for the formation of 1-kestose. The decrease of FOS yield in the presence of glucose in the reaction mixture confirms the inhibitory nature of glucose. The absence of FOS at 40 % initial glucose concentration in the reaction mixture suggests complete inhibition in agreement with the findings of other workers (Steinberg *et al.*, 2002). Glucose was shown to have an inhibitory effect on an exoinulinase enzyme and fructose had no effect on the hydrolysis rate (Zhang *et al.*, 2004). The fructose peak detected by HPLC in Figure 4.12 is a result of invertase contamination and therefore hydrolysis of sucrose in the reaction mixture since a crude fructosyltransferase was used.

The incorporation of ammonium ions slightly enhanced FOS yields. Ammonium nitrate increased the yield of GF_4 by 1.5 fold. In a similar study, Park *et al.*, 1999, found that ammonium nitrate had a 15 times fold increase in FOS levels using a *Bacillus macerans* fructosyltransferase. This can be explained by possible protein precipitation in the reaction mixture. The mechanism of the effect of ammonium ions is not clearly understood (Park *et al.*, 1999).

The fructosyltransferase was activated by some divalent and monovalent ions. Calcium ions are known to stabilise fructosyltransferases from various sources (Ghazi

et al., 2007). The chelating agent EDTA maintained enzyme activity indicating that the fructosyltransferase enzyme does not require divalent metal ions for activity. Heavy metals investigated in this work completely inhibited the fructosyltransferase activity but the mechanism is currently unknown (Ghazi *et al.,* 2007). *N*-bromosuccinimide is reported to be a strong inhibitor of the hydrolase activity, and in order to stop the hydrolysis of the formed FOS, NBS was therefore used primarily to stop the hydrolysis of the formed FOS thereby increasing the reactivity of the fructosyltransferase. The complete inhibition of the fructosyltransferase activity by *N*-bromosuccinimide (NBS) suggest the possible structure of this enzyme and this may point to the presence of Asp / Glu, Trp and Cys residues in the active site of this enzyme (Nakamura *et al.,* 1997).

In an attempt to remove glucose in the reaction mixture a mixed enzyme system was evaluated for its efficacy and there was a marked decrease in the levels of glucose with concomitant increase in FOS yield. There was incomplete elimination of glucose and this is as a result of oxygen limitation and inefficient buffering capacity since pH levels dropped to 4.8 hence affecting glucose oxidase activity (Sheu *et al.,* 2001). Another possible reason for the incomplete removal of glucose is as a result of inactivation of glucose oxidase at high sucrose concentration. In a similar study, Yun and Song, (1993), found that glucose oxidase is inactivated at high sucrose concentration therefore hampering effective glucose removal from the reaction mixture.

4.5 Conclusions

Based on the use of a crude and purified fructosyltransferase for the synthesis of FOS, a number of conclusions can be drawn:

1. RSM with the crude fructosyltranferase generated optimal conditions for the synthesis of FOS.

2. The purified fructosyltransferase did not give higher levels of FOS therefore for industrial synthesis of FOS; it is cost effective to just use the crude fructosyltransferase preparation instead of purifying the enzyme.

3   Ammonium ions in the reaction mixture enhanced the FOS yield under batch conditions. FOS were not synthesised from fructose and glucose as precursors for FOS synthesis

4   There was complete fructosyltransferase inhibition at 40 % initial glucose in the reaction mixture.

5   By using a mixed enzyme system, glucose levels were lowered with an increase in the yield of FOS. The crude fructosyltransferase from Pectinex Ultra SP-L is a potential candidate for biotechnological application for the industrial synthesis of FOS using sucrose as a substrate.

The next chapter explores the production of fructose using a commercial exoinulinase preparation optimised using RSM under batch conditions.

Chapter 5

Production of Fructose from Inulin Hydrolysis by a Crude and Purified Exoinulinase from *Aspergillus ficuum* Optimised using Response Surface Methodology

5.1 Introduction

Exoinulinase (EC 3.2.1.80), a member of glycoside hydrolase family (GHT) 32, is a fructofuranosyl hydrolase that splits fructose off the non-reducing end of inulin (Nagem *et al.*, 2004). There are several sources of exoinulinase enzymes that have been evaluated for their use for the hydrolysis of inulin to fructose. Microbial sources of inulinolytic enzymes are attractive because of the ease of production through solid state and submerged fermentation. In addition, most of the inulinolytic enzymes are thermophilic which allow fructose production at elevated temperatures such as 50 °C (Tomotani and Vitolo, 2007). This is advantageous because it prevents product contamination and enhances inulin solubility. Inulin serves as a relatively inexpensive and abundant substrate for fructose production and consequently is of considerable industrial interest (Nagem *et al.*, 2004).

The structural basis of the catalytic mechanism of an exoinulinase (PDB, 1y9m) isolated from *Aspergillus awomori* has been elucidated by Nagem *et al.*, (2004) (Figure 5.1).
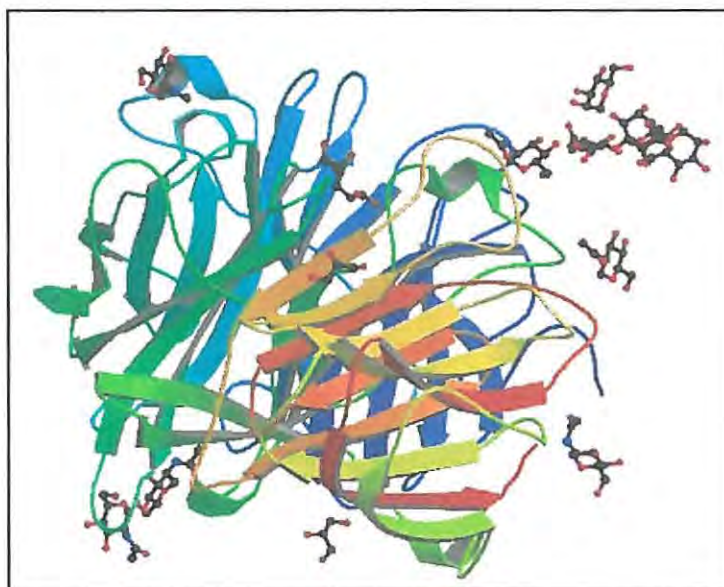
Figure 5.1 Ribbon illustration of the secondary structural elements of exoinulinase with the *N*-terminal belonging to the five-bladed β-propeller fold. The five blades are shown in different colours, blade 1 yellow, blade 2 marine, blade 3 red, blade 4 lime, blade 5 orange. The second domain coloured in slate consists of 12 β-strands arranged in a β-sandwich fold. The short polypeptide connecting the 2 domains is depicted in pink. The *N*-linked oligosaccharides present in the crystal are represented in ball and stick (Nagem *et al.*, 2004).

Generally, it is thought that the enzymatic hydrolysis catalysed by glycoside hydrolases is performed by two catalytic residues of the enzyme, a general acid and a nucleophile. The hydrolysis occurs via two major mechanisms which give rise to either an overall retention or inversion of the anomeric configuration of the substrate. Nagem and co-workers (2004) identified two putative catalytic residues namely Asp41 and Glu241, where the former residue is thought to be the nucleophile and the latter a proton donor. According to Zherebtsov *et al.*, 2003, carboxylic and imidazole groups are involved in catalysis in inulinases isolated from *Aspergillus* sp.

Fructose is widely used in foods and beverages instead of sucrose, and due to the importance of fructose in the food and pharmaceutical industry, it is therefore imperative to find optimal conditions for the production of fructose using enzymes by using cheap and readily available agricultural raw materials such as inulin. The objectives of this research were:

1   to use response surface methodology (RSM) with central composite design (CCD) for the production of fructose from inulin using a crude commercial exoinulinase preparation, by simultaneously varying important multivariate factors such as pH, temperature, time, enzyme concentration, and inulin concentration.

2   to investigate the effect of initial fructose concentration in the reaction mixture on fructose production from inulin hydrolysis by the exoinulinase.

3   to establish the nature and type of fructose inhibition of the exoinulinase.

4   to purify and characterise the exoinulinase and use it for inulin hydrolysis.

5.2 Materials and Methods

5.2.1 Materials

Fructozyme L (Batch number KIN00046) a commercial enzyme preparation isolated from *Aspergillus ficuum* was obtained from Novozymes, South Africa. Fructose Assay Kit (Product code FA-20) was purchased from Sigma-Aldrich, South Africa. All other chemicals and reagents were of analytical grade unless otherwise stated.

5.2.2 Preparation of inulin and crude exoinulinase

The inulin substrate was prepared as previously described in section 2.2.4 in Chapter 2. The commercial crude exoinulinase preparation (20 ml) was dialysed overnight against citrate-phosphate buffer (0.1 M, pH 5.0) with gentle shaking at 4 °C to remove traces of salts and reducing sugars.

5.2.3 Determination of exoinulinase activity

Exoinulinase activity was measured by modification of the procedure by Jing *et al.*, (2003[a]) using pure non-hydrolysed commercial inulin as a model substrate. The assay mixture contained an aliquot (1 ml) of dialysed exoinulinase preparation and the inulin substrate (5 %, w/v, 4 ml) dissolved in citrate-phosphate buffer (0.1 M, pH 5.0). The reaction was done in triplicate. The control tube was prepared by replacing the enzyme solution with the buffer solution and the tube was treated under the same incubation conditions as the experimental tubes. The reaction mixture was incubated (50 °C, 1 h), and the reaction was stopped by boiling for 10 minutes. The reaction products were centrifuged (4 000 x g, 10 min, 4 °C) and then syringe filtered through a 0.45 μm Millipore nylon membrane. Enzyme activity was determined by measuring the amount of fructose liberated from inulin hydrolysis using the Fructose Assay Kit (Product code FA-20), as recommended by the manufacturer (Sigma-Aldrich, South Africa) (Appendix A6) and fructose liberated estimated from a calibration curve (Appendix Figure A.6). One exoinulinase unit was defined as the amount of enzyme catalysing the liberation of 1 μmole of fructose per minute under the experimental conditions.

5.2.4 Preliminary inulin hydrolysis using crude exoinulinase

Approximate conditions for fructose production by the hydrolysis of inulin by a crude exoinulinase were chosen from a preliminary RSM with CCD experimental runs. The critical parameters chosen for fructose production in the first RSM were pH (5.0 to 6.2), temperature (30 to 50 °C), and inulin concentration (50 to 150 g/L) (Table 5.1). Incubation time was 8 h and enzyme dosage was kept at 100 U/ml in all experimental runs. An aliquot (1 ml) of the crude enzyme (100 U/ml) was added to the appropriate

concentration of pure inulin substrate (4 ml). The reaction mixture in all the experimental runs was incubated at the appropriate temperature for 8 h. The control tube containing the boiled enzyme with the inulin substrate was incubated under the same conditions the experimental tubes. Reaction products were boiled for 10 min to stop the enzyme activity and fructose concentration was determined by the fructose assay kit as previously described in section 5.2.3. A calibration curve was generated from pure fructose standard (Appendix A6).

5.2.5 Optimisation of fructose production by the crude exoinulinase

After the preliminary experimental runs, RSM was adopted for optimisation of fructose production by the crude exoinulinase in the second and third RSM runs. The coded levels of the RSM with the CCD were established as previously described in chapter 2 section 2.2.7. Table 5.2 and 5.3 show the parameters that were simultaneously varied. The conditions of the experimental runs were arranged as previously described in Chapter 2 Table 2.2. In the second RSM experimental runs, pH was kept at 5.0 and temperature was maintained at 50 °C (Table 5.2).

Table 5.1 Generation of coded levels for RSM and CCD for 3 parameters, pH, temperature and inulin concentration for fructose production using 100 U/ml crude exoinulinase preparation for 8 h.

Independent Variable	Symbol	Coded Levels		
		-1	0	+1
pH	X_1	5.0	5.6	6.2
Temperature (°C)	X_2	30	40	50
[Inulin] (g/L)	X_3	50	100	150

In the third, RSM experimental runs, enzyme dosage was maintained at 100 U/ml and pH was kept at 5.0 (Table 5.3). The reaction was initiated by adding the appropriate enzyme concentration (1 ml) to the inulin substrate (4 ml). After incubation at the appropriate temperature, the reaction was terminated by boiling the tubes for 10 minutes. Fructose concentration was determined using the Fructose assay kit as described in Appendix A6 and concentrations were read off the standard calibration curve. Any effects of thermal and acid hydrolysis were ruled out after reducing

sugars were not detected after incubating the inulin substrate at 50 °C, at pH 5.0 for the duration of the experiment.

Table 5.2 Coded levels for RSM and CCD for 3 parameters, time, enzyme concentration and inulin concentration for fructose production using crude exoinulinase preparation with pH 5.0 at 50 °C.

Independent Variable	Symbol	Coded Levels		
		-1	0	+1
Time (h)	X_1	4	8	12
[Exoinulinase] (U/ml)	X_2	20	60	100
[Inulin] (g/L)	X_3	50	100	150

5.2.6 Effect of initial fructose concentration on inulin hydrolysis

In order to establish if fructose was acting as an end product inhibitor, the effect of initial fructose concentration was investigated on the yield of fructose produced by the crude exoinulinase. Inulin (10 %, w/v) in citrate-phosphate buffer (0.1 M, pH 5.0) with initial fructose concentration ranging from 0 to 20 % (w/v) was used for fructose production at 50 °C for 1 h with the crude exoinulinase as previously described. This was done in triplicate and the reaction was initiated by adding the crude exoinulinase (1 ml, 100 U/ml) to inulin (10 %, w/v, 4 ml) suspended in citrate-phosphate buffer (0.1 M, pH 5.0) and initial fructose concentration ranging from 5 to 20 % (w/v). The reaction was terminated by boiling for 10 minutes and fructose concentration was determined by the fructose assay kit as previously described.

Table 5.3 Coded levels for RSM and CCD for 3 parameters, time, temperature and inulin concentration for fructose production using 100 U/ml crude exoinulinase preparation with pH 5.0.

Independent Variable	Symbol	Coded Levels		
		-1	0	+1
Time (h)	X_1	24	36	48
Temperature (°C)	X_2	30	40	50
[Inulin] (g/L)	X_3	50	100	150

5.2.7 Type of fructose inhibition on exoinulinase

After observing the end product inhibitory nature of fructose on exoinulinase activity, it was therefore important to establish the nature of fructose inhibition on exoinulinase as described by Whiteley (1997). This was done by establishing the kinetic parameters in the presence and absence of initial fructose (5 %) by using different inulin concentrations (5 to 20 mM). The kinetic parameters, K_m and V_{max} were determined as previously described in section 3.2.10.7 except that the incubation temperature was 50 °C at pH 5.0. The test of the reversibility of the exoinulinase inhibition by fructose was performed by removal of fructose by dialysis following the procedure of Whiteley and Daya, (1995). This was done by performing the reaction using inulin (5 to 20 mM, pH 5.0) in the presence of 5 % initial fructose for 1 h and the reaction mixture was dialysed against distilled water at 4 °C for 24 h. Enzyme activity was determined at the beginning and at the end of dialysis.

5.2.8 Purification of exoinulinase

The crude exoinulinase preparation was purified to electrophoretic homogeneity by a 3 step purification protocol involving 50 % ammonium sulphate precipitation, dialysis and PEG 2000 concentration, and size exclusion chromatography using Sephacryl S-200 as previously described in Chapter 3 section 3.2.5. In order to check the purity of the purified enzyme, SDS-PAGE was performed as previously described in section 3.2.8 (Appendix B1).

5.2.9 Native PAGE

Native PAGE was carried out using the purified exoinulinase to confirm its authenticity as an exoinulinase. Two discontinuous native gels were prepared as previously described in section 3.2.9. The pure band was cut from the gel and the procedure for ascertaining the authenticity of the exoinulinase nature of the purified enzyme followed according to the procedure described in section 3.2.9. Briefly, the band was macerated into small pieces in a minimal volume of distilled water and the resulting solution was used as the enzyme solution. An aliquot of the enzyme

solution (300 μl) was added to 1200 μl of inulin solution (5 %, pH 5.0) at 50 °C for 3 h. The reaction was terminated by boiling and the products were analysed by HPLC.

5.2.10 Physico-chemical characterisation of the purified exoinulinase

The purified exoinulinase was characterised by the following parameters: optimal pH, optimal temperature, pH stability, thermal stability, kinetic parameters. These parameters were performed as previously described in section 3.2.10.

5.2.11 Optimisation of fructose production by the purified exoinulinase

In order to make a comparative analysis of the fructose yield obtained between the crude and purified exoinulinase, the latter was subjected to a fourth and fifth RSM with CCD, as previously undertaken with the crude exoinulinase preparation. In the fourth RSM experimental runs, the following parameters were simultaneously varied, time (4 to 12 h) (X_1), enzyme dosage (20 to 100 U/ml) (X_2), inulin concentration (50 to 150 g/L) (X_3) while the initial pH was kept at pH 5 and all incubations were carried out at 50 °C, (Table 5.2). In the fifth RSM experimental runs, time was varied from 4 to 12 h (X_1), temperature (30 to 50 °C) (X_2) and inulin concentration (50 to 150 g/L) (X_3), and pH and enzyme dosage were kept at 5.0 and 100 U/ml respectively (Table 5.3).

5.2.12 Statistical Analysis

Statistical analysis was performed as stated in Chapter 2 section 2.2.9.

5.3 Results

5.3.1 Determination of exoinulinase activity

Exoinulinase activity in the crude Fructozyme preparation was found to be 117 U/ml.

5.3.2 Preliminary inulin hydrolysis using crude exoinulinase

In order to choose important parameters that are crucial for fructose production from inulin hydrolysis by a crude exoinulinase, 3 factors were chosen, pH (5.0 to 6.2), temperature (30 to 50 °C), and inulin concentration (50 to 150 g/L). The highest fructose concentration (76.19 mg/ml) was achieved after 8 h incubation at pH 5.0, temperature 50 °C and 150 g/L inulin concentration and response surface regression predicted 70.04 mg/ml of fructose (R^2 of 98.67, p = 0.034) under similar experimental conditions (Figure 5.2). The lowest concentration was 23.19 mg/ml in experimental run 2 at 30 °C, pH 6.2 and 50 g/L inulin concentration and response surface regression predicted 28.44 mg/ml (R^2 of 0.81, p = 0.029) under similar experimental conditions.
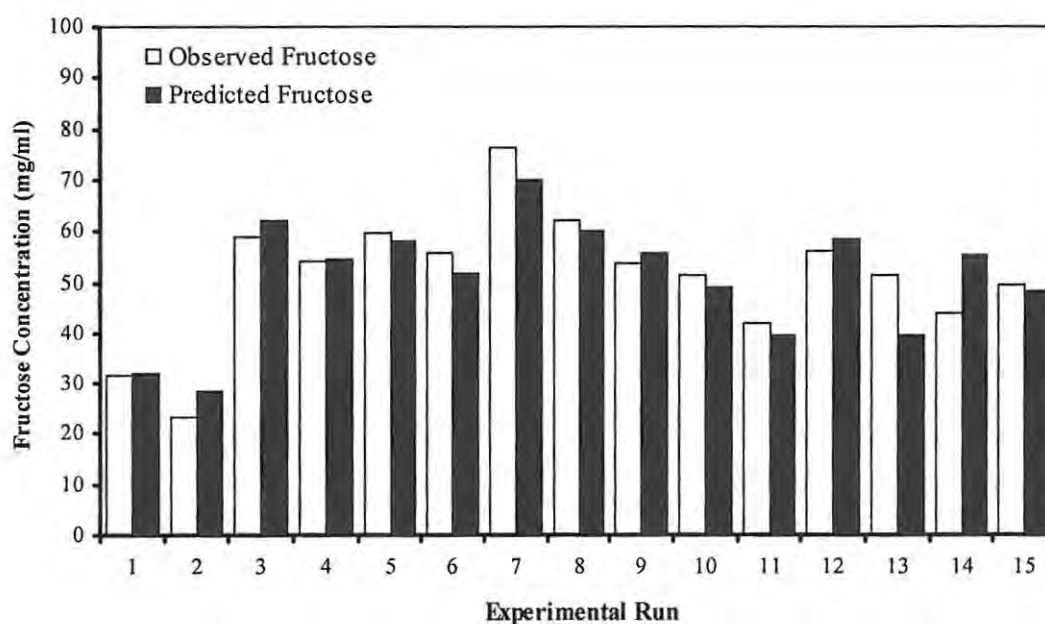
Figure 5.2 Production of fructose from preliminary experimental runs to find important parameters for inulin hydrolysis using RSM for 8 h and crude exoinulinase (100 U/ml). The experimental conditions are shown in chapter 2, Table 2.3.

5.3.3 Optimisation of fructose production by the crude exoinulinase

In the second RSM strategy, 3 parameters were varied, time (4 to 12 h), enzyme concentration (20 to 100 U/ml) and inulin concentration (50 to 150 g/L); pH and temperature were fixed at 5.0 and 50 °C respectively. The highest concentration of fructose (84.51 mg/ml) was produced in experimental run 8 under the following conditions: 12 h incubation, 100 U/ml enzyme dosage and 150 g/L inulin

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concentration (Figure 5.3). Response surface regression was used and under these conditions 84.06 mg/ml of fructose was predicted (R^2 of 0.99, $p = 0.25$) indicating that there were no significant differences between the observed and the predicted fructose yield. The lowest fructose yield was 31.23 mg/ml in experimental run 3 after 4 h incubation with 100 U/ml enzyme concentration, and 50 g/L inulin concentration. Under these conditions, response surface regression predicted 30.28 mg/ml (R^2 of 98.32, $p = 0.087$). Experimental run 14, 5, 6, and 7 also gave high yields of fructose, 83.7 mg/ml, 81.63 mg/ml, 82.08 mg/ml, and 81.04 mg/ml (Figure 5.3).
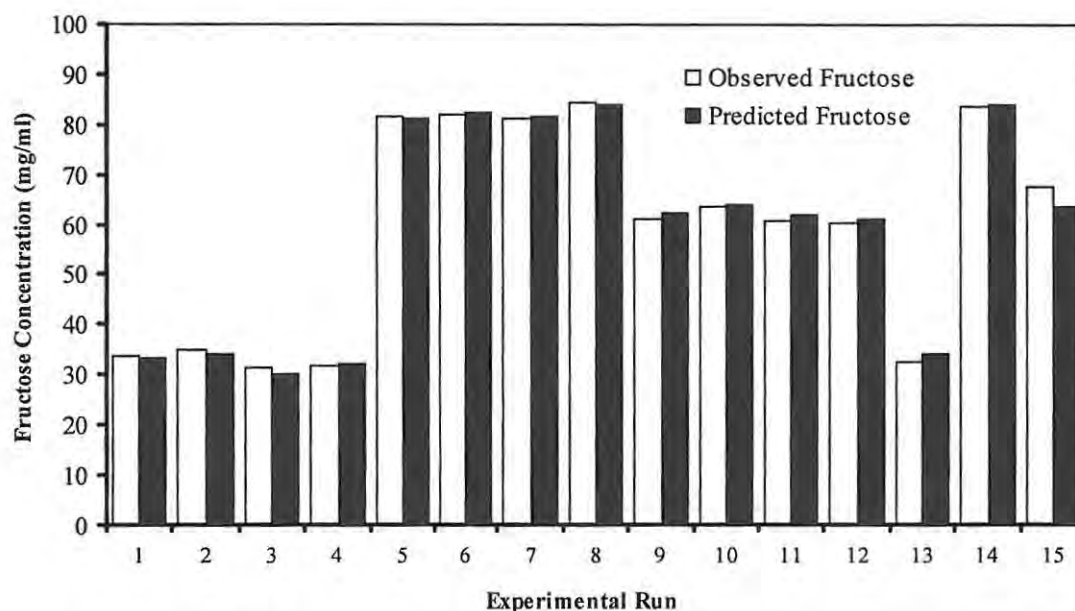
Figure 5.3 Fructose production from controlled inulin hydrolysis by exoinulinase under batch conditions. Coded variables of the central composite design and fructose concentration (mg/ml) produced using RSM with crude dialysed exoinulinase with three coded levels and 3 independent variables with temperature at 50 °C and pH 5.0.

In the third RSM experimental runs, 3 parameters, time (24 to 48 h), temperature (30 to 50 °C) and inulin concentration (50 to 150 g/L) were varied simultaneously. In all the experimental runs, initial pH (5.0) and enzyme concentration (100 U/ml) were fixed. Under these conditions the data generated is presented (Figure 5.4). Experimental run 8 gave the highest fructose concentration (106.40 mg/ml) after 48 h incubation at 50 °C at 150 g/L inulin concentration (Figure 5.4). Response surface regression predicted a maximal of 105 mg/ml of fructose (R^2 of 0.98, $p = 0.096$) under same experimental conditions. Experimental run 14 also gave a high fructose yield (104.92 mg/ml) after 36 h incubation, at 40 °C with 150 g/L inulin concentration. Experimental runs 5, 6, 7 and 9 also gave fairly high fructose yields,

103.32 mg/ml, 81.75 mg/ml, 102.72 mg/ml, and 91.61 mg/ml respectively. The lowest fructose concentration (33.69 mg/ml) was obtained under experimental run 3 after 24 h incubation, 50 °C and 50 g/L inulin concentration.
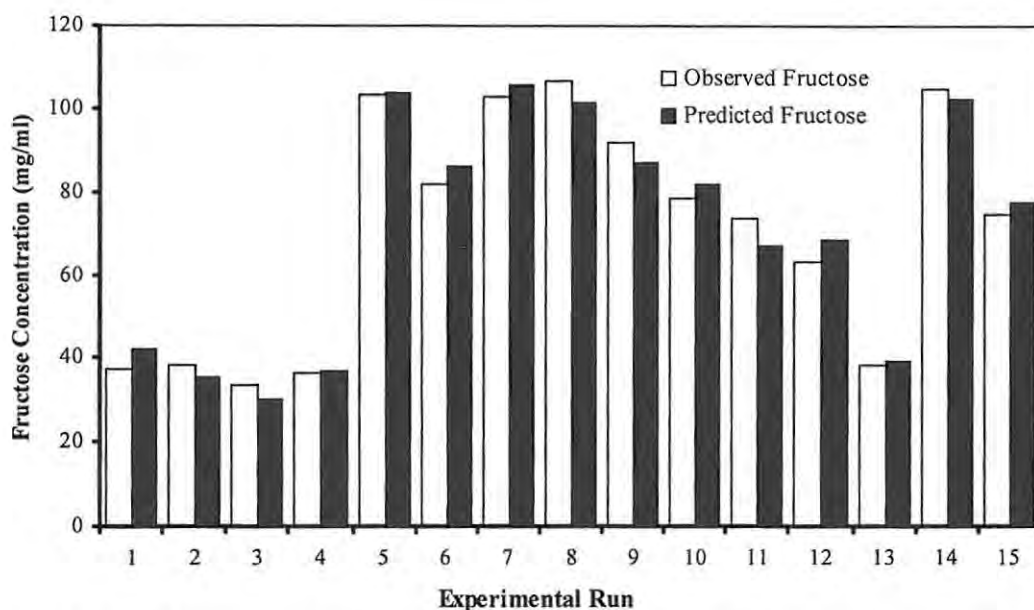
Figure 5.4 Coded variables of the central composite design and fructose concentration (mg/ml) produced using RSM with crude dialysed exoinulinase with three coded levels and 3 independent variables with crude enzyme dosage at 100 U/ml and pH 5.0.

5.3.4 Effect of initial fructose concentration on inulin hydrolysis

The addition of initial fructose to the reaction mixture had a profound effect on the yield of fructose from inulin hydrolysis by the crude exoinulinase indicating that fructose is acting as an end product inhibitor to this enzyme. Linear inhibition was observed and the net fructose yield decreased from 100 ± 4.87 % without initial fructose added to 39.98 ± 1.76 % with 20 % initial fructose in the reaction mixture (Figure 5.5).
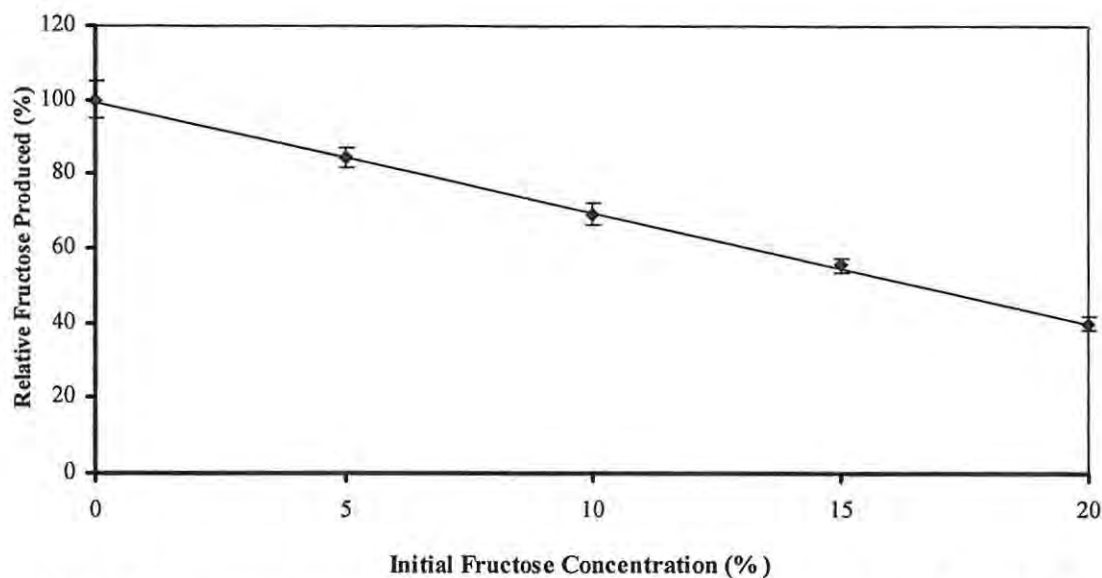
Figure 5.5 Effect of initial fructose concentration on exoinulinase hydrolysis of inulin for the production of fructose. The fructose produced with no added fructose was 48.8 mg/ml and other concentrations were calculated relative to this concentration with no added fructose. Data points represent means ± SD, n = 3.

5.3.5 Type of fructose inhibition on exoinulinase

The kinetic parameters in the presence and absence of fructose were determined. In the presence of initial fructose (5 %), the K_m was 7.3 mM and the V_{max} was 833.3 µmol/min/m and in the presence of 10 % initial fructose, the Km increased to 11.58 mM and the V_{max} remained unchanged at 833.33 µmol/min/ml. In the absence of added initial fructose, the K_m was 4.75 mM and the V_{max} remained unchanged at 833.3 µmol/min/ml (Figure 5.6). The increase in K_m with the increase in initial fructose concentration and the unchanging V_{max} in the presence or absence of the inhibitor indicate that fructose acts as a competitive inhibitor of the exoinulinase. The exoinulinase showed reversible inhibition suggesting that it followed Michaelis-Menten kinetics.

5.3.6 Purification of Exoinulinase

A 50 ml portion of the crude exoinulinase (117 U/ml) was precipitated with 50 % ammonium sulphate saturation at 4 °C and the reconstituted pellet was further purified using Sephacryl S-200 molecular exclusion chromatography. Gradient elution was done using 0 to 3 M NaCl and 5 ml fractions were collected and protein concentration

was determined by the Bradford method. The elution profile is shown in Figure 5.7 where fractions 17 to 26 showed high exoinulinase activity.
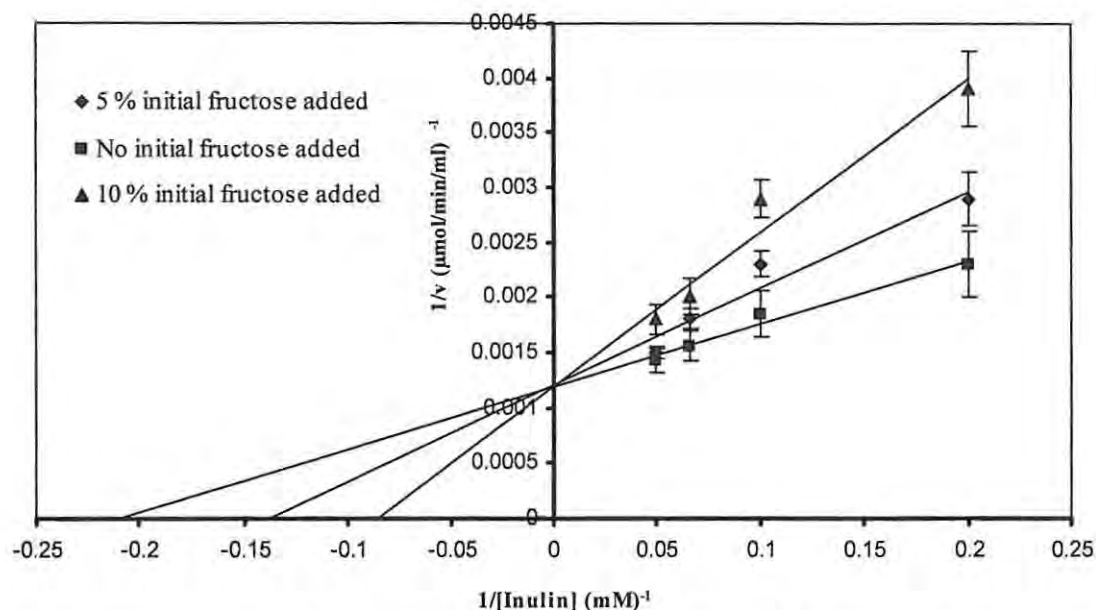
Figure 5.6 Lineweaver-Burk double reciprocal plot of the exoinulinase in the presence and absence of initial fructose (5 %). Each point on the graph represents the mean ± SD of three replicate determinations.

5.3.7 SDS-PAGE

Fractions 17 to 26 showed high exoinulinase activity and were pooled, concentrated by PEG 2 000 and the purity checked by 8 % SDS-PAGE with a 5 % stacking gel as shown in Figure 5.8. The homogenous monomeric exoinulinase band was estimated to be 53 kDa by SDS-PAGE using the molecular weight calibration curve in Appendix C2. A purification table was constructed after the size exclusion purification step. The final exoinulinase activity after Sephacryl S-200 size exclusion chromatography was 123 U/ml with a specific activity of 2 929 U/mg. After the size exclusion purification step, the endoinulinase was purified 4.2 fold with a final yield of 21 % (Table 5.4).
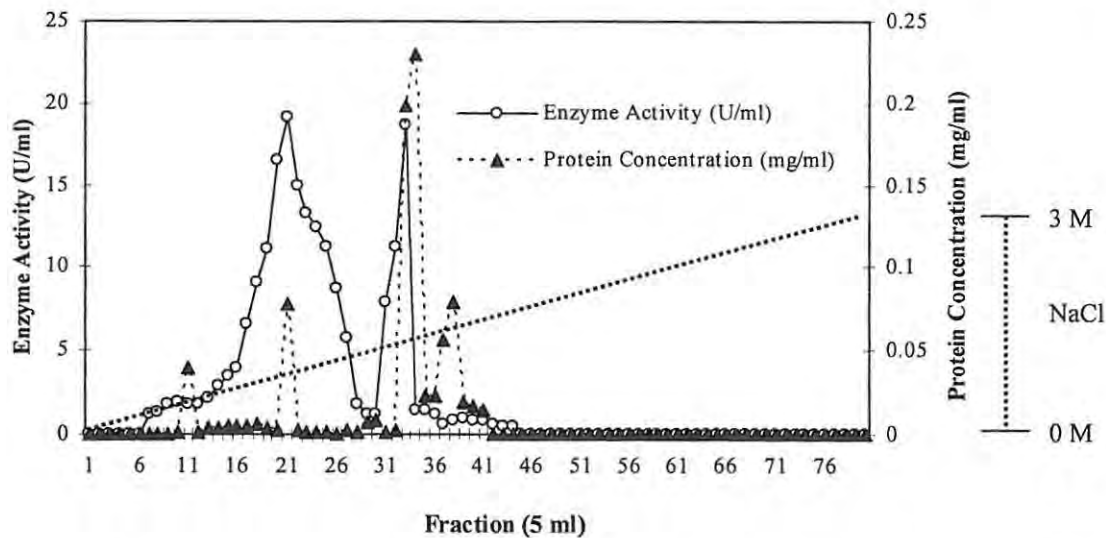
Figure 5.7 Gradient elution (0 to 3 M NaCl) profile of exoinulinase using Sephacryl S-200 molecular exclusion chromatography. Fractions (5 ml) were collected and protein concentration was monitored using the Bradfords method and exoinulinase activity was determined by incubating the enzyme fraction with 5 % inulin at 50 °C for 1 h and reducing sugars were assayed by the fructose assay kit.
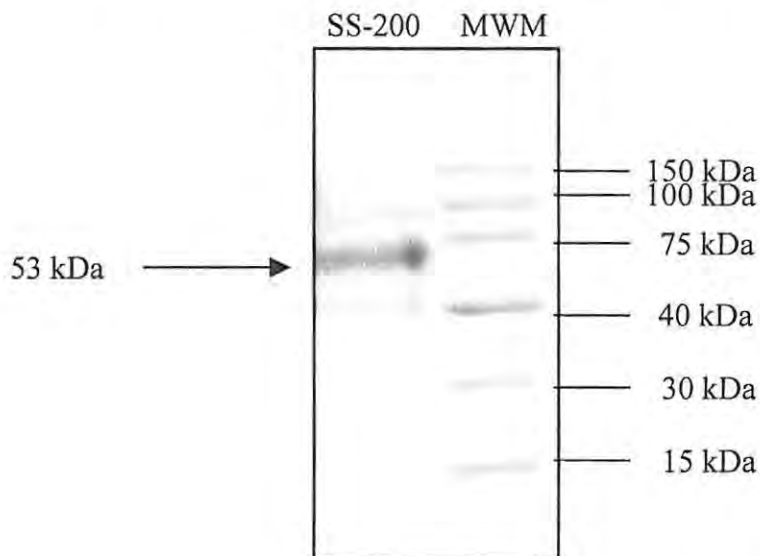
Figure 5.8. SDS-PAGE of the purified exoinulinase using 8 % separating gel and a 5 % stacking gel. Lane SS-200 was loaded with the pooled fractions 17 to 26 from Sephacryl S-200 molecular exclusion chromatography and lane MWM was loaded with molecular weight markers ranging from 15 to 150 kDa. The gel was stained with Coomassie Brilliant R-250 for 30 minutes and destained with glacial acetic acid destaining solution overnight with gentle shaking.

Table 5.4 Purification of exoinulinase from Fructozyme.

Fraction	Volume (ml)	[Protein] (mg/ml)	Total Protein (mg)	Activity (U/ml)	Total Activity (U)	Specific Activity U/mg	Yield (%)	Fold Purification
Crude	50	0.04	2.0	117	5 857	2 929	100	1
AS[a]	10	0.02	0.2	99	990	4 950	17	1.7
SS-200[b]	10	0.01	0.1	123	1 230	12 300	21	4.2

[a]Dialysed pellet after 50 % ammonium sulphate precipitation freeze dried and reconstituted.
[b]Sephacryl S-200 with NaCl linear gradient elution (0 – 3 M).

5.3.8 Native PAGE

The exoinulinase was resolved by native PAGE and the pure band was cut-out to determine exoinulinase activity in order to confirm its authenticity as an exoinulinase (Figure 5.9). HPLC analysis of the reaction products of the pure band confirmed that it was an exoinulinase because it produced fructose, inulobiose, sucrose and traces of 1-kestose in the reaction mixture (Figure 5.9).
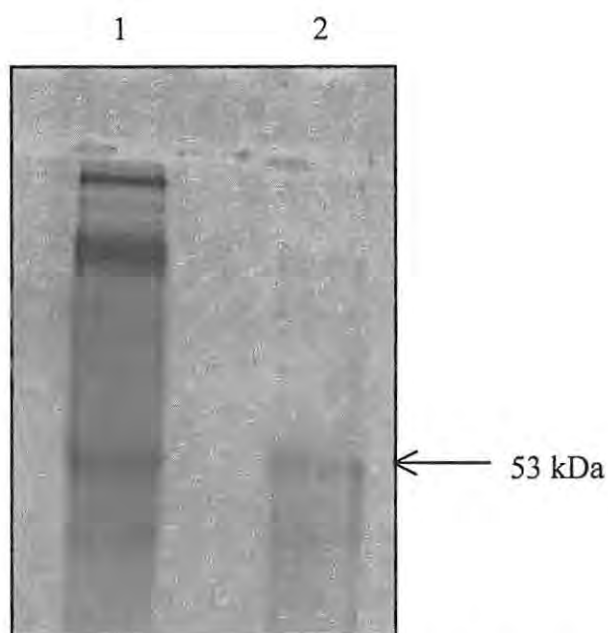
Figure 5.9 Activity PAGE of the pure exoinulinase after Sephacryl S-200 size exclusion chromatography using 8 % separating gel and a 5 % stacking gel. Lane 1, dialysed crude Fructozyme, lane 2, pooled fractions 17 to 26 after Sephacryl S-200 size exclusion chromatography. The gel was stained with Coomassie Brilliant Blue R-250 for 30 minutes and destained with glacial acetic acid destaining solution overnight with gentle shaking.
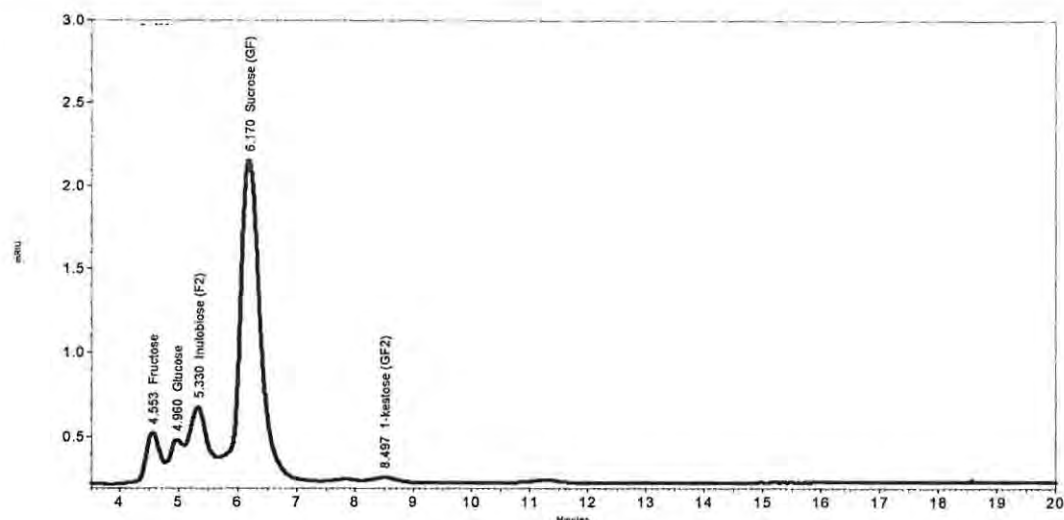
Figure 5.10 HPLC chromatogram of the reaction products of the purified exoinulinase after 3 h incubation period at 50 °C, with inulin (5 %, initial pH 5.0). The sample was analysed by HPLC with RI detection at room temperature and eluted with 73 % acetonitrile in water at a flow rate of 1 ml/min and peak integration was done with 32 Karat Software.

5.3.9. Effect of temperature on the purified exoinulinase

The optimal temperature of the purified exoinulinase was determined to be 50 °C (Figure 5.11). After 1 h incubation, the relative activity was 29.6 ± 2.17 % at 30 °C, 100 ± 5.68 % at 50 °C and 21.8 ± 2.89 % at 80 °C.
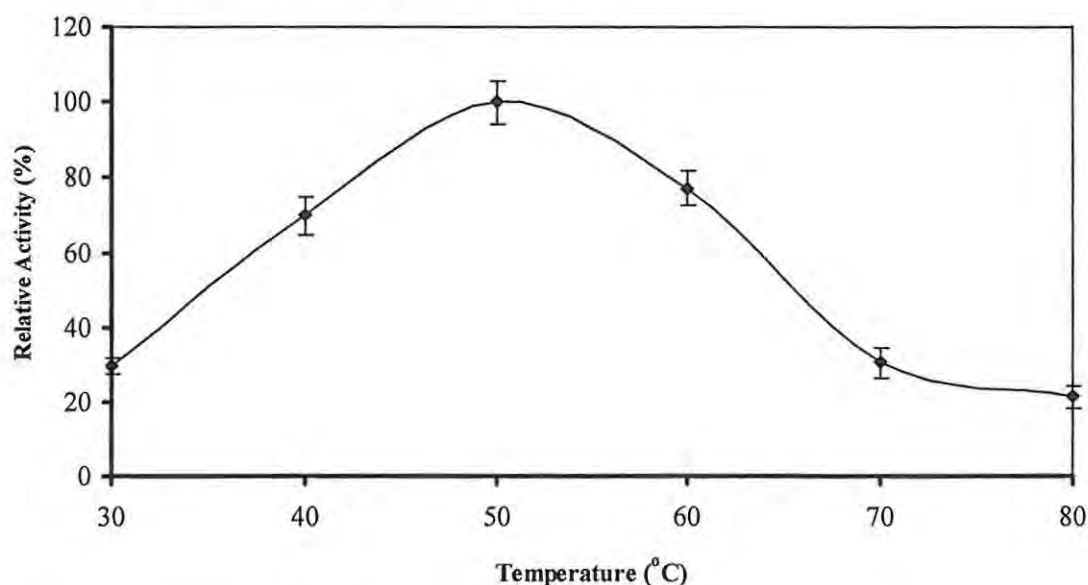
Figure 5.11 Temperature profile of the purified exoinulinase. Data points represent a triplicate determination and error bars on data points represent standard deviations (± SD).

5.3.10 Effect of pH on the purified exoinulinase

The effect of pH on the activity of the purified exoinulinase was determined in the pH range of 3 to 10. The residual activity was 18.29 ± 1.67 % at pH 3 and increased to 38.96 ± 2.98 % at pH 4. The optimal pH was 5.0 (Figure 5.12). The residual activity decreased to 49.56 ± 2.34 % at pH 7 and finally dropped to 7.86 ± 0.89 % at pH 10.
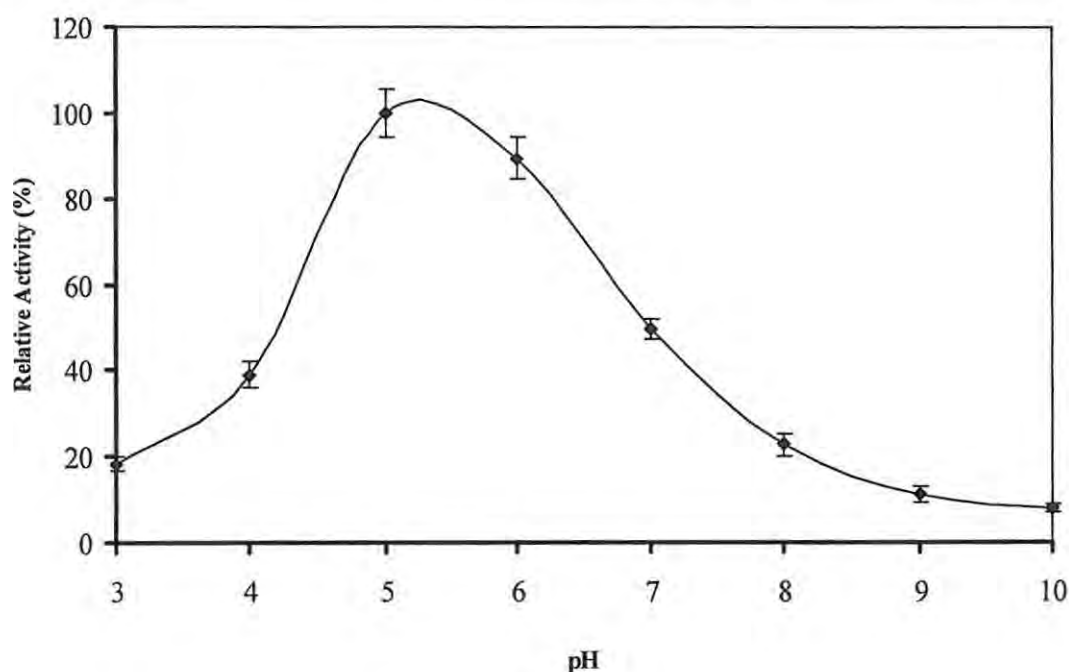
Figure 5.12 Effect of pH on the activity of the purified exoinulinase. Enzyme activity was determined by incubating the purified endoinulinase with 5 % inulin for 1 h in the pH range of 3 to 10 using 0.1 M sodium acetate (pH 3 to 5), citrate-phosphate (pH 6 to 8) and glycine-NaOH (pH 9 to 10). Data points represent the mean of a triplicate determination and error bars on data points represent standard deviations ($\pm$ SD).

5.3.11 Thermal and pH stability

Temperature stability of the purified exoinulinase was determined over 72 h of incubation at 50 °C and samples were periodically withdrawn for analysis (at 6 h interval). Exoinulinase activity was determined as previously described. Figure 5.13 shows the profile generated whereby the enzyme exhibited good thermostability. The exoinulinase was stable at 50 °C after 12 h with 96.17 ± 3.82 % residual activity. After 72 h of incubation, the enzyme had 63.83 ± 3.98 % residual activity which indicates that this enzyme can be used industrially at elevated temperatures for long incubation time without being inactivated.

The purified exoinulinase was investigated for its pH stability (pH 5.0) for 72 h and samples were periodically withdrawn for analysis (at 6 h interval). The purified endoinulinase was highly stable (100 %) at pH 6 for 24 h without any decrease in activity and thereafter the activity started to decrease (Figure 5.13). The residual activity was 90.82 ± 3.89 % after 36 h of incubation at pH 5.0. After 72 h incubation at this pH, the residual activity was 68.42 ± 3.89 %.
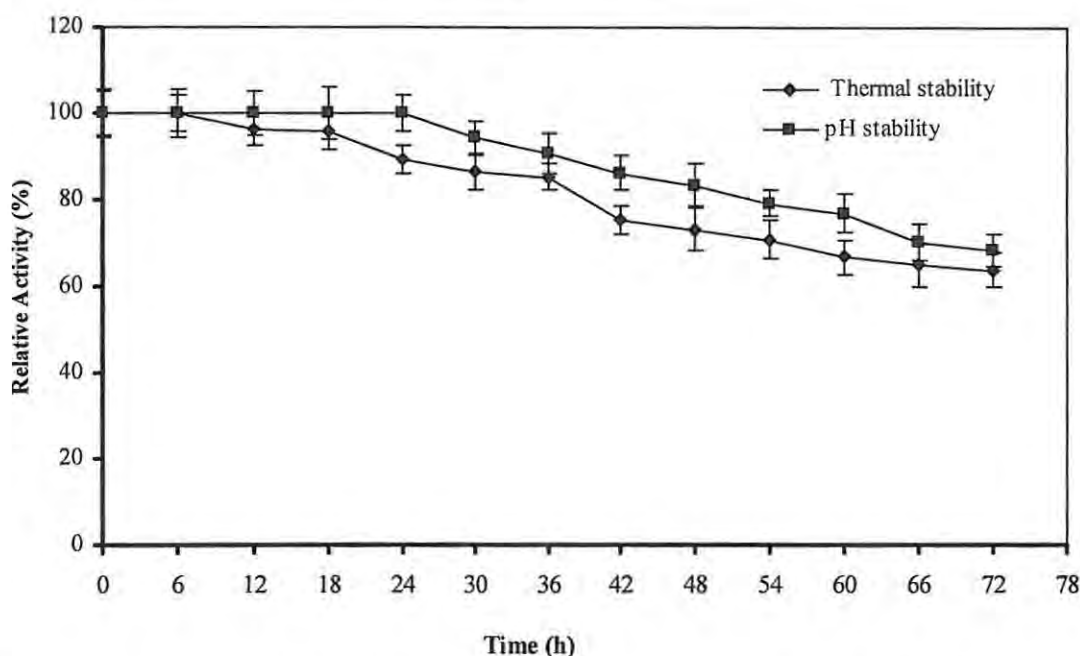
Figure 5.13 Thermal and pH stability of the purified exoinulinase for 72 h at 50 °C and 5.0 respectively. Data points represent the mean of a triplicate determination and error bars on data points represent standard deviations (± SD).

5.3.12 Optimisation of fructose production by the purified exoinulinase

The purified exoinulinase was evaluated for the production of fructose using fourth and fifth RSM using coded levels described for the crude exoinulinase in Tables 5.2 and 5.3 respectively. In the fourth RSM experimental runs, the highest fructose yield by the purified exoinulinase was 83.63 mg/ml in experimental run 14 after 8 h incubation using 60 U/ml enzyme dosage with 150 g/L inulin (Figure 5.14). Response surface regression predicted 82.18 mg/ml (R^2 of 0.99, $p = 0.14$). Experimental runs 5 to 8 produced fairly high yields of fructose, 83.03 mg/ml, 81.73 mg/ml, 77.65 mg/ml, and 77.99 mg/ml respectively. The lowest fructose yield (32.6 mg/ml) in experimental run 4 after 12 h incubation, 100 U/ml enzyme concentration and 50 g/L inulin concentration.
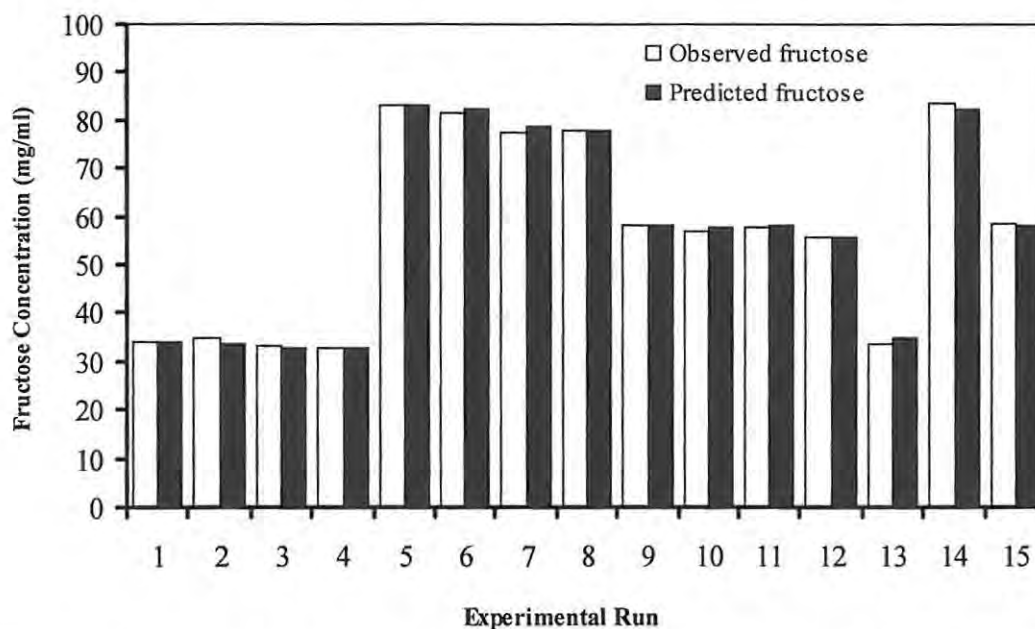
Figure 5.14 Fructose production from controlled inulin hydrolysis by the purified exoinulinase under batch conditions. Coded variables of the central composite design and fructose concentration (mg/ml) produced using RSM with the purified exoinulinase with three coded levels and 3 independent variables with temperature at 50 °C and pH 5.0.

In the fifth RSM experimental runs, the highest fructose yield using the purified exoinulinase was 98.43 mg/ml after 12 h incubation at 50 °C with 150 g/L inulin concentration (Figure 5.15). Response surface regression predicted 96.67 mg/ml (R^2 of 0.99, $p = 0.19$). Regression analysis was performed to fit the response function (fructose yield) with the experimental data. The statistical significance of the response surface regression model equation was checked by one-way ANOVA and the fit value, termed R^2 (determinant coefficient), of the response surface regression model was 0.99, indicating that 99 % of the variability in the response could be explained by the response surface regression equation. One-way ANOVA showed that this model is appropriate in predicting fructose concentrations, ($p < 0.05$). Experimental runs 5, 6, 7, 9, 10, 11, and 12 also produced fairly high fructose yield ranging between 66.14 mg/ml and 89.4 mg/ml. The lowest fructose yield (34.36 mg/ml) was produced in experimental run 2 after 48 h incubation at 30 °C with 50 g/L inulin concentration.
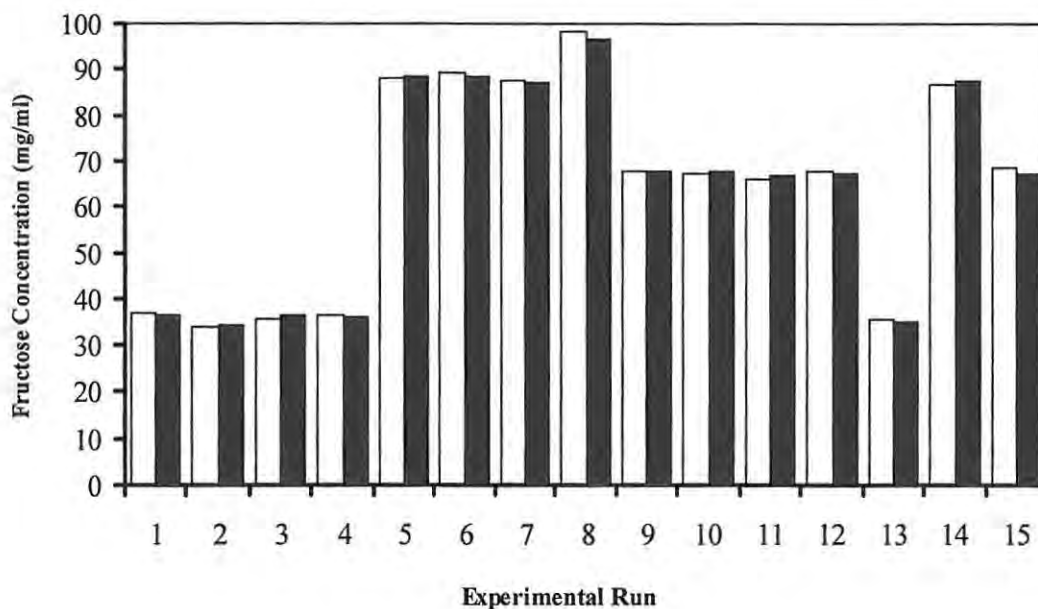
Figure 5.15 Coded variables of the central composite design and fructose concentration (mg/ml) produced using RSM with purified exoinulinase with three coded levels and 3 independent variables with enzyme dosage at 100 U/ml and pH 5.0.

5.4 Discussion

The fructose yield from the controlled hydrolysis of inulin in all the RSM trials was affected by various factors such as pH, enzyme dosage, incubation time, temperature, and inulin concentration. With respect to the second and third RSM, the exoinulinase produced highest fructose concentrations, 84.51 mg/ml and 106.4 mg/ml, respectively. From the purified exoinulinase however, the respective yield of fructose was 83.63 mg/ml and 98.43 mg/ml in the fourth and fifth RSM. Therefore, it appeared obvious and surprisingly that the crude enzyme afforded higher fructose yields than the pure enzyme. The difference in the fructose yield produced by the crude and pure exoinulinase is explained by the fact that the crude exoinulinase possibly has some invertase and endoinulinase activity which also cleave inulin and consequently there is synergism which leads to increased fructose yield.

The addition of initial fructose to the reaction mixture resulted in the decrease of fructose production from 100 % relative fructose produced with no added initial fructose to 39.98 % relative fructose produced at 20 % initial fructose suggesting that fructose is acting as an end product inihibitor to the exoinulinase. The K_m was 11.58 mM in the presence of 10 % added initial fructose, 7.3 mM in the presence of 5 %

added initial fructose and 4.75 mM with no added initial fructose. The high V_{max} and low K_m show the affinity of the exoinulinase for the inulin substrate in the absence added initial fructose. The V_{max} of this exoinulinase (833.3 μmol/min/ml) remained unchanged both in the absence and presence of the inhibitor (fructose) suggesting that fructose is a competitive inhibitor of the exoinulinase with respect to inulin as the substrate (Figure 5.5). Dialysis of the reaction mixture completely removed fructose and showed that the type of inhibition is reversible which shows true Michaelis Menten kinetics for the exoinulinase enzyme. Zhang *et al.*, (2005), showed that glucose was a competitive inhibitor to enzyme activity for the hydrolysis of inulin with a degree of polymerisation not less than 10. Furthermore, these workers established that fructose had no inhibitory effect on the exoinulinase from *Kluyveromyces marxianus* for the hydrolysis of oligosaccharides with a DP $\leq$ 9.

Diaz *et al.*, (2006) assessed the effect of fructose concentration on the initial rate of inulin. According to these workers, the inhibitory nature of fructose can be explained by the reaction equilibrium thermodynamics, or by the diffusional competition between large and small substrate molecules. The inhibitory nature of fructose can be overcome if *in situ* removal of the product takes place by means of a membrane bioreactor (Diaz, *et al.*, 2006).

The exoinulinase was purified to eletrophoretic homogeneity and it was a monomeric band on SDS-PAGE with a molecular weight of 53 kDa. The molecular weight of the purified exoinulinase falls in the range of molecular weight of microbial exoinulinases that have been purified to date (Zhang *et al.*, 2004; Sheng *et al.*, 2007). Native PAGE afforded the confirmation of the exoinulinase nature of this enzyme. The optimal conditions for fructose production were established to be temperature (50 °C), pH (5.0), enzyme dosage (100 U/ml), inulin concentration (150 g/L). A long incubation time is ideal for high yield of fructose for complete hydrolysis of inulin since the enzyme showed good pH and thermostability. Consequently it can be speculated that these conditions could be incorporated into an industrial process for fructose production using this enzyme preparation. In addition, the pH optimum (5.0) observed for the exoinulinase is advantageous for industrial fructose syrup preparations as it prevents undesired colour formation and formation of unwanted by-products such as fructose dianhydrides (Gill, *et al.*, 2006).

5.5 Conclusions

Based on the findings from this research, a number of conclusions can be drawn:

1. Response surface methodology arrived at the optimal conditions for the production of fructose using a crude and purified exoinulinase under batch conditions.

2. The exoinulinase was estimated to be 53 kDa as judged by SDS-PAGE and was confirmed to be an authentic exoinulinase by native PAGE.

3. There was a decrease in the yield of fructose after using a purified exoinulinase for inulin hydrolysis indicating that a crude preparation is suitable for industrial application.

4. Fructose acts as a competitive end product inhibitor to the exoinulinase and the K_m were established to be 11.58 mM, 7.3 mM and 4.75 mM in the presence of 10 %, 5 % added initial fructose and absence of added fructose respectively. The V_{max} remained unchanged at 833.33 μmol/min/ml in the presence and absence of added initial fructose.

The next chapter focuses on general discussion and conclusions from the findings generated in this research.

Chapter 6

General Discussion and Conclusions

6.1 General Discussion and Conclusions

Oligosaccharides are important due to their prebiotic properties as they are selectively fermented by Bifidobacteria. Due to the health benefits of these compounds, the emphasis of this research was on the optimisation of batch enzymatic production of oligosaccharides using simple raw materials such as inulin and sucrose as substrates. The formation of oligosaccharides require a planned optimisation strategy to cut costs and time since the one-factor-at-a-time approach has some inherent shortcomings such as being tedious and the requirement of a large number of experimental runs. Response surface methodology has proved to be the most beneficial optimisation technique in this research in which multivariate factors were simultaneously varied and therefore minimising the number of experimental runs. The full potential of this experimental approach can be hampered by the fact that response surface methodology is only approximate, and if not properly planned, may not give the optimal response. The use of neural networks in combination with RSM provides a promising alternative to the currently available optimisation strategies (Bas and Boyaci, 2007[b]).

Structural determination and identification of inulin and its hydrolysis products presents a major limitation because of its polydispersity. The polydispersity of inulin was therefore a particular challenge to be considered in this research and hampers the accurate determination of the stoichiometry of the reaction products. IOS standards such as inulotriose (F_3), inulotetraose (F_4), inulopentaose (F_5) are not commercially available at present so glucose-linked standards are used and these can introduce some inaccuracies since the retention times of GF_n and F_n are different (Ronkart *et al.*, 2007). Identification of these compounds is generally based on the elution order since the longer chain inulooligosaccharide (IOS) and/or fructooligosaccharide (FOS) elutes later. The separation of F_n and GF_n can be a problem by using HPLC procedures (Heinze *et al.*, 1991; Immermans *et al.*, 1996). In order to avoid errors in quantitative determination of oligosaccharides in food products, proper method development and validation should be done (Ronkart *et al.*, 2007).

The elution of higher IOS/FOS from HPLC columns is technically difficult since they adsorb and get retained on the column and therefore are not resolved. The use of mass spectrometry with electro spray ionisation (MS-ESI) is reasonably sensitive though sugars are not ionised and therefore need derivatisation by forming metal ion sugar adducts. This analytical technique was applied in this research and gave confirmatory information on the identities of the enzymatic reaction products obtained. Other researchers have used HAEPAC-PAD for the analysis of oligosaccharides. Peak areas on the chromatograms do not supply information about the concentrations of the oligosaccharides using this technique. The reason for this is that the response factors of the pulsed electrochemical detector vary significantly with the various oligomers, with lowest responses occurring with oligosaccharides with a high DP (Ronkart *et al.*, 2007; Mabel *et al.*, 2007).

One of the most important prerequisites for the development of a successful biotechnological process for the production of oligosaccharides and fructose is a stable biocatalyst (Catana *et al.*, 2007). The development of a fully enzymatic process thus required the physico-chemical characterisation of 3 key enzymes used in this study, endoinulinase, fructosyltransferase, and exoinulinase. The establishment of thermal and pH stability of the enzymes was a major emphasis of the research. The general stability of fungal inulinase and fructosyltransferases, in particular, has attracted the introduction of these enzymes into biotechnological and industrial applications for the synthesis of novel sugars and fructose. As a consequence, the majority of industrial processes for the production of oligosaccharides and fructose are currently being carried out using fungal fructosyltransferases and inulinases for the conversion of sucrose or inulin substrates to the FOS and IOS intermediates. The chemical approach for the production of oligosaccharides is not attractive because of the attendant problems such as colour formation in the final product and also formation of difructose anhydrides with no sweetening properties (Singh *et al.*, 2007).

A further criterion for determining the feasibility of a commercial bioconversion process is the availability of inexpensive, abundant and readily available starting materials

(Catana *et al.*, 2007). Thus, the cost of the pure non-hydrolysed pure inulin and sucrose for the production of the oligosaccharides had to be considered. The main drawback of using pure inulin is that it is expensive and has reduced solubility for industrial application therefore the use of raw dried chicory is being evaluated in our laboratory (Nemukula and Whiteley, *pers com*). The use of sucrose as a raw material for the synthesis of FOS offers some advantages such as being low cost and promotes transfructosylation at high substrate concentration of 600 g/L without showing any substrate saturation to the enzyme. Moreover, at high sucrose concentrations, hydrolase activity which is intrinsic to the transferase enzyme is limited therefore promoting high yield of FOS with longer chain length.

The IOS reaction product yields obtained using RSM obtained in this research using the crude endoinulinase were relatively low in comparison to the product yields reported by previous researchers working on inulin hydrolysis using enzymes (Kim *et al.*, 1997; Yun *et al.*, 2000). It therefore became apparent during the preliminary experiments that the crude endoinulinase enzyme reaction would be the rate limiting step in the production of high levels of IOS intermediates and subsequently restricting the productivity of the process leading to a necessary task in purifying this enzyme. This was confirmed later by the application of the purified endoinulinase enzyme using RSM in a batch system that resulted in a slightly higher conversion yield in comparison to the crude enzyme. The main reason for this is that the purification removed contaminating enzymes such as invertases in the crude preparation which also hydrolysed inulin randomly therefore lowering the yield of higher DP intermediates.

One of the main thrust of this research was to enzymatically synthesise FOS with high DP. The crude fructosyltransferase, used as the biocatalyst, only synthesised FOS up to DP 5 indicating that the enzyme could not probably accommodate FOS with a longer chain in its active site. It is possible that the absence of synthesis of higher DP FOS is most probably due to the absence of any fructan binding domain in the fructosyltransferase enzyme. Another plausible reason for this phenomenon could be that there might be some residues on the active site of this enzyme which sterically restricts

this and therefore a molecular modelling exercise of this enzyme needs to be done to substantiate this claim. Though the disproportionation reaction mechanism of the transferase enzymes has been demonstrated (Jung, *et al.*, 1989), molecular work to show the structure of fructoslytransferases has not been achieved to date. This work, in the future, might elucidate why this enzyme does not allow the formation of FOS with a chain length greater than DP 5. Purifying the fructosyltransferase did not significantly improve the yield as it did not synthesise GF_4 as was the case with the findings of other workers (Sangeetha *et al.*, 2005[a]).

Hydrolase activity is a problem with fungal fructosyltransferases for the preparation of FOS. As a result, a search of potential inhibitors of hydrolase activity was undertaken. Strong inhibitors of hydrolase activity such as N-bromosuccinimide, barium chloride, and copper sulphate were also found to inhibit transferase activity. Therefore this approach was not successful.

One of the objectives of this research was to identify the optimal reaction conditions for the 3 enzymes from fungal sources with the aim of process development using RSM. The physico-chemical characterisation of the enzymes was achieved after the enzymes were purified to electrophoretic homogeneity. This type of characterisation gave valuable information regarding the intrinsic properties of the enzymes and their stabilities. The pH and thermostability of these enzymes done in separate experiments, also agreed with the optimal conditions established using response surface methodology suggesting that RSM was adequate in establishing optimal conditions.

The pH and temperature optima of the enzymes were in the range reported in the literature for fungal inulinases and fructosyltransferases (Ghazi *et al.*, 2007). The 3 enzymes had pH optima in the range from 5.0 to 6.0 and temperature in the range from 50 to 60 °C and these optimal conditions were advantageous for the production of oligosaccharides and fructose since spontaneous hydrolysis of the substrates occur at acidic pH. The optimal temperatures were high with increased reaction rates and are therefore favoured for industrial processes since they prevent product contamination by

microorganisms. The solubility of inulin is also increased at higher temperatures, promoting the bioconversion of inulin into IOS. Both exo and endoinulinases showed good pH and thermostability for 72 h showing their potential industrial application under these conditions for extended incubation periods.

The purification of the exoinulinase did not significantly improve the yield of fructose using RSM suggesting possible removal of any enzyme synergism in the crude exoinulinase preparation by the purification procedure. The purification procedures described in the literature for the purification of fungal inulinases, in particular, generally involve multiple steps and result in high yields of active enzyme due to their high stability (Vandamme and Derycke, 1983). The crude preparation can be used without any purification for the industrial production of fructose since it was established that for such an application a purification is not cost effective. On the other hand, the use of purified enzymes has the disadvantage of increasing the costs of an industrial process and, in the case of entrapped or encapsulated enzymes, leaching of the purified enzymes may be experienced resulting in the loss of enzyme activity and lowered productivity. Fructose was demonstrated to be an end product inhibitor to the exoinulinase and it may be necessary to evaluate enzyme immobilisation with continuous production to avoid this problem.

To date, various immobilisation strategies have been investigated to improve the enzyme stability and product yield (Yun *et al.*, 2000; Sangeetha *et al.*, 2005[b]). Immobilisation not only has the advantage of allowing the reuse of the biocatalyst, thereby reducing costs, but has been shown to improve the stability of some enzymes (Yun *et al.*, 2000). The best method of immobilisation, in terms of activity retention and durability during storage, was entrapment of the enzyme preparation in polystyrene carrier material. The marked increase in the inulooligosaccharide yield and composition with this form of immobilisation was an important result, since no other reports of increased IOS on immobilisation have been reported. The use of free enzymes for the production of oligosaccharides and fructose using response surface methodology for optimisation as

described in this research was novel and the experimental conditions generated for oligosaccharide and fructose production are amenable to industrial scale up.

6.2 Conclusions

In conclusion, the present study has provided an in depth analysis of the enzymology of the production of oligosaccharides and fructose using simple substrates such as inulin and sucrose. An understanding of the intrinsic enzyme mechanisms for the hydrolysis of inulin as well as the transfructosylation of sucrose will open new vistas in complex polysaccharide research in general. It has been demonstrated in this study that inulooligosaccharide production can be enhanced by purifying the endoinulinase enzymes in order to get high yield of intermediates. Furthermore it is proposed that proper elimination of the build up of side products in the reaction mixture can increase the yield of the target products and therefore minimise product inhibition of the enzyme.

To date no optimisation strategy has been performed for the production of oligosaccharides using response surface methodology and it would be of interest to establish if the optimal conditions found under laboratory-scale RSM applies on a larger scale at industrial level. Using the knowledge gathered in this study it would be possible to further optimise the conditions for the production of IOS/FOS and this will facilitate the industrial production of these products using cheap and readily available substrates such as inulin and sucrose respectively. The consumption of healthy and bifidogenic products is gaining a lot of attention globally and the design of experiments for their production will redress this problem.

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Appendices

Appendix A: Determination of standard curves

A.1 Standard curve for protein concentration determination

Protein concentration was determined using the Sigma Bradford (1976) reagent for protein determination (Product Number B6916). The method is rapid, convenient and produces an equivalent absorbance change for many proteins. When mixed with protein solution a protein dye complex is formed which causes a shift in the dye absorption maximum from 465 to 595. The dye is called Brilliant Blue G-250, and the amount of absorption produced is proportional to the protein concentration. The standard curve based on Bovine Serum Albumin (BSA) gives a linear response (Figure A.1)
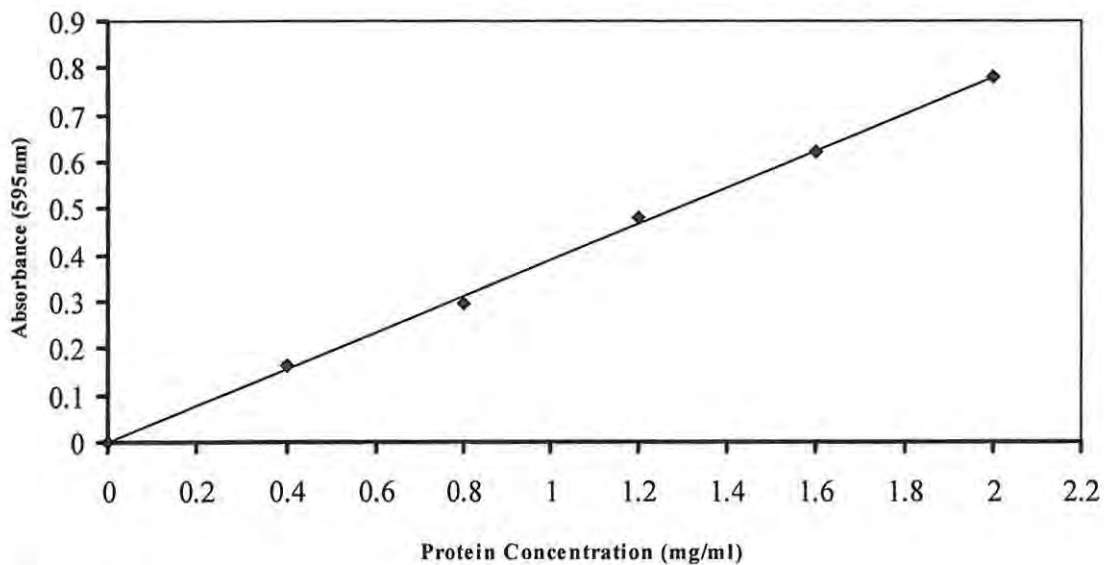

Figure A.1 Protein standard curve using BSA as a protein standard solution.

A.2 Recipe for the preparation of the DNS reagent

The DNS stock reagent was prepared according to the recipe described by Ghose, (1987). The DNS reagent was prepared by mixing distilled water (1 416 ml), 3.5-Dinitrosalicylic acid (10.6 g), sodium hydroxide (19.8 g). The mixture was allowed to dissolve completely before adding Rochelle salts (Na-K tartrate) (306 g), phenol (7.6 ml), Na-Metabisulfate (8.3 g). The mixture was allowed to dissolve completely and then filtered through a Whatman Number 1 filter paper. The DNS stock reagent was stored at room temperature in a cool dry place until needed for use.

A3 Standard curve for the determination of fructose concentration

The DNS method is a popular method used for the determination of reducing sugars. The DNS reagent was prepared as described in Appendix A2. A fructose solution standard aliquot (500 µl) was transferred to 3, 5-Dinitrosalicylic acid (3 ml) and boiled for 10 minutes. The assays were done in triplicate and the test tubes were cooled rapidly under tap water. The tubes were diluted by adding 20 ml of distilled water and absorbance was determined at 540 nm. The standard curve based on fructose concentration gives a linear response (Figure A.2) and was used for calculating endoinulinase activity.
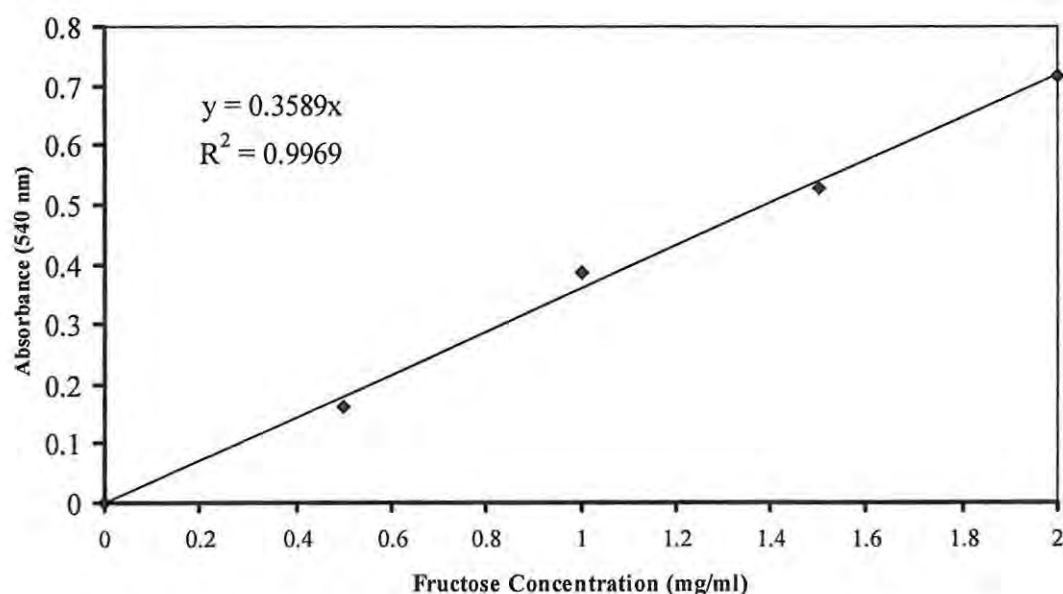

$y = 0.3589x$
$R^2 = 0.9969$

Figure A.2 Reducing sugar standard curve using the DNS method with pure fructose as a standard.

A.4 FOS standard curves by HPLC

Pure authentic FOS were used as standards using HPLC with RI detection. The FOS standards (5 to 20 mM) were prepared in distilled water and peak areas were converted into FOS concentration. The standard curves based on FOS, fructose and glucose concentration gave linear responses (Figure A.3, A4, and A5 respectively) and were used for calculating the concentrations of FOS/IOS in the linear range by HPLC.
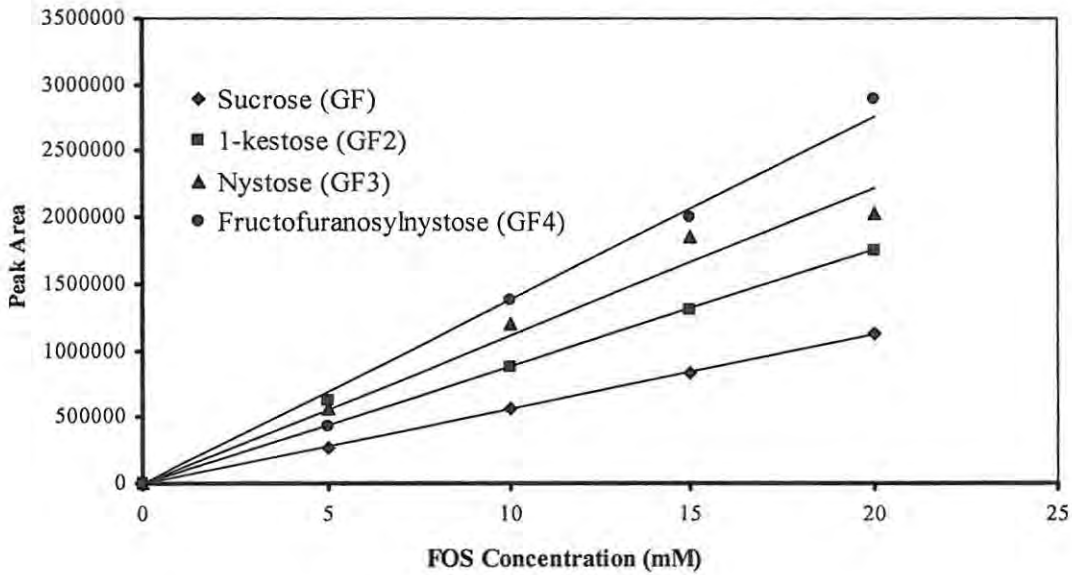
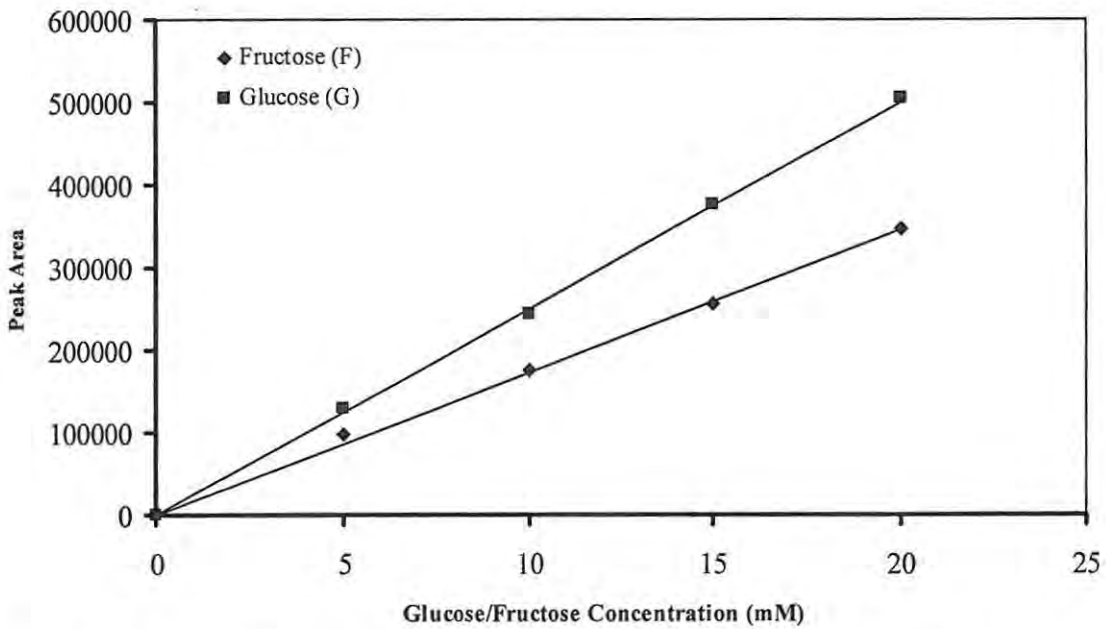**Figure A.3** FOS standards using HPLC with RI detection.

Figure A.4 Glucose and fructose standard calibration curves using HPLC with RI detection.

A.5 Glucose standard curve by the glucose assay kit

Glucose concentration liberated by the fructoslytransferase was determined by the glucose (HK) Assay Kit (Product Code GAHK-20, Sigma-Aldrich).

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Principle:

Glucose is phosphorylated by adenosine triphosphate (ATP) in the reaction catalysed by hexokinase. Glucose 6-phosphate (G6P) is then oxidised to 6 phospho-gluconate in the presence of oxidised nicotinamide adenine dinucleotide (NAD) in a reaction catalysed by glucose-6-phosphate dehydrogenase (G6PDH). During this oxidation, an equimolar amount of NAD is reduced to NADH. The consequent increase in absorbance at 340 nm is directly proportional to glucose concentration.

$$\text{D-Glucose + ATP} \xrightarrow{\text{Hexokinase}} \text{Glucose-6-Phosphate + ADP}$$

$$\text{G6P + NAD} \xrightarrow{\text{G6PDH}} \text{6-Phosphogluconate + NADH}$$
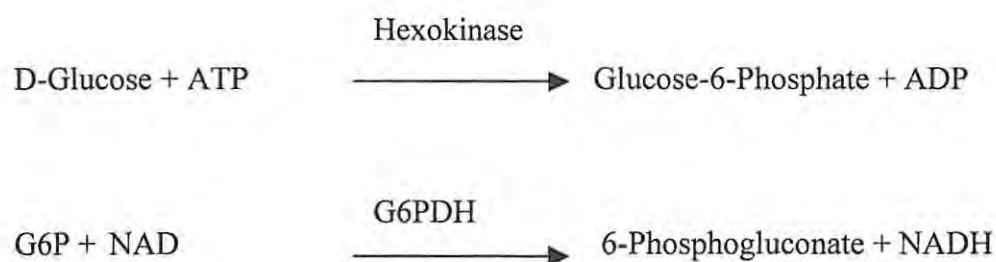
Procedure:

Glucose standard solutions were prepared by diluting the stock D-glucose standard solution (Product Code G 3285) from 0 to 1 mg/ml. The glucose (HK) assay reagent (Product Code G 3293) was prepared and reconstituted as recommended by the manufacturer (Sigma-Aldrich). The sample blank consisted of sample volume (0.2 ml) and distilled water (1 ml). The reagent blank consisted of the glucose assay reagent (1 ml) and distilled water (0.2 ml). The test mixture consisted of sample volume (0.2 ml) and glucose assay reagent (1 ml). The tubes were mixed and incubated at room temperature for 15 min and absorbance was measured at 340 nm. The standard solutions generated a linear response (Figure A.5)

Calculations:

$A_{\text{Total Blank}} = (A_{\text{Sample Blank}} + A_{\text{Reagent Blank}})$

$\Delta A = A_{\text{Test}} - A_{\text{Total Blank}}$

$$\text{mg glucose/ml} = \frac{(\Delta A)(TV)(\text{Molecular Weight of Glucose})(F)}{(\varepsilon)(d)(SV)(\text{Conversion Factor for } \mu g \text{ to mg})}$$

$$= \frac{(\Delta A)(TV)(180.2)(F)}{(6.22)(1)(SV)(1\ 000)}$$

$$= \frac{(\Delta A)(TV)(F)(0.029)}{(SV)}$$

A = absorbance at 340 nm, d = light path (cm), TV = Total Assay volume (ml), SV = Sample Volume (ml), F= Dilution Factor from sample preparation, ε = Millimolar Extinction Coefficient fro NADH at 340 nm, Glucose MW = 180.2 g/mole or equivalently 180.2 µg/µmole, 1 000 conversion factor for µg to mg.
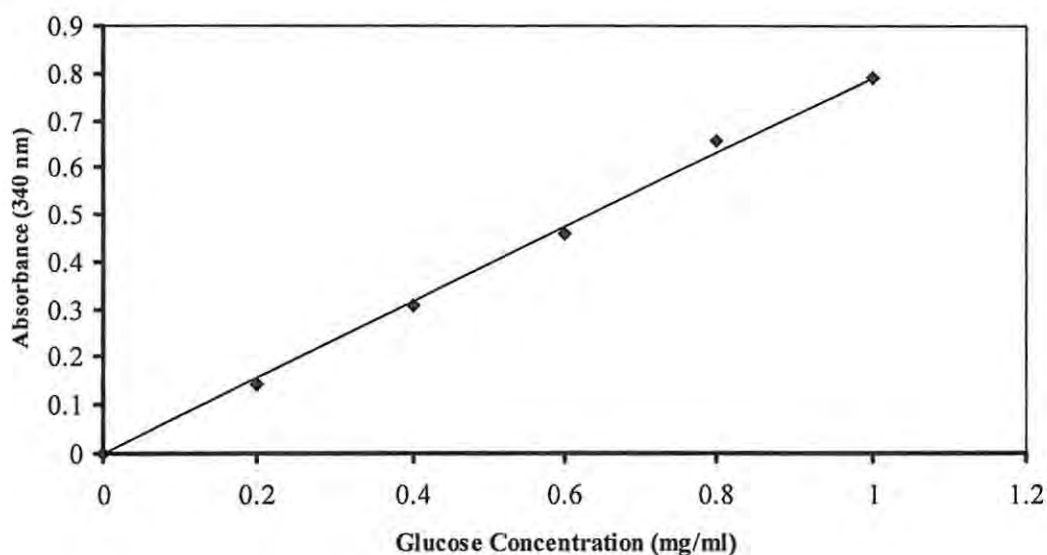

Figure A.5 Glucose standard curve using the glucose assay kit.

A.6 Fructose standard curve by the fructose assay kit

Principle:

Fructose is phosphorylated by adenosine triphospahte (ATP) in the reaction catalysed by hexokinase. Fructose 6-phosphate is converted to glucose 6-phosphate by phosphoglucose isomerase (PGI). Glucose-6-phosphate (G6P) is then oxidised to 6-phosphogluconate in the presence of nicotinamide adenine dinucleotide (NAD) in the reaction catalysed by glucose-6-phosphate dehydrogenase (G6PDH). During this oxidation, an equimolar amount of NAD is reduced to NADH. The consequent increase in absorbance at 340 nm is directly proportional to fructose concentration.

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Fructose + ATP $\xrightarrow{\text{Hexokinase}}$ Fructose 6-Phosphate + ADP

Fructose 6-Phosphate $\xrightarrow{\text{PGI}}$ Glucose 6-Phosphate

G6P + NAD $\xrightarrow{\text{G6PDH}}$ NADH + 6-Phosphogluconate
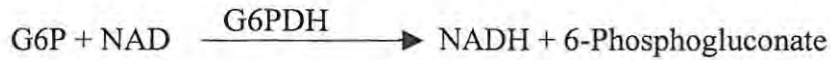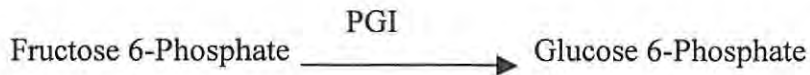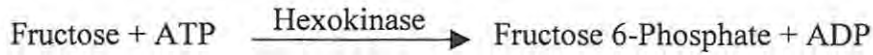
Procedure:

Fructose standard solutions were prepared by diluting the stock D-fructose standard solution (Product code F 2793) from 0 to 1 mg/ml. The PGI (Product code F2668) and the glucose (HK) assay reagent (Product Code G3293) were prepared and reconstituted as recommended by the manufacturer (Sigma-Aldrich). The PGI blank consisted of PGI (0.02 ml), distilled water (0.1 ml) and glucose assay reagent (2 ml). The sample blank consisted of the sample (0.1 ml), distilled water (0.02 ml) and glucose assay reagent (2 ml). The glucose assay reagent blank was made up of distilled water (0.12 ml) and glucose assay reagent (2 ml). The test mixture consisted of PGI (0.02 ml), sample volume (0.1 ml), and glucose assay reagent (2 ml). The tubes were mixed and incubated at room temperature for 15 min and absorbance was measured at 340 nm. The standard solutions generated a linear response (Figure A.6)

Calculations:

$A_{\text{Total Blank}} = (A_{\text{Sample Blank}} - A_{\text{Glucose Assay Reagent Blank}}) + A_{\text{PGI Blank}}$

$\Delta A = A_{\text{Test}} - A_{\text{Total Blank}}$

$$\text{mg fructose} = \frac{(\Delta A)(TV)(\text{Molecular Weight of Fructose})(F)}{(\varepsilon)(d)(SV)(\text{Conversion Factor for } \mu g \text{ to mg})}$$

$$= \frac{(\Delta A)(2.12)(180.2)(F)}{(6.22)(1)(0.1)(1\ 000)}$$

$$= (\Delta A)(F)(0.614)$$

A = absorbance at 340 nm, d = light path (cm), TV = Total Assay volume, SV = Sample Volume, F= Dilution Factor from sample preparation, ε = Millimolar Extinction Coefficient fro NADH at 340 nm
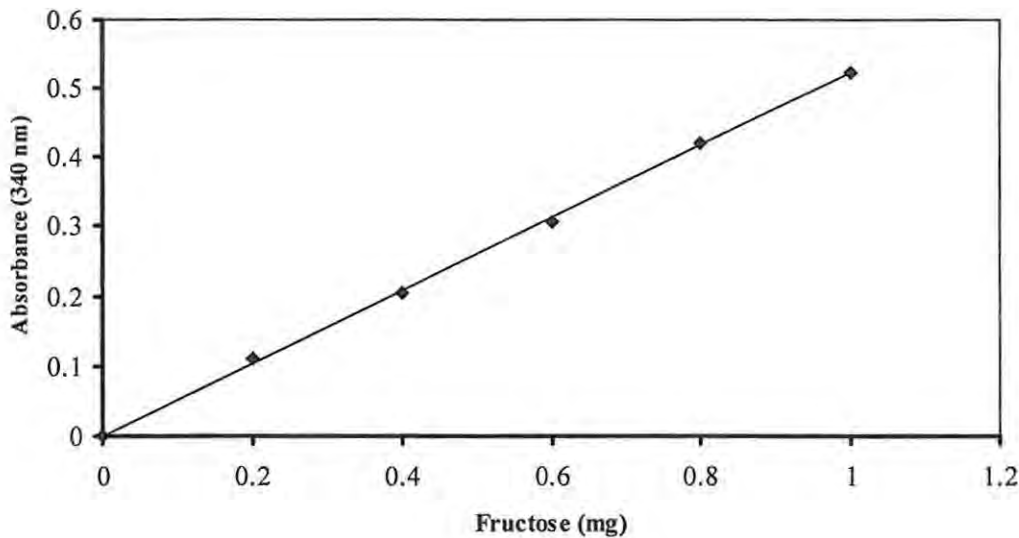
Figure A.6 Fructose standard curve using the fructose assay kit.

Appendix B: Recipe for the preparation of SDS-PAGE and native PAGE gels

B.1 Preparation of gels for SDS-PAGE

Principle: SDS-PAGE is a low cost, reproducible, and rapid method for quantifying, comparing, and characterising proteins. This method separates proteins based primarily on their molecular weights (Laemmli, 1970). SDS binds to the hydrophobic portions of a protein disrupting its folded structure and renders it to exist stably in solution in an extended linear conformation therefore the length of the SDS protein complex is proportional to its molecular weight (Bollag *et al.*, 1996).

Stock solutions were prepared according to the recipe described by Bollag *et al.*, (1996), as follows, 2 M Tris – HCl (pH 8.8, 100 ml), 1 M Tris – HCl (pH 6.8, 100 ml), SDS (10 %, w/v, 100ml), glycerol (50 %, v/v, 100 ml), bromophenol blue (1 % w/v, 10 ml). Working solutions were prepared from the stock solutions as follows:

1. Solution A (Acrylamide solution, 100 ml) 30 % (w/v) acrylamide, 0.8 % (w/v) bis-acrylamide.

2. Solution B (4x Separating Gel Buffer, 100 ml)

3 Solution C (4x Stacking Gel Buffer, 100 ml)

4 Ammonium persulphate (10 %, w/v, 5 ml)

5 Electrophoresis buffer, (1L)

6 5X Sample Buffer (10 ml)

The following solutions were mixed in a small beaker to make up two 8 % separating gel, Solution A (2.7 ml), Solution B (2.5 ml), water (4.8 ml), Ammonium persulphate (10 %, 50 µl), and TEMED (5 µl). The mixture was gently swirled and the gel was introduced into the gel sandwich and 1 to 5 mm of water was layered on top of the separating gel. The gel was allowed to polymerise for 30 to 60 minutes.

The following solutions were mixed in a small beaker to make up two 5 % stacking gels, water (2.3 ml), Solution A (0.67 ml), Solution C (1.0 ml), Ammonium persulphate (10 %, 30 µl), and TEMED (5 µl). The gel was allowed to polymerise for about 30 minutes.

B.2 Preparation of gels for discontinuous native PAGE

For the preparation of native gels, the reducing and protein denaturing agent, SDS was excluded from the 4x separating gel buffer, 4x stacking gel buffer, electrophoresis buffer, 5x sample buffer and 2-mercaptoethanol was excluded from the 5 x sample buffer. Working solutions were prepared from stock solutions as follows:

1. Solution A (Acrylamide solution, 100 ml) 30 % (w/v) acrylamide, 0.8 % (w/v) bis-acrylamide.)

2. Solution B (4x Separating Gel Buffer, 100 ml), (1.5 M Tris-HCl, pH 8.8)

3 Solution C (4x Stacking Gel Buffer, 100 ml), (0.5 M Tris – HCl, pH 6.8)

4 Ammonium persulphate (10 %, w/v, 5 ml)

5 Electrophoresis buffer, (1L)

6 5X Sample Buffer (10 ml)

These solutions were mixed in a small beaker to make up two 8 % separating gel and 5 % stacking gel in the same proportions as described for SDS PAGE in section B1.

Appendix C: Determination of molecular weight of proteins

The rate of migration of a protein during SDS-PAGE is generally proportional to the mass of the protein. A standard curve is generated with proteins of known molecular weight, and the molecular weight of the protein can be extrapolated from this curve (Bollag, *et al.*, 1996). After completion of gel electrophoresis, the distance of migration of the proteins as well as that of the bromophenol blue (tracking dye) is measured from the beginning of the separating gel to the leading edge of a protein band. The R_f values are calculated by dividing the distance of protein migration by the distance of tracking dye migration. The log_{10} of the known protein molecular weights is plotted as a function of their R_f.

C.I Endoinulinase molecular weight calibration curve

The molecular weight of the purified endoinulinase was estimated from the semilogarithmic graph of pure molecular weight markers (peqLab Protein Marker II). The log10 of the molecular weight markers yielded a linear curve (Figure C.1).
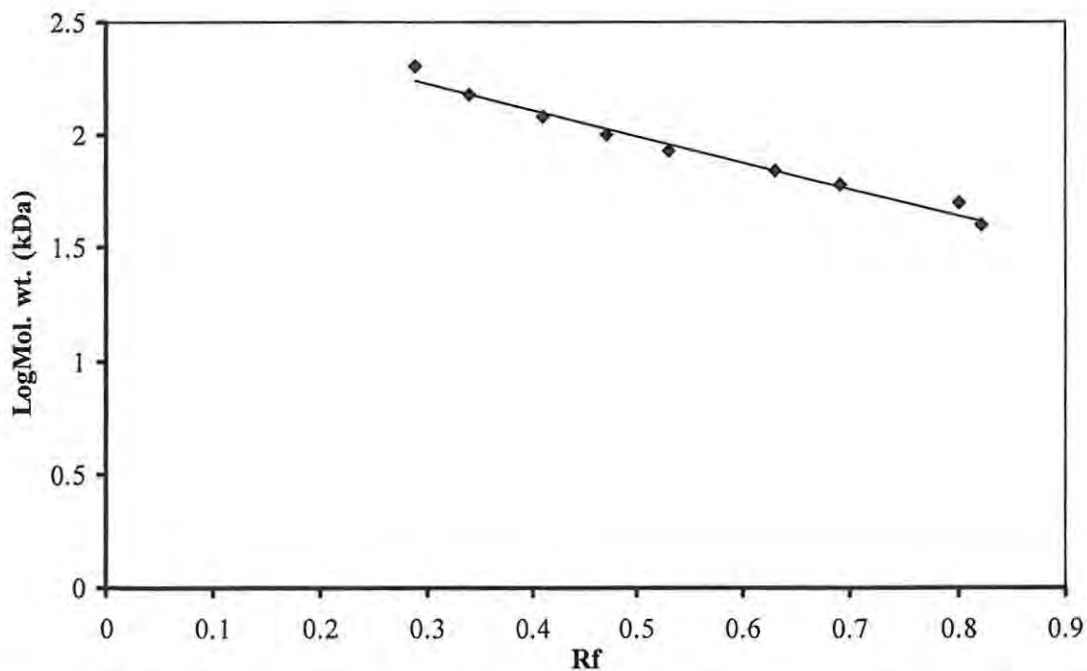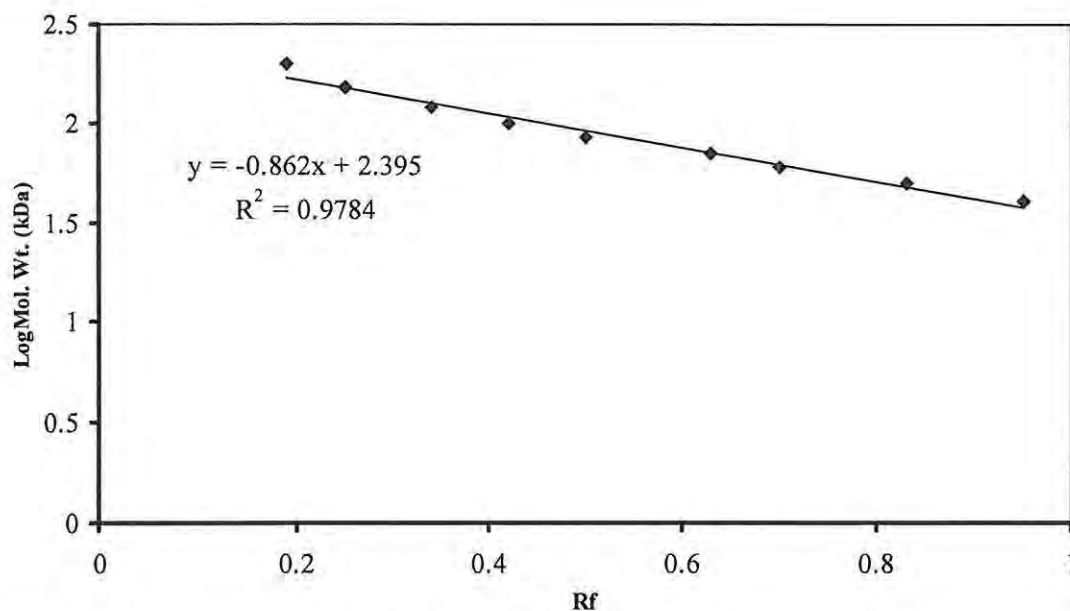

Figure C.1 Molecular weight calibration curve using peqLab Protein Marker II ranging in molecular weight from 15 to 200 kDa.

C.2 Exoinulinase molecular weight determination

The molecular weight of the purified endoinulinase was estimated from the semilogarithmic graph of pure molecular weight markers (Perfect Protein™ Markers, 15 to 150 kDa, Novagem). The log10 of the molecular weight markers yielded a linear curve (Figure C.2).

The graph shows LogMol. Wt. (kDa) on the y-axis (0 to 2.5) versus Rf on the x-axis (0 to 1), with data points forming a linear trend:

$$y = -0.862x + 2.395$$
$$R^2 = 0.9784$$

Figure C.2 Molecular weight calibration curve using Merck Recombinant Molecular weight marker ranging in molecular weight from 15 to 150 kDa.