The synthesis of fructooligosaccharides by the β-fructofuranosidase FopAp from *Aspergillus niger*

Thesis submitted in fulfilment of the requirements for the degree of

MASTERS IN SCIENCE (BIOCHEMISTRY)

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By

Mitchell Kingsley Chido Pindura

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ABSTRACT

Fructooligosaccharides (FOS) are short-chain fructans with a terminal glucose moiety and are found naturally in many plant species. Besides their wide use as an alternative sweetener in food and beverage industry, FOS have shown great potential as neutraceuticals against diabetes, colon cancer and bowel disease. The uses of FOS are dependent on the degree of polymerisation that they exhibit. β-fructofuranosidase (FFase) and fructosyltransferase (FTase) enzymes are capable of synthesing FOS from carbohydrate raw materials such as chicory and sugar beet. The aim of this study was to investigate the synthesis of FOS of a pre-defined chain length, from sucrose, by the enzyme FopAp; a β-fructofuranosidase from *Aspergillus niger*. ATCC 20611. The crude enzyme FopAp was successfully purified, with a yield of 78.20 %, by ammonium sulphate precipitation and anion exchange chromatography. Two protein fractions, named FA and FB were shown to exhibit FFase activity. SDS PAGE analysis revealed two proteins with molecular weights of 112 kDa and 78 kDa, which were identified as a FFase and a hydrolase. Temperature and pH optima of 20 ºC and 9, respectively, were observed for the transfructosylation activity in the FFase. The purified FFase exhibited a half life of 1.5 hrs under optimal conditions. Substrate kinetic studies indicated a high hydrolytic activity at low sucrose concentrations, with V_{max} and K_m of 1.25 µmol/ml/min and 3.28 mM, respectively. Analysis by response surface methodology identified temperature and pH to be significant factors for the production of kestose and nystose, at a 95 % level of confidence. These findings were confirmed by neural networks constructed to identify optimal conditions of FOS synthesis.FOS synthesis was found to be optimal between pH 6 and pH 9 at 25 ºC. The factor of reaction time was found to be insignificant within the selected experimental constraints, for both FOS species. The findings of this investigation are very important as the foundations of a commercially viable synthetic process for the production of FOS.

DECLARATION

I declare that this thesis is my own, unaided work. It is being submitted for the degree of Master of Science (Biochemistry) at Rhodes University. It has not been submitted before for any degree or examination at any other university.

_____

Signed: Mitchell Kingsley Chido Pindura

On this _____ of _____ 20____

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This thesis would not have been possible without you!

TABLE OF CONTENTS

3 D	three dimensional
ACN	acetonitrile
ADP	adenosine di-phosphate
ANN	Artificial neural network
AOAC	association of analytical chemistry
ASD	ammonium sulphate and dialysis
ASP	aspartate
ATP	adenosine tri-phosphate
BCI	brain computer interface
C	crude
CCD	central composite design
CE	catalytic efficiency
CI	confidence interval
cm	centimetre
CNT	carbon nano- tubes
DCA	deoxycholic acid
DNS	dinitrosalicylic acid
DP	degree of polymerisation
ELSD	evaporative light scattering detection
ENZ	ffase enzyme
ESI- MS	electro-spray mass spectrometry
EU	european union
F	sucrose
FDA	food and drug administration
FFase	fructofuranosidase
FOS	fructooligosaccharides
FPLC	fast protein chromatography
FTase	fructosyl transferase
FTIR	fourier transform infrared spectroscopy
G	glucose
g	grams
GC	gas chromatography
GF	sucrose
GF2	1-kestose (kestose)
GF3	1-nystose (nystose)
GF4	fructo-furanosyl nystose
GLU	glutamate
GRAS	generally recognized as safe
HCl	hydrochloric acid
HILIC	hydrophilic interaction liquid chromatography
HK	hexokinase
HPAEC	high performance anion exchange chromatography
HPLC	high performance liquid chromatography
HR	high resolution

hrs	hours
IEC	ion exchange chromatography
Ig A/G	Immunoglobulin A/G
k_{cat}	turnover number
kDA	kilodaltons
k_m	Michealis Menten constant
MALDI-TOF-MS	matrix assisted laser desorption /ionisation-time of flight- mass spectrometry
mFOS	mixture of fructooligosaccharides
mins	minutes
ml	millilitre
mM	millimolar
mol	moles
Mr	molecular mass
MS	mass spectrometry
MWCO	molecular weight cut off
NaCl	sodium chloride
NADP	nicotinamide adenine dinucleotide phosphate
NADPH	reduced nicotinamide adenine dinucleotide phosphate
NASA	national aeronautics and space authority
NEC	necrotizing enteric colitis
NMR	nuclear magnetic resonance
PAD	pulsed amperometric detection
PAGE	polyacrylamide gel electrophoresis
PGI	phosphoglucose isomerase
PGIV	peqGold IV
R	reducing sugars
RDA	recommended daily amounts
RI	refractive index
RSM	response surface methodology
S	substrate
Sc FOS	short chain fructooligosaccharides
SD	standard deviation
SDS	sodium dodecyl sulphate
SEC	size exclusion chromatography
SYNCAN	synbiotics and cancer prevention in humans
TLC	thin layer chromatography
U	units of enzyme activity
Uh	hydrolase activity
USDA	united states department of agriculture
Ut	transfructosylase activity
Utot	total FFase activity
UV/VIS	ultra-violet/visible light spectrum
V	volume
v/v	volume per volume
v_{max}	maximum reaction velocity

w/v	weight per volume
β-ME	β-mercapto-ethanol

CHAPTER 1
GENERAL INTRODUCTION AND LITERATURE REVIEW

1. Structure of FOS and fructans

Fructooligosaccharides (FOS) are short-chain fructans with a terminal glucose moiety and are found in many plant species (Becker and Lorenz, 1978; Jaime *et al.,* 2001). Lewis (1993) and Campbell *et al.* (1997) have also defined FOS to be a mixture of short chain oligosaccharides that are linked by β-(2, 1) glycosidic linkages and possess an α-D-glucose unit on their non-reducing end **(Figure 1.1)**.

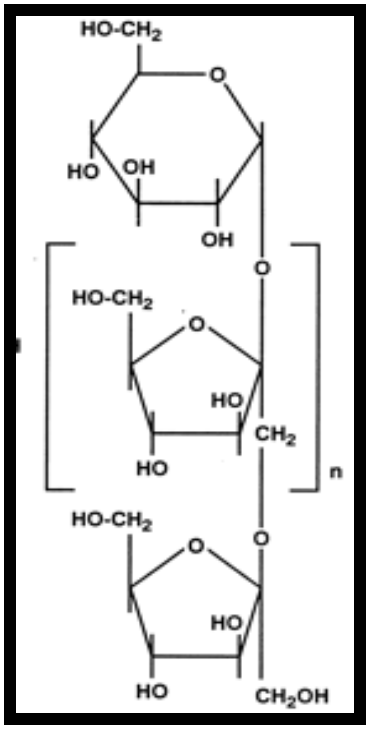


Figure 1.1: General structure of fructooligosaccharides (Where: G-glucose, F- Fructose and m is DP ≥ 2 (adapted from Mutanda, (2007))

Fructans are linear compounds consisting of *β*-(2, 1) linked D-fructofuranose units and one terminal *α*-(1, 2) linked D-glucopyranose unit. Several types of fructans are known and they differ based on their chemical structure as well as the organisms producing them (Corradini *et al.,* 2004). An oligosaccharide is characterized by the degree of polymerisation (DP), type as well as 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 (Prapulla *et al.,* 2000; Roberfroid *et al.,* 1998). Inulin fructans are poly-disperse with a degree of polymerisation ranging from 2 to 60 fructose units

exhibiting β-(2, 1) linkages **(Figure 1.2)**. The degree of polymerisation is defined as the number of fructose units linked to the terminal glucose moiety (Sharma and Gill 2007; Sharma *et al.,* 2006; Van Loo *et al.,* 1995).

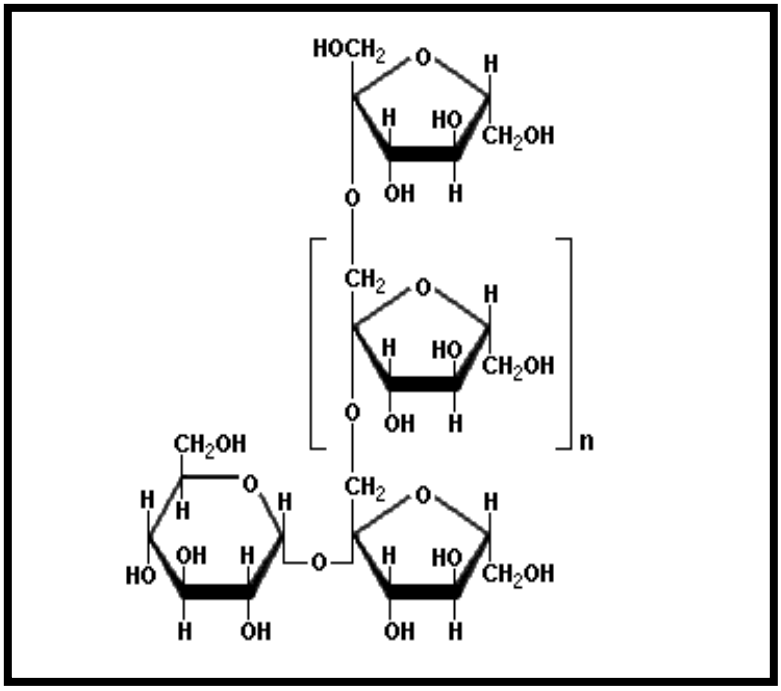


Figure 1.2: General structure of inulin fructans (Where: G-glucose, F- Fructose and n is ≥ 2 (Mutanda, (2007))

Fructooligosaccharides are among the most extensively studied prebiotics and include a diverse family of fructose polymers which vary in length and can either be derivatives of simple fructose polymers or fructose moieties attached to a sucrose molecule (Saulnier *et al.,* 2007). Kestoses are fructans containing glucose and two fructose units **(Figure 1.3)** while keto-*n*-oses are oligomeric fructans of DP ≥ 3 that contain a sucrose unit with the DP designated by a Greek root as in the instances: ketohexose and keto-decaose (Lewis, 1993)

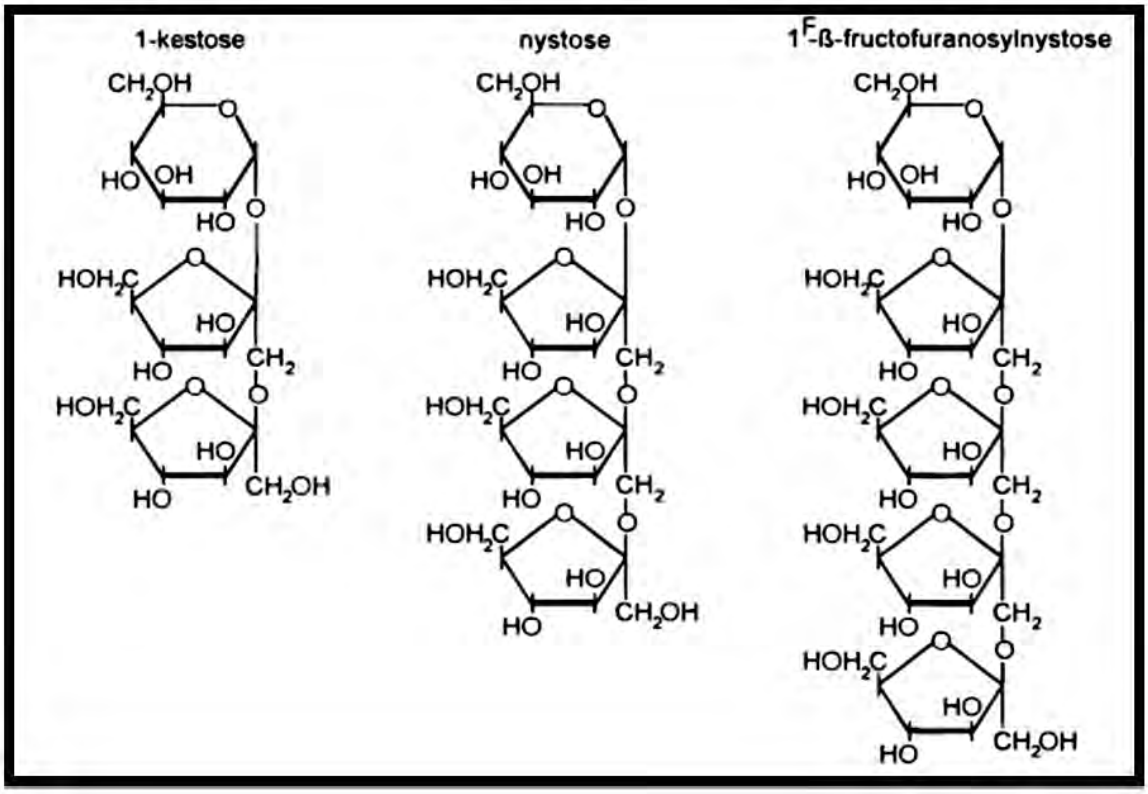


Figure 1.3: Structures of 1-kestose, 1-nystose and 1-β-fructofuranosylnystose (Bornet and Brouns, 2002)

2. Sources of FOS

Fructooligosaccharides are found in trace amounts as natural components in fruits, vegetables and honey (Sangeetha *et al.,* 2005). More than 36 000 vegetable plants accumulate large amounts of poly-fructans as reserve carbohydrate (Sharma *et al.,* 2006; Itaya *et al.,* 2007) and about 15 % of flowering plants have stored fructans in at least one of their organs during their lifetime (Kaur and Gupta, 2002; Hendry, 1993). Fructans have been shown to act as energy reserves to support plant growth during periods of inhibited photosynthetic activity; a function of their nature as polymers of fructose, synthesized from sucrose (Winzeler *et al.,* 1990; Goggin and Setter, 2004).They may also be formed as a result of –ecompatible solute synthesis", by plants in cold or drought conditions, to protect cell membranes from osmotic damage.(Goggin and Setter, 2004; Rathinasabapathi, 2000) Dicotyledonous species store inulin type fructans consisting of linear β (2,1) fructo-furanosyl units while more complex and branched type fructans are more common in monocots (Kaur and Gupta, 2002; Vijn and Smeekens 1999). However,

despite the high fructan content of the aerial parts of many grasses and cereals, the presence of components that can interfere with extraction of FOS, renders them unsuitable for industrial food and non-food applications. On the other hand, species that store fructans in bulbous organs, tubers and roots, provide easily extractible fructans. This limits the sources of fructans to species mainly from *Liliaceae, Amaryllidaceae* and *Compositae* families (De Bruyn *et al.,* 1992; Kaur and Gupta, 2002). Fructooligosaccharides have thus been successfully extracted from fruits and vegetables such as bananas, onions, chicory root, garlic, asparagus, barley, wheat, jícama, tomatoes, and leeks (**Figure 1.4**). The Jerusalem artichoke and its relative, the yacón, have been found to exhibit concentrations of fructooligosaccharides as high as 282 mg/g of dry mass (Campbell *et al.,* 1997).

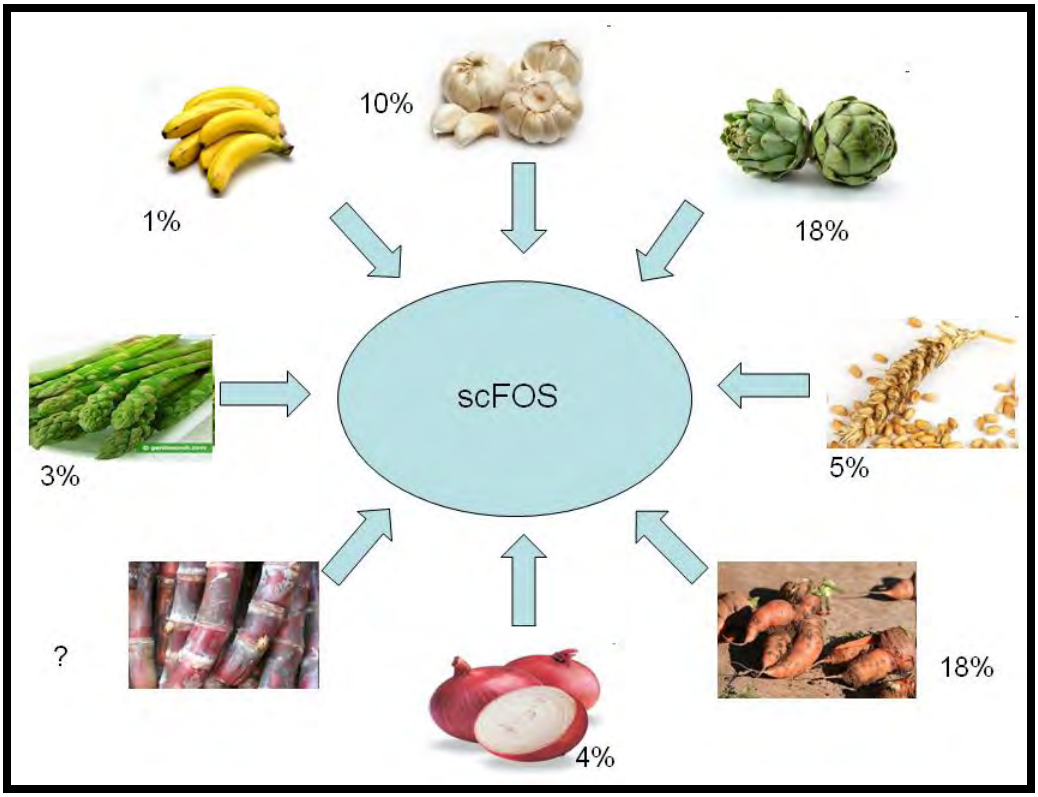


Figure 1.4: Common sources of fructooligosaccharides (FOS) showing their relative use in FOS synthesis. ?- Despite being a ubiquitous food crop, there is limited information on the relative use of sugarcane in FOS synthesis (adapted from Roberfroid *et al.* (1998))

2.1 Sucrose

Sucrose (α-D-glucopyranosyl-1, 2-β-D-fructofuranoside), more commonly known as table sugar is, according to Bieniawska *et al.* (2007), the most common sugar accumulated by plants. It is extensively used as a basic ingredient in the food and pharmaceutical industries due to quality attributes it confers on the sweetness, flavour, texture, shelf-life and function of final products (Lee, 2010) .

2.1.1 Sources of sucrose

Sucrose is the principal photosynthetic export from the leaves to supply the rest of the plant with the carbon and energy needed for both growth and the synthesis of storage reserves (Lunn and MacRae, 2003). Due to its stability and the limited number of enzymes catalysing its degradation, sucrose serves as the major storage reserve in plant organs such as sugarcane *(Saccharum officinarum)* stems, where it comprises 90 % of total sugar; sugar beet (*Beta vulgaris*) roots as well as the fruits of many species (Calsa and Figueira, 2007; Lunn and MacRae, 2003). Sugarcane, being a robust and vigorous tropical plant, with superior growth over most crop species, is the largest source of commercially produced sucrose. Sugar beet is as an alternative source of sucrose in temperate climates though its use is still limited compared to sugarcane (Gerbens-Leenes and Hoekstra, 2009; Wu and Birch, 2007).

2.1.2 Structure of sucrose

The chemical structure of sucrose comprises a fructo-furanose and a gluco-pyranose adjoined by a β-(1-2) glycosidic linkage (**Figure 1.5**) (Kaminski *et al.,* 2008). In its crystalline structure, the sucrose molecule exists in a folded state with two additional hydrogen bonds being formed with oxygen atoms within the molecule. Binding between molecules in the crystal structure also occurs via hydrogen bonds to oxygen atoms (Beevers *et al.,* 1952 in Kroll *et al.,* 2007). Five different structural isomers of sucrose exist. All are composed of glucose and fructose with variations in adjoining glycosidic linkages as follows: α-1,1 (trehalulose), α-1,3 (turanose), α-1,4 (maltulose), α-1,5 (leucrose), α-1,6 (isomaltulose) (Lipski *et al.,* 2010).

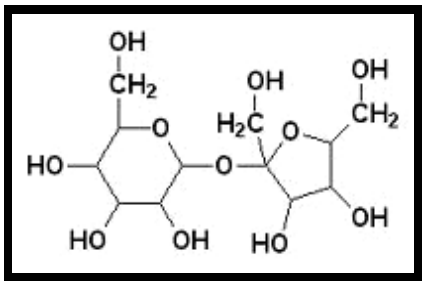


Figure 1.5: Structure of sucrose showing oxygen and hydrogen for bonding

2.1.3 Effects of sucrose on metabolism and human physiology

Despite the obvious benefit of sucrose as a high energy food constituent, it is a well known fact that frequent intake of high levels of sucrose may induce undesirable metabolic and pathologic effects. In the body, sucrose is readily absorbed intestinally and hydrolysed to glucose and fructose, with a consequent increase in blood sugar levels (Lipski *et al.,* 2010). Wong and Sissons (2007), in a study to determine the effects of various nutrients on calcium phosphate deposition, suggested a definite link between cariogenesis and sucrose concentrations in the buccal cavity. Sucrose, as a highly calorific food ingredient has also been directly linked to weight gain. A study on rats, has for the first time, revealed a strong correlation between mitochondrial dysfunction in adipose tissue, impaired metabolism and high sucrose diets (Lipski *et al.,* 2010; Lomba *et al.,* 2010). A scientific statement by Johnson *et al.* (2009), of the American Heart Association, described the obesogenic effect of diets high in sucrose, glucose and high-fructose corn syrup. Also highlighted was the superiority of high fibre products over low fibre (highly refined) products in reducing hyperglycaemia.

2.1.4 Sucrose as a source of FOS

In spite of the evidence provided against the use of sucrose as a dietary constituent, it is unfortunate that there has not been sufficient production of an alternative sweetener that (i) has nutritious properties and (ii) is not harmful to humans in the short or long term (Lipski *et al.,* 2010). Notwithstanding the emergence of a variety of artificial sweeteners such as aspartame, saccharine and sucralose, it is still thought that none possess taste profiles that are comparable to that of sucrose (Cardoso and Bolini, 2010; Trevizam - Moraes and Bolini, 2010). There has been great interest in the large scale synthesis of

FOS from sucrose over the past 20 years. Early works by Hidaka *et al.* (1988), describe the use of an enzyme from *Aspergillus niger* ATCC 20611 to synthesize FOS with inulin-type structures, with sucrose as the substrate. Since then, a variety of yeasts and fungus have shown the great potential in the bio-transformation of sucrose to FOS (Sangeetha *et al.,* 2005). Recent studies by Bekers *et al.* (2008), showed the possibility of linking FOS production using *Zymomonas mobilis* from sucrose substrate (sugar beet) to bio-ethanol production. With the increasing incidence of diabetes, diarrhoea and cancer in developing economies, the synthesis of FOS in sufficient amounts to replace sucrose as the principal sweetener in many food products, may serve as a timely solution. From an economic point of view, there is clearly a need for commercial production of a healthier choice of sweetener without necessarily obliterating the sugar industry which generates enormous amounts of revenue per annum (Maloa, 2001). In South Africa alone, commercial sugarcane, despite the 2008/09 Global Financial Crisis, covers a substantial portion of the eastern coastal area (**Figure 1.6**). Given the *status quo*, there is great potential for a sustainable commercial process which produces functional foods locally while promoting research and technology transfer and not to mention creating revenue through exporting a commodity whose global demand is increasing rapidly (Verschuren, 2007).

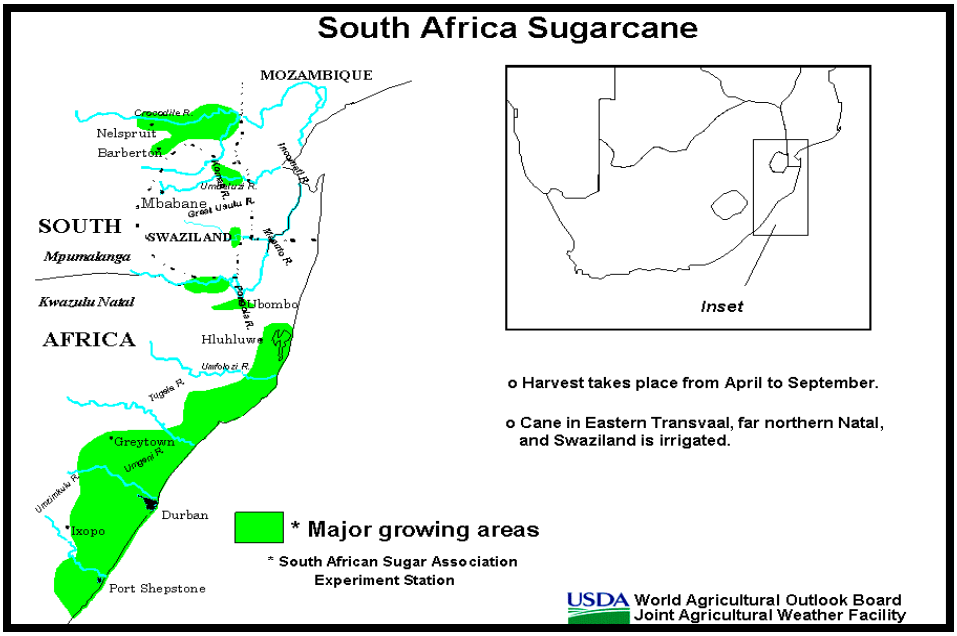


Figure 1.6: Map showing the major sugar growing areas in South Arica (Spectrum Commodities, 2010).

2.2 Other Disaccharides used in the synthesis of FOS

Sucrose analogues have also been evaluated as donor substrates for many bacterial fructosyl transferases (Biedendieck *et al.,* 2007; Seibel *et al.,* 2006). An article by Beine *et al.* (2008), reported the use of sucrose analogues α-Xyl-1, 2-β-Fru and α-Gal-1, 2-β-Fru as substrates of levansucrase enzyme from *Bacillus subtillis*, in the synthesis of novel fructooligosaccharides. Sucrose analogues are believed to yield analogues of the FOS, 1-kestose and 1-nystose, in which a glucosyl residue is exchanged for a glycol-pyranosyl residue of the sucrose analogue (Beine *et al.,* 2008). Zuccaro *et al.,* (2008), have successfully demonstrated this principle on an engineered fructofuranosidase from *A. niger* using engineered sucrose analogues (Man-Fru), (Gal-Fru), (Xyl-Fru).

3 Uses of Fructooligosaccharides

There has been a rapid shift in global perceptions towards nutrition and the strong relationship it has with a healthy state of being (Sangeetha *et al.,* 2005). Foods that provide benefits beyond basic nutrition and are thought to play a role(s) in disease prevention or health promotion are called, Functional Foods (Campbell *et al.,* 1997). A functional food ingredient that has received much attention is FOS (Hussein *et al.,* 1998). FOS (which have one to three units of fructose bound to the, β 2-l position of sucrose) and iso-maltooligosaccharides (in which there is one or more glycosidic bonds with or without α-1, linkages) have received particularly special attention due to their applicability in food and medicine (Yun, 1996). In addition to being stable at neutral pH and at temperatures up to 140°C, they possess useful physico-chemical and physiological properties, which make them widely applicable to food and feed stuffs (Nishizawa *et al.,* 2000). They have good quality sweetness, comparable to sucrose, high fructose corn syrup and other starch derived sugars (Yun, 1996).

3.1 Medical applications

The health benefits derived from the colonic fermentation of FOS in humans are well documented (Gibson *et al.,* 1995). As a result of the β-configuration of the carbons about their glycosidic bonds, FOS resist hydrolysis by salivary and intestinal digestive enzymes: α-glucosidase, maltase, sucrase and salivary amylase (Kaur and Gupta, 2002).

This property along with the fact that they are fermented specifically by certain intestinal anaerobes makes them efficient prebiotics in the lower digestive tract (Boehm *et al.,* 2002; Crittenden and Playne, 1996; Gibson, 1995). According to Gibson *et al.* (2010), a prebiotic is a non digestible, selectively fermented, food entity that can potentially benefit the host organism‘s health by stimulating the growth and/or activity of specific resident bacterial species; a probiotic is a microbial supplement that directly modulates certain beneficial species of intestinal flora of the host organism; and a synbiotic is a combination product of the two. Selective fermentation of any given FOS molecule is closely linked to its degree of polymerization and is thus an important factor to consider before prebiotic use (Biedrzyka and Bielecka, 2004; Macfarlane and Macfarlane, 2007). Furthermore, studies by Suzuki *et al.,* (2006) , revealed that 1-kestose exhibited greater fermentation by bifidobacteria than a recommended mixture of FOS (mFOS) which contained a larger proportion of nystose than 1-kestose.These results introduce the significance of the ratio of FOS within mFOS products on probiotic activity.

Inulin and sucrose based oligosaccharides are candidate carbohydrates for fermentation by bifidobacteria (**Figure 1.7**) in the large intestine (Kaur and Gupta, 2002). *In vivo* demonstrations by Campbell *et al.* (1997) and Hussein *et al.* (1998), showed a marked increase in bifidobacterial colonies in rats that had been administered fructooligosaccharide rich treatments. Similar results have been observed in human populations in Japan, Europe and North America by Mitsouka *et al.* (1987); Kleessen *et al.* (1997) and Buddington *et al.* (1996), though in some of these studies only bifidobacterial colonies were accounted for, leaving a gap in knowledge of the relative effects on other species (Roberfroid *et al.,*1998). Boehm *et al.* (2002), reported a significant increase in bifidogenic colonies and stool frequency in pre-term neonates after a course of bovine milk supplemented by an oligosaccharide mixture (90 % galacto-saccharides and 10 % fructosaccharides). This particular study highlighted the vulnerability of the pre-term neonate‘s alimentary canal to infections borne from complex bacteria and also emphasized that a balanced intestinal micro-flora is pivotal to an adequately healthy state and may reduce the incidence of necrotizing enterocolitis (NEC) (Boehm *et al.,* 2002; Catala *et al.,* 1999). Walker and Duffy (1998) observed that human

breast milk tends to favour predominance of bifidobacterial and/or lactobacillus colonization of the lower intestine. It thus follows that, for a pre-biotic preparation to be used as supplementation in infants, it should exhibit synergistic qualities that favour the growth of both types of microflora, (Boehm *et al.,* 2002).

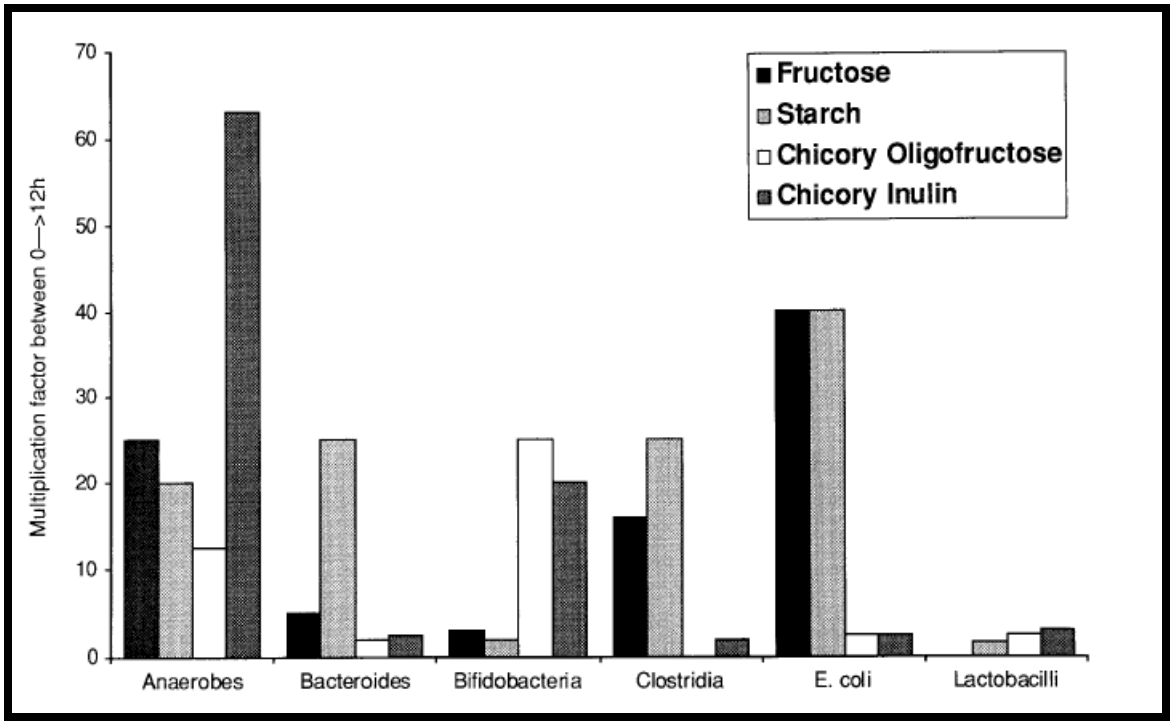


Figure 1.7: Selective fermentation of different carbohydrates by microorganisms (Roberfroid, 2007)

Bifidobacterial production of fatty acids as a result of FOS fermentation, is thought to stimulate intestinal peristalsis and osmotically increase the humidity of the faecal bolus; a classical laxative effect of dietary fibre (Hidaka *et al.,* 1988; Riverourgell, 2001).

The ratio of accumulated acetate to propionate from FOS fermentation has been linked to a reduction in serum- cholesterol levels. Although Jenkins *et al.* (1991), reported an increase of serum cholesterol on intestinal absorption of acetates in rats, a more recent review by Venter, (2007) hypothesized that FOS, which can lower the acetate- propionate ratio, can also reduce the volume of low density lipids in the serum and possibly reduce the risk of coronary heart disease.

Acidification of the colon mucosa by acetate and lactate products of FOS fermentation is also thought to inhibit the proliferation of bacteria that are involved in the aetiology of bowel diseases, thereby reducing the incidence (Gibson *et al.*, 1995; Grizzard *et al.,* 1999). This may be of particular benefit in infants, whose gastro-intestinal pH is not as low as adults and immune systems have not yet fully developed (Riverourgell, 2001). It is also proposed that this build up in organic acids, maybe responsible for observed mucosal hyperplasia, increased wall thickness, and improved blood flow in the intestinal tract (Kaur and Gupta, 2002). Another protective function of FOS stems from the secretion of bacteriotoxins by bifidobacteria against common pathogenic organisms such as *Escherichia coli, Salmonella*, *Shigella* and *Vibrio cholerae* (Fooks and Gibson, 2002).

There is increasing evidence that the addition of FOS to the diet alters gut structure and function, modifies the production of gut-derived hormones and is associated with improved whole body glucose homeostasis even in the absence of disease (Mortensen and Clausen, 1996; Schley and Field, 2002). Prebiotic supplementation in poultry feed has demonstrated an increase in serum immunoglobulin (IgG) and is thus thought to have an immuno-modulatory effect which may reduce the use of antibiotics in farming and promote more ecologically favourable methods (Gibson *et al.,* 2004; Midilli *et al.,* 2008). Some researchers have shown that the ingestion of FOS stimulated intestinal IgA and the production of cytokines by blood mononuclear cells (Hosono *et al.,* 2003; Manning and Gibson, 2004). It has also been observed that consumption of inulin-type fructans increases the phagocytic capacity of macrophages; and preliminary data in different experimental models suggests convincing correlations between FOS consumption and immuno-modulation in lymphoid tissue and the peripheral circulatory system, though more studies are required to confirm these hypotheses (Venter, 2007).

Coudray *et al.* (2003) highlighted the additional benefit of these non-digestible sugars as consistent promoters of magnesium and calcium absorption in different animal species. The enhanced bio-availability of Mg^{+2} is directly linked to higher Mg^{+2} in bone tissue and consequently, a significant reduction in the risk of osteoporosis (Rude *et al.,* 2004).

A study conducted by Rozan *et al.* (2008), on the lifelong effects of FOS on ageing and the general health and wellbeing of rats, reported that subjects fed on a diet of FOS enriched insulin (i) did not become overweight, (ii) remained more active and alert at 24 months of age as compared to the more docile control group and (iii) exhibited a higher survival rate compared to the control group (**Figure 1.8**).

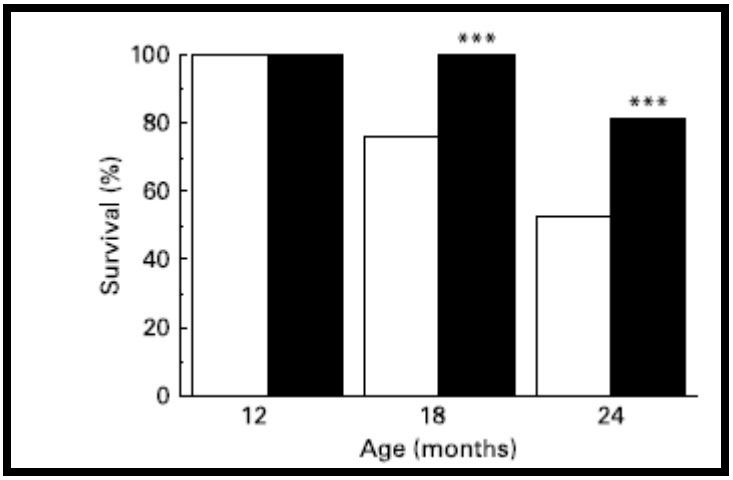


Figure 1.8: Graph of relative survival vs. Age of rats on FOS diet (***) and those on normal diet. (Rozan *et al.*, 2008)

The protective effect of FOS against colon cancer has been observed and well documented. Investigations on mice by Pierre *et* al. (1997) demonstrated a significant reduction in colon cancer as well as a definite development in the gut-associated lymphoid tissue (GALT). It was then proposed that FOS may indirectly stimulate anti-tumour immunity (Hijova *et al.,* 2009; Perrin *et al.,* 1994; Pierre *et al.,* 1997). Pool-Zobel *et al.* (2002) suggested that the carcinogenic effect of low fibre diets can be explained by an increase amount of cytotoxic compounds such as deoxycholic acid (DCA), which induce cell proliferation in the colon. Short chain fatty acids produced by the fermentation of FOS in the intestinal tract create a low pH environment, which suppresses the activity of carcinogen producing enzymes like β-glucuronidase, urease and azo-reductase (Grizard and Barthomeuf, 1999; Reddy, 1998; Teitelbaum and Walker, 2002). Inulin-fructans have also been demonstrated to inhibit azoxymethane induced pre-neoplastic lesion formation in rats (Pretlow *et al.,* 1992 in Grizard and Barthomeuf, 1999). The European Union (E.U.) sponsored project, SYNCAN, has been initiated to

validate the *in vitro* findings on the anti-carcinogenic effect of FOS and other prebiotic compounds. This initiative involves (i) the combination of collected *in vitro* data (ii) further *in vivo* investigations on rats in an attempt to determine the viability of synergistic benefits of selected synbiotics in human subjects; as well as (iii) a human intervention study. Preliminary results show high viabilities of both rifampicin resistant *Lactobacilli* and bifidobacteria within 7 days of feeding, as well as modulation of colon cancer risk parameters in human subjects (Van Loo *et al.,* 2005). Early investigations by Oku *et al.* (1984) in Kaur and Gupta (2002) on rats fed a 10 % FOS diet for 30 days demonstrated a 17 % and 26 % reduction in post-prandial glycaemia and insulineamia, respectively. However, Kaur and Gupta (2002) commented that this effect may be dependent on physiological and disease states of the subject, especially in diabetic cases. More recent publications have indicated the potential benefit of FOS in reducing the risk of diabetes and have linked the short chain fatty acid propionate, to reduced fasting glucose and increased maximum fasting insulin in human subjects (Bornet and Brouns, 2002; Crittenden and Playne, 1996;). The health benefits as well as the supportive evidence for the above claims are summarised in **Table 1.1**.

Table 1.1: Health benefits of FOS (adapted from Roberfroid (2007))

Target Functions or Disease Risk	Supportive Evidence
Lipid homeostasis	Reduced cholestorelemia
Immuno-stimulation	Improved resistance to common infections in children, improved response to vaccines
Gastro-intestinal Endocrinology	Stimulation of production of intestinal hormonal peptides
Inflammatory Bowel Diseases	Improved clinical symptoms, biomarkers and overall disease management
Cancer	Animal data in different experimental models; SYNCAN
Diabetes	Reduces Hyperglycaemia

3.2 Applications in food

The capacity in and extent to which FOS can be used in food preparations is thought to be linked to: (i) the degree of polymerisation as well as (ii) the ratios of FOS within the mixture itself (Crittenden and Playne, 1996). For instance, a mixture of short chain FOS, is more soluble than inulins and therefore maybe preferentially used as a prebiotic additive to yoghurt and other food products (**Table 1.2**) (Roberfroid, 2007; Barreteau *et al.,* 2006).

Table 1.2: (adapted from Gibson *et al.* (2004)

Possible foodstuffs that can be fortified with prebiotics
Dairy products
Beverages and health drinks
Spreads
Infant formulae and weaning food
Cereals
Bakery products
Confectionery, chocolates and chewing gum
Savoury products and soups
Animal feeds
Food supplements
Canned food

Fructooligosaccharides are used specially in combination with or as a substitute for high-intensity artificial sweeteners, such as aspartame, whose sweetness profile and after-taste it improves; a property that also makes them suitable for use in low-calorie diet foods and for individuals with diabetes (Crittenden and Playne, 1996; Franck, 2002; Wiedmann and Jager, 1998). Neosugar (Meioligo®), a new sweetener, was found to be a non-digestible sugar for humans and to be physiologically useful because it increases intestinal flora (Tokunaga *et al.,* 1993). Neosugar is a mixture of FOS: 1-kestose (GF2), nystose (GF3) and fructofuranosyl-nystose (GF4), which is commercially produced from sucrose using an enzyme obtained from *Aspergillus niger* ATCC2611 (Nishizawa *et al.,* 2000). Combinations of FOS, which exhibit sweetness 0.3-0.6 times that of sucrose have also been used as food bulking agents that do not modify the delicate flavour of principle ingredients (Crittenden and Playne, 1996; Franck, 2002).

Another important characteristic which has made FOS appealing is their relatively low calorific value in comparison to sucrose and its alternatives (Roberfroid *et al.,* 1993). This is directly linked to their glycaemic index, which as a result of the human intestine‘s incapacity to digest β-(2, 1) glycosidic linkages is very low (Campbell *et al.,* 1997). A study conducted by Hussein *et al.* (2002) on the percentage recovery of FOS from rats‘ digestive systems showed that most of 25 FOS containing pet-food ingredients investigated exhibited a 100 % ± 10 recovery of FOS. Some authors have even gone as far as describing FOS as dietary fibre and a necessary part of a balanced diet whose intake should be monitored and accounted for (Coudray *et al.,* 2003; Venter, 2007). This opinion has been shared and so widely accepted that the GRAS (Generally Recognized As Safe) status of inulins and FOS has been confirmed by food experts and authorities in Australia, Canada, Japan and the USA (Coussement, 1999; Franck, 2002; Prosky and Hoebregs, 1999). Campbell *et al.* (1997) have commented on the significant nutritional and health related importance of setting up RDA (recommended daily amounts) of FOS from fruit, vegetables and feedstuffs.

The need for global restrictions on the dietary intake of FOS and fructans has been supported by Shepherd and Gibson (2006), who discuss the potential pitfalls of excess ―free fructose” in diets. A link between excess fructans and fructose mal-absorption disorder, has been established, though it does not warrant the removal of the dietary entity, given its ubiquity in food stuffs as well as the extensive range of benefits it provides (Gibson *et al.,* 2010; Shepherd and Gibson, 2006; Sheperd and Gibson 2010). The actual amount of fructans in fruit and vegetable produce may admittedly be a difficult parameter to predict and monitor due to the fluctuations in fructan yield as a result of variations in growth conditions and harvesting times (Campbell *et al.,* 1997; Monti, 2005; Saengthongpinit and Sajjaanantakul, 2005).

Fructooligosaccharides have also been shown to improve ―mouth-feel” characteristics and exhibit technological properties that reduce water activity while ensuring optimal microbial stability of food preparations (Crittenden and Playne, 1996; Franck, 2002; Sangeetha *et al.,* 2005).

4 Quantification and characterization of FOS

Plant carbohydrates comprise a large variety of structures, including homo- and hetero-polysaccharides, neutral and ionic polysaccharides, linear and branched structures, and widespread molecular sizes ranging from a few monosaccharide units to thousands (Manzi and Van Halbeek, 1999). The occurrence of chirality at many carbon atoms, the possibility of anomers, and differences in three dimensional structures, by introducing large numbers of isomers, often leads to difficulties in carbohydrate separation (Ikegami *et al.,* 2008). Hence it is not surprising that the main difficulties associated with the precise detection and accurate quantification of carbohydrates stem from this wide range of chemical properties and the proportionately large range of options that result from them (Guignard *et al.,* 2005;Campa *et al.,* 2006). However, the increasing interest for carbohydrates as holders of essential bio-information, compounded by their newly-found importance in food quality by producers and regulatory authorities, has boosted the need to fully quantify and characterize them through analytical techniques (Campa *et al.,* 2006; Montilla *et al.,* 2006).

According to the FDA (Food and Drug Administration) and the USDA (United States Department of Agriculture), a dietary fibre is material that precipitates in 78 % ethanol as described by AOAC (Association of Analytical Chemists) international method for dietary fibre analysis for nutritional labelling purposes (Prosky and Hoebregs, 1999) This method is not suitable for FOS and inulin as they are only partially precipitated at the prescribed concentration of ethanol(Quemener *et al.,* 1997).It has thus been imperative for the scientific community to establish an efficient, AOAC acceptable method of analysing fructans. In 1995, a method of fructan determination consisting of an enzymatic hydrolysis of inulin coupled with HPAEC (High Performance Anion Exchange Chromatography) as described by Prosky and Hoebregs, (1999), was designed and is implemented to date (**Figure 1.9**). Such a method would not only serve as the means by which to define FOS and inulin as dietary fibre, but would also be a first step towards the establishment of efficient methods to determine the quantity of FOS formed during transfructosylation as well as its degree of polymerisation (Mutanda *et al.*, 2008). Borromei *et al.* (2009b) have since, revisited the AOAC method and put forward a robust

HPEAC-PAD protocol, based on a study of specific FOS in fermented milk, for use in the routine analysis of quality of functional foods containing FOS and IOS as prebiotic ingredients.

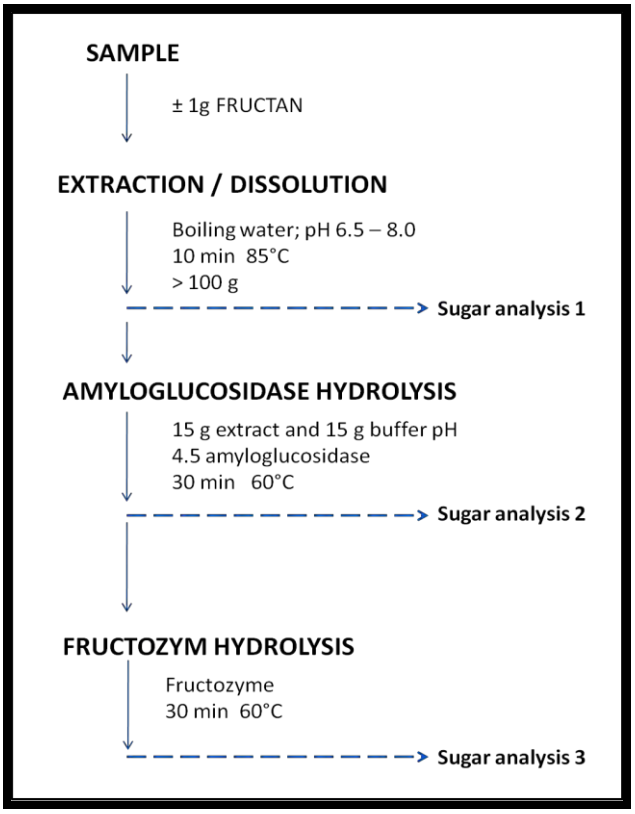


Figure 1.9: Schematic of the AOAC method for fructan determination (Prosky and Hoebregs, 1999)

The fact that most oligosaccharides do not possess a natural chromophore or fluorophore reduces the reliability of most direct spectophotometric and/or spectrofluorimetric methods of quantification (Guignard *et al.,* 2005). A potential solution is to pre-derivatize the molecule(s) of interest by attaching highly fluorescent moieties to them, though, depending on the nature of the method employed, this may be time consuming, sample expensive and may exhibit very limited selectivity and efficiency. It is therefore, desirable to avoid the derivatization of carbohydrates for quantitative measurements (Ikegami *et al.,* 2008). Another main problem associated with the quantification of FOS is the scarcity of authentic commercial standards in sufficient amounts. An alternative method which has been employed is the estimation of peak identity based on elution

profiles: a method that is based on assumptions of elution order and product purity (Corradini *et al.,* 2004; Ronkart *et al.,* 2007).

A robust method that can separate molecules of varying degrees of polymerization and their isomers as well as accurately quantify individual constituents of a mixture without consuming a great deal of time or sample; is simple and whose operation does not require a high level of expertise, is ideal in the production of fructooligosaccharides at a laboratory or industrial scale (Agblevor *et al.,* 2004; Borromei *et al.,* 2009b; Cheng *et al.,* 2006; Ronkart *et al., 2007* and Shiomi *et al.,* 1991). There are a number of methods that have been employed in the quantification of FOS over the past half -century, all of which demonstrate various advantages and disadvantages.

4.1 Separation techniques

4.1 1 HPLC

The use of HPLC systems in fructooligosaccharide quantification is well documented. In fact, HPLC is considered the method of choice (Mutanda, 2007) due to its convenience and simplicity (Cheng *et al.,* 2006). Due to the slight differences in chemical and physical properties between monosaccharide, disaccharides and trisaccharides, HPLC separation is primarily a based on differences in conformation, configuration and bonding mode. Polar bonded phase and resin-based HPLC columns have traditionally been used to separate FOS with elution on the basis of increasing and decreasing molecular weight, respectively (Sangeetha *et al.,* 2005)

The use of R.I. (refractive index) detectors was common in HPLC as they conveniently circumvented the need for derivatization as well as provided non-destructive separation of molecules based on their relative refractive index against a mobile phase under isocratic flow (Guignard *et al.,* 2005; Kenmore *et al.,* 1997; Savadogo *et al.,* 2004). Mutanda (2007) reported the separation and quantification of FOS through a Prevail® polar bonded phase column under isocratic flow using R.I. detection. Downes and Terry (2010) have reported the determination of FOS from onions on a Prevail® column using ELSD (evaporative light scattering detection), in the absence of acetonitrile (ACN) as

mobile phase. This protocol greatly reduces costs by substituting acetonitrile with ethanol, while still obtaining similar concentrations of FOS (Downes and Terry, 2010).
With recent technological advancement, these traditional methods have lost popularity due to low sensitivity, relatively weak separation and limitations in applicability (Kenmore *et al.,* 1997; l'Homme *et al.,* 2001). The use of HPAEC has been preferentially adopted as an efficient method for quantification that enables complete, single step separation of neutral and charged oligo and polysaccharides differing by branch, linkage and positional isomerism (Borromei *et al.,* 2009b;Corradini *et al.,* 2004; Guignard *et al.,* 2005) Coupling HPAEC with PAD (Pulsed Amperometric Detection) allows for an even greater sensitivity and selectivity of quantification and separation, without the time expensive step of pre- derivatization (Guignard et al., 2005). This technique involves the oxidation of carbohydrates on a gold electrode by application of a positive potential. Investigations by Shiomi *et al.* (1990) demonstrated successful separation of FOS isomers by HPEAC on a Carbo-pak® column using PAD. More recent publications by Borromei *et al*. (2009b) have documented the successful determination of FOS in fermented milk as well as comparative studies on different species of onion using the same techniques. PAD and ELSD also have the added advantage of allowing finer mobile phase adjustments to be made via gradient elution (l'Homme *et al.,* 2003). Albeit, PAD has limitations with regards to separation of highly complex natural compounds where co-elution and high baseline noise are exhibited (Campa *et al., 2006;* Guignard *et al.,* 2005; Ronkart *et al.,* 2007). There has also been a great interest within the scientific community to explore the applications of carbon nano-tubes (CNTs) as a means of enhancing the detection of glucose and sugars in general. The popularity of CNTs stems from their high electrical conductivity, high surface area, as well as their good chemical stability (Buratti *et al.,* 2008)

In as much as high performance chromatographic methods have improved the quality and ease of quantification of FOS, they appear to be insufficient in the determination of FOS structure. The use of various forms of mass spectrometry (MS) in tandem with chromatographic techniques has gained a lot of popularity as it is both convenient and easily applicable. Liquid chromatography coupled with ESI-MS (electro-spray mass

spectrometry) has been described as a useful and sensitive method for analysing trace sugars in complex media (Campa *et al.,* 2006; Cheng *et al.,* 2006).The limitations of ESI-MS include; its reduced sensitivity to carbohydrates which necessitates post-column derivatization, the fact that it requires a medium to high level of expertise, its compatibility issues with most methods and that it requires an extra de-salting step (Bruggink *et al.,* 2005; Guignard *et al.,* 2005). Consequently, despite its exploitability in the detection of neutral carbohydrates thereby improving separation selectivity with volatile buffers and increasing sensitivity of the MS detection, relatively few works with derivatization of carbohydrates were found (Campa *et al.,* 2006). Matrix-assisted laser desorption/ionization time-of flight mass spectrometry (MALDI-TOF-MS) has been used in molecular sizing of carbohydrates and can conveniently be combined with other methods such as HPLC, as a powerful tool for the qualitative and quantitative characterization of carbohydrates without derivatization (Borromei *et al.,* 2009a). Ikegami *et al*. (2009) described the use of HILIC (hydrophilic interaction liquid chromatography) coupled with ESI-MS in the enhancement of the detection of underivatized carbohydrates.

4.1.2 Gas Chromatography (GC)

Another simple and relatively cheap technique that has shown considerable potential in the quantification and characterization of FOS is gas chromatography. GC using flame ionised detection (FID) can be coupled with MS, which has demonstrated highly sensitive and selective quantification and molecular identification in analysis (Medeiros and Simoneit, 2007). However, as a result of the high polarity, hydrophilic and low volatility characteristics of carbohydrates, acetate and trimethysilyl derivatives of samples should first be synthesized to allow efficient analysis. This method is not only time consuming but also introduces error due to loss of molecules through heat damage and pre-analytical hydrolysis (Agblevor *et al.,* 2004; Guignard *et al.,* 2005; Montilla *et al.,* 2006).

4.1.3 Thin Layer Chromatography (TLC)

Prapulla *et al.* (2000) described TLC as one of the most simple and cost-effective methods employed in the qualitative analysis of sugars. Early investigations have demonstrated the effective separation of FOS of up to 20 DP in chicory leaves using TLC by carefully manipulating ethyl acetate, propan-1-ol and water ratios in a mobile phase (Collins and Chandorkar, 1971). Since then, Mutanda (2007); Park *et al.* (2001) and Vaccari *et al.* (2001) have documented the use of modern TLC techniques to analyse FOS from inulin hydrolysis, beet molasses and other products. However, this technique is limited to qualitative analysis unless derivatization of samples is performed. Malinowska *et al.* (2010) recently integrated TLC with optical densitometry whereby amounts of sugar were determined by scanning spotted TLC plates and comparing absorbance values against a calibration curve. Although the method exhibited linearity over a wide range of substrate concentration, it still required derivatization of the sample and entailed the use of highly specialized equipment (Malinowska *et al.,* 2010).

4.2 Spectroscopic techniques

4.2.1 Nuclear Magnetic Resonance Spectroscopy (NMR)

NMR is probably one of the most powerful techniques for elucidating the chemical structure of carbohydrates (Prapulla *et al.,* 2000). NMR has been utilised to determine the configuration, position of linkages, branching, and micro-heterogeneity of oligosaccharides (Duus *et al.,* 2000). This technique is not used to separate compounds and thus must be coupled with a non-destructive method such as semi-preparative HPLC (Mutanda, 2007). Nemukula *et al.* (2009) have documented the use of NMR in the characterization of FOS synthesized from sucrose using a fructosyl-transferase from *Aspergillus aculeatus.*

Techniques like HPLC or GC could be used to separate products, but these may be relatively time-consuming and only single-point assays are possible. Multidimensional spectroscopic detectors like Diode-Array UV/VIS, Raman or FTIR spectrometers are capable of monitoring several species simultaneously and continuously (Schindler, 1998; Schindler and Lendl, 1999).

4.3 Other Techniques

Resa *et al.,* (2009) utilized ultrasound velocity assays to analyse the activity i.e. the rate of sucrose consumption, of an invertase in alcoholic fermentation. This technique involves the use of low intensity ultra-sound in non-destructive assays. Separate studies by Contreras *et al.* (1992), reported the superiority of ultrasound velocity over refractive index and density as a means of differentiating monosaccharides in fruit juice. Overall, it is an easily automated, non-invasive technique that allows analysis of opaque and concentrated solutions (Resa *et al.,* 2009).

5. Enzymatic synthesis of FOS

Two different classes of FOS mixtures are produced commercially, based on inulin degradation or sucrose transfructosylation processes (Mutanda, 2007). Inulin can be degraded enzymatically or chemically to a mixture of oligosaccharides with a degree of polymerisation ranging from 1 to 7 (Rocha *et al.,* 2006). This process also occurs to some extent in nature, and these oligosaccharides can be found in a large number of plants, especially in Jerusalem artichoke and chicory (Marx *et al.,* 1997). Even though FOS can be produced by the action of enzymes present in some plants, the industrial production is mainly based on microbial enzymes (Yun, 1996). The enzymes currently used industrially for FOS synthesis are produced by the fungi *Aureobasidium pullulans* (Hayashi *et al.,* 1990; Yun, 1996) and *A. niger* (Fernandez *et al.,* 2007; Hidaka *et al.,* 1988).

5.1 FOS Producing enzymes

The enzymatic synthesis of short chain FOS is hypothesized to be the result of either a sucrose-sucrose fructosyl transferase (EC 2.4.1.99), also known as FTase or β-fructo-furanosidase (EC 3.2.1.26), also known as FFase. Both classes have shown the ability to catalyse the transfer of a fructose molecule from one sucrose molecule to another, giving rise to trisaccharides such as 1-kestose (Hidaka *et al.,* 1988; Kaur and Gupta, 2002). Addition of one or two more fructose molecules to 1-kestose yields nystose and fructosyl-nystose, respectively (Riverourgell, 2001). Polymeric chain elongation in plant fructans is generally thought to be a result of a subsequent step, mediated by 1F and 6F fructan–

fructan–fructosyltransferase (EC 2.4.1.100) (**Figure 1.10**) leading to inulin and levans respectively (Kaur and Gupta, 2002). Transfructosylating and hydrolytic enzymes belong to glycoside hydrolase family 32 (GH32) and GH68 of clan GHj (Alberto *et al.,* 2004; Goosen *et al.,* 2007), and can be found in a variety of plants, bacteria and fungi (Yanai *et al.,* 2001).

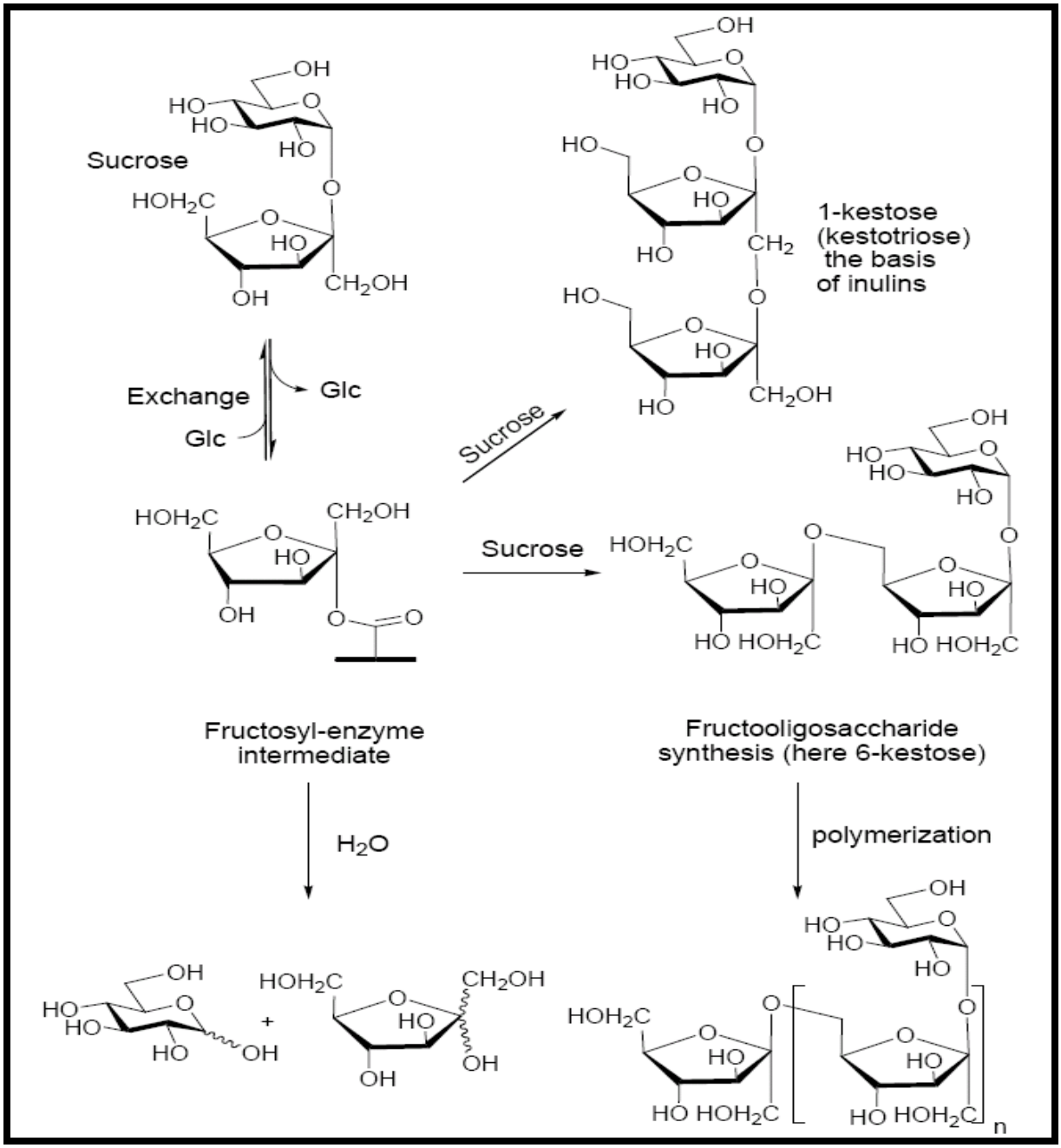


Figure 1.10: Transfructosylation reaction leading to FOS chain-elongation (Martinez-Fleites *et al.,* 2005)

5.1.1 Isolation and purification of β-fructofuranosidases and fructosyl transferases

FOS producing FFases and FTases can be isolated from ubiquitous sources in both plant and microbial populations.(Yun, 1996), reviewed sources of FOS producing enzymes and represented them in **Table 1.3**.

With the increasing interest in the synthesis of FOS, a large number of enzymes that exhibit transfructosylase activity, have been purified from natural and engineered species **(Table 1.4)** with the aim of: (i) understanding their mode of action, (ii) deciphering their genetic coding and (iii) optimizing their production of FOS (Zuccaro *et al.,* 2008; Goosen *et al.,* 2007; Nemukula *et al.,* 2009). Enzymes exhibiting a large range of physical and chemical characteristics have been purified and applied in the synthesis of FOS (Sangeetha *et al.,* 2005).

Table 1.3: Sources of FOS producing enzymes (Yun, 1996)

Plant Sources	Microorganism Sources
Agave americana (agave)	*Aureobasidium pullulans*
Agave vera cruze (agave)	*Aureobasidium sp*
Asperagus officinalis (asperagus root)	*Arthrobacter sp*
Allium cepa (onion bulbs)	*Aspergillus japonicus*
Cichorium intybus (chicory)	*Aspergillus niger*
Crinum longifollium (sugar beet leaves)	*Aspergillus oryzae*
Helianthus tuberosus (Jerusalem artichoke)	*Aspergillus Phoenicis*
Lactuca lativa (lettuce)	*Aspergillus sydowi*
Lycoris radiate (monocot)	*Claviceps purpurea*
Taraxacum offinale (dandelion)	*Fusarium oxysporium*
	Penicillium frequentans
	Penicillium spinulosum
	Phtopthora parasitica
	Scopulariosis sp
	Saccaromyces cerivasae
	Zymomonas mobillis

Table 1.4: Characteristics of transfructosylating enzymes from various sources (Sangeetha *et al.*, 2005)

Source of enzyme	Fold Purif.	Molecular Weight (kDa)	Optimal pH	Optimal Temp (°C)	Stability pH	Stability Temp (°C)
B. macerans EG-6	63.5	66	5.0	50	5.0-7.0	20-50
A-oxydans j17-21	95.5	54	6.5	45	5.0-11.0	20-40
M. laevaniformis ATCC 15953	45.6	64	6.0	30	5.0-7.0	
A.niger ATCC 20611	51.6	340	5.0-6.0	50-60	4.5-10	Up to 60
Arthrobacter sp	405.3	52	6.5-6.8	55	5.5-10	Up to 40
S.salivarius ATCC25975	34.5	125.4	6.0-7.0	37-40	-	-
Microbacterium sp AL-210	98.8	46	7.0	40	7.0-8.0	Up to 40

5.1.2 β-Fructofuranosidase mechanism of action

It has been proposed that the synthesis of FOS is a kinetically controlled reaction that involves (i) the formation of a fructosyl-enzyme intermediate; and (ii) two nucleophiles, water and sucrose, competing for binding with the intermediate **(Equations 1.1 and 1.2**, where, G = glucose, F = fructose. ENZ = FFase enzyme) (Ghazi *et al.,* 2006; Lee *et al.,* 1999). It is also suggested that where either water or sucrose act as a nucleophile, the enzyme behaves predominantly as either a hydrolase to liberate glucose and fructose, or a transfructosylase to yield high DP FOS. This also leads to the possibility of products acting as substrates of hydrolase activity to yield glucose (**Equation 1.3**) (Ghazi *et al.,* 2006). Furthermore, Lee *et al.* (1999) pointed out that the reaction does not solely consist of the re-iterated removal of glucose from, and subsequent fructosylation of, sucrose. It is a complex sequence which may involve synthesized FOS products acting as reactants. This suggests that the enzyme active site has an affinity for, not only sucrose but glucose and FOS as well (Lee *et al.,* 1999).

$$\mathbf{GF} + \mathbf{\mathit{enz}} \rightarrow \mathbf{G} + \mathbf{F\text{-}\mathit{enz}} \quad \textbf{(1.1)}$$

$$\mathbf{F\text{-}\mathit{enz}} + \mathbf{GF}\mathit{n} \rightarrow \mathbf{GF}\mathit{n}_{+1} + \mathbf{\mathit{enz}},\ \mathit{n} \geq 1 \quad \textbf{(1.2)}$$

$$\mathbf{GF}\mathit{i} + \mathbf{GF}\mathit{j} \rightarrow \mathbf{GF}\mathit{i}_{-1} + \mathbf{GF}\mathit{j}_{+1},\ \mathit{i} \geq 1, \mathit{j} \geq 1 \quad \textbf{(1.3)}$$

Accumulation of glucose as a by-product is thought to reduce the yield of FOS, possibly through competitive inhibition. Evidence of this effect has been observed after removal of excess glucose and unreacted sucrose from the reaction mixture yielded a 98 % FOS product (Itaya *et al.,* 2007; Sangeetha *et al.,* 2005). Possible measures to prevent the accumulation of glucose and other low weight carbohydrates include the use of nanofiltration and microfiltration (Díaz *et al.,* 2006; Goulas *et al.,* 2007; Sanz and Martínez-Castro, 2007; Tanriseven and Aslan, 2005). Enzymatic alternatives have also been suggested, such as the use of glucose oxidase or glucose isomerase to convert excess glucose into less inhibiting by-products, glucuronic acid and fructose, respectively (Mislovičová *et al.,* 2009; Tanriseven and Aslan, 2005). The use *Z. mobillis* to ferment excess glucose into ethanol has also been reported (Bekers *et al.,* 2008).

β-fructofuranosidases exhibit both fructosyltransferase and hydrolytic activities. The transferase / hydrolase ratio and consequently, the enzyme‘s ability to produce FOS depend on sucrose concentration. Hydrolysis is dominant where sucrose concentration up to 5.0 g/l, whereas transfructosylation is the principal process when the sucrose concentration is higher than 200 g/l (Fernandez *et al.,* 2007; Zuccaro *et al.,* 2008).

6. Analysis and optimization of FOS synthesis

To effectively synthesize FOS on a laboratory or commercial scale, an optimization step is essential. Various techniques may be employed in the quest for process optima, two of which are described below:

6.1 Experimental design and Response Surface Methodology

Development of new products or the improvement of existing ones, as well as the developments of manufacturing processes to produce them, are crucial activities in modern industry. Statistically designed experiments are a valuable tool in process design and development. Experimental design is widely applied in: process and product characterization, achieving variability reduction as well as process optimization and development to achieve robustness (Montgomery, 1999).

Process optimization entails the manipulation of experimental variables with the ultimate goal of increasing product yield, reducing unwanted bi-products and reducing production or analysis costs (Zeelie, 2010). Traditionally, one at a time factorial designs have been utilized as a means of estimating the significance of a reaction variable's effect, yielding poor or unsatisfactory results, not to mention being time consuming and ultimately expensive, particularly where interaction effects between variables are the basis of the desired response (Mutanda, 2007; Nemukula *et al.,* 2009; Zeelie, 2010).

Response Surface Methodology (RSM) is a collection of statistical and mathematical techniques that are useful in the developing, analysis and optimization of processes (Myers and Montgomery, 2002). This method involves the exploration of the space of the process or independent variables, using statistics and mathematical models to approximate the influence of variables on a response and finally finding the value of variables that favour a desirable response (Carley *et al.,* 2004).

RSM essentially comprises of 4 stages:

- Identification of critical parameters of the process
- Determination of the range of factor levels
- Selection of specific test samples using experimental design
- The analysis of data using statistical / mathematical software for RSM (Mutanda, 2007)

6.2 Artificial Neural Networks (ANN)

An artificial neural network is an algorithm with some of the learning characteristics of a biological brain. "Our brains take the experiences of our five senses and draw conclusions that help us cope with our changeable world" (Jan and Lawshe, 2007).The use of ANNs as predictors of optima in different fields and industries has widened over the past decade (Paliwal and Kumar, 2009). In fact, ANNs are increasingly being used in areas of prediction and classification, where regression models and other related statistical techniques have traditionally been used (Ripley, 1994).

6.2.1 Rationale

The human brain consists of about 10^{10} processing units, called neurons, which operate in parallel in the sense that each neuron is connected to about 10^4 others thereby making the brain a massively parallel computational organ. Each neuron receives inputs, in the form of electrical signals, from other neurons and each connection modifies its input so that the inputs, in a sense, have a threshold. The modification is achieved chemically via neurotransmitters in the brain and the modification process is ongoing, i.e. as the brain learns, the thresholds are adjusted. The pattern of interconnections between neurons and the thresholds on the interconnections constitute the ability of the brain to cope with various cognitive tasks. The brain is therefore a network of neurons acting in parallel – a neural network (Burton, 2010).

The neuron is the fundamental processing unit of the brain. It consists of:

- A branching tree of input connections, called dendrites, which transmit the inputs.
- A cell body, which receives and integrates the inputs and then produces an output in response when the integrated input exceeds some threshold.
- A branching tree of output connections, called an axon, which sends the output response to other neurons.

Therefore, the total weighted sum of the inputs is received by the neuron, which only fires if the weighted sum exceeds a certain threshold. Its output then passes to the next layer of neurons (Burton, 2006; Bishop, 2010). By applying algorithms that mimic the processes of real neurons, the network can ‗learn' to solve many types of problems (Krogh, 2008). A model neuron is referred to as a threshold unit and its function is illustrated in figure 1.11.

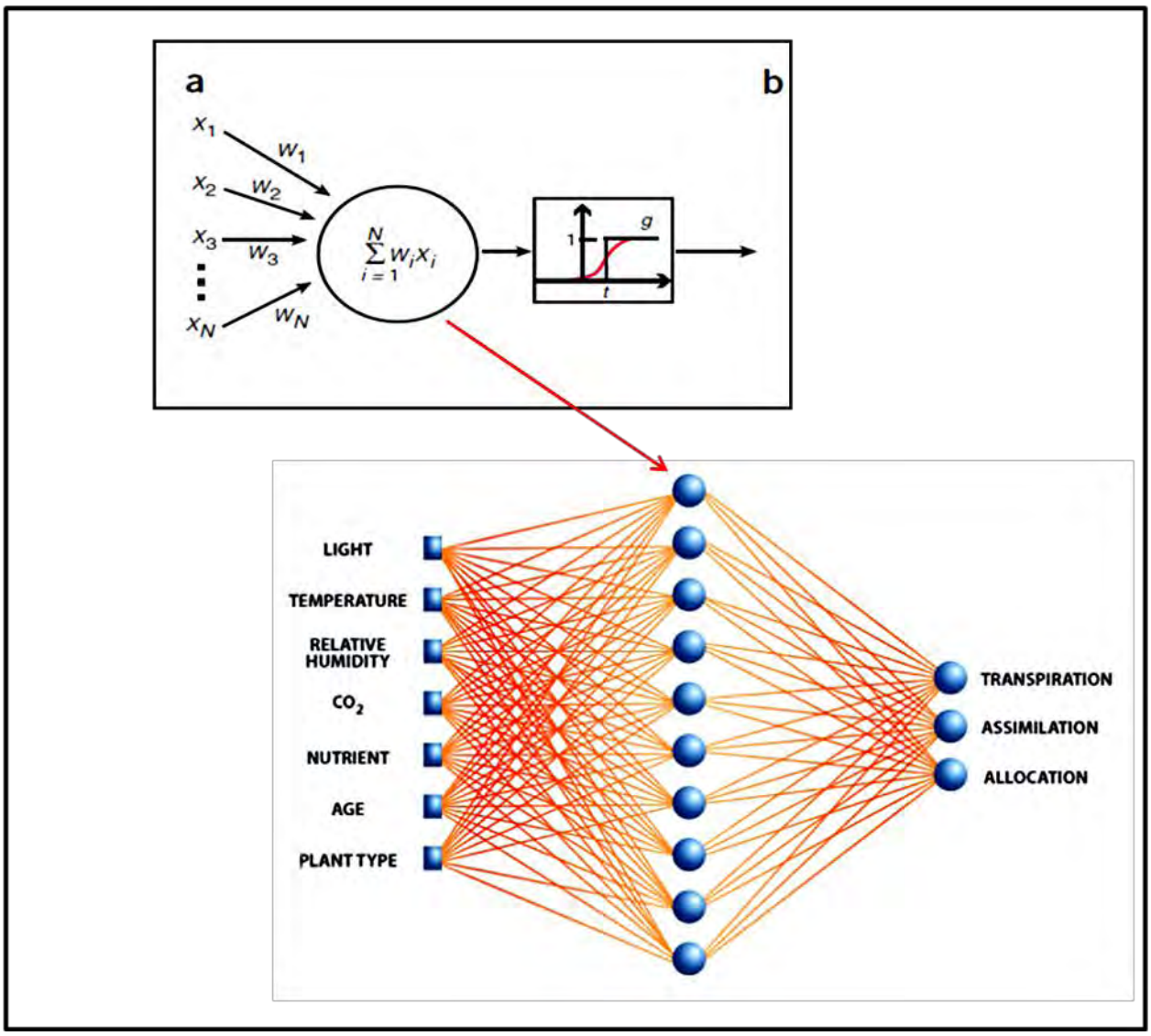


Figure 1.11: A schematic representation of the neural network concept, as applied by NASA (National Aeronautics and Space Authority) AEMC programme (Jan and Lawshe 2007) showing the threshold unit receiving inputs (Xi) of weighting (Wi) until $\sum w_i x_i \geq$ threshold (t) (Krogh, 2008)

6.2.2 Applications

Several types of ANNs can be created, such as, Boltzman's machines, unsupervised networks and Kohonen networks, to name a few. They also range from being single neuron models called perceptrons, to multi-layered neural networks, which are used to give better and more thorough results (Bishop, 2006). Different types of networks have found a variety of applications in most sectors of industry. NET talk, an application of an ANN for machine reading of text, was one of the first widely known applications,

followed by the use of a similar type of network in the field of biology, whose function was to predict protein secondary structure (Qian and Sejnowski, 1988; Sejnowski and Rosenberg, 1987).

In the finance sector, Fanning and Cogger (1998) developed a successful discriminant function against management fraud using ANNs. This network was thought to perform better than earlier models of discriminator functions built using traditional logistics analysis (Paliwal and Kumar, 2009). Artificial neural networks, although thought of by some authors to be relatively ineffective on a global basis as compared to more traditional multivariate statistic methods, proved to be generally better at tackling classification problems, such as predictions on the risk of bankruptcy (Odom and Sharda, 1990).

Other uses of ANNs include neural computation in medical informatics as well as modelling of psychiatric and neurological phenomena. A recent application of neural networks has been the creation of a headset (Emotiv ® Epoc Headset), that reads neuron firing patterns in the brain and creates associations which enable the user to set functions to certain thought patterns (i.e. feeling of going up or down) and emotions (i.e. happy or sad). This particular application has also been discussed by Stamps and Hamam, (2010) as, ~~"a~~ step towards an inexpensive brain computer interface (BCI) control for wheel chair navigation in an enabled world".

7. Problem statement

The evidence presented in the above review reveals a clear link between the applicability of FOS as functional foods, their degree of polymerization and consequent selective fermentation by gut microorganisms. Also highlighted, are the unfavourable effects of sucrose and traditional artificial sweeteners. There is therefore, a need to purify, characterize and optimize the action of enzymes that are involved in the bio-transformation of sucrose into FOS of a pre-defined chain length.

8. Hypothesis

The synthesis of fructooligosaccharides of a pre-defined chain length can be catalysed by a β-fructofuranosidase from *Aspergillus niger* ATCC 20611.

9 Aims

The aims of the present research are to investigate an enzymatic process for the synthesis of FOS from sucrose using a β-fructofuranosidase (FopAp) from *Aspergillus niger* ATCC 20611 as well as to analyse and optimize it using response surface methodology and artificial neural networks.

10 Objectives

- To purify the crude enzyme FopAp from *Aspergillus niger* ATCC20611
- To characterize and determine the kinetic parameters of the enzyme, FopAp
- To use enzymatic assays and/or HPLC to quantify the synthesis of FOS from sucrose using FopAp
- To use the principles of response surface methodology and artificial neural networks to analyse and optimize the synthesis of FOS from sucrose using FopAp

CHAPTER 2
PURIFICATION OF FOPAP

1 Introduction

With the increasing significance of FOS as a functional food in modern society, there has been proportional research interest in enzymes possessing trans-fructosylating activity as well as the organisms that produce them (Nguyen *et al.,* 2005). Investigations to fully understand the physical and chemical properties of FOS producing enzymes are still ongoing. Sangeetha *et al.* (2005) have reviewed the extensive work that has been undertaken to isolate and purify FOS producing enzymes from a variety of plant and microbial sources. Regardless of their differences in subunit structure, chemical susceptibility and substrate specificity, there is general consensus that they all possess transfructolsylase activities (Ghazi *et* al., 2007; Maiorano *et al.,* 2008). The purification of β-fructofuranosidase (FFase) and fructosyltransferase (FTase) enzymes over the past decade has not only shed light on conditions that favour FOS production but has also created the possibility of commercially producing this highly economically and medically beneficial food constituent.

However, controversy still exists with respect to the correct classification as well as the mechanism of action of the above mentioned enzymes (l'Hocine *et al.,* 2000). FTases are thought to only exhibit transfructosylase activity (Ut) whereas FFases have demonstrated both hydrolytic (Uh) and transfructosylating activities. Moreover, both classes of enzymes show great inter and intra species variation in physicochemical character as well as FOS production conditions (Maiorano *et al.,* 2008). The purification of FOS producing enzymes, isolated from different organisms serves well to "fill the gaps" in current knowledge, with particular respect to the mechanism of action by which these enzymes operate and may possibly improve the quality and quantity of FOS produced. Ultimately, the availability of pure enzymes for academic analysis could indeed aid in creating consensus and promoting collaboration between researchers throughout the scientific community (l'Hocine *et al.,* 2001; Maiorano *et al.,* 2008).

1.1 Analytical techniques

The aim of a purification process is not only removal of unwanted contaminants, but also the concentration of the desired protein and the transfer to an environment where it is stable and in a form ready for the intended application (Hedhammer *et al.,* 2004). A variety of techniques are available for the purification of proteins from crude mixtures. The purification of any new enzyme is a problem in itself largely due to the array of choices the researcher is presented with as a starting point (Structural Genomics Consortium, 2008). Four important characteristics manipulated to facilitate the purification process are: solubility, charge, size and specific binding (Pletschke and Beukes, 2010). FOS producing enzymes have been documented to exhibit molecular weights ranging from 86 to 120 kDa and pIs in the range, 4.0-5.5 units. Based on these properties and others, the following techniques were utilised to purify FopAp, from the crude enzyme mixture:

1.1.1. Protein precipitation

Fractional precipitation is frequently used for separation of gross impurities, membrane proteins and nucleic acids. The most popular methods utilized are addition of salts (salting out) and addition of organic solvents. The addition of high concentrations of salt to a solution of protein removes the water of solvation around the hydrophobic patches in protein tertiary structure, thereby allowing hydrophobic interactions to occur, leading to aggregation. Precipitation may be dependent on pH, buffer composition as well as protein concentration. Different proteins will precipitate over a range of ionic strength values as determined by the relationship outlined in equation 2.1, Where, **S** is solubility in water (g/kg), **I** is the ionic strength value in water (mol/kg), **B** and **Ks** are constants for a particular protein at a fixed pH and temperature (Pletschke and Beukes, 2010)

$$\mathbf{Log\ S = B - Ks\ [(I/2)]} \qquad \mathbf{(2.1)}$$

Due to the common occurrence of overlaps in protein precipitation ranges, this technique serves mainly as an initial purification step, protein enrichment and concentration procedure and less as a means of separating a pure protein from a mixture (Ward and

Swiatek, 2009; Burgess *et al.,* 2009). Ammonium sulphate is popularly used because of its innocuous nature to proteins, high solubility in water and low heat of solution which allows for use at conditions of minimal protein degradation. Saturated solutions of ammonium sulphate are less dense than protein and thus allow for easy collection by centrifugation. The concentration of solutions is usually expressed in terms of their percentage saturation, which can be obtained from pre-designed tables (Pletschke and Beukes, 2010).

1.1.2 Ion exchange chromatography (IEC)

Ion exchange chromatography (IEC) is one of the most powerful and widely used forms of chromatography in research, analysis and process scale purification of proteins (Amersham Biosciences, 1999; Pletschke and Beukes, 2010). This technique separates proteins based on charge difference to give a very high resolution separation with high sample loading capacity. Separation is based on the attraction between oppositely charged particles in the sample and the ion exchanger matrix (**Figure 2.1**) and the net charge of these attractions is based on the pH as well as the ionic strength of the mobile phase. Counter ions incorporated in the stationary phase are displaced by appropriately charged proteins of interest, which can in turn be displaced by the addition of large concentrations of positive ions to facilitate protein elution.

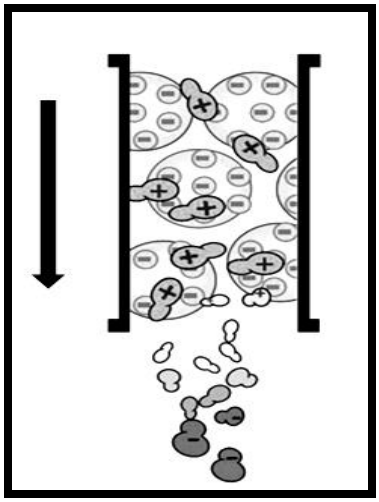


Figure 2.1: Schematic of ion exchange chromatography illustrating the binding of oppositely charged particles in the sample (+) to the ion exchanger (-), while unbound particles are eluted (Hedhammer *et al.,* 2004).

This type of chromatography is ideal for initial capture of protein due to its high capacity, relatively low cost and durability of stationary phases. It can also be used for the ―polishing", of partially purified material (Amersham Biosciences, 1999). The mild conditions under which this technique is performed also offer the advantage of maintaining its native conformation during the chromatographic process (Karlsson *et al.,* 1998).

Additional reasons for the success of IEC include the straight forward separation principle and ease of performance as well as the controllability of the method. Furthermore, ion exchange resins are very robust and can be sanitized in place and re-used over a large number of cycles. However, the main disadvantage of IEC lies in its limited selectivity and applicability where pre-derivatization may be required (Amersham Biosciences, 1999; Jeong *et al.,* 2007).

1.1.3 Size exclusion chromatography (SEC)

Size exclusion chromatography is a non adsorptive method which separates molecules based on their size-controlled permeation through the capillaries of a porous matrix (Hedlund, 2004). Separation is essentially determined by the diameter of the pores within the gel matrix such that molecules that are larger are excluded and eluted first, while the elution of smaller molecules is restricted by the matrix (**Figure 2.2**). Ease of diffusion through the pore system is determined by the hydrodynamic volume, which is the volume displaced by a molecule as it moves through water and is closely linked to its shape (Hedhammer *et al.,* 2004).

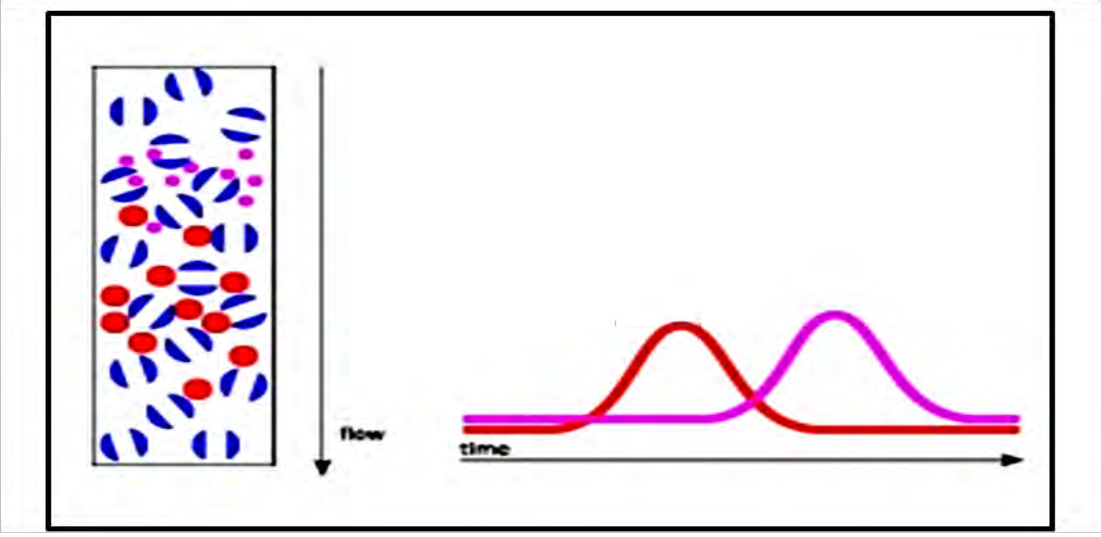


Figure 2.2: Simplified Illustration of separation by particle size in size exclusion chromatography, showing how ● large particles cannot enter pores and are eluted first, while ●smaller particles enter the gel and are eluted later. (Yonemoto, 2006)

The gel matrix usually consists of cross-linked systems of dextran, agarose or polyacrylamide beads whereby pore diameter is essentially determined by the degree of cross-linking exhibited. In analytical SEC, excellent resolution is crucial and is largely determined by the length of the column (Wilson and Walker, 2010). SEC is particularly popular as a final purification method when impurities are low and the target protein has been concentrated by previous steps. It has the principal advantage of preserving the biological activity of samples as well as the ability to separate multimers that may be undetectable by most chromatographic methods (Hedhammer *et al.,* 2004). It is also useful as an analytical means to determine the molecular weight of pure proteins (Pletschke and Beukes, 2010).

1.1.4 Polyacrylamide gel electrophoresis (PAGE)

The electrophoresis technique is based on the migration of charged particles in an electric field (Laemmli, 1970). PAGE can be carried out in denaturing or native (non-denaturing) conditions. The former is useful in the analysis and molecular weight determination of polypeptide subunits while the latter allows for the analysis of the quaternary structure in protein while retaining biological activity (Wilson and Walker, 2010).

Denaturing PAGE involves the treatment of samples with detergents such as sodium dodecyl sulphate (SDS) as well as reducing agents such as β-mercaptoethanol (β-ME), which disrupt quaternary structure to form negatively charged linear molecules and reduces disulphide linkages, respectively. This treatment ensures the uniformity of charge in samples thus facilitating electrophoretic mobility purely by molecular weight. The relative mobility of samples can then be compared to known standards to estimate molecular weights with considerable accuracy (Ahmed, 2005).

Electrophoresis uses conductive gels as support mediums for bio-molecule separation. Polyacrylamide is commonly used in various concentrations to form gels of proportionally variable pore size, making it possible to analyse proteins over a wide range of molecular weights (Wilson and Walker, 2010).

The main aim of this chapter was to purify an enzyme FopAp from crude enzyme broth, using ammonium sulphate precipitation, ion exchange chromatography and size exclusion chromatography. The secondary aim of this chapter was to assay the activity exhibited by FopAp as a result of purification.

2 Materials and methods

2.1 Materials

The crude enzyme, FopAp expressed in *Pichia pastoris* using a synthetic gene from *Aspergillus niger,* was kindly donated by the Department of Microbiology at Stellenbosch University, (Stellenbosch, South Africa). Pre-mixed 30 % Bis-acrylamide, was purchased from Bio-Rad (South Africa). All bench reagents were of analytical grade and were obtained from either Merck Chemicals (South Africa) or Sigma-Aldrich (South Africa). Unless otherwise stated; all reagents were dissolved in Milli-Q water and all experimental work was performed at 0-4 °C. All samples were stored at 0-4 ºC overnight and -20 ºC over longer periods, where appropriate.

Labnet Accublock™ Digital dry baths were obtained from White Scientific (South Africa). Beckman centrifuge (Avanti® J-E) was purchased from Beckman-Coulter (USA).

All HPLC analyses in this chapter were carried out with degassed and filtered HPLC grade solvents. Filtration and degassing were performed by a Milli-vac maxi™ vacuum with a 0.45µm nylon filtration membrane from Millipore (South Africa).

2.2 Ammonium sulphate precipitate

Protein precipitation was carried out as per adaptation of a method by l'Hocine et al., (2000). Solid ammonium sulphate was slowly added to 10 ml of crude extract (low shear force; 4 ºC) until 30 % saturation was achieved. The suspension was then centrifuged (10 000 x *g*, 20 minutes, 4 ºC). The resultant supernatant was then subjected to further ammonium sulphate precipitation until 80 % saturation was attained. Subsequent centrifugation (10 000 x *g*, 30 mins, 4 ºC), yielded a pellet, which was washed thoroughly with distilled water and re-suspended in 10 ml of Tris-HCl buffer (50 mM, pH 9) in preparation for dialysis.

2.3 Dialysis

A Slide-a-lizer® dialysis cassette (10 000 MWCO) (Thermo-scientific, South Africa) was hydrated prior to use as per the manufacturers instruction. Enzyme extract (10 ml) from section 2.2 were carefully injected into the cassette and dialysed overnight against 2 changes of Tris-HCl buffer (50 mM, pH 9, 4ºC). A 1.5 ml aliquot was removed for FFase assays and protein determination. The remainder of the solution was then subjected to ion exchange chromatography.

2.4 Ion exchange chromatography

A Hi-Trap-Q™ HP Sepharose free flow anion exchange column connected to an ÄKTA™ fast protein liquid chromatography (FPLC) system with a UV detector (A_{280nm}) and fraction collector (GE Healthcare Biosciences, USA) was equilibrated using Tris-HCl buffer (50 mM, pH 9, degassed and filtered). Aliquots of the enzyme extract (5 ml) from section 2.3 were then loaded onto the column using a syringe with an adapted tip. After 3 column volumes of equilibration buffer were applied, adsorbed protein was eluted against a linear salt gradient (0.1 - 0.8 M NaCl in Tris-HCl 50 mM, pH 9, degassed and filtered) at a flow rate of 1 ml/min. Fractions were collected every minute. Data viewing was

facilitated by Unicorn™ 5.1 software (GE Healthcare Biosciences, USA). Fractions corresponding to observed peaks were pooled separately and assayed for FFase activity as well as protein concentration before further analysis.

2.5 SDS PAGE analysis

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was performed using an 8 % resolving gel and 5 % stacking gel on mini-PROTEAN apparatus (Bio-Rad, South Africa), according to the method described by (Laemmli, 1970). 10 µl of each sample to be analysed were mixed with 4 µl of a 5X sample buffer (containing 3.55 ml water; 1.25 ml 0.5 M Tris-HCl, pH 6.8; 2.5 ml glycerol; 2.0 ml 10 % (w/v) SDS; 0.2 ml 0.5 % (w/v) bromophenol blue; and 5 % (v/v) β-mercaptoethanol added prior to use) and boiled at 100 °C for 5 minutes. The boiled samples were loaded into the wells and the gel electrophoresed at 150 V for 60 minutes. The SDS-PAGE gels were stained with Coomassie protein stain (0.1 % (w/v) Coomassie brilliant blue G 250; 45 % (v/v) methanol; and 10 % glacial acetic acid) overnight, and treated with a destaining solution (45 % methanol; 45 % distilled water; 10 % glacial acetic acid) for approximately 3 hours. A peqGOLD® protein marker IV (peqLab Biotechnolgie, GmbH) was electrophoresed next to the samples. With the protein marker as a reference, the molecular weights of the bands seen on the SDS-PAGE gels were calculated using a graph of log M_r versus R_f (Appendix A 3), where: R_f was the ratio of the migratory distance of the protein to the migratory distance of the bromophenol blue dye.

2.6 Size exclusion chromatography

A Sephacryl HR-300 (Sigma-Aldrich, South Africa) was packed into a 1.8 x 42 cm column (bed volume: 106.92 cm^3) attached to a Bio-Logic™ LP low pressure chromatography system (Bio-Rad, South Africa) was utilised to confirm the size of the purified protein as well as identify any aggregates that may have been undetected by earlier purification steps. The column was equilibrated with Tris-buffer (50 mM, pH 9) Standards: Dextran Blue (2000 kDa), apoferitin (443 kDa), β-glucuronidase (290 kDa), β-amylase (200 kDa), Alcohol dehydrogenase (150 kDa) and β-galactosidase (125 kDa), were used to construct a calibration curve by plotting log M_r versus K_{av} (Appendix A 2).

5 ml aliquots of enzyme sample from the proposed pure fraction were pipetted onto the column and eluted at a rate of 3 ml/min. Fractions were collected every minute on a Bio-Logic™ fraction collector (Bio-Rad, South Africa) and assayed for protein. Fractions containing protein were pooled and analysed by native PAGE.

2.7 Native PAGE analysis

A 1 ml aliquot was removed from pooled fractions eluted from size exclusion and concentrated by freeze drying in a Labconco Freezone® bench-top freeze dry system (Vacutec, South Africa) followed by re-suspension in 100 µl of Milli-Q water. Native PAGE was performed using a 6.5 % resolving gel and 5 % stacking gel on mini-PROTEAN apparatus (Bio-Rad, South Africa), according to the method described by Laemmli (1970). 10 µl of the sample to be analysed was mixed with 4 µl of a 5X sample buffer (containing 3.55 ml water; 1.25 ml 0.5 M Tris-HCl, pH 6.8; 2.5 ml glycerol; 0.2 ml 0.5 % (w/v) bromophenol blue). The sample was loaded into a well and the gel electrophoresed at 100 V for about 90 minutes. The Native PAGE gel was stained with Coomassie protein stain (0.1 % (w/v) Coomassie brilliant blue G 250; 45 % (v/v) methanol; and 10 % glacial acetic acid) overnight, and treated with a destaining solution (45 % methanol; 45 % distilled water; 10 % glacial acetic acid) for approximately 2 hours.

2.8 Fructofuranosidase assay

The transfructosylating (Ut) and hydrolytic (Uh) activities of FopAp were assayed using a modification of the method by Kurakake *et al*. (2008). 1200 µl of 600 mg/ml sucrose in Tris-HCl buffer (50 mM, pH 9) were incubated with 300 µl of enzyme sample at 30 °C in an Eppendorf ®safe lock™ tube (Merck, South Africa) for 1 hour. Separate tubes, consisting of either 300 µl of enzyme sample or 1200 µl of 600 mg/ml sucrose; made up to reaction volume with Tris-HCl buffer (50 mM, pH 9) were used as enzyme and substrate controls, respectively. The reaction was stopped by incubating for 10 min in boiling water. The reaction mixture was syringe filtered through a 0.22 µl nylon membrane (Millipore, South Africa), diluted appropriately, and then analysed by high performance liquid chromatography (HPLC). The two enzymatic activities were

calculated from amounts of glucose (G) and fructose (F) produced. Total activity (Utot) or (Ut + Uh), which corresponds to the degradation of sucrose, was defined as the amount of enzyme that could produce 1 µmol of glucose from sucrose. Values of Ut and Uh were obtained by multiplying Utot by the transfer ratio (G - F)/G and the hydrolysis ratio F/G, respectively; and they were defined as the amount of enzyme that could hydrolyze 1 µmol of sucrose and transfer 1 µmol of fructosyl residues, respectively. The enzyme assay was carried out in triplicate for each sample.

2.9 High performance liquid chromatography

All HPLC analysis in this chapter was carried out on a Prevail® Carbohydrate ES column (250 x 4.6 mm); guard column (7.5 x 4.6 mm) packed with 5 µm spherical polymer beads, (Alltech inc., USA). The HPLC system consisted of a Beckman 110B Solvent Delivery Module Pump, coupled to a RI detector (Knauer, Germany) and a system organizer (Beckman-Coulter, South Africa). A mixture of acetonitrile (HPLC grade), Milli-Q water, NH_3 (72:27:1)(v/v/v) was used as the mobile phase at a flow rate of 1 ml/min. 20 µl injections were performed manually for each sample All quantities were calculated with reference to a calibration curve of authentic standards (Appendix A 4). Data acquisition and peak integration were performed using 32 Karat® chromatographic software (Beckman-Coulter, USA).

2.10 Protein determination

The protein content was determined using the method described by Bradford (1976). 5 µl of enzyme sample were added to 245 µl of the Bradford reagent in a microtitre plate, incubated at room temperature for 15 mins then read at 595 nm in a Power $Wave_x$™ microtitre plate reader (Biotek Instruments, USA). Protein concentration was calculated in mg/ml using a bovine serum albumin (BSA) standard plot of absorbance versus concentration (mg/ml) (Apppendix A 1)

3 Results

3.1 Purification of crude FopAp

3.1.1 Ammonium sulphate precipitation and dialysis

As an initial purification step, crude enzyme extract was precipitated using solid ammonium sulphate at 30 % followed by 80 % saturation. This was done in order to remove cell debris and very small protein before the protein of interest was precipitated out, thereby simplifying the purification process. After centrifugation, the resultant pellet was washed, re-suspended and dialysed overnight to remove residual ammonium sulphate and debris. The sample was then subjected to ion exchange chromatography on an FPLC system and eluted against a linear salt gradient (0.1-0.8 M NaCl) (**Figure 2.3**).

3.1.2 Ion exchange chromatography

As illustrated in figure 2.3, two peaks were observed in the elution profile of FopAp on Hi-Trap® Q anion exchange chromatography and were named FA and FB. Bradford's reagent at $A_{595\ nm}$ was used to supplement protein detection by UV spectroscopy at $A_{280\ nm}$, revealing an apparent shift in the positions of the eluted protein. All the fractions in each peak were pooled and assayed for protein as well as FFase activity. Both FA and FB exhibited FFase activity thus indicating the possibility of more than one sucrose degrading enzyme in the crude mixture.

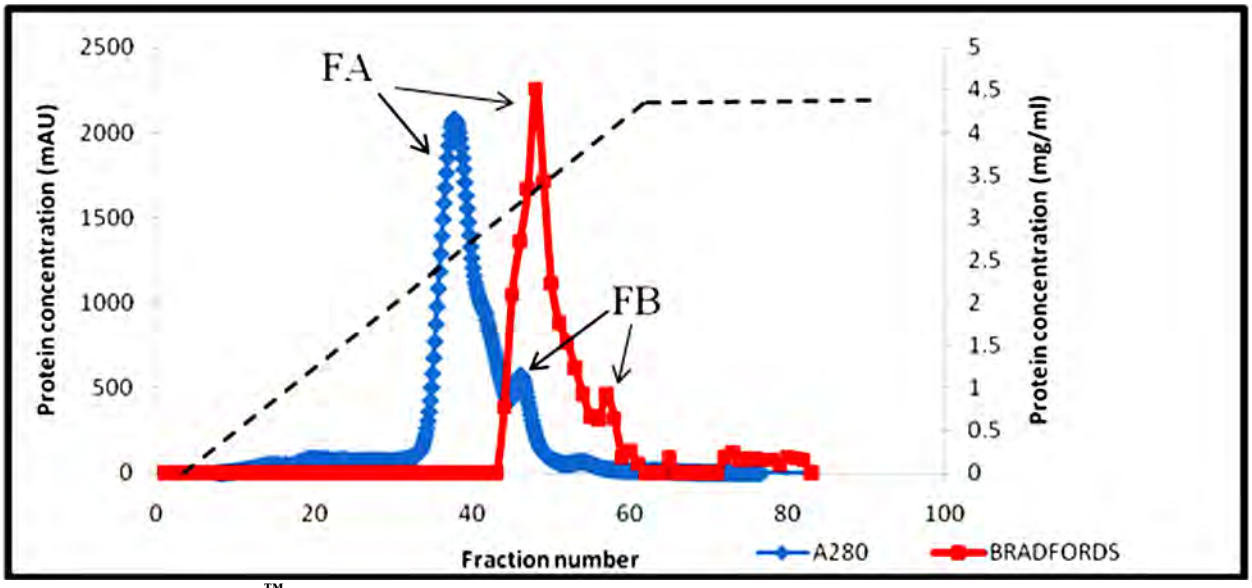


Figure 2.3: Hi-Trap™ Q elution profile of FopAp; eluted with a 0.1-0.8 M NaCl (-----) gradient (flow rate: 1 ml/min, collection: 1 ml/fraction). A_{280nm} (mAU) - ♦, Bradfords (mg/ml) - ■.

3.2 β-fructofuranosidase assay

As a means to elucidate the nature of the enzymes in them, FA and FB were assayed for the production of FOS using HPLC. The range of FOS products (**Figure 2.4** and **2.5**) as well as amounts of glucose and fructose (**Table 2.1**) formed were compared to that of the crude mixture

Table 2.1: Sugars assayed by HPLC in FopAp fractions (FA and FB) at high sucrose concentrations under alkaline conditions and incubated for 60 mins.

	FRUCTOSE (mM)	GLUCOSE (mM)	SUCROSE (mM)
CRUDE	343.07	1627.67	659.75
FA	10.53	449.59	336.95
FB	90.47	268.68	1576.89

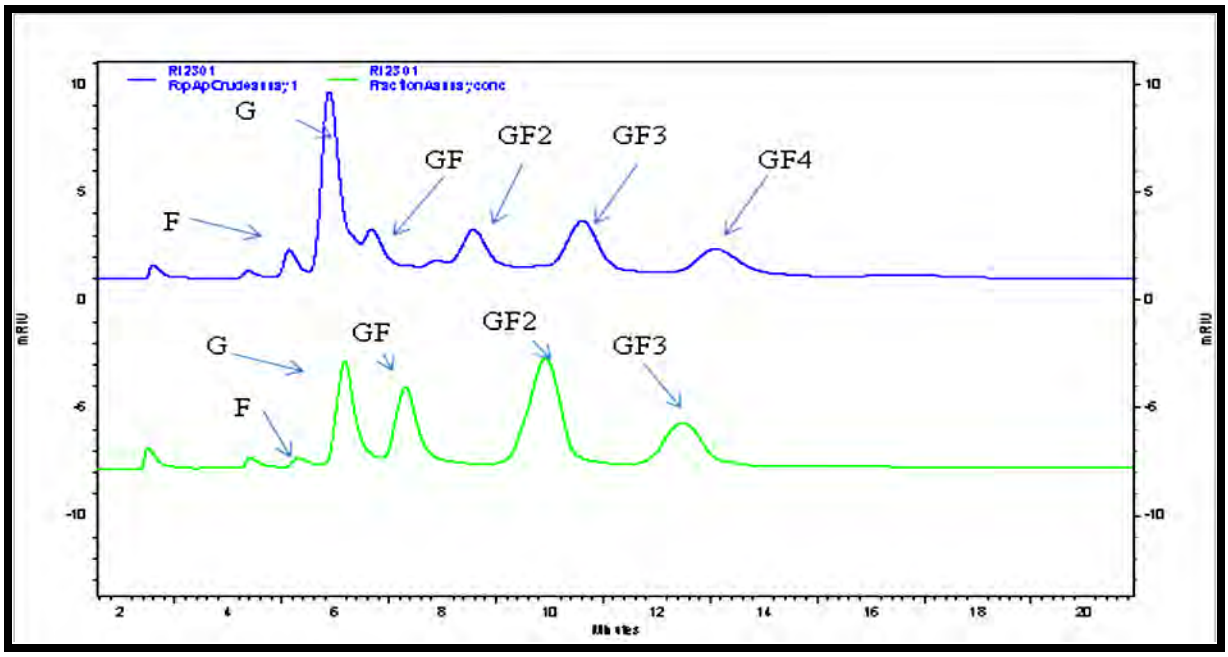


Figure 2.4: Chromatogram showing relative production of glucose, fructose and FOS by FA — and crude FopAp — (where: G- glucose, F- fructose, GF- sucrose, GF2- 1-kestose, GF3- 1-nystose and GF4- fructosyl-nystose)

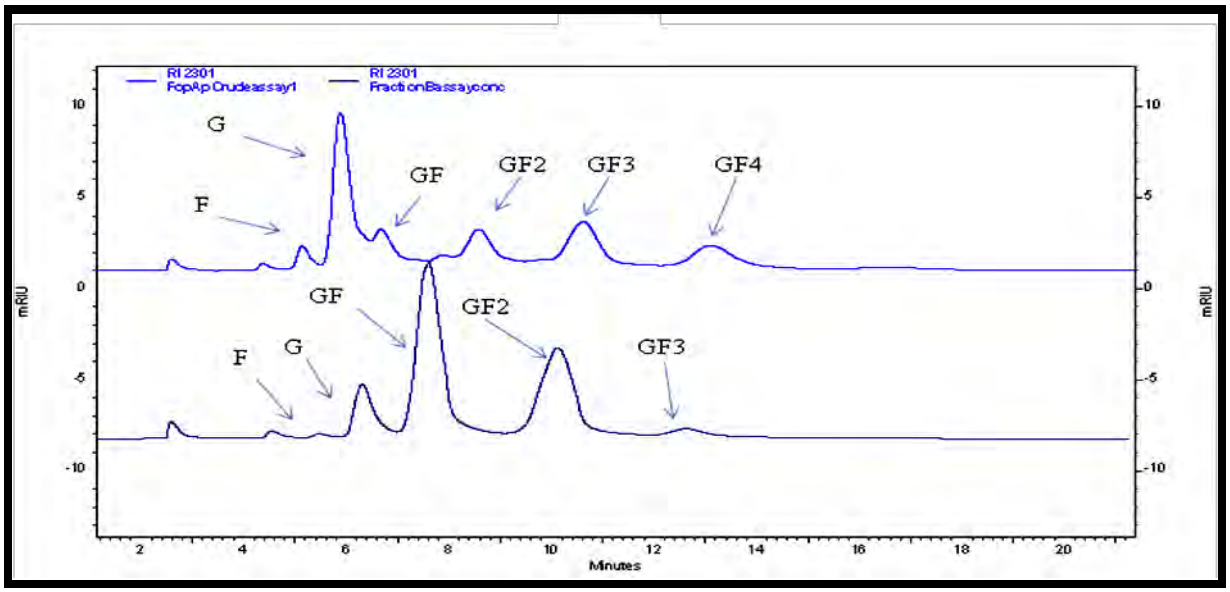


Figure 2.5: Chromatogram showing relative production of glucose, fructose and FOS by FB — and crude FopAp —— (where: G- glucose, F- fructose, GF- sucrose, GF2- 1-kestose, GF3- 1-nystose and GF4- fructosyl-nystose)

Figures 2.4 and 2.5 show that the crude enzyme produced a wider range of FOS products compared to both FA and FB. It is also evident that the crude enzyme degraded more sucrose than FA or FB. The presence of a larger fructose peak in the crude FopAp trace suggests greater hydrolytic activity compared to the purified fractions. However, both FA and FB produced larger amounts of GF2 (1- Kestose) than the crude enzyme thus indicating the prevalence of transfructosylating activity in these samples. Out of the 3 samples, FB exhibited the lowest ability to degrade sucrose. It is noteworthy that reducing sugars were observed in neither the substrate nor enzyme controls. The results of the purification steps as well as those from Bradford assays were collated and are presented in table 2.2.

Table 2.2: Purification of FopAp from *A. niger*. C- crude, ASD- ammonium sulphate precipitation and dialysis; IEC- ion exchange chromatography, U- µmol of glucose produced/minute.

Step	Volume (ml)	Protein (mg/ml)	Total Protein (mg)	Activity (U/ml)	Total Activity (U)	Specific Act. (U/mg)	Purif. Factor	Yield (%)
C	10	11.63	116.33	96.80	968.03	8.32	1.00	100.0
AS	10	3.48	34.80	85.45	854.57	24.55	2.95	88.28
IEC	36	1.55	55.89	21.04	757.49	13.55	1.63	78.20

According to table 2.2, there is a large decrease in protein concentration between the crude (11.63 mg/ml) and subsequent purification steps (3.48 mg/ml and 1.55 mg/ml for AS and IEC, respectively). Also, while the reduction in protein only yields a slight reduction in activity between the crude (96.80 U/ml) and ammonium sulphate precipitation (85.45 U/ml) steps, there is a more significant decrease after the ion exchange step (21.04 U/ml). This trend is also reflected as a reduction in purification factor between the ammonium precipitate and ion exchange steps.

3.3 Determination of the molecular weight of purified FopAp

In order to determine the molecular weights as well as assess the purity of the proteins in FA and FB, denaturing PAGE (8 % SDS PAGE) was performed. It was valid to include samples from the previous purification steps as references in the assessment of purity. All molecular weights were estimated using a calibration curve as described in section 2.5 of this chapter. A scanned image of the Coomasie stained gel is presented in figure 2.6.

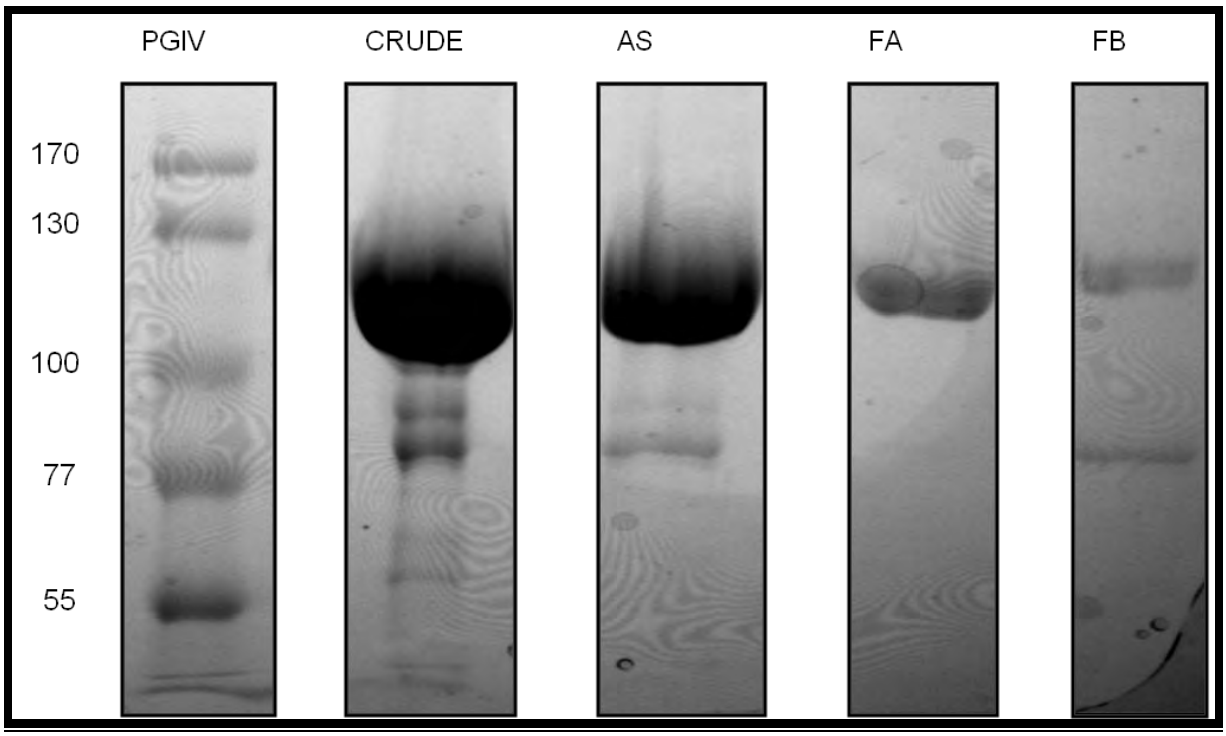


Figure 2.6: 8 % SDS PAGE analysis of FopAp, illustrating the change in purity through purification steps, where: PGIV- peqGold® IV pre-stained marker, AS- ammonium sulphate precipitation. Molecular weights are in kDa

In FA, a homogenous single band was observed at about 112 kDa while FB yielded two bands at 112 kDa and 78 kDa. The band at 112 kDa coincided perfectly with that in FA, differing only in intensity.

3 4 Confirmation of homogeneity and estimation of native molecular weight

After SDS PAGE analysis the FA fraction alone was subjected to size exclusion chromatography as a means of determining the native molecular weight. The protein was eluted at a flow rate of 5 ml\min on a Sephacryl 300 HR column. Fractions were collected every minute and assayed for protein at 595 nm by the Bradford assay. The resultant elution profile is illustrated in figure 2.7.

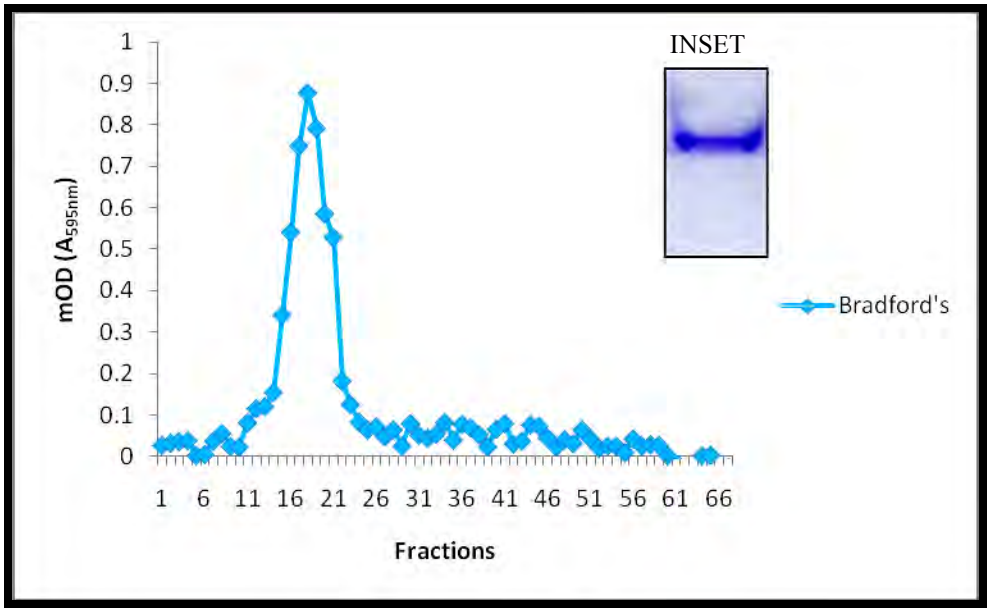


Figure 2.7: Sephacryl S-300 HR chromatogram of FA. Column dimensions: 42 × 1.8 cm; flow rate: 3 ml/min. ♦ A_{595nm} in mOD. Inset : 6.5 % Native PAGE gel stained with 0.1 % (w/v) Coomasie brilliant blue G 250.1- pooled fractions 14-22 from Sephacryl S-300 size exclusion chromatography.

As clearly demonstrated by figure 2.7, FA was eluted as a single peak corresponding to the results a molecular weight of 287 kDa. When analysed by 6.5 % native PAGE, the protein migrated as a single band which, in correlation to the results from SDS PAGE, suggests it to be homo-dimeric **(Figure 2.7: Inset**).

4 Discussion and conclusions

The crude enzyme FopAp was successfully purified to apparent homogeneity with an overall yield of 78 %, a value almost seven times higher than those obtained from early work by Hirayama *et al*. (1989) on *Aspergillus niger* ATCC 20611.The protein in FA was estimated to have a subunit with a molecular weight of 112 kDa on SDS-PAGE (**Figure 2.6**) and an estimated native molecular weight of 287 kDa by size exclusion on a Sephacryl S-300 HR column (**Figure 2.7)**. The molecular weight observed by SDS-PAGE analysis of FA gave similar results to those of proteins purified by Hirayama *et al.*(1989), Nguyen *et al.* (2005) and Zuccaro *et al.* (2008) from *A. niger* ATCC 20611, *A .japonicus* and *A. niger* SKAn1015, respectively; which gave bands at 100 kDa, 120 – 130 kDa and 120 kDa, respectively.

The slight variation in size of β-fructofuranosidases on SDS-PAGE could be attributed, in part to glycosylation, which occurs in varying degrees at the catalytic site and thus may be necessary for activity (Homann and Siebel, 2009). Zuccaro *et al.* (2008) described the deglycosylation of an extracellular fructofuranosidase from *A. niger* SKAn1015, leading to a 50 % reduction in molecular weight; though no evidence of activity post-deglycosylation was documented. There is evidence of an active non-glycosylated atypical invertase from *Candida utilis,* though this particular enzyme was secreted in its non-glycosylated form and exhibited very low invertase activity (Belcarz *et al*., 2002). Consequently, no attempts at deglycosylation were made on the purified enzyme in this work.

The results obtained from native PAGE (**Figure 2.7: Inset**), describe a homo-dimeric protein with a molecular weight of about 287 kDa. This is in agreement with the findings of Hirayama *et al.* (1989), who observed that β-fructofuranosidase of *A. niger* ATCC 20611 showed only one kind of subunit even after treatment with β-mercapto-ethanol, albeit with a size of 340 kDa. Nguyen *et al*. (2005) concluded that the native fructofuranosidase exists as a dimer with an average size of about 91 kDa. This apparently atypical behaviour of β-fructofuranosidases from *A. niger* is thought to result from the occurrence of two or more subunits of the same molecular weight that migrate

as a single band regardless of denaturation (Nguyen et al., 2005). This hypothesis can be supported by work by Rubio and Maldonado, (1995) on *A. niger* from mouldy lemons, which exhibited weights of 47 kDa and 95 kDA on SDS PAGE and gel filtration (Sephadex, G-150), respectively. Sund and Linder (1980), reported similar –subunit masking", in a β-fructofuranosidase from *Streptococcus mitis* that appeared homogenous after PAGE analysis but yielded two identical bands of the same activity, at different pIs (4.05 and 4.25), after gel electro-focusing.

Investigations by l'Hocine and co-workers (2000) and Ghazi *et al*. (2007) on fructosyltransferases from *A.niger* AS0023 and *A. aculeatus,* respectively; have reported proteins as small as 71 kDa and 65 kDa on SDS PAGE, respectively. Two distinct protein bands were observed during the SDS PAGE analysis of FB (**Figure 2.6**); one of which appeared at about 112 kDa, forming a band identical to that observed in FA. The second band appeared at an estimated 78 kDa, which is very similar to the molecular weights of fructosyltransferases reported in literature (l'Hocine *et al.,* 2000; Ghazi *et al.,* 2007). However, in as much as it is helpful, no final conclusions could be drawn from this information alone.

Preliminary investigations were carried out on FA and FB in order to evaluate their ability to synthesize FOS under assay conditions. All the assays performed in this and subsequent chapters, unless otherwise indicated, were based on conditions that promote the highest transfructosylating activity while limiting hydrolase activity thereby creating a high transferase/hydrolase activity ratio (U_t/U_h). Studies by Kurakake *et al*. (1996), revealed that optimal transfructosylating activity can be observed at high sucrose concentrations under alkaline conditions, and thus, based on these findings, all assays were conducted at pH 9 and a sucrose concentration of 600 mg/ml.

The difference observed in the synthesis of FOS by the crude enzyme and FA (**Figure 2.4**) were as expected, as there was a decrease in the range of FOS products formed as well as a proportional increase in one particular transfructosyl product. According to Ghazi *et al.* (2006) the mechanism of the transfructosyl reaction, is limited by the

availability of free fructose, which, where available, would have been transferred to either sucrose or an already formed FOS substrate. The large glucose peak observed in both the crude and purified traces of figure 2.4 is characteristic of transfructosylating activity (Kurakake *et al.*, 2008; Lee e*t al.*, 1999) and its presence at such a magnitude, qualitatively, suggests it to be the dominant process. The presence of a smaller though significant fructose peak (**Figure 2.4**) is indicative of hydrolysis reactions occurring with sucrose. Due to the lack of evidence of autolysis in the substrate control or naturally occurring sugars in the enzyme control, it is safe to assume that all the fructose observed is a direct result of an enzymatic hydrolysis reaction This evidence, supported by the single bands observed in SDS and native PAGE analysis of FA, strongly suggest that FA is a pure, typical fructofuranosidase.

The results obtained in figure 2.4 imply that a synergistic reaction takes place with the crude enzyme and not with FA. On this assumption, it was then postulated that the two components of the crude enzyme had been separated by ion exchange chromatography. One of the components was thought to have been eluted as FB (**Figure 2.3**) and due to the reduction of hydrolase activity observed in FA, in comparison to the crude enzyme, was expected to yield hydrolase activity. More specifically, it was thought that this component was either an active hydrolase subunit of the FopAp enzyme or a co-expressed native invertase from *Pichia pastoris*.

However, assay results of FB do not agree with either of the above suggestions. An unexpected decrease in fructose is observed (**Figure 2.5**). This is accompanied by a remarkably large amount of unreacted sucrose (GF). Also interesting, is the fact that despite the lack of hydrolase activity, a substantial amount of kestose (GF2) is apparently produced. The band observed in SDS-PAGE at 112 kDa (**Figure 2.6**) is thus proposed to be a dilution of the protein in FA, as a result of possible cross contamination during fraction collection and pooling. This is very probable considering how close together the two peaks as per adjustment of the elution profile at A_{280nm} by the Bradford assay (A_{595nm}) (**Figure 2.3**). The measurement of protein concentration at A_{280mn} is specific to homogenous protein and is easily affected by contaminants (Tsaprailis, 2001). Detector

errors are also possible, resulting in mismatching of fractions and protein content. A large portion of the available FPLC and HPLC detectors utilise A_{280nm} to determine protein concentration, therefore as indicated by this research, it is wise to compliment this technique with a more reliable and versatile method such as the Bradford method. It is possible that the activity exhibited by the FB in figure 2.5 is merely a reflection of the diluted activity of the fructofuranosidase in FA; hence the large amounts of residual sucrose and apparently reduced hydrolysis. This however, does not provide an explanation for the apparent loss of synergy between crude and purified FopAp.

Work by l'Hocine *et al.* (2000) on the purification of invertase and fructosyltransferase from *A.niger* AS0023, points out the difficulty in separating fructofuranosidases from transfructosylases, due to their similarities in structure and activity. In addition, l'Hocine and colleagues reported the purification of the fructosyltransferase which did not demonstrate any hydrolytic activity. It is possible that a native invertase from *P.pastoris* may have been co-expressed with the fructofuranosidase and thus would have been responsible for the synergistic results observed in the crude enzyme (**Figure 2.4; 2.5**). SDS-PAGE (**Figure 2.6**) as well as activity and protein assays show that 80% ammonium sulphate precipitation removed a large amount of smaller proteins and conserved the activity of the remaining protein bulk, which at this stage consisted mainly of protein in FA and FB (**Figure 2.6**). This could account for the increased purification factor that resulted from the ammonium purification step (**Table 2.1**) as well as the subsequent drop after separation of the two proteins by ion exchange chromatography. This may be confirmed by the appearance of the band at 78 kDA on SDS PAGE analysis of FB (**Figure 2.6**).

In conclusion, FopAp was successfully purified with relatively high enzyme recovery and was shown, by qualitative investigations on HPLC, to synthesize short chain FOS. A partially purified enzyme mixture was also isolated and was shown to possess transfructosylating activity. It was also shown that under assay conditions, the crude enzyme produces a greater range of products as compared to the purified enzyme, though this is still to be tested over a wider range of conditions. Knowledge of the extent to

which synthesis can be carried out as well as the conditions necessary for the optimal production of FOS is still limited and deeper understanding of the biochemical characteristics of the enzyme is required before this can be ascertained.

CHAPTER 3
CHARACTERIZATION OF FOPAP

1 Introduction

The role of enzymes in synthetic processes is thought to be dependent on a number of variables such as temperature, pH, time, substrate concentration, enzyme concentration and inhibition (Mutanda *et al.*, 2008; Wilson and Walker, 2010). Furthermore, the complexities of synthesis are only but compounded, during commercial production, by the effect of external factors, such as reaction volume, rate of agitation and choice of reactor to name only a few (Santos and Maugeri, 2007). It is thus desirable to have a fairly in depth understanding of an enzyme‘s properties before any process optimisation and/or up-scaling steps are attempted. Once purified, an enzyme is therefore characterized according to its temperature and pH optima, stability as well as kinetic parameters.

FOS producing enzymes from a variety of plant and microbial sources have been extensively characterized. Typically, transfructosylating enzymes have shown temperature and pH optima in the ranges; 30-60 ºC and 4.5-7.0, respectively (Sangeetha *et al.,* 2005). Goosen *et al*. (2007) have reported a novel intracellular invertase from *Aspergillus niger* which retains 50 % of its activity at 25 ºC and has its maximal activity at 37 ºC. Kinetic studies have shown the transfructosylation reaction to be dominant under high concentrations (>450 mg/ml) of sucrose for β-fructofuranosidase enzymes whereas, concentrations as low as 50 mg/ml are sufficient for fructosyltransferases (Ghazi et al., 2006). The synthesis of FOS may also be inhibited non-competitively by glucose (Nemukula *et al.*, 2009; Ghazi et al., 2006).

1.1 Analytical techniques

1.1.1 High performance liquid chromatography (HPLC)

HPLC coupled with PAD, RI or ELS detection, is the most commonly used means of quantifying activity during characterisation. As described in section 4.1.1 of chapter 1, it is the most efficient, reliably accurate and reproducible method available for the quantification of FOS without the expensive and time consuming step of pre-derivatization. It may however, be unavailable as and when required by researchers, especially in a ―developing world" setting. It is consequently necessary to develop

cheaper and simpler methods, without significantly compromising the accuracy and precision of quantification.

1.1.2 Enzymatic assays

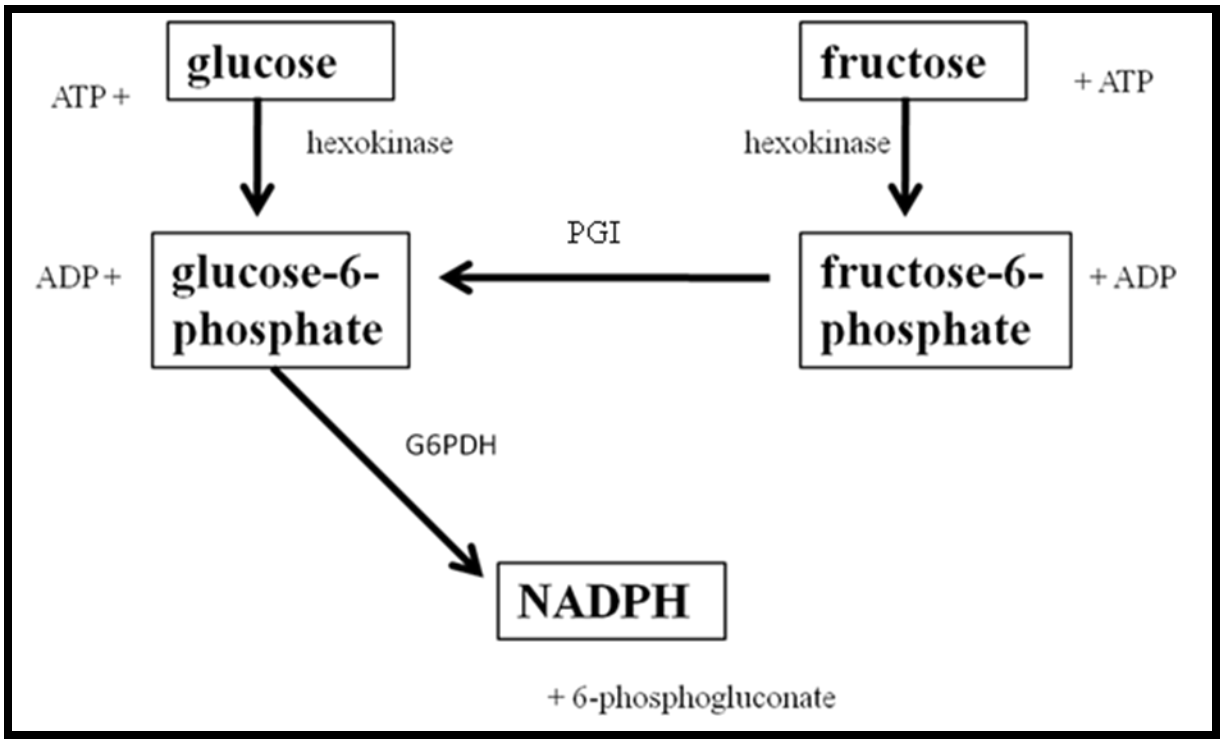
The use of enzymatic methods to detect simple sugars as a means of assaying for Ftase and FFase activity is fairly well documented. Guimarães *et al*. (2009) have reported the use of the dinitrosalicylic acid (DNS) and Rochelle salt (Miller, 1959) to quantify reducing sugars produced by the action of β-fructofuranosidases on agro-industrial residues. This method was shown to provide accurate and reproducible results though it did not offer clarity on the extent of transfructosylase or hydrolase activities in the reactions studied. Reddy *et al.* (2010), in an investigation to screen for high transferase β-fructofuranosidase producers in filamentous fungi, have improved the latter method by coupling the DNS reaction to the glucose oxidase-peroxidise assay for glucose determination. This method managed to quantify the amount of glucose produced as well as the total reducing sugars, the difference of which yielded the amount of fructose produced. Equations 3.1a and 3.1b describe the relationship between total reducing sugars (R), glucose (G) and fructose (F), as determined by the DNS and glucose oxidase-peroxidase methods, respectively, where: (F) - free fructose and (F‘) - transferred fructose. It was thus successful in shedding insight on the amounts of the transfructosylase and hydrolase activities. However, this method suffers in its reproducibility, especially where a large number of samples are being investigated, as it involves a relatively large number of manipulations and may be affected by other oxygen species in the reaction medium (Breuil and Sadler, 1985). It may also have limitations in its applicability to kinetic studies.

$$\mathbf{R - G = F} \qquad \mathbf{(3.1a)}$$

$$\mathbf{F' = G - F = 2G - R} \qquad \mathbf{(3.1b)}$$

Another possible option is the use of a different spectrophotometric assay, whereby the concentrations of glucose and fructose where determined using a combination of hexokinase (HK) and phosphoglucoisomerase (PGI) assays respectively. Glucose and fructose can be enzymatically phosphorylated and linked to NADP reduction to NADPH

by glucose-6-phosphate dehydrogenase (G6PDH) (Ernst, 1972, Cairns *et al*., 1987). NADPH can easily be detected at $A_{340\ nm}$ and has a 1:1 stoichiometry with glucose and consequently, with fructose as well. This principle is illustrated in scheme 3.1. The concentrations of glucose and fructose are then translated from the change in absorbance prior to and following, the addition of PGI, respectively. The main disadvantage of this method is that it is both expensive and time consuming, since it was designed for use in 1 ml cuvettes.



Scheme 3.1: Reactions involved in the enzymatic assay of β-fructofuranosidase using hexokinase and phosphoglucoisomerase (Ernst, 1972)

Thus far, the crude enzyme FopAp was purified to yield two fractions FA and FB whose protein contents show apparent molecular weights of 112 kDa and 78 kDa, respectively. Both fractions have shown potential to degrade sucrose and form FOS (Chapter 2). The main aim of the current chapter was to characterize both FA and FB, based on their pH and temperature optima, thermal stability, as well as kinetic properties. A secondary aim of this chapter was to assess the effectiveness of a hexokinase-PGI assay, adapted for use in microtitre plates, as a substitute for HPLC in enzyme characterisation.

2 Methods and Materials

2.1 Materials

Authentic 1-nystose and 1-kestose standards as well as acetonitrile (HPLC grade), glucose (HK) assay kits and phospho-glucose isomerase were purchased from Sigma-Aldrich (South Africa). All bench reagents were of analytical grade and were obtained from either Merck Chemicals (South Africa) or Sigma-Aldrich (South Africa). Unless otherwise stated; all reagents were dissolved in Milli-Q water and all experimental work was performed at 0-4 °C. Where appropriate, samples were stored at 0-4 ºC overnight and -20 ºC over longer periods.

Labnet Accublock™ Digital dry baths were obtained from White Scientific (South Africa). All HPLC in this chapter was carried with degassed and filtered HPLC grade solvents. Filtration and degassing were performed by a Milli-vac maxi™ vacuum with a 0.45µm nylon filtration membrane from Millipore (South Africa). All spectrophotometry was performed on a Synergy™ HT Multi-Mode microplate reader (Biotek Instruments, USA).

2.2 Determination of the effect of temperature on FFase activity

Sucrose (1200 µl, 6 mg/ml) in Tris-HCl buffer (50 mM, pH 9) was incubated with 300 µl of appropriately diluted enzyme sample over a range of temperatures (20 - 60 °C) in Eppendorf ®safe lock™ tubes (Merck, South Africa) for 1 hour. Separate tubes, consisting of either 300 µl of enzyme sample or 1200 µl of 6 mg/ml sucrose; made up to reaction volume (1500 µl) with Tris-HCl buffer (50 mM, pH 9) were used at each temperature, as enzyme and substrate controls, respectively. The reaction was stopped by incubating for 10 min in boiling water. The reaction mixture was then syringe filtered through a 0.22 µm nylon membrane (Millipore, South Africa), diluted appropriately and then subjected to enzymatic assay (**Section 2.5**). Incubation at each reaction condition was carried out in triplicate.

2.3 Determination of the effect of pH on FFase activity

Sucrose (1200 µl, 6 mg/ml) in; 50mM citrate-phosphate buffer (ph 4 – 7) or 50mM Tris-HCl (pH 8 – 9), were incubated with 300 µl from the appropriate enzyme fraction (FA or FB) at 20 ºC in Eppendorf® safe lock™ tubes (Merck, South Africa) for 1 hour. Separate tubes, consisting of either 300 µl of appropriate enzyme sample or 1200 µl of 6 mg/ml sucrose; made up to reaction volume (1500 µl) with appropriate buffer (50mM, pH range 4 – 9) were used as enzyme and substrate controls, respectively. The reaction was stopped by incubating for 10 min in boiling water. The reaction mixture was then syringe filtered through a 0.22 µm nylon membrane (Millipore, South Africa), diluted appropriately and then subjected to enzymatic assay (**Section 2.5**). Incubation at each reaction condition was carried out in triplicate.

2.4 Assessment of the thermal stability of FA and FB

FA and FB were incubated, in the absence of substrate at optimal temperature, for periods ranging from 1 - 24 hours. At the end of the selected time periods,1200 µl of 6 mg/ml sucrose in Tris-HCl buffer (50 mM, pH 9) were incubated with 300 µl of appropriately diluted enzyme sample at 20 ºC in Eppendorf ®safe lock™ tubes (Merck, South Africa). Separate tubes, consisting of either 300 µl of enzyme sample or 1200 µl of 6 mg/ml sucrose; made up to reaction volume (1500 µl) with Tris-HCl buffer (50 mM, pH 9) were used, at each time interval, as enzyme and substrate controls, respectively. All reactions were stopped by incubating for 10 min in boiling water. The reaction mixture was then syringe filtered through a 0.22 µm nylon membrane (Millipore, South Africa), diluted appropriately and then subjected to enzymatic assay as described in section 2.5 of this chapter. All treatments at each time period were carried out in triplicate.

2.5 Determination of FFase activity using HK/PGI assay

The glucose assay reagent (1.5 mM NAD, 1.0 mM ATP, 1.0 U/ml hexokinase, 1.0 U/ml G6PDH) (Sigma-Aldrich, South Africa), was made up according to the manufacturer‘s specifications. This assay was adapted for use in UV microtitre plates. 150 µl of glucose reagent were added to 10 µl of appropriately diluted sample and read at $A_{340\ nm}$ for 35 mins until no further change in absorbance was observed. 10 µl of diluted phospho-

glucose isomerase (60 U/ml) (Sigma-Aldrich, South Africa) were then added to the mixture and read at $A_{340\ nm}$ for 30 mins until no further change in absorbance was observed. The total change in absorbance for both enzymatic steps was noted. All assays were completed in triplicate and read against a sample blank (sample and distilled water), reagent blanks (glucose reagent/PGI and distilled water) at 25 ºC. The concentrations of glucose and fructose were calculated as instructed by the manufacturer in equation 3.2, where: d – length of the light path (cm), V_t – total volume, V_s – sample volume, F – dilution factor from sample preparation and ε – millimolar extinction coefficient for NADH at $A_{340\ nm.}$ The transfructolsylase (Ut) and hydrolase (Uh) activities were subsequently calculated as described in Chapter 2, section 2.8.

$$\textbf{Concentration} = (\Delta A_{340\ nm})\ (V_t)\ (F) / (\varepsilon)\ (d)\ (V_s) \qquad (3.2)$$

The results from the investigations conducted in section 2.2 – 2.4 were confirmed by subjecting each sample to HPLC analysis on a Prevail® Carbohydrate ES column (250 x 4.6 mm); guard column (7.5 x 4.6 mm) packed with 5 µm spherical polymer beads, (Alltech inc., USA). The HPLC system comprised of a Waters 2695 separation module coupled to an ELSD 2000 detector (2.2 L/min gas flow, 80 ºC oven temperatures) (Alltech inc., USA). Data processing was facilitated by Empower Pro chromatography software (Waters Inc, USA). Sample injection (10 µl) was carried out by a Waters 717 auto-sampler (Waters Inc, USA).

2.6 Determination of the kinetic parameters of FA and FB

Sucrose (40 µl, 0.55 mM – 5.5 mM) in Tris-HCl-buffer (50 mM, pH 9) were pipetted into separate wells in a UV microtitre plate. Glucose assay reagent (300 µl) was then added to all occupied wells. The reaction was initiated by the addition of 10 µl of enzyme. Absorbance at $A_{340\ nm}$ was read at 30 sec intervals for 5 mins. The initial rate of reaction at each concentration was determined based on the proportion of glucose produced and was used, to construct suitable kinetic plots. All readings were blanked against wells containing only the enzyme, glucose assay reagent and distilled water.

3. Results

3.1 Determination of the effect of temperature on FFase activity

In order to establish the temperature optima of FA and FB as well as identify any temperature related differences between the activities of the proteins in both fractions, an investigation on the effect of increasing temperature on FFase activity was carried out. In an attempt to assess its effectiveness in the characterization of FFases, the HK/PGI assay was employed to assay for activity and the results thereof are presented in figures 3.1 – 3.4.

In FA, increasing temperature leads to an apparent reduction of transfructosylase activity, as well as a converse, increase in hydrolase activity. This trend is also reflected in the decrease in Ut/Uh ratio between 20 ºC and 60 ºC (**Figure 3.1**). However, the total FFase activity is relatively unaffected by the increase in temperature within the same temperature range (20 ºC - 40 ºC). An apparent increase in Utot, albeit slight, can be observed in the range 40 ºC to 60 ºC (**Figure 3.2**).

Similar trends can be observed in the temperature profiles of FA and FB (**Figure 3.1-3.4**). However, despite having significantly lower activity, FB shows higher proportions of hydrolase activity than FA and hence demonstrates low Ut/Uh ratios within the same temperature range (**Figure 3.3**). FA and FB exhibit apparent temperature optima, at 20 ºC and 60 ºC, with regard to transfructosylase activity and hydrolase activity respectively.

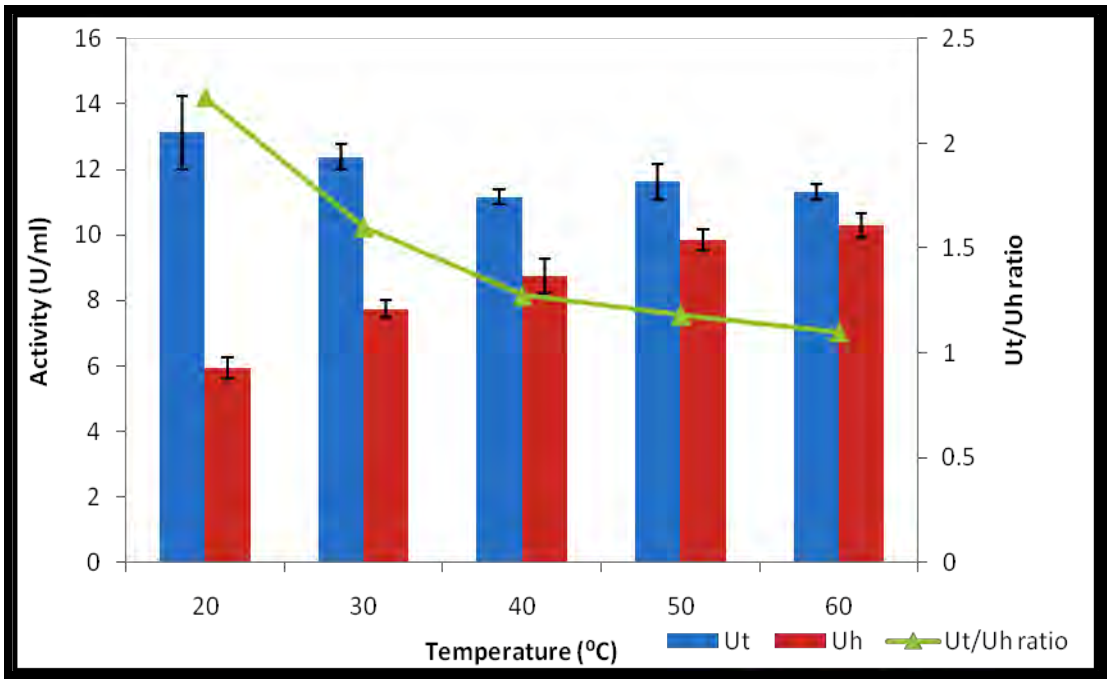


Figure 3.1: Graph showing the temperature profile of FA. (Values are represented as means ± SD, *n* = 3) (U- µmol/min)

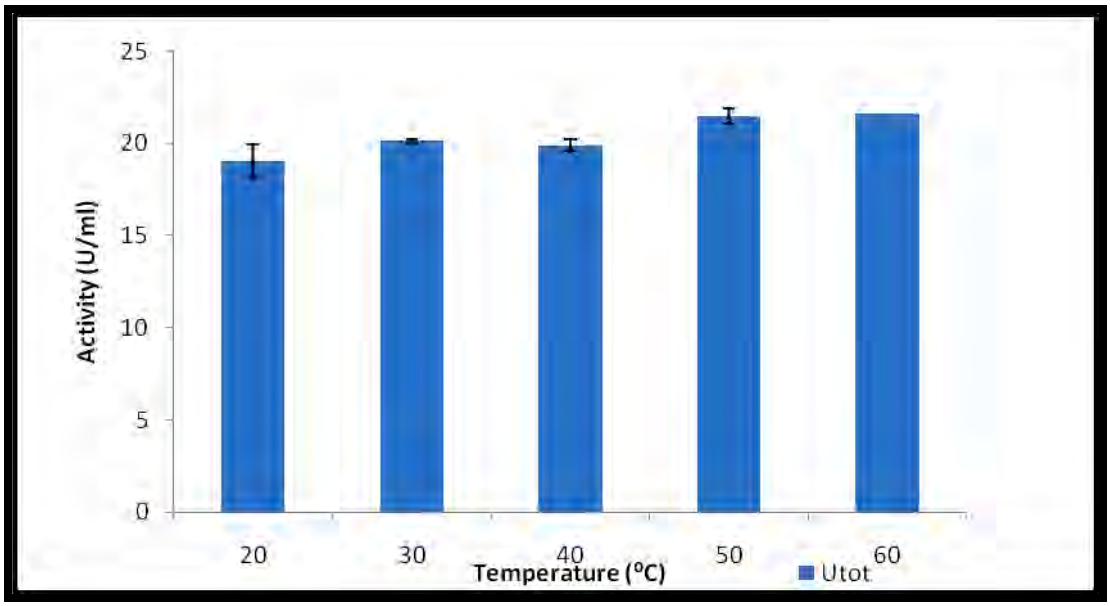


Figure 3.2: Graph showing the relationship between ■total activity (Utot) of FA and temperature. (Values are represented as means ± SD, *n* = 3)(U- µmol/min)

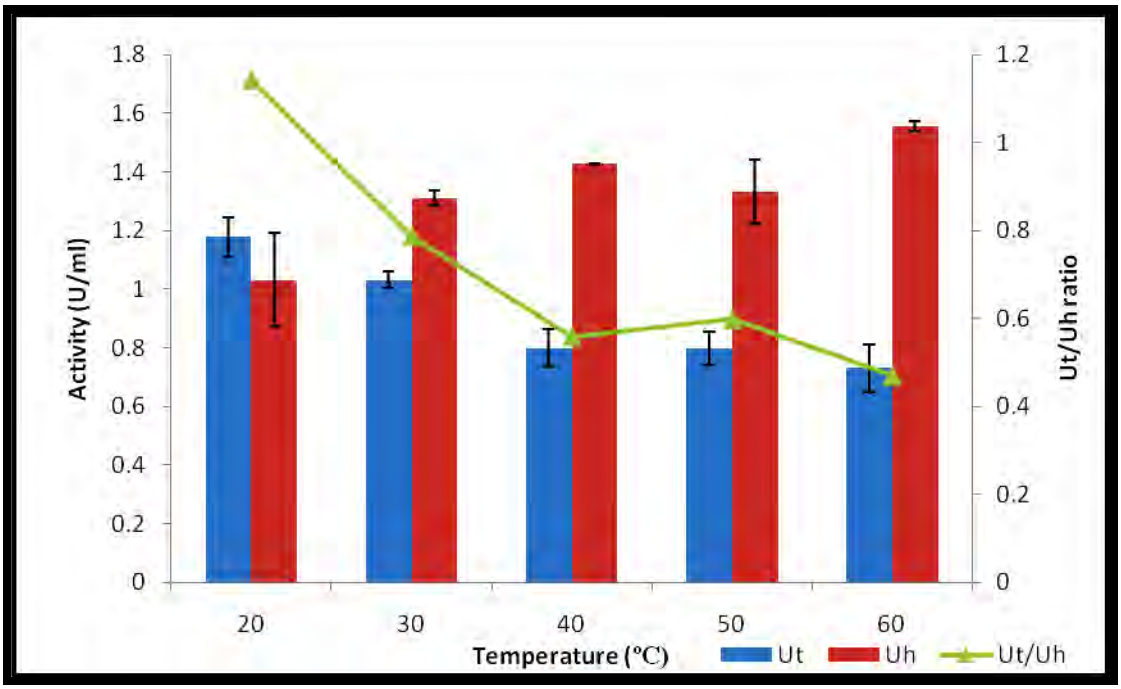


Figure 3.3: Graph showing the temperature profile of FB. (Values are represented as means ± SD, *n* = 3)(U – µmol/min)

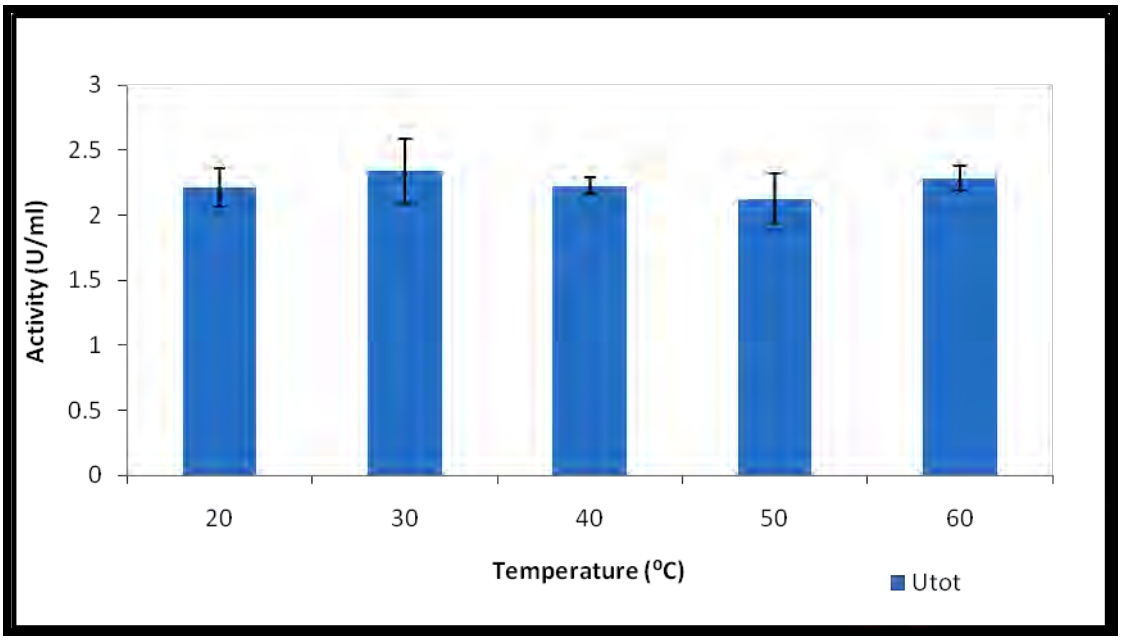


Figure 3.4: Graph showing the relationship between ■total activity (Utot) of FB and temperature. (Values are represented as means ± SD, *n* = 3)(U= µmol/min)

3.2 Determination of the effect of pH on FFase activity

The effect of pH on the catalytic properties of the proteins in FA and FB was determined in the range 4 – 9 at a temperature optimum for the transfructosylase activity of both enzyme fractions. The results obtained are represented in figures 3.5-3.8.

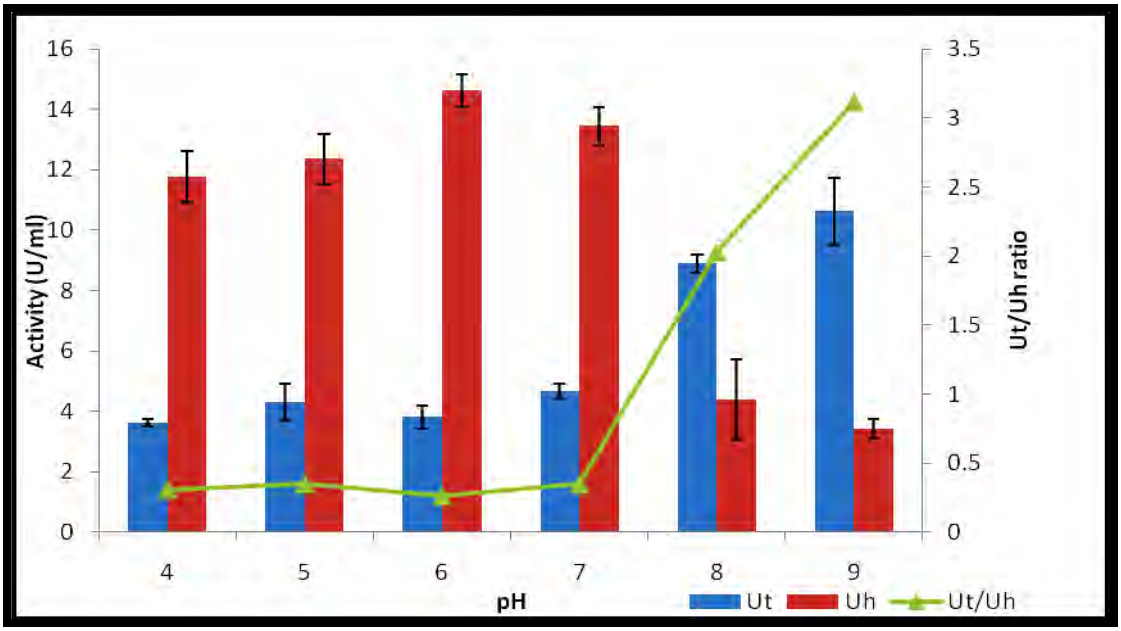


Figure 3.5: Graph showing the pH profile of FA. (Values are represented as means ± SD, *n* = 3)(U-µmol /min)

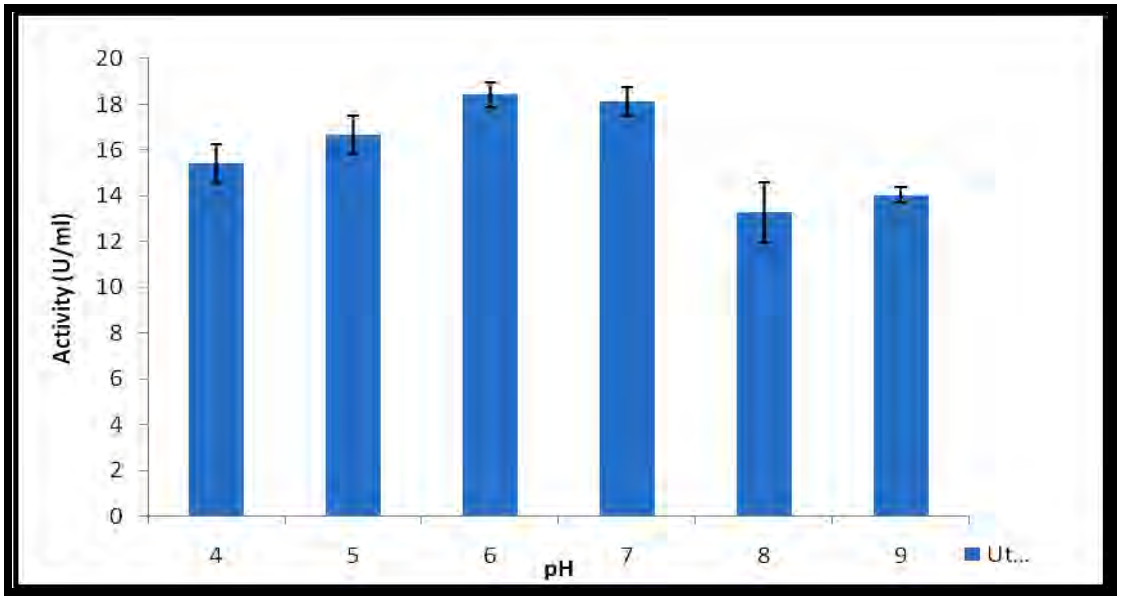


Figure 3.6: Graph showing the relationship between ■total activity (Utot) of FA and pH. (Values are represented as means ± SD, *n* = 3)(U- µmol/min)

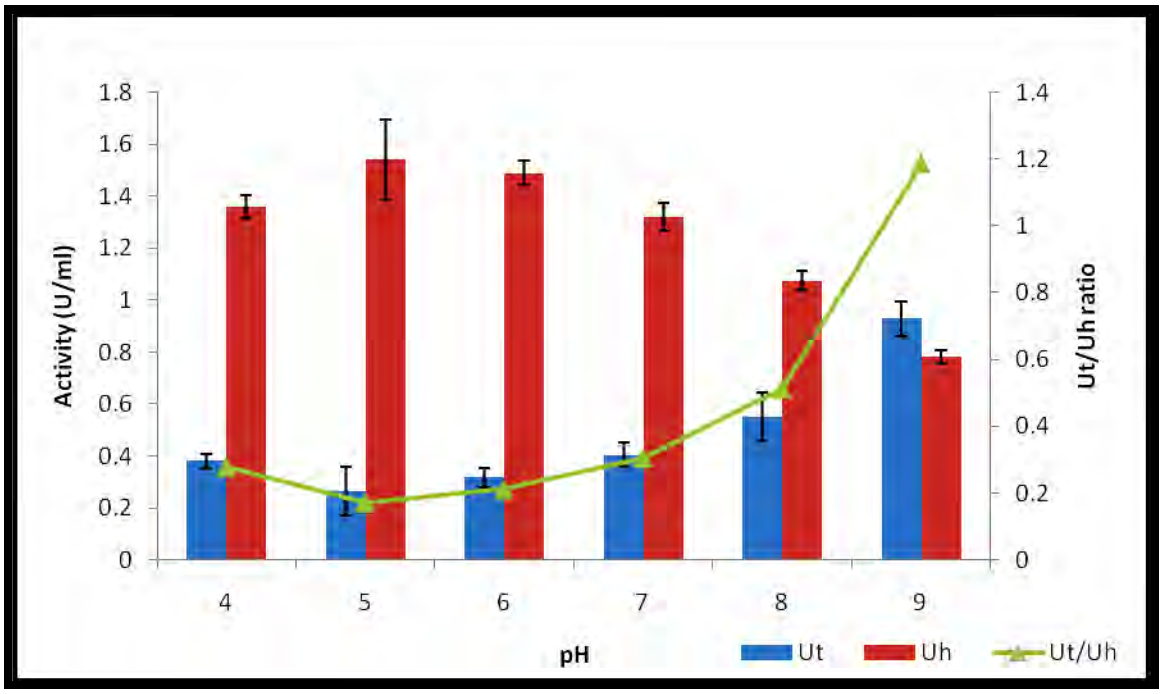


Figure 3.7: Graph showing the pH profile of FB. (Values are represented as means ± SD, *n* = 3)(U- µmol/min)

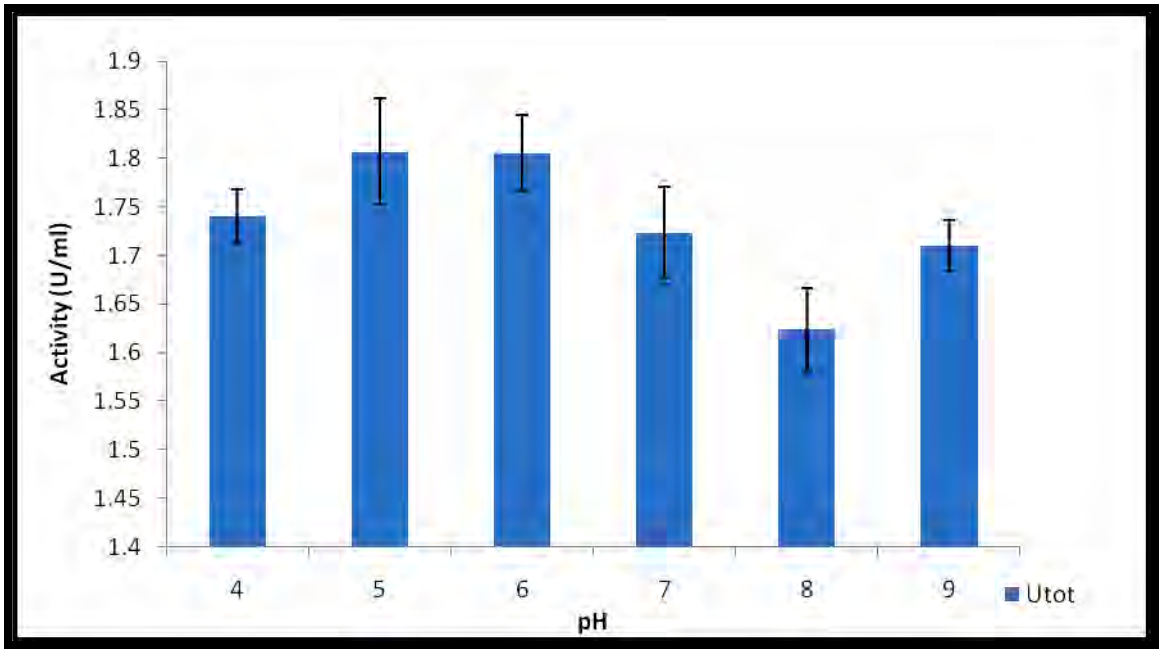


Figure 3.8: Graph showing the relationship between total activity (Utot) of FB and pH. (Values are represented as means ± SD, *n* = 3)(U- µmol/min)

FA and FB exhibit similar trends with respect to their pH profiles (**Figures 3.5 and 3.7**). There is a general increase of transfructosylase activity with increasing alkalinity. Hydrolase activity appears to increase proportionally with pH until about pH 5 and then

decreases dramatically at more alkaline conditions. The prevalently hydrolytic nature of FB is also apparent as hydrolase activity is still greater than transfructosylase activity at pH 8 (**Figure 3.7**). Figures 3.5 and 3.7 also demonstrate increasing Ut/Uh ratios with increasing alkalinity. FA and FB both show apparent optima at pH 9 and in the range pH 5 - 6, with regards to transfructosylase and hydrolase activity, respectively (**Figures 3.5 and 3.7**).

There is a significant increase in total FFase activity between pH 4 and pH 6, with a decreasing trend thereafter (**Figures 3.6 and 3.8**). This suggests that pH has a more significant effect on FFase activity than temperature. It also reveals a reduction of FFase activity at pH 9 and an apparent optimum at pH 6.

3.3 Assessment of the thermal stability of FA and FB

After the determination of pH and temperature optima it was also necessary to subject the enzyme to a thermal stability assessment under optimal conditions. At this stage, the optimal conditions for transfructosylase activity were used. Figures 3.9 and 3.10 show the results obtained

Figure 3.9 shows that FA loses activity rapidly after the first hour of incubation. The observed rate of thermal denaturation seems to decrease after the third hour of incubation, leaving the curve to ~~"t~~ail off" asymptotically. FB exhibits the same general trend in loss of activity (**Figure 3.10**). FA and FB exhibit half lives ($t_{1/2}$) of about 1.5 hours and 1.7 hours, respectively.

Both transfructosylase and hydrolase activities in FA and FB seem to be lost at a proportional initial rate. However, the hydrolase with 37.70 % residual activity remains significantly higher than the transfructosylase activity (19.65 %) after the third hour of incubation (**Figures 3.9 and 3.10**). In FB, this trend continues until the sixth hour of incubation when 37.19 % of hydrolase activity remains in comparison to 8.98 % of transfructosylase activity. The former finally decreases uniformly with transfructosylase activity, at twelve hours of incubation (**Figure 3.10**).

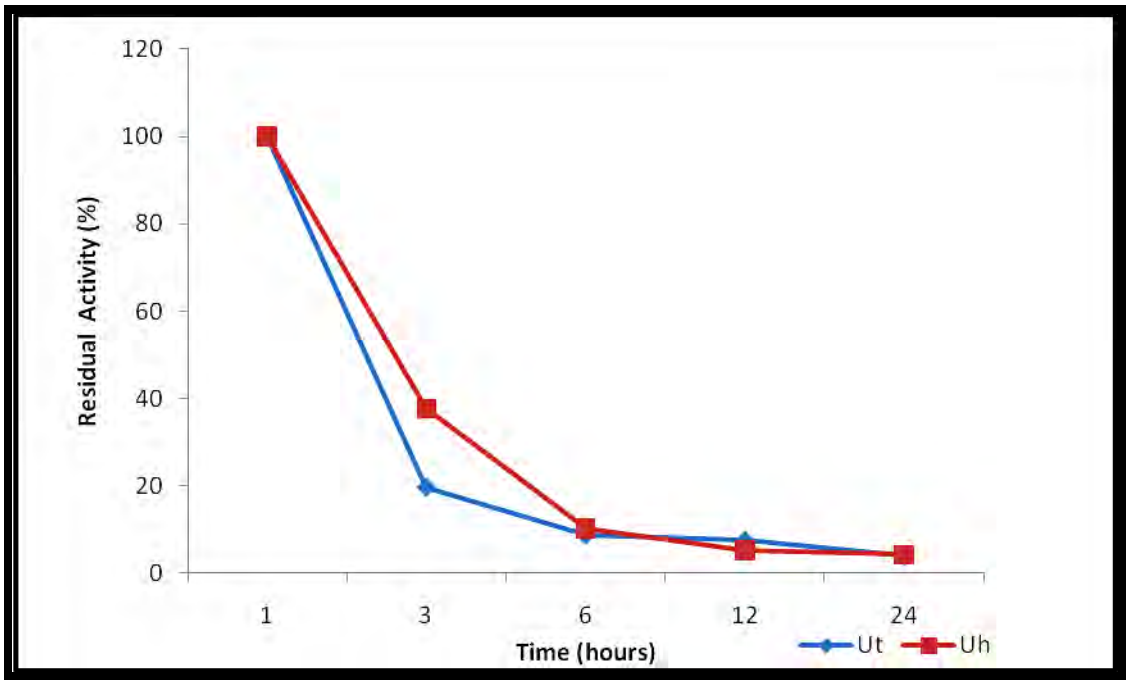


Figure 3.9: The thermal stability profile of FA at 20 ºC (Values are represented as means ± SD, *n* = 3)

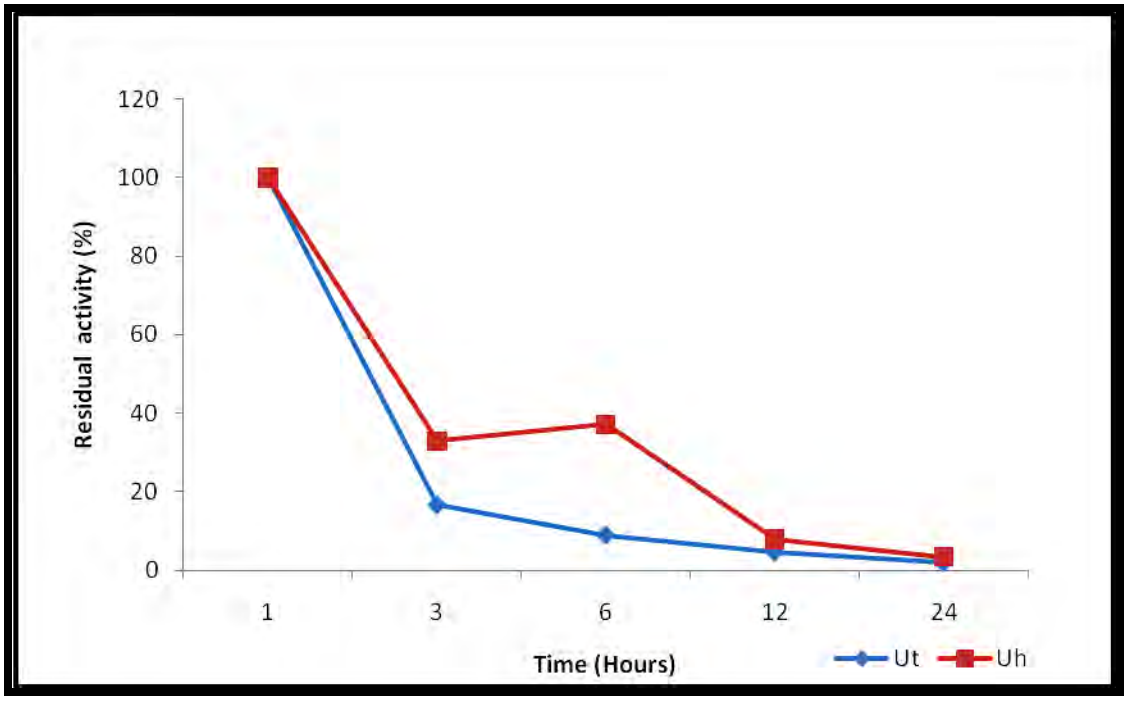


Figure 3.10: The thermal stability profile of FB at 20 ºC (Values are represented as means ± SD, *n* = 3)

3.4 Determination of the kinetic parameters of FA and FB

In order to gain insight on the affinity for sucrose exhibited by both FA and FB proteins, both enzyme fractions were assumed to follow Michaelis-Menten kinetics. Linearised

forms of the Michaelis-Menten equation (**Equation 3.3**), namely the Lineweaver-Burk and Hanes-Woolf plots (**Equation 3.4-3.5; Figures 3.11-3.14**), where: *v*- velocity, [S] - substrate concentration, K_m - Michaelis-Menten constant, V_{max} - maximum reaction velocity were utilised to obtain all kinetic data. Catalytic activity was assessed in terms of turnover number (k_{cat}) which together with catalytic efficiency were calculated using equations 3.6-3.7.(**Table 3.1**), where: [E].- enzyme concentration

$$v = V_{max} \bullet [S]/ [S] + K_m \quad (3.3)$$

$$1/v = 1/V_{max} + K_m/ V_{max} \bullet 1/ [S] \quad (3.4)$$

$$[S]/v = 1/V_{max} \bullet [S] + K_m/ V_{max} \quad (3.5)$$

$$k_{cat} = V_{max}/ [E] \quad (3.6)$$

$$\text{Catalytic efficiency} = k_{cat}/ K_m \quad (3.7)$$

Table 3.1: Kinetic parameters of FA and FB on sucrose substrate as determined by Lineweaver-Burk and Hanes-Woolf plots; Where C.E. - Catalytic efficiency and the units for V_{max}, K_m, k_{cat} and C.E. are µmol/ml/min, mM, /min and /mM/min, respectively.

	Lineweaver-Burk				Hanes-Woolf			
	V_{max}	K_m	k_{cat}	C.E.	V_{max}	K_m	k_{cat}	C.E.
FA	0.74	1.41	0.15	0.10	1.25	3.28	1.50	0.47
FB	0.73	1.49	0.31	0.21	1.26	2.6	1.08	0.42

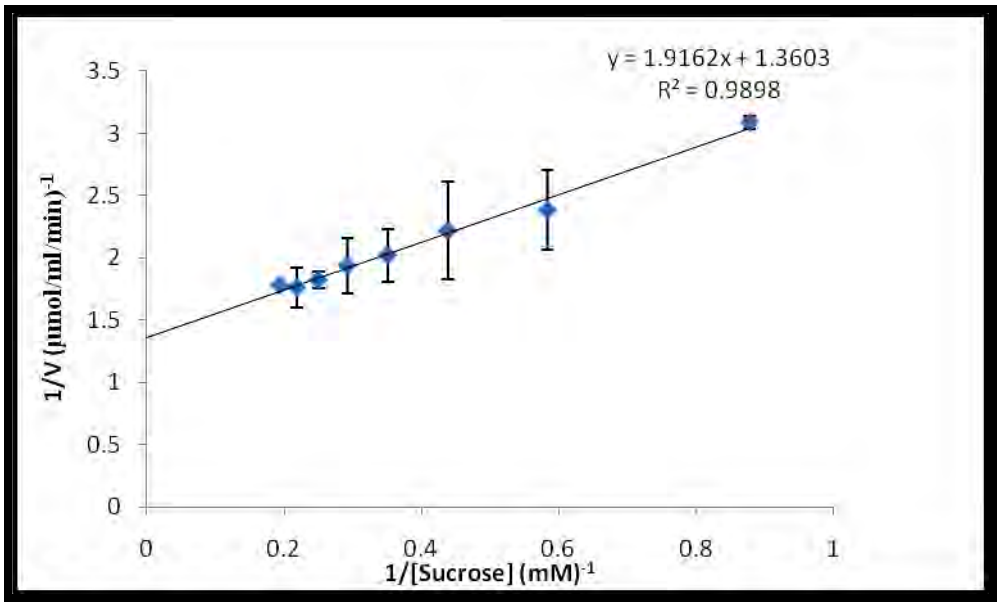


Figure 3.11: Lineweaver-Burk plot of FA with sucrose as substrate (Values are represented as means ± SD, *n* = 3)

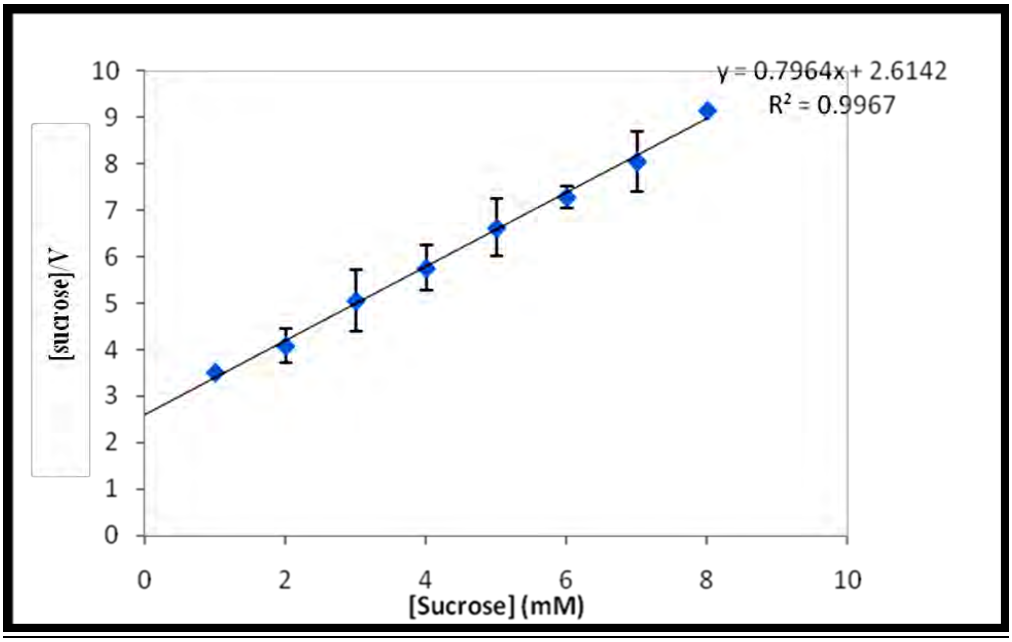


Figure 3.12: Hanes-Woolf plot of FA with sucrose as substrate (Values are represented as means ± SD, *n* = 3)

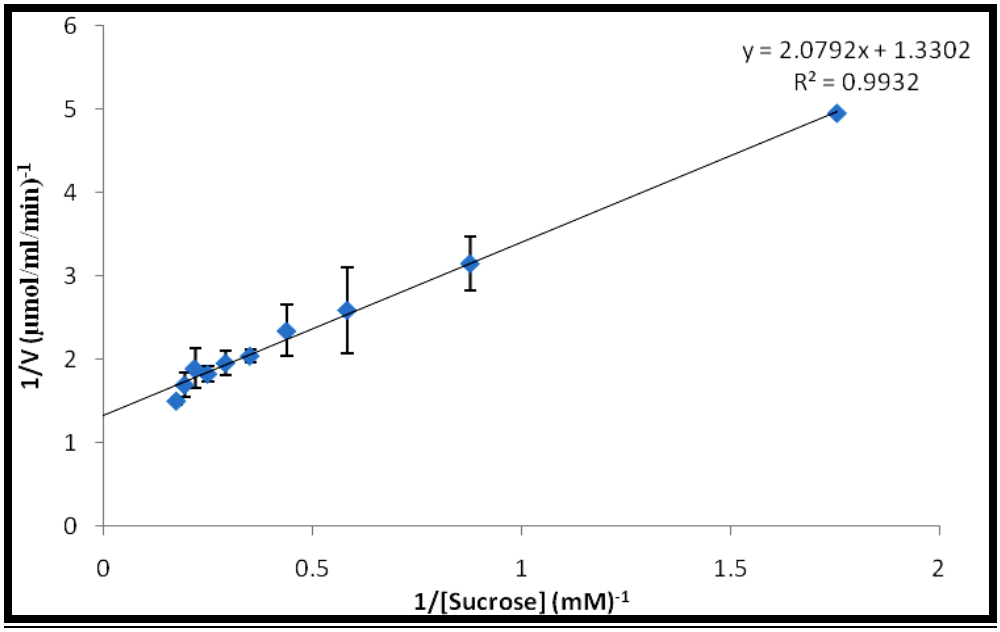


Figure 3.13: Line weave-Burk plot of FB with sucrose as substrate (Values are represented as means ± SD, *n* = 3)

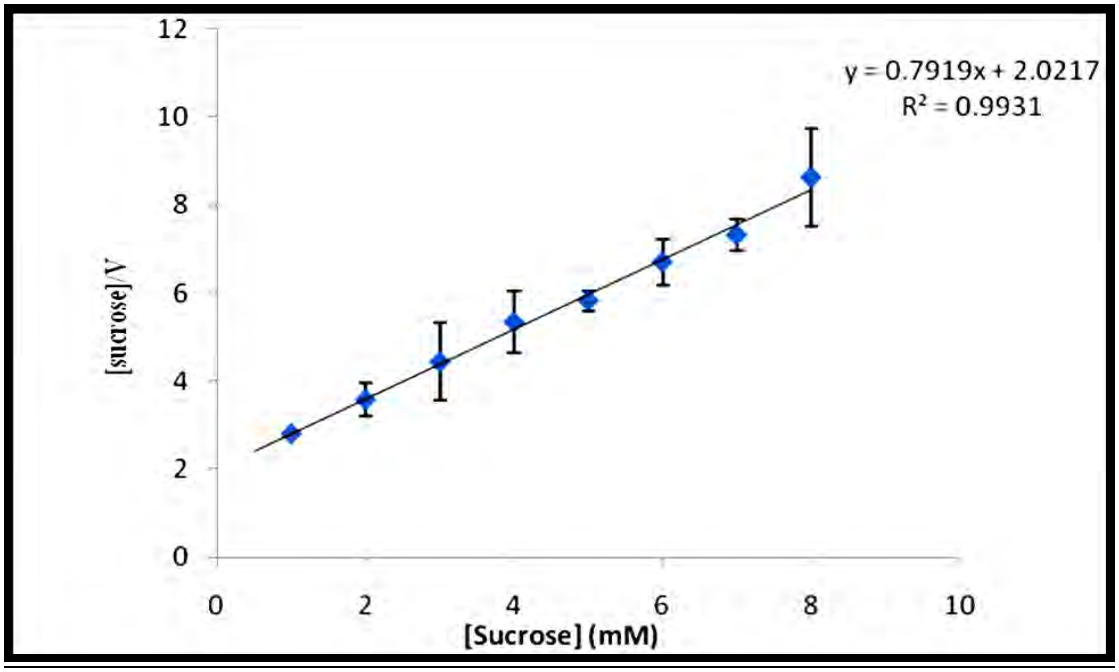


Figure 3.14: Hanes-Woolf plot of FB with sucrose as substrate (Values are represented as means ± SD, *n* = 3)

4. Discussion and conclusions

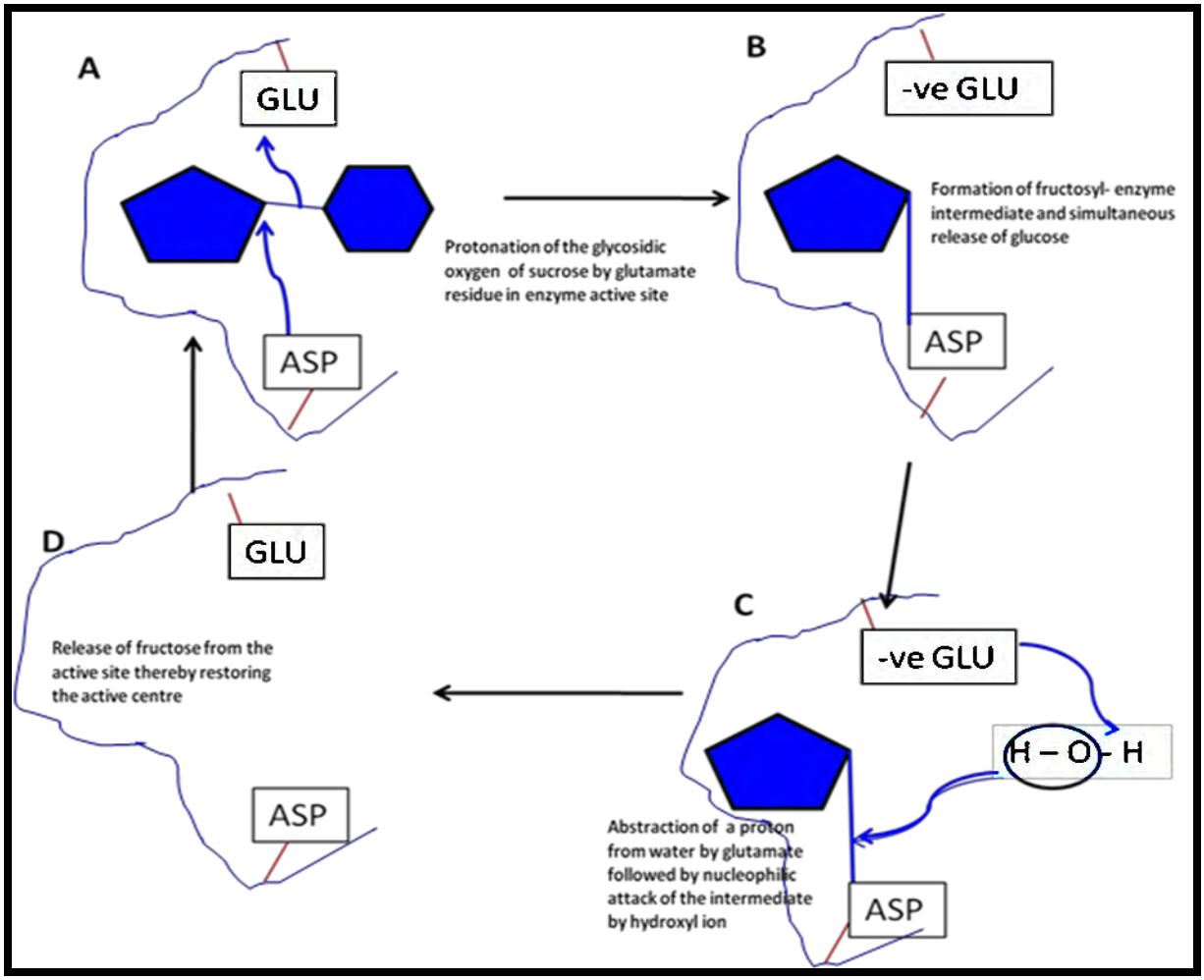
FA was shown to exhibit an apparent temperature optimum of 20 ºC and 60 ºC with regards to transfructosylase activity and hydrolase activity, respectively (**Figure 3.1**). An apparent optimum for total FFase activity was observed at 60 ºC. This, however, only showed as a slight inflexion on the temperature profile thus leading to a deduction that temperature did not have a pronounced effect on total FFase activity (**Figure 3.2**). Work carried out on a purified β-fructofuranosidase from *Aspergillus niger* ATCC20611 by Hirayama *et al*. (1989) showed a temperature optimum at 50 ºC and no activity at 20 ºC. However, the reaction was carried out at pH 5.0 using a 10 % (w/v) sucrose solution which differ from the conditions described in this research (**Section 2.2**) and may consequently account for the difference in results. It may also be useful to note that the assay utilised by Hirayama *et al.* (1989), relied on the concentration of kestose produced, as a marker for transfructosylase activity. In our opinion, this may be a source of error as kestose has been shown to act as a substrate of the enzyme under investigation and thus results may not only represent transfructosylase activity but also hydrolysis (Ghazi *et al.,* 2006*;* Hirayama *et al.,* 1989*).*

There is evidence of considerable variation in optimal conditions for FFase and FTase activity observed between wild type and recombinant strains as well as between species (Sangeetha *et al.*, 2005; Chen *et al.,* 2009). Nguyen *et al*. (2005) reported a fructosyltransferase from *A. japonicus* that exhibited an optimal activity at 60 ºC while retaining 60 % of its transferase activity at 20 ºC. A novel intracellular invertase from *A.niger* has also been reported to show an optimal temperature in the range of 37 – 40 ºC while retaining 80 % of its total activity at 20 ºC (Goosen *et al*., 2007).

The optimal pH of both purified fractions (FA and FB) was observed at pH 9 and pH 5 with regards to transfructosylase and hydrolase activity, respectively (**Figures 3.5 and 3.7**), while the optimum for total FFase activity was noted in the range of pH 5- pH 6 (**Figure 3.6 and 3.8**). The latter is in agreement with results obtained by Hirayama *et al*. (1989), Nguyen *et al.* (2005) and Gutiérrez-Alonso *et al.* (2009), who reported pH optima of 5.5, 6 and 5, from independent investigations on FOS producing enzymes from *A. niger* ATCC20611, *A. niger* IMI303386 and *Rhodotorula diarenensis,* respectively. Lee and Sturm (1996) have reported an invertase, extracted from carrot (*Daucus carota*) tissue, with a pH optimum of 8. However, most investigations reported in the literature, including those mentioned previously, only document the effect of pH on the total activity exhibited while insufficient information has been presented on the effect of pH on the activities of the individual components of FFase activity. As the main aim of this research was to establish and investigate the FOS forming potential of FopAp, it was crucial to understand the main parameters that individually affected both transfructosylase and hydrolase activities.

FOS producing invertases possess a common defining feature in the form of, two critically located acidic residues, which make up the catalytic machinery responsible for the cleavage of glycosidic bonds. These two invariant carboxylic residues have been identified in yeast invertase as an aspartate (ASP) located close to the N terminus and a glutamate (GLU), which acts as a nucleophile and an acid/base catalyst, respectively (Alberto *et al*., 2004; Reddy and Maley, 1996). As described in section 5.1.2 of chapter 1, the proposed mechanism for fructofuranosidase action is initiated by the protonation of

the glycosidic oxygen in sucrose by glutamate, thereby allowing nucleophillic attack by the aspartate residue to occur and consequently form the fructosyl-enzyme intermediate, which is only broken down after abstraction of a proton from water by the glutamate residue to yield fructose (**Scheme 3.2**) (Chen *et al.*, 2009; Reddy and Maley, 1996).



Scheme 3.2: Schematic of sucrose hydrolysis in fructofuranosidase active site (adapted from Chen *et al.*, 2009)

Figures 3.5 and 3.7 illustrate the increase of hydrolytic activity at pH 5; while figures 3.6 and 3.8 show the concomitant increase of total activity, at the same pH. It has thus been suggested that the higher activity at lower pH may be attributed to the spontaneous protonation of the glycosidic oxygen without the action of glutamate (Chen *et al.*, 2009). This effect however, does not account for any increase in transfer reactions between sucrose and free fructose. It is thus plausible that, assuming that FopAp follows the trend

of other FOS producing enzymes, at these conditions hydrolysis dominates to yield an excess of free fructose thereby contributing to a higher total activity but not necessarily greater production of FOS. Conversely, it is then possible that FOS production may be optimal at conditions that do not promote excessive hydrolysis activity and supply enough substrate to promote transfer of fructose from the fructosyl intermediate to a protonated acceptor, i.e. mildly alkaline conditions at high sucrose concentration. This could explain the reduction in hydrolase and increase in transfructosylase activities at pH 9. Transfructosylase activity is thought to be governed by the degree of competition between water and sucrose as nucleophilic acceptors (Ghazi *et al*., 2006; Lee *et al*., 1999). In light of this evidence, it is understandable that the transfructosylase activity may not be greatly affected by changes in temperature, and that the observed increase in hydrolase activity with temperature may merely be due to increased reaction energy at particulate level.

The thermal stability profile of FA (**Figure 3.9**) reveals that purified FopAp is not very stable at room temperature, with only 50 % activity left over after 1.5 hours. This is uncommon though not frequently documented. Rodriguez et al. (1995) have written about an invertase from *Pichia anomala* which exhibited a temperature optimum of 37 °C and was unstable at temperatures above 32 °C, with a half-life of 15 mins. These findings, exacerbated by degradation in freeze-thaw cycles may also offer an explanation for the loss of activity observed in FA and FB throughout this research.

Another interesting result highlighted in this investigation was the apparent prevalence of hydrolase activity in FB compared to FA at the same conditions of pH and temperature, illustrated by the smaller range of Ut/Uh ratios exhibited in figure 3.3. This behaviour suggests the presence of another hydrolytic component in FopAp and thus supports the initial hypothesis made on the origin and nature of the 78 kDa protein observed in FB (**Section 3, chapter 2**). This is also supported by thermal stability studies as depicted in figures 3.9 and 3.10. A significant difference can be observed between the thermal stability profiles of FA and FB after 6 hours of incubation. Although there is no considerable difference between the transfructosylase activities at this time interval, there

is a significantly less hydrolase activity in FA than FB. This activity may be from a hydrolytic protein which possesses significantly different thermal-stability characteristics from those exhibited in FA. Information on the half-life of FopAp may be useful in the design of commercial reaction and storage protocols for both crude and purified enzymes. A very short half life could necessitate frequent ~~–e~~nzyme reloading" during commercial synthesis, making the process quite costly. Mansour and Dawoud (2003) have suggested a possible solution in the form of immobilisation of invertase on celite and polyacrylamide matrices as a means of enhancing pH and temperature stability.

Substrate kinetics on FA and FB using sucrose were estimated by Lineweaver-Burk and Hanes-Woolf plots. The kinetic parameter values obtained from FA (V_{max} = 1.25 µmol/ml/min; K_m = 3.28 mM) and FB (V_{max} = 1.26 µmol/ml/min; K_m = 2.6 mM) (**Table 3.1**) are comparable to those exhibited by hydrolytic fructofuranosidases such as those from *Scopulariopsis bravicaulis* (V_{max} = 0.26 µmol/ml/min; K_m = 3 mM) and *Fusarium oxysporium* (V_{max} = 0.78 µmol/ml/min; K_m = 4.4 mM) as investigated by Hatakeyama *et al*. (1996) and Nishizawa *et al*. (1980). The findings in table 3.1 were much lower than those observed by Nishizawa *et al.* (2000) from *A.niger* ATCC20611 (V_{max} = 508 µmol/ml/min; K_m = 70 mM). These differences and similarities are probably due to low concentration of sucrose that was utilised in the investigation (0.5 – 5.5 mM).

The low K_m values obtained from the kinetic plots (**Table 3.1**) imply stronger binding of the substrate to the enzymes active site. The lower K_m observed in FB may be a result of higher sucrose binding by the 78 kDa protein discussed earlier in this section. Nishizawa *et al.* (2000) discussed that a lower K_m may be associated with a greater number of fructose units within the substrate molecule. This could be true for both fractions but more so for FB, as the competition between the hydrolytic enzymes may have promoted the hydrolysis of FOS as well as sucrose.

The Lineweaver-Burk plots (**Figures 3.11 and 3.13**) for FA and FB produced values of K_m and Vmax that were lower than those obtained by the Hanes-Woolf plots (**Figures 3.12 and 3.14**). Consequently, larger differences were observed in the k_{cat} as well as

catalytic efficiency values. The Lineweaver-Burk plot (**Equation 3.4**) has been reported to introduce errors as it changes the weighting of values when mathematical transformations are made (Lineweaver, 1984). It however is still considered credible as a means of representing data though is not recommended for the calculation of kinetic parameters (Liao *et al*., 2005). The use of the Hanes-Woolf plot is therefore recommended due to its superior credibility as observed in this research.

In conclusion, purified FopAp was extensively characterized to reveal apparent temperature optima at 20 °C and 60 °C for transfructosylase and hydrolase activities, respectively. It was also concluded that temperature may not have a marked effect on the total FFase. On the other hand, both fractions exhibited observed pH optima at 9 and 5 for transfructosylase and hydrolase activity, respectively. The significant effect of pH was also apparent in the total activity with an observed optimum of pH 5. It is essential to acknowledge that FOS production occurs through the synergistic action of hydrolase and transfructosylase activities and thus a characterization of both activities may be necessary to locate a point of synergy. Purified FopAp showed to be a thermo-labile protein with a half life of 1.5 hrs, a K_m of 3.28 mM and a V_{max} of 1.25 µmol/ml/min. However, these results may not be used as final conditions for FOS synthesis. Synthesis of bio-molecules is a complex process where, amongst other things, factor interactions play a large role. It is thus necessary to further investigate the findings of this chapter using a multivariate approach so as to identify and manage interactions before ultimately optimising synthesis.

It has also been concluded that FB does indeed comprise of a diluted protein from FA (112 kDa) as well as a hydrolytic component (78 kDa), which is possibly from *Pichia pastoris*. The use of the HK/PGI enzymatic assay proved to produce reproducible and comparable results for the characterization of purified FopAp fractions FA and FB. Its use is by no means superior to the conventional and widely accepted application of HPLC methods but can definitely offer a reliable substitute in cases where the former is unavailable. The results obtained by the assay were comparable to those seen in the

literature using HPLC (Hatakeyama *et al.,* 1996; Hirayama *et al.,* 1989; Nishizawa *et al.,* 1980).

CHAPTER 4
ANALYSIS OF FOS SYNTHESIS USING RESPONSE SURFACE METHODOLOGY

1. Introduction

As discussed in previous chapters, the identification of optimal conditions for the synthesis of FOS is difficult due to the large number of process parameters to be considered not to mention the vast array of possible interactions that can occur between them. The occurrence of error as a result of "uncontrolled factors" such as the physical (e.g. fatigue) and mental (bias) state of the researcher, only adds to the complexity of the process. It is thus desirable to have an understanding of these underlying factors and more so a reliable method of analysing them. The principle of experimental design allows one to carry out randomized and appropriately blocked investigations without time consuming repetition of experimental runs. As a result, experimental design has become a popular method of obtaining large amounts of information from a minimal amount of experiments, with substantial statistical credibility (Zeelie, 2010; Myers and Montgomery, 2002).

Response surface methodology (RSM) is a combination of mathematical and statistical techniques used for developing and optimising chemical and biological processes (Chaibva, 2010). Factorial design and response surface analysis are vital tools in the determination of optimal process conditions. Factorial design of a limited set of variables is superior to conventional "one-at-a-time" methods, which frequently fail to locate optimal conditions as they do not consider the potential interactions between factors (Santos and Maugeri, 2007). Furthermore, factorial design also allows one to estimate the effect of a factor at several levels of other factors thus yielding valid results over a range of conditions (Myers and Montgomery, 2002). Response surface modelling can be used to analyze how sensitive optimal conditions are to the changes in various parameters and are consequently of great importance in the development and application of robust synthetic and analytical techniques (Zeelie, 2010). However, the use of RSM is limited to the analysis of those processes without complex interaction effects. Considerable time may be necessary to investigate such interactions, thereby negating the main advantage of response surface methods (Chaibva, 2010).

There is evidence of the extensive use of these novel techniques in various scientific disciplines. For example, Mutanda *et al.* (2008) have reported the use of a central composite design (CCD) to optimize the synthesis of FOS from inulin using endoinulase enzymes from *Aspergillus niger*. Nemukula *et al.,* 2009 also demonstrated the successful utilisation of a central composite design to optimize the synthesis of FOS from sucrose using a fructosyltransferase from *Aspergillus aculeatus*. Another study by Santos and Maugeri (2007) reported the use of a fractional factorial design in the synthesis of FOS using an inulinase from *Kluyveromyces marxianus*. Through the use of Taguchi's arrays, Ghasemi *et al*. (2008) have used response surfaces to optimize the production of bacteriorhodopsin by *Halobacterium salinitarium* PTCC 1685. Response surface methodology has also been used as a chemometric approach to determine the photolytic rates of organic compounds in natural waters (Giokas and Vlessidis, 2006).

1.1 Analytical techniques

1.1.1 Factorial experimental design

This technique involves the planning of experiments in a manner that confers statistical validity of all measurable outcomes thereby ensuring the collection of appropriate data and ultimately resulting in objective and valid conclusions (Montgomery, 2002). All critical parameters (independent input variables) under investigation are called factors, while their measurable units are referred to as levels. Choosing the correct factors to incorporate into a factorial design is not a simple task and may involve running a number of screening designs to identify those factors that are directly involved in the process under investigation as well as the levels at which they are most influential. Those factors not selected are then kept constant (Otto, 1999).

All the levels of factors to be investigated are assigned coded values (**Equation 4.1**, where x_i is the coded value, X_i is the actual value, X_{cp} is the centre point value and ΔX_i is the difference between X_i and X_{cp}) to allow plotting of values with different units on the same axes. Coding yields low level (-1), centre point (0) and high level (+1) values for all input variables. Factorial designs structured in an orthogonal manner allow for

randomization of samples and when appropriately blocked, minimize both known and unknown uncontrollable factors (Otto, 1999; Zeelie, 2010).

$$\mathbf{X_i = (X_i - X_{cp}) / \Delta X_i} \qquad \mathbf{(4.1)}$$

In order to understand their effectiveness in covering the ~~"t~~heoretical experimental space" governed by a set of input variables, orthogonal designs can be depicted as a hyper-cube (**Figure 4.1**). Two commonly utilised designs are the central composite (CCD) and Box-Behnken designs and their suggested hyper-cubes are illustrated in figure 4.1. The CCD is a more robust design compared to the Box-Behnken especially where a large number of variables are being investigated. The CCD is a combination of a full or fractional factorial design with a star design which shares the same central point. The axial points (star points) utilised in CCDs are additional factor levels that infer better statistical properties to the design as well as pronouncing the observed response within the hypercube. Axial points are also thought to allow for the estimation of curvature as well as the approximation of new extremes of high and low levels for all factors under investigation (Chaibva, 2010). The CCD may however, prove to be time consuming, especially where a large number of variables are under investigation particularly due to the additional axial point runs that must be conducted (Otto, 1999). The Box-Behnken design performs more replicates at the centre than classical factorial or central composite designs, thereby allowing fewer runs to be made without affecting statistical validity.

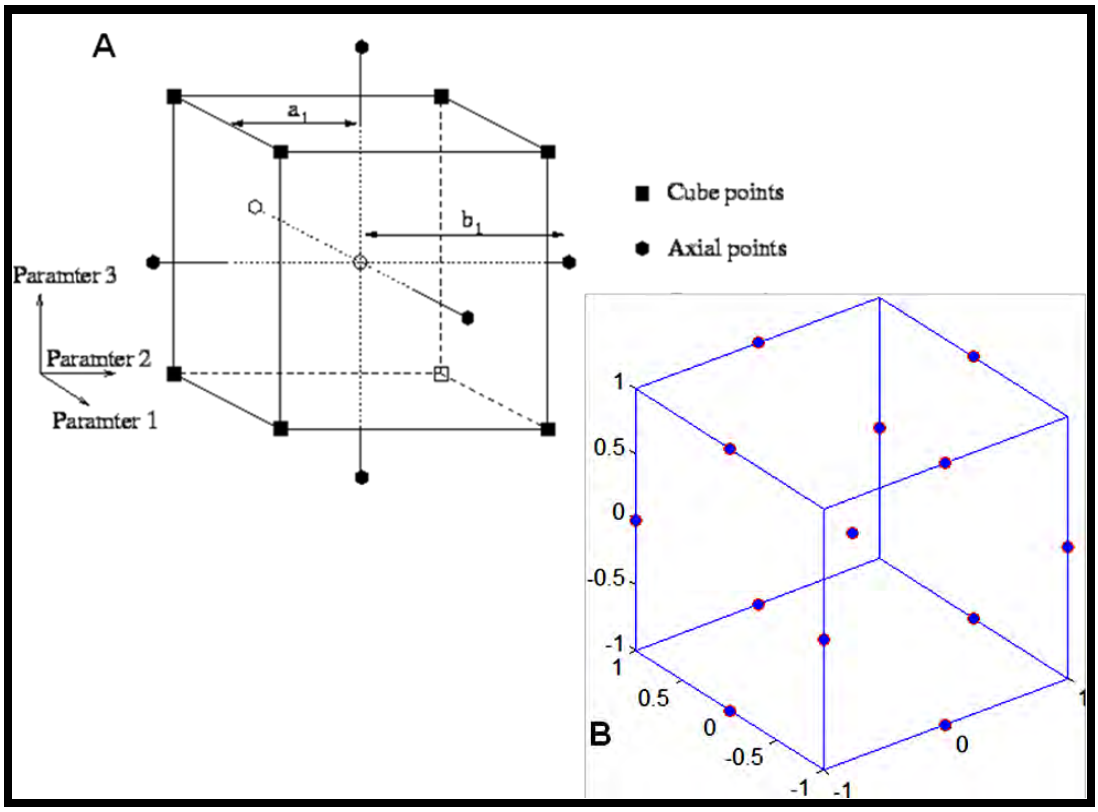


Figure 4.1: Design hyper cubes for, (A) central composite design showing the combination of factorial (cube) and star (axial) designs; (B) Box-Behnken design with experimental points in a spherical arrangement, equidistant from the centre point.

1.1.2 Mathematical modelling

Following the execution of statistically designed experiments, the implementation of RSM involves the subsequent generation of mathematical equations and graphical outcomes to describe a complete picture of the variability of a response as a function of previously defined input factors. Empirical data is fitted to statistical or mathematical models that are used to predict process performance and to optimize defined critical response variables (Singh *et al*, 2004). The polynomial models that are generated from statistically designed experiments can be used to summarize empirical data and to predict the relationship between input and response variables. The polynomial equations may be first, second or third order in nature though only second order models are usually applied in industrial processes (Draper and Lin, 1996; Mutanda *et al.*, 2008; Nemukula *et al.,* 2010). Equations 4.2 and 4.3 show typical first and second order polynomial models where; y= estimated response, x_i/x_j = input factors, β_0= constant that represents the

intercept, β_i/ β_j= coefficients of first order terms, β_{ii} =coefficients of second order terms and β_{ij} = coefficients of second order interaction terms.

$$y = \beta_0 + \beta_i x_i + \beta_j x_j + \ldots + \varepsilon \qquad (4.2)$$

$$y = \beta_0 + \beta_i x_i + \beta_j x_j + \beta_{ij} x_i x_j + \ldots + \varepsilon \qquad (4.3)$$

First order models are useful in investigations whereby the crucial variables are expected to exhibit minimal variability and no interactions with each other. Conversely, second order models are generally used as linear (**Equation 4.3**) and/or quadratic (**Equation 4.4**) equations to describe a response where crucial variables are expected to vary extensively throughout the experimental domain and exhibit significant interactions (Chaibva, 2010).

$$y = \beta_0 + \beta_i x_i + \beta_j x_j + \beta_{ij} x_i x_j + \beta_{ii} x^2_i + \beta_{jj} x^2_j + \ldots + \varepsilon \qquad (4.4)$$

The effect of two or more input variables on a measurable response as described by a particular polynomial model can then be graphically analysed as a three dimensional response surface or a two dimensional contour plot.

The main aim of this chapter is to investigate the synthesis of FOS by the enzyme FopAp using principles of experimental design and response surface methodology. The primary objective was to explore the experimental space and identify the significant variables in the synthesis of FOS by FopAp and fit a mathematical model for the process in an attempt to identify suitable conditions for the optimal synthesis of FOS by the enzyme FopAp.

2 Methods and materials

2.1 Materials

Authentic 1-nystose and 1-kestose standards were purchased from Sigma-Aldrich (Germany). All bench reagents were of analytical grade and were obtained from either Merck Chemicals (South Africa) or Sigma-Aldrich (South Africa). Unless otherwise stated; all reagents were dissolved in Milli-Q water and all experimental work was

performed at 0-4 °C. All samples were stored at 0-4 ºC overnight and -20 ºC over longer periods, where appropriate.

Labnet Accublock™ Digital dry baths were obtained from White Scientific (South Africa). All HPLC in this chapter was carried out with degassed and filtered HPLC grade solvents. Filtration and degassing were performed by a Milli-vac maxi™ vacuum with a 0.45µm nylon filtration membrane from Millipore (South Africa). Unless otherwise stated, all the data and word processing in this chapter was done Microsoft ® Office tools (Microsoft® inc., USA)

2.2 Initial investigation of significant factors using a Classical 2^3 factorial design

In order to identify the significant factors involved in the synthesis of FOS, factor effects and interactions were assessed at high and low factor levels. A classical (2^3) factorial design was generated using Design Expert™ software (version 8.0.4, Stat-ease®, USA). Three factors (temperature, pH and time) were incorporated into the design matrix at two levels each (+1 and – 1) as described in tables 4.1 and 4.2. Enzyme (300 µl) and substrate concentration (600 mg/ml) were both kept constant throughout this investigation. The factors were chosen following their use in characterisation studies (**Chapter 3**), so enabling a comparison to be made between the findings of the two chapters. Experimental runs were carried out accordingly. All reactions were terminated and subjected to HPLC sample preparation as described in previous chapters.

Table 4.1: Factors and levels selected for 2^3 design

FACTORS	LEVELS	
	-1	+1
TEMPERATURE (ºC)	20	40
pH	3	9
TIME (MINS)	30	60

Table 4.2: Classical factorial design(2^3)

RUN	CODED FACTOR LEVELS		
	TEMPERATURE	pH	TIME
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

Aliquots (10 µl) of sample from each experimental run were then injected onto to a Prevail® Carbohydrate ES column (250 x 4.6 mm); guard column (7.5 x 4.6 mm) packed with 5 µm spherical polymer beads, (Alltech inc., USA).The mobile phase comprised of 70% acetonitrile at a flow rate at 0.5 ml/min. The HPLC system comprised of a Waters 2695 separation module coupled to an ELSD 2000 detector (2.2 L/min gas flow, 80 ºC oven temperature) (Alltech inc., USA). Data processing was facilitated by Empower Pro chromatography software (Waters Inc, USA). Peak areas were converted to concentration by means of a calibration curve constructed as described in section 2.6 of chapter 2 (**Appendix A 4**). Sample injection (10 µl) was carried out, in triplicate, by a Waters 717 auto-sampler (Waters Inc, USA).

2.3 Analysis of FOS synthesis using a Box-Behnken design (3^3)

The results obtained from the 2^3 design, in combination with prior knowledge from enzyme characterisation studies and literature, were used to construct a second factorial design within a narrower experimental domain. The centre points for temperature and time were carried forward from the best runs in the 2^3 design while the centre values for pH were obtained from the literature as proposed optima. Using Design Expert software™, a 3^3 Box-Behnken design was selected for three factors (temperature, pH and

time) at three levels (+1, 0, -1) as illustrated in tables 4.3 and 4.4. The selected design consisted of 17 factor combinations and 5 replications at the centre point. Experimental work was then carried out accordingly, keeping the same constants as described in 2.2 of this chapter. Reaction termination and HPLC analysis were also carried out as described in section 2.2. This investigation was carried out for both the crude and purified forms of FopAp.

Table 4.3: Factors and levels selected for 3^3 Box-Behnken design

FACTORS	LEVELS		
	-1	**0**	**+1**
TEMPERATURE(ºC)	**15**	**20**	**25**
pH	**5**	**5.5**	**6**
TIME (MINS)	**30**	**60**	**90**

Table 4.4: 3^3 Box-Behnken design for FOS synthesis

RUN	CODED FACTOR LEVELS		
	TEMPERATURE	**pH**	**TIME**
1	+1	-1	0
2	0	0	0
3	-1	0	-1
4	0	0	0
5	-1	-1	0
6	-1	0	+1
7	0	+1	+1
8	0	0	0
9	0	-1	-1
10	+1	+1	0
11	0	+1	-1
12	+1	0	+1
13	-1	+1	0
14	0	0	0
15	+1	0	-1
16	0	0	0
17	0	-1	1

2.4 Data analysis

Empirical data obtained from the 2^3 and Box-Behnken designs was analysed using the regression modelling function in Design Expert™ software. Mathematical models were employed to fit the experimental data and were subsequently assessed to determine which of the input factors had a significant impact on measured responses by minimising the PRESS for each model. The use of PRESS is appropriate for working towards a regression model that provides good predictive capabilities for an experimental domain. When PRESS is evaluated in data modelling, if there are *n* runs in the data set, then the equation is assessed by fitting ($n - 1$) runs to the model under investigation. The difference between a recorded experimental data value and the value predicted by the model with ($n - 1$) data points is termed the prediction residual and PRESS is the sum of the squares of the prediction residuals for each point in a data set (Stat-ease, 2008). Models were evaluated using stepwise regression by minimising the PRESS statistic for each coefficient in the model. A Student's *t*-test was used to evaluate whether a coefficient was statistically different from zero with a 5 % level of significance (Stat-ease, 2008) and only significant factors were included in the evaluation of models selected for further investigation. Two types of model diagnostic plots, *viz*., the actual data *vs*. predicted data and studentised residuals *vs.* predicted data graphs, were constructed to validate the goodness of fit of the proposed models to experimental data (**Appendix B 2**). The plot of experimental data *vs*. predicted response data generated from a model is particularly useful for detecting. The studentised residual values provide an indication of the differences in magnitude between observed and predicted responses from a mathematical model and these are plotted *vs.* the predicted values of the response parameters under investigation.

3. Results

3.1 Factorial design (2^3)

An initial investigation was carried out using a randomized 2^3 factorial design. The results obtained from the HPLC analysis of the 2^3 design were collated and are presented in table 4.5. Figure 4.2 shows an HPLC chromatogram of run 8 (at 20ºC, pH 9, 30 mins) of the classical factorial design, which shows the highest total FOS yield (13.17 mM).

Table 4.5: Design summary for 2^3 design

	CODED FACTOR LEVELS			RESPONSES		
Run	A:time (Mins)	B:temp (°C)	C:pH	[kestose] (mM)	[nystose] (mM)	total [FOS] (mM)
1	1	1	-1	0	0	0
2	1	-1	-1	0.05	2.09	2.15
3	1	1	1	0.10	9.63	9.72
4	-1	1	1	0.06	6.64	6.70
5	-1	-1	-1	0.01	0.00	0.01
6	1	-1	1	0.11	8.56	8.68
7	-1	1	-1	0.00	0.00	0.00
8	-1	-1	1	0.24	12.93	13.17

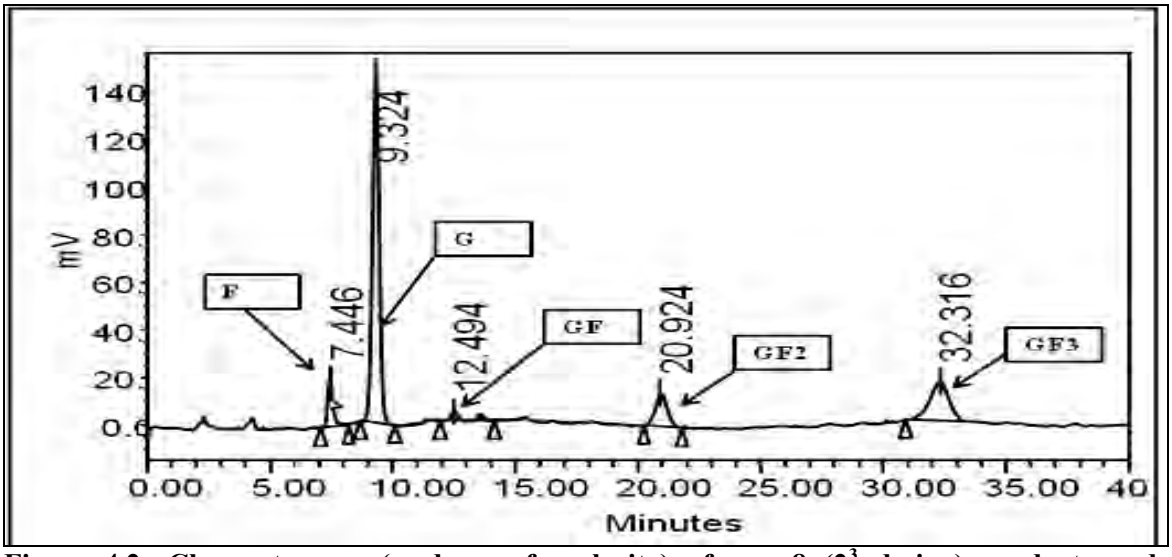


Figure 4.2: Chromatogram (re-drawn for clarity) of run 8 (2^3 design) products and retention times; where F = fructose at 7.45 mins, G = glucose at 9.32 mins, GF = sucrose at 12.49 mins, GF2 = kestose at 20.92 mins and GF3 = nystose at 32.32 mins. (All retention times reduced to 2 dp.)

Design Expert™ software was used to evaluate the significance of the temperature, time and pH on the synthesis of kestose and nystose by the crude enzyme FopAp. A linear mathematical model was fitted to the data by regression analysis (**Appendix B 1**) and only significant factors were included in this model. Temperature and pH were found to have a significant effect on the production of kestose within the selected experimental constraints; with ~~"p~~-values" of 0.111 (90 % CI) and 0.0229 (95 % CI), respectively. However, within the chosen experimental range, no significant interactions were

observed between temperature and pH. The model exhibited an ―F-value" of 7.12 which implies that the model was indeed significant. There is only a 3.44 % chance that this value occurred due to noise. This model exhibited a PRESS of 0.030. It was also observed that only pH illicited an effect on the amount of kestose produced. This was confirmed at the 95 % CI with a ―p-value" of 0.0007 and an ―F-value―of 39.61.There is only a 0.07 % chance of this value occuring due to noise.The PRESS for this model was 42.81which is relatively low and thus confirms its significance. The predicted and adjusted R-squared values for both models were seen to be in agreement, suggesting that uncontrollable factor effects had been minimised. Equations 4.5 and 4.6 can be used to describe the main effects on kestose and nystose concentration (mM), respectively, where B = the effect of temperature and C = the effect of pH.

$$\textbf{[Kestose]} = 0.072 - 0.033\,B + 0.055\,C \qquad (4.5)$$

$$\textbf{[Nystose]} = 4.98 + 4.46\,C \qquad (4.6)$$

Figure 4.3 illustrates the proposed models for FOS synthesis based on the 2^3 design. Surface (A) describes the effect of pH and time on nystose concentration, though, it is apparent that the latter has a negligible effect on nystose concentration. Surface (B) describes kestose concentration as a function of temperature and pH. The slope of the surface shows significant temperature and pH effects on kestose production.

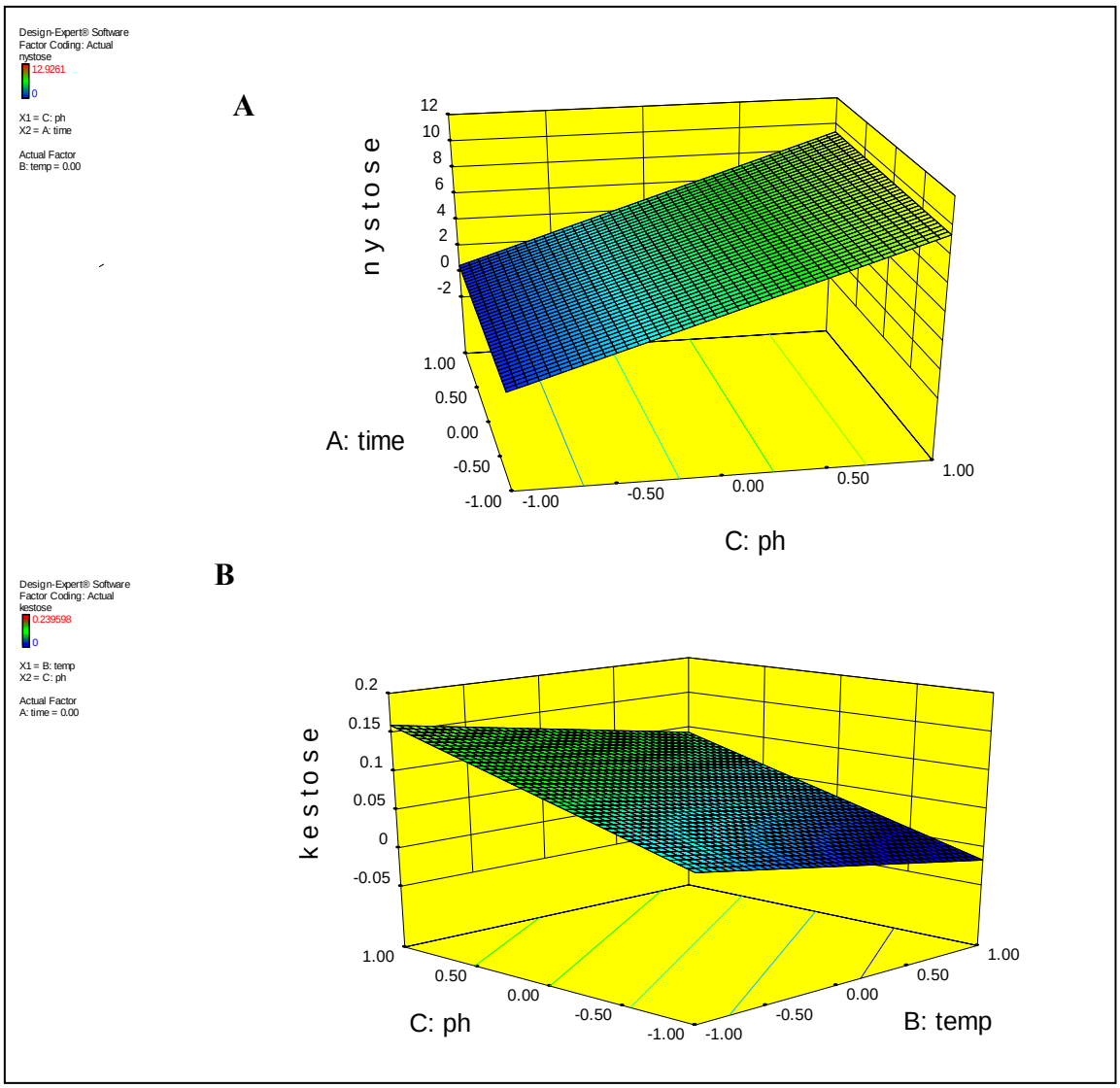


Figure 4.3: 3D response surfaces illustrating the main effect models fitted to (A) nystose and (B) kestose synthesis , based on the 2^3 factorial design.

3.2 Box-Behnken design

Using the results of the 2^3 design as well as prior knowledge from characterisation studies, a Box-Behnken design was constructed to navigate the experimental space using more restrictive constraints. Both the purified and crude enzymes were used in this investigation. Table 4.6 and 4.7 summarize the designs which used the purified and crude enzymes, respectively.

Table 4.6: Design summary of the Box-Benkhen design with purified FopAp

	CODED FACTOR LEVELS			RESPONSES		
Run	**A:pH**	**B:Temp (ºC)**	**C:time (Mins)**	**[kestose] (mM)**	**[nystose] (mM)**	**total [FOS] (mM)**
1	1	-1	0	0.29	5.94	6.23
2	0	0	0	0	0	0
3	-1	0	-1	0	0	0
4	0	0	0	0.19	10.7	10.89
5	-1	-1	0	0.44	4.98	5.42
6	-1	0	1	0.6	0	0.6
7	0	1	1	0	0	0
8	0	0	0	0	0	0
9	0	-1	-1	0	0	0
10	1	1	0	0.2	6.22	6.42
11	0	1	-1	0	0	0
12	1	0	1	0	0	0
13	-1	1	0	0.06	1.8	1.86
14	0	0	0	0	0	0
15	1	0	-1	0.14	4.03	4.17
16	0	0	0	0	0	0
17	0	-1	1	0	0	0

HPLC analysis showed a significantly higher amount of FOS products (Max. = 22.83 mM) than observed in the 2^3 design (Max. = 13.17 mM). Also observed, was a large difference between the crude and purified enzyme runs. HPLC analyses of run 4 from each design reveal an approximately 1.62-fold difference in FOS production between the crude (17.61 mM) and purified forms of FopAp (10.89 mM) (**Tables 4.6 and 4.7**). It is noteworthy that run 4 was the best run for the purified enzyme investigation and only the 5^{th} best in the crude enzyme investigation

Table 4.7: Design summary of the Box-Behnken design with crude FopAp

Run	CODED FACTOR LEVELS			RESPONSES		
	A:pH	B:Temp (°C)	C:time (Mins)	[kestose] (mM)	[nystose] (mM)	total FOS (mM)
1	1	-1	0	0.75	14.07	14.82
2	0	0	0	0.56	22.27	22.83
3	-1	0	-1	0.48	14.96	15.44
4	0	0	0	0.51	17.10	17.61
5	-1	-1	0	0.37	3.46	3.83
6	-1	0	1	0.46	21.44	21.91
7	0	1	1	0.34	14.79	15.13
8	0	0	0	0.45	18.33	18.77
9	0	-1	-1	0.67	13.29	13.97
10	1	1	0	0.46	16.14	16.60
11	0	1	-1	0.58	19.89	20.47
12	1	0	1	0.50	17.96	18.46
13	-1	1	0	0.48	12.48	12.96
14	0	0	0	0.30	14.32	14.61
15	1	0	-1	0.52	19.22	19.74
16	0	0	0	0.56	14.92	15.48
17	0	-1	1	0.65	13.12	13.77

By comparing figures 4.4 and 4.5 there is a significant difference in peak areas and peak heights for FOS products. The lack of FOS products observed in most of the runs performed with the purified FopAp (**Table 4.6; Figure 4.4**) made model fitting impossible as no significant factors were identified. Consequently, no further work was performed on purified FopAp.

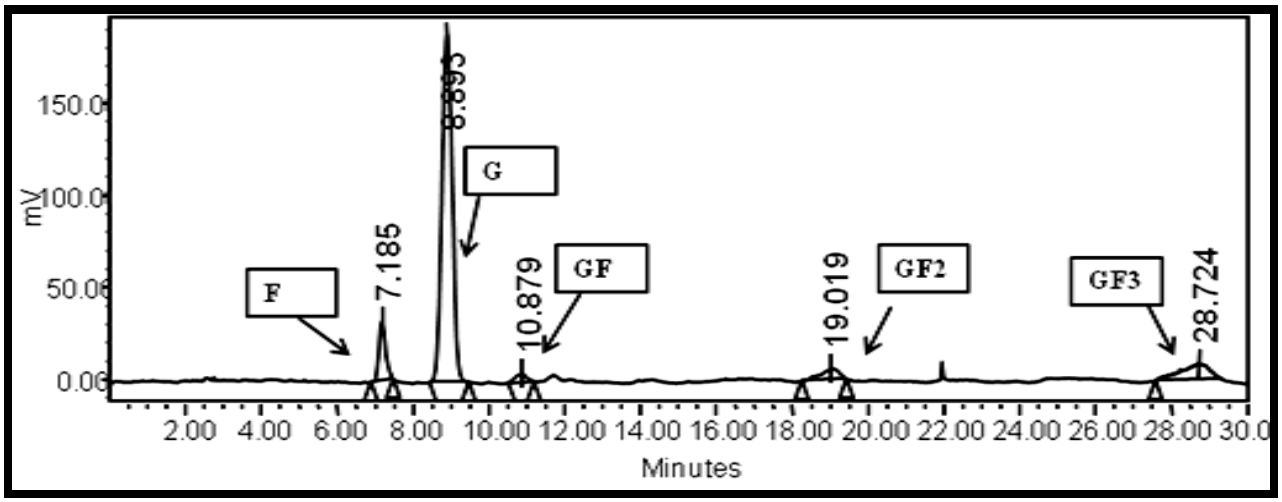


Figure 4.4: Chromatogram of run 4 (Box-Behnken design, FA purified enzyme) products and retention times; where F = fructose at 7.19 mins, G = glucose at 8.90 mins, GF = sucrose at 10.88 mins, GF2 = kestose at 19.02 mins and GF3 = nystose at 28.72 mins. (All retention times reduced to 2 dp.)

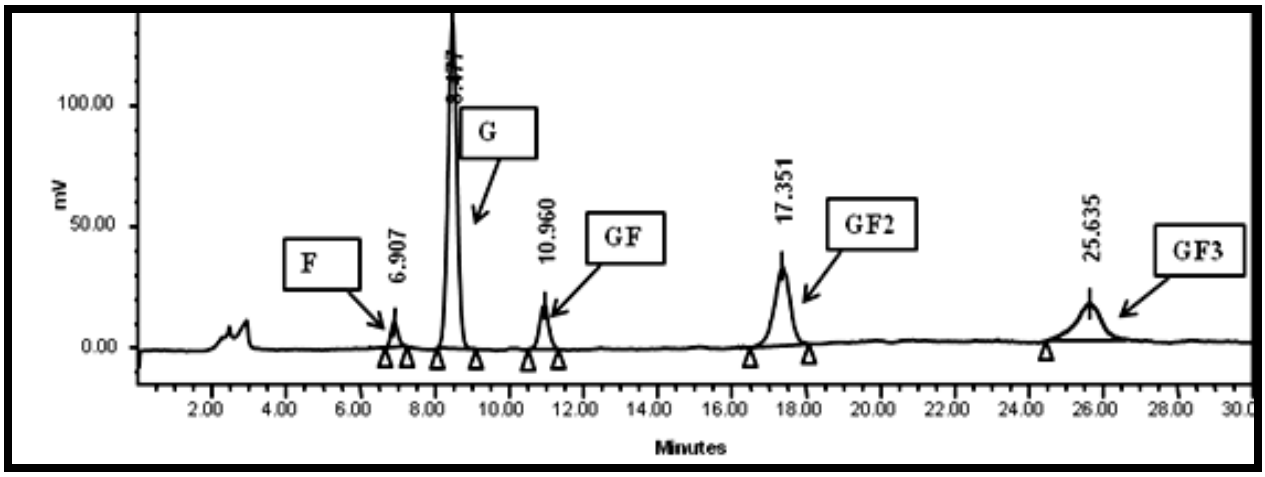


Figure 4.5: Chromatogram of run 4 (Box-Behnken design, crude enzyme) products and retention times; where F = fructose at 6.91 mins, G = glucose at 8.12 mins, GF = sucrose at 10.96 mins, GF2 = kestose at 17.35 mins and GF3 = nystose at 25.63 mins. (All retention times reduced to 2 dp.)

Design Expert™ software was used to fit a quadratic model on the data obtained by the Box-Behnken design on crude FopAp. Within the adjusted constraints, temperature and pH were found to significantly affect kestose concentration, with ~~"p~~-values~~"~~of 0.12 (90 % CI) and 0.04 (95 % CI), respectively. An interesting observation was the increase in the effect of temperature at the adjusted pH range (5 – 6). The interaction effect of pH and temperature was found to be significant, with a ~~"p~~-value" of 0.05 (90% CI). The

whole model exhibited an ―F-value" of 6.29 and a PRESS of 0.44, thus confirming its significance and usability in defining the experimental space.

For the synthesis of nystose, a 2fl model was fitted .In this model, the effect of temperature and its ―self-interaction" (temperature^2) were found to be the only significant factors,with ―p-values" of 0.06 (90 % CI) and 0.02 (95 % CI).The ―F-value" and PRESS of the model were found to be 5.37 and 268.18, respectively, both of which satisfy the criteria to accept the model and render it significant and usable. Equations 4.7 and 4.8 were then generated to define the main effects on the concentration (mM) of each FOS species;where A = the effect of pH, B = the effect of temperature .

$$\textbf{[kestose]} = 0.051 + 0.055\,A - 0.074\,B - 0.10\,AB \qquad (4.7)$$

$$\textbf{[nystose]} = 17.84 + 2.42\,B - 4.43\,B^2 \qquad (4.8)$$

Figure 4.6 illustrates the relationships described in equations 4.7 and 4.8 as 3 dimensional surface plots. These relationships appear more complex than those observed in the 2^3 factorial design. The presence of quadratic interactions is also very apparent and is evident in the curvature of the given surfaces.

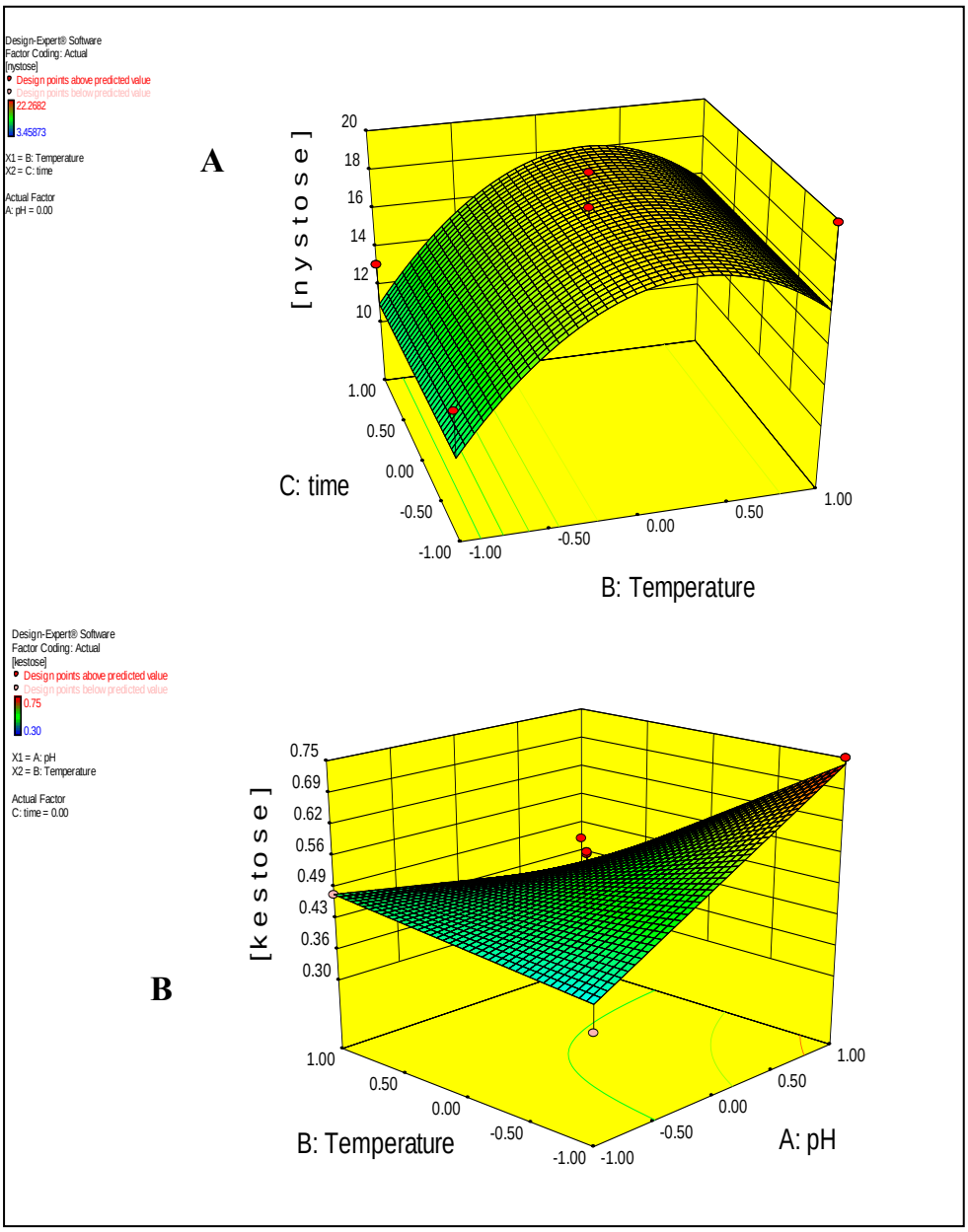


Figure 4.6: 3D response surfaces illustrating the main effect models fitted to (A) nystose and (B) kestose synthesis, based on data from the Box-Behnken design (crude enzyme).

4. Discussion and conclusion

The principles of experimental design and response surface methodology were implemented successfully to analyse the synthesis of FOS by the enzyme FopAp. By using the 2^3 factorial design as an initial method to scan the experimental space, it was observed that for the production of kestose, only the pH and temperature played a

significant role. From equation 4.5 it can be deduced that an inversely proportional relationship exists between the concentration of FOS and temperature. An increase in the magnitude of temperature has very little effect on FOS production. From the initial investigation it was deduced that a low temperature is preferable. These findings are in agreement with those observed in chapter 3 of this research using the purified enzyme fraction, FA which showed optimal transfructosylase activity at 20 °C. Work done by Hirayama *et al.* (1989), on the synthesis of FOS, by a fructofuranosidase from *Aspergillus niger* ATCC 20611, has reported optimal enzyme activity at 60 °C. However, the results from the current research thus far, show that this may not necessarily be optimal for FOS production. A close look at the effect of temperature in the range 15°C to 30 °C using a Box-Behnken design shows that more kestose is produced at 15°C than at higher temperatures while nystose production peaks at 25 °C (**Figure 4.6**).This suggests the possibility of a favourable industrial process that occurs at room temperature (25°C) circumventing excessive energy costs.

The effect of pH has been shown to be very significant in the synthesis of both kestose and nystose. Using the 2^3 design, the highest production of total FOS was observed at pH 9 and 20 °C (**Table 4.5**) while a negligible concentration was observed under acidic conditions. Further investigations using the Box-Behnken design at lower pH (5 – 6) showed a marked increase in the production of both kestose and nystose (**Table 4.7**) as alkalinity increased. As proposed in section 4 of chapter 3, it seems more likely that in the range between pH 6 and 9 sufficient protonation of sucrose may occur to propagate the release of fructose while maintaining the competitive advantage of sucrose over water, thereby allowing the formation of more FOS than both extremely acidic and alkaline conditions (Ghazi *et al*., 2006; Lee *et al*., 1999).

Despite the apparently low significance of temperature as an independent factor in the synthesis of kestose, it has a more noticeable effect in its interactions. The quadratic model for the synthesis of nystose (**Equation 4.8**) describes not only temperature but more so its interaction with itself, as significant factors at 95 % confidence interval. The production of kestose within the constraints studied in this research is also governed in

part by a temperature-pH interaction at the 90 % confidence interval. It is thus safe to say that one cannot, at this stage discard temperature as a variable until a ~~"h~~ierarchy" of effects, based on investigations with factors not yet covered in this research, is established.

From the current study it is also apparent that the use of the Box-Behnken design yielded a more detailed representation of factor interactions involved in the synthesis of FOS. This may be attributed to the higher number of factor levels used and the consequently larger amount of runs performed; thus enabling the approximation of quadratic interactions (**Figure 4.6; Equation 4.8**). In our opinion, two level designs such as the 2^3 factorial design employed in this chapter, may rather be suited for the screening of insignificant factors prior to an in depth analysis.

A comparative analysis of the crude and purified FopAp could not be completed due to the low activity of FA in this investigation. This is probably due to the short half life of the purified enzyme (**Section 3.3 of chapter 3**). Evidence of minimal activity was observed in table 4.5, though the concentration of FOS produced was insufficient and too erratic to be statistically viable.

RSM has the potential disadvantage of being cumbersome due to the large amount of factors that one may have to test for significance. Nevertheless, it offers the greater advantage of being statistically viable while enabling the collection of large amounts of data (Chaibva, 2010). This study has analysed the synthesis of kestose and nystose by the enzyme based on the effects of temperature, pH and to a lesser extent, time. The information obtained will be integral in the continual process of developing a commercially applicable means of FOS synthesis from the enzyme FopAp. However, more factors are still to be investigated, before optimization and up-scaling can be carried out. These include enzyme concentration, substrate concentration and up-scale volume.

CHAPTER 5
OPTIMIZATION OF FOS SYNTHESIS USING ARTIFICIAL NEURAL NETWORKS

1. Introduction

According to Edgar (1996), the upsurge in digital technology, amplified by current thinking to "bring theory into practice", has led to the emergence and recognition of computer modelling applications as areas of great priority for the future. It is becoming even more widely accepted that quantitative mechanistic knowledge may be insufficient for analysis of bio-processes and may require supplementation with more modern, statistical, qualitative and fuzzy knowledge forms (Feyo de Azevedo *et al.,* 1997). More specifically, the limitations in the predictive ability of conventional mechanistic methods in process biochemistry are well known and are largely attributed to the intrinsic non-linear nature of cellular metabolic processes. The application of artificial neural networks (ANNs) in process biochemistry is thus an increasingly popular option for predictive optimization as they are thought to be superior due to their flexibility in representing dynamic multivariate systems (Otto, 1999; Chen *et al.,* 2000).

1.1. Analytical techniques

The concept of artificial neural networks and their applications has already been reviewed in section 6.2 of chapter one. However, for the purposes of this chapter, it is important to understand the principle by which "learning and training" in ANNs is carried out.

The building blocks of ANNs are process units, otherwise known as process elements (PEs). These units are arranged into layers in which each PE receives an input that is then weighted according to weighting factors and the resulting quantities are summed. A transfer function is then carried out on the weighted sum and the subsequent value passed onto the next processing element. The transfer function maps the possibly infinite summation of PE input to a predefined range, which is the output. The training phase of a network requires paired input-output data. The input is fed into the network, transferred through the network layers then ultimately; the network calculates a predicted output. A comparison is then made between the predicted output and the actual output as observed experimentally. Adjustments in the connection weights between PEs are then made so as to reduce the observed discrepancy, the degree and frequency of such adjustments over a series of runs being governed by "the learning rule", until a desired accuracy and precision are achieved. This principle is known as back propagation (Liu *et al*, 1999).

Provided that the experimental space has been sufficiently navigated by the input-output pairs that make up the training set, a neural network should be able to predict an output given a random combination of viable inputs and the converse should also be true. The quality of prediction may vary between different types of ANNs, depending on the arrangement of layers within each network as well as the direction of its connections (Feyo de Azevedo *et al*., 1997)

As described in section 6.2 of chapter 1, a variety of neural networks can be constructed and utilised for a myriad of functions. An ANN commonly used in biochemical research is the feed-forward network. A feed-forward network is characterized by a unidirectional set of connections which only allows the movement of signals from subordinate layers to those ahead of them as illustrated in figure 5.1 (Otto, 1999).

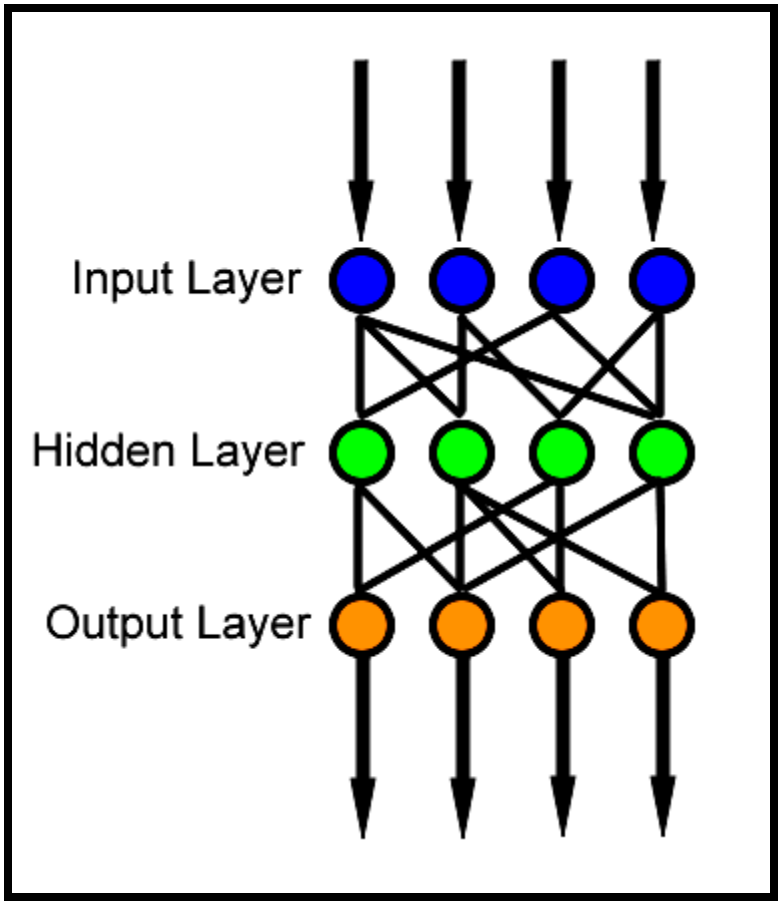


Figure 5.1: Schematic of a feed forward network

Different combinations of FOS of a defined chain length are required for a variety of uses in foodstuffs and neutraceutical products (Biedrzyka and Bielecka, 2004). Also, a minimal amount of residual sucrose is desirable if these products are to be suitable for diabetics, young children and the elderly. As one of the main aims of this research is to analyse and optimize the synthesis of FOS of a pre-defined length, the primary objective in this chapter was to find a relationship between the inputs and outputs of the FOS synthetic process, by construction and training of an ANN. The second objective was to introduce the use of the trained ANNs to predict conditions that may favour desirable amounts of the process outputs: kestose, nystose and residual sucrose. It should also be emphasised that the focus this chapter is not on the programming or mathematical aspect of ANNs but merely to highlight the potential of their application in the FOS synthetic process.

2. Methods and materials

2.1 Materials

Authentic 1-nystose and 1-kestose standards were purchased from Sigma-Aldrich (Germany). All bench reagents were of analytical grade and were obtained from either Merck Chemicals (South Africa) or Sigma-Aldrich (South Africa). Unless otherwise stated; all reagents were dissolved in Milli-Q water and all experimental work was performed at 0-4 °C. All samples were stored at 0-4 ºC overnight and -20 ºC over longer periods, where appropriate

Labnet Accublock™ Digital dry baths were obtained from White Scientific (South Africa). All HPLC in this chapter was carried with degassed and filtered HPLC grade solvents. Filtration and degassing were performed by a Milli-vac maxi™ vacuum with a 0.45 µm nylon filtration membrane from Millipore (South Africa). All data processing and mathematical programming was performed on Matlab, version R2010a (Math Works®, USA).

2.2 Data collection

Sucrose (1200 µl, 600mg/ml) in citrate-phosphate buffer (50 mM) were reacted with 300 µl of crude FopAp over a temperature range of 20-60 ºC, a pH range 3.85-9.85 and at reaction times ranging from 30-120 minutes, using the one-at-a-time factorial method. All reactions were carried out in triplicate. Reaction termination, sample preparation and HPLC were then carried out as described in previous chapters. All relevant data was collated and utilised as the training set for the ANN.

2.2.1Data processing

The dataset utilised comprised of 28 instances, with five input attributes and five output attributes. The input attributes are as follows:

1. Amount of sucrose (mg/ml).
2. Reaction temperature (ºC).
3. Reaction time (minutes).
4. pH
5. Enzyme concentration

As the concentration of sucrose as well as enzyme used in each experiment was constant, these input attributes were excluded during construction. Consequently, in the neural network, attributes 2-4 are the only inputs and together are used to describe the experimental space.

The output attributes are as follows:

1. Fructose(mM)
2. Glucose(mM)
3. Unreacted Sucrose (GF)(mM)
4. Kestose (GF2)(mM)
5. Nystose (GF3)(mM)

For the sake of simplicity, the products of interest were chosen as attributes 3 to 5. Attributes 1 and 2 were excluded as targets for the neural network since knowledge of

their optimal values was not a priority at this stage though they may be included in future work.

2.2.2 Descriptive statistics

All the attributes were characterized based on their statistical properties and correlations. The patterns of distribution were also noted and recorded. Tables 5.1 and 5.2 describe the statistical parameters of the dataset while figure 5.2 illustrates the distribution patterns of the output attributes throughout the 27 samples.

Table 5.1: Statistical analysis of process attributes (GF- sucrose, GF2- kestose, GF3- nystose)

	Temp (ºC)	Time (mins)	pH	GF (mM)	GF2 (mM)	GF3 (mM)
Min	20	30	3.85	5.88	2.14	5.98
Max	60	120	8.95	143.52	87.66	56.69
Mean	20	30	3.85	5.88	2.14	5.98
St Dev	10.37	35.98	1.425	27.00	17.74	12.93

Table 5.2: Correlation of input and output variables (GF- sucrose, GF2- kestose, GF3- nystose)

	Temp	Time	pH	GF	GF2	GF3
Temp	1	-0.1864	-0.11093	0.414429	0.065914	-0.08628
Time		1	0.456598	-0.08786	0.010926	0.117469
pH			1	-0.52017	-0.02278	0.387575
GF				1	-0.25749	-0.08452
GF2					1	0.040623
GF3						1

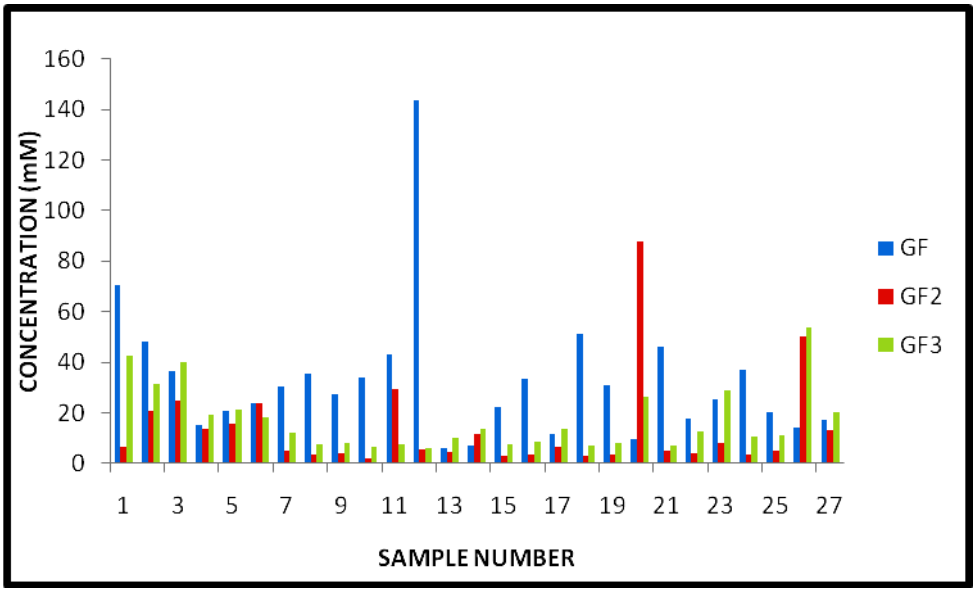


Figure 5.2: The relative distributions of GF, GF2 and GF3 throughout the 27 reactions

2.2.3 Encoding

The input/output patterns and targets were then encoded in their original form. All attributes were applied as real-values and without normalisation as the R2010a version of Matlab does so automatically using the ~~"m~~apminmax" function when the ~~"n~~ewff" function is called.

2.3 Training the Neural Network

Owing to the small dataset, it was not appropriate to divide the data up into training and test sets. Therefore the network was trained on 26 samples and tested on one sample. There was no need to use a validation set due to the minimal risk of over-training associated with small data sets. Usually too many output variables can confuse the neural network, resulting in poor training. Thus, three separate feed-forward neural networks were created, each being trained on one particular output attribute.

The stopping criterion specified was the maximum number of epochs for which the network may train. This number was decided upon using trial and error, by selecting certain values and watching the graph of the performance function (which is the mean squared error). The maximum number of epochs was chosen to be 1000, which is higher

than necessary, in order to make sure that the network received as much training as possible.

The training function plays a role in avoiding pre-mature termination of the learning process (i.e. before 1000 epochs have elapsed). In the current research, the training function ‘trainscg’ (the Scaled Conjugate Gradient back-propagation function in Matlab®) was used, as it results in the best performance on the test example.

The network architecture also plays a role in the performance of the neural network. The number of neurons in the first and second layers for the three neural networks was decided on by using three separate supervising scripts, which tested different combinations of numbers of neurons and selected the combination that resulted in the best performance of the network on the test sample. For the first network, which trained only on the GF data, 20 neurons in layer 1 and 14 neurons in layer 2 were chosen; for the network that is trained only on the GF2 data, 16 neurons in layer 1 and 11 neurons in layer 2 were chosen; and for the network which trains only on the GF3 data, 20 neurons in layer 1 and 12 neurons in layer 2 were chosen All scripts used the Matlab® transfer functions ‘tansig’, ‘logsig’ and ‘purelin’ for the input, first and second layers respectively.

2.4 Testing the Neural Network

A separate testing script was written to test the performance of the three neural networks after training. The test script began by using the neural network trained in the training script to simulate on the training and test data. Two measures of assessment for the neural network were used, both of which were calculated using the neural network toolkit in Matlab®. The first, an R-squared statistic on all the data (training set plus the test sample) and the second, a variable called the ‘dtest’, which measures the distance between the target output (the actual output for the test sample) and the activation output (the output which the network estimates for the test sample). A desirable activation being as close as possible to the target, and thus a low dtest value indicates a good fit. Visual representation of the data was also used to assess the performance of the network, by

plotting the activation and target values for all the data on one graph, using circles for the targets and stars for the activations (**Appendix C 1**). After each of the10 training runs, each of the neural networks underwent testing on all 27 data instances. Appropriate adjustments in weights and dedicated neurons were made after each run.

2.5 Simulating FOS synthesis on the Network

After satisfactory training of the neural networks (i.e. ANNs have learned the relationships between the input conditions and the outputs GF, GF2 and GF3), they were used in simulation scripts programmed to optimize GF, GF2 and GF3 and report back on the input conditions which achieve optimal values. Three simulation scripts were written. The first, aimed at minimising GF, the second to maximise GF2 and the third to maximise GF3. For these scripts a ―brute force" method was used. This technique creates a multi-dimensional array with many different output values associated with varying input values (Burton, 2010). The maximum (or minimum) output value is found in the array and the corresponding input values are then recovered. A fourth simulation script was written to find optimal conditions for the ratios GF: GF3 and GF2:GF3. The objective was to find the conditions that minimise the first ratio (such that a high GF3 output coincides with a low GF output) and maximises the second ratio (such that high GF2 output coincides with high GF3 output).

3. Results

3.1 Training the neural network

The randomised nature of this process is reflected in the variation among the results. Consequently, many training runs had to be performed for each of the three neural networks, before satisfactory results were obtained. Once established, excellent networks were saved for future use for any simulations, and they performed very well. Figures 5.3-5.8 and tables 5.3-5.5 show the training results for each of the three scripts. ANN 1, ANN 2 and ANN 3 where trained only on GF, GF2 and GF3 output data, respectively.

Table 5.3: Result summary for ANN 1 (GF) training, n=10

Average	602	0.86425	37.39925
Max	928	0.9999	98.5727
Min	472	0.4875	1.1057
Median	495	0.94805	27.90175
St Dev	183.3194	0.173997	36.12674
	# of epochs	**R-squared statistic**	**dtest**

Figure 5.3: R-squared statistics for ANN 1 (GF) over 10 training runs

Figure 5.4: The d-test values for ANN1 (GF) over 10 training runs

Figure 5.3 demonstrates that the best run had an R-squared statistic (on the training set and test example) of 0.9999. Training stopped after 494 epochs owing to the minimum

gradient criterion, and dtest was 1.1057 (**Figure 5.4**). The network that produced this run was saved as –goodnet1" and used in all future simulations.

Table 5.4: Result summary for ANN 2 (GF2) training, n=10

Average	557.3	0.96785	13.73433
Max	644	0.9997	31.1166
Min	336	0.8817	1.4971
Median	585	0.98895	10.6546
St Dev	99.07803	0.039446	9.686341
	# of epochs	**R-squared statistic**	**Dtest**

Figure 5.5: R- squared statistics for ANN2 (GF2) over ten training runs

Figure 5.6: dtest values for ANN2 (GF2) over10 training runs

The best run in ANN2 had an R-squared statistic (on the training set and test example) of 0.9997 (**Figure 5.5**). Training stopped after 584 epochs owing to the minimum gradient

criterion, and dtest was 1.4971 (**Figure 5.6**). The network that produced this run was saved as –goodnet2" and used in all future simulations.

Table 5.5: Result summary for ANN3 (GF3 training), n= 10

Average	**521.2**	**0.88099**	**19.23033**
Max	**761**	**1**	**35.9848**
Min	**425**	**0.7025**	**0.015**
Median	**494.5**	**0.8946**	**20.63915**
St Dev	**100.3215**	**0.109586**	**12.83209**
	# of epochs	**R-squared statistic**	**dtest**

As illustrated in figure 5.7, the best run had an R-squared statistic (on the training set and test example) of 1. Training stopped after 564 epochs owing to the minimum gradient criterion, and dtest was 0.015 (**Figure 5.8**). The network that produced this run was saved as –goodnet3" and used in all future simulations.

Figure.5.7: R-squared statistics for ANN3 (GF3) over 10 training runs

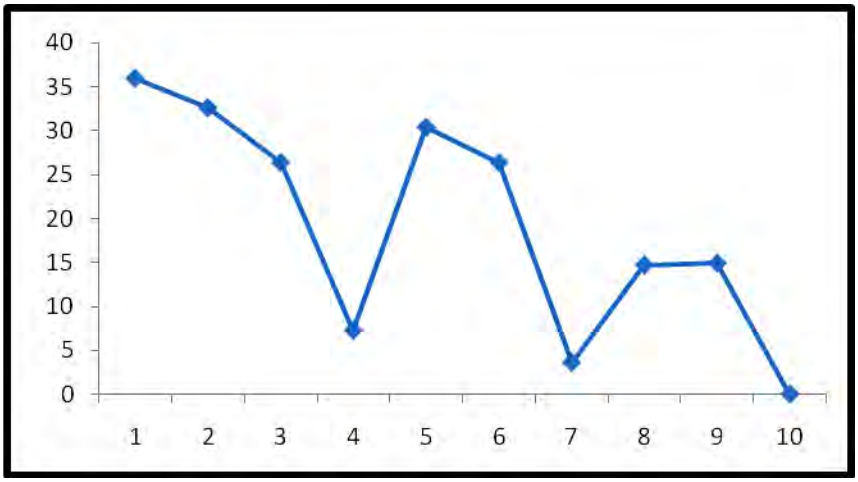


Figure 5.8: dtest values for ANN3 (GF3) over 10 training runs

3.2 Simulation Results:

The trained neural networks were used to simulate desirable values and ratios of their allocated process outputs as instructed by written simulation scripts. The networks were also programmed to give feedback on the corresponding conditions for obtained output values.

ANN1:

The first simulation script (sugars_sim1.m) used goodnet1.mat (the good neural network found during training) to find the maximum possible value of 1/GF (i.e. the minimum GF value), and retrieved the appropriate input conditions (temperature, time and pH) which resulted in the optimum. The results obtained were as follows:

Best (lowest) amount of GF: 0.05 mM
Corresponding to
Temperature: 40 °C
Time: 63.78 mins
pH: 3.67

ANN 2:

The second simulation script (sugars_sim2.m) used goodnet2.mat to find the maximum possible value of GF2, as described for ANN 1. The results obtained were as follows:

Best (highest) amount of GF2: 178.17 mM
Corresponding to:

Temperature: 60 ºC
Time: 111.11 mins
pH: 9.44

ANN 3:

The third simulation script (sugars_sim3.m) used goodnet3.mat to find the maximum possible value of GF3, as described in the previous instances. The results obtain were as follows:

Best (highest) amount of GF3: 88.83 mM
Corresponding to
Temperature: 20 ºC
Time: 54.44 mins
pH: 9.11

*Maximising Ratios***:**

The ratios simulation script (sugars_sim_ratios.m) used all three good networks (i.e. goodnet1.mat, goodnet2.mat and goodnet3.mat) to maximise the ratios GF3:GF2 and GF: GF3, and retrieved those input conditions (temperature, time and pH) which resulted in these optima. The results obtained were**:**

The maximum ratio **GF3:GF2** is **1:2.63**
Corresponding to
Temperature: 25 ºC,
Time: 50.00 mins
pH = 9.00

The maximum ratio **GF: GF3** is **1:1718.54**
Corresponding to
Temperature: 20 ºC,
Time: 57.78 mins
pH: 9.44

The results of optimisation are compared graphically in figures 5.9-5.11:

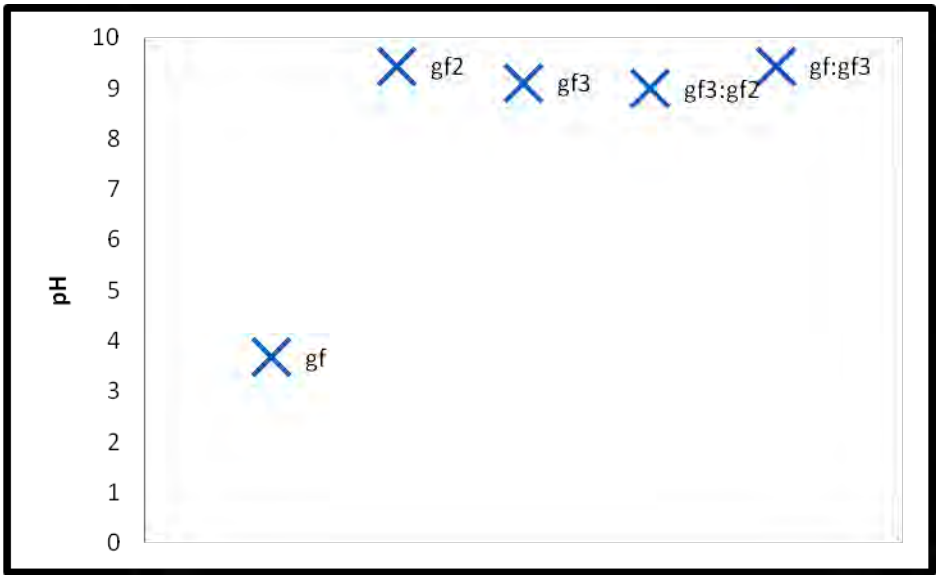


Figure 5.9: Optimal pH values for best GF, GF2, GF3, (GF3:GF2) ratio and (GF: GF3) ratio

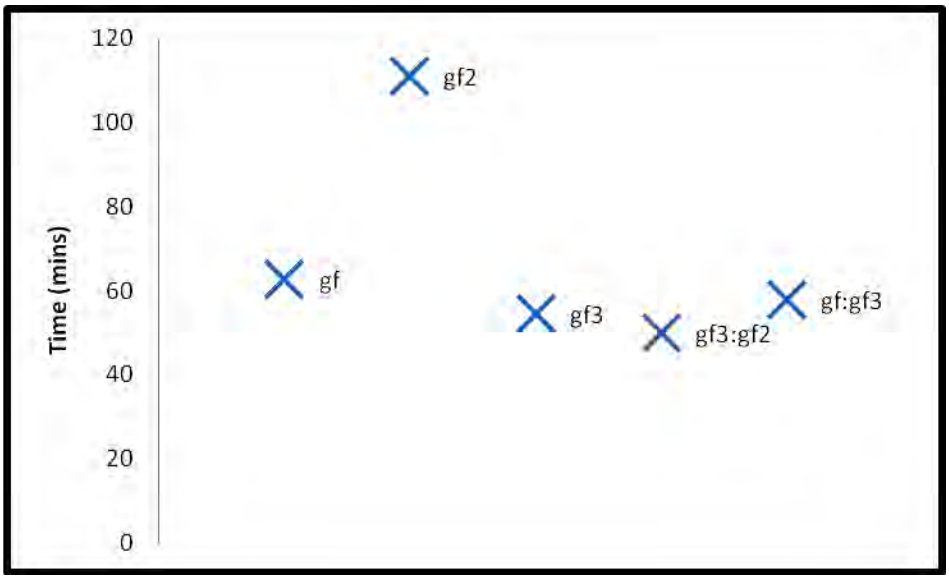


Figure 5.10: Optimal reaction times for best GF, GF2, GF3, (GF3:GF2) ratio and (GF: GF3) ratio

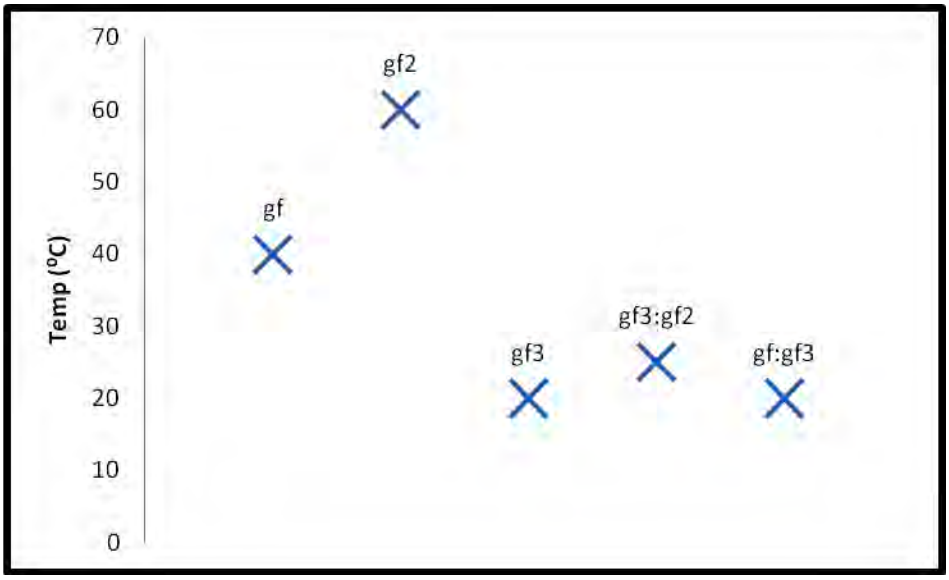


Figure 5.11: Optimal temperature values for best GF, GF2, GF3, (GF3:GF2) ratio and (GF: GF3 ratio)

4. Discussion and conclusions

The biggest challenge in creating the neural networks used in this research was the task of training them adequately on such a small dataset. However, we were finally able to find networks which had trained well on the training data and performed very well on the testing. These were saved as the ―good nets" and were considered satisfactory in their prediction ability and thus their suitability for use in the simulation scripts. In fact, the trained neural networks in this study had very little difficulty in reproducing information from the domain of the training set. The learning capacity of the ―good nets" is comparable, with regard to predictive precision, to more complex networks used in biochemical applications as described by Feyo De Azevedo *et al*. (1997) and Baş and Biyaci. (2007). It may be noteworthy that, although they can predict the output values for new input patterns with a degree of certainty, the neural networks have only been trained on 26 examples; therefore it is currently impossible for them to know how the outputs will be distributed for inputs which they have not been exposed to. Thus it is most likely that the neural networks' prediction ability would improve significantly if one was to obtain more data and retrain the network on a larger dataset.

The neural network approach sheds much needed insight on the subject of optimizing the synthesis of FOS and also serves as an auxiliary confirmation to previously investigated methods in this research. On considering figure 5.9, it is apparent that the desired outputs are mainly optimized around pH 9, the only exception being GF (residual sucrose), which is minimised at a pH of 3.67. This is congruent with findings from chapter 3, as supported by the proposed mechanism of retaining invertases (Ghazi, 2006). These findings also highlight the fact that attaining a universal optimum for the desirable variable levels may be more difficult a task than expected.

Largely due to health concerns, the reduction of residual sucrose within the final reaction products is as pivotal to the commercial application of FOS as the optimization of the dietary fibres themselves (Lipski *et al*, 2010). It is then somewhat puzzling, as to what means can be employed to secure these two variables at desirable levels within the same reaction. However, it is also visible that GF3 is optimized at a time of around 60 minutes, except for GF2, which is apparently optimized at 111.11 minutes (**Figure 5.10**). This difference in optimal reaction time for the different FOS species may be, as previously suggested, ideal for industrial production of tailor-made FOS preparations. It is important to explain that, the variables whose optima were investigated in this study, despite being crucial to the commercial synthesis of FOS, are not the only significant output attributes but were chosen particularly for the sake of simplicity.

Based on observations made by Liu *et al.* (1999) and Baş and Biyaci (2007), the incorporation of RSM generated data to the current neural networks, may be a powerful modification, adding both volume to the training data set as well as adequate navigation of the experimental space. However, the exclusive use of feed forward networks as predictive tools has been criticized by Feyo De Azevedo *et al.* (*1997*), due to their ~~–s~~tatic" learning mechanism. Although this is not evident in our research, it may be considered in future research, to employ more dynamic networks such as partially recurrent networks in order to have a more accurate model of the bio-synthetic process.

It is arguable as to whether or not the predicted optima will be sustained if a larger data set is utilized to retrain the neural networks. As pertinent as this concern may be, it can by no means undermine the capability of the "good nets" in navigating the current experimental domain, as confirmed by the R-squared statistic, dtest and consequent agreement with previous methods in this research. In fact, given the current lack of information on the subject within the public domain, the findings of this research may well be sure steps towards the development and application of functional artificial intelligence in commercial FOS synthesis and bio-synthetic processes in general.

CHAPTER 6
GENERAL DISCUSSION, CONCLUSIONS AND FUTURE RECOMMENDATIONS

Advances in the knowledge of the relationship between nutrition and health, augmented by the need for an improved quality of life across all age groups, have seen the emergence of functional foods and neutraceuticals as both essential dietary constituents as well as potentially lucrative commodities (Sirő *et al.,* 2008). The production of food constituents such as FOS at a commercial scale not only has the potential to provide much needed nourishment and reduce the incidence of lifestyle associated diseases, but also offers a means of "greener" energy in an integrated industrial system with bio-ethanol production (Bekers *et al.,* 2008; Sangeetha *et al.,* 2005; Sirő *et al.,* 2008). Research into the commercialisation of FOS synthesis as well as organisms involved in it, is therefore an imperative investment for governments and concerned organisations in the developing world, where raw materials are readily available and dire need is apparent.

The main aim of this research was to investigate the synthesis of FOS by a β-fructofuranosidase from *Aspergillus niger* ATCC20611, as a foundation towards the development of an efficient commercial bio-process. The purification of the crude enzyme was carried out successfully (**Table 2.2**) to yield two fractions, FA and FB, which both exhibited transfructosylase activity (**Figures 2.4 and 2.5**). SDS PAGE analysis of fractions revealed the presence of two proteins, concluded to be a β-fructofuranosidase and a hydrolase; exhibiting molecular weights of 112 kDa and 78 kDa, respectively (**Figure 2.6**). The homogeneity of FA, which contained the β-fructofuranosidase, was confirmed by size exclusion chromatography on a Sephacryl S-300 HR column as well as native PAGE, exhibiting a native molecular weight of 287 kDa (**Figure 2.7**). These findings are typical of FOS producing enzymes as investigated by Hirayama *et al.* (1989), Nguyen *et al.* (2005) and Zuccaro *et al.* (2008), though the occurrence of predominantly hydrolase activity in FB is thought to be a result of co-expression of an invertase from *Pichia pastoris*. Substrate kinetic studies on both fractions later confirmed the higher hydrolytic capacity and substrate affinity of the proteins in FB compared to that in FA (**Table 3.1**). It is strongly suggested that in future work, FopA-gene-free *P.pastoris* should be used as a negative control to ascertain the presence of native invertases and determine the extent of background activity.

Investigations on the effect of temperature and pH on both FA and FB yielded optima of 20 ºC, pH 9; and 60 ºC; pH 5, (**Figures 3.1, 3.3 3.5 and 3.7**) for transfructosylase and hydrolase activities, respectively. Most FOS producing enzymes discussed in the literature, exhibit temperature optima between 40 ºC and 60 ºC; and pH optima in the range 5 – 6 (Sangeetha *et al.,* 2005). These results are in agreement with results obtained from investigations conducted on the effect of temperature and pH on the total activities of the proteins in FA and FB (**Figures 3.2, 3.4, 3.6 and 3.8**). Due to this discrepancy between the various activities, it is of great concern to us that characterization studies based on total activity may not be sufficient determinants of the FOS synthesing potential of candidate enzymes. Ghazi *et al.* (2006) and Kurakake *et al*. (2008) have highlighted the significance of a high transfructosylase: hydrolase ratio with regards to FOS production. This, supported by the proposed mechanism of fructofuranosidase catalysis, as outlined by Chen *et al*. (2009), fortifies the need to investigate the individual activities of FOS producing enzymes if actual optima are to be ultimately achieved.

Response surface methodology, through experimental design, allowed the effect of the above described conditions to be simultaneously investigated and quantified. pH was found to have a profound effect on both kestose and nystose concentrations over a wide range of temperature values (**Equation 4.5 and 4.6**). An interesting quadratic interaction of temperature was observed between pH 5 and 6. This effect was linked to the concentration of nystose under these conditions at a 95 % confidence interval. Optimal regions were obtained based on 2^3 factorial, as well as Box-Behnken designs. By elimination of unfavourable conditions, it was thus proposed that the highest yields of FOS (kestose and nystose), may be obtained in the temperature range 20 ºC – 25 ºC, and pH range 6 - 9 (**Figures 4.3 and 4.5**). However, it is important that more investigations be carried out within these constraints, before any final conclusions are drawn.

An important observation made throughout this research was the higher feasibility of the use of crude enzyme to produce FOS on a large scale. As shown in preliminary HPLC analysis (**Figures 2.4 and 2.5**) and response surface analysis (**Figures 4.4 and 4.5**), larger concentrations of both kestose and nystose can be observed from the crude enzyme

compared to FA, after an hour of reaction. This could possibly serve as evidence of synergy between the β-fructofuranosidase and hydrolase components of the crude enzyme, though further work must be carried out to maintain this hypothesis. In addition, thermal stability studies indicated high instability at optimal temperature and pH, with a half life of 1.5 hrs for the pure β-fructofuranosidase (**Figure 3.9**). This loss of stability on purification is supported by observations made by Mutanda (2007) on a fructosyltransferase from *Aspergillus niger* and, in our opinion, strongly motivates for the exclusive use of crude FopAp in future attempts at synthesis.

Previous studies have utilized the principles of experimental design and response surface methodology in the study of FOS. A central composite design has been used to optimize the synthesis of FOS by fructosyltransferases from *A.niger* and *A.aculeatus* (Mutanda *et al.,* 2008; Nemukula *et al.,* 2009). Santos and Maugeri (2007) have also utilized the technique as a method of analysis in the synthesis of FOS from sucrose, by an inulinase from *Kluyveromyces marxianus*. Response surface methods are able to graphically describe the experimental domain as a function of its most significant variables. In the current research, the temperature, time, and pH were chosen initially though only the former two were deemed significant and fitted to a mathematical model (**Appendix B**). It is suggested that any reduction of FOS synthesis with time, in the constraints provided, may well be a sign of inhibition by glucose (Ghazi *et al.,* 2006; Santos and Maugeri, 2007).

Very little work has been done on the application of artificial neural networks in FOS synthesis. Attempts at using ANNs to ~~"m~~map out" the experimental domain of, and optimize pharmaceutical formulations have been carried out by Chaibva (2010), with limited success. A large data set of inputs from which to train an ANN is essential, if forecasts outside of a training domain are to be made (Burton, 2010). In this research, a small set of training data was produced using the ~~"o~~ne at a time" factorial technique. In retrospect, this was not particularly ideal because it limited the variety of factor combinations that could be taught to the network and by so doing, also reduced the number of training samples available to it. It was thus very difficult to train the ANNs to

a satisfactory level of competence. However, a few good networks that showed excellent correlation with the training set were finally discovered, but even these could only navigate the process within the training domain (**Figures 5.3-5.7**).

In our opinion, the use of mathematical modelling and artificial intelligence in the analysis and optimisation of a bio-process, revealed technical advantages and disadvantages of both techniques. RSM provides a large source of statistically viable data, as well as much needed insight on process intricacies that conventional methods can not show. It can however, prove to be cumbersome and entails a series of calculated trial and error operations before accurate predictions of optima can be made. Artificial neural networks can readily assimilate data and, with relatively little effort, can predict optima with considerable accuracy. ANNs however suffer due to their mechanised manner of learning. They use simple mathematical functions to identify patterns and relationships without process knowledge. This at times results in the prediction of optima that are not scientifically possible (Feyo de Azevedo *et al.*, 1997). It is therefore one of our future recommendations that an integrated approach comprising of a dataset determined and validated by response surface methodology, be incorporated into a less ~~"~~static" neural network. This may aid to circumvent the short comings of both techniques.

In the near future, it may be necessary to use a 2^5 screening design and incorporate additional factors such as enzyme and sucrose concentration as well as those used in this study. This, albeit cumbersome, may yield a more complete picture of process variables before optimisation steps are taken.

References

Agblevor, F.A.; Murden, A. and Hames, B.R. (2004), Improved method of analysis of biomass sugars using high-performance liquid chromatography, *Biotechnology Letters,* 26 ,15, 1207-1211.

Ahmed, H. 2005, *Principles of reactions of protein extraction, purification and characterisation,* CRC press*,* Florida*, USA.*

Alberto, F.; Bignon, C.; Sulzenbacher, G.; Henrissat, B. and Czjzek, M. (2004), The three-dimensional structure of invertase (beta-fructosidase) from *Thermotoga maritima* reveals a bimodular arrangement and an evolutionary relationship between retaining and inverting glycosidases, The *Journal of Biological Chemistry,* 279, 18, 18903-18910.

Amersham Biosciences, 1999, *Protein purification (handbook),* Amersham Pharmacia Biotech, Upssala, Sweden.

Barreteau, H.; Delattre, C. and Michaud, P. (2006), Production of oligosaccharides as a promising new food additive generation, *Food Technology and Biotechnology,* 44, 3, 323-333.

Baş, D. and Boyaci, I. H. (2007), Modeling and optimization II: comparison of estimation capabilities of response surface methodology with artificial neural networks in a biochemical reaction, *Journal of Food Engineering* 78, 846–854.

Becker, R. and Lorenz, K. (1978), Saccharides in proso and foxtail millets, *Journal of Food Science,* 43, 5, 1412-1414.

Belcarz, A.; Ginalska, G.; Lobarzewski, J. and Penel, C. (2002), The novel non 16 glycosylated invertase from *Candida utilis* the properties and the conditions of 17 production and purification, *Biochemistry and Biophysics Acta*, 1594, 40-53.

Beevers, C.A.; McDonald, T.R.R.; Robertson, J.H. and Stern, F. (1952), The crystal structure of sucrose, *Acta Crystallographica,* 5, 5, 689-690.

Beine, R.; Moraru, R.; Nimtz, M.; Na'amnieh, S.; Pawlowski, A.; Buchholz, K. and Seibel, J. (2008), Synthesis of novel fructooligosaccharides by substrate and enzyme engineering, *Journal of Biotechnology,* 138, 1-2, 33-41.

Bekers, M.; Upite, D.; Kaminska, E.; Danilevics, A.; Linde, R.; Grube, M. and Viesturs, U. (2008), Production of bio-ethanol and fructans from semi-products of sugar beet processing using *Zymomonas mobilis*, *Proceedings of the UEAA General Assembly and the Associated Workshop: Renewable Energy Resources, Production and* Technologies, Latvia, 28-31 May 2008.

Biedendieck, R.; Beine, R.; Gamer, M.; Jordan, E.; Buchholz, K.; Seibel, J.; Dijkhuizen, L.; Malten, M. and Jahn, D. (2007), Export, purification, and activities of affinity tagged *Lactobacillus reuteri* levansucrase produced by *Bacillus megaterium* , *Applied Microbiology and Biotechnology,* 74, 5, 1062-1073.

Biedrzyka, E. and Bielecka, M. (2004), Prebiotic effectiveness of fructans of different degrees of polymerization, *Trends in Food Science and Technology,* 15, 3-4, 170 - 175.

Bieniawska, Z.; Paul Barratt, D.H.; Garlick, A.P.; Thole, V.; Kruger, N.J.; Martin, C.; Zrenner, R. and Smith, A.M. (2007), Analysis of the sucrose synthase gene family in *Arabidopsis*, *The Plant Journal,* 49, 5, 810-828.

Bishop, C.M. (2006), *Pattern recognition and machine learning,* Springer, New York.

Boehm, G.; Lidestri, M.; Casetta, P.; Jelinek, J.; Negretti, F.; Stahl, B. and Marini, A. (2002), Supplementation of a bovine milk formula with an oligosaccharide mixture increases counts of faecal bifidobacteria in preterm infants, *Arch. Dis. Child. Fetal Neonatal Ed*., 86, 178-181.

Bornet, F.R.J. and Brouns, F. (2002), Immune-stimulating and gut health-promoting properties of short-chain fructo-oligosaccharides, *Nutrition reviews,* 60, 10, 326 - 334.

Borromei, C.; Careri, M.; Cavazza, A.; Corradini, C.; Elviri, L.; Mangia, A. and Merusi, C. (2009a), Evaluation of fructooligosaccharides and inulins as potentially health benefiting food Ingredients by HPAEC-PED and MALDI-TOF MS , *International Journal of Analytical Chemistry,* 2009, 1 -10.

Borromei, C.; Cavazza, A.; Merusi, C. and Corradini, C. (2009b), Characterization and quantitation of short-chain fructooligosaccharides and inulooligosaccharides in fermented milks by high-performance anion-exchange chromatography with pulsed amperometric detection, *Journal of Separation Science,* 32, 21, 3635- 3642.

Brueil, C. and Sadler, J.N. (1985), Limitations of using the D-glucose oxidase peroxidase method for measuring glucose derived from lignocellulosic substrates, *Biotechnology Letters*, 7, 3, 191-196.

Bruggink, C.; Wuhrer, M.; Koeleman, C.A.; Barreto, V.; Liu, Y.; Pohl, C.; Ingendoh, A.; Hokke, C.H. and Deelder, A.M. (2005), Oligosaccharide analysis by capillary-scale high-pH anion-exchange chromatography with on-line ion-trap mass spectrometry, *Journal of chromatography, Analytical technologies in the biomedical and life sciences,* 829, 1-2, 136-143.

Buddington, R.; Williams, C.; Chen, S. and Witherly, S. (1996), Dietary supplement of neosugar alters the fecal flora and decreases activities of some reductive enzymes in human subjects, *American Journal of Clinical Nutrition,* 63, 5, 709-716.

Buratti, S.; Brunetti, B. and Mannino, S. (2008), Amperometric detection of carbohydrates and thiols by using a glassy carbon electrode coated with Co oxide/multi-wall carbon nanotubes catalytic system, *Talanta,* 76, 2, 454-457.

Burgess, R.R. (2009), Protein precipitation techniques, *Methods in Enzymology,* 463, 331-342.

Burton, M (2010),Neural Networks Course Notes, Department of Mathematics, Rhodes University, *Personal Communications.*

Calsa, T.Jr and Figueira, A. (2007), Serial analysis of gene expression in sugarcane (*Saccharum spp*.) leaves revealed alternative C4 metabolism and putative antisense transcripts, *Plant Molecular Biology,* 63, 6, 745-762.

Campa, C.; Coslovi, A.; Flamigni, A. and Rossi, M. (2006),Overview on advances in capillary electrophoresis-mass spectrometry of carbohydrates: A tabulated review, *Electrophoresis,* 27, 11, 2027- 2050.

Campbell, J.M.; Bauer, L.L.; Fahey, G.C.; Hogarth, A.J.C.L.; Wolf, B.W. and Hunter, D.E. (1997a), Selected fructooligosaccharide (1-kestose, nystose, and 1F-ß-fructofuranosylnystose) composition of foods and feeds, *Journal of Agricultural and Food Chemistry,* 45, 8, 3076 -3082.

Cardoso, J.M.P. and Bolini, H.M.A. (2007), Different sweeteners in peach nectar: ideal and equivalent sweeteners, *Food Research International,* 40, 10, 1249-1253.

Carley, K.M.; Kamneva, N.Y. and Reminga, J. (2004), *Response surface methodology*, CASOS - Center for Computational Analysis of Social and Organizational Systems, Carnegie Mellon University, USA

Catala, I.; Butel, M.J.; Bensaada, M.; Popot, F.; Tessedre, A.C.; Rimbault, A. and Szylit, O. (1999), Oligofructose contributes to the protective role of bifidobacteria in experimental necrotising enterocolitis in quails, *Journal of Medical Microbiology,* 48, 1, 89-94.

Chaibva,F.A. (2010), *The use of response surface methodology and artificial neural networks for the establishment of a design space for a sustained release salbutamol sulphate formulation,* PhD edn Rhodes University, Grahamstown.

Chen, T.; Huang, Y.; Yang, C.; Yang, C.; Wang, A. and Sung, H. (2009), Insights into the catalytic properties of bamboo vacuolar invertase through mutational analysis of active site residues, *Phytochemistry,* 70, 25-31.

Cheng, C. Tsai, H.R. and Chang, K.C. (2006), On-line cut-off technique and organic modifier addition aided signal enhancement for trace analysis of carbohydrates in cellulase hydrolysate by ion exclusion chromatography-electrospray ionization mass spectrometry, *Journal of chromatography.A,* 1119, 1-2, 188-196.

Collins, F.W. and Chandorkar, K.R. (1971), Thin-layer chromatography of fructo-oligosaccharides , *Journal of Chromatography A,* 56, 163- 167.

Contreras, N.I.; Fairely, P.; McClements, D.J. and Povey, M.J.W. (1992), Analysis of the sugar content of fruit juices and drinks using ultrasonic velocity measurements, *International Journal of Food Science and Technology,* 27, 5, 515-529.

Corradini, C.; Bianchi, F.; Matteuzzi, D. Amoretti, A. Rossi, M.and Zanoni, S. (2004), High-performance anion-exchange chromatography coupled with pulsed amperometric detection and capillary zone electrophoresis with indirect ultra violet detection as powerful tools to evaluate prebiotic properties of fructooligosaccharides and inulin, *Journal of Chromatography A,* 1054, 1-2, 165- 173.

Coudray, C.; Demigne, C. and Rayssiguier, Y. (2003), Effects of Dietary Fibers on Magnesium Absorption in Animals and Humans, *Journal of Nutrition,* 133, 1, 1-4.

Coussement, P.A.A. (1999), Inulin and oligofructose: safe intakes and legal status, *Journal of Nutrition,* 129, 7, 1412.

Crittenden, R.G. and Playne, M.J. (1996), Production, properties and applications of food-grade oligosaccharides, *Trends in Food Science and Technology,* 7, 11, 353-361.

De Bruyn, A.; Alvarez, A.P.; Sandra, P. and De Leenheer, L. (1992), Isolation and identification of O-beta-D-fructofuranosyl-(2-->1)-O-beta-D-fructofuranosyl-(2-->1)-D-fru ctose, a product of the enzymic hydrolysis of the inulin from *Cichorium intybus*, *Carbohydrate research,* 235, 303-308.

Díaz, E.G. Catana, R. Ferreira, B.S. Luque, S. Fernandes, P. and Cabral, J.M.S. (2006), Towards the development of a membrane reactor for enzymatic inulin hydrolysis, *Journal of Membrane Science,* 273, 1-2, 152-158.

Downes, K. and Terry, L.A. (2010), A new acetonitrile-free mobile phase method for LC-ELSD quantification of fructooligosaccharides in onion (*Allium cepa L.*), *Talanta,* 82, 1, 118-124.

Draper, N.R. and Lin, D.K. J. 1996, *Response surface designs,Design and analysis of experiments*, Handbook of statistics, North-Holland, Amsterdam.

Duus, J.Ø.; Gotfredsen, C.H. and Bock, K. (2000), Carbohydrate structural determination by NMR spectroscopy: modern methods and limitations, *Chemical Reviews,* 100, 12, 4589-4614.

Edgar, T.F. (1996), Modeling and control - back to the future, part L, *CAST Communications* 19, 1, 7-12.

Ernst, R. and Ardilli, J. (1972), Enzymatic quantitative determination of hexoses, singly and in mixtures with their oligosaccharides, *New Phytologist,* 71, 2, 307-315.

Fanning, K.M. and Cogger, K.O. (1998), Neural network detection of management fraud using published financial data, *Intelligent Systems in Accounting, Finance and Management,* 7, 1, 21-41.

Fernandez, R.C.; Ottoni, C.A.; da Silva, E.S.; Matsubara, R.M.; Carter, J.M.; Magossi, L.R.; Wada, M.A.; de Andrade Rodrigues, M.F.; Maresma, B.G. and Maiorano, A.E. (2007), Screening of beta-fructofuranosidase-producing microorganisms and effect of pH and temperature on enzymatic rate, *Applied Microbiology and Biotechnology,* 75, 1, 87-93.

Feyo de Azevedo, S.; Dahm, B. and Oliveira, F. R. (1997), Hybrid modeling of biochemical processes comparison with the conventional approach, *Computers Chemical Engineering,* 21, 751-756.

Fooks, L.J. and Gibson, G.R. (2002), Probiotics as modulators of the gut flora, *The British Journal of Nutrition,* 88, 39-49.

Franck, A. (2002), Technological functionality of inulin and oligofructose, *British Journal of Nutrition,* 87, S2, S287.

Gerbens-Leenes, P.W. and Hoekstra, A.Y. (2009), The water footprint of sweeteners and bio-ethanol from sugar cane, sugar beet and maize, *Value of Water Research Report Series*, 38, UNESCO-IHE, University of Twente, Enschede, The Netherlands.

Ghasemi, M.F.; Shodjai-Arani, A. and Mazami, N. (2008), Optimization of bactereriorhodospin production by *Halobacterium salinarium* PTCC 1685, *Process Biochemistry,* 43, 10, 1077-1082.

Ghazi, I.; Fernandez-Arrojo, L.; Gomez De Segura, A.; Alcalde, M.; Plou, F.J. and Ballesteros, A. (2006), Beet sugar syrup and molasses as low-cost feedstock for the enzymatic production of fructo-oligosaccharides, *Journal of Agricultural and Food Chemistry,* 54, 8, 2964-2968.

Gibson, G.R.; Probert, H.M.; Loo, J.V.; Rastall, R.A. and Roberfroid, M.B. (2004), Dietary modulation of the human colonic microbiota: updating the concept of prebiotics, *Nutrition Research Reviews,* 17, 2, 259-275.

Gibson, G.R.; Beatty, E.R.; Wang, X. and Cummings, J.H. (1995), Selective stimulation of bifidobacteria in the human colon by oligofructose and inulin, *Gastroenterology,* 108, 4, 975-982.

Gibson, G.R.; Scott, K.P.; Rastall, R.A.; Tuohy, K.M.; Hotchkiss, A.; Dubert-Ferrandon, A.; Gareau, M.; Murphy, E.F.; Saulnier, D.; Loh, G.; Macfarlane, S.; Delzenne, N., Ringel, Y.; Kozianowski, G.; Dickmann, R.; Lenoir-Wijnkoop, I.; Walker, C. and Buddington, R. (2010), Dietary prebiotics: current status and new definition , *Food Science and Technology Bulletin: Functional Foods,* 7, 1, 1-19.

Gibson, P.R.; Newnham, E.; Barrett, J.S.; Shepherd, S.J. and Muir, J.G. (2006), Review article: fructose malabsorption and the bigger picture, *Alimentary Pharmacology & Therapeutics,* 25, 4, 349 -363.

Giokas, D.L. and Vlessidis, A.G. (2006), Application of a novel chemometric approach to the determination of aqueous photolysis rates of organic compounds in natural waters, *Talanta,* DOI: 10. 1016/j.talanta.2006.03.060.

Goggin, D.E. and Setter, T.L. (2004), Fructosyltransferase activity and fructan accumulation during development in wheat exposed to terminal drought, *Functional Plant Biology,* 31, 1, 11.

Goosen, C.; Yuan, X.L.; van Munster, J.M.; Ram, A.F.; Van der Maarel, M.J. and Dijkhuizen, L. (2007), Molecular and biochemical characterization of a novel intracellular invertase from *Aspergillus niger* with transfructosylating activity , *Eukaryotic Cell,* 6, 4, 674-681.

Goulas, A.; Tzortzis, G. and Gibson, G.R. (2007), Development of a process for the production and purification of α- and β-galactooligosaccharides from *Bifidobacterium bifidum* NCIMB 41171, *International Dairy Journal,* 17, 6, 648-656.

Grizard, D. and Barthomeuf, C. (1999), Non-digestible oligosaccharides used as prebiotic agents: mode of production and beneficial effects on animal and human health, *Reproduction, Nutrition, Development,* 39, 5-6, 563-588.

Guignard, C.; Jouve, L.; Bogeat-Triboulot, M.B.; Dreyer, E.; Hausman, J.F. and Hoffmann, L. (2005), Analysis of carbohydrates in plants by high-performance anion-exchange chromatography coupled with electrospray mass spectrometry, *Journal of Chromatography.A,* 1085, 1, 137-142.

Guimara˜es, L.S.; Somera, A.S.;Terenzi,; de Lourdes Teixeira de Moraes Polizeli, M. and Atı´lio Jorge, J. (2009), Production of b-fructofuranosidases by *Aspergillus niveus* using agroindustrial residues as carbon sources: characterization of an intracellular enzyme accumulated in the presence of glucose, *Process Biochemistry,* 44, 237-241.

Gutierrez-Alonso, P.; Fernandz-Arrojo, L.; Plou, F.J. and Fernadez-Lobato, M. (2009), Biochemical characterization of a β-fructofuranosidase from *Rhodotorula dairenesis,* with transfructosylating activity, *Federation of European Microbiological Societies,* DOI:10.1111/j.1567.

Hayashi, S.; Nonokuchi, M.; Imada, K. and Ueno, H. (1990), Production of a fructosyl-transferring enzyme by*Aureobasidium sp*. ATCC 20524, *Journal of Industrial Microbiology,* 5, 6, 395 - 399.

Hedlund, H. Size –exclusion chromatography, In: Simpson, R.J. (ed) (2004) *Purifying Proteins for Proteomics,* Cold Spring Harbor Laboratory Press, New York.

Hendry, G.A.F. (1993), Evolutionary origins and natural functions of fructans: a climatological, biogeographic and mechanistic appraisal, *New Phytologist,* 123, 1, 3-14.

Hidaka, H.; Hirayama, M. and Sumi, N. (1988), A fructooligosaccharide-producing enzyme from *Aspergillus niger* ATCC 20611, *Agricultural and Biological Chemistry,* 52, 5, 1181-1187.

Hijova, E.; Chmelarova, A.; Bomba, A. and Zitnan, R. (2009), Prebiotic foodstuffs and their health benefits in experiment, *Bratislavske Lekarske Listy,* 110, 9, 523-525.

Hirayama M.; Sumi, N and Hidaka H (1989), Purification and properties of a fructooligosaccharide-producing β-fructofuranosidase from *Aspergillus niger* ATCC 20611.*Agricultural Biochemistry*, 53, 667–673.

Homann, A. and Seibel, J. (2009), Towards tailor-made oligosaccharides—chemo-enzymatic approaches by enzyme and substrate engineering, *Applied Microbiology and Biotechnology,* 83, 209-216.

Hosono, A.; Ozawa, A.; Kato, R.; Ohnishi, Y.; Nakanishi, Y.; Kimura, T. and Nakamura, R. (2003), Dietary fructooligosaccharides induce immunoregulation of intestinal IgA secretion by murine Peyer's patch cells, *Bioscience, Biotechnology, and Biochemistry,* 67, 4, 758-764.

Hussein, H.S.; Campbell, J.M.; Bauer, L.L.; Fahey, G.C.; Hogarth, A.J.; Wolf, B.W. and Hunter, D.E. (1998), Selected fructooligosaccharide composition of pet-food ingredients, *The Journal of Nutrition,* 128, 12, 2803-2805.

Ikegami, T.; Tomomatsu, K.; Takubo, H.; Horie, K. and Tanaka, N. (2008), Separation efficiencies in hydrophilic interaction chromatography , *Journal of Chromatography.A,* 1184, 1-2, 474-503.

Itaya, N.M.; Asega, A.F.; Carvalho, M.A.M. and Figueiredo-Ribeiro, R.d.C.L. (2007), Hydrolase and fructosyltransferase activities implicated in the accumulation of different chain size fructans in three *Asteraceae* species, *Plant Physiology and Biochemistry,* 45, 9, 647-656.

Jaime, L.; Martín-Cabrejas, M.A.; Mollá, E.; López-Andréu, F.J. and Esteban, R.M. (2001), Effect of Storage on Fructan and Fructooligosaccharide of Onion(Allium cepaL.), *Journal of Agricultural and Food Chemistry,* 49, 2, 982 - 988.

Jan, D. and Lawshe, C. (2007), 13 February 2007-last update*, Advanced Environmental Monitoring and Control Program* [Homepage of NASA jet propulsion laboratory,California Institute of Technology], [Online]. Available: http://aemc.jpl.nasa.gov/activities/bio_regen.cfm [2010, 10/17].

Jenkins, D.J.; Wolever, T.M.; Jenkins, A.; Brighenti, F.; Vuksan, V.; Rao, A.V.; Cunnane, S.C.; Ocana, A.; Corey, P. and Vezina, C. (1991), Specific types of colonic fermentation may raise low density lipoprotein cholesterol concentrations, *American Journal of Clinical Nutrition*, 54, 141, 147.

Jeong, H.J.; Jeong, J. B.; Kim, D.S. and de Lumen, B. (2007), Inhibtion of core histone acetylation by the cancer preventative peptide Lunasin, *Journal of Agricultural Food Chemistry*, 55, 3, 632-637.

Johnson, R.K.; Appel, L.J.; Brands, M.; Howard, B.V.; Lefevre, M.; Lustig, R.H.; Sacks, F., Steffen, L.M.; Wylie-Rosett, J. and American Heart Association Nutrition Committee of the Council on Nutrition, Physical Activity,and Metabolism and the Council on Epidemiology and Prevention (2009), Dietary sugars intake and cardiovascular health: a scientific statement from the American Heart Association, *Circulation,* 120, 11, 1011-1020.

Kaminski, K.; Kaminska, E.; Hensel-Bielowka, S.; Chelmecka, E.; Paluch, M. Ziolo, J.; Wlodarczyk, P. and Ngai, K.L. (2008), Identification of the molecular motions responsible for the slower secondary (beta) relaxation in sucrose, *The journal of physical chemistry B,* 112, 25, 7662-7668.

Karlsson, E. and Ryden, L. Ion-Exchange Chromatography, in Janson, J.C., Ryden, L, (eds) (1998) *Protein Purification:Principles, High-Resolution Methods and Applications*, Wiley-VCH, Canada.

Kaur, N. and Gupta, A.K. (2002), Applications of inulin and oligofructose in health and nutrition, *Journal of Biosciences,* 27, 7, 703 -714.

Kenmore, C.; Erskine, S. and Bornhop, D. (1997), Refractive-index detection by interferometric backscatter in packed-capillary high-performance liquid chromatography, *Journal of Chromatography A,* 762, 1-2, 219- 225.

Kleessen, B.; Sykura, B.; Zunft, H. and Blaut, M. (1997), Effects of inulin and lactose on fecal microflora, microbial activity, and bowel habit in elderly constipated persons, *American Journal of Clinical Nutrition,* 65, 5, 1397-1402.

Krogh, A. (2008), What are artificial neural networks, *Nature Biotechnology,* 26, 2, 195-197.

Kroll, J., Darmo, J. and Unterrainer, K. (2007), Terahertz optical activity of sucrose single-crystals, *Vibrational Spectroscopy,* 43, 2, 324-329.

Kurakake, M.; Ogawa, K.; Sugie, M.; Takemura, A.; Sugiura, K. and Komaki, T. (2008). Two types of fructofuranosidases from*Aspergillus oryzae* KB, *Journal of Agriculture and Food Chemistry,*56, 591-596.

Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of the bacteriophage T4. *Nature,* 227, 680-685.

H. S. and Sturm, A. (1996), Purification and characterization of neutral and alkaline invertases from carrot, *Plant Physiology*, 112, 4, 1513-1522.

Lee, J.W.; Thomas, L.C. and Schmidt, S.J. (2011), Investigation of thermal decomposition as the cause of the loss of crystalline structure in sucrose, glucose, and fructose, *Journal of Agricultural and Food Chemistry*, 59, 2, 684-701.

Lee, W., Chiang, C. and Tsai, P. (1999), Kinetic modeling of fructo-oligosaccharide production catalyzed by immobilized ß-fructofuranosidase , *Industrial and Engineering Chemistry Research,* 38, 7, 2564 2570.

Leiti, J.T.C.; Martinelli, P.; Murr, F.E.X. and Jin, K. Study of the inulin conentration by physical methods, *Proceedings of the 14th International Drying Symposium,* Sao Paulo, 22-25 August 2004B: 868-875.

Lewis, D.H. (1993), Nomenclature and diagrammatic representation of oligomeric fructans - a paper for discussion, *New Phytologist,* 124, 4, 583-594.

L'Hocine, L.; Wang, Z.; Jiang, B. and Xu, S. (2000). Purification and partial characterization of fructosyltransferase and invertase from *Aspergillus niger*, *Journal of Biotechnology,* 81, 73.

l'Homme, C.; Peschet, J.; Puigserver, A. and Biagini, A. (2001), Evaluation of fructans in various fresh and stewed fruits by high-performance anion-exchange chromatography with pulsed amperometric detection, *Journal of Chromatography A,* 920, 1-2, 291- 297.

Liao, F.; Zhu, X.; Wang, Y. and Zuo, Y. (2005), The comparison of the estimation of enzyme kinetic parameters by fitting reaction curve to the integrated Michaelis-Menten rate equations of different predictor variables, *Journal of Biochemical and Biophysical Methods,* 62, 1, 13-24.

Lineweaver, H. (1985), The determination of enzyme dissociation constants, *Current Contents (Citation Classic),* 11.

Lipski, A.; Rhimi, M.; Haser, R. and Aghajari, N. (2010), Structure/Function relationship of sucrose isomerases with different product specificity, *Journal of Applied Glycoscience,* 57, 219-228.

Liu, C.H.; Hwang, C.F., and Liao, C.C. (1999), Medium optimization for glutathione production by *Saccharomyces cerevisiae*. *Process Biochemistry*,34, 17–23.

Lomba, A.; Milagro, F.I.; Garcia-Diaz, D.F.; Campion, J.; Marzo, F. and Martinez, J.A. (2010), A high-sucrose isocaloric pair-fed model induces obesity and impairs NDUFB6 gene function in rat adipose tissue, *Journal of Nutrigenetics and Nutrigenomics,* 2, 6, 267-272.

Lunn, J.E. and MacRae, E. (2003), New complexities in the synthesis of sucrose, *Current Opinion in Plant Biology,* 6, 3, 208-214.

Macfarlane, G.T. and Macfarlane, S. (2007), Models for intestinal fermentation: association between food components, delivery systems, bioavailability and functional interactions in the gut, *Current opinion in Biotechnology,* 18, 2, 156-162.

Maiorano, A.E.; Picoli, R. M.; Sabino da Silva, E. and Filomena de Andrade Rodrigues, M. (2008), Microbial production of fructosyltransferases for synthesis of pre-biotics, *Biotechnology Letters,* 30, 1867–1877.

Malinowska, E.; Krzyczkowski, W.; Łapienis, G. and Herold, F. (2010), Densitometric determination of carbohydrates: Application to purification and molecular weight determination of polysaccharide from *Hericium erinaceum* mushroom, *Food Research International,* 43, 4, 988-995.

Maloa, B.M. (2001), Sugar cane: a case as development crop in South Africa, *Proceedings of SARPN conference on Land Reform and Poverty Alleviation in Southern* Africa, Pretoria, 4-5 June 2001.

Manning, T.S. and Gibson, G.R. (2004), Microbial-gut interactions in health and disease. Prebiotics, *Best Practice and Research.Clinical Gastroenterology,* 18, 2, 287-298.

Mansour, E.M. and Dawood, F.M. (2003), Immobilization of invertase on celite and on polyacrylamide by an absorption procedure, *Journal of the Science of Food and Agriculture,* 83, 446-450.

Manzi, A.E. and Van Halbeek, H. *Principles of structural analysis and sequencing of glycans* in Cummings, R.; Esko, J.; Freeze, H.; Hart, G. and Marth,J.(eds) (1999) *Essentials of Glycobiology,* 2nd edn, Cold Spring Harbor Laboratory Press, New York.

Martinez-Fleites, C.; Ortiz-Lombardia, M.; Pons, T.; Tarbouriech, N.; Taylor, E.J., Arrieta, J.G.; Hernandez, L. and Davies, G.J. (2005), Crystal structure of levansucrase from the Gram-negative bacterium *Gluconacetobacter diazotrophicus* , *The Biochemical Journal,* 390, 19-27.

Marx, S.P.; Nosberger, J. and Frehner, M. (1997), Seasonal variation of fructan-beta-fructosidase (FEH) activity and characterization of a beta-(2-1)-linkage specific FEH from tubers of Jerusalem artichoke (*Helianthus tuberosus*), *New Phytologist,* 135, 2, 267-277.

Medeiros, P.M. and Simoneit, B.R. (2007), Analysis of sugars in environmental samples by gas chromatography-mass spectrometry , *Journal of Chromatography.A,* 1141, 2, 271-278.

Midilli, M.; Alp, M.; Kocabach, N.; Muglah, O.H.; Turan, N.; Yilmaz, H. and Cakir, S.d. (2008), Effects of dietary probiotic and prebiotic supplementation on growth performance and serum IgG concentration of broilers, *South African Journal of Animal Science,* 38, 1, 21-27.

Miller G.L. (1959), Use of dinitrosalisylic acid reagent for determination of reducing sugar. *Analytical Chemistry,* 31, 426-428.

Mislovičová, D.; Turjan, J.; Vikartovská, A. and Pätoprstý, V. (2009), Removal of d-glucose from a mixture with d-mannose using immobilized glucose oxidase, *Journal of Molecular Catalysis B: Enzymatic,* 60, 1-2, 45-49.

Mitsouka, T.; Hidaka, H. and Eida, T. (1987), Effects of fructooligosaccharides on intestinal microflora, *Die Nahrung,* 31, 427-436.

Montgomery, D.C. (1999), Experimental design for product and process design and development, *Journal of the Royal Statistical Society: Series D (The Statistician),* 48, 2, 159 -177.

Monti, A. (2005), Growth, fructan yield, and quality of chicory (*Cichorium intybus L.*) as related to photosynthetic capacity, harvest time, and water regime , *Journal of Experimental Botany,* 56, 415, 1389-1395.

Montilla, A.; Ruizmatute, A.; Sanz, M.; Martinecastro, I. and Delcastillo, M. (2006), Difructose anhydrides as quality markers of honey and coffee, *Food Research International,* 39, 7, 801-806.

Mortensen, P.B. and Clausen, M.R. (1996), Short-chain fatty acids in the human colon: relation to gastrointestinal health and disease , *Scandinavian Journal of Gastroenterology.Supplement,* 216, 132-148.

Mutanda, T. 2007, *Purification, characterisation and application of inulinase and transferase enzymes in the production of fructose and oligofructose*, PhD edn, Rhodes University, Grahamstown.

Mutanda, T.; Wilhelmi B.S, and Whiteley C.G.(2008), Response surface methodology:Synthesis of inulooligosaccharides with an endoinulinase from *Aspergillus niger*. *Enzyme Microbiology Technology,* 43, 362-368.

Myers, R.H. and Montgomery, D.C. 2002, *Response surface methodology: process and product optimization using designed experiments,* Wiley-interscience, Hoboken, New Jersey, USA.

Nemukula, A. 2008, *Purification and characterisation of fructosyltransferase for the synthesis of short chain fructooligosaccharides*, Msc edn, Rhodes University, Grahamstown.

Nemukula, A.; Mutanda, T.; Wilhelmi, B.S. and Whiteley, C.G. (2009), Response surface methodology: Synthesis of short chain fructooligosaccharides with a fructosyltransferase from *Aspergillus aculeatus*, *Bioresource Technology,* 100, 6, 2040-2045.

Nguyen Q.D.; Szabo,´ J.M.R.; Bhat, M.K.and Hoschke A.P. (2005),Purification and some properties of b-fructofuranosidase from *Aspergillus niger* IMI303386. *Process Biochemistry*, 40, 2461–2466.

Nishizawa, M.; Maruyama, Y. and Nakumara, M. (1980), Purification and characterization of invertase isoenzymes from *Fusarium oxysporum*, *Agricultural Biochemistry*, 44, 489–498.

Nishizawa, K.; Nakajima, M. and Nabetani, H. (2000), A forced-flow membrane reactor for transfructosylation using ceramic membrane, *Biotechnology and Bioengineering,* 68, 1, 92-97.

Odom, M.D. and Sharda, R. (1990), A neural network method for bankrupcy detection, *IJCNNI International Joint Conference on Neural Networks 1990*17-21 June 1990.

Otto, M. 1999, *Statistics and computer application in analytical chemistry*, Chemometrics. Wiley-VCH, Germany.

Paliwal, M. and Kumar, U.A. (2009), Neural networks and statistical techniques: A review of applications, *Expert Systems with Applications,* 36, 1, 2-17.

Park, J.;Oh, T.and Yun, J. (2001), Purification and characterization of a novel transfructosylating enzyme from *Bacillus macerans* EG-6, *Process Biochemistry,* 37, 5, 471-476.

Perrin, P.; Cassagnau, E.; Burg, C., Patry, Y.; Vavasseur, F.; Harb, J.; Le Pendu, J.; Douillard, J.Y.; Galmiche, J.P. and Bornet, F. (1994), An interleukin 2/sodium butyrate combination as immunotherapy for rat colon cancer peritoneal carcinomatosis, *Gastroenterology,* 107, 6, 1697-1708.

Pierre, F.; Perrin, P.; Champ, M.; Bornet, F., Meflah, K. and Menanteau, J. (1997), Short-chain fructo-oligosaccharides reduce the occurrence of colon tumors and develop gut-associated lymphoid tissue in Min mice, *Cancer Research,* 57, 2, 225-228.

Pletschke, B and Beukes, N, (2010), Protein Purification course notes, Department of Biochemistry; Rhodes University, *Personal Communications*

Pool-Zobel, B.; van Loo, J.; Rowland, I. and Roberfroid, M.B. (2002), Experimental evidences on the potential of prebiotic fructans to reduce the risk of colon cancer, *British Journal of Nutrition,* 87, 2, 273.

Prapulla, S.; Subhaprada, V. and Karanth, N. (2000), Microbial production of oligosaccharides: A review, *Advances in Applied Microbiology,* 47, 299 - 343.

Pretlow, T.P.; Ann O'Riordan, M.; Pretlow, T.G. and Stellato, T.A. (1992), Aberrant crypts in human colonic mucosa: Putative preneoplastic lesions, *Journal of Cellular Biochemistry,* 50, 16, 55- 62.

Prosky, L. and Hoebregs, H. (1999), Methods to determine food inulin and oligofructose, *Journal of Nutrition,* 129, 7, 1418.

Qian, N. and Sejnowski, T.J. (1988), Predicting the secondary structure of globular proteins using neural network models, *Journal of Molecular Biology,* 202, 4, 865-884.

Quemener, B.; Thibault, J. and Coussement, P. (1997), Integration of inulin determination in the AOAC method for measurement of total dietary fibre, *International Journal of Biological Macromolecules,* 21, 1-2, 175-178.

Rathinasabapathi, B. (2000), Metabolic Engineering for Stress Tolerance: Installing Osmoprotectant Synthesis Pathways, *Annals of Botany,* 86, 4, 709 - 716.

Reddy, B.S. (1998), Prevention of colon cancer by pre- and probiotics: evidence from laboratory studies, *The British Journal of Nutrition,* 80, 4, S219-23.

Reddy, P.P.; Reddy, G.N.S. and Sulochana, M.B. (2010), Screening of β-fructofuranosidase producers with high transfructosylation activty and its 3^2

experimental run studies on reaction rate of enzyme, *Journal of Biological Sciences,* 10, 3, 237-241.

Resa, P; Elvira, L.; Sierra, C. and Espinosa, F.M.d. (2009), Ultrasonic velocity assay of extracellular invertase in living yeasts, *Analytical Biochemistry,* 384, 1, 68-73.

Ripley, B.D. (1994), Neural networks and related methods for classification, *Journal of the Royal Statisitical Society,* 56, 3, 409-456.

Riverourgell, M. (2001), Oligosaccharides: application in infant food, *Early Human Development,* 65, S43- S52.

Roberfroid, M. (2007), Prebiotics: The Concept Revisited, *Journal of Nutrition,* 137, 3, 830-837.

Roberfroid, M.; Gibson, G. and Delzenne, N. (1993), The Biochemistry of Oligofructose, a Nondigestible Fiber: An Approach to Calculate Its Caloric Value, *Nutrition Reviews,* 51, 5, 137-146.

Roberfroid, M.B.; Van Loo, J.A. and Gibson, G.R. (1998), The bifidogenic nature of chicory inulin and its hydrolysis products, *The Journal of Nutrition,* 128, 1, 11-19.

Rocha, J.R.; Catana, R.; Ferreira, B.S.; Cabral, J.M.S. and Fernandes, P. (2006), Design and characterisation of an enzyme system for inulin hydrolysis, *Food Chemistry,* 95, 1, 77-82.

Rodríguez, J.; Pérez,J.A.; Ruiz,T. and L. Rodríguez**.**(1995), Characterization of the invertase from *Pichia anomala, Journal of Biochemistry*, 306,235-239.

Ronkart, S.N.; Blecker, C.S.; Fourmanoir, H.; Fougnies, C.; Deroanne, C.; Van Herck, J.C. and Paquot, M. (2007), Isolation and identification of inulooligosaccharides resulting from inulin hydrolysis, *Analytica Chimica Acta,* 604, 1, 81-87.

Rozan, P.; Nejdi, A.; Hidalgo, S.; Bisson, J.F.; Desor, D. and Messaoudi, M. (2008), Effects of lifelong intervention with an oligofructose-enriched inulin in rats on general health and lifespan, *The British Journal of Nutrition,* 100, 6, 1192-1199.

Rubio, M.C. and Maldonado,M.C. **(**1995). Purification and characterization of invertase from *Aspergillus niger*, *Current views in, Microbiology*, 31, 80–8.

Rude, R.K.; Gruber, H.E.; Norton, H.J.;Wei, L.Y.; Frausto, A. and Mills, B.G. (2004), Bone loss induced by dietary magnesium reduction to 10% of the nutrient requirement in rats is associated with increased release of substance p and tumor Necrosis Factor-α, *Journal of Nutrition,* 134, 1, 79-85.

Saengthongpinit, W. and Sajjaanantakul, T. (2005), Influence of harvest time and storage temperature on characteristics of inulin from Jerusalem artichoke (L.) tubers, *Postharvest Biology and Technology,* 37, 1, 93-100.

Sangeetha, P.; Ramesh, M. and Prapulla, S. (2005), Recent trends in the microbial production, analysis and application of Fructooligosaccharides, *Trends in Food Science and Technology,* 16, 10, 442- 457.

Santos A.M.P. and Maugeri F. (2007), Synthesis of fructooligosaccharides from sucrose using inulinase from *Kluyveromyces marxianus*. *Food Technology Biotechnology,* 45, 181-186.

Sanz, M.L. Martínez-Castro, I. (2007), Recent developments in sample preparation for chromatographic analysis of carbohydrates, *Journal of Chromatography A,* 1153, 1-2, 74-89.

Saulnier, D.M.; Molenaar, D.; de Vos, W.M.; Gibson, G.R. and Kolida, S. (2007), Identification of prebiotic fructooligosaccharide metabolism in Lactobacillus plantarum WCFS1 through microarrays, *Applied and Environmental Microbiology,* 73, 6, 1753-1765.

Savadogo, A.; Ouattara, C.A.T.; Savadogo, P.W.; Barro, N.; Ouattara, A.S. and Traore, A. (2004), Identifying exopolysaccharides-producing *lactobacillus* from Burkina faso fermented milk, *African Journal of Biotechnology,* 3, 3, 189-194.

Schindler, R. (1998), Determination of enzyme kinetics and chemometric evaluation of reaction products by FTIR spectroscopy on the example of ß-fructofuranosidase, *Vibrational Spectroscopy,* 16, 2, 127 -135.

Schindler, R. and Lendl, B. (1999), Simultaneous determination of enzyme activities by FTIR-spectroscopy in a one-step assay, *Analytica Chimica Acta,* 391, 1, 19-28.

Schley, P.D. and Field, C.J. (2002), The immune-enhancing effects of dietary fibres and prebiotics, *British Journal of Nutrition,* 87, 2, 221.

Seibel, J.; Moraru, R.; Gotze, S.; Buchholz, K.; Na'amnieh, S.; Pawlowski, A. and Hecht, H.J. (2006), Synthesis of sucrose analogues and the mechanism of action of Bacillus subtilis fructosyltransferase (levansucrase), *Carbohydrate Research,* 341, 14, 2335-2349.

Sejnowski, T.J. and Rosenberg, C.R. (1987), Parallel networks that learn to pronounce english text, *Complex Systems,* 1, 145-168.

Sharma, A.; Kainth, S. and Gill, P. (2006), Inulinase production using garlic (*Allium sativum*) powder as a potential substrate in *Streptomyces sp*, *Journal of Food Engineering,* 77, 3, 486-491.

Sharma, A.D. and Gill, P.K. (2007), Purification and characterization of heat-stable exo-inulinase from Streptomyces sp, *Journal of Food Engineering,* 79, 4, 1172-1178.

Sheperd, S.J. and Gibson, P.R. (2010), 22 October-last update*, Evidence-Based Dietary Management of Functional Gastrointestinal Symptoms: More Than Just Fructose and Fructans* [Homepage of Blackwell], Available: http://www.medscape.com/viewarticle/716634_6 [2010, 10/22] .

Shepherd, S.J. and Gibson, P.R. (2006), Fructose malabsorption and symptoms of irritable bowel syndrome: guidelines for effective dietary management, *Journal of the American Dietetic Association,* 106, 10, 1631-1639.

Shiomi, N.; Onodera, S.; Chatterton, J. and Harrisson, P. (1991), Separation of fructooligosaccharide isomers by anion-exchange chromatography, *Agricultural and Biological Chemistry,* 55, 5, 1427-1428.

Singh, B.; Kumar, R., and Ahuja, N., (2004), Optimizing drug delivery systems using systematic design of experiments. part I: fundamental aspects, *Critical Reviews in Therapeutic Drug Carrier Systems*, 22, 1, 27–105.

Sirő, I.; Ka´polna,E.; Ka´polna, B. and Lugasi, A. (2008), Functional food: product development, marketing and consumer acceptance—A review, *Appetite,* 51, 456-467.

Spectrum Commodities (2010), 09/21-last update*, South Africa: Sugar*. Available: http://www.spectrumcommodities.com/education/commodity/sb.html [2010, 10/21] .

Stamps, K. and Hamam, Y. (2010), Brain Informatics; Towards inexpensive BCI control for wheelchair navigation in the enabled environment – A hardware survey, *Brain Informatics,* 6334, 336- 345.

Structural Genomics Consortium, (2008), Protein production and purification, *Nature Methods,*5, 2, 135-146.

Sund, M.L. and Linder, L (1980), Cell surface bound β-fructofuranosidase (invertase) of the oral bacterium *Streptococcus mitis, Archives of Oral Biology,* 25, 8, 575-578.

Suzuki, N.; Aiba, Y.; Takeda, H.; Fukumori, Y. and Koga, Y. (2006), Superiority of 1-kestose, the smallest fructo-oligosaccharide, to a synthetic mixture of fructo-oligosaccharides in the elective stimulating activity on bifidobacteria, *Bioscience and Microflora,* 25, 3, 109-116.

Tanriseven, A. and Aslan, Y. (2005), Immobilization of Pectinex Ultra SP-L to produce fructooligosaccharides, *Enzyme and Microbial Technology,* 36, 4, 550-554.

Teitelbaum, J.E. and Walker, W.A. (2002), Nutritional impact of pre- and probiotics as protective gastrointestinal organisms, *Annual Review of Nutrition,* 22, 107-138.

Tokunaga, T.; Nakada.; Y. Tashiro, Y.; Hirayama, M. and Hidaka, H. (1993), Effects of fructooligosaccharides intake on the intestinal microflora and defecation in healthy volunteers, *Bifidus - Flores, Fructus et Semina,* 6, 143-150.

Trevizam Moraes, P.C.B. and Bolini, H.M.A. (2010), Different sweeteners in beverages prepared with instant and roasted ground coffee: Ideal and equivalent sweetness, *Journal of Sensory Studies,* 25, 215-225.

Tsaprailis, G. 20 December 2001- last update, *Colorimetric methods in protein determination,* [Homepage of the University of Arizona, College of Science], [Online] Available: http:www.biochem.arizona.edu/classes/bioc463a/info/lecturenotes/colorimetric, [2010, 03/ 16].

Vaccari, G.; Lodi, G.; Tamburini, E.; Bernardi, T. and Tosi, S. (2001), Detection of oligosaccharides in sugar products using planar chromatography, *Food Chemistry,* 74, 1, 99-110.

Van Loo, J.; Clune, Y.; Bennett, M. and Collins, J.K. (2005), The SYNCAN project: goals, set-up, first results and settings of the human intervention study, *The British Journal of Nutrition,* 93, S91-8.

Van Loo, J.; Coussement, P.; De Leenheer, L.; Hoebregs, H. and Smits, G. (1995), On the presence of Inulin and Oligofructose as natural ingredients in the western diet, *Critical Reviews in Food Science and Nutrition,* 35, 6, 525 - 552.

Vandamme, E.J. and Derycke, D.G. (1983), Advances in Applied Microbiology in , ed. A.I. Laskin, Academic Press, 139.

Venter, C.S. (2007), Prebiotics: An update, *Journal of Family Ecology and Consumer Sciences,* 35, 17-25.

Verschuren, P.M. (2007), Functional Foods: Scientific and Global Perspectives, *British Journal of Nutrition,* 88, 2, 126.

Vijn, I. and Smeekens, S. (1999), Fructan: more than a reserve carbohydrate?, *Plant Physiology,* 120, 2, 351-360.

Walker, W.A. and Duffy, L.C. (1998), Diet and bacterial colonization: role of probiotics and prebiotics, *The Journal of Nutritional Biochemistry,* 9, 12, 668-675.

Ward, W. and Swiatek, G. (2009), Protein Purification, *Current Analytical Chemistry*, 5, 21, 85, 105.

Wiedmann, M. and Jager, M. (1998), Synergistic sweeteners, *Food Ingredients and Analysis International,* 19, 51-56.

Wilson, K. and Walker, J.2010, *Principles and Techniques of Practical Biochemistry*, Cambridge University Press, United Kingdom.

Winzeler, M.; Dubois, D. and Noesberger, J. (1990), Absence of fructan degradation during fructan accumulation in wheat stems, *Journal of Plant Physiology,* 136, 324-329.

Wong, L. and Sissons, C.H. (2007), Human dental plaque microcosm biofilms: effect of nutrient variation on calcium phosphate deposition and growth, *Archives of Oral Biology,* 52, 3, 280-289.

Wu, L. and Birch, R.G. (2007), Doubled sugar content in sugarcane plants modified to produce a sucrose isomer, *Plant Biotechnology Journal,* 5, 1, 109-117.

Yanai, K.; Nakane, A.; Kawate, A. and Hirayama, M. (2001), Molecular cloning and characterization of the fructooligosaccharide-producing .beta-fructofuranosidase gene from *Aspergillus niger* ATCC 20611, *Bioscience, Biotechnology, and Biochemistry,* 65, 4, 766-773.

Yonemoto, I. (2006), 26 March 2006-last upload, *Size exchange chrom-png,* [Wiki-premed.com], [Online]. Available: wikipedia.org/wiki/files/sixeexchrom.png. [2010, 10/12].

Yun, J. (1996), Fructooligosaccharides—Occurrence, preparation, and application, *Enzyme and Microbial Technology,* 19, 2, 107 117.

Zeelie. B(2010),Optimization: three easy methods Lecture notes, Port Elizabeth Technikon .*Personal Communications.*

Zuccaro, A.; Gotze, S.; Kneip, S.; Dersch, P. and Seibel, J. (2008), Tailor-made fructooligosaccharides by a combination of substrate and genetic engineering, *Chembiochem : A European Journal of Chemical Biology,* 9, 1, 143-149.

Appendix A: Standard curves

A1. Bradford standard curve

A protein standard curve (**Figure A1**) was generated using a modified Bradford protein assay (Bradford, 1976). Bovine serum albumin (BSA) was used as a standard with commercial Bradford reagent (Sigma-Aldrich, South Africa). Various concentrations of BSA were prepared ranging from 0 to 1.4 mg/ml. The standard curve was generated using 5 µl and 245 µl of sample and Bradford reagent, respectively. The protein samples were mixed with Bradford reagent and allowed to stand at room temperature for 15 minutes before reading the absorbance at 595 nm.

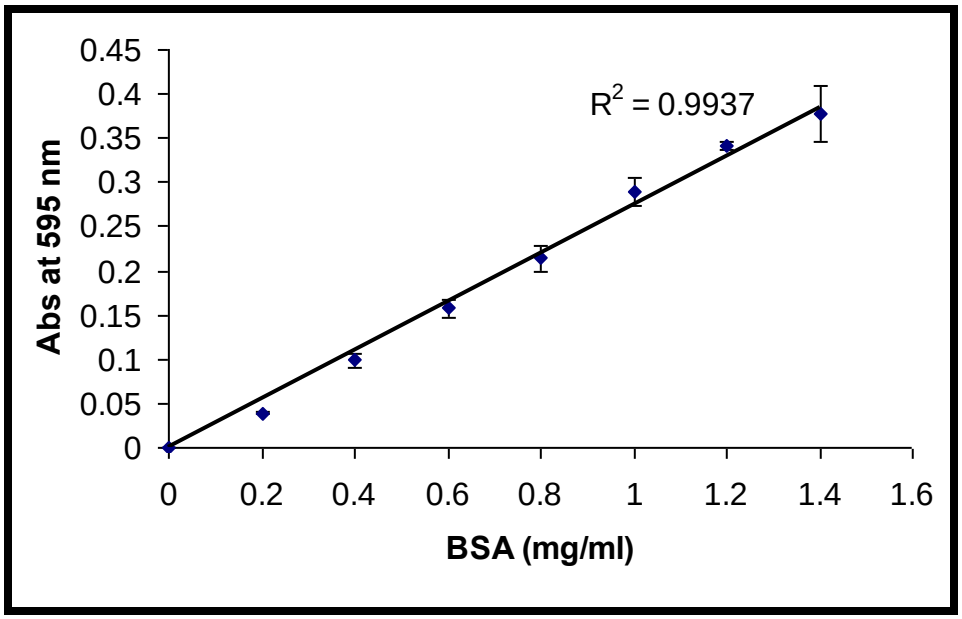


Figure A1: Bradford standard curve for protein concentration

A2: Sephacryl S-300 HR standard curve

The standards: Dextran Blue (2000 kDa), apoferitin (443 kDa), β-glucuronidase (290 kDa), β-amylase (200 kDa), Alcohol dehydrogenase (150 kDa) and β-galactosidase (125 kDa), were made up to a concentration of 20 mg/ml. Each standard (1 ml) was loaded separately onto a Sephacryl S-300 HR chromatography column and eluted using 20 mM Tris-HCl, pH9.0. The elution volume of each standard was used to calculate K_{av} and calibration curve was generated (**Figure A.2**).

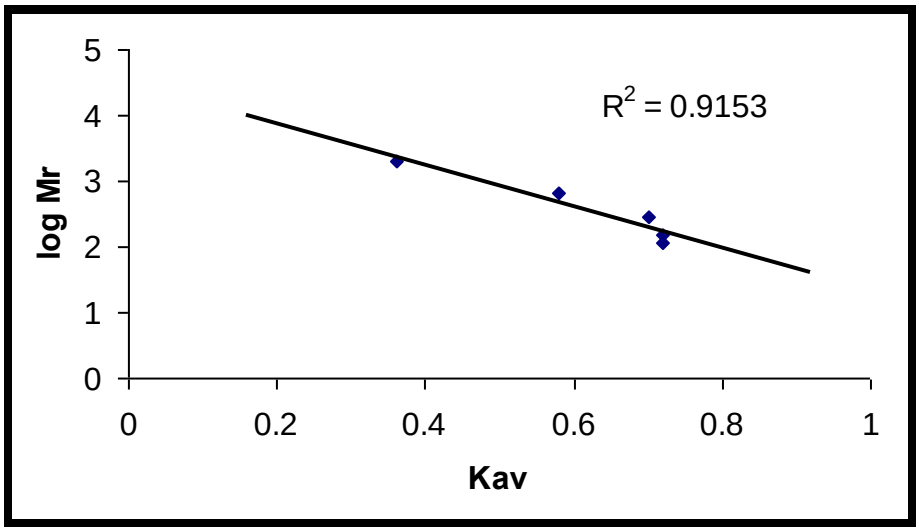


Figure A 2: Standard curve for Sephacryl S-300 HR column

A 3: Electrophoresis standard curves

A peqGOLD® IV protein marker was electrophoresed on a 10 % SDS-PAGE gel and the distance migrated by each standard was measured. The R_f values for each protein band was calculated and a standard curve of log M_r versus R_f was calculated (**Figures A.3**).

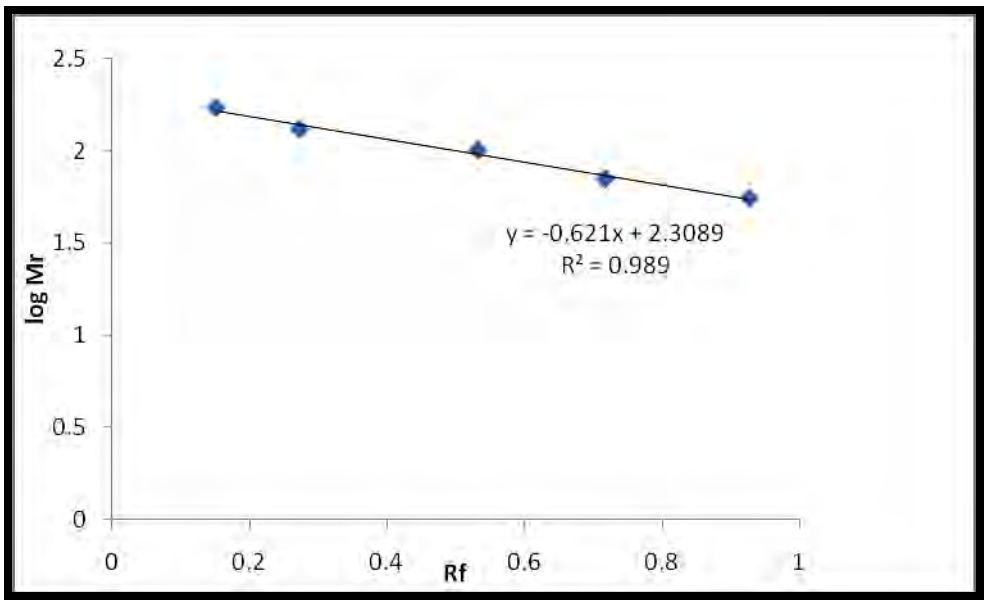
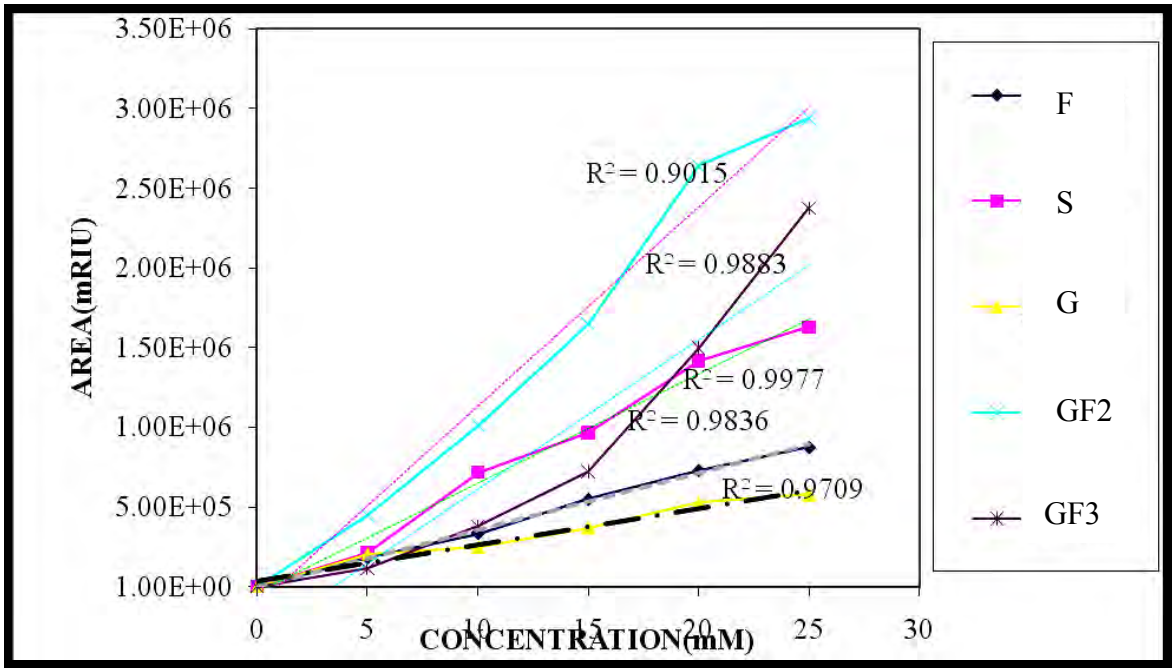


Figure A 3: Electrophoresis standard curve

A 4: Sugar standard curves for HPLC

The sugar standard curves were generated with concentrations of fructose, glucose, sucrose, nystose and kestose between 0 and 25 mM. 10 and 20 µl volumes of each

concentration of standard, for ELS and RI detection, respectively, were injected onto a Prevail® ES column at a flow rate of 1ml/min. A mobile phase of 70 % acetonitrile was used for both detectors. All peak integrations were performed manually using 32 Karat® (Beckman, USA) and Empower Pro® (Waters, USA) software, fro RI and ELS detection, respectively. Standard curves were plotted as peak area vs. concentration (**Figure A4** and **A5)**.



Figugre A4: Sugar standard curve for HPLC with refractive index detector

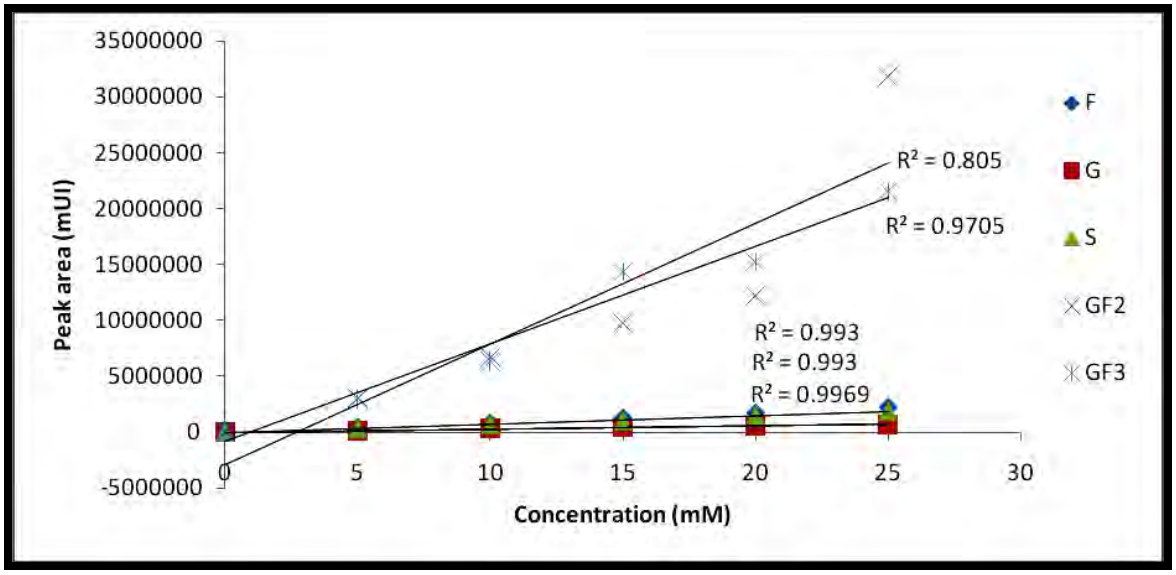


Figure A 5: Sugar standard curve for HPLC with ELS detector

Appendix B: Regression analyses of experimental designs

B1: ANOVA by partial sum of squares method

Following experimental procedure based on the 2^3 factorial and Box-Behnken designs, the data obtained was fitted to a mathematical model using Design Expert® software. ANOVA was performed using the partial sum of squares and is presented in tables B1-B4.

Table B 1: Regression analysis on the effect of temperature and pH on the synthesis of kestose as per the 2^3 factorial design

[kestose]
ANOVA for selected factorial model
Analysis of variance table [Partial sum of squares - Type III]

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F	
Model	0.033197	2	0.016599	7.120414	0.0344	**significant**
B-temp	0.008707	1	0.008707	3.735211	0.1111	
C-pH	0.02449	1	0.02449	10.50562	0.0229	
Residual	0.011656	5	0.002331			
Cor Total	0.044853	7				

Table B 2: Regression analysis on the effect of pH on the synthesis of nystose as per the 2^3 factorial design

[nystose]
ANOVA for selected factorial model
Analysis of variance table [Partial sum of squares - Type III]

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F	
Model	158.974	1	158.974	39.6121	0.0007	**significant**
C-pH	158.974	1	158.974	39.6121	0.0007	
Residual	24.0797	6	4.01328			
Cor Total	183.054	7				

Table B 3: Regression analysis on the effect of pH and temperature on the synthesis of kestose,as per the Box-Behnken design

[kestose]						
ANOVA for Response Surface Reduced 2FI Model						
Analysis of variance table [Partial sum of squares - Type III]						
Source	**Sum of Squares**	**df**	**Mean Square**	**F Value**	**p-value Prob > F**	
Model	0.10834	3	0.03611	4.11449	0.0295	**significant**
A-pH	0.02408	1	0.02408	2.74302	0.1216	
B-Temperature	0.04346	1	0.04346	4.95164	0.0444	
AB	0.0408	1	0.0408	4.6488	0.0504	
Residual	0.1141	13	0.00878			
Lack of Fit	0.06467	9	0.00719	0.58151	0.7708	**not significant**
Pure Error	0.04943	4	0.01236			
Cor Total	0.22244	16				

Table B 4: Regression analysis on the effect of temperature on the synthesis of kestose as per the Box-Behnken design

[nystose]						
ANOVA for Response Surface Reduced Quadratic Model						
Analysis of variance table [Partial sum of squares - Type III]						
Source	**Sum of Squares**	**df**	**Mean Square**	**F Value**	**p-value Prob > F**	
Model	130.009	2	65.0045	5.37114	0.0186	**significant**
B-Temperature	46.8335	1	46.8335	3.86972	0.0693	
B^2	83.1755	1	83.1755	6.87257	0.0201	
Residual	169.436	14	12.1025			
Lack of Fit	129.132	10	12.9132	1.28159	0.4371	**not significant**
Pure Error	40.3036	4	10.0759			
Cor Total	299.445	16				

B2: Diagnostic plots

As a measure of the goodness of fit of fitted mathematical models, predicted vs. actual plots as well as residual vs. predicted plots were constructed (**Figures B 5** and **B 6**).

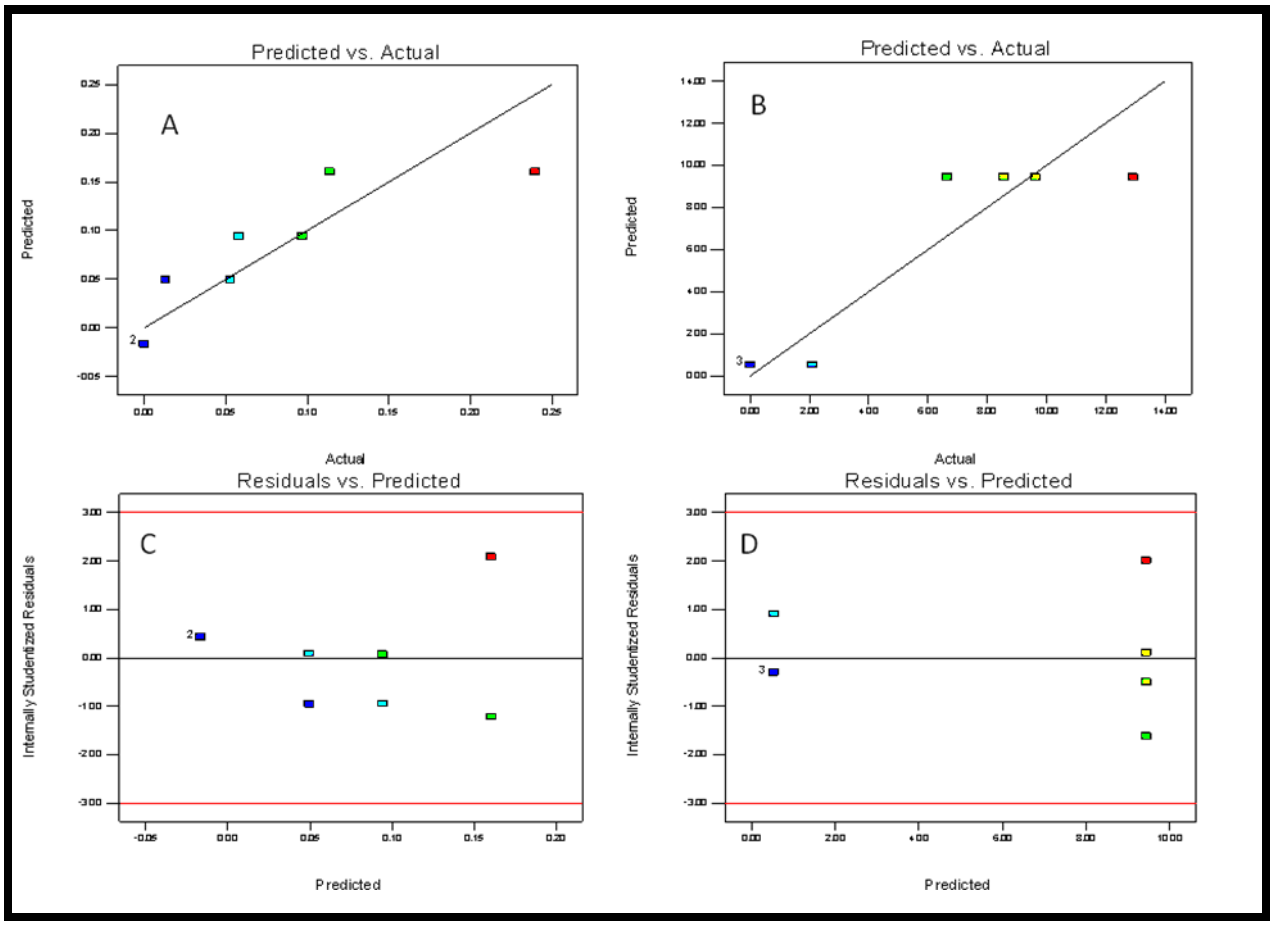


Figure B 1: Diagnostic plots for the 2^3 design using crude enzyme, showing predicted vs. actual plots for (A) kestose, (B) nystose and residuals vs predicted plots for (C) kestose and (D) nystose.

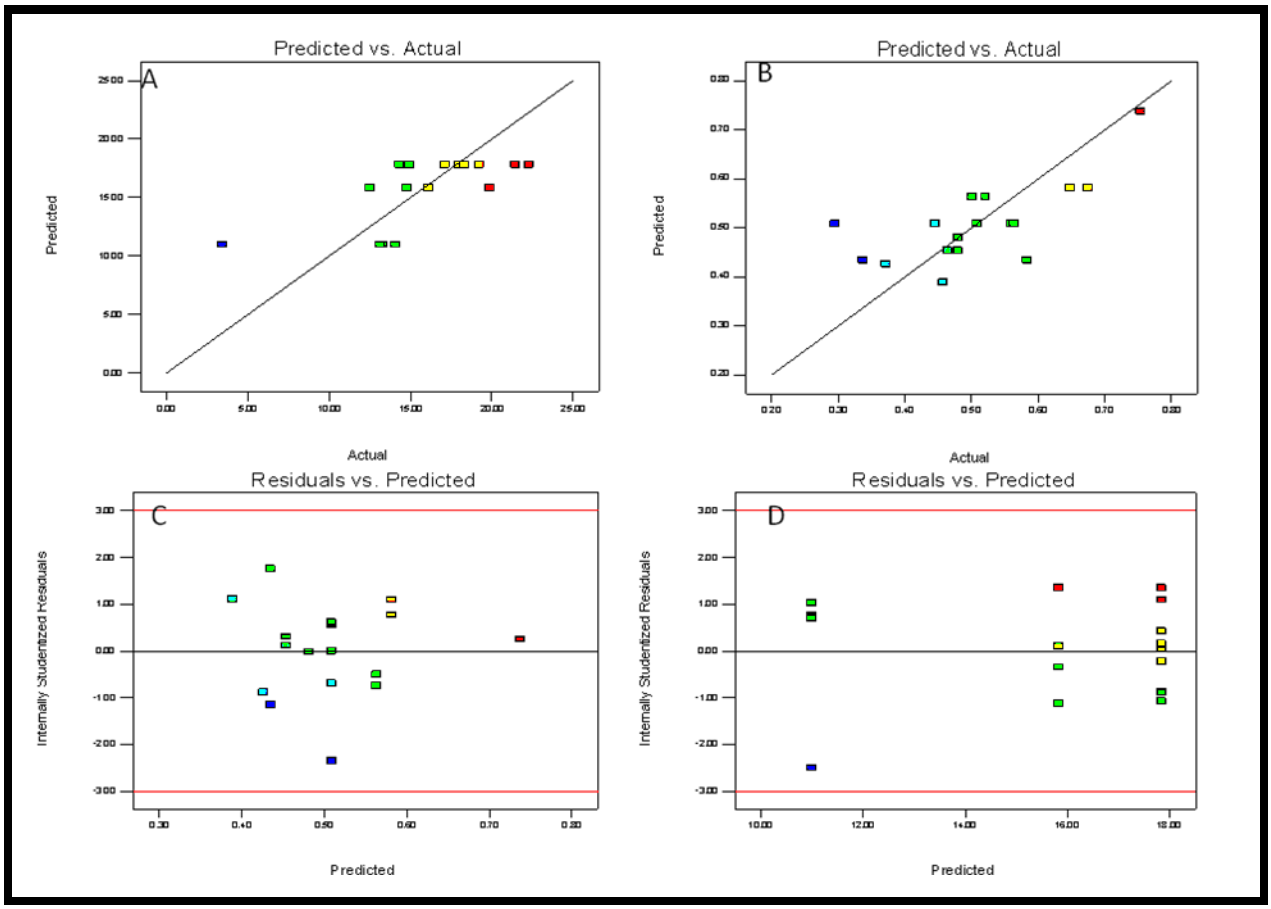


Figure B 2: Diagnostic plots for the Box-Behnken design using crude enzyme, showing predicted vs. actual plots for (A) kestose, (B) nystose and residuals vs predicted plots for (C) kestose and (D) nystose.

Appendix C: Target/activation plots for neural networks

During the training stage of neural network development, target/activation plots were constructed to compare how well the neural network output (activation), correlated with targets set as per input data (target).This served as a test of how well the networks had learnt to navigate the selected experimental space. The network would be given the same conditions used in the training set and expected to produce the corresponding output values, as illustrated in figures C 1 and C 2. Table C1 shows the raw data that was used in the training of all neural networks.

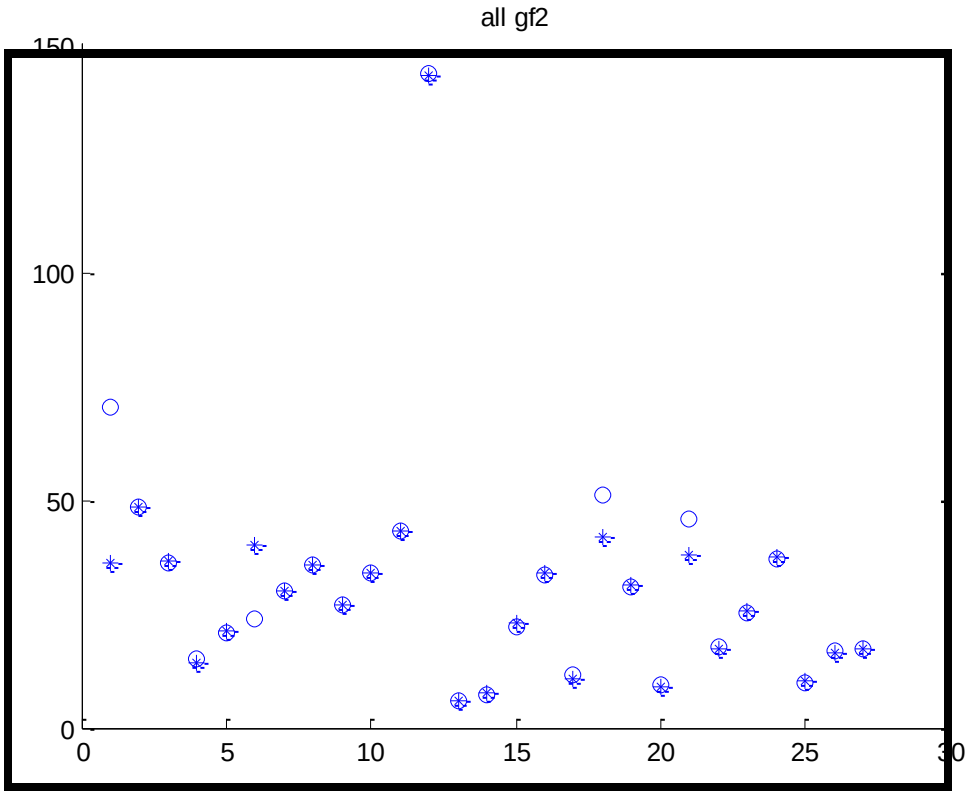


Figure C 1: Target/activation plot of goodnet 1 before an intermediate training stage. ○ = target and * = activation. The y- axis represents the target levels (mM) while the x- axis is the data set.

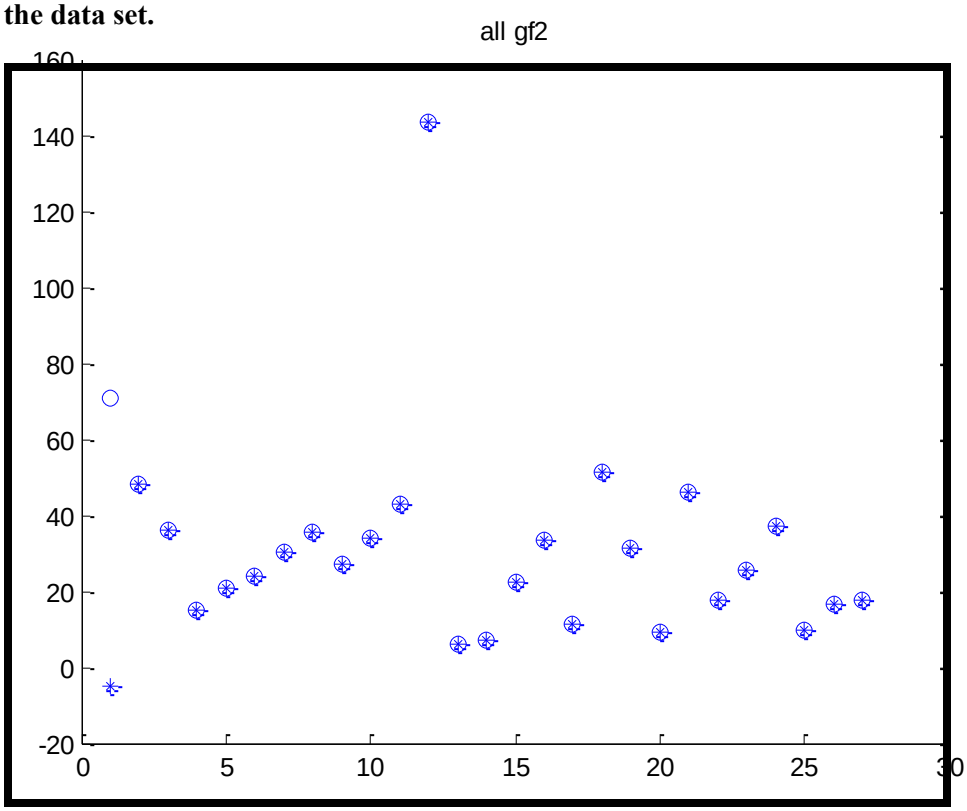


Figure C 2: Target/activation plot of goodnet 1 after neural modifications (increased epochs and neurons/layer). ○ = target and * = activation. The y- axis represents the target levels (mM) while the x- axis is the data set.

Table C1: Raw data for neural network training

					mM *	(µmol/ml)			
Sucrose	TEMP	TIME	PH		fructose	glucose	sucrose*[b]	gf2	gf3
600	20	30	5.8		84.83398	1694.955	70.57507	6.67502	42.82481
600	20	60	5.8		128.55	2141.478	48.36511	20.90415	31.52111
600	20	120	5.8		263.9347	1821.254	36.31161	24.9753	39.81059
600	30	30	5.8		163.7206	1441.149	15.05621	13.70066	19.33794
600	30	60	5.8		133.3909	848.1669	20.94921	15.72922	21.28618
600	30	120	5.8		169.0229	1796.863	23.88372	23.87971	18.38343
600	40	30	5.8		211.2372	1083.302	30.21186	5.192917	11.89167
600	40	60	5.8		238.7495	918.2956	35.57061	3.488639	7.483242
600	50	30	5.8		197.8589	910.3107	27.16418	3.984569	8.129459
600	50	60	5.8		354.9345	818.1918	33.79749	2.142025	6.59582
600	60	30	5.8		250.2476	672.0667	43.01202	29.5929	7.52137
600	60	60	5.8		666.3425	1852.124	143.5292	5.354042	5.981976
	pH	TIME	TEMP		fructose	gluose	sucrose	gf2	gf3
600	3.85	30	30		441.6155	1137.234	5.881388	4.605914	10.24194
600	3.85	60	30		363.6222	985.8927	7.116539	11.67495	13.52189
600	3.85	120	30		448.4299	959.6225	22.24566	3.175228	7.583205
			30						
600	5.1	30	30		328.5979	1039.822	33.38842	3.54149	8.548012
600	5.1	60	30		273.7752	1004.771	11.43019	6.593917	13.72821
600	5.1	120	30		517.0721	1300.51	51.29632	2.920304	6.951882
600	6.55	30	30		351.0899	926.7697	31.06169	3.255401	7.859577
600	6.55	60	30		191.0204	1008.912	9.352666	87.66353	26.34191
600	6.55	120	30		477.1059	1141.153	45.95674	5.039504	6.956689
600	7.8	30	30		280.4019	956.4972	17.78839	3.843622	12.4186
600	7.8	60	30		206.0497	1105.449	25.40535	7.835116	28.67618
600	7.8	120	30		393.2583	1162.288	36.96102	3.38847	10.4476
600	8.95	30	30		194.3364	934.8859	9.771423	3.968072	23.39426
600	8.95	60	30		123.7553	905.013	16.7939	38.28881	56.69705
600	8.95	120	30		273.5353	1437.995	17.52863	4.444014	16.19444