SYNTHESIS, DETECTION AND QUANTIFICATION OF INULOOLIGOSACCHARIDES AND FRUCTOOLIGOSACCHARIDES BY EXTRACELLULAR AND INTRACELLULAR INULINASE AND FRUCTOSYLTRANSFERASE ENZYMES ISOLATED FROM COPROPHILOUS FUNGI

by

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Submitted in fulfilment of the academic requirement for the degree of Master of Science (MSc) in the Discipline of Microbiology, School of Life Sciences, College of Agriculture, Engineering and Science at the University of KwaZulu-Natal, Durban, South Africa.

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As the candidate's supervisor, I have approved this dissertation for submission.

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Preface

The experimental work depicted in this dissertation was carried out in the Discipline of Microbiology, School of Life Sciences, College of Agriculture, Engineering and Science at the University of KwaZulu-Natal, Durban, South Africa from August 2016 to June 30 2018, under the supervision of Dr Taurai Mutanda and Prof Samson Mukaratirwa. These studies represent authentic work of the author and have not otherwise been submitted in any form for any diploma, degree or to any tertiary institution. Where use has been made of the work of others it is duly referenced in text.

College of Agriculture, Engineering and Science

Declaration 1 – plagiarism

I, Jeff Okinda Ojwach declare that:

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Declarations 2 – Conferences and Publications

This Masters work formed an interface between Microbiology and Indigenous Knowledge System (IKS) and resulted in two journal articles. One manuscript under IKS-Food security is in preparation and one article in preparation is targeted for submission to the journal "Biocatalysis and Agricultural Biotechnology." The study also culminated in a poster presentation at the College of Agriculture, Engineering and Science at the University of KwaZulu-Natal, Durban South Africa and local conference in Muldersdrift Pretoria at the Society for South African Microbiology (SASM 2018).

Ojwach J., Mutanda T, Kaya H, Chinsamy M and Mukaratirwa, S. 2018: Current Perspectives on Indigenous Knowledge Systems in relation to Food Security in sub-Saharan Africa. A Systematic Review. Target Journal: *Journal of Social Science* (In Preparation).

Ojwach J., Mukaratirwa S., and Mutanda, T: Screening, Morphotaxonomic and Molecular Identification of Indigenous Coprophilous Fungi for the Biocatalytic Conversion of Sucrose into Biofunctional Prebiotics. Target Journal: *Biocatalysis and Agricultural Biotechnology* (In Preparation).

Ojwach J., and Mutanda T: Screening Coprophilous Fungi for Fructosyltransferase and Inulinase Biocatalysts for Potential Biotechnological Applications. Poster Presentation at the College of Agriculture Engineering and Science Research Day on 26 October 2017 T block, Westville Campus (Appendix J), Awarded First Prize.

Ojwach J., and Mutanda T: Morphotaxonomic and Molecular Identification of Coprophilous Fungi with Extracellular Fructosyltransferase and Hydrolase Activities for Production of Biofunctional Prebiotics. Oral Presentation at a local conference organized by the South African Society of Microbiology SASM 2018 on 4-7 April 2018, Misty Hills Hotel and Conference Center, Muldersdrift, South Africa.

Signed: 

Abstract

Exploration of fungal biodiversity capable of producing fructosyltransferase and inulinase enzymes in significant amounts is crucial for the production of oligofructans. Indigenous coprophilous fungi are predominantly sustainable bioresources, harbouring novel enzymes with potential industrial and biotechnological applications. Fructosyltransferase (Ftase) and inulinase are gaining considerable attention due to their capability to synthesise biofunctional nutraceuticals with low calories and health benefits when ingested in recommended dosages. Hence, due to several health benefits associated with prebiotics, bioprospecting for coprophilous fungi as unique bioresources of fructosyltransferase and inulinase was imperative. The present study therefore focused on the collection of herbivore dung from various terrestrial habitats in KwaZulu-Natal Province, South Africa whereby sixty-one (61) indigenous coprophilous fungal strains were isolated after repeated purification to monoculture. The axenic fungal strains were identified using morpho-taxonomic keys and molecular identification by 18S rDNA sequencing where *Neocosmospora* spp, *Trichoderma* spp., *Aspergillus* spp and *Fusarium* spp. were dominant. The fungal strains were subsequently assessed for their ability to produce extracellular and intracellular Ftase and inulinase enzymes. During the preliminary screening, the culture filtrate was examined for transfructosylating and hydrolytic activity using 2,3,5-triphenyl tetrazolium chloride (TTC) as a chromogenic marker and Lugol's iodine solution, respectively. Zones of hydrolysis on 30 fungal isolates were observed on the TTC assay plates in diameters ranging from 15 mm to 30 mm, representing high extracellular Ftase activity. The formation of clear zones following addition of iodine solution on inulin rich media indicated the presence of inulinolytic activity. Secondary screening involved DNS assays of eight (8) isolates that secreted high concentrations of Ftase while six (6) different fungal strains showed <50 % inulinase: invertase ratio. The final screening step was tertiary screening where products of biocatalysis were qualitatively detected by thin layer chromatography to visualize saccharide spots of fructooligosaccharides and inulooligosaccharides. HPLC analysis of Ftase and inulinase reaction products revealed and further confirmed that coprophilous fungi harbour fructosyltransferase and inulinase enzymes. The crude extracellular fructosyltransferase enzyme was partially purified by 9.3-fold with a yield of 7.3 % and a specific activity of 2465.5 U mg^{-1} after a three-step procedure involving $(NH_4)_2SO_4$ fractionation, dialysis and ion exchange chromatography. The apparent molecular weight of this Ftase was estimated by SDS-PAGE to be approximately 70 kDa. Zymogram analysis under non-reducing conditions

showed the enzyme migrating as a polydisperse aggregate yielding broad band of approximately 100 kDa. The enzyme further exhibited an enhanced activity at a broad pH range of 4.0 – 8.0 and optimal activity at a temperature range of 40 °C – 80 °C, while the enzyme was stable at pH 8.0 and between 40 °C – 60 °C, respectively. Under these conditions, the enzyme remained stable retaining 95 % residual activity after incubation for 6 h. The presence of metal ions such as Hg^{2+} and Ag^{2+} inhibited Ftase activity while, Ca^{2+}, Mg^{2+} and K^{+} at 1 mM increased the enzyme activity, with stabilization observed with Na^{+}, Zn^{2+} and $Cu^{2+.}$ With sucrose as the substrate, the enzyme kinetics fitted the Michaelis-Menten model. The K_m, V_{max} and k_{cat} values were 2.076 mM, 4.717 µmole min^{-1}, and 4.7 min^{-1}, respectively with a catalytic efficiency of 2.265 µmole min^{-1}. *In vitro* antioxidant potential of FOS by 1,1 - diphenyl-2-picryl hydroxyl (DPPH) assay, ferric reducing antioxidant power (FRAP) assay and nitric oxide (NO) radical inhibition yielded IC_{50} of 6.71 µg/ml, 1.76 µg/ml and IC_{25} of 0.27 µg/ml, respectively. Free radical scavenging and inhibition activities showed a concentration-dependent antioxidant activity with no significant differences with oligosaccharide standards ($p < 0.01$). However, vitamin C was significant in FRAP and NO assays. These results clearly demonstrated that an indigenous coprophilous fungus is a potential new reservoir of salient biotechnological enzymes that can be exploited for the production of prebiotics for subsequent biotechnological applications.

Dedication

This dissertation is dedicated to my lovely parents, Mr and Mrs Ojwach for their unconditional love and support.

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I thank the Almighty God for the gift of life and enabling myself to come here to South Africa to pursue my MSc qualification. Moreover, the completion of this study could not be possible without the input of my supervisors and distinguished academics Dr Taurai Mutanda and co-supervised by Prof Samson Mukaratirwa. I am grateful for their advice, guidance, mentorship belief in me, and for supporting me financially during hard times through the course of my research.

Special gratitude to the staff and my fellow postgraduate students in the Discipline of Microbiology at Westville Campus for their support. I would like to thank Mr Basil Naidoo for the orders and purchase of reagents, vehicle and consumables for my research work. I pay gratitude to my Lab 3 colleagues for assistance and companionship during this research period in South Africa.

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Finally, I would like to thank my uncle Prof S. Ojwach for the support he has given me and his family to come pursue my postgraduate studies. Special mention to my parents Mr and Mrs Ojwach, my siblings and the support of my church Calvary Covenant Centre in Nairobi for their prayers and support.

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Abbreviations

bp – Base pair

kDa - Kilodalton

BSA – Bovine serum albumin

PCR – Polymerase chain reaction

SDS – PAGE – Sodium dodecyl sulphate-polyacrylamide gel electrophoresis

Native – PAGE – Non-reducing/non-denaturing polyacrylamide gel electrophoresis

FOS – Fructo-oligosaccharide

IOS – Inulo-oligosaccharide

Ftase – Fructosyltransferase

Ffase – β-fructofuranosidase

scFOS – Short-chain fructooligosaccharide

GOS – Galacto-oligosaccharide

IMO – Isomalto-oligosacharide

MOS – Malto-oligosaccharide

XOS – Xylo-oligosaccharide

GRAS – Generally regarded as safe

FOSHU – Foods for specialized health use

GF_2 – 1-Kestose

GF_3 – Nystose

GF_4 – Fructofuranosyl nystose

F – Fructose

G – Glucose

S – Sucrose

DP – Degree of polymerization

DNS – 3,5-Dinitrosalicylic acid

DEAE – Diethyl-amino ethylene

SmF – Submerged fermentation

SSF – Solid-state fermentation

1 – SST – Sucrose: sucrose fructosyl transferase

1 – FFT – Fructan: fructan fructosyl transferase

DPPH – 1,1 diphenyl-2-picrylhydrazyl

FRAP – Ferric reducing antioxidant power

NO – Nitric oxide

ROS – Reactive oxygen species

·OH – Hydroxyl radical

iNOS – Inducible nitric oxide synthase

VLDL – Very low density lipoprotein

UHFS – Ultra high fructose syrup

k_{cat} – Catalytic constant

K_m – Michaelis-Menten constant

QFF – Q Sepharose® Fast Flow

V_{max} – Maximum rate of reaction

BLAST – Basic Local Alignment Search Tool

dNTP – Deoxynucleotide triphosphate

DNA – Deoxyribonucleic acid

PDA – Potato dextrose agar

MEA – Malt extract agar

CMA – Corn meal agar

CDA – Czapek dox agar

CPB – Citrate phosphate buffer

HPLC-RI – High performance liquid chromatography with refractive index detector

TLC – Thin layer chromatography

Cu - Copper

Hg – Mercury

Mg – Magnesium

Ag - Silver

K – Potassium

Ca – Calcium

$MgSO_4.7H_2O$ – Magnesium sulphate heptahydrate

GFn – Sucrose with n ranging from 2 to 10

GPS – Global positioning system

ANOVA – Analysis of variance

FAO – Food and agricultural organization

FDA – Food and drug administration

® - Registered trademark

pH – Pondus hydrogenii

pI – Isoelectric point

pKa – pH at which an acid is half dissociated

sp. – Species

TEMED – *N,N,N,N* –teramethyl-ethylenediamine

List of mathematical symbols

Micromole - µmol

Microliter - µl

Standard deviation - ±

Greater than or equal to - ≥

Less than or equal to - ≤

Millimetre – mm

Micrometre - µm

Microgram - µg

Millilitre - ml

Minutes - min

Seconds - s

Degree Celsius - °C

Hour - h

Gram per litre – g/L

Millimolar – mM

Nanometer – nM

Revolutions per minute – rpm

Weight per volume – w/v

Volume per volume – v/v

Parts per million – ppm

Glossary

Expression	Description
1-kestose	A carbohydrate sugar consisting of 2 parts fructose and 1 part glucose
Nystose	A carbohydrate sugar comprising of 3 parts fructose and 1 part glucose
1^F-fructoffuranosyl nystose	A carbohydrate sugar containing 4 parts fructose and 1 part glucose
β-D-fructofuranosidase	An enzyme with both transferase and hydrolase activity
Fructosidase	An alternative name for β-D-fructofuranosidase
Invertase	An alternative name for β-D-fructofuranosidase (Swiss Institute of Bioinformatics, 1998)

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CHAPTER 1: Introduction

1.1 General Introduction

Food has long been used to improve health, but more recently the knowledge of interrelation between food and health is now used to revamp food (MacAulay *et al.,* 2005, Dominguez *et al.,* 2014). Due to advances and desires in food technology and the emerging scientific evidence linking diet to disease, there is a need to address consumption of functional foods with health-promoting properties besides basic nutrition (Cassani *et al*., 2018). The design of food products that confer health-promoting properties is emerging and there is a growing acceptance that functional food can lead to disease prevention, well-being and treatment (Roberfroid, 1999a, Goldberg, 2012). Ideally, all food is functional in that, they provide energy and nutrients necessary for growth and survival (Roberfroid, 2000). The term functional food was first coined by Gibson and co-workers (Gibson and Roberfroid, 1995, Gibson *et al*., 1999) in that food supplements with health properties help in gut manipulation and composition towards a salutary regimen. These foods provide additional physiological benefit that may promote health or prevent disease (Hasler, 1996). This increases the proliferation of endogenous *Bifidobacterium* and *Lactobacillus* composition by fermenting short chain fatty acid (SCFAs) in the colon by creating a prebiotic effect (Femia *et al.,* 2002). Prebiotics are defined as non-digestible food ingredients that affect the host by selective stimulation of growth and/or of one or a limited number of bacteria in the gut and thus improves health (Gibson *et al.,* 2004). Prebiotics therapies have been claimed to cure gut related illness like constipation, insulin resistance, diarrhoea, obesity, probably type 2 diabetes and some cardiovascular disease associated with dyslipidaemia (Younis *et al.,* 2015). Fermentation features that have led to the emphasis on prebiotics go beyond basic nutrition as they also include also nutraceutical effects. For a food ingredient to be considered a prebiotic, it must resist gastric metabolism and hydrolysis from enzymatic activity. Secondly, the oligomers must be fermented by intestinal microbes and also stimulate the activity of selective bacteria in the colon associated with well-being (Barreteau *et al.,* 2006). Thirdly, they must have high rate of proliferation and affinity for adherence to intestinal mucosa (Gibson, 1999). In addition to these prebiotic effects, these food ingredients are still pursued due to their nutraceutical effects. The term nutraceutical was first conceived by S. DeFelice from nutrition and pharmaceutical to give a much needed identity and legitimacy to this amorphous nomenclatural area (DeFelice, 1992, DeFelice, 1995). It

is described as food that has health or medical benefits including prevention, treatment or disease control. Such products may range from dietary supplements like oligosaccharides, isolated nutrients and specific diets to genetically engineered designer foods, herbal products and processed foods (Kalra, 2003, Pandey *et al.*, 2010, Pearson, 2018). Specifically, these food products include oligosaccharides which are said to be dietary carbohydrates which play a more fundamental role as functional ingredients compared to probiotics, sugars, polyunsaturated fatty acids and peptides (Khodaei and Karboune, 2016). The prebiotic compound can also be applied to their fortification of different food products and develop food with high nutritional values (Panesar *et al.*, 2014). The requisite end products of carbohydrates metabolism are short-chain fatty acids majorly butyric, acetic and propionic acid, which are consumed by host organism as a source of energy (Al-Sheraji *et al.,* 2013). Sources of prebiotics such as non-digestible carbohydrates and oligosaccharides are plants like chicory, asparagus, onions, leeks and Jerusalem artichoke (Kalyani Nair *et al.*, 2010). Microbes are also widely documented as an alternative source of oligosaccharides production (Belorkar and Gupta, 2016, Michel *et al.,* 2016a). Current knowledge of carbohydrate fermentation in the gut, microbiome denote that health benefits of dietary fibre and prebiotics are not limited to stimulation of selective bacteria but attributed to the capacity of intestinal microbiomes for bioconversion of oligosaccharides to short-chain fatty acids (SCFA) (Yan *et al.*, 2018). Currently, prebiotics are recognized as oligosaccharides and polysaccharides, which are also included in the definition of fermentable Oligo-, Di-, Mono-saccharides and Polyols (FODMAPs) such as short-chain carbohydrates and dietary fibres (Gearry *et al*., 2009). Oligosaccharides are sugar combinations with the degree of polymerization (DP_3-DP_{10}) and are from plant inulin or produced commercially from sucrose as substrate (Hernández *et al.,* 2018). In the first approach, inulin is randomly cleaved from chicory by microbial endoinulinase (EC 3.2.1.7) yielding oligofructosides (Mutanda *et al*., 2014b). In the second approach sugar cane is fructosylated to GF_2, GF_3 and GF_4 by *β*-fructofrunosidases (EC 3. 2. 1. 26) or *β*-fructosyltransferases (EC 2.4.1.100) from fungal species of *Aureobasidium* and *Aspergillus* (Ganaie *et al.,* 2014, Bali *et al.,* 2015). Prebiotic oligosaccharides are mainly produced in three different ways, that is: isolation from plant sources (Roberfroid *et al.*, 1998, Scholz-Ahrens and Schrezenmeir, 2002), enzymatic degradation of polysaccharides (Prapulla *et al.,* 2000, Antosova and Polakovic, 2001), and microbial production or enzymatic synthesis (Al-Sheraji *et al.,* 2013). A combination of probiotics and prebiotics are used together to take advantage of synergic effects in food application and biotechnology and the

mixture is called synbiotic (Ziemer and Gibson, 1998). Due to health effects of functional foods, including their nutraceutical effect, this has led to numerous studies on food grade oligosaccharides which include fructooligosaccharides (FOS), inulooligosaccharides (IOS), xylo-oligosaccharides (XOS), and galactooligosaccharides (GOS) among the classes of prebiotics (Sangeetha *et al*., 2005b, Goulas *et al*., 2007, Lecerf *et al*., 2012). In order to produce foodborne FOS and IOS, microbial enzymatic syntheses remains attractive and desirable, as they are less toxic and hazardous to the environment, emit fewer emission and by-products, operates at low temperatures and biocatalysts can be reused easily. Furthermore, enzymes are more specific and utilize raw materials (Tomotani and Vitolo, 2007, Silvério *et al*., 2018). In this study, the feasibility of a novel approach to bioprospecting for indigenous coprophilous fungi from herbivore dung for transferase and inulinase enzymes was explored. To our comprehension, this is the first report on isolation of autochthonous coprophilous fungi as producers of fructosyltransferase and inulinase for potential biotechnological application.

1.2 Aims

The following aims and objectives of this study were pursued:

1.2.1 Sampling of coprophilous fungi from herbivore dung from various terrestrial environments for intracellular and extracellular inulinase and fructosyltransferase (Ftase) enzymes for IOS and FOS production.

1.2.2 Purification of fungi to axenicity by conventional microbiological techniques and investigation of inulinolytic and Ftase activity from a wide range of coprophilous fungal strains.

1.2.3 Identification of fungi using morpho-taxonomic keys and molecular biology tools and selection of the best fungal strain showing potential for highest inulinase and fructosyltransferase activity.

1.2.4 Partial purification of the crude enzyme of either Ftase or inulinase, characterize the enzyme and determine its enzyme kinetics properties.

1.2.5 Demonstration of potential biotechnological applications of the partially purified Ftase or inulinase enzyme.

1.3 Objectives

1.3.1 To collect samples of fresh pieces of herbivore dung in sterile bags and isolation of coprophilous fungi using normal microbial plating techniques.

1.3.2 To screen for unique inulinase and fructosyltransferase from fungal extracts from herbivore dung using a chromogenic technique for transfructosylation and inulinolytic activity. To further identify the pure fungal strains using morpho-taxonomic techniques and molecular biology tools (18S rDNA) and to select the best fungal strain.

1.3.3 To detect, separate, identify and quantify FOS and IOS using thin layer chromatography (TLC) and high performance liquid chromatography (HPLC).

1.3.4 To purify, characterize the enzyme, and determine its physicochemical and biochemical properties such as pH, temperature optima and stability. To determine the effects of different metal ions and compounds on enzyme activity.

1.3.5 To estimate the molecular weight of either Ftase or inulinase enzyme by SDS-PAGE and perform zymogram analysis by native-PAGE.

1.3.6 To determine enzyme kinetics K_m, V_{max} and k_{cat} values for the partially purified enzyme by using sucrose or inulin as the substrate in citrate-phosphate buffer at optimal pH and temperature.

1.3.7 To investigate the potential biotechnological applications of IOS or FOS as food supplements and their antioxidant properties.

CHAPTER 2: Literature Review

2.1 What are Oligosaccharides?

Fructo-oligosaccharides (FOS) are natural food products with beneficial health effects to the human colon when consumed in recommended dosages, by selectively stimulating the proliferation of *Bifidobacteria* and *Lactobacilli* while concurrently suppressing the growth of potentially pathogenic microbiota such as Clostridia (Al-Sheraji *et al*., 2013, Zhang *et al*., 2017a). FOS are oligosaccharides of fructose consisting of a glucose unit (G) connected with fructosyl units (F) at the *β*-(2-1) position of sucrose (Ganaie and Gupta, 2014, Bersaneti *et al*., 2018). It is for this reason that FOS have received particular attention as biofunctional food products. Due to their biofunctional properties, FOS can safely be consumed as alimentary additives, are safe for diabetics, are non-cariogenic as nutraceutical compounds, and are therefore termed prebiotics (L'Hocine *et al*., 2000, Saminathan *et al*., 2011, Dominguez *et al*., 2014, Ganaje *et al*., 2014, Mutanda *et al*., 2014b). FOS are short-chain carbohydrates, which are not digested in the upper part of the gastrointestinal tract (GIT), therefore they are also referred to as non-digestible oligosaccharide (NDO) (Al-Sheraji *et al*., 2013, Benkeblia, 2013). The linkage type between their monosaccharide residues distinguishes FOSs from other polysaccharides. They consist of fructan oligomers mainly of 1- kestose (GF_2), nystose (GF_3) and 1^F- fructofuranosyl nystose (GF_4) and are termed prebiotics (Panesar *et al*., 2014, Meyer *et al*., 2015). Prebiotics are compounds that selectively stimulate proliferation of gut microbiota in the colon by inhibiting pathogenic microbes, protonation of potentially toxic ammonia and amines, diminution of total cholesterol in the blood, relieving constipation, triglyceride and phospholipids (Brownawell *et al*., 2012). The human colon is one of the mostly colonized and metabolically active organ in the human body. It presents different bacterial composition and variability is largely due to different physicochemical conditions like favourable pH, slow transit time and nutrient availability in the gut (Roberfroid, 1999b, Brownawell *et al*., 2012). The human body does not have the necessary enzymes to hydrolyze *β*-glyosidic linkages of sugars consumed and as such, non-digestible oligosaccharides (NDO) can ferment these sugars creating a prebiotic effect (Zhao and Cheung, 2011). Prebiotics also display secondary functions like mineral absorption, synthesis of vitamin B-complex, immune system activation and non-cariogenecity (Tanriseven and Gokmen, 1999, Ganaie *et al*., 2013). The human gut ferments a range of carbohydrates that pass the ileum and are available for fermentation in the colon (Dominguez *et al*., 2014). Additionally, FOS have generated a great demand in the

global food market and are generally regarded as safe (GRAS). Synthesis of FOS occurs through the catalytic action of transfructosylating enzymes, which are classified into two categories. *β*-D-fructofuranosidase (Ffase) (EC 3.2.1.26) and fructosyltransferases (Ftase) (EC 2.4.1.9) (Lorenzoni *et al*., 2014, Bali *et al*., 2015). The Ffase enzyme possess both hydrolytic and transfructosylating activity as it releases glucose molecule from sucrose by cleaving the *β*-1, 2-glyosidic linkage and thereby shifting the fructosyl group to sucrose forming FOS products (Fernández *et al*., 2004). Ftases exhibit a high transfructosylating activity by catalyzing the transfer of fructosyl moiety from one sucrose molecule to another in order to produce higher FOS units as major products (Bali *et al*., 2015). These enzymes occur in many higher plants like *Cichorium intybus* and *Helianthus tuberosus* that produce high levels of Ftase such as sucrose: sucrose fructosyltransferase (1-SST, EC.2.4.1.99) and fructose: fructose 1-fructosyltransferase (1-FFT, EC 2.4.1.100) (Nemukula *et al*., 2009). Microorganisms such as fungi e.g. *Aspergillus niger* ATCC 20611, *Aspergillus niger* strain AN 166, *Aspergillus foetidus*, *Aspergillus oryzae* CFR 202 and *Aureobasidium pullulans* CFR 77 have been largely documented to produce enzymes with both hydrolytic and transfructosylating activities (Michel *et al*., 2016b). Bacterial strains have also been reported to produce Ftase for FOS production but only a few species have been mentioned which include *Bacillus macerans*, *Lactobacillus reutri*, *Streptococcus mutans* and *Zymomonas mobilis* (Russell *et al*., 1983, Burne *et al*., 1987, Shiroza and Kuramitsu, 1988, Cheetham *et al*., 1989, Park *et al*., 2001, van Hijum, 2004, Michel *et al*., 2016b). FOS consist mainly of 1-kestose (GF_2), nystose (GF_3) and 1-*β*-D-fructofuranosyl nystose (GF_4) which have 1-3 fructose units bond to the *β*-2-1 position of sucrose (Sangeetha *et al*., 2005b, Manosroi *et al*., 2014).

Numerous species of microorganisms such as *Aspergillus niger, Aspergillus oryzae, Aspergillus japonicas* and *Aureobasidium pullulans* have been studied for their ability to produce FOS. Strains of bacteria, yeasts and moulds have been reported, but the most studied and investigated are fungi belonging to the genera *Aureobasidium*, *Aspergillus* and *Penicillium* (Hirayama *et al*., 1989, Park and Almeida, 1991, Hayashi *et al*., 1992, Yun *et al*., 1992, Yun *et al*., 1997a, Yun *et al*., 1997b, Ganaie *et al*., 2013). For a food ingredient to be classified as a prebiotic, it must not be hydrolyzed or absorbed in the upper part of the gastrointestinal tract, it must be a selective substrate specific to beneficial bacteria commensal to the colon, it must be able to alter the colonic microbiota of healthier individuals and should induce systemic flow beneficial to individual health (Glenn and Roberfroid, 1995, Prapulla *et al*., 2000, Roberfroid, 2000). Inulin-type and non-digestible

oligosaccharides are among the group of products reported to have important health benefits and their functionality as a food ingredient (Khodaei and Karboune, 2018). They provide a dietary and prebiotic effect on human health (Delzenne *et al*., 2007).

The demand for functional food is growing and efforts are being made to identify and screen novel microorganisms that are stable and have the desired activity to produce Ftases and inulinases. The present study, therefore, focuses on exploiting new fungal strains with robust transfructosylating and inulinolytic activity for the synthesis of FOS and IOS, respectively.

2.1.1 Overview of Oligosaccharides

Oligosaccharides form part of new functional foods with great potential to improve health due to their physicochemical characteristics. They are classified as oligosaccharides, since they contain 3 - 10 simple sugar moieties that are linked together (Kothari *et al*., 2014). Commercially, there are approximately 12 classes of food grade oligosaccharides produced (Table 2.1). Most food grade oligosaccharides are produced from simple sugars such as lactose or sucrose (Han *et al*., 2009). They can also be produced from the controlled hydrolysis of starch or other polysaccharides (Murphy, 2001). Oligosaccharides produced from these enzymatic reactions are not homogeneous, but rather contain oligosaccharide mixtures of different molecular weights, glycosidic linkages and monosaccharide residues (Crittenden and Playne, 2002). Food grade oligosaccharides are not pure products as they contain mixtures with varying degrees of polymerization, disaccharide and monomer sugars (Crittenden and Playne, 1996). Oligosaccharides are carbohydrates with a low molecular weight and degree of polymerization. Carbohydrates are the main group forming oligosaccharides and their monosaccharide units are glucose, galactose, fructose and xylose (Crittenden and Playne, 1996). The concept of non-digestible oligosaccharides emanates from the survey that carbon atoms of the monosaccharide have some disposition that make glycosidic bonds resistant to hydrolytic activity of enzymes in the human intestine (Sangeetha, 2003). Oligosaccharide stability differs according to classes depending on sugar residues present, and their anomeric configuration. Predominantly β-linkages are stronger and stable than α-linkages, and hexoses are more strongly linked than pentoses (Voragen, 1998). Furthermore, pyranoses and deoxysugars are more strongly linked than furanoses. They differ from FOS as they can be hydrolyzed resulting in loss of nutritional and physicochemical properties (Mudgil and Barak, 2013). They also have high moisture retaining capabilities, preventing excessive drying and low

water activity that inhibits microbial contamination (Mussatto and Mancilha, 2007). A review by Sako *et al*., (1999) has reported the caloric value of non-digestible oligosaccharide estimated at 1.5- 2.0 kcal/g average 45 % digestible carbohydrate like sucrose (Sako *et al*., 1999).

Table 2.1: Different types of food grade oligosaccharides (Playne and Crittenden, 1996, Murphy, 2001, Crittenden and Playne, 2002)

Type of Oligosaccharide	Sources	Industrial production
Lactosucrose		Synthesis from lactose and sucrose either by β-fructofuranosidase (E.C. 3.2.1.26) or levansucrase (E.C. 2.4.1.10)
Lactulose		Synthesis of lactose
Galacto-oligosaccharides	Breast milk	Synthesis of lactose
Malto-oligosaccharides		Synthesis from starch
Isomalto-oligosaccharides	Dextran and maltose	Dextran hydrolysis
Fructo-oligosaccharides	Fruits and vegetables such as garlic, onions, asparagus.	Synthesis of sucrose by Ftase or Ffase
Inulo-oligosaccharides	Chicory roots, and leaves	Enzymatic hydrolysis of the polysaccharide inulin by endo-inulinases
Soybean oligosaccharides	Soybean	
Xylo-oligosaccharides		Polyxylans hydrolysis
Cyclodextrin	Vegetables such as potato	Starch synthesis
Glycosyl sucrose		
Gentio-oligosaccharides		Produced from starch via a combination of hydrolytic and transglucosylase enzymes

2.1.2 Chemical Structure of FOS

FOS are generally known as inulin-type and are short-chain fructooligosaccharides containing D-fructose of linear polymers and oligomers connected by *β*-(2-1) linkages bonds (Vogt *et al*., 2015). A glucose molecule typically resides at the end of each fructose chain where it is linked by an α-(1-2) bond like in sucrose (Wang, 2015). Inulin is a highly polymerized fructan with chain length ranging from 2 - 60 units and an average degree of polymerization (DP) of 25 with its molecular distribution ranging from 11 to 60 monomers (Niness, 1999, Barclay *et al*., 2010, Mensink *et al*., 2015). While, FOS are fructan with DP ranging from 2 to 10 (Cassani *et al*., 2018). They are depicted by the formula GF_n and constitute a series of homologous oligosaccharide gleaned from sucrose (Yun, 1996a, Yun, 1996b). FOS are short-chain oligosaccharides and members of the fructan group having a general glucose unit linked to several fructose units. Fructans present in nature can be distinguished based on glycosidic linkages where fructose residues are linked together. They can be divided into three distinct groups. The first group are inulin where fructose units are linked through *β*-(2-1) bond, the second group are levans, which are linear fructans and the fructose units are linked via a *β*-(2-6) bond and the third group graminan fructan of mixed type which has both *β*-(2-1) and *β*-(2-6) linkages bonds between fructose units (Ponce S *et al*., 2008). FOS is mainly used for short inulin-type fructose with a terminal glucose moiety bound to the *β*-(1-2) glycosidic bonds (Antošová and Polakovič, 2001).

Chain length or degree of polymerization has a vital role in inulin biofunctionalities. ~~Biofunctional attributes of inulin and oligofructose are attributed to their chain length~~. Inulin has a longer chain length than oligofructose which makes it less soluble and it forms inulin microcrystals when sheared in water or milk (Niness, 1999). Oligofructose is defined by IUB-IUPAC Joint Commission on Biochemical Nomenclature as a fructose oligosaccharide containing 2-10 monosaccharide residues connected by glycosidic linkages (Flamm *et al*., 2001b). Oligofructose are short chain oligomers that display similar functional properties to glucose syrup or sugar. Its solubility is higher than sucrose and accounts for 30 - 50 % of sugars. Oligofructoses, have numerous nutritional properties such as providing crispiness to low-fat cookies, acts as a binder in nutritional or granola bars (Cherbut, 2007). Since inulin and oligofructose have desirable functional properties they are used together and offer dietary fibre effect leading to reduced caloric effect in foods than typical carbohydrates because they possess *β*-(2-1) bonds linking fructose molecule (Niness, 1999). FOS are substantially composed of 1-kestose, 1-nystose and 1-*β*-

fructofuranosyl nystose. The chemical structures of oligosaccharides are depicted by Dominguez *et al*. (2014) showing 1-kestose (Figure 2.1), 1-nystose (Figure 2.2), 1-*β*-fructofuranosyl nystose (Figure 2.3).

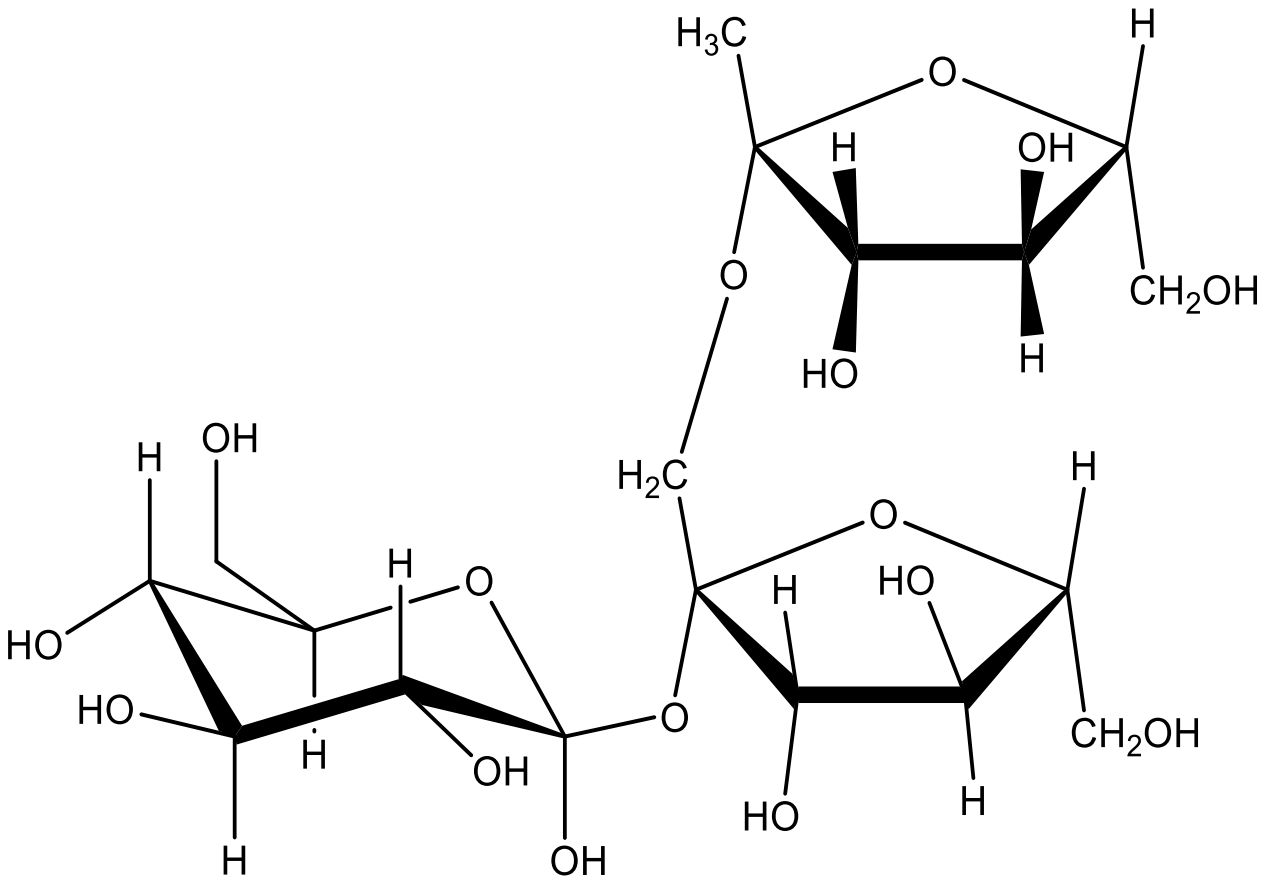


Figure 2.1: Structure of 1-kestose (GF_2) adapted from (Dominguez *et al.*, 2014).

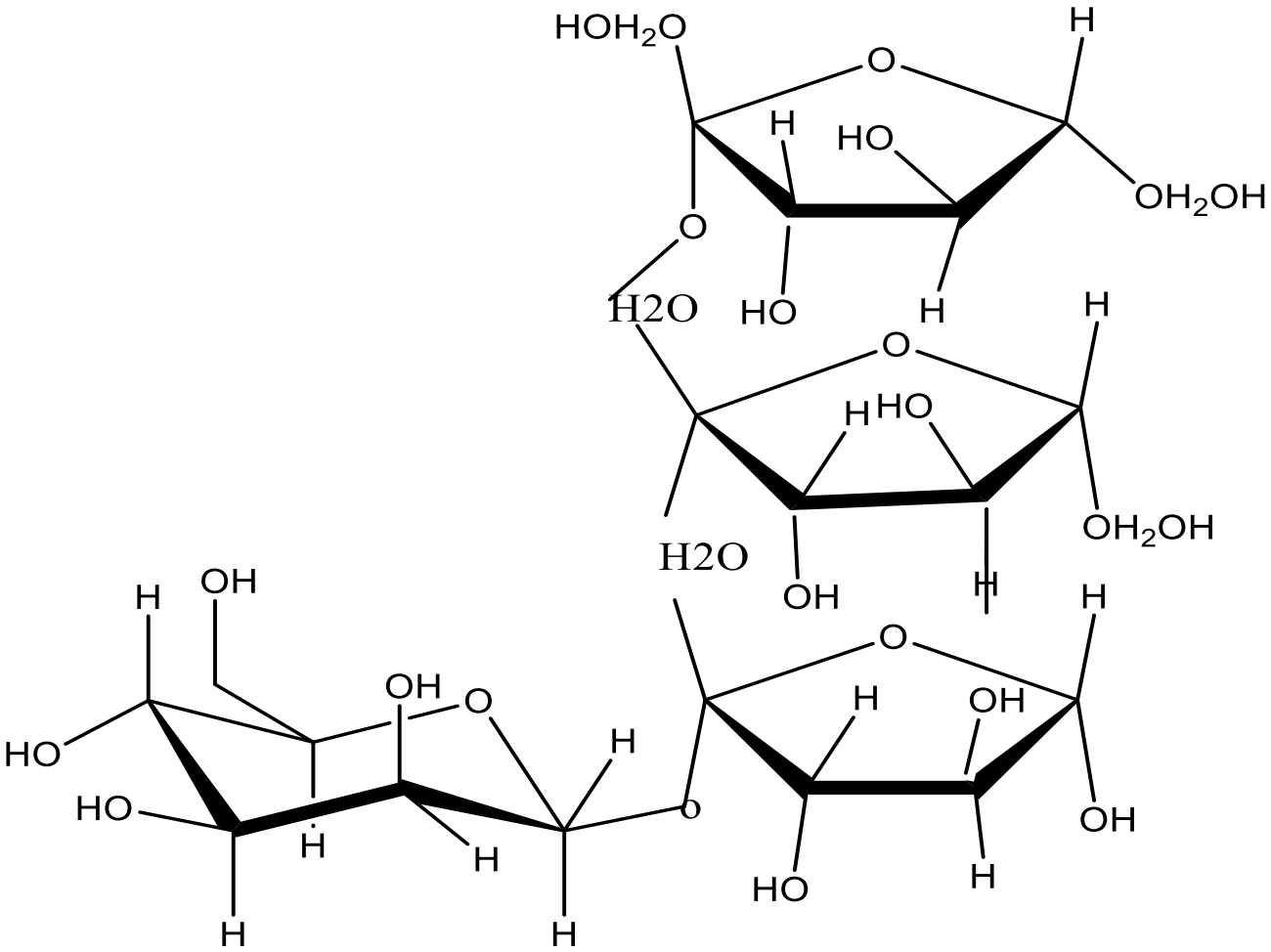


Figure 2.2: Structure of 1-nystose (GF_3) adapted from (Dominguez *et al*., 2014).

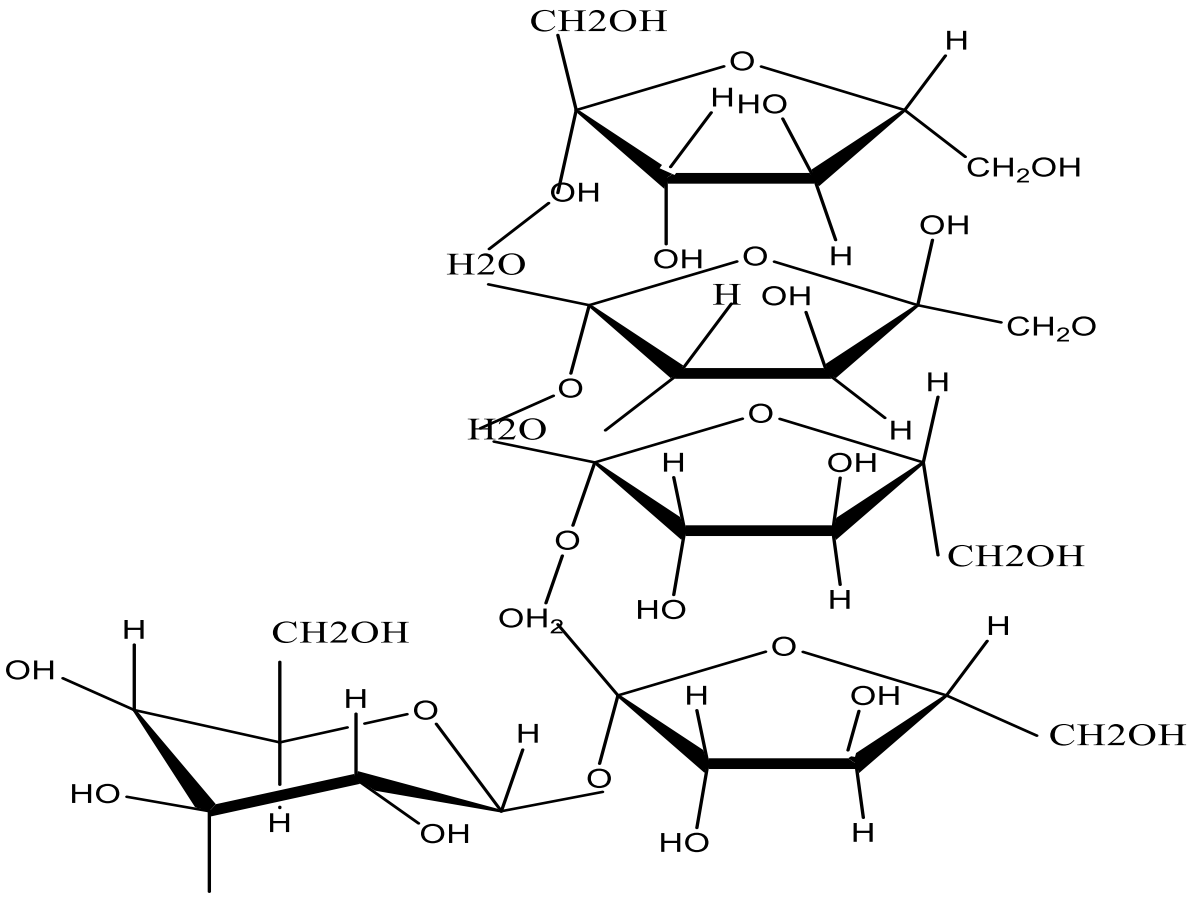


Figure 2.3: Structure of 1-*β*-fructofuranosyl nystose (GF_4) adapted from (Dominguez *et al.*, 2014).

2.1.3 Physicochemical and Functional Properties of Oligosaccharides

Oligosaccharides have biofunctional and physicochemical properties that make them desirable for consumption as food ingredients or supplements. Incorporation of oligosaccharides enriches the rheological and physiological characteristics of foods (Alméciga-Díaz *et al*., 2011). This is predominantly due to their undermentioned specific properties: They are water soluble, slightly sweeter than sucrose (0.3 - 0.6 times), but the sweetness is dependent on the degree of polymerization, chemical array and level of mono and disaccharides present in the mixture (Mussatto and Mancilha, 2007, Ur Rehman *et al*., 2016). The viscosity of fructo-oligosaccharides solution is relatively higher than that of mono-and disaccharide (sucrose) at the same concentration (Prapulla *et al*., 2000). They are more viscous due to their higher molecular weight (Patel and Goyal, 2010). They have humectant properties, which alter the amount of browning in food by recasting the freezing temperature of some foods (Voragen, 1998). They control microbial

contamination by absorbing water since they act as a drying agent due to their moisture-retaining capabilities (Roberfroid and Slavin, 2000b). FOS have higher thermal stability than sucrose, moreover, they are stable within normal pH range of foods (pH 4.0 - 7.0) (Prapulla *et al*., 2000). Their stability is dependent on ring form, sugar residue content, anomeric configuration and linkage type. Principally, β-linkages are stronger compared to α-linkages while hexoses are strongly linked than pentoses (Patel and Goyal, 2010). Oligosaccharides are used as low cariogenic sugar substitutes as they are inactivated by mouth enzymes or in the upper gastrointestinal tract to form acid or polyglucans due to their physicochemical characteristics of being less sweet, thus making it suitable for consumption by diabetics (Kwak and Jukes, 2001, Roberfroid, 2007a). They show immoderately high structural diversity than oligonucleotides and oligopeptides (Raman *et al*., 2005). They are also used as bulking up components with sucralose, aspartame, and phenylalanine providing effects similar to dietary fibres (Crittenden and Playne, 1996). Moreover, most oligosaccharides have shown strong inhibition of starch retrogradation leading to improved body and mouth feel (Topping and Clifton, 2001, Wang *et al*., 2015).

2.2 FOS Occurrence

FOS derived from sucrose are produced in many higher plants as reserve carbohydrates. The plants include asparagus, garlic, chicory, sugar beet, Jerusalem artichoke, onion, wheat, and tomatoes while some are found in trace amounts in edible fruits like banana (Figure 2.4) (Roberfroid *et al*., 1998, Flamm *et al*., 2001b, Sangeetha *et al*., 2005b, Dominguez *et al*., 2014). FOS concentration is minimal in these sources and is limited by changing seasonal conditions (Michel *et al*., 2016b). FOS and IOS can be produced using three methods, either from extraction from inulin-rich plant material, or by enzymatic synthesis of sucrose, and/or degradation of inulin by enzyme hydrolysis, respectively (Kango and Jain, 2011, Fernandes *et al*., 2013, Rawat *et al*., 2017b). The majority of FOS like inulin which are food ingredients are synthesized through enzymatic degradation of plant polysaccharides or synthesized from sucrose by fructosyltransferase activity (Flamm *et al*., 2001a). Inulin as a food ingredient with nutritional health-promoting features together with its hydrolytic products, is generally regarded as safe (GRAS) (Kumar *et al*., 2015, Singh *et al*., 2016b).

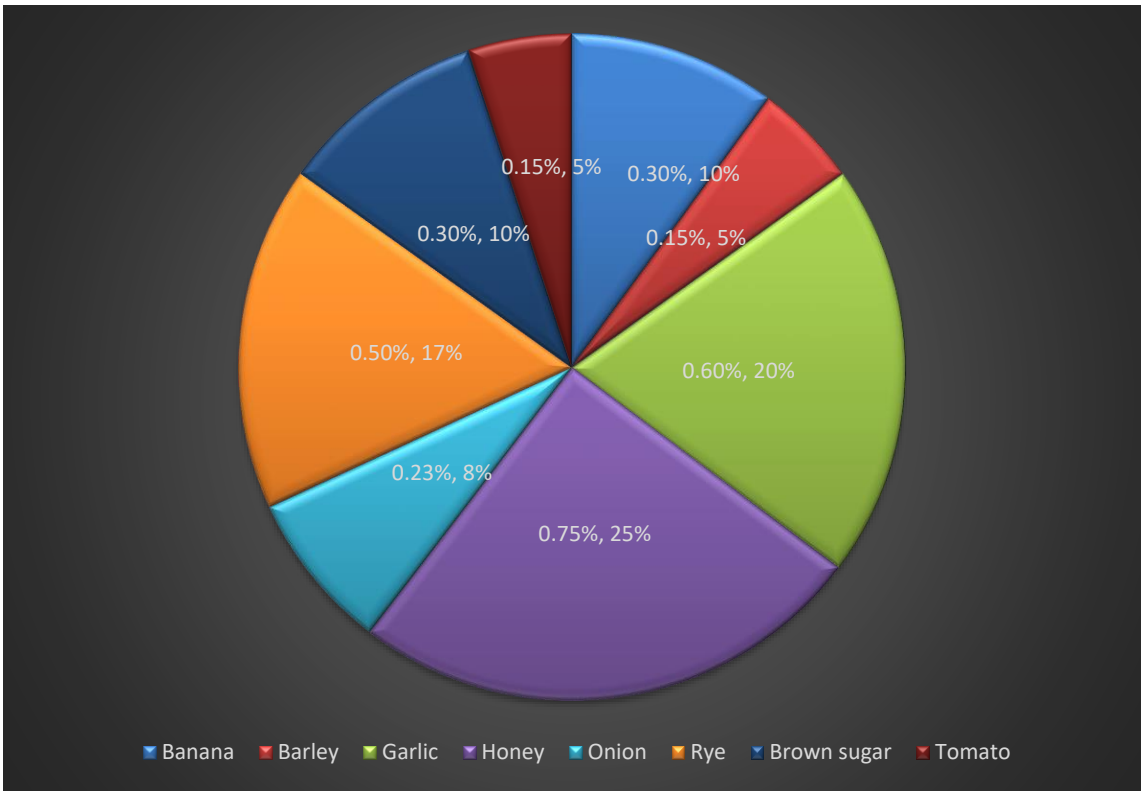


Figure 2.4: FOS concentration in some natural foods mentioned according to the data of the Environmental Protection Agency Dietary Risk (EPA 1984) (Sangeetha, 2003).

Chicory species (*C. intybus*), Jerusalem artichoke (*H. tuberosus*), dahlia (*Dahlia pinnata*) and yacon (*Polymnia sonchifolia*) are the major sources of inulin as inulin content in this plants range from 10 % to 20 % of fresh weight (Apolinário *et al*., 2014); Notwithstanding the fact that at harvest, the roots of chicory contain 75 % inulin on a dry matter basis as compared to Jerusalem artichoke. Moreover, chicory is a more reliable source of inulin than other plant sources due to less variability in crop yield annually (Monti *et al*., 2005, Lucchin *et al*., 2008). Inulin derived from plants are polydisperse and from different mixtures of carbohydrates with varying chain length and molecular weights depending on their growth conditions (Mutanda *et al*., 2014b).

FOS and their derivatives can also be derived from microbial sources, as they are isolated from plants. FOS are synthesized by a wide array of enzymes like inulinases and fructosyltransferases in large-scale industrial production. It has been reported to be difficult to source this enzyme from plants hence the need for a microbial approach, which can be bacteria, yeast or fungi (Sangeetha

et al., 2005b, Mutanda *et al*., 2014b). Various microbial and plant sources of IOS and FOS are presented in Table 2.2.

Table 2.2: Microbial and plant sources of FOS synthesizing enzymes

Fungal source	Plant source	Bacterial source
Aureobasidium pullulans *Aureobasidium sp.* *Aspergillus oryzae* *Aspergillus japonicas* *Aspergillus niger* *Aspergillus phoenics* *Aspergillus phoenics* *Aspergillus foetidus* *Aspergillus sydowi* *Calviceps purpurea* *Fusarium oxysporum* *Penicillium frequentans* *Penicillium spinulosum* *Phytophthora parasitica* *Penicillium citrinum* *Scopulariopsis brevicaulis* *Saccharomyces cerevisiae* (Voragen, 1998, L'Hocine *et al*., 2000, Sangeetha, 2003, Sangeetha *et al*., 2005b, de Oliveira Kuhn *et al*., 2013, Mutanda *et al*., 2014b, Singh and Chauhan, 2017)	*Agave vera cruze* *Agave americana* *Asparagas officinalis*(asparagus roots) *Cichorium intybus* (Chicory) *Allium cepa* *Crinum longifolium* (Sugar beet) *Helianthus tuberosus* (Jerusalem artichoke) *Lactuca sativa* *Lycoris radiate* *Taraxacum officinale* (Sangeetha, 2003, Sangeetha *et al*., 2005b, Dominguez *et al*., 2014, Mutanda *et al*., 2014b)	*Lactobacillus reuti* *Arthrobacter sp* *Bacillus macerans* *Zymomonas mobilis* *Pseudomonas* sp.(Kalil *et al*., 2001, Sangeetha *et al*., 2005b, Catana *et al*., 2007, Singh *et al*., 2017a)

2.3 Enzymes used for FOS Production

Plants and microbes are documented as major sources of inulinases and fructosytransferases for the production of FOS and inulooligosaccharides, respectively. Moreover, FOS such as 1-kestose, nystose and fructofuranosyl nystose are synthesized by a wide array of enzymes for industrial application (Mutanda *et al*., 2014b). Industrially salient FOS consist mainly of 1-kestose, nystose and fructofuranosyl nystose (Prapulla *et al.*, 2000). Conventionally, chemical methods have been used for the synthesis of oligosaccharides but have posed several disadvantages. These methods use hazardous and expensive chemicals. They are laborious since they require rigid monitoring due to multiple reaction steps. More importantly, they result in low yield with high costs of production, which is inherently undesirable (Feizi *et al*., 2003). This has prompted a move to a more feasible microbial production system which is increasingly attractive for the industrial production of biofunctional prebiotics (Prapulla *et al*., 2000, Schmid *et al*., 2001).

FOS is produced by transfer of fructose residues to sucrose molecules by the action of fructosyltransferase (E.C.2.4.1.9) or *β*-fructofuranosidase (E.C.3.2.1.26) and inulinase enzymes, respectively (Table 2.3) (Prapulla *et al.*, 2000, Guío *et al.*, 2009). Inulinases are divided into two subclasses mainly due to their different mode of action. Exoinulinases (EC: 3.2.2.80) which cleave fructose from the non-reducing sugar end of inulin through hydrolysis and is mainly used in the synthesis of ultrahigh fructose syrup. Endoinulinases (EC:3.2.1.7) hydrolyses inulin into inulooligosaccharides. Inulooligosaccharides produced from inulin are suggested to have corresponding physiological functions to FOS with variations in their degree of polymerization (Cho and Yun, 2002). Numerous strains of fungi, yeast and bacteria such as *Aspergillus niger, Aspergillus ficuum, Arthrobacter* sp, *Penicillium purpurogenum*, *Bacillus macerans* and *Streptococcus mutans*, respectively, are sources of endoinulinases (Shiroza and Kuramitsu, 1988, Park *et al*., 2001). Moulds are the most prominent groups producing endoinulinases and interestingly, few fungal species have both exo and endoinulinase properties (Singh *et al*., 2016b).

Table 2.3: Synopsis studies of microbes used for FOS production and yield produced

Microorganism	Enzyme	Optimal condition	Substrate (g/L sucrose)	Yield (%)	Reference
Aspergillus niger AS 0023	β-fructofuranosidase (EC2.1.4.9) free enzymes Extracellular ftase Intracellular ftase	40 – 60 °C, pH 6.0 -8.5 Sucrose 40 - 70 %	500	54	(L'Hocine *et al.*, 2000)
Aspergillus japonicus	β-fructofuranosidase (EC 3.2.1.26) free enzymes. Intra and extracellular ftase Extracellular ftase Extracellular ftase	55 °C, pH 5.5, Sucrose 65 %	400	55.8	(Wang and Zhou, 2006)
Aspergillus oryzae CFR 202	Fructosyltransferase (EC 2.1.4.9) free enzymes Extracellular ftase	55 °C, pH 5.5, 24 h Sucrose 55%	600	58	(Sangeetha, 2003) (Sangeetha *et al.*, 2004)
Penicillium citrum	Neo-fructosyltransferase free mycelia	50 °C, 40 h - 100 rpm Sucrose 70 %	700	55	(Lim *et al.*, 2005a, Lim *et al.*, 2005b)
Rhodotorula sp.	Extracellular β-fructofuranosidase and fructosyltransferase	72 °C – 75 °C, pH 4.0, 65 °C – 70 °C, 48 h	500	48	(Hernalsteens and Maugeri, 2008)
Zymomonas mobilis	levansucrase	24 h	500 - 600	24 - 32	(Bekers *et al*., 2002)

Table 2.3b: Continuation

Microorganism	Enzyme	Optimal condition	Substrate (g/L sucrose)	Yield (%)	Reference
Aspergillus sp. N74	Fructosyltransferase (EC 2.1.4.9)	pH 5.5 temp 60 ºC at 350 rpm sucrose con 70% w/v	700	57	(Sánchez *et al*., 2008, Sánchez *et al*., 2010)
Bacillus macerans EG-6	Fructosyltransferase (EC 2.4.1.9) free enzymes fructosyltransferase	50 °C, pH 5.0 - 7.0, 100 h	500	33	(Park *et al*., 2001)
B. macerans EG-6		37 °C, pH 6.0, 40 h	500	GF_4 (42.3)	(Woo Kim *et al*., 1998)
Aureobasidium pullulans CFR 77	Fructosyltransferase (EC 2.1.4.9) free enzymes Extracellular ftase	55 °C,pH5.5, 9 - 24 h Sucrose 80 %	200	59	(Lateef *et al*., 2007a, Lateef *et al*., 2007b) (Chi *et al*., 2009b)
Aureobasidium pullulans CCY-27-1-1194	Extracellular and intracellular fructosyltransferase	55 °C, pH 5.5, 48 – 72 h	350	52 - 56	(Antosova *et al*., 2003)
Penicillium purpurugenum	Extracellular and intracellular fructosyltransferase	30 °C, pH 5.5, 720 h	10	58	(Dhake and Patil, 2007)

Table 2.3c: Continuation

Microorganism	Enzyme	Optimal condition	Substrate (g/L sucrose)	Yield (%)	Reference
Aspergillus japonicus	β-fructofuranosidase	28 °C, pH 5.5, rpm 200, 72 h	150 - 180	55.2	(Chen and Liu, 1996)
Aspergillus aculeatus	Ftase from commercial enzyme: Pectinex Ultra SP-L	60 °C, pH 5.0 – 7.0, 24 h 60 °C, pH 6.0, 16 h	600 600	60.7 88	(Ghazi *et al*., 2005) (Ghazi *et al.*, 2007) (Nemukula *et al.*, 2009)
Penicillium expansum	β-fructofuranosidase	60 °C, pH 5.0 – 6.5,	200	GF_2 80 %, GF_3 19 %, GF_4 1%	(Prata *et al.*, 2010)
Aspergillus foetidus NRRL 337	Extracellular fructosyltransferase (EC 2.4.1.9)	40 °C – 45 °C, pH 5.0, 120 h	260 - 470	26% - 47 %	(Hang *et al*., 1995)
Penicillium citrium FERM P-15944	B-fructofuranosidase	30 °C, pH 4.0, 100 rpm, 72 h	100	57	(Hayashi *et al*., 2000)

2.4 Fungal Fructosyltransferases

Fungal Ftases have a molecular mass ranging between 180,000 - 600,000 Da and are homopolymers with two to six monomeric units (Maiorano *et al*., 2008). Fructofuranosidase isolated from *Aspergillus oryzae* is a monomer with a molecular weight of 87000 - 89000 da (Antošová and Polakovič, 2001, Dominguez *et al*., 2014). Several studies on features of transfructosylation enzymes produced by some species of *Aspergillus* and *Aureobasidium* produced a maximum yield of FOS after media cultivation and the isolated enzyme displayed both hydrolytic and transferase activity. Yoshikawa *et al.*, (2006) reported Ftase from the cell wall of *Aureobasidium pullulans* with high transferase activity. The Ffase 1 was not hindered by glucose but those of Ffases II-V were inhibited by the presence of glucose during the reaction. In fungi, Ffase 1 plays a major role in FOS formation while Ffase IV has a strong hydrolytic action that may degrade FOS in fungi (Dominguez *et al.*, 2014). A number of fungi such as species of *Aspergillus*, *Aureobasidium,* and *Penicillium* are known to produce both intracellular and extracellular *β*-fructofuranosidase and fructosyltransferase enzymes, respectively (Barthomeuf and Pourrat, 1995, Chi *et al*., 2009b, Kurakake *et al*., 2009, Lateef *et al*., 2012, Mashita and Hatijah, 2014, Xu *et al*., 2015, Ademakinwa *et al*., 2017). Fungal enzymes are reported to produce high yields of FOS (Yun, 1996b). Predominantly, *Aspergillus* species have received particular interest. *A. niger* and *A. oryzae* have been exploited for biotechnological programs for enzyme production since they have GRAS status (Maiorano *et al*., 2008). Other microorganisms such as *Penicillium rugulosum* (Barthomeuf and Pourrat, 1995), and *Aspergillus phoenicis* CBS 294.80 which produces a thermostable inulinase for industrial fructose production also produces a sucrose-1^F-fructosyltransferase, SFT (E.C 2.4.1.99) (Van Balken *et al*., 1991). Fungal Ftases have been the focal point as numerous studies on industrial biotechnology have described the isolation and screening of intra- or extracellular fructosyltransferase. *Aspergillus japonicus*, with five other mould strains, were selected after a screening exercise revealed their ability to produce transferase enzymes (Chen and Liu, 1996). In addition, (Madlov *et al.*, 2000) investigated two strains of *A. pullulans* and *A. niger*. After the screening exercise, the microorganisms revealed transfructosylation ability by their catalytic action of fructosyltransferase. Afterwards (Fernandez *et al*., 2007), reported screening of seventeen different filamentous fungi grown in batch cultures and compared their ability to produce *β*-fructofuranosidase and fructosyltransferase. The findings

revealed three strains of *A. niger* ATTC 20611, IPT-615 and *A. oryzae* IPT-301 as potential candidates for industrial Ftase production.

Screening of new fungal isolates is always a difficult and laborious procedure due to a number of considerations (Maiorano *et al*., 2008). However, numerous reports still exist on screening fungi for biotechnological applications. A presumptive and indirect colourimetric plate assay was employed for screening a filamentous fungus for transfructosylation ability. The method was to simultaneously determine the release of fructose and glucose from sucrose biotransformation. A glucose oxidase-peroxidase reaction using phenol and 4-aminoantipyrine was used for glucose determination. Fructose dehydrogenase oxidation in the presence of tetrazolium salt was used for fructose determination. The formation of a pink halo was observed which revealed the presence of glucose, while blue halo formation revealed and further confirmed the presence of fructose and transfructosylation activity (Dominguez *et al*., 2006b). Other studies on screening fungal and yeast species for Ftase production have been reported as they are a more feasible and economic source of biocatalytic enzymes (Guimarães *et al.,* 2006, Maugeri and Hernalsteens, 2007, Ganaie *et al*., 2013, Lama, 2017). Based on this evaluation, fungal Ftases are more desirable than plant and bacterial Ftases for large-scale production of FOS. This is due to their physicochemical characteristics like minimal loss of enzyme activity, by-product inhibition, and low molecular weight to allow for easier separation from product. The scope still exists to unravel the new sources of fungal Ftases for screening, identification and purification. This study therefore reports on the screening and production of FOS by extracellular and intracellular inulinase and Ftase isolated from coprophilous fungi.

2.5 Purification, Characterization and Properties of Ftase and Inulinase Enzymes

Purification and characterization of Ftases are crucial in order to understand their mode of action including ~~its~~ kinetic properties. The use of purified Ftases is preferable when compared to crude Ftase for the synthesis of FOS. Theoretically, the purified enzymes give higher Ftase activity and FOS production (Sangeetha, 2003, Kovács *et al*., 2013). However, the mechanism of reaction of FOS does not necessarily lead to the expected theoretical maxima of about 60 % due to end product inhibition caused by glucose accumulation. Several studies on FOS production have reported a yield of 60 % or more using purified Ftases (Ghazi *et al*., 2007, Nemukula *et al*., 2009). Furthermore, minimal time is required and higher yields of FOS are achieved by using the purified

Ftase instead of the crude Ftase (Trollope, 2015). Many authors have reported the purification and characterization of microbial Ftases and inulinases (Gill *et al.*, 2004, Fujishima *et al*., 2005, Lateef *et al.*, 2007b, Chen *et al*., 2009). Numerous reports on the purification of extracellular Ftases and inulinases produced by microbes deal with the conventional methods of centrifugation, salt/organic precipitation or ultrafiltration and/or ammonium sulphate precipitation, followed by ion-exchange and gel filtration chromatography (Ettalibi and Baratti, 1987, Chen *et al*., 2009, Singh and Singh, 2010). In some instances, fast protein liquid chromatography, hydrophobic interaction chromatography and preparative electrophoresis have been used (Uhm *et al*., 1999). Intracellular inulinase and Ftases need the usual step of cell wall disruption followed by sonication and centrifugation. Ftases and inulinase of fungal origin are mostly extracellular in nature, and generally, exo-acting due to challenges of cell disruption encountered with intracellular enzymes (Fernandes and Jiang, 2013). Moreover, endoinulinases purified from fungal and bacterial strains have been extracellular in nature. However, few reports of intracellular endoinulinase in *Kluyveromyces* sp. Y-85 and *Aspergillus niger* strain 12 have been documented (Wei *et al*., 1997, Nakamura *et al*., 2001).

Detailed purification procedures have been reported for fungal Ftases by (Wei *et al*., 2014). A novel fructosyltransferase capable of synthesizing sucrose 6-acetate from glucose 6-acetate and sucrose was purified by successive chromatographies to homogeneity from *Aspergillus oryzae* ZZ-01. The enzyme was purified to 17-fold with a yield of 30 % on Q-Sepharose FF, phenyl-Sepharose FF and Sephacryl S-200 HR columns (Ratnam *et al*., 2010). Purification of Ftase from the crude extract of *Aspergillus niger* AS 0023 up to nine steps by successive chromatogarphies on DEAE Sephadex A-25, Sepharose 6B, Sephacryl S-200, and concanavalin A-Sepharose 4B columns has been reported (L'Hocine *et al.*, 2000). The yield of FOS increased to 8 % using the purified enzyme (L'Hocine *et al*., 2000, Sangeetha, 2003). A transferase from *Aspergillus aculeatus* sp. was purified from a crude extract of Pectinex Ultra SP-L (Nemukula *et al*., 2009). The enzyme was purified 7.1 fold with a yield of 22.3 % and specific activity of 486.1 U mg^{-1} after dialysis using 30 % PEG and DEAE-Sephacel chromatography. From the enzymatic elongation of sucrose, GF_2 and GF_4 were the main FOS products after 4 h and 8 h of reaction, respectively.

Biochemical properties of the microbial Ftase enzyme such as pH and temperature profiling as well as stability have been determined and reported (Nemukula *et al*., 2009). An invertase was

partially purified by dialysis followed by DEAE-column chromatography from the crude extract of *Aspergillus terreus*. The enzyme was purified 8 fold with a yield of 76 %. The enzyme was stable at the temperature range of between 20 °C – 40 °C and pH range of 3.0 – 5.0 (Shaker, 2015).

Inulinases extracted from microbial sources have been purified and characterized (Ettalibi and Baratti, 1987, Kushi *et al*., 2000). An exoinulinase was purified from a commercially available broth (Fructozyme ®) obtained from the submerged fermentation of *Aspergillus ficuum*. The extract was treated with 50 % ammonium sulphate, and subsequently dialysed overnight followed by successive chromatographies of size exclusion on Sephacryl S-200 and ion exchange. The enzyme was purified 4.2 fold with a yield of 21 % and a specific activity of 12,300 U mg^{-1} protein. The molecular weight was estimated to be approximately 63 kDa by SDS-PAGE and the enzyme biochemical and physicochemical characteristics were evaluated (Mutanda *et al*., 2009). In another study, an inulinase was purified from a marine bacterium identified as *Bacillus cereus* MU-31. The inulinase was conventionally purified with 80 % ammonium sulphate, then dialysed, followed by anion exchange chromatography on DEAE-cellulose column. The enzyme was purified to homogeneity and it gave a single band and the molecular weight was estimated to be approximately 66 kDa by SDS-PAGE. The purified inulinase was characterized for optimum fructose production. The enzyme was purified 25-fold with a yield of 27.3 % and a specific activity of 1636.3 U/mg (Meenakshi *et al*., 2013). Additionally, the extracellular endoinulinase extracted from *Xanthomonas oryzae* No. 5 was purified by ammonium sulphate fractionation, followed by column chromatography on Phenyl-Sepharose and DEAE-Sephacel. The enzyme was purified 29-fold with a yield of 5.5 % and a specific activity of 1372 U/mg of protein. The purified endoinulinase gave a single band on SDS with molecular weight estimated to be 139 kDa. The enzyme biochemical and physicochemical properties revealed pH and temperature optima of 7.5 and 50 °C, respectively. The enzyme was stable at pH and temperature ranges of 6.0 – 9.0 and 45 °C respectively. The enzyme further obeyed the Michaelis-Menten kinetics with K_m of 16.7 g/l and V_{max} of 12.1 g/l.h (Cho and Yun, 2002).

2.6 Inulooligosaccharides

Due to increasing demand for basic nutrition, significant attention is being paid to biofunctional foods with health-promoting properties. Beside basic nutrition, the functionality of food with high product value and nutraceutical effect is in great demand (Mutanda *et al*., 2014b, Singh *et al*.,

2018). These reasons have led to the production of inulooligosaccharides (IOS), which is a class of prebiotics. According to a Global Industry Analysis (GIA), it was reported that the prebiotic market rose to nearly USD 3 million, and £ 1 billion in the USA and Europe, respectively in 2015 (Bali *et al*., 2015). Another report by the Leatherhead Food Research (LFR) predicted a global increase in demand for functional food market by 25 % from 2019 onwards compared to the previous years, with estimates reaching USD 54 billion (Abdul Manas *et al*., 2018). Overwhelming consumer consciousness for healthier food has heightened the fast growth of the functional food market of FOS and IOS. Inulin as a substrate can be regarded as a promising source for oligosaccharide production (Singh *et al*., 2016b). IOS produced from inulin hydrolysis are reported to have homogeneous biochemical and physiological functions as compared to FOS (Yun *et al*., 1997c, Roberfroid, 2005, Roberfroid, 2007b). Inulin has been reported to have a high degree of polymerization (DP) ranging between 2 to 60 fructose units with a few reports indicating a high-DP as 100 (Yun *et al*., 1997c, Niness, 1999). Inulin with high DP has shown good prebiotic potential (Singh *et al*., 2016b). This is due to their resistance to digestion by the gut enzymes because of the presence of fructose in their *β*-configuration (He *et al*., 2014). However, the DP varies from different plant species, the age of plants, climatic conditions, harvesting periods and inulin-rich plant organic material (Singh *et al*., 2016b). Inulin serves as reserve carbohydrate of vegetable and plant polysaccharides in *Compositae* and *Gramineae* (Vandamme and Derycke, 1983). It is accumulated in the underground roots and tubers of dahlia *(Dahlia pinnata*), chicory (*Cichorium intybus*), Jerusalem artichoke (*Helianthus tuberosus*), asparagus (*Asparagus racemosus*) and dandelion (*Taraxacum officinale*) as illustrated in (Figure 2.6) (Rawat *et al*., 2015). Inulin consists of linear chains of *β*-(2-1)-D-fructosyl fructose links terminated by a glucose residue via a sucrose-type linkage at the reducing end. Regioselective-reaction and mode of action of inulin with inulinases release fructose units or inulooligosaccharides as illustrated in Figure 2.5 (Chi *et al*., 2009a, Singh *et al*., 2017a). There are several types of fructans such as inulin, levan, phlein, kestoses, kesto-*n*-oses and graminan (Mutanda *et al*., 2014b). However, inulin fructan is a potential substrate for the production of ultra-high fructose syrup (UHFS). The partial hydrolysis of inulin using endoinulinases yields oligofructose with an average DP of 4. Lower DP oligosaccharides are composed of inulobiose (F2), inulotriose (F3), inulotetraose (F4) and prebiotic IOS (Ganaie *et al*., 2014, Rawat *et al*., 2015, Trollope, 2015).

Inulin represents an inexpensive, abundant, renewable raw material and the hydrolytic product of inulin consist of fermentable sugars (Hughes *et al*., 2017). Value-added products such as bioethanol, 2,3-butanediol, UHFS, single-cell protein, single cell oil, high-optical purity L-acetate and citric acid are produced by microbial inulinolytic enzymes (Xu *et al*., 2016).

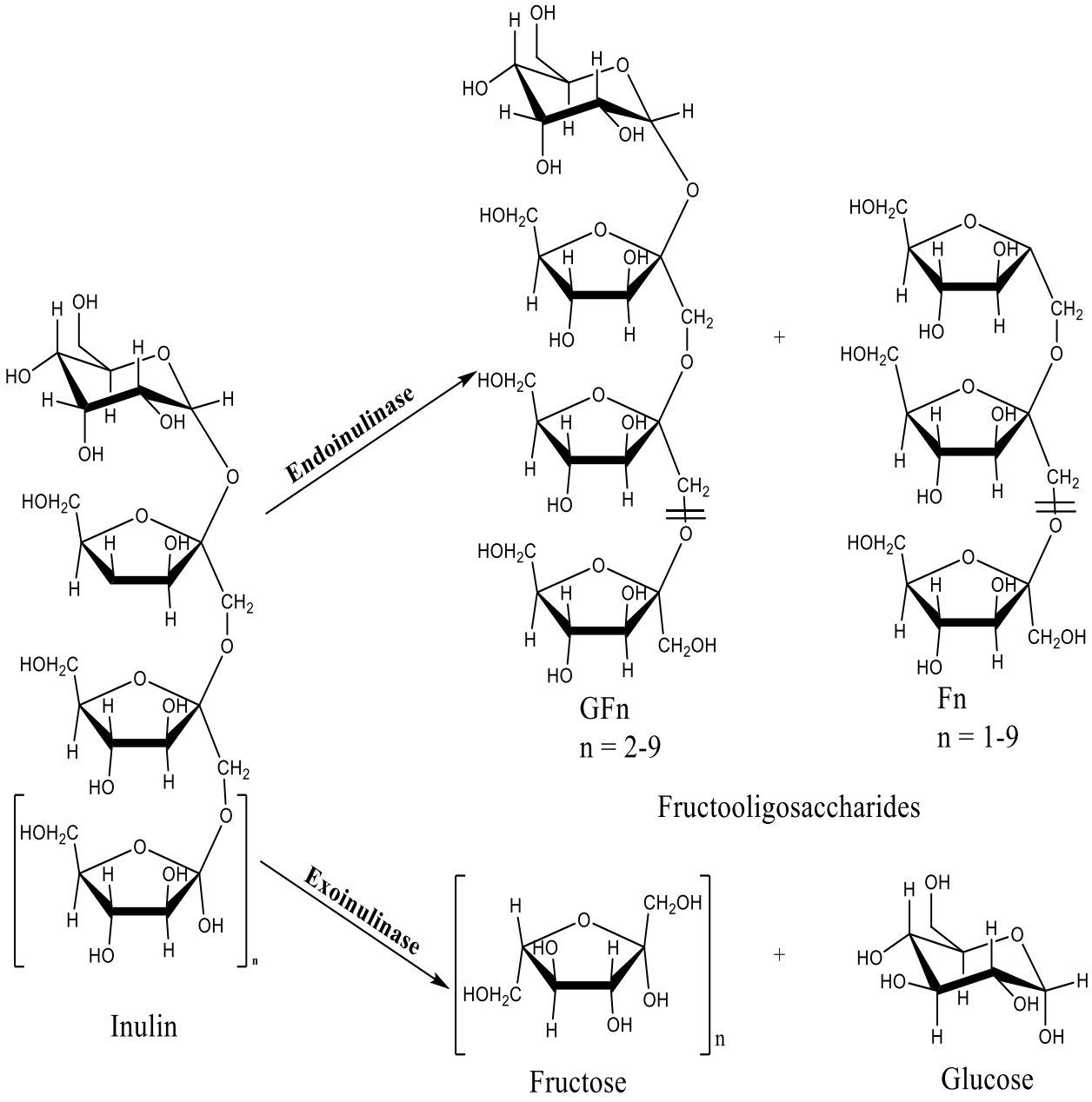


Figure 2.5: Degradation pattern of inulinase on inulin. Adapted from (Roberfroid *et al.*, 1998) (Singh and Singh, 2010, Singh *et al*., 2017a).

Figure 2.6: Photographs of inulin producing plants A and B chicory flowery plants and its storage roots (*Cichorium intybus*), C, D and F Jerusalem artichoke (*Helianthus tuberosus*), and E onions. Adapted from (en.wikipedia.org/wiki/chicory, www.thespruce.com/how-to-grow-organic-jerusalem-artichokes-2539639).

Inulin-type fructans have desirable properties similar to FOS such as low sweetness intensity, as they are a third sweeter as sucrose and this feature is important in foods restricted to sucrose because of its high sweetness (Apolinário *et al*., 2014). Secondly, IOS has low levels of calories that are rarely absorbed by the upper part of the gut and consequently are not used up as an energy source making them safe for consumption by diabetics. Thirdly, IOS are noncariogenic, that is, they are unused by *Streptococcus mutans* to form acids and *β*-glucan which is insoluble and a major cause of dental caries (Rawat *et al*., 2017b). Fourth, inulin-type fructans act as prebiotics as they encourage the growth of bifidobacteria and suppress the growth of potentially putrefactive microbes in the digestive tract (Mutanda *et al*., 2014b, Khuenpet *et al*., 2017). These properties improve gut functions as illustrated in (Figure 2.7). The gut microflora was evaluated before inulin intake as shown in Figure 2.7 and after inulin intake as illustrated in Figure 2.8 showed proliferation of bifidobacteria.

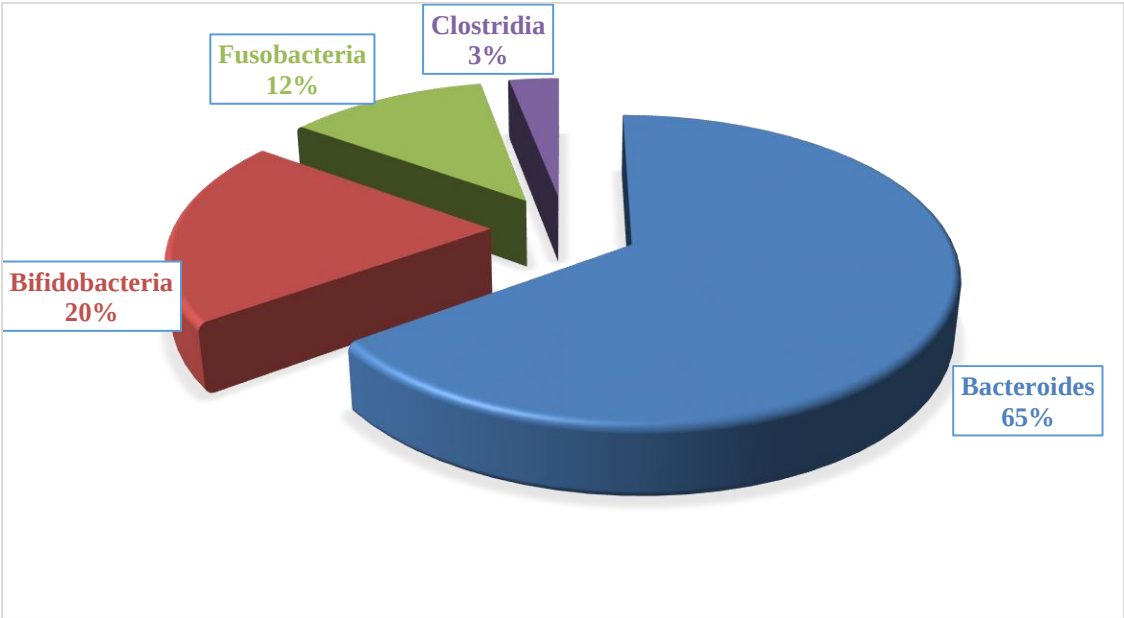


Figure 2.7: Prevalence of pathogenic microbes before uptake of inulin. Adapted from (Gibson and Roberfroid, 1995, Gibson *et al*., 1999, Roberfroid, 1999b, Roberfroid, 1999a).

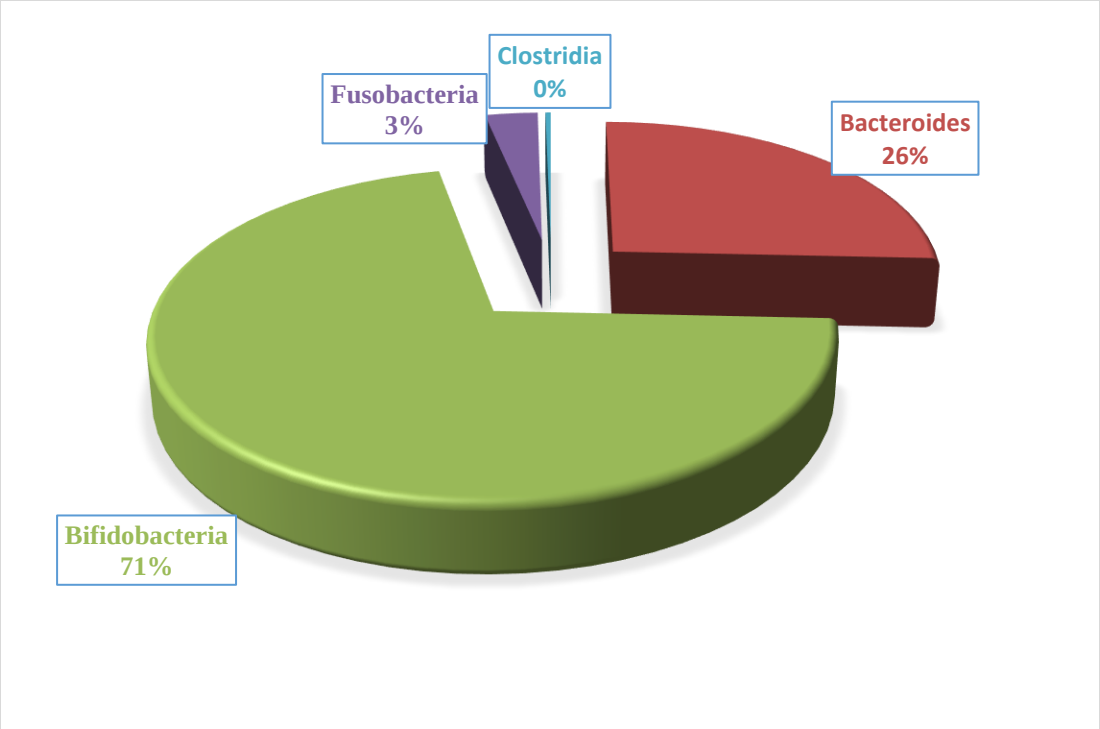


Figure 2.8: Proliferation of *Bifidobacteria* after inulin intake showing the prebiotic effect of inulo-oligosaccharide. Adapted from (Gibson and Roberfroid, 1995, Gibson *et al*., 1999, Roberfroid, 1999b, Roberfroid, 1999a).

FOS production can be achieved through controlled fructan hydrolysis. About 15% of flowering plant species contain fructose-based polysaccharides, which is a major reserve of fructans base. Due to their differences in glycosidic linkages, they can be classified into different types, the linear inulin being the best studied and most characterized (Bezerra *et al*., 2017). Inulin consists of *β*-(2-1)-linked fructose units terminating at the tail end with a glucose residue annexed through sucrose-type linkage (Sheng *et al*., 2007). Action of inulinase on the *β*-(2-1) linkage in inulin can yield either fructose or IOS (Kango and Jain, 2011). The exoinulinase (*β*-D-fructanfructohydrolase, EC 3.2.1.80) catalyse the removal of terminal fructose residue to yield fructose as the main residue, while the endoinulinase (2,1-*β*-D—fructanfructohydrolase, EC 3.2.1.7) act randomly by hydrolysing inulin internal linkages to liberate short-chain IOS such as inulobiose, inulotriose, inulotetrose and inulopentaose as the main products (Pouyez *et al*., 2012, Altunbaş *et al*., 2013, Rawat *et al*., 2015).

Microorganisms are the best sources of commercial inulinase because of their high yields and easy cultivation for the production of fructose and IOS (Dinarvand *et al*., 2017). Fructose was conventionally produced from starch, including α-amylase, amyloglucosidase, and glucose isomerase action liberating about 45 % fructose. In contrast, fructose formation from inulin is a single enzymatic step yielding up to 95 % fructose (Vandamme and Derycke, 1983, Meenakshi *et al*., 2013). Due to these drawbacks of numerous enzymatic steps associated with chemical hydrolysis of inulin to fructose such as low yields, high temperatures and the high cost of production, microbial inulinase is desirable (Singh *et al*., 2018). A panorama of bacterial producing inulinases for industrial application has been reported (Singh *et al*., 2017a). Bacterial strains such as *Bacillus* sp., *Streptomyces* sp., *Arthrobacter* sp., and *Clostridium* sp., have been reported to produce an acceptable amount of inulinase (Allais *et al*., 1987, Drent *et al*., 1991, Keto *et al*., 1999). These bacterial strains have the ability to survive adverse environmental conditions like acidity, alkalinity, high temperature and salinity (Pikuta *et al*., 2007). Due to the low yield of inulinase recovered, only a few investigations for industrial applications has been carried out. It is for this reason that fungal inulinases are a more feasible and appealing microbial source for production of inulinase enzymes.

2.6.1 Microbial Exoinulinases

Inulin is a polyfructan containing linear *β*-(2-1) linked polyfructose chain and is considered the most suitable substrate for IOS production. It is also considered a renewable source of raw material in fructose syrup manufacturing and FOS production (Naidoo *et al*., 2015). It is insoluble in water due to variations in chain length elongation and molecular weight, which varies between 3500 – 5500 Da. Microbial inulinase (2,-++1-*β*-D-fructan fructohydrolase EC, 3.2.1.80) catalyses inulin hydrolysis by cleaving D-fructose from non-reducing sugar *β*-2,1 end of inulin. Inulinase (I) and invertase (S) activity are used to characterize exoinulinases using the (I/S) ratio criteria to determine their affinity to sucrose or inulin substrates (Moriyama *et al*., 2002). Microorganisms involved in exoinulinase production include species of *Penicillium, Aspergillus, Kluyveromyces, Sporotrichum, Cryptococcus, Pichia, Cladosporium, Bacillus, Pseudomonas, Xanthomonas, Sporotrichum* and *Candida* (Pandey *et al*., 1999, Gao *et al*., 2007).

2.6.2 Microbial Endoinulinases

Microbial endoinulinases (2,1-*β*-D-fructan-fructan hydrolase, EC3.2.1.7) act on the internal linkage of inulin to randomly form intermediates such as inulotriose, inulotetraose and inulopentaose (Mutanda *et al*., 2014b). It is noted that similarities exist between exoinulinases and endoinulinases and this makes it difficult to separate by these conventional methods. However, native-PAGE has been proposed as an efficient tool to separate enzymes displaying similar characteristics (Jing *et al*., 2003a). Endoinulinase that is free from invertase or exoinulinase activity has been investigated and reported to hydrolyze inulin internal linkages, and thus producing several oligosaccharides which are soluble dietary fibre with low caloric value (Cho and Yun, 2002).

2.7 Functionalities and Applications of Fructooligosaccharides

2.7.1 Fructooligosaccharides as Prebiotics

Prebiotics are biofunctional food supplements that stimulate the selective growth of *Lactobacilli* and *Bifidobacteria* in the gut leading to improved health (Figure 2.9). Prebiotics create an unfavourable environment to harmful invasive pathogens by stimulating *Lactobacilli* and *Bifidobacteria* proliferation (Kaprasob *et al*., 2018b). The intestinal bacteria ferment FOS and produce large compounds of short-chain fatty acid that result to acidic conditions in the colon which colonize adhesive sites and secrete bacteriostatic peptides (Cummings and Macfarlane, 2007). The probiotic bacteria survive harsh acid conditions and are adherent to mucosal walls of the gut by producing organic acids like lactic acid which are inhibitors of many pathogenic microorganisms hence improving gut health (Durieux *et al*., 2001).

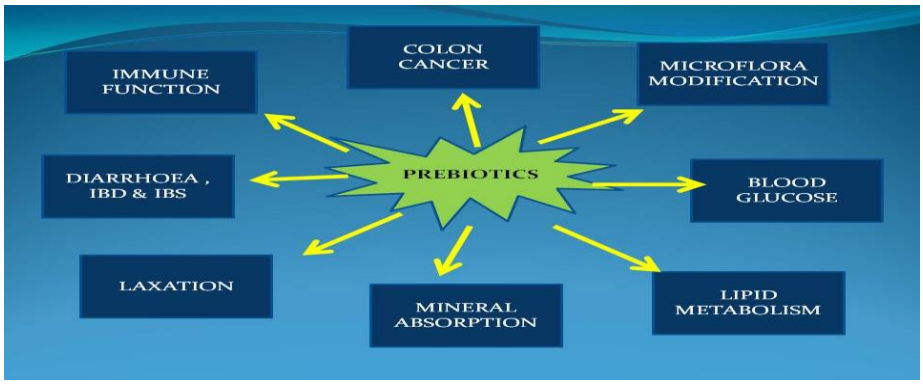


Figure 2.9: Beneficial impacts of *Bifidobacteria* accumulation in the colon. Adapted from (Gibson and Roberfroid, 1995).

2.7.2 Dietary Fibre Effect

Dietary fibre has been defined as plant or carbohydrate analogs that are not easily hydrolysed in the upper part of the small intestines (Champ *et al*., 2003). They contain edible plant cell polysaccharides remnants associated substances that are resistant to enzyme hydrolysis by the human gut (AACC, Report 2001). This definition recognizes partial or complete fermentation in large bowel as a crucial part in the metabolism of dietary fibre. There is an increasing evidence that supplementation of diet with fermentable fibre alters the gut function and structure either by modification or production of gut-derived hormones which are associated with improved glucose homeostasis (Schley and Field, 2002). Moreover, oligosaccharides, which are associated with dietary fibre both portray beneficial physiological characteristics and show similarity with dietary fibre intake (Cherbut, 2007, Candela *et al*., 2011). Some important physiological effects are the bioavailability of minerals and aids in lipid metabolism, which contributes to the reduction of risks associated with colon cancer and cardiovascular disease (Kalyani Nair *et al*., 2010). They also contribute to faecal bulking either by attracting their own water or through their own mass (Roberfroid, 2005). They can be incorporated into food and drinks as they provide caloric dilution in viscous drinks and diets (Flamm *et al*., 2001b).

2.7.3 Role as an Anticancer Agent

Diets containing high protein, high animal fat concentrations and with low dietary fibre concentration have been associated with colonic cancer (Sabater-Molina *et al*., 2009). However, FOS contributes indirectly to colon cancer prevention. Oligofructose administration has been found to decrease genotoxicity. Some bacterial commensal of the colon are carcinogenic and tumour promoters as a result of food metabolism (Azcárate-Peril *et al*., 2011). In the gut, there exist two types of fermentation after ingestion of food proteolytic and saccharolytic where the latter is more favourable due to metabolic by-products formed such as acetate, short-chain fatty acid (SCFAs), propionate and butyrate (Van Loo, 2004). When a model system of the human gut was investigated after feeding of galactooligosaccharides (GOS) there was a considerable depreciation of nitroreductase. This is a metabolic activator and carcinogenic substance that also decreases indole and isovaleric levels (Al-Sheraji *et al*., 2013). According to a study conducted by Kim *et al*. (1982), butyrate has been found to have antitumor characteristics, also up-regulates apoptosis, therefore, contributing to the prevention of colon cancer by promoting cell differentiation (Dominguez *et al*., 2014). In another study reported by (Bali *et al*., 2015),

consumption of FOS was observed to reduce an intestinal tumour while increasing development of lymphoid nodules in the gut-associated lymphoid tissue (GALT). In addition, propionate has chemoprevention properties that induces an anti-inflammatory effect on colon cancer cells (Scharlau *et al*., 2009). Another study looked at the effect of starch administration to human flora-associated rats (Bird *et al.*, 2000). There was decrease in ammonia levels and β-glucuronidase with the observation of high-levels caecal butyrate. Butyrate plays a dual role in maintaining a healthy epithelium as well as providing energy for colonocytes proliferation and thereby reversing colonic atrophy which is a feature of low-fibre diets (Al-Sheraji *et al*., 2013). Furthermore, the decrease in AOM-induced colorectal cancer in F344 rats by being fed on oligofructose diet has also been evident with an indication of anti-cancer properties (Bali *et al*., 2015).

2.7.4 Role in Mineral Absorption

To expand the knowledge of FOS in improving mineral absorption, several mechanisms have been explained. The consumption of FOS has been elucidated in several experimental animals. The dietetic fibre binds to or sequesters minerals reducing their absorption in the ileum (Sabater-Molina *et al*., 2009). The sequestered minerals along with fermented soluble fibre become available in the colon and the high concentration of SCFAs from colonic fermentation of oligofructose increases the solubility of calcium and magnesium ions (Roberfroid and Delzenne, 1998). The stimulation of magnesium and calcium absorption was also observed in dogs, while in adult animals mineral absorption was stimulated by the consumption of recommended dosages of starch or inulin. Moreover, there was a significant increase in calcium absorption if there was a combination of the two (Scholz-Ahrens *et al*., 2007). Bioavailability of FOS occurs largely in the colon. This is due to fermentation by commensal microorganisms (Bornet *et al*., 2002). Short-chain fatty acid decreases luminal pH, leading to acidic environment favouring mineral solubility of Ca^{2+}, Mg^{2+}, Fe^{2+} that maintain a homeostatic balance between $Fe^{2+}$and Zn^{2+} (Freitas *et al*., 2011, Dominguez *et al*., 2014). A study on gastrectomized experimental animals was fed with oligosaccharides and it was seen to increase iron uptake, a key mineral in anaemic conditions. FOS uptake was also seen to prevent osteopenia in rats as calcium ions stored in bones was easily absorbed (Bali *et al*., 2015). Numerous benefits emanate from intestinal calcium and magnesium uptake such as an increase in bone Mg^{2+} content consequently leading to a reduction in risks of osteoporosis (Gibson *et al*., 2004). Furthermore, high levels of magnesium ions have been implicated in assuaging asthma symptoms in young people (Abrams *et al*., 2007).

2.7.5 Role in Lipid Metabolism

Animal studies carried out in mice have shown oligofructan and inulin or a non-digestible but fermentable oligomer of *β*-D-fructose obtained by inulin hydrolysis to display physiological effects on cholesterol. This significantly lowers serum triglyceride levels by decreasing postprandial cholesterolemia and triglyceridemia by 15 % and 50 % respectively (Kok *et al*., 1996). It was on the finding of a lipogenic decline in enzyme activity and very low-density lipoprotein (VLDL) which contains the highest amount of triglycerides particle that contribute to this effect (Yamamoto *et al*., 1999). Moreover, FOS fermentation increases propionic acid in the intestinal mucosa and in turn, reduces levels of triacylglycerol (TAG) and associated hypercholesterolemia LDL and VLDL (Bali *et al*., 2015). In human studies using inulin and oligofructose as food, supplements in normal and hyperlipidaemic conditions displayed no effect on serum level cholesterol or triglyceride. However, three investigations showed a slight reduction in triacylglycerol, while four inspections cholesterol and triacylglycerol lowered significantly. It was concluded by (Saad *et al*., 2013a) that inulin appears to be more suitable to oligofructose in reducing triglyceridemia while in animal studies both oligofructose and inulin were equally active. Based on these findings, prebiotics were shown to have an effect on hepatic lipid metabolism (Saad *et al*., 2013b). In a study of diabetic rats, simple carbohydrates were replaced with xylooligosaccharides (XOS) in their diets and there was a drastic drop in serum cholesterol and TAG in diabetic rats while liver triacylglycerol increased to commensurate levels to that observed in healthy rats (Imaizumi *et al*., 1991). The findings were attributed to lipogenic enzyme inhibition resulting from prebiotic fermentation in the gut by the action of propionate (Al-Sheraji *et al*., 2013).

2.7.6 Role in Defence Mechanism and Immune Regulation

Emphasis on functional foods is made as it is claimed to bolster the immune system. Fermentation of saccharolytic metabolites resulting from the dietary intake is closely associated to be in contact with gut lymphoid tissues, which covers a majority of the intestinal immune system. Products of FOS fermentation may modulate the gut-associated lymphoid tissue, (GALT) as well as the systemic immune system (Schley and Field, 2002). A concept of immunity suggested by Saad *et al.* (2013) is that innate immune response can be activated by sugar moieties interacting synergistically with innate receptors on host plasma membrane in dendritic cells and macrophages. B-glucose oligosaccharide activates immune reactions by binding to macrophages receptors.

Orally ingested oligofructose and inulin are claimed to modulate immune system parameters like IL- 10 and IFN-γ natural killer cells activity, lymphocyte proliferation, intestinal IgA and increase polymeric immunoglobulin receptor expression in ileum and colon regulation. Consumption of prebiotics fibre induces bifidogenic microflora as a result of short-chain fatty acid from fibre fermentation and direct contact with cytoplasmic components with immune cells (Saad *et al*., 2013b).

2.7.7 Antioxidant Effect

Antioxidants are natural or synthetic compounds that may delay or prevent oxidative stress caused by physiological oxidants (Sies, 1997, Finkel and Holbrook, 2000, Carocho and Ferreira, 2013). Conventionally the antioxidants are divided into two groups: the antioxidants that scavenge directly for active free radicals such as reactive oxygen species (ROS) or reactive nitrogen species (RNS) and antioxidants that inhibit oxidative stress (Guo *et al*., 2003, Lama, 2017). Free radicals are customarily unsteady and originate from nitrogen (RNS), oxygen (ROS) and, sulfur (Reactive Sulphur Species: RSS) (Amir Aslani and Ghobadi, 2016). ROS, RNS, and RSS generation both in their radical and/or non-radical forms occur in humans and animal cells because of metabolic and physiological processes (Kunwar and Priyadarsini, 2011). Moreover, ROS-induced free radical whether from exogenous or endogenous sources can be injurious to the body cell biomolecules, cause impairment to cell functions, oxidative stress or apoptosis (Fang *et al*., 2002). Free radicals have also been implicated in numerous pathologies including cardiovascular complications, neurodegenerative disorders as well as oncogenic complications (Battin and Brumaghim, 2009). Intake of inulin-type oligosaccharides, vitamin C, vitamin E and carotenoids have been found to have the potential to minimize the harmful effects of reactive species (Amir Aslani and Ghobadi, 2016). Dietary intake of antioxidants such as tocopherol, carotenoids and ascorbate are difficult to disentangle through epidemiological studies from other vital vitamins and ingredients in fruits and vegetables. Nevertheless, several studies published suggest that antioxidants are a major remedy for endogenous damage to DNA, lipids and proteins. Antioxidants play a key role in immune system activation. The proliferation of B and T cells, natural killer cells and lymphokine-activated killer cells that protect the body's defence mechanism from pathogens (Ames *et al*., 1993). Supplementation with dietary antioxidants counteracts the oxidants and thereby boosting the complement system.

2.7.8 Dosage and Side Effects

A daily dosage of 8.8 g/day of γ-cyclodextrin has been recommended to be sufficient for gut tolerance. However, the dosage of non-digestible oligosaccharides tends to vary depending on their action on *Bifidobacteria* in the colon. Some authors have reported FOS<15 g/day while others claim that 10 g/day is sufficient to create a bifidogenic effect (Morris and Morris, 2012). In some studies where an individual's indigenous *Bifidobacteria* is low like in adults a 2.5 g/day has been recommended (Brusaferro *et al*., 2018, Kerry *et al*., 2018, Mall and Peter, 2018). Dosages of non-digestible oligosaccharides are also compared to galactooligosaccharides (GOS) and xylooligosaccharides (XOS) and prescription of 8-10 g/day appear to be recommended. In conclusion, the acceptable limit of dosage is difficult to predict as it varies in individuals as some have reported loose diarrhoea, flatulence and intestinal discomfort on ingesting large quantities (Mussatto and Mancilha, 2007).

2.7.9 Applications of FOS in the Global Market

FOS interest as functional food has led to their industrial applications in the food and beverage industry. In beverages, they are used in cocoa, fruit drinks, infant formulas and powdered milk as supplements. Also as probiotics, yoghurt and yoghurt drink to create synbiotic products. Other current applications include puddings and sherbets, desserts such as jellies, confectioneries like chocolate, biscuits, pastries spread like jam, marmalades and meat products such as fish paste and tofu (Crittenden and Playne, 1996, Mussatto and Mancilha, 2007).

There is increasing demand for prebiotics worldwide, which is estimated to be around 167,000 tones and 390 million euros. The total cost of developing functional foods is estimated to be between 1 - 1.5 million euros while the cost of marketing and product value may surpass it. Many European countries like France, the United Kingdom, the Netherlands, and Germany produce nearly two-thirds of functional dairy products sales in Europe (Mishra *et al*., 2018). For Germany, alone volumes from 1995 to 2000 have increased considerably to 317 million euros synbiotics contributing 228 million. The most popular FOS are of the inulin type non-digestible oligosaccharide, lactulose and resistant starch. Companies that produce FOS from sucrose around the world include Meiji Seika Kasha Limited in Japan has been known for producing enzymes from *A. niger* and it had a merger with Beghin-Meiji industries in France to increase FOS

production which is marketed as Actilight^{R} and also with USA based GTC for nutrition. In a 2011 report, Meiji holdings presented its annual financial records with reports of net sales amounting to 21 million euros (Dominguez *et al*., 2014).

Wako pure chemicals and Sigma-Aldrich in Japan and USA manufacture FOS oligomers such as 1-kestose (GF_2), nystose (GF_3) and 1F-β-fructofuranosyl nystose (GF_4), respectively. The USA also manufactures inulin from derived products such as chicory roots under the trade name Raftiline^{R} and Raftilose^{R}, respectively (Singh *et al*., 2016b).

Based on this statistics, it is clear that the field of prebiotics and FOS studies should be explored further for more biotechnological and pharmaceutical applications. Due to their strong physicochemical properties that show anti-cancer effects combined with the numerous health benefits mentioned, it is prudent to expand studies on FOS and IOS in order to unravel novel sources.

2.8 Coprophilous Fungi

Coprophilous fungi are described as the dung loving fungi, (copro: dung; philous: loving) (Farouq *et al*., 2012). They occur on dung substratum and due to their dung-loving nature are sometimes referred to as fimicolous species (Eliasson, 2013). Herbivore dung is an extremely complex substrate comprising of ingested vegetation, gut microbiota and a diversity of supplementary components ranging from soil particles, hair follicles to organic and inorganic sediments (Harrower and Nagy, 1979). Variations in dung composition is in most cases influenced by the herbivore diet such as leaves of dicotyledonous plants, fruits, seeds, barks and pollen (Shahack-Gross, 2011). According to (Dix, 2012) the excrement of coprophilous have been characterized as either optional or obligate. The obligate coprophilous contain spores that require the action of gut enzymes to break their dormancy, while facultative coprophilous don't need to go through animal digestive tract to sporulate and germinate (Richardson, 2001b). Coprophilous fungi are a group of saprophytic fungi adapted to life on dung and faecal pellets of herbivores (Sarrocco, 2016b). Most of these fungi rely on terrestrial warm-blooded herbivores to complete their life cycle (Baker *et al*., 2013). Coprophilous fungi play a prominent role in herbivore dung decomposition and are therefore crucial in nutrient recycling in the ecosystem (Aluoch *et al*., 2017). They have special features that make them reappear on dung such as higher pH, value usually above 6.5, high

moisture content as well as readily available nutrients in large quantities in the dung substrates and high carbohydrates in their biomass (Abdullah, 1982, Kuthubutheen and Webster, 1986a). When herbivores graze on vegetation they ingest spores of which some are coprophilous while others from non-coprophilous fungi along with vegetation. The spores of non-coprophilous are killed by high temperature and gastric juices in the GIT while spores of coprophilous survive gut hydrolysis and are passed out germinate, grow and fruit on dung (Harper and Webster, 1964, López-Sáez and López-Merino, 2007). They grow on herbage where by good fortune may be eaten by a herbivore, then pass through the animal digestive system to continue their life cycle (Bell, 1983). However, any dung can yield fungi but herbivore dung have been regarded as the best source of coprophilous fungi. These fungi have a cosmopolitan distribution as they occur in many herbivore species around the world (Johnson *et al*., 2015, Calaça and Xavier-Santos, 2016).

Coprophilous are classified into different morphological keys. Key one and two (MJR) are said to belong to coprophilous ascomycetes that are a very diverse group with many species yet to be discovered. The second key includes the original plectomycete key (RW). This key contains fungi not only biased on herbivore but also occur in horn, hair, cadavers and carnivore dung. The third key is (RW, p52) belongs to basidiomycetes of dung associated debris. The fourth key (MJR, p63) is of zygomycetes and was found to appear first on freshly dropped dung but soon disappear. However, it can be prolonged by plating a small portion on enumeration media like potato dextrose agar (Richardson and Watling, 1997).

Herbivore dung is a rich substratum of coprophilous fungi (Figure 2.10) and supports high species diversity. Fruiting bodies of dung fungi appear in succession mostly following the sequence of Zygomycotina, Ascomycotina and Basidiomycotina (Weber and Webster, 1998, Richardson, 2001b). Dung fungi play a vital role in mineralization and decomposition of herbivore dung while some display few modifications peculiar to their habitat. Coprophilous fungi growing in some dung in their natural habitat are illustrated on (Figure 2.10).

2.8.1 Fungal Succession on Herbivore Dung

Freshly voided dung, on incubation in moist chamber, displays a host of fruiting bodies in succession, with zygomycets the first to appear after two to three days. The species of *Mucor*, *Pilaria* and *Pilobolus* dominate the first phase (Santiago *et al*., 2011, Pinto *et al*., 2012, de Souza *et al*., 2017). This is succeeded by apothecial Ascomycota after six to seven days that include the

genera of *Coprobia, Ascobolus*, *Podospora, Saccobolus* (Richardson, 1972). The perithecial ascomycetes of *Chaetomium* and *Podospora* are mainly observed after nine to ten days on herbivore dung. These species persist for 3 – 4 weeks until the appearance of basidiocarps of *Coprinus, Sropharia, Conocybe,* and *Panaeolus* (Caretta *et al*., 1998, Richardson, 2002, Doveri, 2011). The timing and succession pattern is dependent on herbivores species and dung droppings. Nutrient utility is always considered as a fundamental factor influencing succession of coprophilous fungi (Webster and Weber, 2007). The proteins, sugars and starch are mainly utilized by species of *Mucorales*, which have been termed as sugar fungi (de Souza *et al*., 2017). Cellulose component is consumed by ascomycetes and the basidiomycetes exhaust both lignin and cellulose in dung substrate (Webster and Weber, 2007). The presence of reproductive structures such as ascocarps, or basidiocarps and sporangiophores forms the basis of fungal succession while the development of mycelial sequence may not be the same (Yadav, 2011). From an ecological standpoint, fungal succession in its habitat is influenced by resident microbes competing which influences the actual time and appearance of fruiting bodies (Krug *et al*., 2004). In the succession sequence basidiomycetes have been observed as not only the last group to appear as some ascomycetes of genera *Podospora, Coprotus* and certain Gymnoascaceae have been seen to develop fruiting bodies even after 35 – 50 days of incubation (Bell, 1983).

2.8.2 Coprophilous Diversity and Taxonomic Studies

Most species of coprophilous fungi have a cosmopolitan distribution worldwide (Watling, 2005, Mueller, 2011). However, certain coprophilous fungi are restricted to specific areas. Some species are more common in the northern latitude than the southern latitude and *vice versa* (Richardson, 2001b). The diversity of coprophilous community tends to decrease with increasing latitude, and it is vast in the tropics (Lundqvist, 1972, Richardson, 2001b). Most hitherto works on coprophilous have been reported in Europe (Denmark, Italy), North America and Latin America (Doveri, 2011, Calaça *et al*., 2014, de Souza *et al*., 2017, Loughlin *et al*., 2018). Some studies have also been reported around East Africa, Asia with few taxonomic studies in South Africa (Mungai *et al*., 2012a, Mungai *et al*., 2012b, Ndlela and Schmidt, 2016). Fungal frequencies and species richness in most instances are correlated with geographical origin, sampling intensity and interest and expertise of the mycologist. Therefore, in the present study, the focus was not on fungal ecology, taxonomy or fungal succession even though an overview of coprophilous fungi is reported *sensu lato.* The present study evaluated the ability of dung fungi to produce important biocatlytic

enzymes that may potentially have industrial significance in production fructo-oligosaccharide and inulo-oligosaccharide, respectively.

Figure 2.10: Coprophilous fungi growing on dung substratum of some herbivores. Adapted from https://www.google.com/search?q=coprophilous+fungi.

2.8.3 Adaptations of Coprophilous Fungi on Herbivore Dung

2.8.3.1 Phototropism and violent spore-discharge:

There are various adaptations that enable coprophilous fungi to adapt to their habitat. Phototrophic response determines the direction of where spore mass will be projected. In most coprophilous fungi, the spore-bearing structures such as sporangiospore in Zygomycetes, conidiophores of hyphomycetes, asci and basidia in higher fungi get phototrophic orientation towards light and are usually supplemented by violent spore discharge (Ingold, 1953, Fischer *et al*., 2004). This phenomenon of violent spore discharge is demonstrated by *Pilobolous* the first coprophilous fungus to have its whole genome sequenced (Viriato, 2008). The spores are ingested with herbage and survive herbivore gut metabolism are egested out back to the environment to continue the life cycle.

2.8.3.2 Adhesive projectiles:

Ascospores of coprophilous are armoured with gelatinous appendages as extensions of their spores. These projectiles provide the necessary attachment to the fungi that hold on to herbage and consumed by other herbivores before being blown away by wind or washed with water (Richardson, 2008b). Their cell wall fragmentation lead to the formation of primary appendages, whereas secondary appendages are formed by exudation through spore in the cell wall (Yadav, 2011).

2.8.3.3 Mucilaginous spores:

Most species of Ascomycota have elaborate or brief mucilaginous sheaths which aid in attachment of spores to the substrata (Gareth Jones, 2006). Species of *Sodaria* and *Podospora* have gelatinous ascospores that favour adherence of propagules to adjacent vegetative environment (Doveri, 2014).

2.8.3.4 Spore-wall pigmentation:

The spore walls of coprophilous fungi are often pigmented and protect them from exposure to excess UV radiation while on vegetation (Krug *et al*., 2004, Richardson, 2008a). Numerous coprophilous species especially of the Ascomycota are thick-walled and contain pigments that protect their spores from excessive UV radiation. This characteristic accounts for long time survival in the soil and consequently their presence in sedimentary samples (van Asperen, 2017).

2.8.3.5 Resistance to gut metabolism of herbivore:

Passage of spore through the gut of herbivore is necessary to facilitate spore germination of dung fungi (Richardson, 2008a). Passage of spores in the herbivore alimentary canal leads to stimulation of spore when they are passed out as they remain unhydrolyzed by the enzymatic action of the gut following physical and chemical reaction in the animal GIT (Kuthubutheen and Webster, 1986a, Kuthubutheen and Webster, 1986b). When animals egest dung, the spore are triggered to germinate on vegetation. Fungi that survive gut metabolism have been referred to as "true coprophilous"(Larsen, 1971, Eliasson, 2013).

2.9 Why Coprophilous Fungi?

Farouq *et al*., (2012) reported that fungi that grow on herbivore dung are full of fibre from dung biomass and have potential cellulase activity (Farouq *et al*., 2012). Cellulose is a linear glucose polymer linked by β-1,4-glycosidic bond forming a large component of plant biomass. Cellulase

has two major enzymes which are endo-*β*-D-glucanase (EC3.2.1.4) which catalyzes cellulose hydrolysis by randomly splitting sugar moieties of the molecule. The other enzyme exo-*β*-D-glucanase (EC3.2.1.19) *β*-glucosidase can hydrolyze cellulose to glucose (Sohail *et al*., 2009).

Herbivore dung contains a high amount of readily available complex carbohydrates made up of cellulose, hemicellulose, pectin, lignin and high nitrogen content. In addition, they have a high moisture content, vitamin, growth factors, pH closer to neutral and minerals (Richardson, 2002, Sarrocco, 2016a). The ruminal ecosystem represents the most potent fibrolytic fermentation system known. It is composed of a diverse population of obligate anaerobic fungi, bacteria and protozoa (Selinger *et al*., 1996). Fungi in the rumen produce potent fibrolytic enzymes that have the ability to degrade recalcitrant plant cell wall polymers. The gut metabolism of herbivores is specifically adapted for highly specialized microbial processing of complex plant polysaccharides ingested (Selinger *et al*., 1996). Since dung is egested along with, plant cells and interwoven matrix of plant polymers from rumen incomplete digestion and consequently microbes on dung use them up. The array of enzymes population in the rumen is not only from microbial diversity but also from the multiplicity of fibrolytic enzymes produced by individual microbes (Selinger *et al.,* 1996). The complex carbohydrates including cellulose and lignin in dung biomass, therefore displays unexplored reservoir as it can produce substrates with potential transfructosylating activities. Cellulases have been used for several years in food industries for wine fermentation, wastewater treatment, textile production and detergent formulation (Jahangeer *et al*., 2005). In another study, extracellular hydrolases were isolated from the dung of Koala and were characterized comprehensively with potential biotechnological applications. Enzymes such as endoglucanases (EC 3.2.1.4 cellulase), *β*-glucosidase (EC 3.2.2.1.21) and xylamase (EC 3.2.1.8 endo1-4-*β*-xylanase) displayed desirable characteristics sought after for industrial exploitation (Peterson *et al*., 2011). Need for additional applications should be investigated. Several investigations involving herbivore dung have demonstrated potential for enzyme production for industrial and biotechnological applications (Table 2.4).

Coprophilous fungi, which grow on herbivore dung appear to encode an unexpectedly high number of enzymes with potential to degrade lignin. The filamentous coprophilous of *Podospora anserina* and *Sordaria macrospora* have been implicated in lignin degradation (Poggeler, 2011). Degradation of plant biomass is a major challenge towards the production of biological

compounds. The coprophilous ascomycete *Podospora ansenira* has had its whole genome sequenced and it is well delineated to be used as a model organism to study various biological mechanisms. The sequence revealed a wide array of enzymes targeting lignin and plant carbohydrates (Couturier *et al.*, 2016). The coprophilous basidiomycete *Coprinus* sp. was investigated for its ability to produce extracellular peroxidase. After preliminary screening, three strains namely *Coprinus cinerreus* UAM 4103, IFO 30116 and UAMH 7907 revealed potential peroxidase activity (Ikehata and Buchanan, 2002, Anh *et al*., 2007). The effectiveness of the cultivated *C. cinerreus* peroxidase was evaluated for its ability to remove phenolic and other aromatic compounds from wastewater (Ikehata *et al*., 2004). Phenols and phenolic are constituents of industrial effluents which impact water bodies causing toxicity on flora and fauna (Nair *et al*., 2008). Additionally, herbivore dung also offers novel opportunities for antibiotic discovery from microbes that compete for the nutrient-rich ephemeral source (Bills *et al*., 2013). Antimicrobial and biochemical studies on microbes isolated from herbivore dung samples can open new vistas in comprehending coprophilous fungi as an emerging, cheap and readily available bioresource (Teo and Teoh, 2011). Sohail *et al.*, (2009) has studied species of *Aspergillus* such as *Aspergillus niger* and reported to produce cellulose under solid state fermentation. It also produces large quantities of endoglucanase and *β*-glucosidase, but with relatively low exoglucanase and some high-level protein (Sohail *et al.*, 2009). In numerous reports, *Aspergillus* sp. have been documented to have high fructosyltransferase (Ftase) activity and since it displays high cellulase activity, a correlation can be drawn with similarities to coprophilous fungi.

A lot of work involving the study of coprophilous fungi has boarded on fungal taxonomy, fungal ecology and coprophilous succession (Ebersohn and Eicker, 1992, Ebersohn and Eicker, 1997, Monteiro *et al*., 2011). Even though taxonomic studies are a vital tool to contribute to better comprehension of dung-inhabiting fungi, the main objective of this study was to source for novel fructosyltransferase and inulinase enzymes.

Table 2.4: Recent investigations of herbivore dung as a source of enzymes

Source of dung	Aim of the study	Preliminary investigation	References
Giraffe, zebra and impala	To evaluate the faeces of wild herbivores in South Africa as a potential source of hydrolytically active microbes	Dung from three indigenous herbivores in Pietermaritzburg, South Africa were sampled. Soil and faecal droppings was measured by triphenyltetrazolium chloride and fluorescein diacetate for hydrolase and dehydrogenase activity respectively. Cellulose, amylase and protease producers were determined by viable plate count on solid agar media containing cellulose, skim milk, starch and Tween 80. Zebra dung displayed the highest hydrolytic activity confirming potential target for new hydrolytic enzyme.	(Ndlela and Schmidt, 2016)
Cow dung from India	A review on cow dung as a cheap available bioresource.	Cow dung contains high diversity of microbial population. Due to this characteristic, it is feasible to obtain microbial enzymes with potential biocatalytic application that can be harnessed to produce enzymes from its high microbial diversity. *Bacillus* sp. from cow dung is capable of producing cellulose, carboxymethyl cellulose and cellulose.	(Gupta *et al.*, 2016)
Cow dung used as substrate	To produce a protease from dung for enzyme bioprocess	In the study, a halo-tolerant-alkaline protease from *Halomonas* sp. PVI was produced under solid-state fermentation. Cow dung serves as a good substrate for enzyme production of detergent-stable dehairing protease by alkaphilic *B subtilis.* Dehairing process was important as it eliminated use of hazardous sodium sulphide.	(Vijayaraghavan *et al*., 2012, Vijayaraghavan and Vincent, 2012)

Table 2.4: Continuation

Source of dung	Aim of the study	Preliminary investigation	References
Cow dung	Statistical optimization of fibrinolytic enzyme	Considering its cheap and readily available cow dung was used as substrate for production of fibrinolytic enzyme from *Pseudoalteromonas* sp. under solid-state culture. The newly protease producing *Pseudoalteromonas* sp. has been reported by various researchers as a potential producer of thrombolytic enzyme. Hence, in the reported study it was worthwhile to screen *Pseudoalteromonas* sp. for fibrinolytic enzyme secretion and statistical model of central composite design employed for enzyme production	(Vijayaraghavan and Vincent, 2014)
Koala faeces	Screening dung from koala species for enzymes production	Thirty-seven (37) fungal strains isolated from koala faeces were identified by molecular tools of 18S rDNA whereby, they were amplified and sequenced. The enzymes extracted from the fungi were screened for various enzyme production such as xylanase, protease, ligninase and endoglucanase. Using plate agar technique one third of the fungi displayed a halo indicating presence of amylase and tannase activity. Some isolates degraded crystalline cellulose while others displayed lipase activity. It was concluded that koala dung could be harbouring wide array of biocatalytic enzymes capable of breaking down recalcitrant substrates.	(Peterson *et al.,* 2009)
Cow dung	Investigate potential of enzyme production from herbivore dung	A potent bacteria *Bacillus* sp. Identified by 16S rDNA was isolated from cow dung. On preliminary screening, the strain showed potential to produce a thermotolerant endoglucanase (CMCase). The strain was purified 8.5 fold with recovery of 39.5 % and characterized for different parameters including temperature, effect of metal ions, chemicals and pH stability. The enzyme in this strain could be applied for bioconversion of lignocellulosic biomass into fermentable sugars.	(Sadhu *et al.,* 2013)

2.10 Rationale of the Study

Several studies have found that human gut flora can be modulated with prebiotics to increase in numbers and activity of *Bifidobacteria*. However, there are positive outcomes against specific disease conditions (Tuohy *et al*., 2003). The search for new sources of enzymes with potential for pharmaceutical and biotechnological applications remains as a contested field of research as there is a growing awareness of benefits and market for functional foods.

Many works have reported of the availability of FOS in trace amounts in fruits, vegetables and some plant products. Therefore, to harness large amounts of FOS yields the route of microbial enzymatic activity of Ftase is more desirable. Enzymes are the most proficient biocatalysts offering a more competitive approach compared to chemical hydrolysis. In addition, environmental awareness has led to processes that are cleaner, safer and more eco-friendly such as biocatalysis. These are compounded by overwhelming consumer awareness and health benefits of fructose, inulooligosaccharides and fructooligosaccharide consumptions. Due to increase in the market value of functional foods, industrial enzymes are preferred alternative for large-scale oligosaccharide production at minimal cost. Herbivorous dung represents a cheap and readily available bioresource that can offer an alternative source of obtaining microbes such as coprophilous fungi.

Researchers have reported Ftase production from different microbial sources like fungi and bacteria with the former proving more economically viable, feasible and sustainable (Gupta and Prakash, 2017). Enzyme cost remains a fundamental factor in the economy of biocatalysed processes. Higher specific activity influences the scale of the required enzyme dosage in the synthesis of FOS (Trollope, 2015). The present study involved bioprospecting for autochthonous coprophilous fungi with potential for producing hydrolytic and transferase enzymes that can be exploited for biotechnological upstream and downstream applications. This study further investigated the synthesis, detection and quantification of inulo-oligosaccharides (IOS) and fructo-oligosaccharides (FOS) by extracellular and intracellular inulinase and fructosyltransferase enzymes isolated from different strains of coprophilous fungi.

Screening for fungal strains for the enzymatic production of FOS and IOS is yet to be explored satisfactorily and not established.

2.11 Hypothesis of Study

It was hypothesized that autochthonous strains of coprophilous fungi can yield high amounts of fructosyltranferase (Ftases) and inulinase enzymes for the production of biofunctional fructo-oligosaccharides (FOS) and inulo-oligosaccharides (IOS), respectively, with antioxidant properties.

CHAPTER 3: Screening, Morphotaxonomic and Molecular Identification of Indigenous Coprophilous Fungi for the Biocatalytic Conversion of Sucrose and Inulin into Biofunctional Prebiotics

3.1 Abstract

Indigenous coprophilous fungi were explored for their ability to produce fructosyltransferase (Ftase) and inulinase enzymes for the biocatalytic conversion of sucrose and inulin into fructo-oligosaccharides (FOSs) and inulo-oligosaccharides (IOSs), respectively. The present study involved collection of herbivore dung from various terrestrial habitats in KwaZulu-Natal province, South Africa, whereby sixty-one (61) indigenous coprophilous fungal strains were isolated and purified to monoculture. The axenic fungal strains were identified to belong to the genera *Neocosmospora*, *Trichoderma*, *Aspergillus*, *Mucor* and *Fusarium* via morpho-taxonomic keys and molecular identification by 18S rDNA sequencing. During preliminary screening, the culture filtrate was examined for transfructosylating and hydrolytic activity using 2,3,5-triphenyl tetrazolium chloride (TTC) as a chromogenic marker and Lugol's iodine solution respectively. Zones of hydrolysis on 30 fungal isolates were observed on the TTC assay plates in diameters ranging from 15 mm to 30 mm, representing high extracellular Ftase to intracellular activity. Staining and clearing zones formed after addition of iodine solution on inulin rich media indicated the absence and presence of inulinolytic activity respectively. Secondary screening involved DNS assays of submerged culture and eight (8) isolates secreted high concentrations of Ftase while six (6) different fungal strains showed <50% inulinase: invertase ratio. The final screening step was tertiary screening involving qualitative detection of biocatalysis products by TLC to visualize saccharide spots of FOSs and IOSs. High performance liquid chromatography coupled with refractive index detection (HPLC-RI) and analysis of Ftase and inulinase reaction products revealed and further confirmed that coprophilous fungi harbour fructosyltransferase and inulinase enzymes for potential biotechnological application for the industrial production of biofunctional prebiotics.

Keywords: Coprophilous, Fructosyltransferase, Inulinase, Fructooligosaccharides, Inulooligosaccharides, HPLC-RI.

3.2 Introduction

Recently, functional foods have been gaining interest due to their physiological bioactive components aside from basic nutrition (Reddy *et al*., 2010, Mutanda *et al*., 2014b). Among these, fructo-oligosaccharides (FOSs) and inulo-oligosaccharides (IOSs) are more desirable due to their low caloric value, anti-cariogenecity factor, decreased levels of phospholipid triglycerides, cholesterol and protonation of potentially toxic ammonia and amine (Zhang *et al*., 2017a, Hernández *et al*., 2018, Kaprasob *et al*., 2018a, Kumar *et al*., 2018b, Nobre *et al*., 2018). These biofunctional compounds are also useful in facilitating the gut to absorb calcium and magnesium ions and more fundamentally, their ability to promote proliferation of *Bifidobacteria* in the intestinal flora while concomitantly suppressing the growth of pathogenic and deleterious bacteria (Ganaie *et al*., 2013, Wang *et al*., 2017). FOS are comprehended as inulin-type oligosaccharide and they are not susceptible to hydrolysis by the human or animal digestive enzymes hence they are considered as prebiotics as they survive gut metabolism and metabolism of these compounds occurs in the colon (Wang *et al*., 2017). The degree of polymerization (DP) influences the speed of fructan fermentation by the gut microbes in the large intestine. Fermentation of FOS primarily occurs in the colon while degradation of inulin chain length elongation mostly occurs in the distal colon (Hernández *et al*., 2018). Commercially, the biocatalytic conversion of sucrose to produce FOSs is mediated by two major enzymes, an invertase (*β*-fructofuranosidases fructohydrolase, FFase, EC 3.2.1.26) which catalyze the hydrolysis of sucrose as well as the transfructosylation from sucrose (Xu *et al*., 2015). Sucrose and inulin hydrolysis is a result of invertase and inulinase activity respectively (Chand *et al*., 2017), while sucrase (sucrose fructosyltransferase, FTase, EC 2.4.1.9) is another enzyme possessing transfructosylating activity to produce FOSs (Gujar *et al*., 2018), which are oligosaccharides containing a single terminal glucose moiety (Sánchez *et al*., 2008). FOS are mainly composed of 1-kestose (GF_2), nystose (GF_3), and 1^F- *β*- fructofuranosyl nystose (GF_4) in which fructose units are bound at the *β*-(2-1) position of sucrose molecule (Sangeetha *et al*., 2005a, Sangeetha *et al*., 2005b, Fernandes *et al*., 2018). To efficiently continue production of Ftase and inulinase there is a need for bioprospecting for novel and sustainable biosources not commonly explored. It is for this reason that increased demand for prebiotics is driving search for novel biosources of FOSs and IOSs producers with differentiated biofunctionalities (Nobre *et al*., 2018). Numerous health benefits of non-digestible carbohydrates via gut fermentation provide energy and nutrient supply to the host and this represent a good

environment for immune regulation, mineral absorption and increased expression of short-chain fatty acids (Younis *et al*., 2015). For the production of prebiotics, two methods are widely used that is; hydrolysis of inulin by inulinase and sucrose biotransformation by Ftase (Gujar *et al*., 2018). Acid or chemical hydrolysis was previously used but has posed several disadvantages due to formation of colored products with no sweetening capacity, formation of di-fructose anhydride and also requirement for high temperatures, which hinder effectiveness of the biocatalyst. These high temperatures may decrease final yield and lead to high energy economy (Díaz *et al.*, 2006). It is for this reason that microbial enzymes are considered as superior alternative sources of Ftase and inulinase enzymes. Enzyme based processes operate at low temperatures, produce less toxic pollutant waste to the environment and have fewer emissions and by-products compared to the conventional chemical process (Tomotani and Vitolo, 2007). The increase in market value of FOS along with other desirable prebiotic properties has propelled the need for novel microbial sources (Ganaie *et al*., 2013, Yan *et al*., 2018). Screening of new strains for Ftase and inulinase enzymes is a tedious and complicated exercise due to a number of evaluations. Nevertheless, researchers have reported on screening of different strains of microbes such as yeast, fungi and bacteria for Ftases and *β*-Ffase production at high sucrose concentration (Hidaka *et al*., 1988a, Yun, 1996b, Bañuelos *et al*., 2005, Dominguez *et al*., 2006a, Guimarães *et al*., 2006, Lateef *et al*., 2008, Yoshikawa *et al*., 2008, Mussatto *et al*., 2009b, Nascimento *et al*., 2016, Rawat *et al*., 2017a, Singh *et al*., 2017a). To the best of the researchers knowledge, there is no report of transferase or inulinase enzymes production from coprophilous fungi. Coprophilous fungi are dung loving fungi that grow and sporulate on herbivore dung (Perrotti and van Asperen, 2018). They assimilate nutrients, that are unused when food passes through the herbivore digestive tract and help in decomposition and recycle nutrients back to the environment (Calaça *et al*., 2014, Calaça and Xavier-Santos, 2016). Herbivorous dung represent a rich reservoir of a cheap and readily available bioresource for growth and manipulation of vital microbes such as fungi that produce transferase and hydrolytic enzymes. Preliminary research has demonstrated that coprophilous fungi can produce enzymes for applied biotechnological upstream and downstream processing (Ndlela and Schmidt, 2016, Lee *et al*., 2018, Mata *et al*., 2018). Therefore, the main aim of the current study was to isolate and screen indigenous coprophilous fungi from various terrestrial habitats in and around KwaZulu-Natal. Consequently, their ability of production, transfructosylating and inulinolytic activities of Ftase and inulinase enzymes was evaluated through a three step screening

exercise. Furthermore, their reaction products were quantified by HPLC-RI, in order to select the best FOS or IOS producer for biotechnological application. The selected strain was identified using morphological taxonomic keys, and by PCR amplification of 18S rDNA sequences and phylogenetic analysis of close nucleotide homology was determined.

3.3 Materials and Methods

3.3.1 Reagents

Triphenyl tetrazolium chloride (TTC), inulin, glucose, fructose (Sigma Aldrich, St Louis), FOS standards 1,1,1- kestotetraose, 1,1- kestopentaose, 1-kestose (Megazyme, Ireland). Sucrose (anhydrous) D-Fructose (Sigma Aldrich), 3,5 Dinitrosalicylic acid, Bradford reagent, NaCl, KCl, $MgSO_4$, $FeCl_3$, K_2Cl, TLC Silica gel 60 F_{254} (Merck, Germany). Czapek Dox Media (CD) (Oxoid), bovine serum albumin (BSA) (Merck, South Africa), Potato dextrose agar, bis-acrylamide, acetonitrile (HPLC grade) (Sigma Aldrich St Louis), Whatman No. 1 filter paper 240mm. Inulin from chicory was purchased from (Sigma Aldrich, St Louis) and Malt extract agar was supplied by Merck, Darmstadt, Germany. All other reagents were supplied by reputable scientific suppliers and were of analytical grade unless stated otherwise.

3.3.2 Sampling and Collection of Herbivore Dung

Sampling of herbivore dung was carried out in three different locations in KwaZulu-Natal Province, South Africa. The herbivore dung samples were collected in North-West KwaZulu-Natal at Ukulinga Research Farm in Pietermaritzburg. The farm covers an area of 400 hectares situated near the University of KwaZulu-Natal, with central coordinates of 29.6627 ºS, 30.4050 ºE. In this site, herbivore dung was collected from three species of domesticated herbivores namely cow, goat and horse and immediately put in clearly labelled sterile plastic bags. The second collection site was Tala game reserve, 30 km from Durban and the area spans nearly 3000 hectares of pristine natural plains characterized by a mix of acacia thorn veld, open grassland and natural wetlands with central coordinates 29.8257 ºS, 30.5416 ºE. The dung collected were from rhino, hippo, giraffe, waterbuck, bush back, impala and zebra. The third sampling site was Phinda game reserve situated in Northern KZN, about 350 km from Durban between Mkuze Game Reserve and greater St. Lucia Wetland Park. The park covers an area of 170 km^2 covered with savannah, bushveld and open woodland with massive flora and fauna. Its central coordinates are 27.8476º S, 32.3335º E. The dung sampled were from white rhino, inyala, buffaloes, elephant, red duiker, common duiker

and zebra. This brought a total of seventeen different dung types, ranging from browsers and grazers with foregut and hind gut fermenters respectively. During collection, all sites were marked with GPS and were used to generate a map showing sample collection points as depicted in (Figure 3.0). All dung samples were put in plastic bags and transported to the Department of Microbiology Laboratory, University of KwaZulu-Natal. In the laboratory, the dung were cultured in moist chambers under ambient conditions near window seals for alternating light and temperature for twenty-five days. The dung was placed in labelled large transparent glass Petri plates 100 mm by 15 mm lined with paper towel and moistened with sterile distilled water. The Petri plates were positioned on window seals to allow sunlight for sporulation during day time (Aluoch *et al*., 2015). The set up was monitored daily for sporulation and fruiting bodies were picked by a sharp needle, and placed on a microscope slide with a drop of water and observed on compound light microscope at 100X magnification (Nikon eclipse ATI with camera Nikon DS-Fil). Morphological features of coprophilous such as size, shape height and width of sporangium, sporangiophore and sporangiospore were observed with a scale set at 100 µm using NIS Element D version 4.60 software. Unique features and characteristics of fruiting bodies including morphological descriptions were analysed using different morphological keys as developed by Brummelen, Bell and Doveri (Brummelen, 1967a, Bell, 1983, Doveri, 2014).

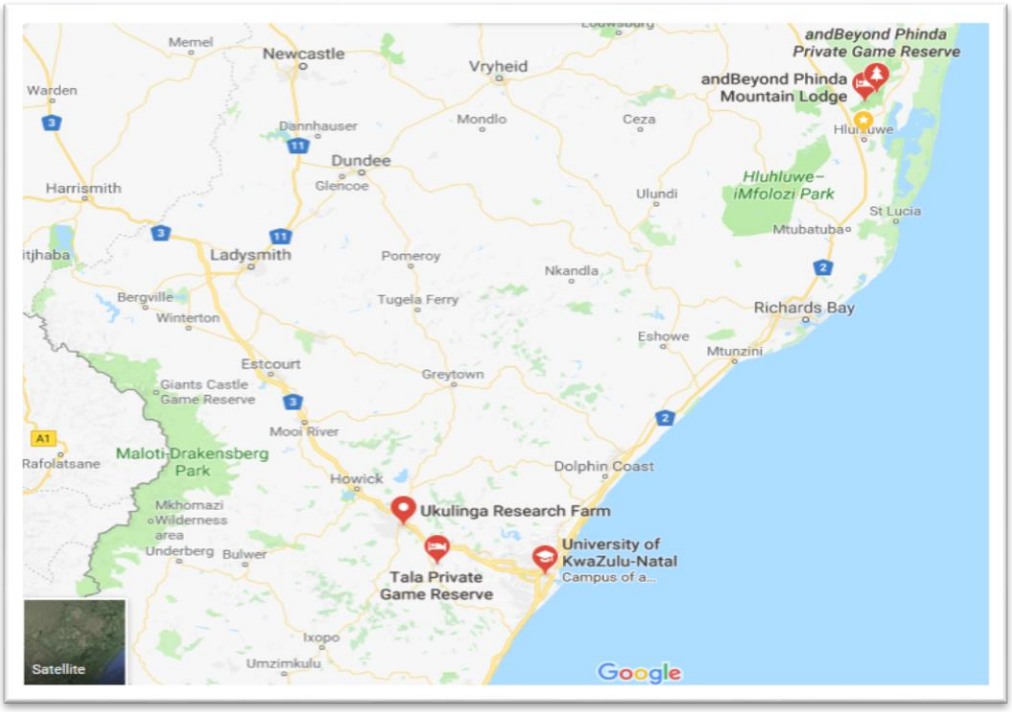


Figure 3.0: A map of KwaZulu-Natal showing different sampling sites around the province.

3.3.3 Purification and Morphotaxonomic Analysis

Small pieces of dung samples of between 2-4 mm^2 were cultured on solid agar of PDA, MEA, CMA, CDA and broth media of MEB and PDB for isolation of fungi and incubated at ambient room temperature in order to initiate fruiting and microbial growth. Dung samples were rehydrated in Petri-plates with moist filter paper until the synnemata was formed on dung surface (Xu *et al*., 2018a). Conidia were dissected and further sub-cultured on PDA for observation of microbial succession and fungal cultures were further sub-cultured on PDA in order to obtain pure axenic microbial isolates (Bridge *et al*., 2008, Farouq *et al*., 2012). The pure fungal isolates were cultivated for 72 h at temperatures ranging between 24 °C and 28 °C and screened for both extracellular and intracellular enzyme activity using suitable enzyme assays. Fungal isolates growing on dung substrate and on the solid agar media were then examined under a stereo microscope (Nikon AZ 100, camera Nikon DS-Fi3) using NIS Element D software version 4.60 for conidia, mycelium, hyphae and other morphological characteristics with keys according to published literature under 400X and 1000X magnification. The pure fungal cultures obtained were

subsequently submitted for DNA extraction, PCR amplification of 18S rDNA and sequencing for molecular studies (Schabereiter-Gurtner *et al*., 2001, Farouq *et al*., 2012, Arif and Saleem, 2017).

3.3.4 Molecular Identification of Coprophilous Fungi

DNA was extracted from pure axenic cultures of fungal isolates obtained from the actively growing colonies using a Zymo kit ZR Fungal/Bacterial DNA MiniPrep™ (Irvine CA92614, USA) according to manufacturer's instructions with some minor modifications. Briefly, 50 - 100 mg (wet biomass weight) of fungal isolates were suspended in 200 µl of water or isotonic buffer Phosphate buffer saline (PBS), (pH 7.4, 0.1M). A 750 µl aliquot of lysis solution was added to the tube secured with a bead beater. This mixture was centrifuged at 10,000 x g for 1 min at room temperature. About 400 µl of the supernatant was transferred to Zymo-spin™ in collection tube and centrifuged at 7000 x g for 1 min. A 1200 µl volume of fungal DNA binding buffer was added to the filtrate. This was then transferred to a centrifuge tube and prewashed with DNA buffer in a new collection tube. All prewashed buffers were combined in a micro centrifuge and DNA elution buffers added directly to the column matrix and centrifuged at 10,000 x g for 30 sec to elute the fungal DNA. The quality and purity of the DNA was checked using the NanoDrop™ 200 (UV-Vis Spectrophotometer) technique and ultra-pure genomic DNA concentration was stored at -20 ºC until used for PCR amplification.

3.3.5 Polymerase Chain Reaction and Phylogenetic Analysis

The PCR reaction was performed in a total reaction mixture of 25 µl volume containing 2 µl of the genomic DNA buffer. The PCR buffer prepared contained 750 mM Tris-HCL 200 mM $(NH_4)_2SO_4$, 0/1 %Tween 20 2.5 mM $MgCl_2$ 0.16 mM dNTPs (Embong *et al*., 2008). The PCR mixture of 15 DNA isolates consisted of 37.5 µl buffer, 15 µl $MgCl_2$, 10 mM dNTPs (7.5µl), oligonucleotide ITS4 (5'TCCTCCGCTTATTGATATGC3') forward and ITS5 (5'GGAAGTAAAAGTCGTAACAAGG3') reverse primers, *Taq* DNA Polymerase (3 µl), H_2O 252 µl and fungal DNA (2µl). The DNA amplification was performed in a T100™ Thermal Cycler (BIO-RAD, USA) under the following conditions (Alvarez-Navarrete *et al*., 2015). Initial denaturation was at 95 °C for 4 min, 25 cycles of denaturation at 95 °C for 30 sec, annealing at 53 °C for 45 sec and elongation at 72 °C for 8 min. A final elongation step was done at 72 °C for 8 min. Agarose gel electrophoresis of the amplified DNA was resolved on 1.5 % agarose gel (Seakem ® LE Agarose) with molecular weight markers used to estimate the molecular weight of

the amplified regions. The products were run at 100 volts for 45 min and later stained with ethidium bromide (0.5 µg/ml) and visualised under UV transilluminator in G: Box F3 system (Cambridge, United Kingdom) (Ferrer *et al*., 2001). The PCR products were visualized by electrophoresis separation on 1.5 % (w/v) TAE agarose gels and sent for sequencing (Inqaba Biotechnical Industries (Pty) Ltd, South Africa). The overlapping fragments were assembled using Chromas Lite (version 2.1) and compared against GenBank database by using Basic Local Alignment Search Tool (BLAST) (http://blast.ncbi.nlm.nihgov/Blast.cgi). The obtained nucleotide sequence was searched for homology in the NCBI nucleotide database in order to find the nearest homology of target enzymes. The distances generated were computed on the basis of the model reported by (Posada, 2008). A phylogenetic tree was constructed from the sequence alignments by the Clustal W programme using the neighbour- joining method with the help of MEGA 6 software (Tamura *et al*., 2013) and the bootstrap value was set at 1000 replicates.

3.3.6 Microorganisms and Culture Conditions

The axenic fungal cultures were stored at 4 ºC in PDA. Three disc of mycelium (8 mm in diameter) were cut on the edge of each fungal colony and transferred to Czapex dox broth (Oxoid, UK) the inoculation medium containing sucrose 30 %, $NaNO_3$ 3 %, $MgSO_4.7\ H_2O$ 0.5 %, K_2HPO_4 1 %, yeast extract 5 %, $FeSO_4.7H_2O$ 0.01 %, KCl 0.5 %, and incubated for 7 days at 28 ºC while shaking at 200 rpm. The initial pH was 6.5. After 7 days the broth was filtered with a mutton cloth and further filtered with Whatman filter paper No. 1 in order to remove all residues. The filtered liquid broth was used as a source of crude enzyme while the fungal biomass that remained after filtration was used for intracellular enzyme production (Dominguez *et al*., 2012).

3.3.7 Inulinase Culture Conditions

Inulinase chemical defined medium was developed from the modified method of Nakamura and Hoashi (1997). The basal medium contained inulin 5 g/l, yeast extract 15 g/l, KCl 0.5 g/l, $MgSO_4.7\ H_2O$ 0.5 g/l, $FeSO_4.7H_2O$ 0.01 g/l, $(NH_4)_2HPO_4$ 5 g/l, (initial pH 6.5) and agar (for a solid medium), 20 g/l. Pure axenic fungal strains were cultured on inulin agar medium in order to observe growth and sporulation. They were maintained at 28 ºC for 5 days on inulin agar plate. Isolates that showed growth were transferred to inulin basal medium and were grown for 5 days at 28 ºC at 200 rpm agitation for inulinase and invertase assay (Nakamura *et al*., 1997). This was the

source of extracellular crude, and intracellular crude was obtained from the fungal biomass after harvesting and extracted as described in the intracellular Ftase production.

3.3.8 Rapid Screening of Transferase and Hydrolase Production

Preliminary screening with a set of sixty-one uncharacterized crude fungal extracts was done using the plate screening assay technique. Zones of hydrolysis on the plates indicated transferase activity (Xu *et al*., 2018a). The rapid transferase assay was performed using the water soluble triphenyl tetrazolium chloride (TTC) reagent (0.1% TTC [w/v] in 0.5 M NaOH). In the presence of reducing sugars, the TTC is reduced to a red water insoluble compound, triphenyl formazan. Using a sterile cock borer, wells were aseptically made on Petri plates containing Czapex dox agar (Sigma Aldrich, South Africa) which is a selective fungal media that contains sucrose as a sole carbon source at pH 7. An aliquot (300 µl) of the cell free supernatant of the crude extracellular and intracellular enzyme extract were loaded into the wells and incubated overnight at 25 ºC. Staining was carried out by spraying with the TTC reagent 1 ml to the agar plate and the plate was incubated in the dark for 30 min and subsequently washed with 0.1 M acetate buffer (pH 5). The appearance of zones of hydrolysis (halo) around the well and colour intensity was suggestive of extracellular and intracellular Ftase production. The quantity of triphenyl formazan formed was directly proportional to the quantity of reducing sugars liberated (Reddy *et al*., 2010, Belorkar *et al*., 2015) All solutions were freshly prepared before use. The isolates that displayed colour intensity of TTC on agar and showed higher zones of hydrolysis were selected for secondary screening. Secondary screening by quantitative enzyme assays was performed on the selected strains. For inulinase primary screening, the 61 fungal isolates were cultured for 7 days at 28 ºC on inulin rich agar media (chemically defined media described on inulinase production) containing inulin as a sole carbon source. Colonies that displayed rapid growth and attained large colony diameters were evaluated on colony size and appearance (Sirisansaneeyakul *et al*., 2007). Fungal isolates that grew on inulin media were considered to have hydrolase activity and were therefore selected for secondary screening (Singh *et al*., 2013).

3.3.9 Harvesting of Crude Fungal Enzyme Extract

Fungi isolates from the primary screening stage displaying high zones of hydrolysis on sucrose rich agar media and fungi that grew on inulin rich agar media were selected for secondary screening involving quantitative transferase and hydrolase assays. The production of extracellular

transferase was prepared by cutting agar blocks (8 mm^2) from seven day old cultures of fungi grown at 28 °C on PDA plates and inoculated into Erlenmeyer conical flasks (250 ml) containing 200 ml of medium for specific transferase production (Shahriarinour *et al*., 2011). The cultivation media contained (w/v): sucrose 30 %, $NaNO_3$ 3 %, $MgSO_4.7\ H_2O$ 0.5 %, K_2HPO_4 1 %, yeast extract 5 %, and $FeSO_4.7H_2O$ 0.01 % having an initial pH of 6.8. Volumes of 200 ml of this media were dispersed in 250 ml Erlenmeyer flasks and autoclaved at 121 °C for 15 min. Three blocks from the seven day pure inoculum were transferred into the sterile media and flasks were incubated at 28 °C in a shaking incubator at 200 rpm for 120 h. Media contents were filtered through a Whatman filter paper No. 1 and the cell-free culture filtrate was used as a source of crude extracellular enzyme without any further purification (Ganaie *et al*., 2013).

3.3.10 Preparation of Crude Intracellular Fungal Ftase Extract

After harvesting the crude fungal supernatant post fermentation, the fungal biomass that remained after decantation was re-suspended in 50 ml of cold distilled water for ultrasonication at 200 W. Sonication was done at 5 ºC and cell suspension was kept on ice during disruption in order to prevent unfolding and enzyme denaturation due to heat dissipation. The ultrasonic energy was pulsed 0.5 s active and passive interludes for reduction of free radicals formation. After sonication, cell lysate was centrifuged at 4 ºC and the crude was used as a source of intracellular enzyme (Ganaie and Gupta, 2014).

3.3.11 Transferase Assay

Due to the large number of samples, enzyme reactions were performed in Armadillo PCR Plate, 96-well, clear wells microtitire plates (ThermoFisher Scientific, South Africa). The Ftase activity was determined by incubating 50 µl of the crude enzyme solution with 250 µl of 5 % sucrose (w/v) in 0.1 M citrate phosphate-buffer (pH 6.5) at 60 °C for 30 min in a water bath (Ganaie and Gupta, 2014). The microtitre plates were sealed with adhesive PCR plate seals before incubation (Kračun *et al*., 2015). At the end of incubation, the reaction was stopped by inserting microtitre plate in boiling water for 10 min in order to stop the enzyme reaction. The resultant deactivated transferase i.e. reducing sugars liberated was analyzed by dinitrosalicylic acid (DNS) method (Miller, 1959). An aliquot of 250 µl of DNS was added to the reaction mixture and boiled for a further 5 min and absorbance was measured in a microtitre plate reader at 540 nm (Synergy ™ Neo 2 Multi-mode microplate reader, BioTek, Vermont, USA). The calibration curve was used to estimate the amount

of reducing sugars liberated (Appendix B). One unit of fructosyltransferase (Ut) and hydrolase (Uh) activities were defined as the amount of enzyme that released one µmole of glucose per ml per min under the assay conditions (Nemukula *et al*., 2009).

3.3.12 Fungal Biomass Estimation

The fungal biomass of high inulinase and transferase producers were determined as the dry cell weight (mg ml^{-1}). The mycelia that was obtained by filtration of the fermentation broth was washed with deionized water, dried at 105 °C for 4 h and then weighed (Wang *et al*., 2017).

3.3.13 Screening for Inulinase Producing Fungi

The fungal isolates were screened for inulinase production on the basis of hydrolytic zones on inulin-rich medium and the action pattern of enzyme on inulin like formation of clear halo zones around fungal mycelia. Inulinolytic fungal strains were further screened using quantitative enzyme assays (Singh *et al*., 2013). Lugol's iodine assay was used for primary screening. The fungal isolates were grown on enrichment media containing inulin as carbon source at 28 ºC for 5 days. The Petri plates were flooded with Lugols iodine solution comprising of 1 % iodine, (w/v) and 1.5 % potassium iodide (w/v) for 5 min. The plates were washed thrice with sterile distilled water and left open for 30 min. The formation of clear zones of hydrolysis was considered positive for inulinase production.

3.3.14 Preparation of Crude Intracellular Fungal Inulinase Extract

Fungal blocks were cut from agar plates on inulin rich media and inoculated in liquid basal media with inulin as the sole carbon source. They were further incubated for 5 days at 28 ºC, with shaking speed of 200 rpm at pH 6.5. Post fermentation the crude fungal supernatant was decanted. The fungal biomass that remained after decantation was re-suspended in 50 ml of deionized water for ultrasonication at 200 W. Sonication was done at 5 ºC using Omni Sonic Ruptor 400 Ultrasonic Homogenizer-220v and cell suspension was kept on ice during disruption in order to prevent unfolding and enzyme denaturation due to heat dissipation. The ultrasonic energy was pulsed 0.5 s active and passive interludes for reduction of free radicals formation. After sonication, cell debris was centrifuged at 10 000 x g for 20 min at 4 ºC and the supernatant was used as intracellular crude for inulinase (Chen *et al*., 2011). The samples were withdrawn periodically and were analyzed for inulinase (I) and invertase (S) activity (Dinarvand *et al*., 2017).

3.3.15 Determination of Inulinase Activity

The crude enzyme extract (1 ml) was mixed and reacted with 3 ml of the substrate 1 % w/v inulin from chicory (Sigma Aldrich, 12255 Lot SLBQ7169V) in 0.1 M citrate phosphate buffer (pH 6.5). Inulinase activity was estimated using the DNS method of Miller (1959). The inulinase activity was assayed by measuring the amount of reducing sugar released from the reaction mixture. The assay mixture was prepared by adding 1 ml of enzyme extract to 1% (w/v) inulin 3 ml suspended in 0.1 M citrate-phosphate buffer pH 6.5 at 60 °C for 30 min in triplicate. The control test tube was made up of 3 ml inulin substrate with 1 ml buffer. The reaction mixture in the 5^{th} tube was made up of 3 ml of citrate-phosphate buffer and 1 ml enzyme and was used to calculate the actual amount of reducing sugar in the assay tubes. The enzyme activity was terminated by boiling for 5 min. An aliquot of 500 µl of the enzyme reaction mixture was treated with 3 ml of DNS reagent and the tubes were boiled for 5 min at 100 °C in a water bath and cooled to room temperature using running tap water. The tubes were diluted with distilled water (20 ml) and absorbance was read at 540 nm (Mutanda *et al*., 2009). Inulinase activity was defined as the quantity of enzyme liberating one micromole of reducing sugar per min under standard assay conditions. The calibration curve previously drawn using pure D-fructose standard (0 - 2.0 mg/ml) was used to estimate the amount of reducing sugar produced.

3.3.16 Determination of Invertase Activity

The crude enzyme extract (1 ml) was mixed with 3 ml of 5 % (w/v) sucrose as substrate (analytical grade) suspended in 0.1 M citrate-phosphate buffer pH 6.5. The mixture was incubated and analyzed for reducing sugar as previously described for the inulinase assay. One unit of invertase activity was defined as one micromole of reducing sugars liberated per min per ml under the assay conditions (Sirisansaneeyakul *et al*., 2007).

3.3.17 Tertiary Screening

Products showing high transferase and inulinase activities from the secondary screening stage were selected and evaluated for qualitative analysis using thin layer chromatography (TLC). The products were separated by TLC Silica gel 60 F_{254} (Merck, Germany), and then compared to standard solutions of glucose, fructose, sucrose and authentic oligosaccharide standards with short chain degree of polymerization (D_P) (Dp_3, Dp_4 and Dp_5). The syringe filtered carbohydrate samples of (5 µl) of the reaction mixture were spotted on the TLC Silica gel 60 F_{254} plate and then

developed with a solvent system of butanol-acetic acid-water (5:3:2 v/v/v) as the mobile phase in a TLC tank at ambient laboratory temperature (Reiffová and Nemcová, 2006, Singh *et al*., 2016a). To get clear separation of the reaction products, the TLC plate was left to saturate completely with the mobile phase. The plates were then air dried and sprayed with *p*-anisaldehyde reagent and dried in an oven at 110 °C for 10 min and spots visualized (Mutanda *et al*., 2009, Mutanda *et al*., 2015).

3.3.18 Quantification of FOS/IOS Produced

End products (FOS/IOS) of enzymatic reaction were analysed by high performance liquid chromatography, coupled with a refractive index detector (HPLC-RI), YL 9100 system, manual injector, Pinnacle 11 Amino 3 µm, 150 x 4.6 mm column (Sigma Aldrich, South Africa) with 73 % acetonitrile as mobile phase (v/v) in milliQ distilled water (Mutanda *et al*., 2008b). Samples and mobile phase were degassed and filtered before injection. The injection volume of the sample was 20 µl from the vial. The column temperature was maintained at 80 °C inside a column chamber with the mobile phase flow rate set at 1.0 ml/min using isocratic elution. Standard curves of glucose and fructose were plotted from a concentration range of (0 – 10 mg/ml). A stock (10 mg) of each standard of oligosaccharide GF_2, GF_3, GF_4 were weighed separately in 10 ml volumetric flask fully dissolved and top up to volume and used to quantify peak areas of samples by comparing retention times (Lim *et al*., 2005b). The method was validated based on linearity, precision and accuracy of analytes by plotting calibration curves using authentic standards to quantify reducing sugars of transferase and hydrolysis products. The samples were run in triplicate for accuracy and precision with relative standard deviations calculated (Yang *et al*., 2011).

3.3.19 Statistical Analysis

One-way ANOVA at 95 % confidence level ($\alpha = 0.05$) was used to analyse the data generated in all the experiments. Statistical tests were performed by GraphPad prism trial Version 7 for windows (GraphPad software, San Diego, California USA). The values were expressed as means ± standard deviations.

3.4 Results and Discussion

3.4.1 Sampling and Isolation of Coprophilous Fungi

Industrial enzymes extracted from fungi present a great opportunity for the production of biofunctional foods with health promoting properties. Bioprospecting for salient fungal strains

with novel properties from unexplored terrestrial habitats is crucial in order to obtain robust industrial biocatalysts. Therefore, dung samples were collected from diverse terrestrial habitats in and around the province of KwaZulu-Natal, South Africa. Herbivore dung was collected from domesticated herbivores, wild herbivores and mega herbivores. The sampling sites were selected based on the herbivore population density and the feeding habits since some were grazers while others were browsers. This is because there are different fungal components in different herbivore dung related to specific herbivore feeding habits. As herbivores graze, they ingest conidia and spore alongside vegetation in which some propagules are geophilic, endocoprophilous and phylloplane fungi (Caretta *et al*., 1998). Sampling was done on fresh dung droppings in the early hours of the day as they contain fresh biomass that was wet and had some vegetative propagules. Some of the isolates taxa have already been reported in other countries like genera of *Podospora,* and *Mucorales*, (de Souza *et al*., 2017). The sampling was done from December 2016 to June 2017 that is in the summer, autumn and before the start of winter in KwaZulu-Natal with average temperature about 25 ºC. The average environmental temperatures were within the optimal range for fungal growth. The first strains from preliminary screening showing considerable zone of hydrolysis are presented (Table 3.1). A large diversity of fungal species enjoy higher richness during warmer and humid conditions than in dry conditions (Masunga *et al*., 2006). Fungal isolation is a necessary prerequisite for obtaining pure cultures for fructosyltransferase production under solid state fermentation and submerged fermentation (Muñiz-Márquez *et al*., 2016). In this study, sixty-one (61) fungal species (Appendix D) were isolated and grown on different agar media, (PDA, MEA, CMA) and cultivated on fermentation broth media (Fernandez *et al*., 2003). Ten herbivore dung types were sampled at Ukulinga Research Farm, seven herbivores at Tala game reserve, and eight at Phinda game reserve. Twenty-seven (27) pure isolates were obtained from dung from Tala game reserve, nineteen (19) isolates from the dung from Phinda game reserve and fifteen (15) isolates from the dung from Ukulinga game reserve. All axenic isolates were maintained and preserved on PDA at 28 °C. Fungal succession was observed on the moist damp chambers under ambient lab condition. The fungi showing great potential after preliminary screening were selected. However, the interest of this research was not to focus on fungal taxonomy, fungal diversity, fungal ecology or fungal succession but rather on the best fungal strains that produced the target transferase or inulinase enzymes for potential biotechnological application.

Table 3.1: Selected fungal strains from initial screening showing considerable zones of hydrolysis after the TTC assay

Species Number	Herbivore species	Sampling site	Assigned number	Zone of hydrolysis (mm)
2	White rhino	Tala game reserve	XWRP-2	23
48	Buffalo dung	Phinda game reserve	XOBP-48	25
10	Goat dung	Ukulinga research farm	XGOU-10	17
11	Goat dung	Ukulinga research farm	XGOU-11	20
12	Giraffe dung	Tala game reserve	XGFT-12	18
13	Inyala dung	Phinda game reserve	XIOP-13	15
14	Giraffe dung	Tala game reserve	XGFT-14	9
42	Elephant	Phinda game reserve	XOEP-42	21
21	Buffalo	Phinda game reserve	XOBP-21	23
22	Impala dung	Tala game reserve	XIMT-22	18
23	Impala dung	Tala game reserve	XIMT-23	23
26	Cow dung	Ukulinga research farm	XOCU-26	9
27	Cow dung	Ukulinga research farm	XOCU-27	24
28	Cow dung	Ukulinga research farm	XOCU-28	9
30	Zebra	Tala game reserve	XZBT-30	9
43	Elephant	Phinda game reserve	XOEP-43	26
33	Hippopotamus	Tala game reserve	XOHT-33	19

3.4.2 Primary Screening

In the primary screening exercise, an optimized and economic procedure of radial diffusion staining with triphenyl tetrazolium chloride (TTC) was undertaken. This staining procedure is rapid, simple and reproducible (Joshi *et al*., 2004). The principle is that TTC is reduced by the transferase enzyme into a red insoluble formazan. Viable isolates stain deep red with a clearing halo, while the less stained indicate no significant hydrolysis. A major advantage of this technique is that it provides a clear and high contrast resolution (Joshi *et al*., 2004).

It was noted that the color of TTC formazan fade with time but was stable in the first 72 h. However, a high contrast differentiation of hydrolytic enzymes was obtained and provided reliable measurements which corresponded with the enzyme activity of transferase and inulinase enzymes (Chang *et al*., 1999). The 61 pure fungal strains were inoculated in the basal medium with sucrose as a sole carbon source. To determine the transferase activity, the zone diameter was measured as shown in (Table 3.1). Only 30 strains shown in Table 3.1 were considered from the large sample size of 61 strains as these strains showed considerable colour intensity and halo a characteristic of sucrose hydrolysis. The colour intensity and zones of hydrolysis were compared as shown in Figure 3.1.

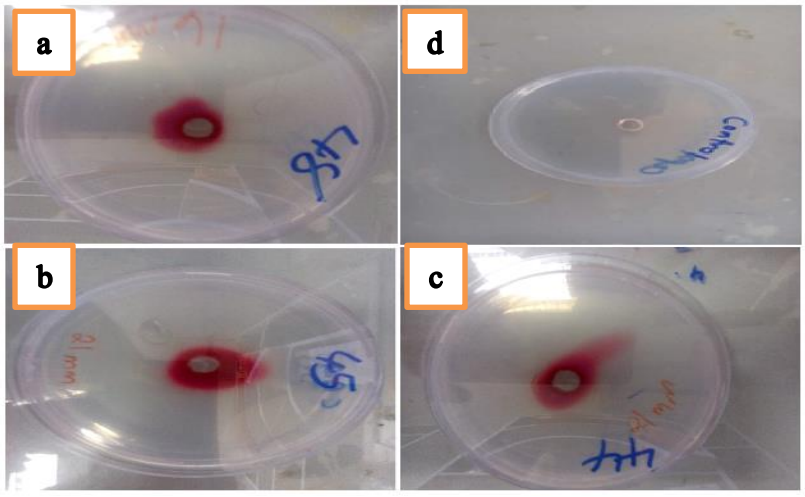


Figure 3.1: Hydrolytic activity of isolates 48 (a), 45 (b), 44 (c) showing halos formed after hydrolysis and control sample (d) with no halo.

The strains chosen ranged between 15 ± 0.10 to 30 ± 0.10 mm. The largest zones of hydrolysis was recorded for isolates named XOBP - 48 (25 mm), XWRP - 2 (23 mm), XOBP - 21 (23 mm), XOEP - 42 (21 mm), XOEP - 43 (21 mm), while the smallest zones of hydrolysis was recorded in the isolates named XZBT – 30 (9 mm), XOCU - 28 (9 mm) (Table 3.1). The diameters of the zones of hydrolysis obtained in the current study correspond to research findings previously reported (Reddy *et al*., 2010). The other 31 isolates did not give any considerable zones so they were excluded from the rest of the study. The screening exercise is in agreement to research work reported by (Belorkar *et al*., 2015) where they screened a large sample size of fungi and bacteria for Ftase production. In this exercise, filamentous fungi showed greater potential for Ftase production in consensus to reports by several authors about filamentous fungi producing inulinase and transferase enzymes (Yun, 1996b, El-Naggar *et al*., 2014, Kowalska *et al*., 2017). The coprophilous filamentous fungi selected from the primary screening exercise were subsequently subjected to secondary screening. There was imperceptible intracellular activity of fructosyltransferase from the primary screening exercise. This could be attributed to the challenge of cell disintegration even after sonication. Therefore, even the vibrations of microbial cells did not necessarily lead to cell disruption to leach out proteins or enzymes that may be remotely located in microbial cells (Lateef *et al*., 2007a). Moreover, intracellular enzymes have been applied more in immobilized cells. Despite the superiority of immobilization, enzyme column, matrices and stability of the enzyme remains a complex industrial process (Hernalsteens and Maugeri, 2010b).

3.4.3 Secondary Screening for Ftase

The 21 filamentous fungal strains selected for secondary screening were cultivated in Czapex dox broth, with sucrose as a sole carbon source under submerged fermentation. Depending on their hydrolysis, isolate XWRP - 2, from white rhino, XOBP - 48, XOBP - 21, from buffalo dung, XOEP - 42, XOEP - 43, XOEP - 44, from elephant dung, XOBT - 46, from bushback, XGOU - 10, from goat dung, XOHU - 36, XOHU - 37, from horse dung (Figure 3.2) showed high Ftase activity**.** Isolate from buffalo dung had the highest transferase activity of 529.5 U/ml followed by isolate 2 from white rhino with 426.9 U/ml. The enzyme activity of isolate XOCU - 28 from cow dung was determined to be 184.7 U/ml. These findings are in line with enzyme activity reported in a screening exercise by other workers who reported highest extracellular Ftase activity from *Penicillium* sp. to be 313.13 U/ml (Nascimento *et al*., 2016). Additionally, a study of an inulinase producing fungus of *Aspergillus awamari* MTCC 2879 produced 107.8 U/ml of inulinase activity,

(Rawat *et al*., 2016) while *Aspergillus niger* ATCC 26011 produced 7.9 U/ml of inulinase activity (Rawat *et al*., 2015). This demonstrated that the strain named as XOBP - 48 from buffalo dung was a potential candidate for transferase production. A novel thermotolerant endoglucanse (CMCase) that was identified originating from the genus *Bacillus* isolated from cow dung was also seen as a potent transferase producer with total activity of 113.6 U/ml from crude and was later purified to 8.5 fold and characterized (Sadhu *et al*., 2013). This investigation showed that herbivore dung harbor fungal strains that can be harnessed for production of transferase and inulinase enzymes.

The average values of Ftase activity produced by the coprophilous fungi are presented in triplicate with standard deviations in (Figure 3.2).

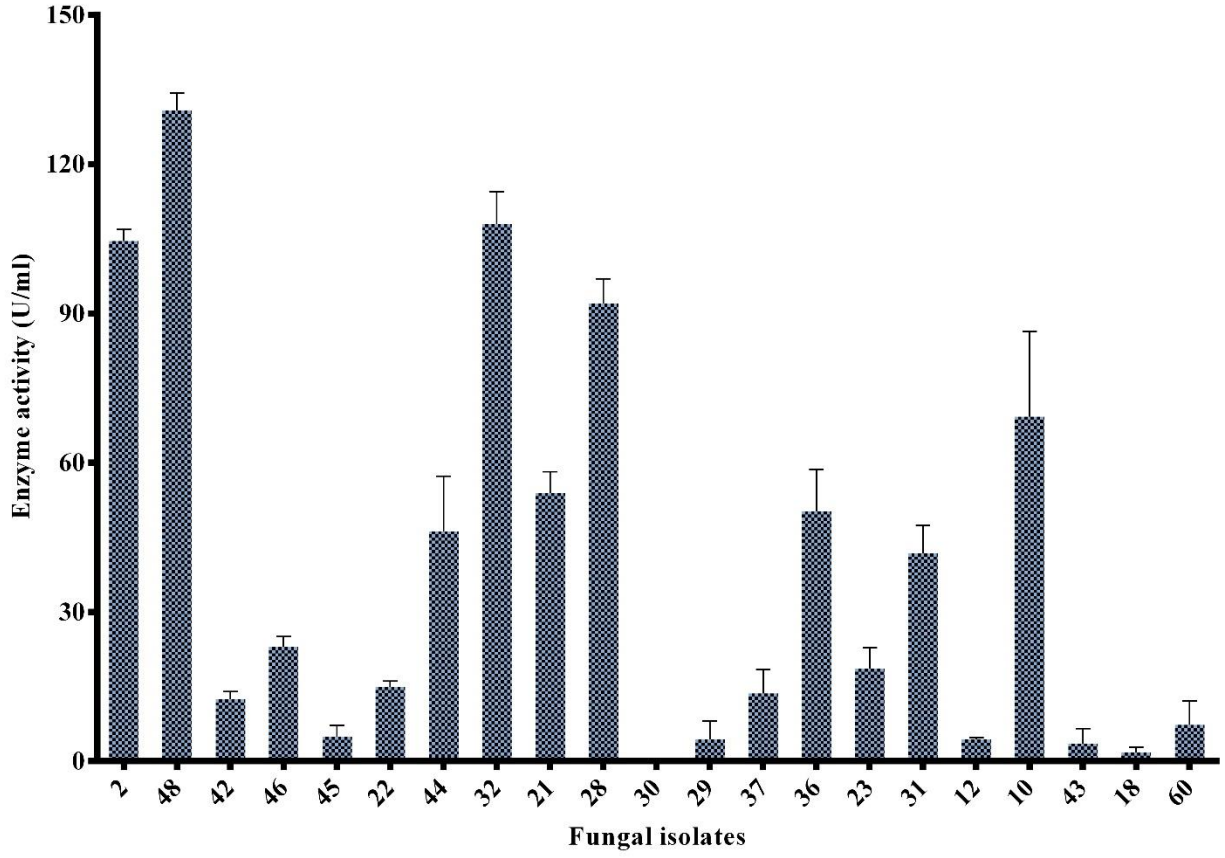


Figure 3.2: Fructosyltransferase activities of selected coprophilous fungal strains showing zones of hydrolysis using sucrose as substrate for the Ftase enzyme.

3.4.4 Tertiary Screening of Ftase and Inulinase Producing Fungi

It was necessary to perform qualitative analysis of the transferase reaction products in order to establish the nature of the enzyme and profile of the product composition and degree of polymerization (DP). Isolate XWRP - 2, XOBP - 48 and XOCU - 28 were selected as they displayed high transferase activities after qualitative TLC (Figure 3.3). Various studies on prebiotics dietetics have reported TLC as an efficient method for the detection of fructooligosaccharides (Reiffová and Nemcová, 2006). Fructooligosaccharides of various degrees of polymerization were detected from the intestinal tract of monogastric animals with butanol-ethanol- water (5:3:2 v/v) as the mobile phase. In this study, after performing the DNS assay the best enzyme producers were selected after enzyme quantification (Figure 3.3). The Ftase enzyme reaction products showed presence and preponderance of 1-kestose (DP_3), nystose (DP_4) and fructofuranosyl nystose (DP_5) along with glucose produced at the end of the reaction.

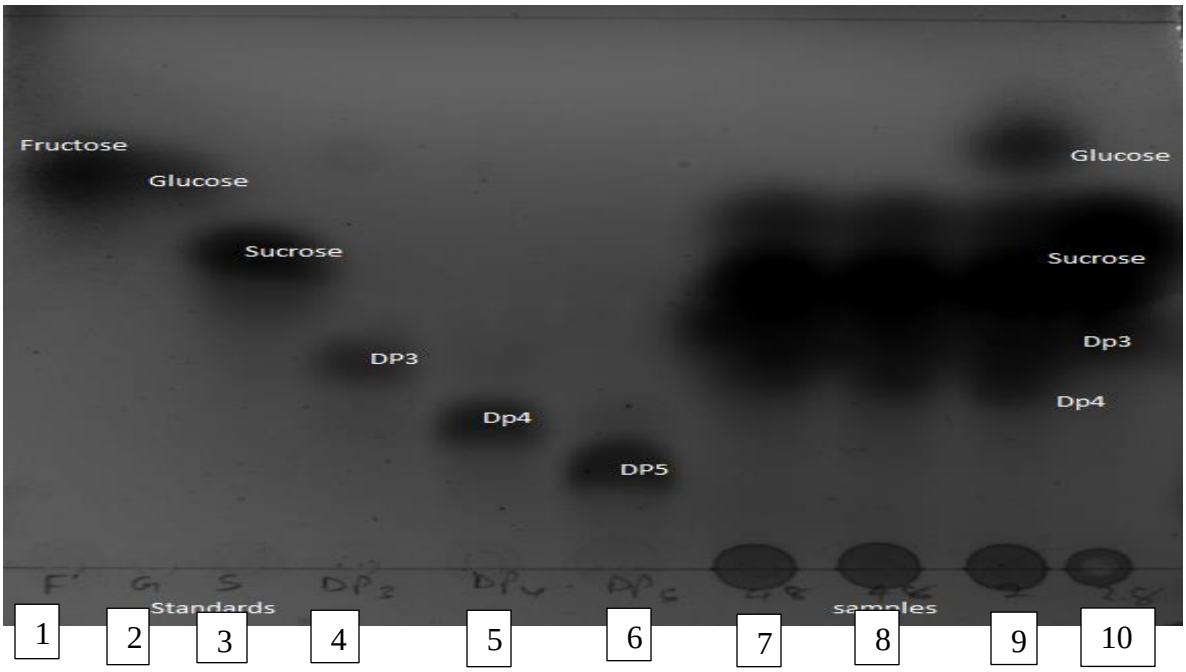


Figure 3.3: TLC profile of transferase enzyme reaction products of fungal samples XWRP - 2, XOBP - 48 and XOCU - 28 and selected sugar standards of fructose(1), glucose (2), sucrose (3) and 1- kestose (4), nystose (5), and fructofuranosyl nystose (6) XOBP – 48 (7), 8, XWRP – 2 (9), XOCU – 28 (10).

The fungal extracellular crude preparations showed the transfructosylating nature of the enzymes. The mechanism is such that FTase catalyzes the transfer of a fructosyl group to sucrose by mediating a polymerization reaction where the DP maximum by transfer of fructosyl unit from higher molecular mass fructans and converts sucrose to short chain-FOS (Michel *et al*., 2016c). The fungal isolate XOBP - 48 from the buffalo dung was selected as the potent strain for further study based on the studious three step screening exercise. The isolate displayed a considerable zone of hydrolysis, produced the highest enzyme activity and yielded short chain oligosaccharides on tertiary screening. Due to different dung sources, it was necessary to further quantify the reaction products further by high performance liquid chromatography (HPLC) and investigating their potential industrial applications.

3.4.5 Isolation and Screening of Inulinase Producing Fungi

In order to uncover a native coprophilous fungi that may be a potential degrader of the polyfructan inulin, a total of sixty-one (61) pure fungal isolates were cultured on Petri plates containing inulin as a sole carbon source. They were subsequently evaluated based on their growth, appearance and colony sizes. After 7 days of cultivation on inulin agar, 10 fungal isolates showed considerable radial colony growth rates, an indication of inulinase activity. Other 51 fungal strains obtained exhibited weak to moderate growth on agar plates without observable halo zones. A prime factor that influences inulinase production is the composition of the culture medium as inulinase has been widely documented to be an inducible enzyme (Rawat *et al*., 2015). This technique is a common and effective method for screening large number of bacteria for endoinulinase production (Singh *et al*., 2013). Moreover, this technique has been reported for screening fungi, yeast, molds and actinomycetes (Sirisansaneeyakul *et al*., 2007, Mansouri, 2017). The ten isolates 44, 49, 42, 45, 48, 37, 9, 14, 48, 2 were screened with iodine Lugol's solution by flooding the plate and left open in order to enhance visibility. Strains 49, 44, 42 showed considerable zone of hydrolysis on inulin rich media (Figure 3.4). Interestingly, this phenomenon was also observed in screening marine derived yeast and fungi from marine ecosystems (Li *et al*., 2011). Staining using iodine solution resulted in brown to black colour where inulin has been degraded. Strains that formed clear halo around the colony were recorded as inulinolytic, whereas strains that did not form clear zones was recorded as non-inulinolytic and were excluded. This technique is simple, rapid and was found to be effective for screening inulin hydrolyzing enzymes while no dye-labelled substrates are needed

which require more time for binding and are very expensive (Bonciu *et al*., 2011, Li *et al*., 2011, Singh *et al*., 2013).

Figure 3.4: The hydrolytic zone of selected fungal strains after staining with Lugol's iodine solution.

3.4.6 Secondary Screening of Inulinase and Invertase Activity

The results of the selected fungal isolates were further quantified by performing inulinase and invertase enzyme assays in liquid culture media supplemented with pure inulin (Figure 3.5). The hydrolytic activity of crude culture filtrates towards inulin (I) or sucrose (S) was measured to calculate the (I/S) ratio. Extracellular inulinase activity was evident after primary screening, while intracellular activity produced insignificant results that were not considered substantial for industrial application. A considerable amount of fungal isolates revealed potential to produce extracellular inulinase activity to intracellular. Numerous studies have reported on production of extracellular inulinases from fungi, yeast and more recently a panorama of bacterial species (Keto *et al*., 1999, Jing *et al*., 2003c, Chi *et al*., 2009a, Meenakshi *et al*., 2013, Singh *et al*., 2017a).

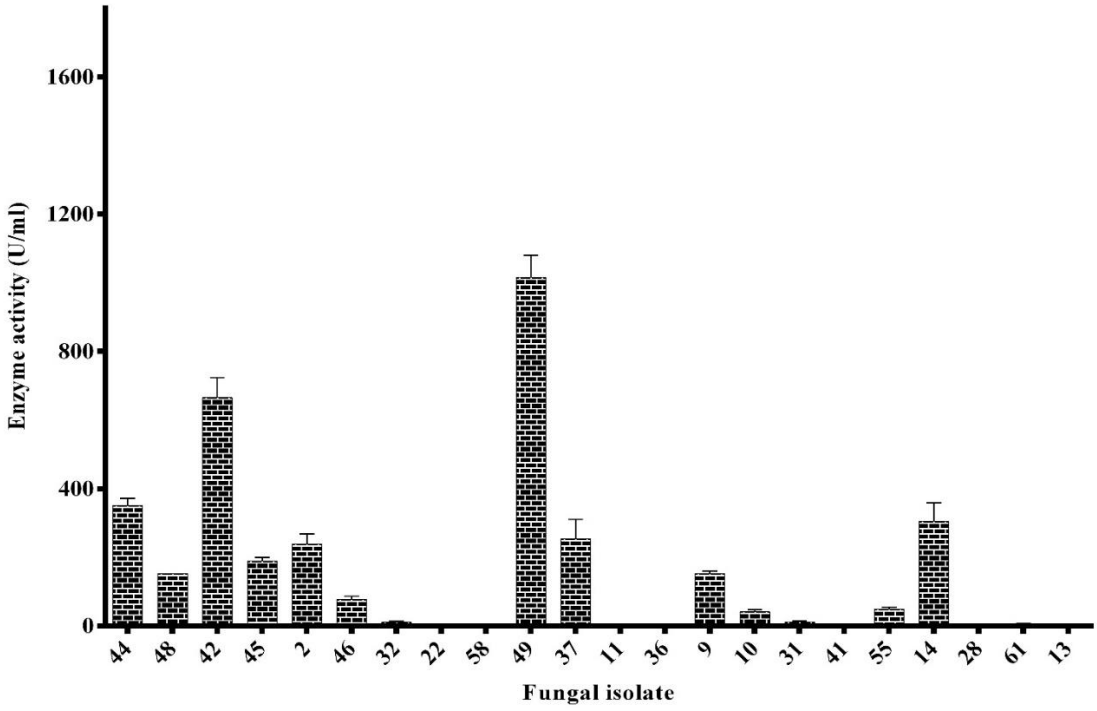


Figure 3.5: Inulinase activity of fungal strains after performing the DNS assay and extrapolated from fructose standard curve.

Strain XOGU - 49 from goat dung in Ukulinga research farm yielded the highest extracellular inulinase activity of 989 U/ml, followed by strain XOEP - 42 (698 U/ml) from elephant dung from Phinda game reserve and strain XOEP - 44 from elephant dung (351.9 U/ml) (Figure 3.5). The inulinase activity recorded in this study was comparable to the selected fungal strains previously reported (Singh *et al*., 2017b). The yeast *Kluyveromyces marxianus* recorded a specific activity of 420 IU/mg. Bacterial strains *Streptomyces* sp., *Bacillus* sp., *Clostridium thermoautotrophicum*, *Xanthomonas oryzae* and *Sphingobacterium* were found to produce inulinase enzymes (Singh *et al*., 2017a). In a separate study, the filamentous fungi, *A. niger* and *Penicillium oxalicum* BGPUP-4 produced inulinase enzymes with activity of 111 U/ml and 208 U/ml respectively (Kowalska *et al*., 2017, Singh and Chauhan, 2017).

Invertase activity was carried out to determine if the fungi had more invertase activity, hence inulinase : invertase ratio was determined (I/S) (Table 3.2). Other workers have also used the (I/S) ratio to define the substrate preference of the crude enzyme and to predict their exo or endo type of activities (Vandamme and Derycke, 1983, Ettalibi and Baratti, 1987, Nakamura *et al*., 1997).

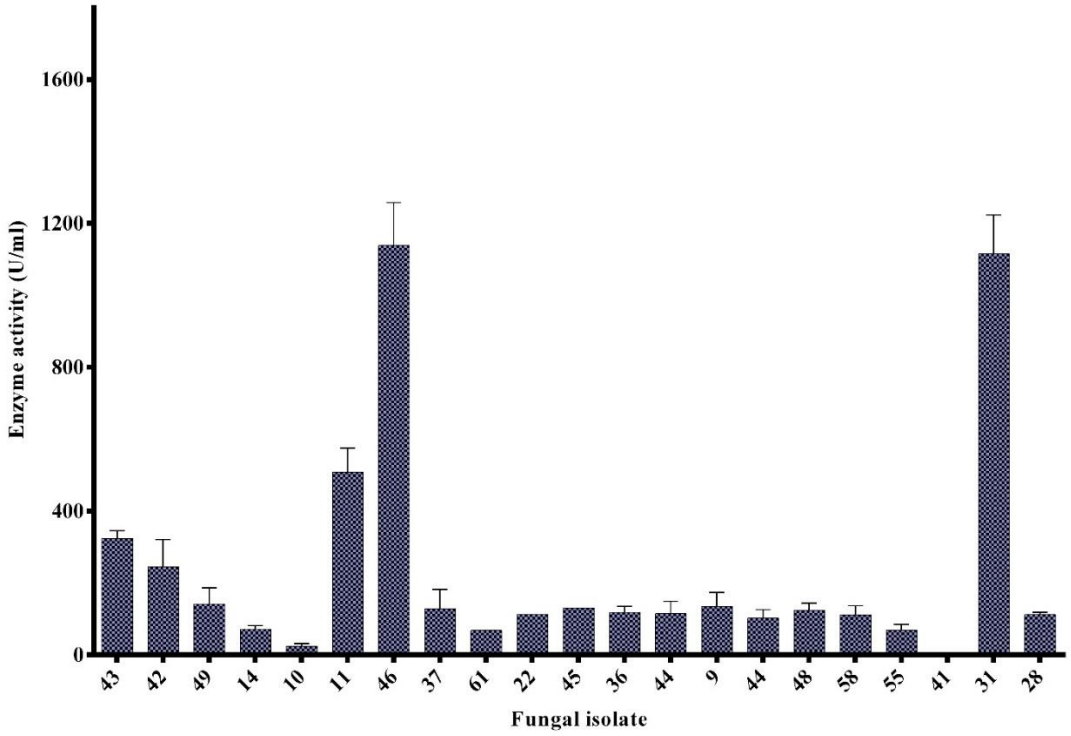


Figure 3.6: Invertase assay of selected fungi showing high inulinase activity following DNS enzyme assay.

High invertase activity was recorded in isolate XZBT - 31 from zebra dung from the Tala game reserve, isolate XOBT - 46 from buffalo dung from the same farm followed by isolate XGOU- 11 from goat dung in Ukulinga research farm (Figure 3.6). Inulinase/ invertase ratio was calculated for all isolates that were screened for inulinase activity as shown in (Table 3.2) to determine if the best strain had a higher inulinase than invertase activity in order to make a valid selection. Inulin hydrolysis (200 g L^{-1}) by the crude enzyme showed lower invertase than inulinase and was therefore selected for further analysis in tertiary screening to establish if they were endo or exo inulinase producers by visualizing the spots for reacting products on TLC. After 7 days of batch culture, an increase was noted in the biomass of fungi producing high invertase activity. Isolate XOBT - 46 and isolate XZBT – 31 exhibited fungal biomass of 7.1 and 5.3 mg ml^{-1} of dry weight respectively. Fungal biomass of isolates XOBP – 48, 11, 44 were subsequently higher. Increase in biomass may be probably attributed to reduction of substrate inhibition and enhanced mass transfer effect. Yun *et al*.,(199) also detected enhancement in glucosyltransferase and fructosyltransferase production from the fungus *A. pullulans* by fresh substrate feeding (Yun *et al*., 1997b).

Table 3.2: Production of inulinase and invertase and their respective I/S ratio for all selected coprophilous fungal isolates after 5 days of batch fermentation.

Isolate	Enzyme activity U/ml		I/S	Fungal biomass (mg ml^{-1})
	Inulinase (I)	Invertase (S)		
XOEP - 44	351.90	154.16	2.28	5.1
XOBP - 48	152.96	130.77	1.77	4.8
XOEP - 42	698.93	370.33	1.89	5.0
XORP - 45	189.43	131.48	1.44	3.2
XOBT - 46	176.39	1165.57	0.15	7.1
XOGU - 49	989.61	198.81	4.98	6.3
XOHU - 37	217.06	100.29	2.16	5
XGOU - 11	33.81	552.48	0.06	6.1
XOHU - 36	75.81	125.81	0.60	5.9
XGOU - 9	160.69	176.84	0.91	4.9
XOEP - 10	45.97	28.0	1.64	5.5
XZBT - 31	11.71	1088.31	0.01	5.3
XOZP - 55	52.61	83.28	0.63	5.1
XGFT - 14	345.29	81.86	4.22	5
XOCU - 28	44.87	118.01	0.38	5.3
XOIP - 61	89.08	68.40	1.30	4.9

[a] E = Elephant dung, C = cow dung, I = inyala dung, Z= Zebra dung, G = Giraffe dung

[b] P = Phinda game reserve, U = Ukulinga game reserve, T = Tala game reserve

[c] *I* = inulinases, S = invertases, I/S = inulinase/invertase ratio

In a previous investigation by (Chand *et al*., 2017) the yeast *Saccharomyces cerevisiae* was reported to have a high invertase activity (416 U/ml) and this confirms the findings in this study whereby filamentous fungi showed higher inulinase activity than invertase (Guimarães *et al*., 2006).

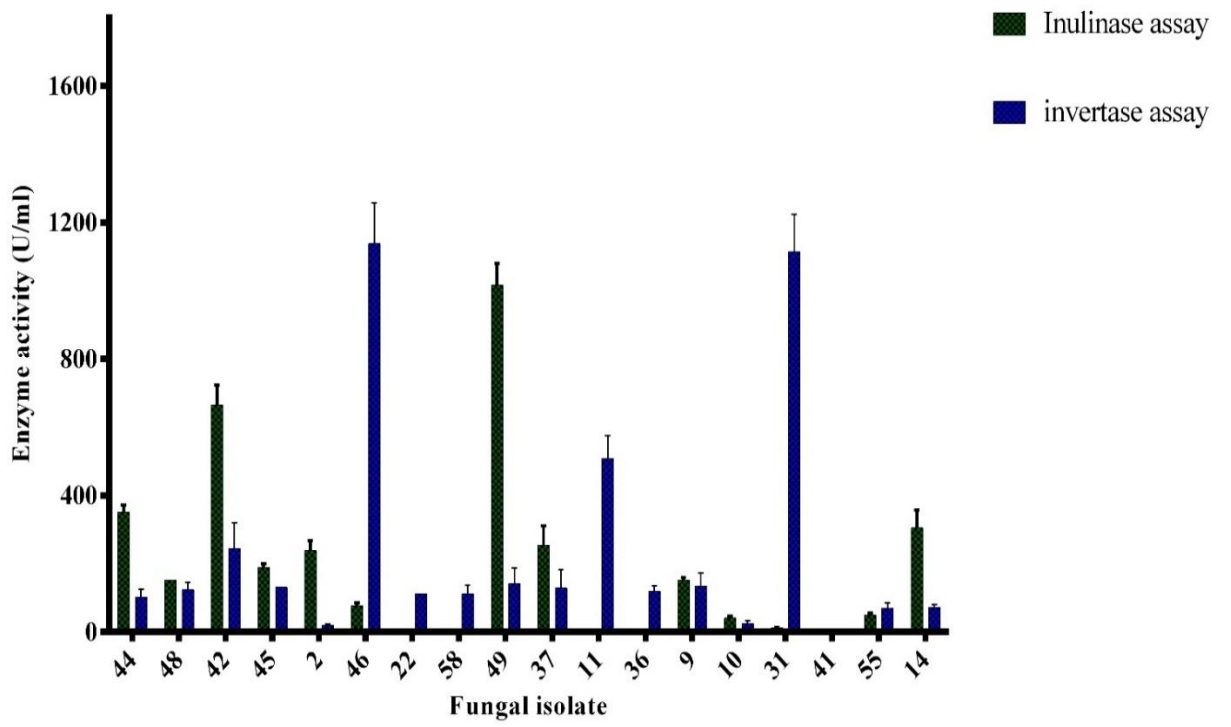


Figure 3.7: Selected strains showing comparative activities of inulinase and invertase producing fungi. Fungal inulinase:inveratse ratio (I/S) of 18 strains.

Isolates XOGU - 49, XOEP - 44, XORP - 45, XOEP – 42 exhibited remarkable potential as inulinase producers (Figure 3.7). This is despite having higher I/S ratio contrary to what is reported in previous studies. In earlier studies, the reported ratios ranging between 0.1 to 0.3 proved that the enzyme was a true inulinase (Mansouri, 2017). Furthermore, it is generally acceptable from

literature that I/S ratio characterizes the inulinases enzymes (Wei *et al*., 1997). If the I/S ration is higher than 10^{-2}, the enzyme is considered an inulinase, while for invertase the ratio is lower than 10^{-4} (Saber and El-Naggar, 2009). Findings from the current study corresponded with studies by (AbdAl-Aziz *et al*., 2012) where they reported very high I/S ratio of between 5.862 to 7.596 U/ml two fold higher than findings from the current study. These findings are attributed probably to the high amount of inulinase units secreted by potent inulinase strains with similarity to this study. Fungal strains 44, 49, 45 and 42 were selected for TLC analysis as they showed high potential for inulinase production with a range of 1.44 to 4.98 I/S ratio. In a recent study by Mansouri *et al*. (2017) they reported screening of filamentous fungi of the genera *Penicillium* which indicated (I/S ratio of 6.7) in submerged fermentation with pure inulin. Another new fungal species, *Penicillium subrubescens* (FBCC 1632) produced inulinase and invertase activities of 7.7 and 6.1 U/ml respectively, with an I/S ratio of 1.3 and was exo- inulinase in nature. Further investigations of *P. trzebinskii* and *P. janczewskii* were also reported with I/S ratio of 2.6 and 1.2 respectively (Mansouri, 2017). In studies conducted earlier on filamentous *Penicillium* sp. TN-88 after a screening exercise. It showed high inulinase productivity of 9.9 U/ml with I/S ratio of 11.2 in inulin liquid culture media (Nakamura *et al*., 1997). Findings from the current screening exercise draws insurmountable similarities with these earlier reports.

3.4.7 Tertiary Screening

Thin layer chromatography was used for qualitative anaysis of inulin hydroysis products from the 4 fungal strains. The hydroysed reaction mixture of isolate XOEP - 44, XOEP - 42, showed distinct correspondence to fructose standard and small amount of glucose in the hydrolysate, a characteristic of exoinulinase as depicted in Figure 3.8. Likewise HPLC anaysis revealed fructose liberation in camparison with standards.

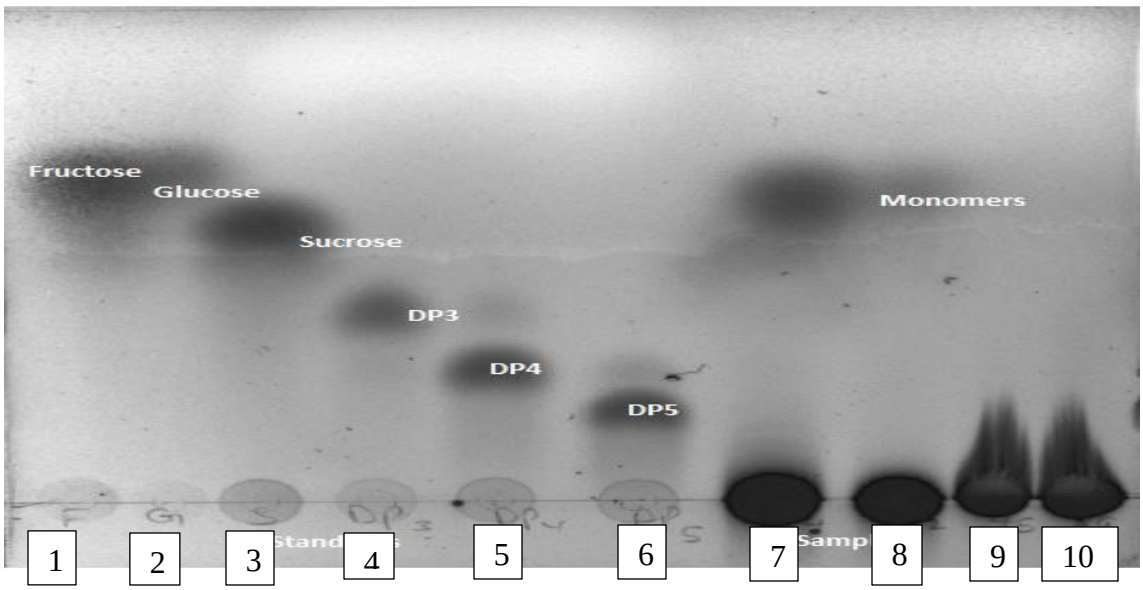


Figure 3.8: TLC showing inulin hyrolysis reaction products of crude fungal enzyme from strains. Lanes: fructose (1), glucose (2), sucrose (3), 1-kestose (4), nystose (5), fructofuranosyl nystose (6) sample XOEP – 44 (7), XOGU – 49 (8), XOEP – 42 (9), and XORP – 45 (10).

Analysis of the inulin hydrolysis products demonstrated the exoinulinase nature of the enzymes from the 4 fungal strains. Microbial exoinulinase hydrolyze the terminal linkage of inulin to yield fructose as the main hydrolysis product (Mansouri *et al*., 2013). There was no presence of oligosaccharides on the TLC plate detected further confirming the exoinulinae nature of the fungal enzymes. Some authors have reported fungal species like *Penicillium subrubescens*, *A. tubingenesis* (Trivedi *et al*., 2012) and *Chrysosporium pannorum* (Liu *et al*., 2010) to be exoinulinase in nature. Additionally, a similar screening exercise revealed a high exoinulinase producing strain of *Aspergillus candidus* (NCIM 83). The purified preparation of inulinase from the fungus produced only fructose as the only product of inulin hydrolysis (Kochhar *et al*., 1999). It was therefore paramaount to quantify the reaction products showing high Ftase and inulinase potential after tertiary screening. Furthermore, the selected fungal strains showing Ftase and inulinase potential were identified via morphological keys developed.

3.4.8 Quantification of Oligosaccharides by HPLC-RI

Reaction products were analyzed by HPLC-RI for monosaccharides namely fructose, glucose; and FOS namely 1-kestose, nystose and 1^F- fructofuranosylnystose. The selectivity of reaction

products examined (Figure 3.9) was determined by correlation of retention times (t_r) obtained for standards and was compared to to samples which showed high resolutions and seperation (Zielinski *et al*., 2014).

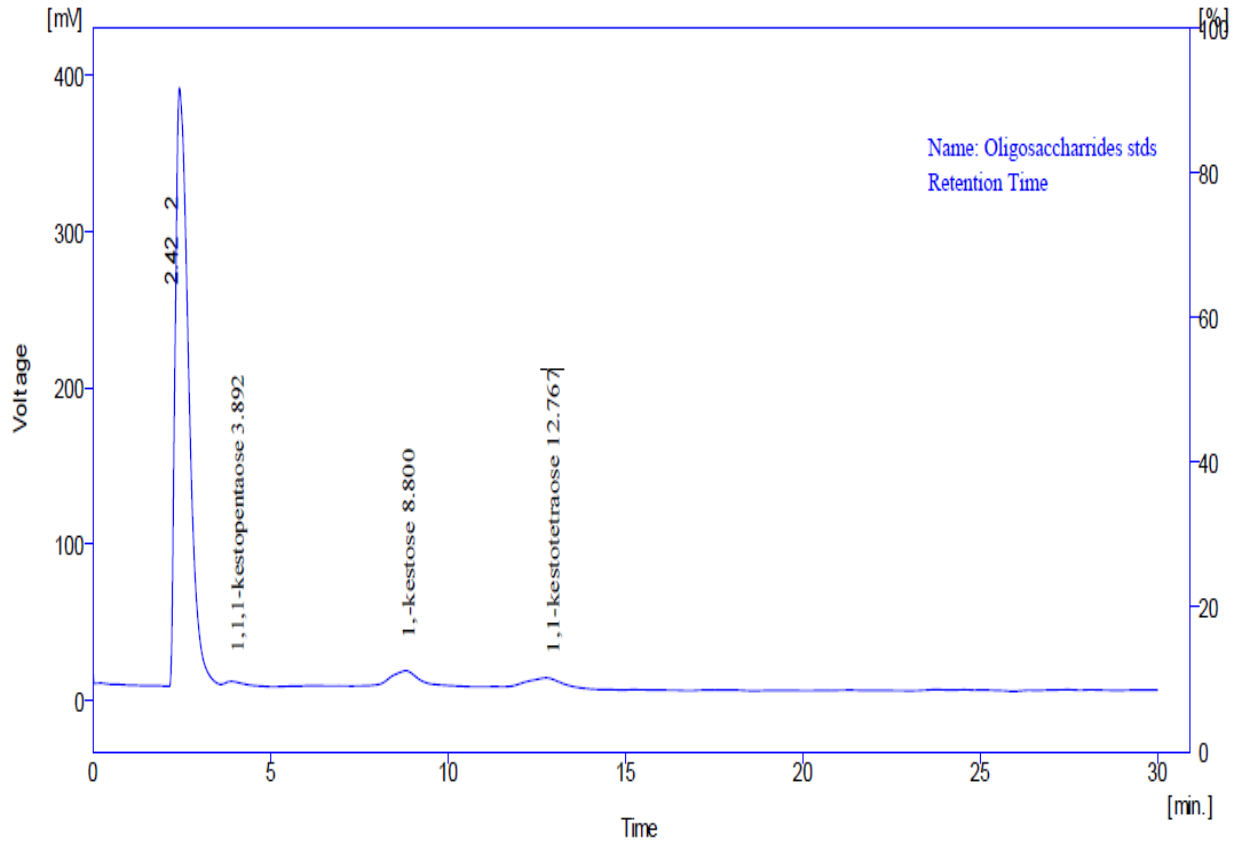


Figure 3.9: HPLC of FOS standards of different degrees of polymerizitaion (DP) DP_3, DP_4,DP_5 with retention times.

The concentration of monosaccharides (Figure 3.10) was calculated from the reaction products using the lenearity of the of calibration curves obtained by linear regression ($R \geq 0.9991$), considering the peak area for each sugar vs the concentration of each sugar (mg/ml) (Appendix F). This calibration correlation was of similar magnitudes in validated methods obtained by (Correia *et al*., 2014) using HPLC-RI detector for FOS analysis and all linear calibration curves were statistically significant with slope values of ($P \leq 0.001$).

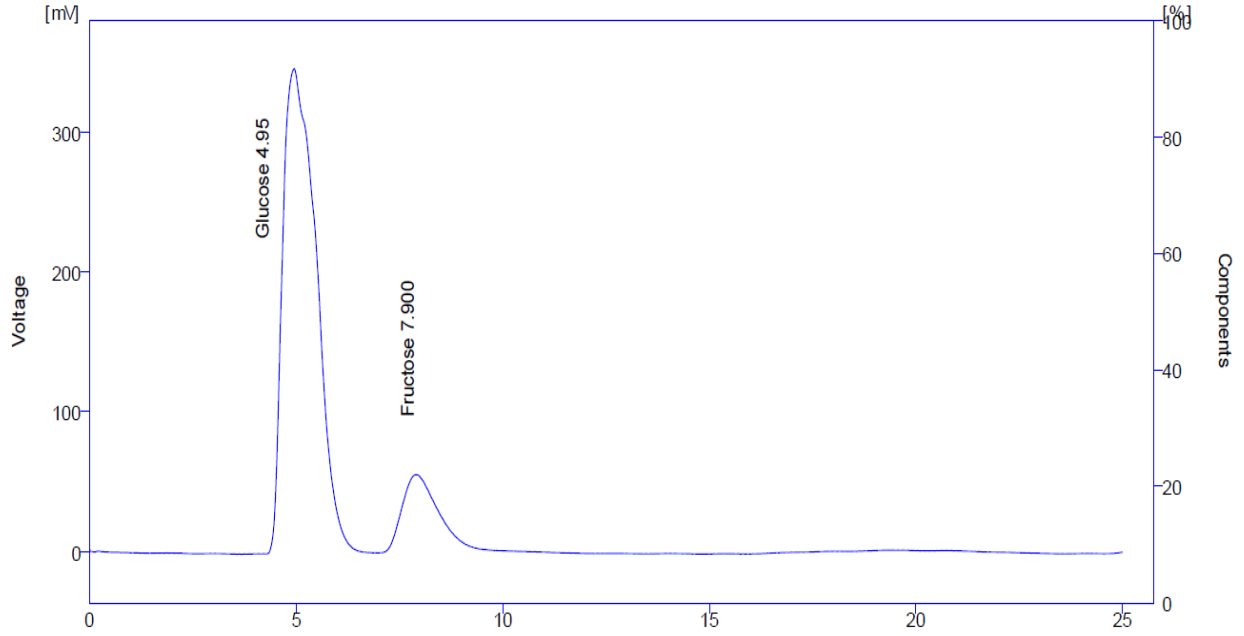


Figure 3.10: Chromatogram of selected monosaccharides standards with their retention times.

The analysis of FOS by HPLC-RI showed the retention time of 1,1,1-kestopentaose as 3.892 min (Figure 3.9) which matched that of reaction products produced by sample XWRP - 2 from Phinda game reserve (Figure 3.11). There was also glucose liberation and thede results corroborated that of earlier TLC results on transferse production as depicted in Figure 3.3 that showed higher production of glucose liberation of 30 mg/ml extrapolated from glucose standard curve from sucrose elongation. From Table 3.3 it is evident that although strain XWRP – 2 liberated high concentrations of glucose, Figure 3.11 strain XOBP - 48 from buffalo dung yielded high level FOS of 1,- kestose and 1,1,- kestotetraose and glucose (Figure 3.12). The production of short chain FOS by isolate XOBP – 48 is a desirable characteristic of oligosaccharide production. From the chain elongation of sucrose to form oligomers, with lower degree of polymerization and the high enzyme activity this strain represented a good candidate for biofunctional poduction of fructooligosaccharides. Other corresponding samples they showed lower peak areas (Appendix H). The fungus strain XOBP- 48 was selected as the highest Ftase producer in the study based on its high oligosaccharide production as previuously confirmed by TLC results (Figure 3.3).

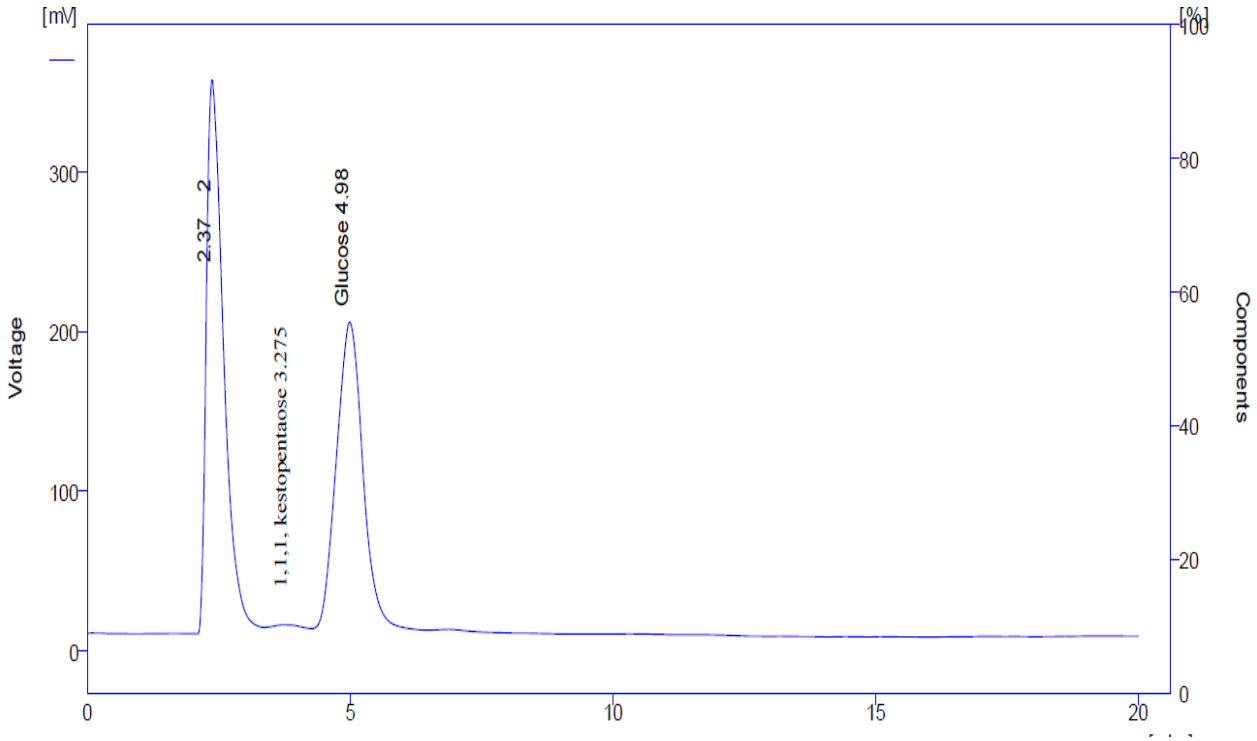


Figure 3.11: Chromatogram of transferase reaction of sample XWRP – 2 sample from white rhino dung in Phinda game reserve identified to molecular level as *T. asperellum* sp.

Table 3.3: Quantification of monosaccharide (glucose) produced from HPLC reactions.

Sample	Concentration (mg/ml)
XWRP - 2	30.958
XOCU - 28	8.711
XOBP - 48	10.835
XZBT - 32	5.657

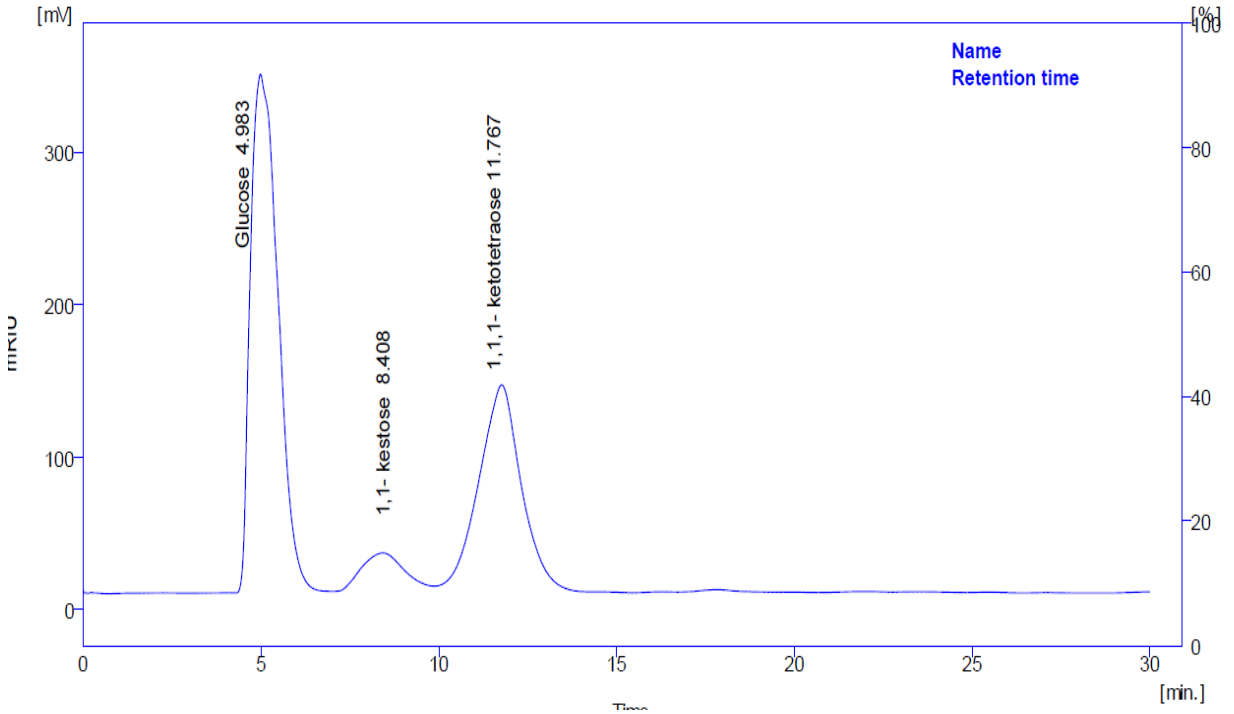


Figure 3.12: HPLC of reaction products of crude enzymes from selected fungal strain XOBP - 48 isolated from buffalo dung from Phinda game reserve that showed highest amount of fructooligosaccharide production.

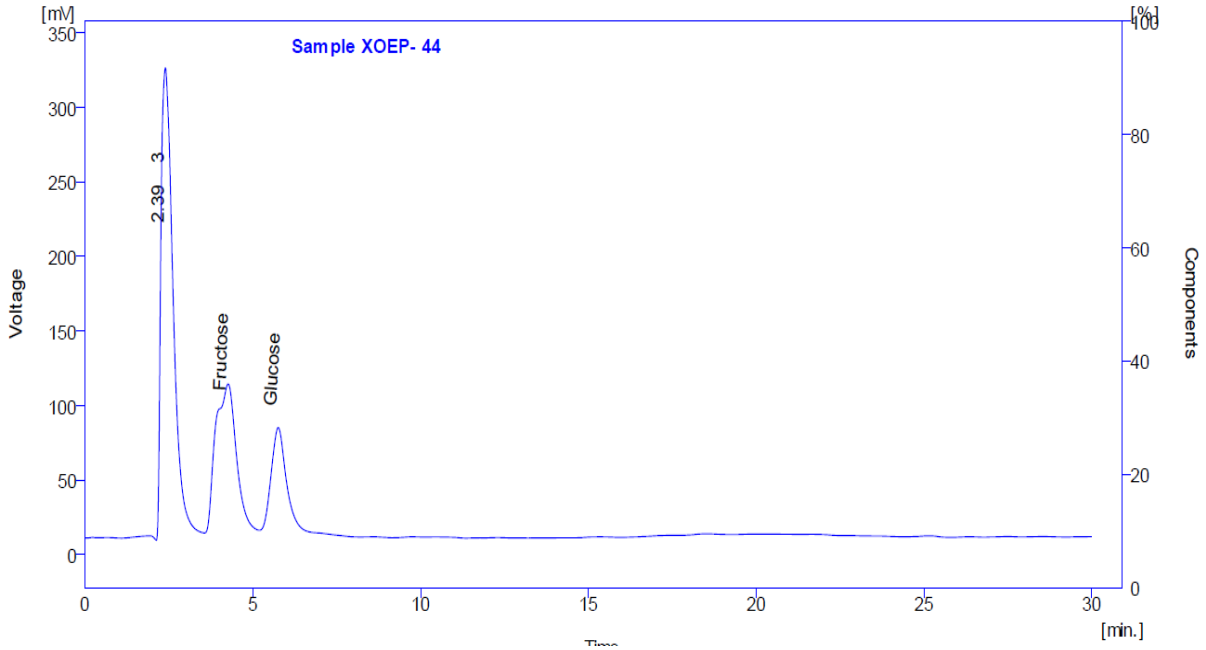


Figure 3.13: HPLC of reaction products of crude enzymes from selected fungal strain XOEP – 44 isolated from elephant dung from Phinda game reserve exhibited exo-inulinase like activity.

For inulooligosaccharide production, the fungus strain XOEP - 44 (Figure 3.13) showed high level of fructose production with no oligosaccharide present indicating the exoinulinase nature of the fungal enzyme (Fernandes and Jiang, 2013). Although the fungus strain XWRP – 2 exhibited high glucose concentarion and 1,1,1- kestopentaose from Figure 3.11 strain XOBP - 48 had the most abunadnt oligomers and 1- kestose was degraded with concomitant increase in glucose concentration as by-product. This study exhibited a simialr trend as previously reported (Correia *et al*., 2014). Equimolar glucose and 1-kestose proportions were produced until satuaration of substrate (Hernández *et al*., 2018). Fructosyl moiety of sucrose was transferred to 1-kestose and liberated nystose which was not elonagted further during incubation. In fungal strain XOBP – 48, there was a futher 1-kestose elongation while in strain XWRP – 2, 1-kestose hydroysis meant total FOS value had reached maximum. Sample XOBP – 48 exhibited higher biological activity and could have high market value. This HPLC technique was found suitable due to simplicity, linearity accuracy and sensitivity as the method demonstrated to be reliable as reported in other studies (Parpinello and Versari, 2000). This is not withought the challenges of carbohydrates due to heterogeneity and subtule differences in their structure (Kailemia *et al*., 2014) and absence of high DP standards (DP >6) and problematic co-elution of high polymers (Mutanda *et al*., 2015). In conclusion, strain XOBP – 48 can be considered a promising candidate for production of extracellular fructosyltransferase for the synthesis of short-chain FOS.

3.4.9 Morphological and Molecular Identification of Fructosyltransferase and Inulinase Producing Strains

Based on the screening creteria adopted, for the different dung types, coprophilous fungi were characterized morphologically. Due to a large number of fungal species cultured in moist chambers, it was logical to only focus on molecular identification of potential strains producing Ftase and inulinase enzymes. However, morphological diversity of various species have been highlighted in (Appendix E). Few selected strains showing high Ftase and inulinase activity were identified by 18S rDNA gene of the fungal isolates. Morpho-taxonomic characterization of selected dung producing transferase and inulinase enzymes appeared in succession as zygomycetes were the first to appear, followed by ascomycetes and lastly by basidomycetes (Table 3.4). This fungal succession has been mentioned in previous studies (Richardson, 2001a, Santiago *et al*., 2011). A high diversity of zygomycetes was observed as the genera of *Mucor* and *Pilobolus* were dominant. In Ascomycetes which appeared second, the observed taxa was from the genera of

Ascobolus and *Saccobolous* (Figure 3.14). This fungal succession was observed in a similar study of coprophilous succession in Sao Paulo and Rio de Janeiro, Brazil (Santiago *et al*., 2011). Basidiomycotina from buffalo dung was also observed in the latter stages of coprophilous fungal succession.

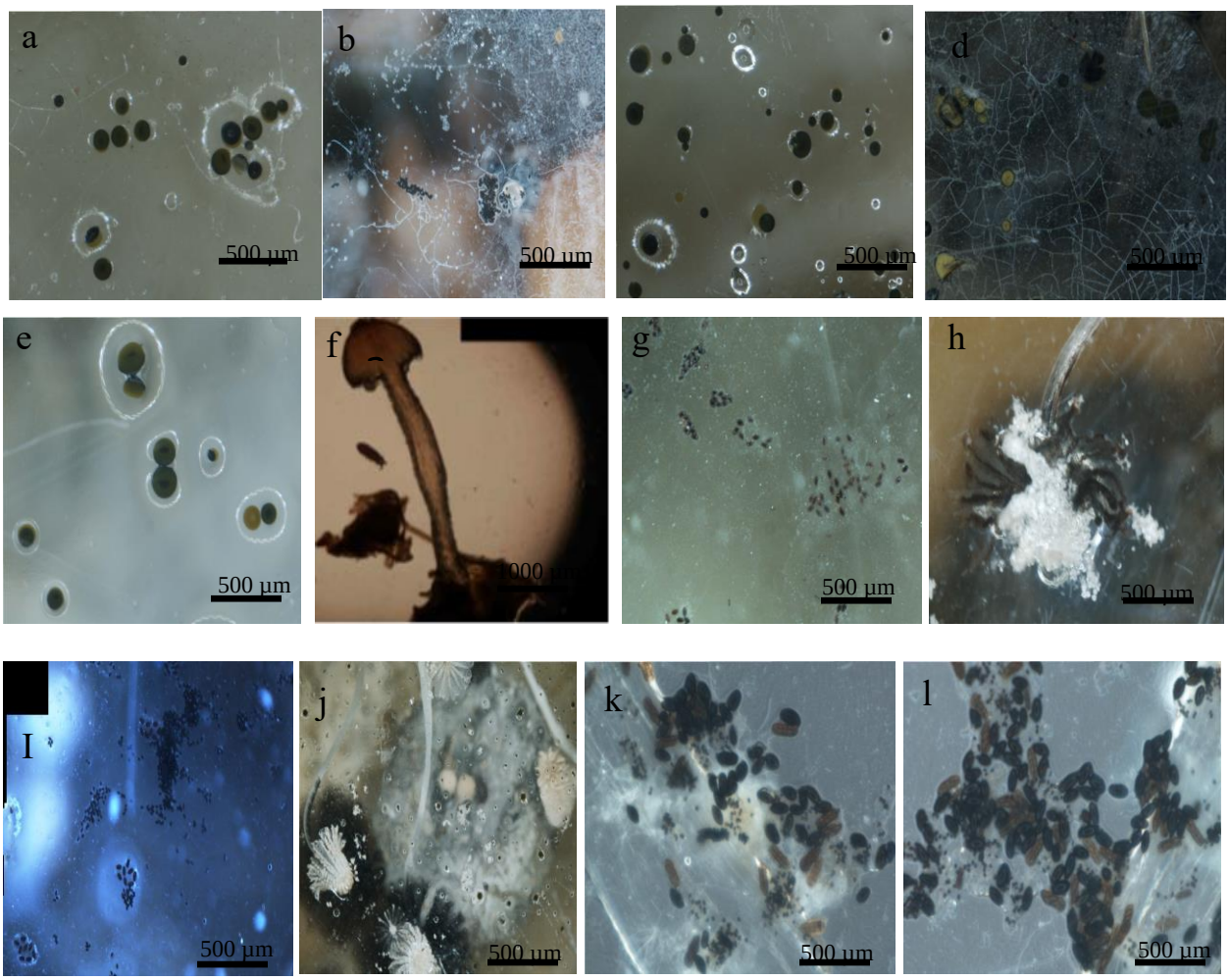
Colonies of fungi grew rapidly on MEA and PDA at 28 ºC. Initialy, colony colour was whitish and later greyish to yellow on strain XOBP - 48, XOCU - 28, and reddish in isolate XOEP – 42, respectively.

Table 3.4: Frequency of occurrence of each coprophilous fungi recorded in herbivore dung from the three sampling sites in KwaZulu-Natal, South Africa.

Species	Habitat		
	Ukulinga research farm	Tala game reserve	Phinda game reserve
Zygomycota			
Pilobolus crystallinus	7	6	3
Mucor circinelloides	3	4	4
Mucor	3	5	6
Ascomycotina			
Ascobolus	6	4	7
Saccobolus	4	3	3
Thelebolus caninus	1	0	0
Podospora	3	6	1
Sodaria fimicola	1	0	1
Sporormiella sp	0	0	1
Basidiomycota			
Coprinus sp.	0	0	1

Diverse species of coprophilous were observed with about 41 species of zygomycetes in the three sampling sites. The taxa of *Pilobolous* and *Mucorales* was observed in Ukulinga research farm, and Tala game researve. This could be due to horse and zebra population as many *Pilobolus* spp. have been previously reported on this dung type (Viriato, 2008, Santiago *et al*., 2011). The remaining species were from the phylum Ascomycotina. The genera of *Ascobolus* in the

Ascobolacea family had a wide distribution of coprophilous fungi, whereas *Saccobolus* were observed where they had similar fruiting bodies but differed in spore characters. Moreover, there was even distribution of *Ascobolous* in the three sampling sites as the relative number of dung piles showed a unimodal distribution in all three habitats. Both these genera have been found to occur worlwide in herbivore dung (Melo *et al*., 2014). The species contained fleshy apothecia, usually with villose disk due to their protruding asci bearing dark coloured ascospores (Figure 3.14 p, k, i). Some species morphology are characterized in Table 3.5. The genus *Coprinus* was only seen on few dung piles cultures of buffaloe in Phinda game reserve. However, *Sporormiella* spp. was only observed from dung piles of elephants from Phinda game reserve and this taxa was also reported in Botswana in elephant dung (Masunga *et al*., 2006).



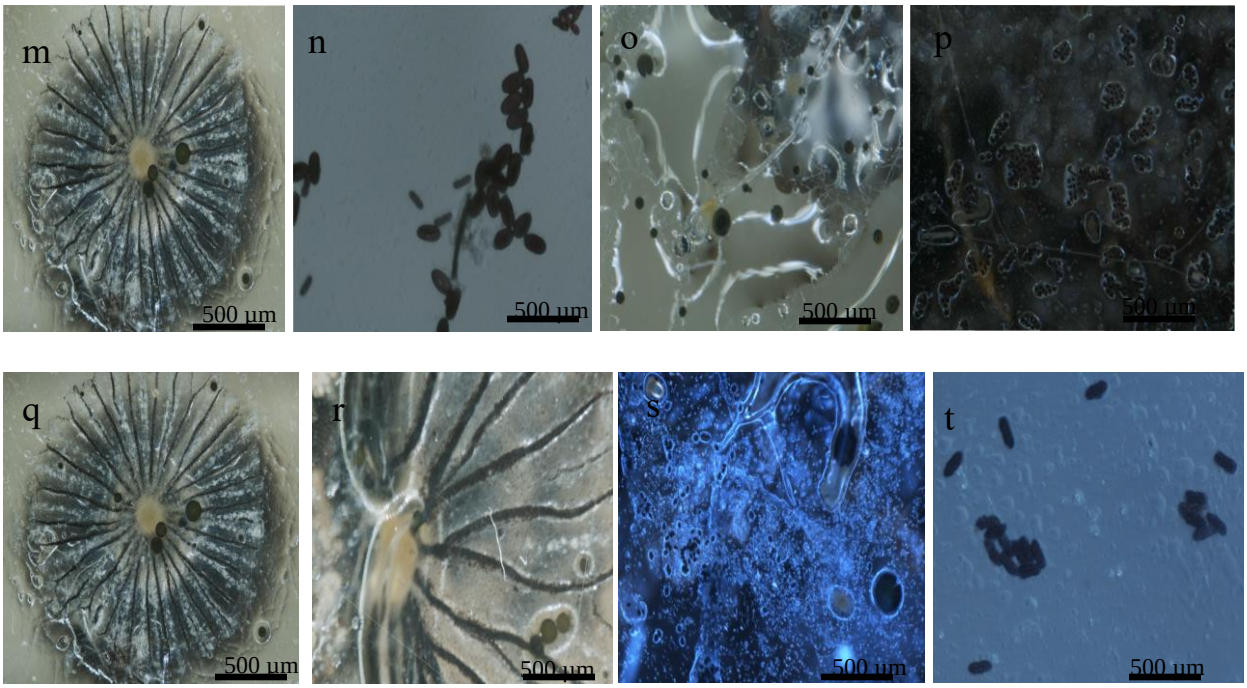


Figure 3.14: Morphology of selected coprophilous strains a = common ducker *Ascobolus*, b=Saccobolus, c=*Ascobolus*, d=*Mucorales* from zebra, e=inyala, f=stromata dissected from horse dung *Podospora oedipus*, g, h, I, j, k, l, m, n, o sporangiospores with appendages, p other herbivores from the three sampling sites. Isolate s and t from hippo dung. Scale bar a – t representing 500 µm excluding f scale which is 1000 µm.

Table 3.5: Morphological characteristics of selected genera of coprophilous isolated in the province of KwaZulu-Natal, South Africa

Phylum	Ascomycota		Ascomycota and Zygomycota	Ascomycota
Ascobolus	Asci with ascospores in diffent stages of maturity. 400 – 700 µm diameter. Asci 55 -90 x 25, usually 8 – spore but only with two to six maturing ascospores (Figure 3.14 a) (Doveri, 2014)	*Ascobolus* spp. Ectal excipilum, paraphysese embedded in hyaline gelatinous material. Ascomata 0.50 – 0.75 mm. Ascospores 11.5 – 13 x 5 – 7 µm dotted or with coarsely knobbed clusters or pigments (Figure 3.14 d) (Bell, 1983, Doveri, 2014).	Asci with ascospors in different maturity stages. Ascomata usually larger because obconical. Cylindric-clavate Asci, Asci smooth or dotted (Figure 3.14 e) (Brummelen, 1967b, Bell, 2005)	*Ascobolus* sp. Observed in horse dung. Superficial ascomata, clavate asci, ascospores (4.5 x 1.5 µm) (Figure 3.14 i) (Brummelen, 1967b). *Ascobolus* sp. Ascomata 0.7 – 1 mm, purple smooth, regullar margin 25 – 28.5 x 12 – 13.5 µm (Figure 3.14 n) (Brummelen, 1967b).
Pilobolus			Sporangiospore variable in length 5 – 15 mm long (Figure 3.14 f) (Doveri, 2011).	
Mucor				Spherical sporangia to oblong tophocyts, 500 – 575 x 200 – 230 µm (Figure 3.14 m) (Mungai *et al*., 2011, Lee *et al*., 2018).
Saccobolus	Ascomata 0.7 – 1mm, purple, smooth, regullar margin 25 – 28.5 x 12 – 13.5 µm (Figure 3.14 b) (Brummelen, 1967b)			

From the data obtained in this study there was great species diversity in and around KwaZulu-Natal, but most of them poorly studied so far. It is conceivable that some isolated strains in KwaZulu-Natal province from the genus *Mucor* could be novel. However, further taxonomic investigations are imperative to offer plausible exposition. Morpho-taxonomic identification using stereo microscope and light microscope alone is challenging, tedious and insufficient due to vast coprophilous diversity reported (Calaça *et al*., 2014, Calaça and Xavier-Santos, 2016, de Souza *et al*., 2017). Species variation was observed and this could be attributed to animal nutrition, geographical location intra and inter-specific competitions of insects that may influence dung mycology. Since the dung piles were cultured under laboratory conditions, it is permissible that these variables can be disregarded and fungal mycology can be ascribed to animal nutrition. Some taxa were from ruminants and others from non-ruminants, while other taxa was from mega herbivores like hippopotamus and elephant. From Phinda game reserve different taxa of hind gut fermenters were observed but it was from buffaloe dung fungus that the highest transferase activity was noted. This could be attributed to the observations made that in ruminats digestive enzymes work longer on fungal spores (Santiago *et al*., 2011). Species cultural and morphological attributes were used to characterize the specimens in this study. Most species had ascomata growing superficially on dung ardoning either uncinate hairs or flexious spiral coils connected to the dung substrate (Bell, 2005). In some species of Basidiomycota wide ostioles at the apex were surrounded by terminal hairs. This characteristic was also observed by Bell, (2005) as the terminal hairs covered the osticular orifice hindering visibility in the chamber (Bell, 1983, Bell, 2005). This research suggest that herbivore dung forms a good substrate for zygomycetes and more fundamentally, this research work could have potentially yielded new taxa being reported for the first time in South Africa.

3.4.10 PCR Identification

Molecular identification of 18S rDNA was successfully done on the 14 selected Ftase and inulinase producers. The DNA fragments were amplified between 500 bp - 600 bp as reported in literature for diverse fungal species (White *et al*., 1990, Bakri *et al*., 2009, AbdAl-Aziz *et al*., 2012). Fungal DNA was amplified with consesus universal fungal primers ITS 4 and ITS 5 which are reported widely in literture, consequently yielding PCR products (Figure 3.15), (White *et al*., 1990, Ramnath *et al*., 2014). Seven of the fourteen (14) isoltes were from the genus *Aspergillus* which have been reported widely as inulinase and Ftase producers (Jing *et al*., 2003b, Sanchez *et al*.,

2008, Zeng *et al*., 2016, Kowalska *et al*., 2017). Other species also identified as *Trichoderma asperellum*, *Neocosmospora* sp, *Fusarium solani* have been reported as xynalase and inulinase producers (Alvarez-Navarrete *et al*., 2015). Noteworthy, two strains one from horse dung which showed 70% similarity to *Trichordema harzianum* and another strain similar to *Mucor cicinelloides* (Table 3.6) could be possibly novel. However, further investigation is integral to ascetain the authenciticity of this claim. The study strain (48) has been highlighted in colour.

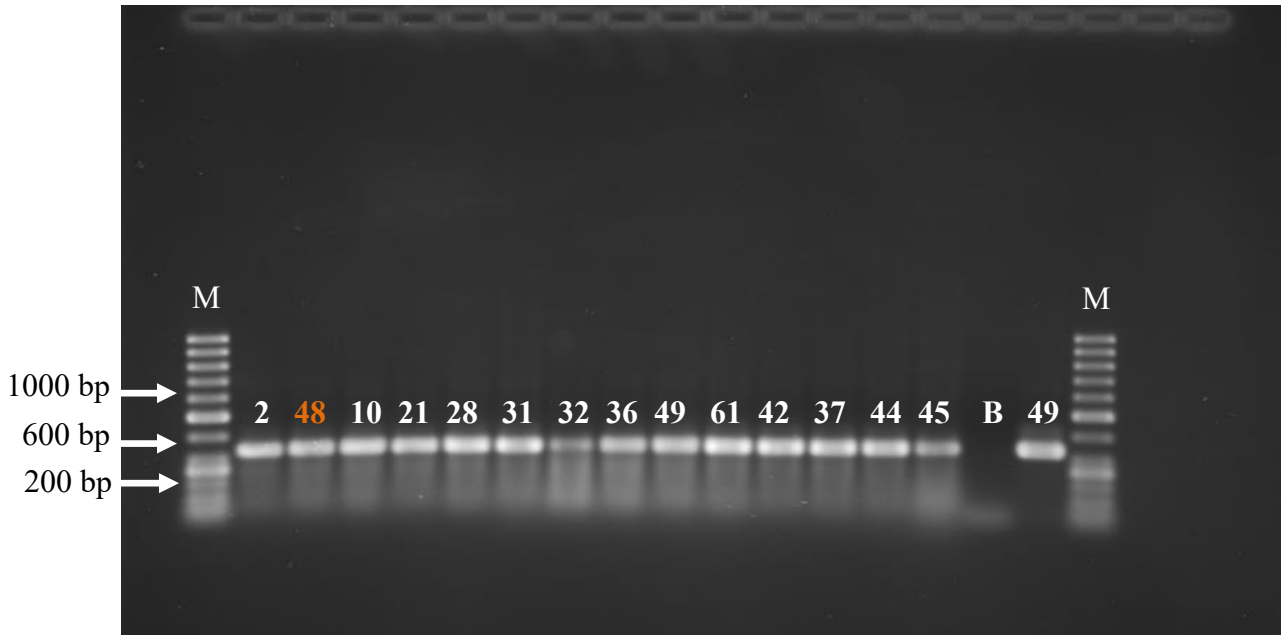


Figure 3.15: Amplified PCR products. *Lane M* = represents DNA molecular weight marker, numbers indicate the amplified DNA samples of fungal isolates *Lane B* = control sample. Numbers 2, 48, 10, 21, 28, 31, 32, 36, 49, 61, 42, 37,44, 45, and 49 represent the isolate number identified by PCR via 18S rDNA.

Agarose gel electrophoresis revealed amplification in the region of 550 - 600 bp as reported in fungal isolates (AbdAl-Aziz *et al*., 2012, Alwakeel, 2013). Nucleotide sequencing confirmed the size of profound PCR product as the sequence analysis of ITS region of rDNA showed significant alignments 99 - 100 %. Nonetheless, two strains indicated a sign of possible novel strains with 70 % and 84 % sequence homology respectively (Table 3.6) against nucleotide data available on the

NCBI GenBank using BLAST tool. The study strain XOBP - 48 was succesfuly identified to species level as *Aspergillus niger* and was sent to NCBI and assigned GenBank accession number MH445969. This strain has been reported widely to have transfructosylating and hydrolytic activity (Hidaka *et al*., 1988b, L'Hocine *et al*., 2000, Nguyen *et al*., 2005, Zeng *et al*., 2016). However, from the present study this is the first report of isolation of *Aspergillus niger* MH445969 from coprophilous fungus for bioconversion of sucrose to FOS.

Among different taxa of fungi, there exist wide intraspecific variation and the ITS regions have been used successfully as barcode markers in identification of filamentous fungi. Though, traditional methods of identification are still in use, further studies are paramount to reconcile the morphological and molecular conceptions of different genera and phylum (Lee *et al*., 2018, Loughlin *et al*., 2018, Mata *et al*., 2018).

Table 3.6: Molecular identification of selected fungal strains after morphological identification compared similarity from GenBank

Species Number	Herbivore species	Fungal Species	Accession number
2	White rhino	*Trichoderma asperellum* 100 %	KY025555
10	Goat dung	*Neocosmospora* sp. FSSC 99 %	LT746275
21	Buffalo	*Aspergillus niger* strain AY795 100 %	MG2503971
28	Cow dung	*Aspergillus welwitshiace* 1063 100 %	KT826630.1
31	Zebra	*Trichoderma asperellum* strain 3 100 %	KY025555.1
32	Zebra	*Aspergillus welwitshiace* 1063 100 %	KT826630.1
36	Horse	No significant similarity possible novel sp 70 % similarity to *Trichordema harzianum*	KY381968.1
37	Horse	*Trichordema asperellum* strain 3	KY025555.1
42	Elephant	*Fusarium solani* V0R6	KX621960.1
44	Elephant	*Aspergillus niger* strain AY795 100 %	MG250397.1
45	Red ducker	*Aspergillus niger* strain AY795 100 %	MG250397.1
48	**Buffalo**	***Aspergillus niger* strain AY795 100 %**	**MH445969**
49	Goat	Possible novel strain similar to *Mucor circinelloides* 84 % similarity	FJ441017.1
61	Inyala	*Aspergillus aculetus* 4F 100 %	KY848352.1

In a previous study by Nascimento *et al*., (2016) fungal strains of *Aspergillus*, *Penicillium* and *Trichoderma* spp. were evaluated for Ftase production. The ascomycete *P. citreonigrum* (UMR 4459) produced the highest yield of 227.56 ± 4 U/ml. The study also showed *Trichoderma* sp. as a potential Ftase producer. In this study, strain XWRP – 2 which was identified to species level as *Trichoderma asperellum* was found as the second candidate showing potential for Ftase production. Intriguing these studies draws some similarities from the present study (Nascimento *et al*., 2016).

3.4.11 Phylogenetic Analysis

Molecular Phylogenetic analysis was generated based on sequence homology of the ITS regions. Only enzymes hydrolyzing sucrose were considered i.e. fructosyltransferase and *β*-fructofuranosidases while those harboring inulinases were excluded. A rooted phylogenetic tree (Figure 3.16) was constructed using the neighbor-joining method of 18S rDNA for Ffase and Ftase producers. A similar approach was used by (Trollope, 2015) to characterize fungal phylogeny with 1000 bootstrap corrected by Jukes and cantor models (Posada and Crandall, 1998). ITS rDNA fragments were in the region of 1800 and 600 bp consistent with literature reported (Ghazala *et al*., 2016). Based on nucleotide homology and phylogenetic analysis *Aspergillus sydowii* GenBank accession number AJ289046.1 and *Aspergillus niger* AB046383.1 were found as the nearest homology to the present study strain of XOBP – 48 and placed within the same clade, while other close homologous were strains of *Penicillium oxalicum* GXU20 KP178534.1 and *Aureobasidium pullulans* NAC8 KX023301.1, respectively (Figure 3.16). This study strain 18S rDNA gene sequence was submitted to GeneBank database and assigned accession number MH445969. The fungus was used as the only suitable potent strain for further investigation in the laboratory for subsequent Ftase production, purification, characterization and biotechnological applications. Ultimately, the molecular techniques applied in the present study exhibited a high specificity and sensitivity for microbes' identification from diverse hierarchical taxonomic levels. Likewise, the PCR technique for gene amplification coding for rRNAs and sequence comparison, offer new and rapid tool for filamentous fungi identification.

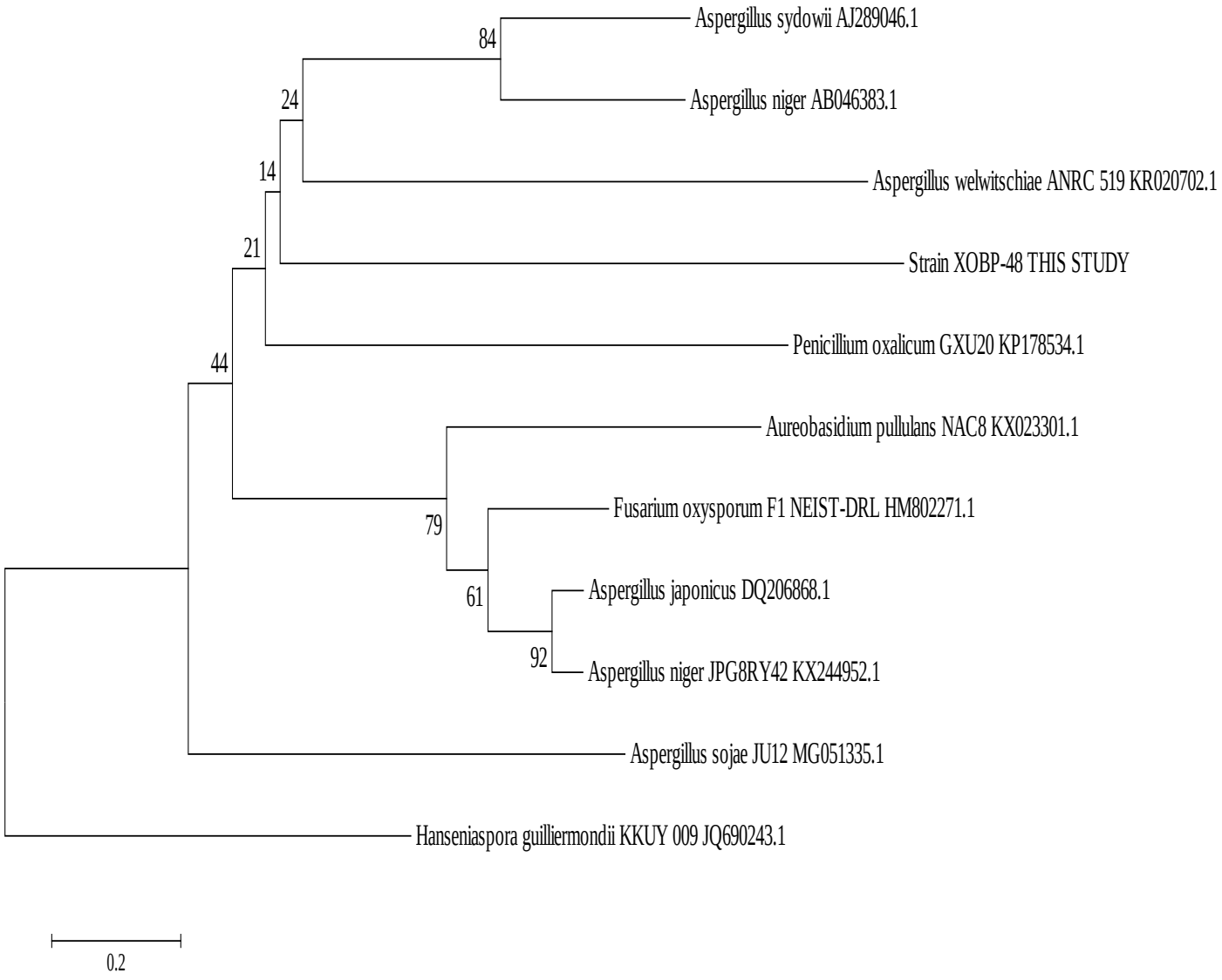


Figure 3.16: Phylogenetic neighbour-joining tree constructed based on 18S rDNA gene sequences of *Aspergillus niger* XOBP - 48 (study strain) indicating relatedness to other fructosyltransferase and *β*-fructofuranosidase producing fungal strains. Number at branching nodes are bootstrap percentages (based on 1000 replications). Yeast strain *Hanseniaspara guilliermondi* KKUY 009 GenBank accession number JQ690243.1 was used as an outgroup. Scale bar, 20 % sequence divergence.

3.5 Conclusion

Bioprospecting for autochthonous coprophilous fungi from various terrestrial habitats in and around KwaZulu-Natal, South Africa, revealed coprophilous strains with unique transferase and inulinase properties amenable for biotechnological exploitation. Of the sixty-one isolated fungal strains, eight strains were selected after the rigorous screening exercise based on rapid screening and hydrolysis of sucrose on sucrose and inulin agar media, respectively. The isolates that displayed high transfructosylating activity and production of short-chain FOS of GF_3, GF_4 and GF_5 were evaluated. Coprophilous fungus isolate XOBP - 48, which was identified to species level as *Aspergillus niger* assigned GenBank accession number MH445969 showed a good combination of high extracellular transferase activity, higher zones of hydrolysis in TTC assay and reacting products on TLC showed presence of monomeric glucose and oligosaccharides production compared to all other sixty (60) strains. HPLC results further revealed and confirmed its high content of oligosaccharide production of GF_3 and GF_4 from continued sucrose elongation. The findings of this research gave a clear indication that coprophilous fungi isolated from various terrestrial habitats is a novel reservoir of Ftase and inulinase enzymes, which have potential biotechnological applications.

CHAPTER 4: Partial Purification and Characterization of an Extracellular Fructosyltransferase Enzyme Extracted from an Indigenous Coprophilous Fungus

4.1 Abstract

The present study focused on partial purification and characterization of an extracellular fructosyltransferase (Ftase) enzyme extracted from an indigenous coprophilous fungus. The crude extracellular enzyme was purified 9.3-fold with a yield of 7.3 % and a specific activity of 2465.5 U mg^{-1} after a three-step procedure involving $(NH_4)_2SO_4$ fractionation, dialysis and ion exchange chromatography. The apparent molecular weight of this enzyme was estimated by SDS-PAGE to be approximately 70 kDa. Zymogram analysis under non-denaturing conditions showed the enzyme migrating as a polydisperse aggregate yielding a broad band of approximately 100 kDa. The enzyme further exhibited a carbohydrate content of 27.3 % by phenol-sulfuric acid method. The Ftase showed enhanced activity at a broad pH range of 4.0 – 8.0 and optimal activity at temperature range of 40 °C – 80 °C, while the enzyme was stable at pH 8.0 and between 40 °C – 60 °C, respectively. Under such conditions, the enzyme remained stable, retaining 95 % residual activity after incubation for 6 h. The Ftase activity was curtailed by the presence of metal ion inhibitors Hg^{2+} and Ag^{2+} while Ca^{2+}, Mg^{2+} and K^{+} at 1 mM activated the enzyme activity. The partially purified enzyme was stabilized with Na^{+}, Zn^{2+} and Cu^{2+}. With sucrose as a substrate, the enzyme kinetics fitted the Michaelis-Menten model. The K_m, V_{max} and k_{cat} values were 2.076 mM, 4.717 µmole min^{-1}, and 4.7 min^{-1}, respectively with a catalytic efficiency of 2.265 µmole min^{-1}. This study demonstrated that a partially purified extracellular fructosyltransferase extracted from a coprophilous fungus is economically feasible for the enzymatic synthesis of biofunctional fructooligosaccharides.

Keywords: Coprophilous, Fructooligosaccharides, Fructosyltransferase, Partial purification, Enzyme kinetics, *Aspergillus niger.*

4.2 Introduction

The production of biofunctional food products that confer both nutritional and health benefits has received greater attention over the last decade (Pearson, 2018). These foods with nutraceutical health properties are of oligosaccharide in nature and are termed prebiotics (do Prado *et al*., 2018). The main classes of prebiotics are: fructooligosaccharides (FOS), inulooligosaccharides (IOS), galactooligosaccharides (GOS), xylooligosaccharides (XOS), isomaltooligosaccharides (IMOS), and mannanooligosaccharides (MOS) with the latter being novel and less studied (Dhake and Patil, 2007, Rai, 2016, Mano *et al*., 2018). Among these prebiotics it is the fructo-oligosaccharides (FOS) that have received much attention and interest due to their excellent biological and functional properties as food additives, dietary fibres and high market value (Maiorano *et al*., 2008, Mutanda *et al*., 2014b, Adhikari *et al*., 2018). The low-calorie FOS, besides their prebiotic effects, help in regulation of lipid metabolism, reduction of cholesterol level, inhibit the growth of harmful bacteria and improve mineral absorption in the gut (Singh *et al*., 2017c).

FOS are oligofructans in nature containing a single glucose moiety. They are mainly made up of 1-kestose (GF_2), nystose (GF_3) and 1^F – fructofuranosyl nystose (GF_4), where fructosyl units (F) are bound at the β–(2 – 1) linkage of sucrose molecule (Hayashi *et al*., 2000, Chen *et al*., 2011, Rodríguez *et al*., 2011, Perna *et al*., 2018). FOS are produced by the action of enzymes found in some plants mainly *Helianthus tuberosus*, and *Cichorium intybus* (Koops and Jonker, 1996, Vijn *et al*., 1997, Apolinário *et al*., 2014, Mutanda *et al*., 2014a, Ur Rehman *et al*., 2016, Maicaurkaew *et al*., 2017). FOS also are produced by microbial enzymes for example bacterial enzymes. However, fungal sources have proven more appealing due to their higher yield in FOS production, either through fructosyl transfer and elongation of sucrose or hydrolysis of sucrose (Dominguez *et al*., 2014, de Almeida *et al*., 2018, Fernandes *et al*., 2018, Kawee-ai *et al*., 2018, Lincoln and More, 2018). Commercial FOS production via sucrose biotransformation with transfructosylating activity using fungal enzymes has been widely reported (Maiorano *et al*., 2008, Sánchez *et al*., 2010, Zhang *et al*., 2017b). Fungi from diverse habitats have been purified, identified and characterized for their potential to produce industrial enzymes that can synthesize FOS. A strain of *Aspergillus niger* ATCC 20611 was the first fungus reported by (Hidaka *et al*., 1988b) to achieve maximum FOS conversion from sucrose producing a yield of between 55 - 60 % (w/v) oligosaccharides. Likewise, *Aureobasidium pullulans* sp. was the first fungus described to have

potential industrial applications (Yun *et al*., 1992, Yun, 1996b, Sanchez *et al*., 2008). FOS production occurs by enzymatic elongation of sucrose by microbial enzymes namely fructosyltransferases (Ftases, E.C.2.4.1.9) and β – fructofuranosidases (Ffases, E.C.3.2.1.26) (Bali *et al.*, 2015). Ftases possess high transfructosylating activity by catalyzing fructosyl moiety transfer from one sucrose molecule to another. This leads to fructo-oligosaccharides with a higher degree of polymerization (Dp) (Maiorano *et al*., 2009, Bali *et al.*, 2015). However, Ffases possess both hydrolytic and transfructosylating activity. Their mechanism of action involves cleaving of the β – 1,2 linkage of sucrose thereby releasing a glucose and a fructose molecule (Hidaka *et al.*, 1988b, Sangeetha *et al*., 2005b, Bali *et al*., 2015). Fructo-oligosaccharide production, purification and characterization has been widely reported. Fungi from the genera of *Aspergillus, Penicillium, Aureobasidium* and *Fusarium*, have been purified and characterized as potential producers of transferase and hydrolytic enzymes (Hirayama *et al*., 1989, Park and Almeida, 1991, Hayashi *et al*., 1992, Yun *et al*., 1997a, Yun *et al*., 1997b, Hayashi *et al*., 2000, Ghazi *et al*., 2007, Guimarães *et al*., 2007, Plou *et al*., 2009, Lorenzoni *et al*., 2014, Wei *et al*., 2014, Xu *et al*., 2015, Castillo *et al*., 2017, Fernandes *et al.*, 2018).

Despite aforementioned extensive studies of Ffases and Ftases from diverse microorganisms, it is still necessary to identify novel sources of transferase and hydrolase enzymes. It is for this reason that bioprospecting for indigenous coprophilous fungi producing fungal Ftases from various terrestrial habitats in KwaZulu-Natal province South Africa was undertaken. Coprophilous fungi are dung loving fungi that grow and sporulate on dung substrates (Nguyen *et al*., 2017, Mata *et al.*, 2018). The fungus *A. niger* was isolated from axenic culture of coprophilous ascomycete and was found capable of producing fungal Ftase (Chapter 3). This in turn, has fueled investigation to rigorously purify and characterize the Ftase for use as a biocatalyst to produce products with high specific functions. Thus, it is the rationale of this chapter, based on the need to fathom mechanistic influences of enzyme kinetics, temperature, pH, activators and inhibitor on enzyme activity for a tailored specific Ftase. The aim of the present study involves the production of fructosyltransferase by *A. niger* MH445969 under submerged fermentation with sucrose as the main carbon source in the media. Further partial purification, biochemical characterization, carbohydrate concentration and enzyme kinetic properties of Ftase were evaluated. To the best of the researcher's knowledge, this is the first report on the isolation, partial purification and characterization of a fructosyltransferase enzyme isolated from indigenous coprophilous fungus in South Africa.

4.3 Materials and Methods

4.3.1 Reagents

HiTrap QFF column, GE Healthcare, UK, Whatman No. 1 filter paper 240 mm, dialysis tube (SnakeSkin^{TM}, Rockford, USA), Coomassie brilliant blue G-250 (Sigma Aldrich), bovine serum albumin (BSA), Tris (2- hydroxymethyl -2- methyl -1,3-propanediol), D-sucrose (Merck South Africa), 3,5 dinitrosalicylic acid (DNS), Bradford reagent, Sodium dodecyl sulphate (SDS), acrylamide, bis-acrylamide, ammonium persulfate (APS), methanol, glacial acetic acid, *N*,*N*,*N*,*N* – tetramethylethylenediamine (TEMED) were purchased from Sigma-Aldrich, South Africa. Broad-range SDS-PAGE was Page Ruler™ Plus Prestained Protein Ladder, Thermo Scientific, South Africa. Native molecular weight marker used was SERVA native marker liquid mix BN/CN PAGE. All other chemicals and reagents were supplied by reputable scientific suppliers and were of analytical grade unless stated otherwise.

4.3.2 Microorganism Selection

Fresh herbivorous dung was sampled from various terrestrial habitats in and around KwaZulu-Natal province, South Africa. The dung was transported to the laboratory and incubated in damp chambers for the growth and sporulation of coprophilous fungi. After isolation and screening of sixty-one (61) axenic fungal cultures as previously described (Chapter 3), the strain named XOBP - 48 was selected based on its performance of highest transfructosylating ability of sucrose to FOS. The fungus was identified morphologically and by 18S rDNA molecular sequence of its internally transcribed spacer regions (ITS 4 and ITS 5) (White *et al*., 1990, Ramnath *et al*., 2014) to species level as *Aspergillus niger* and was assigned GenBank accession number MH445969 from NCBI database. Phylogenetic analysis was done by Clustal W program and revealed close homology of phenotypic characteristics with other fructosyltransferase and β – fructofuranosidase producing fungi (data Chapter 3). Coprophilous fungus isolated from buffalo dung in Phinda game reserve was selected as the study strain after a rigorous screening exercise for Ftase production (Chapter 3). The pure axenic isolate was preserved on potato dextrose agar (PDA), Czapex dox agar (CDA) and malt extract agar (MEA) at 4 ºC (Xu *et al*., 2015, Ademakinwa *et al*., 2017).

4.3.3 Cultivation Conditions and Crude Enzyme Preparation

The pure fungal isolate of *Aspergillus niger* MH445969 was maintained on PDA. Pre-culture fermentation medium composition was (g/L): sucrose 30 g $L^{-l,}$ yeast extract 10 g L^{-1} $NaNO_3$ 10 g

L^{-1}, KCl 0.5 g L^{-1}, K_2HPO_4 0.5 g L^{-1}, $MgSO_4.7H_2O$ 0.5 g L^{-1}, NH_4Cl 0.05 g L^{-1} with initial pH of 6.5 prepared in 250 ml Erlenmeyer flasks before sterilization at 121 ºC for 15 min. The main culture for enzyme production was carried out in 250 ml flask containing 100 ml of the medium and incubated at 28 ºC for 72 h on a shaker incubator at 200 rpm agitation. Uninoculated medium served as control and was incubated under the same condition as the culture (Park *et al*., 2001, Sánchez *et al*., 2010, Farid *et al*., 2015).

4.3.4 Extracellular Enzyme Extraction

The fungal mycelia was harvested by filtration using a mutton cloth after 72 h of cultivation. The remaining cell debris was collected by further filtration using a Whatman No.1 filter paper and washed twice with 100 mM citrate-phosphate buffer, pH 6.5. All the filtrate broths were pooled together and homogenized by centrifugation at 10 000 x g for 15 min, and the supernatant was used as the source of extracellular crude enzyme (Nguyen *et al.*, 2005, Alvarado-Huallanco and Maugeri Filho, 2011).

4.3.5 Enzyme Isolation and Purification

All the enzyme purification steps were carried out at 4 ºC. The crude enzyme solution (100 ml) was first treated with solid ammonium sulphate (30% - 80 % saturation) at 4 °C with gentle agitation for 4 h. The protein pellet was harvested by centrifugation at 10 000 x g for 20 min at 4 ºC and was re-suspended in 10 ml citrate-phosphate buffer (100 mM, pH 6.5) and dialyzed overnight against the same buffer. Dialysis of the suspension was done using a (SnakeSkin™, Rockford, USA) dialysis tubing (10 kDa molecular weight cut off [MWCO]). The crude enzyme was filtered through 0.45 µm filters (Whatman) in order to prevent clogging of the column in further purification steps. The protein concentration and enzyme activity were measured on the concentrated sample before application on the AKTA™ Purifier 100-950 system. The ionic 5 ml HiTrap Q FF column, GE Healthcare, UK (5 ml) was pre-equilibrated with 100 mM phosphate buffer solution which was adjusted to pH 6.5 (Mutanda *et al.*, 2009, Jeza *et al.*, 2018). The unbound proteins that remained were washed from the column with the same buffer until the absorbance at A_{280nm} of the eluate reached baseline. The bound proteins were eluted stepwise with a linear gradient (0 - 3.0 M) NaCl in citrate-phosphate buffer (100 mM, pH 6.5) at a flow rate of 1 mL min^{-1}. Fractions (5 ml) were collected for protein monitoring and analysis of active fractions for transferase activity (Mutanda *et al*., 2008b). At every purification step, (1 ml) of active fractions

was collected for SDS-PAGE and assay of transferase activity. The elution profiles and purification table were plotted and constructed respectively (Mabel *et al.*, 2008). The partially purified enzyme was characterized for optimal pH, temperature, effect of metal ions and the pH and temperature stability of the enzyme was determined (Sharma and Gill, 2007, Oyedeji *et al.*, 2017, Fernandes *et al.*, 2018). The purity and molecular weight of the enzyme were analysed by both SDS and native-PAGE.

4.3.6 Fructosyltransferase Assay

The reaction mixture used to determine the Ftase activity consisted of 5 % (w/v) sucrose (2000 µl), resuspended in 100 mM in citrate-phosphate buffer, pH 6.5 and was incubated with 1000 µl of enzyme extract at 60 °C for 20 min (Nemukula *et al*., 2009). The reaction in the test tube was terminated by boiling for 10 min. DNS (2 ml) was added to the reaction mixture and absorbance was measured at 540 nm using a spectrophotometer (Agilent Cary 60 UV-Vis spectrophotometer, South Africa). The reducing sugar liberated was estimated by the DNS assay and extrapolation from a glucose standard curve (0 - 5mg/ml) by the method of (Miller, 1959). One unit of enzyme activity was defined as being equivalent to one µmole of reducing sugar produced per min under the standard assay conditions (Álvaro-Benito *et al*., 2007, Zeng *et al*., 2016).

4.3.7 Determination of Protein Concentration

An aliquot (100 ml) of the protein sample was transferred to 3 separate test tubes with the addition of 3 ml of the Bradford reagent (Bradford, 1976). The reaction mixture was vortexed to mix homogeneously and allowed to stand for 10 min for complete reaction and colour development. The samples were transferred to cuvettes and absorbance was measured spectrophotometrically at 595 nm with a Cary 60 UV- Vis spectrophotometer, (Agilent Technologies, South Africa). Deionized water (100 µl) was mixed with 3 ml Bradford reagent as a blank. Protein concentration was estimated by extrapolation from a standard curve (Appendix B) generated using BSA as an authentic standard protein.

4.3.8 Gel Electrophoresis and Molecular Weight Estimation

Different fractions of the partially purified enzymes and molecular weight markers (MWM) ranging from 10 to 250 kDa were applied to 12 % SDS-PAGE for 1 h at 100 V following the method previously described (Laemmli *et al*., 1970, Laemmli, 1970). SDS-PAGE was performed

using a separating gel (12 %) and a stacking gel (5 %) (Appendix C) on a Mini-PROTEAN Tetra electrophoresis tank (BIO-RAD, California, USA). The gels were stained with 1.2 g L^{-1} (w/v) Coomassie brilliant blue G-250, followed by destaining in 20 % (v/v) methanol:10 % (v/v) acetic acid:70 % dH_20 (v/v). The staining was performed by carefully immersing the gel in the staining solution on a horizontal rotator for about 30 min at 25 °C. The gel was removed and immersed in the destaining solution and placed on the same shaker for 30 min changing the destaining solution 2-3 times in order to visualize the clear bands with no blue background. The image was captured using a UV transilluminator G: Box system (Bio SYNGENE). The distance moved by the enzyme was measured and its corresponding molecular weight was determined from the standard curve of mobility (R*f*) vs log MW.

4.3.9 Zymogram Analysis

The native PAGE gel was prepared using a 8 % resolving gel and a 5 % stacking gel (Mutanda *et al*., 2008b). A 20 µl aliquot of the sample of the active fractions with 5 µl native loading dye was applied to each well and electrophoresis was run for 1 h at 100 V. The R*f* value of the enzyme was estimated by calculating the distance moved by each protein and the distance moved by the marker and the corresponding molecular size was calculated from the calibration curves. The recombinant, highly purified, coloured SERVA native marker liquid mix BN/CN PAGE was used as the standard non-reducing marker in order to estimate the molecular weight (MW) of Ftase (Mutanda *et al*., 2009, Nemukula *et al*., 2009). A graph was plotted of mobility vs. log molecular weight of standards (Appendix I). To confirm the Ftase nature of the partially purified enzyme, the band from the gel was cut out and tested for transferase activity with 5 % w/v sucrose under the assay conditions described for Ftase.

4.3.10 Measurement of Carbohydrate Concentration

The carbohydrate content of the reaction mixture catalysed by the partially purified enzyme was determined by the colorimetric method of phenol-sulphuric acid with glucose as a standard (Dubois *et al*., 1956). The end products of the Ftase reaction (1 ml) was pipetted into a test tube and 1 ml of 5 % (w/v) phenol was added. Concentrated sulphuric acid (5 ml) was rapidly added to the mixture and homogenized by vortexing (Masuko *et al*., 2005). The tubes were incubated in a water bath for 10 min at 25 °C for colour development and the absorbance was measured spectrophotometrically at 485 nm using a spectrophotometer (Agilent Cary 60 UV-Vis

spectrophotometer, South Africa) (Hayashi *et al.*, 1992, Nielsen, 2010). The blank sample included substituting FOS end products with 100 mM citrate-phosphate buffer at pH 6. The carbohydrate content was extrapolated from a glucose standard curve (0 – 5 mg/ml) (Dubois *et al.*, 1956). All the experiments were carried out in triplicate.

4.3.11 Biochemical Characterization of the Partially Purified Ftase

4.3.11.1 Determination of Optimal pH and pH Stability

To determine the effect of pH on Ftase activity, the partially purified enzyme (500 µl) was incubated with 2500 µl sucrose 5 % (w/v) in various ranges of pH from 2.0 to 12.0 at 55 ºC for 30 min. The buffer solutions included (pH 2 – 3) acetate, (pH 4 – 5) acetate buffer, (pH 6 – 7) citrate-phosphate buffer, (pH 8- 9) Tris- HCL, (pH 10) glycine, and (pH 11 – 12) glycine - NaOH solution. The molarity of all buffers used was 100 mM. The stability of the partially purified enzyme at the optimal pH was performed from pH 3 – 12 at a temperature of 50 ºC for 6 h and the enzyme activity of the reaction products was estimated by determining residual activity as previously described in the assay for transferase. Aliquots were removed periodically (at 1 h intervals) and analyzed for transferase activity for a maximum period of 6 h. The control was considered at 100 % residual activity (Chen *et al*., 2009, Nemukula *et al*., 2009).

4.3.11.2 Determination of Optimal Temperature and Temperature Stability

The effect of temperature on Ftase activity was determined by incubating the partially purified enzyme (500 µl) with 2500 µl of the substrate sucrose (5 % w/v) for 30 min at temperature ranges between 30 °C to 80 °C at the optimal pH using citrate-phosphate buffer (pH 6.5, 100 mM). The protein concentration was determined by the Bradford method (Bradford, 1976), with minor modifications and protein concentration was estimated by extrapolation from a BSA standard curve (0 – 5 mg/ml). The enzyme thermostability was investigated at optimal temperature (40 ºC – 60 ºC) and optimal pH by incubating the partially purified enzyme with the substrate (Mutanda *et al.*, 2009). The reaction mixture without heat treatment served as control at 100 % under the standard assay conditions described for Ftase (Lincoln and More, 2018).

4.3.11.3 Determination of Kinetic Parameters

The partially purified enzyme was incubated at 60 °C with varying substrate concentrations (0 - 50 µg/ml) in citrate-phosphate buffer (pH 6.5, 100 mM) and allowed to react for 30 min in order

to determine the amount of reducing sugars liberated. The reciprocal values of the rate of the substrate hydrolysis (1/v) were plotted against the reciprocal value of substrate concentrations (1/[S]), (Naidoo *et al*., 2015) and the amount of product liberated at the endpoint was measured to establish the transferase activity (Ghazi *et al.,* 2007). The kinetic parameters of the enzyme K_m, which is the affinity constant and V_{max}, which, is the maximum initial velocity, were calculated after the partially purified enzyme reached saturation. The k_{cat} value and catalytic efficiency of the partially purified enzyme were determined and the equations were derived from the measured data. The data generated was plotted using Origin 8, File (Version 8.725) pro software (OriginLab Corporation, Northampton, MA, USA) fitting the Michaelis–Menten equation (Dowd and Riggs, 1965, Álvaro-Benito *et al*., 2007, Naidoo *et al.*, 2015).

4.3.11.4 Effects of Metal Ions on Fructosyltransferase Activity

Different concentrations of metal ions 1mM and 10 mM of $MgSO_4$, $MgCl_2$, NaCl, $CaCl_2$, $AgNO_3$, $FeSO_4$, $CuSO_4$, $ZnCl_2$, $HgCl_2$ and KCl were investigated for their effects on the activity of the partially purified Ftase. The partially purified enzyme (89.4 U) was re-incubated with the metal ions for 30 min at optimal pH (50 mM, citrate-phosphate buffer, pH 6) and optimal temperature (55 °C), and the assay performed as previously described. The mixture without addition of any metal ion was considered as a control with residual activity of 100 % (Gonçalves *et al.,* 2016).

4.3.12 Statistical Analysis

One-way ANOVA at 95 % confidence level ($\alpha = 0.05$) was used to analyze generated data from all the experiments. The statistical analyses was determined by fitting the data using ORIGIN v. 8 Pro software (OriginLab Corporation, Northampton, MA, USA). All the experiments were performed in triplicate and expressed as mean ± standard deviations.

4.4 Results and Discussion

4.4.1 Purification of Extracellular Ftase

The extracellular crude fructosyltransferase (1229.43 U) produced under submerged fermentation conditions in liquid media containing sucrose as sole carbon was purified initially by ammonium sulphate fractionation. The crude extracellular Ftase was subjected to different fractions of $(NH4)_2SO_4$ (30 % - 80%) to determine its saturation limit. These phenomena were attempted by various researchers where they purified fungal Ftases with ammonium sulphate as the first

purification step (Shiomi, 1982, Wang and Rakshit, 2000, Park *et al*., 2001, Dahech *et al.*, 2012, Wei *et al.*, 2014). The extracellular crude enzyme lost considerable activity after initial saturation at 70 %, resulting in about 7 % transferase activity as it proved difficult to precipitate the enzyme at a low salt concentration with the purification fold of 1.32 (Table 4.1).

Table 4.1: Summary of partial purification procedure of an extracellular fructosyltransferase extracted from *A. niger*, a coprophilous fungus

Purification step	Volume (ml)	Protein (mg ml $^{-1}$)	Total protein (mg)	Enzyme activity (U/ml)	Total activity (U)	Specific activity (U mg $^{-1}$)	Purification fold	Yield (%)
Crude	100	0.047	4.667	12.29	1229.43	263.45	1	100
Ammonium sulphate precipitation (30 – 80 %)[a]	10	0.023	0.233	8.1	81.02	346.58	1.32	6.6
Dialysis	10	0.005	0.046	8	80.38	1728.73	6.6	6.5
Ion exchange chromatography QFF	5	7.2528 x 10 $^{-3}$	0.036264	8.9	89.41	2465.50	9.3	7.3

Results represent the mean ± SD of experiment conducted in triplicate

[a]Dialysed protein after 70 % $(NH_4)_2SO_4$ precipitation

In order to fully precipitate, the crude maximal saturation of 70 % salting was found to be optimal. This could be probably due to the hydrophilic nature of the enzyme or a presence of competitive inhibitors at their active sites. Competitive inhibitors are structurally similar with the substrate and compete for the same active site of the enzyme (Arsalan and Younus, 2018). A similar observation was observed in a partial purification of transferase enzyme from *Aspergillus foetidus* where $(NH4)_2SO_4$ precipitation was optimal at 75 % salt concentration (Wang and Rakshit, 2000). In a similar report, purification of Ftase from asparagus yielded 5.6 % activity after ammonium sulfate saturation (Shiomi and Izawa, 1980). Overnight dialysis of the concentrated protein with 100 mM citrate-phosphate buffer, pH 6.5 at 4 °C resulted in the increase of specific activity and purification fold to about 1729 U mg^{-1} and 7 respectively. This characteristic nature of the enzyme was also reported by Goncalves *et al.,* (2016) using ethanol precipitation for the purification of an extracellular *β*-D-fructofuranosidase from *Fusarium graminearum*, which showed a 3 fold increase and relatively lower total activity (Gonçalves *et al.*, 2016). Furthermore, the reconstituted pellet was fractionated on anion exchange chromatography, HiTrap QFF column. Gradient elution with 0 to 3 M NaCl resulted in the removal of large contaminating proteins. Fractions were eluted and resulted in two broad peaks for both unbound proteins (a) fractions A_1- A_{10} and bound protein (b) fractions (B_3 – B_7) (Figure 4.1). This purification step was successful since the specific activity increased from 1728.7 to 2465.5 U mg^{-1} and purification fold of 9.3 with a recovery of 7.3 % (Table 4.1).

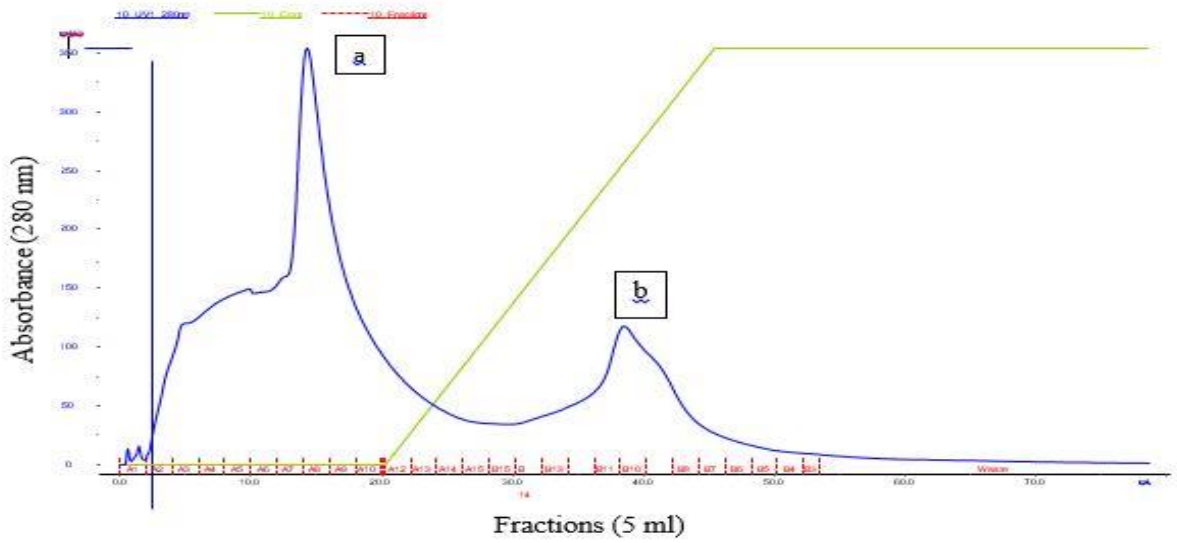


Figure 4.1: Elution profile of fungal proteins using anion exchange chromatography with HiTrap QFF Sepharose Fast Flow resin: The dialyzed enzyme was loaded into the anion exchange column and equilibrated with 100 mM citrate-phosphate buffer (pH 6). The adsorbed enzyme was eluted by linear gradient of 0 - 3 M NaCl using the same buffer, and the effluent was collected as fractions of 5 ml at a flow rate of 1 ml/min. The absorbance was monitored at 280 nm.

This elution profile revealed that not all the protein eluted is the target enzyme as some were non-adsorbed as large contaminants. A noteworthy occurrence was also noted in chromatography steps in the purification of *A. niger* AS0023 and *A. pullulans* using DEAE-Sephadex G200 where Ftase and invertase were eluted as broad asymmetric peaks. This behaviour is quite a characteristic of polydisperse glycoproteins as previously reported by other workers (L'Hocine *et al*., 2000, Lateef *et al*., 2007b). There are numerous reports on the production and purification of Ftase and Ffase from *Aspergillus* sp. However, the purification yield of Ftase obtained in the present study was higher compared to an extracellular *β* – fructofuranosidase from *Aspergillus niveus* purified by DEAE-cellulose and Sepharose CL-6B where purification fold was 8.5 and 5.2 % yield, respectively (Fernandes *et al.*, 2018). In a more recent study, an exo-inulinase enzyme of diesel-degrading *Paenibacillus* D9 sp. was purified with a similar column using HiTrap QFF (GE Healthcare, UK). The specific activity of the exo-inulinase from *Paenibacillus* D9 sp. was found to be 4333 IU/mg with a lower yield of 7.3 % and 4.3 fold purification (Jeza *et al.*, 2018).

Generally the purification of Ftases and β - fructofuranosidases in multiple steps such as three-stages (Chen *et al.*, 2011), four-stages (Ende and Laere, 1993, Xu *et al*., 2015), five-steps (Hirayama *et al*., 1989), six-steps (Park *et al.*, 2001, de Almeida *et al.*, 2018), or as many as nine steps (Shiomi, 1982, L'Hocine *et al*., 2000), have revealed yields lower than 30 %, notwithstanding the numerous purification steps that are very costly and time-consuming. It is also obvious from previous reports and the present study that the higher the purification factor, the lower the enzyme recovery. A novel fructosyltransferase from *Lactobacillus reuteri* was purified with two steps involving Nickel resin chromatography and anion exchange column chromatography (Resource-Q), with 5.8 purification fold and a 7 % recovery (Van Hijum *et al.*, 2002). More recently, it is reported that an invertase from *Aspergillus terreus* sp. was partially purified by two steps involving dialysis and DEAE-column chromatography. The purification fold was 8.21 and the recovery was 76.04 % (Shaker, 2015). From this study, a more efficient and cost-effective purification steps that will minimize microbial contamination, and increase purification fold and recovery is proposed for potential large-scale purification of extracellular fungal Ftase. Moreover, the higher specific activity and purity of the Ftase preparation could be advantageous. Apart from the laboratory scale, experimentation, the enzyme could be used to achieve higher immobilization efficiency and volumetric activity. The process of preparation of pure Ftases encompasses mostly three to nine purification steps with recovery of between 2.5 to 37 % (Hayashi *et al*., 1992, Ende and Laere, 1993, Song and Jacques, 1999, L'Hocine *et al*., 2000, Yoshikawa *et al*., 2007). However, the application of these complex protocols would be uneconomical on the industrial scale. Furthermore, the use of partially purified Ftase is desirable than crude enzyme as they exhibit a higher transfructosylating activity to yield short-chain FOS (Huang *et al*., 2016). The partially purified Ftase could be sufficient for preparing immobilized biocatalysts which can be reused for maximal transferase activity. This can offer a good prospect for industrial scale-up of FOS production (Antošová *et al*., 2008, Vaňková *et al*., 2008).

This outcome is corroborated by studies carried out on a partially purified Ftase (EC 2.4.1.9) from *Rhodotorula* sp. LEB-V10 in free and immobilized form. The Ftase demonstrated prodigious prospective for industrial FOS production hence, the justification of using partially purified Ftase over crude enzyme.

4.4.2 Determination of Molecular Weight and Activity of the Partially Purified Ftase

Denaturing electrophoresis (SDS-PAGE) on 12% polyacrylamide gel showed three bands on Lane 4 (Figure 4.2) indicating that partial purification of the enzyme was achieved since other contaminating proteins were eliminated. The partially purified transferase enzyme was resolved by non-denaturing PAGE and it formed an aggregate of around 70 - 100 kDa (Figure 4.3). In a more recent study, a plant Ftase from Jerusalem artichoke was partially purified by successive anion exchange and affinity chromatographies. On 12 % SDS-PAGE two protein bands were visualized and the molecular weights was estimated to be approximately 66 and 25 kDa (Ngampanya *et al*., 2016).

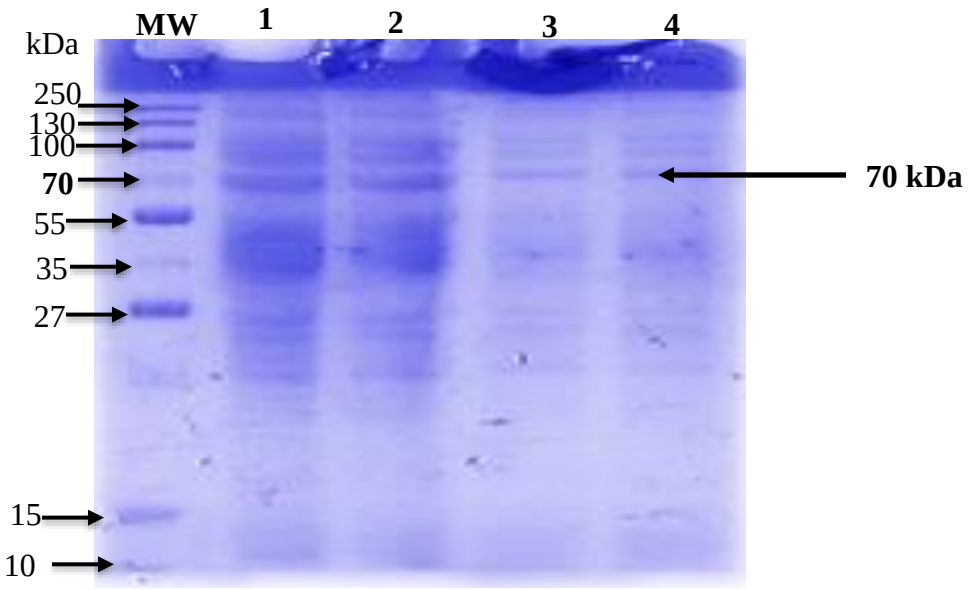


Figure 4.2: A 12 % SDS-PAGE of the partially purified fructosyltransferase. Lane 1 crude enzyme, Lane 2 ammonium sulphate fractionation pellet, Lane 3 dialyzed fraction, Lane 4 was loaded with pooled fractions B3 to B7 from ion exchange chromatography using HiTrap QFF column. Lane *MW* was loaded with Page Ruler™ Plus Prestained Protein Ladder, ranging from 10 to 250 kDa. The gel was stained with Coomassie Brilliant R-250 for 30 min and destained with methanol, glacial acetic acid and water for over 6 h.

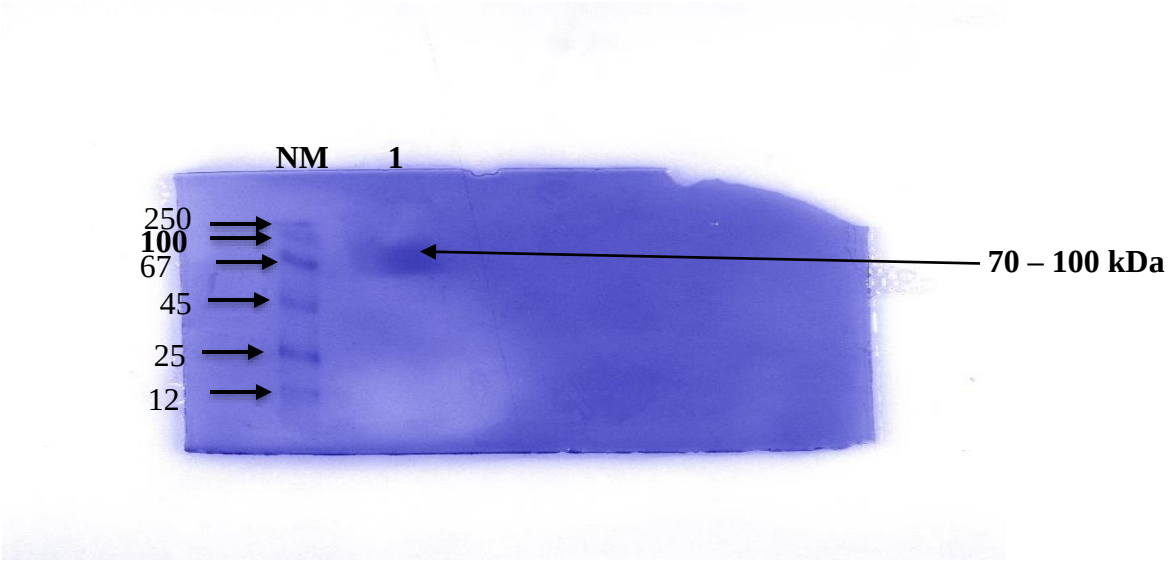


Figure 4.3: Native PAGE of the partially purified fructosyltranasferase enzyme after ion exchange chromatography using 8 % resolving gel and 5 % stacking gel. Lane *NM* native marker, Lane 1 pooled fractions of bound proteins B3 – B7 eluted from Akta Purifier HiTrap QFF Column.

A previous report on the purification of an extracellular *β* - fructofuranosidase from *Fusarium graminearum* two protein bands of 94 kDa and 66 kDa, were detected after SDS-PAGE. The finding was that the protein was heterodimeric (Gonçalves *et al*., 2016). Oligomeric aggregation was noted in native-PAGE in the purification of a fructosyltransferase isolated from *A. niger* fungus. The enzyme aggregated in its subunits that formed due to high carbohydrate content (L'Hocine *et al*., 2000). Other partial purification attempts of invertases isolated from *A. nidulans* and *A. terreus* resulted in more than two bands on SDS-PAGE (Chen *et al*., 1996, Giraldo *et al*., 2014). In the purification of *A. niger* Ftase, the native form of the enzyme seemed to have undergone association-dissociation and aggregated to form oligomers as dimer or tetramer (Nguyen *et al*., 2005). To confirm whether the clear band between 70 kDa - 100 kDa (Figure 4.3) possessed transferase activity or it was just a contaminating protein; the unstained portion of the native gel containing the band corresponding to the Coomassie stain was cut from the non-denaturing gel and subjected to Ftase assay. The band was resuspended in 100 mM citrate-phosphate buffer pH 6.0 and reconstitution of the enzyme activity following native PAGE was done according to a method previously reported (Rothe and Maurer, 1986). Reconstitution was done by rinsing the gels in renaturation buffer (50 mM Tris-HCl, pH 7.5, 1 mM EDTA). The

renaturation was done at 4 °C for 1 h in order to remove staining before gels were cut (Bhown and Claude Bennett, 1983, Spanos and Hübscher, 1983). The results of Ftase assay shown in Table 4.2 indicate that the band tested on native-PAGE gel displayed considerable Ftase activity. Hence, it can be deduced that the studied strain of *A. niger* from coprophilous fungus could have multiple presence of enzymes displaying Ftase like activity. The molecular weight (MW) was confirmed by calculating the size of the protein on SDS-PAGE (Appendix I) from the extrapolation of the graph of log MW vs mobility.

4.4.3 Estimation of Ftase Molecular Weight on SDS-PAGE

The relative migration distance (Rf) of the protein bands on SDS-PAGE was measured. The band displaying high activity was around 4 mm, with the gel length being 68 mm after electrophoresis. To ensure accurate MW estimation the log of molecular weight of the Page Ruler™ Plus Prestained Protein Ladder MW 10 to 250 kDa was used as the standard protein marker (Appendix I). The Rf of the unknown protein from ion exchange chromatography and protein marker were determined. The protein mobility was divided by mobility of the ion front.

Rf = (distance to band)/ (distance to dye front)

The molecular weight was determined from the standard curve of mobility (Rf) vs log MW (Appendix I). Rf of 0.59 = 1.85 = 70 kDa.

Table 4.2: The fructosyltransferase activity of bands obtained on native PAGE gel.

Fructosyltransferase assay	Enzyme activity (U/ml)
Band on native-PAGE 70 - 100 kDa	9.34 ± 0.12
Band on native-PAGE	0.86 ± 0.14

The molecular weight was determined to be around 70 kDa as shown in Figure 4.2 and the zymogram analysis was effective in identifying enzymes with identical properties. The molecular weight of fungal fructosyltransferases has been extensively reported to be between 35 kDa to 120 kDa (Antosova and Polakovic, 2001, Hernalsteens and Maugeri, 2010a). The aforementioned method was used in previous studies of partially purified extracellular Ftase from *Candida* sp. The enzyme was partially purified and was found to possess both transfructosylating and hydrolytic

activity (Hernalsteens and Maugeri, 2010a). The purification factor was low, though the purification procedure was efficient as some variety of proteins were still evident in the pooled fractions (Hernalsteens and Maugeri, 2010a). Interestingly, in another study involving partial purification of inulinase from *A. ficuum* the multiple bands of enzymes on SDS and native PAGE were visualized after successive chromatographies. The enzymes displayed similar properties. Hence it was difficult to separate or distinguish the endo from exo acting inulinase by conventional methods. The eight bands on native-PAGE were assayed for inulinase activity. All bands present on native gel displayed inulinase activities to a different extent. Hence it was deduced that there may be a presence of multiple forms of enzymes in *Aspergillus* displaying inulinase like activities (Jing *et al*., 2003b).

4.4.4 Carbohydrate Analysis

The end product after enzyme reaction was found to contain about 27.27 % of total sugar content after biotransformation of sucrose to FOS by the partially purified enzyme. In the presence of sulfuric acid, phenol can be used to quantify sugar colorimetrically, with their methyl derivatives, oligosaccharides and polysaccharides (Dubois *et al*., 1956). The phenol-sulfuric acid method was suitable to give an estimation of total sugar content of the end product of the Ftase reaction. The colour produced by phenol at constant concentration was considered proportional to the amount of reducing sugar liberated. The carbohydrate content of an extracellular *β*-fructofuranosidase of *A. japonicus* fungus was reported to be 20 % (w/w) similar to *β*-fructofuranosidase enzyme of *Aspergillus niger* which was 17 % (Nguyen *et al*., 2005). Additionally, the *Aureobasidium* sp. Ffase reported by Hayashi *et al.* (1992) produced 30 and 53 % of sugar content respectively than the present Ftase enzyme (Hayashi *et al*., 1992). Moreover, several researchers have applied this method to determine the concentration of oligosaccharides complex type carbohydrates and glycoproteins in FOS synthesis (Lo *et al*., 1970, Liu *et al*., 1973, Rao and Pattabiraman, 1989, Saha and Brewer, 1994, Cuesta *et al*., 2003, Robinson *et al*., 2018, Zavřel *et al*., 2018).

4.4.5 Biochemical Characterization of the Partially Purified Fructosyltransferase

4.4.5.1 pH Optima and Stability

The partially purified fructosyltransferase from the fungus *Aspergillus niger* showed optimum activity at pH 6.0 and the relative activities declined below pH 4.0 and above pH 7.5, respectively (Figure 4.4). The optimal pH values of Ftase (AaFt32A) from *A. aculeatus* were 5.0 – 6.0 (Huang

et al., 2016). The optimal pH from *A. aculeatus* from Pectinex Ultra SP-L were 5.0 – 7.0 with no significant activity recorded below pH 3.5 and above 9.5 (Ghazi *et al.*, 2007). A bioactive chitosan-oligosaccharide from newly isolated *Aspergillus griseoaurantiacus* had an optimal pH 4.5 (Shehata *et al.*, 2018). Ftases, inulinases and invertases from other fungal species have reported optimal pH range of 5.5 to 7.0 similar to the present study (Fernández *et al.*, 2004, Sánchez *et al.*, 2010, Magadum and Yadav, 2018). Therefore, the optimal pH value of around 6.0 to 6.5 suggests that the pKa values of the crucial amino acids at the active sites of the enzyme are in the range of between 3.0 and 6.5. Hence, the most likely amino acids in the active region could be probably glutamic acid, aspartic acid and histidine (Nemukula, 2008). These amino acid compositions may affect the ionization in solution, which is desirable for application in food industries.

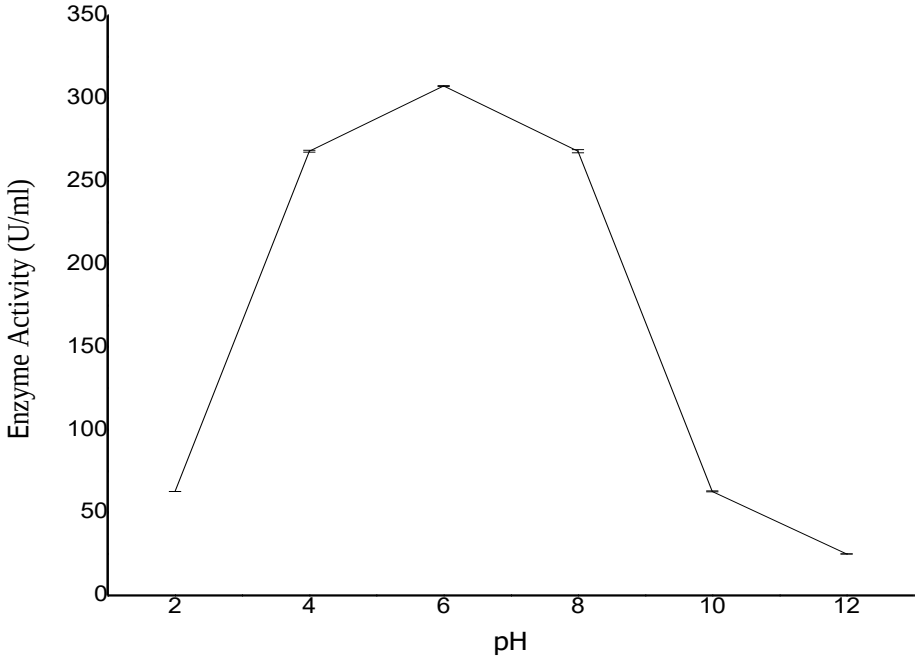


Figure 4.4: Optimal pH of fructosyltransferase enzyme incubated over a time course of 6 h with 5 % (w/v) sucrose at 50 °C. Standard assays were performed at each pH to determine the pH with the highest activity of the partially purified Ftase. The error bars indicate the standard deviations of three replicates.

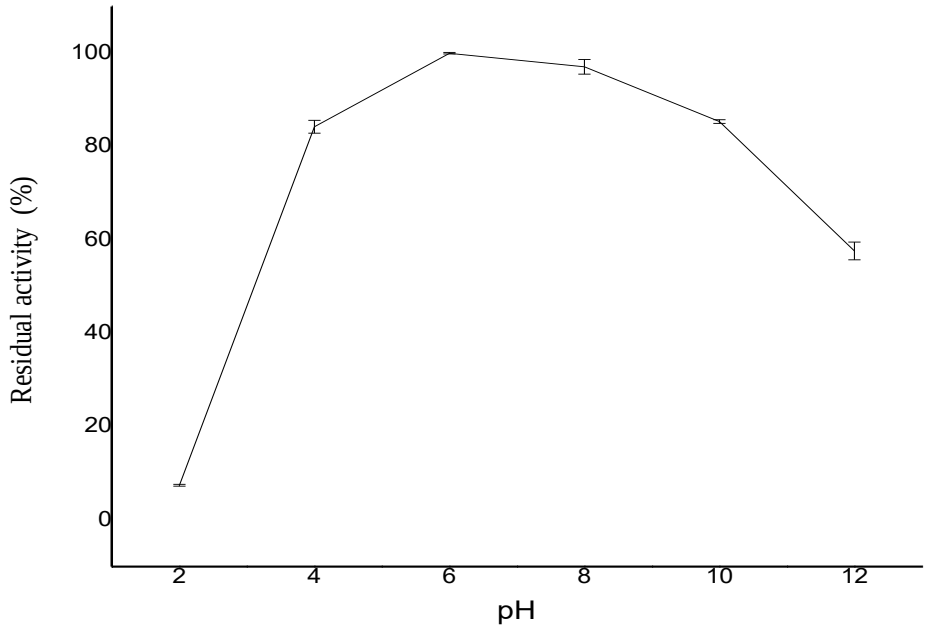


Figure 4.5: pH Stability of fructosyltransferase enzyme incubated over a time course of 0 – 6 h with 5 % sucrose at optimal temperature. Standard assays were performed at each pH range and residual activity determined. The *error* bars indicate the standard deviations of three replicates.

The fungal strain producing Ftase was stable between pH 4.5 to 8.0 for 6 h. The enzyme retained 97 % of its residual activity at the temperature ranging between 50 °C and 60 °C (Figure 4.5). This characteristic of stability in the neutral pH range was also comparable to Ftase tolerance of *A. oryzae*, a fungus reported to produce extracellular transferase under submerged fermentation (Sangeetha *et al*., 2004, Wei *et al.*, 2014). The enzyme activity depreciated from around pH 8.5-12.0 and this can be attributed to deprotonation of important amino acid on active primary and secondary protein sites required for enzyme stabilization (Dhake and Patil, 2007, Gonçalves *et al.*, 2016). The extracellular fructosyltransferase from *A. pullulans* showed stability at pH 4.5 - 7.0 and optimum activity was at pH 5.5 (Yun *et al.*, 1997a, Yun *et al.*, 1997b). Interestingly these findings differ with that of *Aspergillus phoenicis* with tansfructosylating and hydrolytic activity that exhibited an optimal pH value of 8.0 (Fernández *et al.*, 2004).

4.4.5.2 Temperature Optima and Stability

Fructosyltransferase was active over a broad temperature range of 40 °C - 65 °C and exhibited a thermostable nature. However, a slight decline in activity was evident above 60 °C as depicted in Figure 4.6. These results are similar with previous reports of sucrose biotransformation to FOS by *Aspergillus* sp. N74 free cells where optimum pH was at 5.5 and temperature optima for maximal activity was between 50 °C - 60 °C (Sánchez *et al*., 2008, Sánchez *et al*., 2010). Another fungal fructosyltransferase produced by *Aspergillus oryzae,* and *Aspergillus niger*, have been reported widely to operate under similar optimal temperature and pH in submerged fermentation (Hidaka *et al*., 1988a, Hidaka *et al*., 1988b, Sangeetha *et al*., 2005a, Sangeetha *et al*., 2005b).

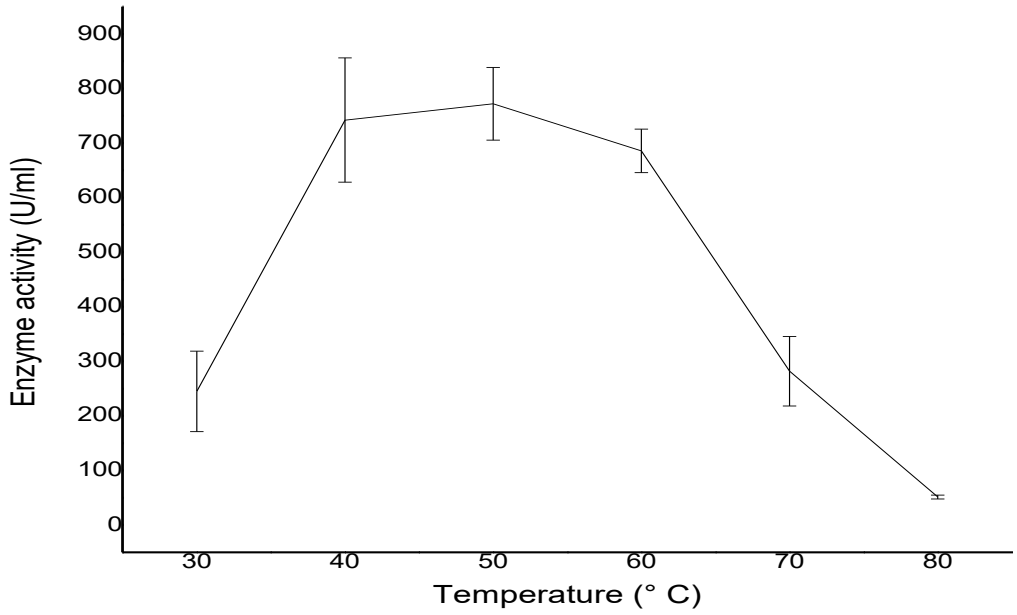


Figure 4.6: Optimum activity of the enzyme assayed at different temperature ranges from 30 °C-80 °C, over a time course of 6 h to monitor optimal temperature with highest transfructosylating activity. The *error* bars indicate the standard deviations of three replicates.

The optimal temperatures of Ftase from *A. oryzae* and *A. aculeatus* (AcFT) was 60 °C for maximal transferase activity (Virgen-Ortíz *et al.*, 2016), whereas the bacterium *Streptococcus salivarus*

optimal temperature was reported at 37 °C for maximal transferase production (Jacques, 1999). These results are comparable to the present study and it can be deduced that *Aspergillus niger* isolated from coprophilous fungus can be advantageous due to their high heat tolerance and high optimum temperature with a wide range of pH activity. High-heat resistance, high temperature stability and high optimum temperatures are advantageous for industrial applications. These properties are fundamental in large-scale FOS production as they reduce the risk of microbial contamination, lower viscosity and increase substrate solubility (Virgen-Ortíz *et al*., 2016).

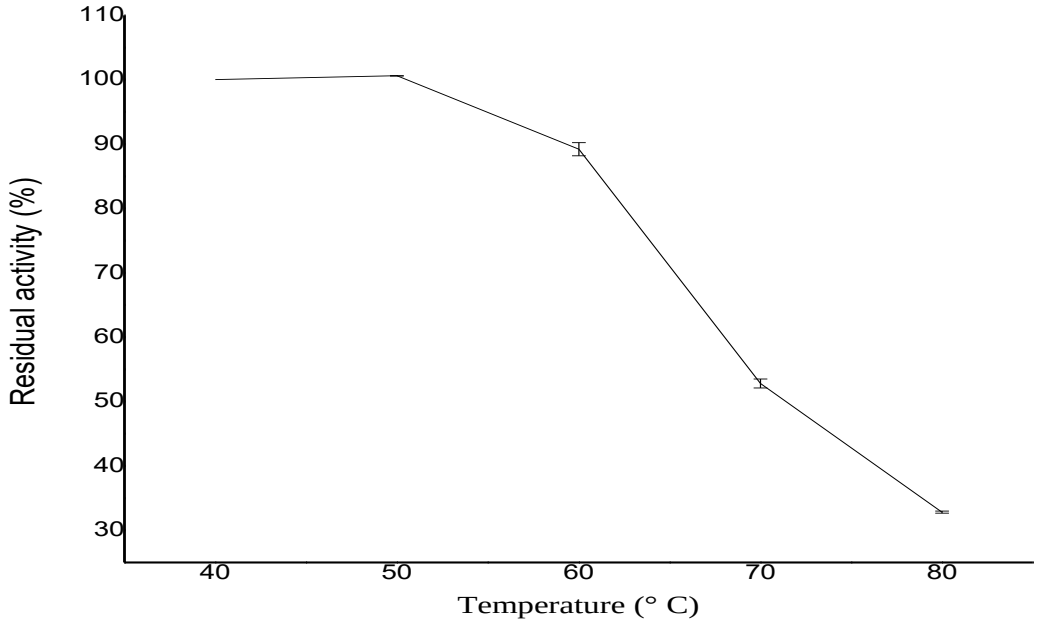


Figure 4.7: Temperature stability of *A. niger* Ftase activity. Ftase was assayed in terms of residual activity after enzyme incubation time course of 6 h. The *error* bars indicate the standard deviations of three replicates.

The partially purified Ftase showed maximal thermal stability over a wide temperature range of temperature of 40 °C to over 60 °C, maintaining more than 80 % of its residual activity during a time course of 6 h (Figure 4.7). Glycosylation could be one reason ascribed to why this transferase enzyme is maintaining high thermostability at a higher temperature than other reported fungal and

bacterial transferases (Giraldo *et al*., 2012). Ftases that can operate at high temperatures are crucial in reducing possibilities of microbial contamination, enhance mass-transfer ability, and reduce the viscosity of the substrate (Nigam, 2013). An invertase from *S. cerevisiae* used for the synthesis of FOS showed thermostability between 30 °C - 50 °C and activity depreciated as incubation time progressed and temperature increased. At 70 °C, there was no activity (Chand *et al*., 2017). This is similar to the present study where the stability of the enzyme was evident from 40 °C to 55 °C. The Ftase activity depreciated from 60 °C and completely lost activity at 80 °C (Figure 4.7). A comparative investigation by Amaya *et al.* (2006) on thermal stability of free and immobilized enzymes of *S. cerevisiae* showed that at 50 °C both enzymes preserved approximately 80 % of their activity. Their activities decayed after an increase in temperature above 60 °C and they suffered complete denaturation at 70 °C (Amaya-Delgado *et al.*, 2006). Aguiar-Oliveira *et al.* (2011) characterized an Ftase from *Rhodotorula* sp. by ethanol precipitation and immobilization. Thermostability of the free enzyme was observed at 62 ºC and later increased to 66 ºC after immobilization. Enzymatic activation of both immobilized and free enzyme was observed after 15 min at 52 ºC but greater activation was reached at pH 4.5, and temperature range of 47 ºC - 51 ºC. However, the stability of the immobilized enzyme was at pH 6.0, while the free enzyme was more stable at a temperature of over 52 ºC (Aguiar-Oliveira and Maugeri, 2011). A number of reports have described temperature ranges of microbial fructosyltransferase in the range of 50 ºC - 60 ºC (Ghazi *et al*., 2007, Perna *et al*., 2018) while few have reported temperature optima of between 65 ºC - 70 ºC (Hernalsteens and Maugeri, 2008). Thermal stability of *A. pullulans* fungus exhibiting fructosyltransferase activity was monitored over 8 h and the biocatalyst was inactivated at temperatures above 60 °C even at high saccharide concentration. The stabilizing effect of concentration of the saccharide on transferase activity was established to be 55 °C (Madlová *et al.*, 2000). These results corroborated with those obtained in the current study. Ftase with special characteristics such as high thermal and pH stability and are desirable for biotechnological applications as an average optimum temperature of 50 °C is reported. Nevertheless, a temperature of 60 °C has been recommended as effective for industrial application (Mutanda *et al*., 2009, Nigam, 2013).

4.4.5.3 Effects of Metal Ions

The effect of different metal ions on Ftase activity was determined at concentrations of 1 mM and 10 mM. There was enhancement of fructosyltransferase activity by Ca^{2+}, $MgSO_4$, K^+ at 10 mM

whereas there was no significant effect of Ftase activity by Fe^{2+} (Table 4.3). The effect of other metal ions at lower concentrations such as Na^{+}, Zn^{2+}, K^{+} and Cu^{2+} were associated with stabilization of the enzyme. These phenomena could be attributed to the significant action at the three-dimensional (3D) structures and key residues of fructosyltransferase active sites (Pons *et al*., 2004). The 3D structures of Ftases are better conserved than most amino acid sequences (Pons *et al*., 2000). In an earlier study, a *β*-fructofuranosidase from the thermophilic fungus *Sporotrichum thermophile* demonstrated the ability to catalyze the synthesis of 6-kestose. Metal ions such as Ca^{2+}, Fe^{2+} and Mg^{2+} were used to optimize transfructosylating activity (Katapodis *et al.*, 2003). These metal ions stimulated transferase and hydrolytic activity.

Table 4.3: Effect of metal ions on enzyme activity of fructosyltransferase extracted from an indigenous coprophilous strain of *Aspergillus niger*

Metal ions	Relative activity (%)[a]	
	1 mM	10 mM
Control (Ftase)[b]	100 ± 0.00	100 ± 0.001
$MgSO_4$	66.37 ± 0.6	107.72 ± 0.2
$MgCl_2$	57.7 ± 0.38	73.75 ± 0.26
Na^{+}	80.17 ± 0.15	94.93 ± 0.83
Ca^{2+}	94.3 ± 0.36	101.7 ± 0.74
Ag^{2+}	1.54 ± 0.7	0.53 ± 1.91
Fe^{2+}	119.3 ± 0.64	83 ± 0.15
Cu^{2+}	77.21 ± 0.53	64.75 ± 1.98
Zn^{2+}	85.62 ± 0.75	68.36 ± 0.87
Hg^{2+}	1.48 ± 0.44	0.04 ± 0.18
K^{+}	74.23 ± 0.33	102.21 ± 0.54

[a] The relative activity of Ftase was determined with addition of various metal ions at optimal pH and temperature (pH 6.5, 100 mM, 55 °C).

[b] The relative activity of enzyme (pH 6.5, 100 mM) without addition of metal ion.

There was a complete inhibition of Ftase activity by Hg^{2+} and Ag^{2+} and a very low activity of 0.04 U/ml and 0.53 U/ml was recorded, respectively. Similar observations were made for *A. japonicas* (Hayashi *et al.*, 1992), *A. niger* for *β*-fructofuranosidase production (Nguyen *et al*., 2005). The presence of heavy metals limited enzyme activity using *A. niger* as an extracellular recombinant fructosyltransferase (Guo *et al.*, 2016). From Table 4.3 Hg^{2+} and Ag^{2+} show almost 95 % inhibition of activity. These heavy metals are reported to interact with sulfhydryl groups in Ftases and invertases, thereby leading to conformational changes and enzyme precipitation (Lincoln and More, 2018). In other reports, Hirayama and Hidaka found that Cu^{2+}, $Mn^{2+,}$ Zn^{2+} and $MgSO_4$ enhanced enzyme activity of *A. niger* ATCC 20611 Ftase (Hirayama *et al.*, 1989).

4.4.5.4 Determination of Enzyme Kinetics

Kinetic parameters of the partially purified Ftase were measured from the initial enzyme reaction rate. A Lineweaver-Burk plot was used by applying the Michaelis-Menten constant (K_m) as shown in (Eq. 1). In this equation, the reciprocal values of substrate hydrolysis (1/v) were plotted against substrate reciprocal concentration (1/[S]) in order to determine both (V_{max}) maximum enzyme velocity and (K_m). The turnover number also ascribed as the catalytic constant of the substrate-enzyme reaction represents the number of reactions catalyzed by each active site and was calculated by (Eq. 2). Enzyme catalytic efficiency was determined by (Eq. 3), (Alvarado-Huallanco and Maugeri Filho, 2011, Naidoo *et al.*, 2015).

$$\frac{1}{vo} = \left(\frac{Km}{vmax}\right)\left(\frac{1}{[S]}\right) + 1/vmax \qquad /1/$$

$$kcat = vmax/[E]t \qquad /2/$$

$$Catalytic\ efficiency = kcat/Km \qquad /3/$$

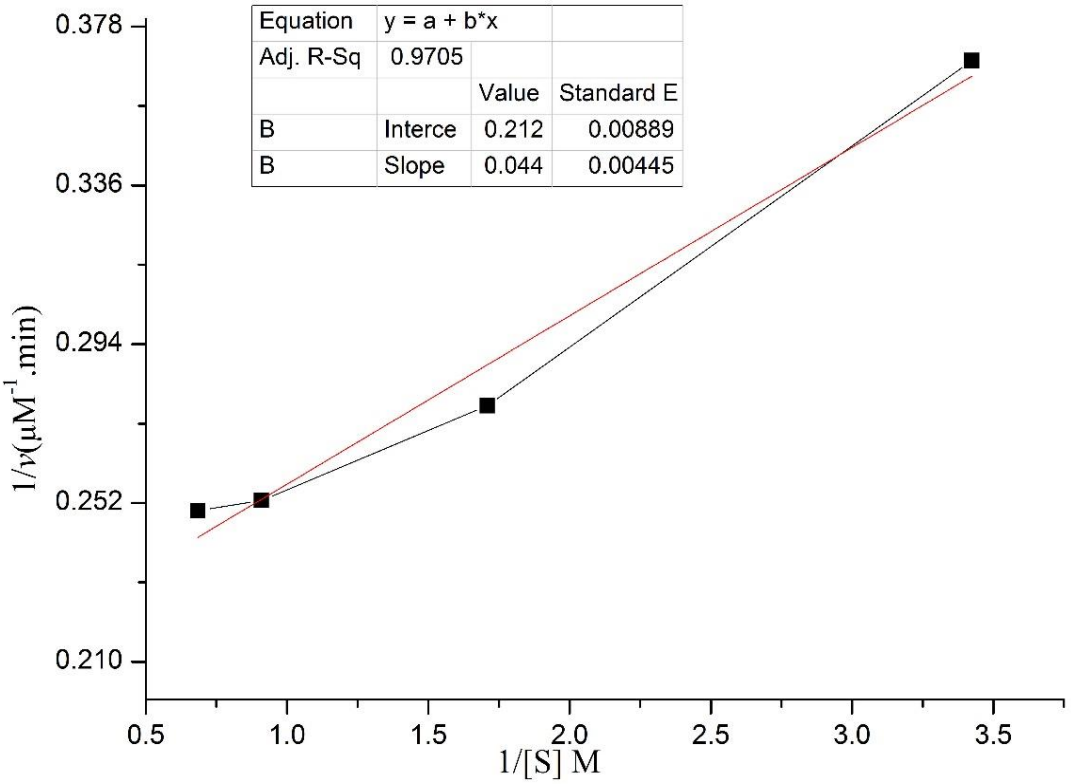


Figure 4.8: Lineweaver-Burk plot for fructosyltransferase activity. The partially purified enzyme was incubated with sucrose (0 – 50 µg/ml) at an optimal temperature of 55 °C and citrate-phosphate buffer 100 mM, pH 6 for 30 min. The reciprocal values of (1/v) were plotted against the concentration of the substrate (1/[S]), and K_m determined by fitting the data using ORIGIN 8 v. 8 Pro.

The enzyme kinetics with sucrose as substrate were determined by Lineweaver-Burk plot from (Figure 4.8) and K_m value was 2.08 mM, V_{max} was 4.72 µmole min^{-1}, while k_{cat} was 4.7 min^{-1}. The catalytic efficiency of the enzyme was found to be 2.26 µmole min^{-1}. In other reports fructosyltansferase enzyme from *Aspergillus aculeatus* with a molecular weight of 135 kDa the kinetic models were formulated by parameter estimation using time course simulations with the help of COPASI ® v. 4.8 software, the K_m was found to be between 0.438 – 10.622 µmole/min (Kashyap *et al*., 2015). In this study, Ftase enzyme demonstrated lower K_m values than other reports on kinetic characterization involving transferase and invertase enzymes. An optimized

thermostable invertase from *Aspergillus niger* yielded K_m and V_{max} of 21.93 mM and 35.71 U/min/ml respectively (Oyedeji *et al.*, 2017). A fungal species of *Aspergillus aculeatus* produced an extracellular fructosyltransferase with K_m, V_{max} and k_{cat} values of 2267 mM, 134 µmol/min/mg of protein, respectively and showed lower turnover of 37.3 s^{-1} (Huang *et al.*, 2016). Ftase from *Fusarium graminearum* exhibited a K_m value of 31.6 mM (Gonçalves *et al*., 2016). Other studies reported K_m of 35.67 mM and V_{max} 3.98 µmole/min (L'Hocine *et al*., 2000). Interestingly, the K_m values of a fructosyltransferase enzyme from *Rhodotorula* sp. using sucrose as a substrate, the enzyme displayed a rather sigmoid behavior. The enzyme fitted the Hill's model, where v is the rate of reaction, V_{max} was the limiting reaction rate which gave a V_{max} value of 2.36 µmole/min (Hernalsteens and Maugeri, 2008). A *β*-fructofuranosidase from yeast strain of *Schwanniomyces occidentalis* with transfructosylating activity reported almost identical kinetic parameters to the coprophilous *A. niger* used in the current study. The enzyme displayed K_m values of 4.9 mM, k_{cat} 1.4 min^{-1} and $\frac{kcat}{Km}$ 1.64 $\mu M^{-1}s^{-1}$ using sucrose as the substrate (Álvaro-Benito *et al.*, 2007). This K_m value was twice higher than the present study, which indicate that Ftase from *A. niger* has a higher affinity for sucrose than most invertases. The K_m value denotes the amount of substrate required to achieve half the maximal initial velocity. In addition, it gives an indication of enzyme affinity to the substrate (Sadhu *et al.*, 2013). Because of the lower K_m value, the turnover number, and the catalytic efficiency denoted by the ratio $kcat/Km$ shows that *A. niger* isolated from indigenous coprophilous fungus in the present study is a potential new reservoir of transferases. This strain also represents a better candidate for commercial exploitation like large-scale FOS production than most Ftases reported.

In a previous study lyophilization of Ftase by LEB –V 10, *Rhodotorula* sp. revealed kinetic studies carried out by either a partially purified fructosyltransferase or an enzyme purified to homogeneity converted sucrose to FOS at low cost and results were practically similar. The enzyme was also thermal stable with good transfructosylating activity and proved a good candidate for biotechnological application in partially purified form (Aguiar-Oliveira and Maugeri, 2012). In another similar study by (Alvarado-Huallanco and Maugeri Filho, 2011) a fructosyltransferase was also produced from *Rhodotorula* sp. in both purified and partially purified form for fructooligosaccharide production. The purified Ftase yielded 54 % FOS after synthesis while the partially purified enzyme yielded 50 % FOS under the same conditions. This was attributed mainly

to the presence of contaminants in partially purified enzyme due to hydrolytic enzymes like invertases or β-fructofuranosidase, which may have increased GF_3 and fructose production. However, in their enzyme kinetics, both purified and partially purified enzyme exhibited competitive glucose inhibition and high affinity to their substrates. Notwithstanding, we can deduce that it is desirable to purify enzymes to homogeneity, but in this study there was no significant difference in their prediction of FOS production either with the purified or partially purified enzyme. Therefore, further investigation of their crystal structures is proposed in the formation of oligosaccharides using fructosyl donor like sucrose. To determine catalytic efficiency and mechanism including molecular docking sites and other mathematical models to aid in predicting the ligand active sites of unknown protein structure interaction in Ftases (Xu *et al*., 2018b)

4.5 Conclusion

In the present study, a transferase enzyme isolated from indigenous coprophilous fungus was partially purified and biochemically characterized. The partially purified enzyme exhibited a high transfructosylating activity to catalyze sucrose to produce FOSs. The enzyme was purified with a three-step process involving ammonium sulphate precipitation, dialysis and anion exchange chromatography. The transferase was purified to 9.3 fold purification with a specific activity of 2465.50 U mg^{-1} with yield recovery of 7.3 %. The enzyme showed remarkable broad pH stability (pH 4.5 – 8.0) and was found to be thermally stable at high temperature. The optimal temperature stability was between 40 °C – 60 °C, retaining over 80 % residual activity above 65 °C and remained active at 70 °C for 6 h. The enzyme was not adversely affected by the presence of metal ions with the exception of $HgCl_2$ and $AgCl_2$ causing inhibition of about 95 %. In addition, Ftase was activated by Fe^{2+} and Mg^{2+}. Moreover, the enzyme displayed exceptional kinetic properties presenting a low K_m value of 2.0755 mM, V_{max} of 4.717 µmole min^{-1} and k_{cat} value 4.7 min^{-1}. The enzyme recorded a catalytic efficiency of 2.2645 µmole min^{-1}, with a relatively high carbohydrate content of 27.27 %. The electrophoretic mobility suggested that the purification process was partial, displaying three protein bands in the pooled fractions from 70 – 100 kDa. However, the purification process showed a satisfactory yield recovery and purification fold than most fructosyltransferase enzymes previously reported. A conventional renaturation technique was used to estimate the molecular weight of the partially purified Ftase to be around 70 kDa. Zymogram

analysis showed the oligomeric aggregation of the protein. Further applications are imperative to combine this approach with better resolution techniques for a greater utility in the determination of molecular weight in partially purified enzymes. Consequently, from the present study, the enzyme demonstration of high affinity to the substrate, high pH and thermal stability could be advantageous to industrial processes. Additionally this study offers a plausible explanation on the justification of using partially purified Ftase. In conclusion, coprophilous fungi are an emerging reservoir of highly efficient biocatalytic enzymes underexplored.

CHAPTER 5: Biotechnological Applications of Fructooligosaccharides Synthesized by a Partially Purified Fructosyltransferase Enzyme Extracted from an Indigenous Coprophilous *Aspergillus niger*

5.1 Abstract

Besides basic nutrition, fructooligosaccharides (FOS) are bioactive oligofructans with nutraceutical health benefits when consumed in recommended dosages. Fructosyltransferase (Ftase) assays for FOS production via sucrose biotransformation were carried out using a partially purified Ftase extracted from an indigenous coprophilous *Aspergillus niger* GenBank accession number MH445969. The end products of fructosyltransferase activity yielded monomeric glucose, 1- kestose (GF_2), and 1,1– kestotetraose (GF_3). High performance liquid chromatography coupled with a refractive index detector (HPLC-RI) was used to confirm and identify the oligosaccharides liberated by comparing their retention times with pure authentic FOS standards of 1- kestose (GF_2), 1, 1 – kestotetraose (GF_3), and 1, 1, 1 – kestopentaose (GF_4). Quantification of the FOS liberated was done by integrating the peak areas using YL Clarity/9300 software on the HPLC system. Three experimental models were used to determine the antioxidant activities of FOS by comparing their properties with pure FOS standards using vitamin C as an authentic antioxidant standard. Free radical scavenging activity by 1,1 - diphenyl-2-picryl hydroxyl (DPPH) assay, ferric reducing antioxidant power (FRAP) assay and nitric oxide (NO) radical inhibition yielded IC_{50} of 6.71 µg/ml, 1.76 µg/ml and IC_{25} of 0.27 µg/ml respectively. The antioxidant activity of the FOS were determined at concentrations of 15 µg/ml, 30 µg/ml, 60 µg/ml, 120µg/ml, and 240 µg/ml. The radical scavenging and inhibition activities showed a concentration-dependent antioxidant activity with no significant differences with oligosaccharide standards $p < 0.01$ though vitamin C was significant in FRAP and NO assays. These results demonstrated that FOS have potential antioxidant properties and can therefore be exploited for biotechnological applications as nutraceuticals.

Keywords: Coprophilous fungus, Fructooligosaccharides, Antioxidants, DPPH, FRAP, Nitric Oxide.

5.2 Introduction

Over the last decade, enzymology has emerged as a major area in biotechnology research (Klibanov, 1997, Kirk *et al*., 2002). Exploitation of industrial enzymes isolated from microorganisms is associated with low energy consumption, biofuel production and new sources of biofunctional foods, thereby presenting socio-economic, pharmaceutical and medical solutions (Aguiar-Oliveira and Maugeri, 2012, Volkin *et al*., 2015). From these industrial processes, biofunctional foods with prebiotic effects have received the most attention due to their health promoting properties in the human gut alongside basic nutrition (Witschinski *et al*., 2018). The human gut is a complex microbial reservoir and is residence to numerous microbiota with a plethora of both probiotic and potentially pathogenic bacterial communities (Gibson *et al*., 2010). The human gastrointestinal track (GIT) is one of the most microbially diverse, colonized and metabolically active region of the human body (Kralj *et al*., 2018, López-Sanz *et al*., 2018). These microbes occupy specific regions in the host GIT, thereby maintaining a homeostatic intestinal ecosystem (Schell *et al*., 2002).

Endogenous secretions and gastrointestinal microorganisms degrade available substrates like polymeric starch as well as dietary fibers (polysaccharides, with degree of polymerization, DP ≥ 10) such as xylan and pectins. Moreover, other lower concentrations of carbohydrates are fermented in the colon that include oligosaccharides DP_3 – DP_9 (Gibson *et al*., 2010, Lecerf *et al*., 2012). Furthermore, FOS are the most studied prebiotics because they are non-digestible carbohydrates that promote growth of beneficial bacteria in the host gut leading to improved health while concurrently suppressing the proliferation of potentially pathogenic bacteria (Roberfroid and Slavin, 2000a, Gibson *et al*., 2010, Patel and Goyal, 2011, Guerreiro *et al*., 2016). Beside nutritional benefits, they are also found to modulate the immune status by decreasing oxidative stress or increase antioxidant potential and improve colonic morphology by scavenging for free radicals (Guerreiro *et al*., 2016, Abasubong *et al*., 2018, Choudhary *et al*., 2018, Kumar *et al*., 2018a). An antioxidant is a molecule stable enough to donate a proton or electron to neutralize a free radical, significantly delays or prevents oxidation of cellular damage through free radical scavenging property (Lobo *et al*., 2010, Varghese *et al*., 2018).

Dietary antioxidants are considered vital nutraceuticals due to their health properties and are widely assimilated in food industries as inhibitors of liquid peroxidation, and excessive free radical

scavenging. They also prevent other malignants that are cytotoxic including scavenging for reactive oxygen species (ROS) that may trigger redox homeostatic disturbance leading to cell degeneration (Guerreiro *et al*., 2016, Poprac *et al*., 2017, Czarnocka and Karpiński, 2018). They also survive upper gut transit and are fermented in the colon (Wang *et al*., 2007, Aparadh *et al*., 2012, Corrochano *et al*., 2018). Reactive oxygen species (ROS) are generated from the body metabolic pathways and are by-products of biological reactions. ROS may cause oxidative stress when in excess and damage cell membranes. This may lead to decrease in membrane fluidity, lipid peroxidation, DNA mutation that may induce cancer and other degenerative maladies due to excessive free radicals (Ruby *et al*., 1995, DeFeudis *et al*., 2003, Lucas-Abellán *et al*., 2011). During certain pathophysiological conditions, or when antioxidants deficiencies occur, these control mechanisms are insufficient, and oxidant by-products may lead to oxidative stress, thereby causing DNA cell damage, lipid peroxidation or apoptosis (De Beer *et al*., 2017). ROS are produced either endogenously or exogenously (Galano *et al*., 2018). In endogenous mechanism, the mitochondrion is the source where incomplete oxygen metabolism produces ROS such as hydroxyl radicals (HO·), superoxide radicals, anion and nitric oxide as by-products (Poprac *et al*., 2017). The exogenous mechanisms is whereby anthropogenic activities which may be carcinogenic such as cigarrete smoking, excess dietary with iron or copper, industrial effluents pollution, and UV irradiation are predisposing to oxidation (Bouayed and Bohn, 2010, Shibamoto, 2017).

Hydroxyl radicals are regarded as the most oxidative free radicals and because of their strong oxidizing potential, hydroxyl radicals play a key role in the degradation and biotransformation of organic compounds (Das and Roychoudhury, 2014, Takeda *et al*., 2017). This is because the hydroxyl radicals lead to a more toxic reactive oxygen species in biological systems causing adverse pathological response (Sanchez-Moreno, 2002, DeFeudis *et al*., 2003, Molyneux, 2004, Lucas-Abellán *et al*., 2011). Therefore, it is imperative to propose antioxidant remedies to address this fundamental challenge. Several synthetic and natural antioxidants from plants such as flavonoids and phenolic compounds have been widely reported in literature. These antioxidants scavenge for free radicals and inhibit nitric oxide (Rao, 1997, DeFeudis *et al*., 2003, Sumanont *et al*., 2004, Chen and Yan, 2005, Kumaran and Karunakaran, 2006, Lim and Quah, 2007, Valko *et al*., 2007, Ak and Gülçin, 2008, Hou *et al*., 2015, Russo *et al*., 2015, Benzie and Devaki, 2017, Motohashi *et al*., 2017, de Francisco *et al*., 2018, Kellett *et al*., 2018).

Of the synthetic antioxidants that have been reported, such as butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT), have been found to have deleterious effects on human health such as damage to the liver and carcinogenesis in animals (Lobo *et al*., 2010). It is for this reason that natural non-toxic antioxidants are produced by fructosyltransferase enzymes via sucrose biotransformation yielding FOS which are highly compatible with dietary fiber. Several attempts have been made to determine antioxidant activity of fructooligosaccharides from *Helianthus tuberosus,* a plant rich in polydisperse inulin. From the various genetic variants of (FW) for the variety 'Bergly' 20 mg g^{-1} to 37 mg g^{-1} for 'Kirkeoy' tested, 30 % to 40 % of total carbohydrates were FOS and the rest were disaccharides and low levels of fructose (Seljåsen and Slimestad, 2005). In another *in vitro* study, the antioxidant potential of *Cichorium glandulosum* seed extracts for radical scavenging activity of DPPH, ABTS, hydroxyl radicals was investigated (Yao *et al*., 2013).

To date, studies on microbial fructosyltransferase for the production of bioactive FOS for biotechnological applications like antioxidants are scanty and require more plausible explanations (Pejin *et al*., 2014, Mano *et al*., 2018). The main aim of this study was to determine the antioxidant properties of fructooligosaccharides produced by a partially purified fructosyltransferase (EC 2.4.1) enzyme extracted from a coprophilous fungus, *Aspergillus niger*. In order to identify, confirm and quantify the prebiotics produced, it was necessary to monitor enzyme reaction end products using HPLC-RI. Numerous, different substrates system compositions and analytical screening tests have been employed to evaluate effectiveness of antioxidants. This include chemical, instrumental or sensory methods. The effectiveness of antioxidants is measured by monitoring the inhibition of oxidation of a suitable substrate after oxidation under standard conditions (Sánchez-Moreno *et al*., 1999, Sanchez-Moreno, 2002). Three known and validated methods of 1, 1 - diphenyl- 1-picryhl-hydrozyl (DPPH) assay (Oyaizu, Molyneux, 2004), ferric reducing anti-oxidant power (FRAP) assay (Kumaran and Karunakaran, 2006, Veenashri and Muralikrishna, 2011, Shaikh *et al*., 2018) and nitric oxide radical scavenging activity (Nakagawa and Yokozawa, 2002, Sumanont *et al*., 2004, Hofseth, 2008, Nambiar *et al*., 2017) were evaluated for their antioxidant effectiveness in determining oxidative inhibition, and free radical scavenging potential of FOS.

5.3 Materials and Methods

5.3.1 Reagents

Dimethyl sulfoxide (DMSO), 1,1-diphenyl-2-picryl-hydrazyl (DPPH), 2,4,6-tris(2-pyridyl)-s-triazine (TPTZ), trichloroacetic acid (TCA), Potassium hexacyanoferrate (111), $C_6FeK_3N_6$, methanol, Sigma Aldrich, South Africa. Ferric chloride hexahydrate, $FeCl_3.6H_2O$, (*N*-(1-naphthyl) ethylenediamine dihydrochloride (NED or Griess reagent), 10 % tricarboxylic acid, ethanol, Merck, Darmstadt, Germany). PDA, Czapex Dox agar, FOS standards GF_2, GF_3, GF_4 (Megazyme), sucrose (anhydrous), D-Fructose, KCl, $MgSO_4.7H_2O$. (Sigma Aldrich, St Louis, Missouri). All other chemicals and reagents were supplied by reputable scientific suppliers and were of analytical grade unless stated otherwise.

5.3.2 Microorganism, Culture Conditions

After the screening exercise as previously described, the coprophilous fungus strain XOBP – 48 was selected for further study. This fungal strain was obtained from buffalo dung in Phinda game reserve and identified to species level as *Aspergillus niger* assigned GenBank accession number MH445969. The isolate was preserved on Czapex-Dox agar and potato dextrose agar (PDA) in Petri dishes and incubated at 25 ºC.

5.3.3 Fermentation Media and FOS Production

FOS production was carried out in 250 ml Erlenmeyer flasks containing 100 ml of liquid basal media with sucrose as a sole carbon source. The medium was constituted as follows (% w/v): sucrose (30), $NaNO_3$ (3), KCl (0.05), $MgSO_4.7.H_2O$ (0.5), K_2HPO_4 (0.5), yeast extract (10), $FeSO_4.7H_2O$ (0.01) (Sangeetha *et al*., 2005a, Mussatto *et al*., 2009b, Prata *et al*., 2010). Three fungal blocks (8 mm^2) were cut from agar plates and inoculated into the flask with liquid basal medium. The flasks were incubated at 28 ºC at 200 rpm for 5 days for the production of extracellular fructosyltransferase. The crude extracellular enzyme was partially purified in a three-step purification procedure as previously described (Chapter 4). The partially purified extracellular enzyme was used for FOS production using sucrose (5 % w/v) suspended in 100 mM citrate-phosphate buffer (pH 6.0) as a substrate. All experiments were carried out in triplicate and reaction mixture end products were analyzed by HPLC coupled to (RI) detector. The HPLC model was a YL 9100 system (Techno Lab System), column (Pinnacle II Amino 3 μm, 150 × 4.6 mm), flow

rate 1.0 ml/min, mobile phase acetonitrile – 70 %: H_2O – 30 % with (0.04 % w/v) ammonium hydroxide, isocratic elution (Correia *et al*., 2014, Manosroi *et al*., 2014, Tihomirova *et al*., 2016). The retention times of FOS produced by the reaction of the partially purified Ftase were compared to those of pure standards of 1- kestose, 1,1- kestopentaose, and 1,1,1- kestotetraose respectively (Mussatto *et al*., 2009a).

5.3.4 Determination of Antioxidant Potential of Fructooligosaccharides

5.3.4.1 DPPH Radical Scavenging Activity

Total free radical scavenging activity of prebiotic end products and pure FOS standards (GF_2, GF_3 and GF_4) were carried out as described briefly by the modified method of (Oyaizu, Ak and Gülçin, 2008) compared to Vitamin C as a standard antioxidant. An aliquot of 500 µl of a 0.3 mM solution of 1,1- diphenyl-2-picryl-hydrazyl (DPPH) dissolved in methanol (analytical grade 99 %) was added to 1 ml of the prebiotic end products at different concentrations from (15, 30, 60, and 240 µg/ml). These solutions were mixed with curcumin solution and incubated for 30 min in the dark at room temperature. The absorbance was measured spectrophotometically at 517 nm against a blank lacking the free radical scavengers (Manosroi *et al*., 2014, Shah *et al*., 2016). The DPPH results were expressed as a percentage of the control (blank) with the following formula:

% Inhibition = [(Abs of control - Abs of sample)] / Abs of control] x 100

IC_{50} which denotes amount of FOS end product the reaction required to reduce initial concentration of DPPH radical by 50 %. It was calculated from a graph plotted between % scavenging versus concentration of the sample (Lim and Quah, 2007).

5.3.4.2 Preparation of DPPH

Methanolic solution of DPPH (0.1 mM): 40 mg of DPPH was dissolved in 100 ml methanol. The mixture was then diluted with 100 ml of distilled water to obtain a stock solution of methanol/water (50:50 v/v). The DPPH working solution of absorbance values 0.75 - 0.80 at 525 nm was prepared by diluting 200 ml of DPPH stock solution with approximately 800 ml methanol/water (50:50 v/v) (Serpen *et al*., 2012, Manosroi *et al*., 2014).

5.3.4.3 Ferric Reducing Antioxidant Power (FRAP) Assay

The FRAP solution was prepared by diluting 10 mM aqueous solution of TPTZ with 20 mM ferric chloride in 300 mM sodium acetate buffer (pH 3.6) in the ratio 1:1:10 (v/v/v) (Serpen *et al*., 2012, Ahmed *et al*., 2017). The ferric reducing anti-oxidant power method of (Lim and Quah, 2007) was slightly modified to measure the reducing capacity of fructooligosaccharides. Prebiotic extract (500 µl) each or FOS end product of (15, 30, 60, 120 and 240 µg/ml) were incubated with 500 µl of 0.2 M sodium phosphate buffer (pH 6.6) and 1 % (w/v) potassium ferricyanide at 50 °C for 30 min. After incubation for 30 min reacting products were acidified by addition of 500 µl 10 % (w/v) trichloroacetic acid. Thereafter, 500 µl of the acidified sample was mixed with 500 µl distilled water and 200 µl of $FeCl_3$ 0.1 % (w/v). The absorbance was read against a blank spectrophotometically at 700 nm. Antioxidant activity was estimated by measuring the increase in absorbance as a result of formation of ferrous ions from the FRAP reagent containing TPTZ and $FeCl_3.6H_2O$ (Veenashri and Muralikrishna, 2011). The results were expressed as ascorbic acid equivalent antioxidant activity.

5.3.4.4 Nitric Oxide Scavenging Assay

Nitric oxide (NO) scavenging activity was estimated according to the modified method of (Lalhminghlui and Jagetia, 2018). Sodium nitroprusside (5 mM) in saline phosphate buffer (pH 7.2, 100 mM) was mixed with different concentrations of chloroform, ethanol (analytical grade) or FOS end products and incubated at 50 ºC for 2 h. The samples were withdrawn and mixed with Griess reagent 1 % sulfanilamide, 2 % H_3PO_4 and 0.1 % *N*-(1-naphthyl)-ethylenediamine dihydrochloride. Measurement of absorbance of the intense pink colour that formed (chromophore) during diazotization of nitrite with sulfanilamide and the coupling with NED was done at 546 nm using a spectrophotometer (Cary 60 UV-VIS, Agilent Technologies) (Nambiar *et al*., 2017). Nitric oxide inhibition was determined with Vitamin C as a standard and the results were expressed in antioxidant Vitamin C scavenging equivalent using the formula: NO scavenging activity (%) = $(A_0 – A_1) /A_0 \times 100$

Where A_0 is absorbance of the control, and A_1 is sample absorbance (Nishaa *et al*., 2012). All reactions were performed in triplicate.

5.3.5 Statistical Analysis

All the experiments were carried out in triplicate unless otherwise specified. The data obtained was presented as mean ± standard deviation (mean ± SD) using MS Excel one way ANOVA, with ($p < 0.05$) significance level. Calibration curves of the standards used were considered significant if $R^2 \geq 0.99$. The IC_{50} values of ROS scavenging activity were calculated from the curves of % inhibition verses log antioxidant concentration.

5.4 Results and Discussion

The partially purified fructosyltransferase from *Aspergillus niger* was used for FOS production via sucrose elongation to yield FOS and monomeric glucose. The end products were analyzed by HPLC coupled to a refractive index detector as depicted on the chromatogram profiles in Figure 5.2. Different standards of oligosaccharides exhibited the retention times as shown in Table 5.1 of 1, 1, 1 – kestopentaose, 1- kestose and 1,1- kestotetraose at 3.892, 8.800 and 12.767 respectively. The end products of fructosyltransferase assay involving the partially purified Ftase showed FOS at peak of retention times of 4.983, 8.408 and 11.767 of glucose, 1- kestose and 1, 1 - kestoteraose respectively in (Figure 5.2).

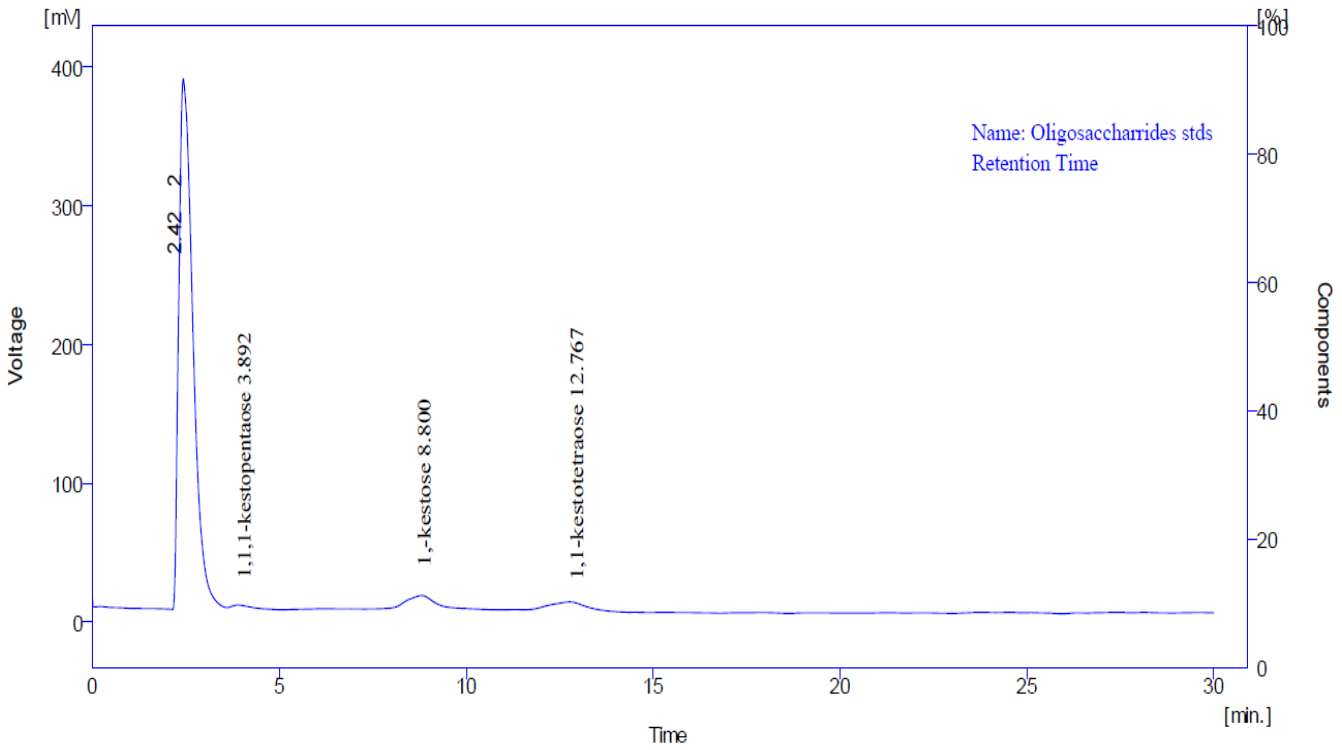


Figure 5.1: Chromatogram showing retention times of oligosaccharides standards

To determine the effect of antioxidants on oligosaccharides, it was necessary to identify the FOS yield in the Ftase assay as produced by transfructosylating ability of sucrose. Different, authentic oligosaccharide standards of 1,1,1-kestopentaose, 1-kestose and 1,1-kestotetraose (Figure 5.1) revealed different retention times on HPLC. It was therefore imperative to establish from the Ftase assay the oligomers produced. A similar method of HPLC qualitative analysis was employed in fortification of fruit juices containing fructooligosaccharides (Renuka *et al*., 2009).

Table 5.1: Table showing the retention times and peak areas of oligosaccharide standards.

Result Table (Uncal - Oligosaccharrides 25042018Oligosaccharrides 2504201825_04_2018 - Channel 1)

	Reten. Time [min]	Area [mV.s]	Height [mV]	Area [%]	Height [%]	W05 [min]	Compound Name
1	0.217	4.622	0.374	0.0	0.1	0.21	
2	2.425	10076.800	382.532	83.5	93.6	0.42	
3	3.892	149.582	3.511	1.2	0.9	0.64	1,1,1- kestopentaose
4	6.233	133.357	1.241	1.1	0.3	2.08	
5	8.800	852.318	11.300	7.1	2.8	0.92	1- kestose
6	12.767	678.768	7.435	5.6	1.8	1.23	1,1 - kestoteraose
7	21.308	24.453	0.382	0.2	0.1	0.57	
8	23.758	100.456	0.814	0.8	0.2	2.04	
9	27.458	24.190	0.524	0.2	0.1	0.93	
10	28.142	17.719	0.491	0.1	0.1	0.63	
	Total	12062.265	408.603	100.0	100.0		

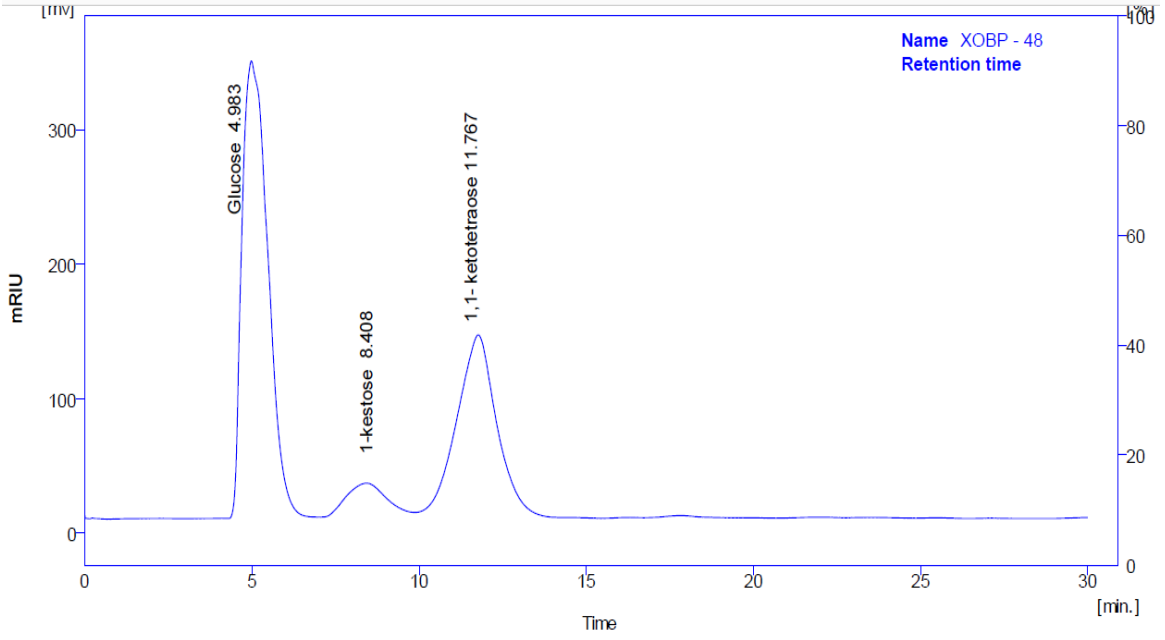


Figure 5.2: FOS and glucose liberated from the reaction of the partially purified transferase with sucrose as substrate and analyzed on HPLC. The retention times were compared to those of standards.

Table 5.2: Table showing retention times and peak areas of FOS products released after transferase activity of sucrose.

Result Table (Uncal - 00000752 - Channel 1)

	Reten. Time [min]	Area [mV.s]	Height [mV]	Area [%]	Height [%]	W05 [min]	Compound Name
1	0.233	6.494	0.539	0.0	0.1	0.15	Study strain XOBP -48
2	4.092	80.259	0.640	0.2	0.1	1.03	
3	4.983	18474.493	340.436	55.6	66.6	0.90	Glucose
4	8.408	2376.360	26.676	7.1	5.2	1.43	1- kestose
5	11.767	11708.143	136.784	35.2	26.8	1.27	1,1 - kestoteraose
6	16.308	59.604	1.037	0.2	0.2	1.02	
7	17.817	280.755	2.482	0.8	0.5	1.38	
8	22.008	199.654	1.190	0.6	0.2	3.32	
9	25.500	39.853	0.683	0.1	0.1	1.13	
10	27.133	21.251	0.370	0.1	0.1	0.78	
	Total	33246.866	510.837	100.0	100.0		

The coprophilous fungus (study strain) showed potential to synthesize FOS by producing 1-kestose (GF_2), 1,1-kestoteraose (GF_3) and monomeric glucose (Figure 5.2) which displayed a crucial antioxidant role of scavenging for free radicals and inhibition of nitric oxide radicals. There was pronounced decrease of glucose production to GF_2 (Table 5.2) and this could be attributed to inhibition from glucose accumulation. A similar model proposed by Yun (1996) showed pronounced decrease in the FOS concentration from GF_2 to GF_4 suggesting the enzyme acceptor site could be presumptively sizeable to accommodate up to GF_4 (Yun, 1996b). Withal, the production of GF_3 (nystose) has been reported to produce higher biological activity than other oligomers (Zduńczyk *et al.*, 2005).

5.4.1 DPPH Radical Scavenging Activity Assay

The antioxidant ability of fructooligosaccharides was investigated along with other standard oligomers by the DPPH assay. Free radical scavenging activity was calculated from a decrease in absorbance and discoloration of the DPPH stable radical caused by antioxidant due to hydrogen donation to form non-radical DPPH-H (Molyneux, 2004, Chen and Yan, 2005). This transformation led to a color change from purple to yellow and appearance of yellow color was stoichiometrically related to a number of electrons gained and was measured at 517 nm (Dave, 2009, Nishaa *et al*., 2012). The presence of antioxidant activity of the FOS end product was evident as it was able to reduce DPPH (Figure 5.3). Additionally, oligosaccharides standards used showed that there was no significant difference with the synthesized FOS ($p < 0.05$), IC_{50} of FOS sample was 6.7073 µg/ml and oligosaccharides standards of GF_3, GF_4 showed relative higher IC_{50} of (7.0479 µg/ml) but not significantly different. This calculation was extrapolated from the IC_{50} curve of Vitamin C of % inhibition scavenging vs log concentration shown in (Figure 5.4). The IC_{50} is the amount of antioxidant needed to decrease the initial concentration of DPPH by 50 % (Lim and Quah, 2007, Mopuri *et al*., 2017). The FOS sample showed good free radical inhibition even at lower concentration of 30 µg/ml. In addition to these, the increase in free radical scavenging activity of FOS was concentration dependent as at 120 and 240 µg/ml scavenging activity was higher. Although the content of bioactive compounds and antioxidant activity of FOS end products was low compared to oligosaccharides and vitamin C, it is vital to remark that they are interesting sources of dietary fibre which can supplement fibre-enriched food.

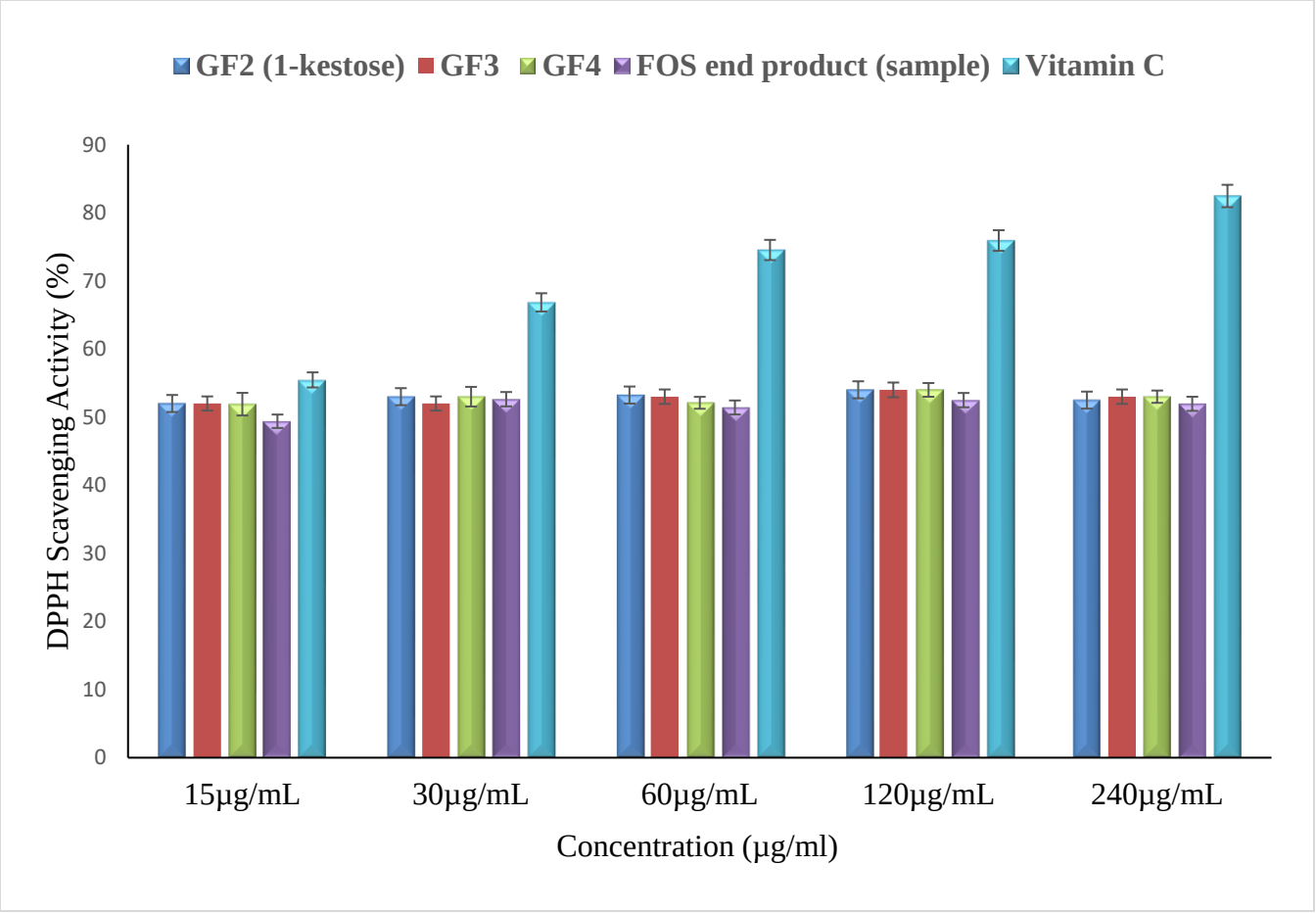


Figure 5.3: DPPH radical scavenging activity of FOS end products of bioconversion of sucrose to oligosaccharides by fructosyltransferase enzyme isolated from coprophilous fungus compared to known oligosaccharide standards with Vitamin C as equivalent antioxidant. (The values are the mean percentage ± S.D from three independent experiments).

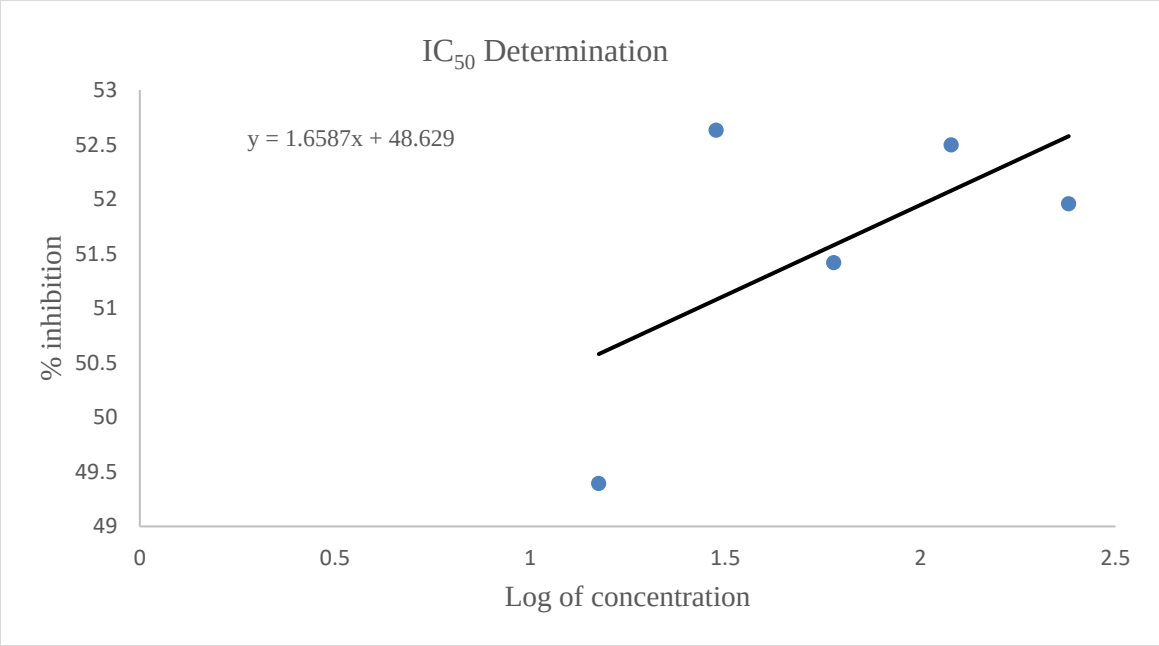


Figure 5.4: Calibration curves using standard oligomers.

Other fibre sources compared to dietary antioxidants such as cellulose, glucomannans, wheat bran and others lack intrinsic antioxidant activity and bioactive compounds (Fuentes-Alventosa *et al*., 2009). These aforementioned facts, together with functional properties reported such as stimulation of gut enzyme, bifidogenic effect and prebiotic effects make FOS synthesized from industrial enzymes attractive. From this assay, FOS was seen capable of scavenging free radical through inhibition as DPPH showed strong absorption maximum at 517 nm. The DPPH assay reveal that GF_2, GF_3 and GF_4 have scavenging properties similar to vitamin C, which is a standard antioxidant. Moreover, the FOS end product concentration dependent characteristic could be inferred or imply that high prebiotic consumption is imperative to offer nutraceutical effect. In conclusion, the acute administration of FOS could possibly overcome the body's reaction to exogenous antioxidants during appropriate therapies in certain pathophysiological conditions associated with oxidative stress. Herein we provide compelling *in vitro* evidence that FOS end product along with other oligomers have capacity to scavenge ·OH. These prebiotics have potential to offer protection against oxidative stress.

5.4.2 Ferric Reducing Antioxidant Power (FRAP) Assay

FRAP assay has been used to evaluate the antioxidant ability of FOS to reduce Fe^{3+} to Fe^{2+}. This is measured with the formation of a coloured complex with TPTZ and read spectrophotometrically (Uppin *et al*., 2018). This method is based on electron transfer and is regarded as an accurate measure of total antioxidant power, since total reducing power is elucidated as sum of reducing power of individual compounds contained in the sample (Ahmed *et al*., 2017). FRAP activity of the end-product reaction showed FOS (Figure 5.5) had effective reducing power but the oligosaccharide standards were slightly higher with no significant difference ($p < 0.01$). At different concentrations from (15 µg/ml - 240 µg/ml) FOS demonstrated powerful reducing ability in this redox-linked colorimetric reaction and it was concentration dependent ($p < 0.01$). The IC_{50} value of FRAP extrapolated from the IC_{50} curve of oligosaccharide (Figure 5.6) was at 1.7603 µg/ml. Ferrous ion reduction of Fe^{3+} complex of tripyridyltriazine ($Fe(TPTZ)^{3+}$) to more intensely coloured Fe^{2+} complex ($Fe(TPTZ)^{2+}$) demonstrated the electron donor property of FOS for neutralizing free radicals by forming stable products (Aparadh *et al*., 2012, Russo *et al*., 2015). Hence, they exhibit potential to donate electrons that scavenge free radicals in the actual biological and food systems. This antioxidant property was seen in other phenolic compounds *in vitro* (Ak and Gülçin, 2008). FRAP assay is a suitable method because it is quick, simple and relatively inexpensive for measuring antioxidant activity as a wide range of samples can also be tested simultaneously (Benzie and Devaki, 2017). This method was used to evaluate the antioxidant activity of a specific green tea extract that contained selenium. FRAP assay demonstrated that the extract could enhance the viability and growth of beneficial *Lactobacillus* using both pure and mixed bacterial cultures *in vitro*. Hence, the green tea extract was able to evince prebiotic potential as an antioxidant (Molan *et al*., 2009).

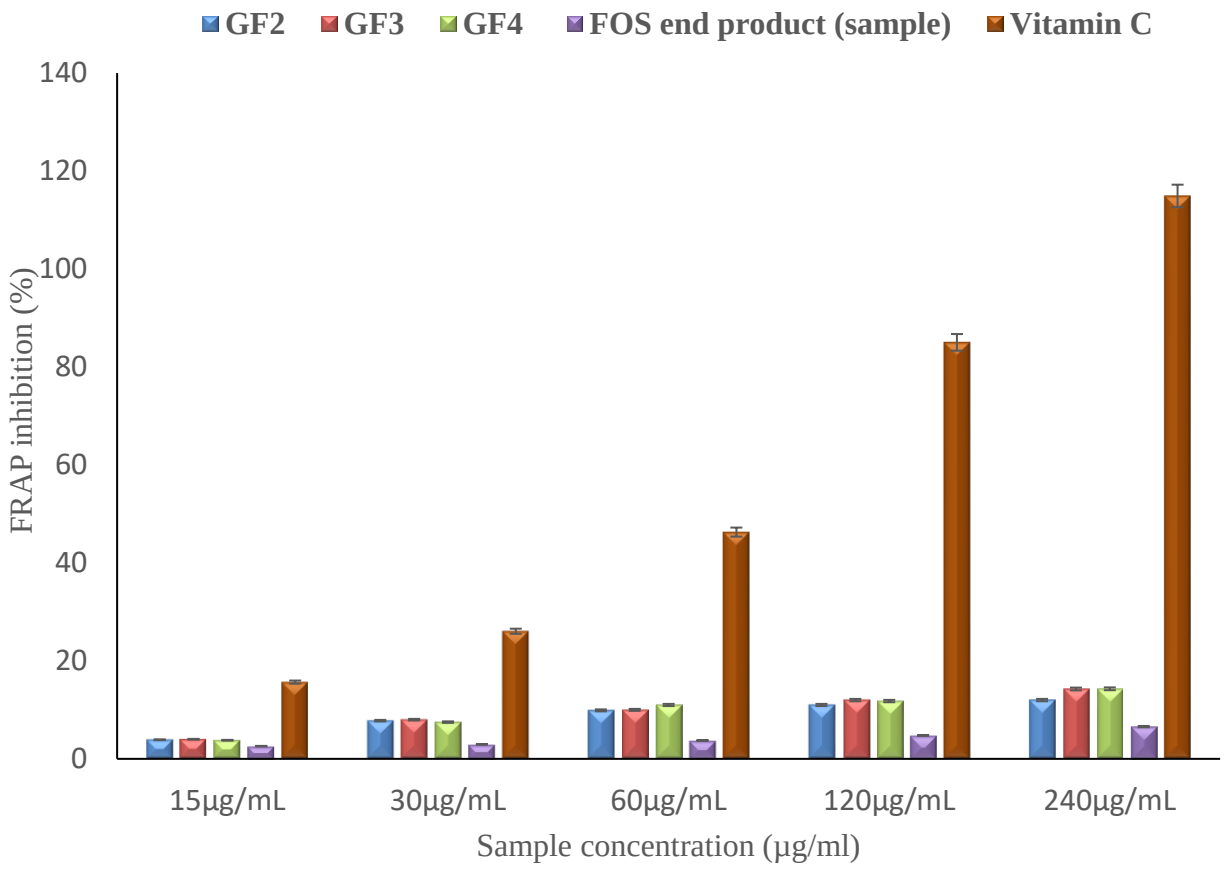


Figure 5.5: Total ferric reductive potential of different concentrations (15µg/ml – 240µg/ml) of FOS end products, oligosaccharide standards and reference antioxidant vitamin C from spectrophotometric detection of Fe^{3+} – Fe^{2+} transformation.

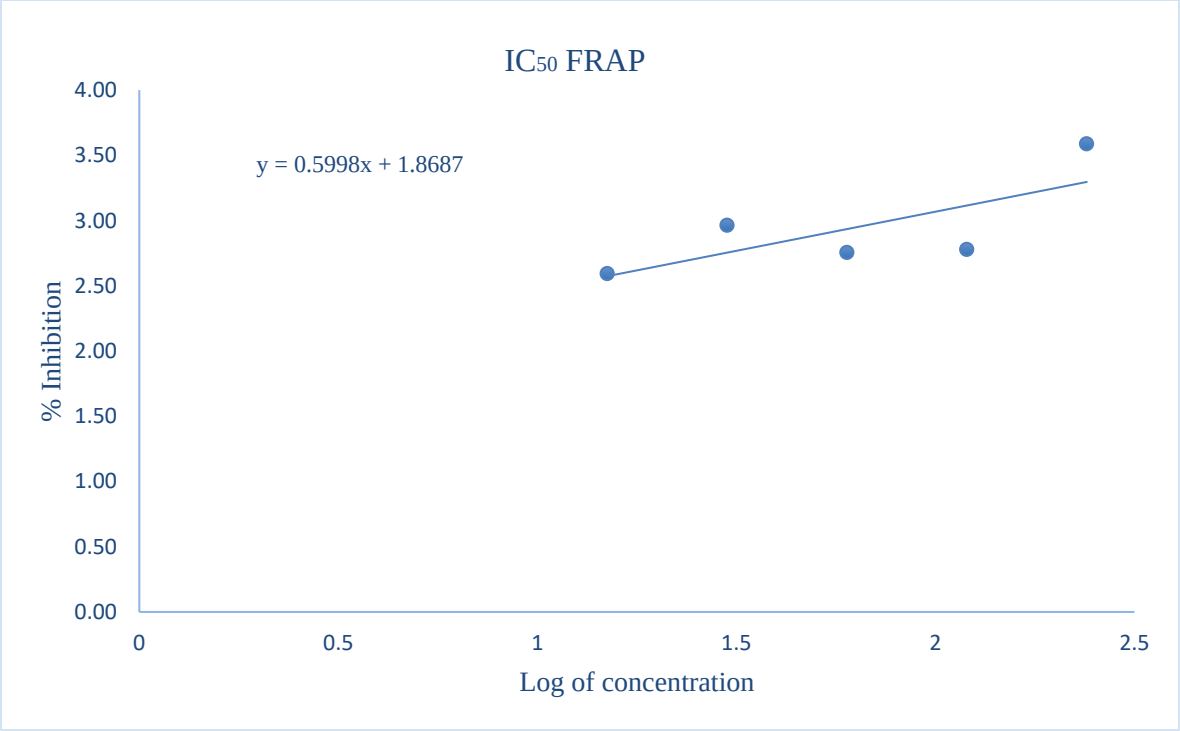


Figure 5.6: IC_{50} determination between ferric ions and FOS.

5.4.3 Nitric Oxide (NO) Scavenging Assay

The ability of FOS to inhibit nitric oxide production *in vitro* is shown in Figure 5.7. FOS exhibited a relatively lower NO scavenging activity as the IC_{50} was not reached with the tested concentrations as it fell slightly below 50 % of total inhibition. Consequently, the results were expressed in IC_{25} (µg/ml), equivalent with values of 0.2705 µg/ml which were significantly lower compared to Vitamin C 6.1 µg/ml. GF_3 oligosaccharide showed higher nitrite oxide inhibition potential as it correlates with the current study that nystose exhibit higher biological activity than 1- kestose and it is crucial to optimize its production (Correia *et al*., 2014, Mutanda *et al*., 2015). Incubation of solution of sodium nitroprusside with PBS resulted in linear dependent nitrite production, which was reduced by FOS sample. Scavenging of NO was present in all samples but the potency to inhibit nitrites of NO_2, N_2O_4 and N_3O_4 varied. This can be attributed to stability of FOS to donate electrons. Structural features are also responsible for scavenging properties for prebiotics like difficulty in cleaving glycosidic linkages in free hydroxyls (Shang *et al*., 2018). Suppression of released NO may be partially attributed to direct NO scavenging as the end product of FOS decreased the amount of nitrite *in vitro.*

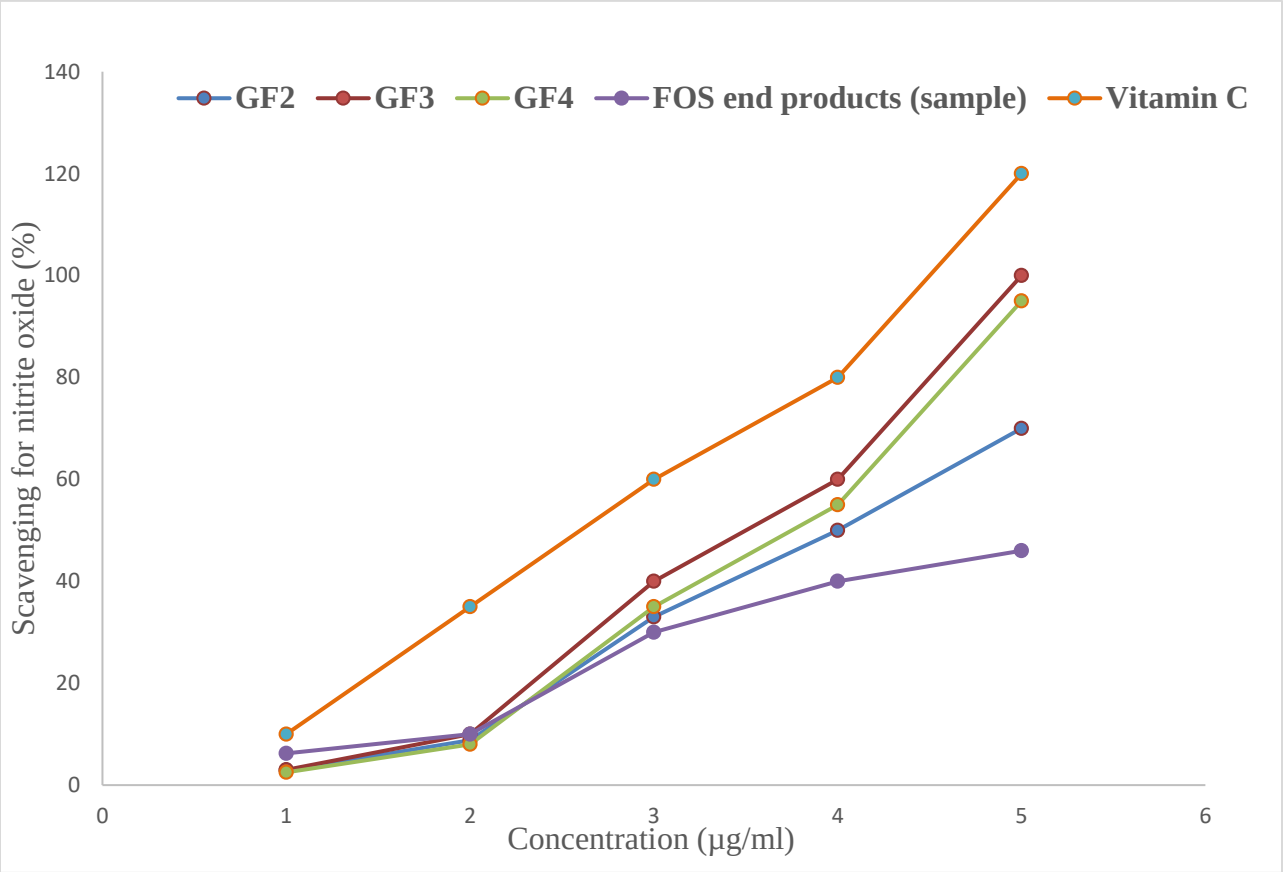


Figure 5.7: Nitric Oxide scavenging antioxidant activity with oligosaccharide standards and potent vitamin C used as standard antioxidant.

Sodium nitroprusside is known to decompose in aqueous solution at physiological pH 7.2 producing NO (Brüne, 2003). Under aerobic conditions, NO reacts with O_2 liberating nitrates and nitrites which are stable products, and their quantities can be determined by Griess reagent (Marcocci *et al*., 1994). The Griess reaction is based on a two-step diazotization reaction in which acidified NO^-_2 produces a nitrosating agent that react with sulfanilic acid to produce diazonium ion. This ion is subsequently coupled to *N*–(1-napthyl) ethylenediamine to form chromophoric azo-derivative absorbing at 546 nm (Ferreres *et al*., 2012).

The nitric oxide radical is toxic after the reaction with oxygen and the FOS end product sample reduced NO along with the standard antioxidant in concentration dependent manner. Similarly in another study investigating FOS antioxidant potential, muffin was incorporated with oligosaccharide from *Emblica officianalis* and exhibited scavenging of NO radical in a concentration dependent manner (Nambiar *et al*., 2017). Nitric oxide is a vital bioregulatory molecule with numerous physiological effects that include neural signal transduction, anti-tumor activity, anti-cancer properties and antimicrobial activity. Low concentration as presented in Figure 5.7 is sufficient to inhibit or scavenge free radicals (Kumaran and Karunakaran, 2006).

5.4.4 Bioactivities of Fructooligosaccharides as Antioxidants

Fructooligosaccharides (FOS) are non-digestible oligosaccharides fructans, and are sucrose derived as they contain several fructose units and a common glucose residue (Pejin *et al*., 2014). FOS are prebiotics which have increasing health benefits beside basic nutrition where they stimulate growth of *Bifidobacteria* and *Lactobacilli* in colonic microflora (Dominguez *et al*., 2014, Mutanda *et al*., 2014b, do Prado *et al*., 2018). Other FOS benefits that have been claimed include reduction in serum cholesterol, increase in magnesium and calcium absorption, vitamin B production, and immune stimulation among others (Sangeetha, 2003, Maiorano *et al*., 2008, Mutanda *et al*., 2008a, Mussatto *et al*., 2009b, Zhu, 2017, Silvério *et al*., 2018, Yan *et al*., 2018). These health benefits have brought significant attention to interrogate or explore their antioxidant potential. FOS antioxidant property has not been elucidated and information on this nutraceutical remains scanty. Few polysaccharides have been tested and like fructofuranan such as *Plantago lanceolate* were found inactive against peroxidation (Pejin *et al*., 2014). There is evidence that reactive oxygen species and free radicals formed under physiological conditions and are not being eliminated by endogenous system induce oxidative stress to the cell (Uppin *et al*., 2018). These accumulation may lead to cytotoxicity and other malignant neoplasia like atherosclerosis, cancer, cardiovascular disease, mild cognitive impairment, alcohol induced liver disease and Parkinson's disease (Yamaguchi *et al*., 1998, Dave, 2009). Free radicals are consequence of various metabolic activities and their excess production is a major cause of illness or cellular damage (Lalhminghlui and Jagetia, 2018). Free radicals are atoms with unpaired number of electron formed when O_2 reacts with free molecules. These interaction may cause damage to cell membrane poor cell function or even apoptosis a process that can be mitigated by prebiotic antioxidant (Lobo *et al*., 2010). Free radical scavenging activity is vital due to harmful effects it causes in biological systems

(Huyut *et al*., 2017). FOS radical scavengers have shown potential to react with free radicals and this model can be useful in termination of peroxidation chain reaction which is the primary product of lipid oxidation (Ak and Gülçin, 2008). When end product of transferase assay were added to a medium containing stable DPPH, free radicals were reduced by decolonization to form non radical DPPH-H upon donation of H proton (Alam *et al*., 2013, Huyut *et al*., 2017). In other studies, over expression of NO synthase in vascular endothelial cells has been reported to cause iNOS (inducible nitric oxide synthases) expression in macrophages leading to cytotoxicity, autoimmune disease inflammatory responses or organ destruction (Rao *et al*., 2016). FOS could alter biological activity in the human body as a potential scavenger of free radicals. FOS as an antioxidant may also act directly on nitric oxide radical N = O and related ROS such as H_2O_2 and ferryl ions. In addition, it may indirectly decrease free radicals formation such as OH, N = O probably through inhibition or suppression of expression of genes such as c-FOS, c-JUN and c-MYC which are implicated in transcriptional induction (Hussain *et al*., 2003). In this study, FOS from a partially purified Ftase has demonstrated inhibitory activity against NO production and it is a promising candidate for inducing iNOS inhibitory activity. These results can be inferred to recommend FOS prebiotics as inhibitors of NO production creating therapeutic response in management of inflammatory diseases. The relationship between *in vivo* and *in vitro* models remain to be established to make the *in vitro* results substantial. More, biological active *in vitro* and *in vivo* models need to be investigated synchronously to correlate their health claims.

5.5 Conclusion

The ability of prebiotic oligosaccharides to reduce DPPH to DPPH-H by donating protons, reduction of Fe^{3+} to Fe^{2+} through FRAP assay and reduction of nitrite oxide by scavenging for free radicals indicates a remarkable antioxidant property to relieve oxidative stress and other reactive oxygen species. Further, and more detailed studies on the chemical composition of these prebiotic oligomers using different experimental models like Trolox equivalent antioxidant capacity (TEAC) assay, Liquid peroxidation, Oxygen radical absorbance capacity (ORAC) assay, and metal chelating activity, Superoxide anion radical scavenging (SO) assay and Hydroxyl radical scavenging (HO) assay need to be explored. In addition, *ex vivo* and *in vivo* studies are paramount in order to fully corroborate and comprehend their potential nutraceutical and biotechnological application, as it will aid in characterization of fructo-oligosaccharides as biological antioxidants.

CHAPTER 6: Conclusion and Recommendations

6.1 Conclusion

The present study has demonstrated the prominence of bioprospecting for autochthonous coprophilous fungi from herbivore dung. The dung was sampled from domesticated and wild herbivores from various terrestrial habitats in the province of KwaZulu-Natal, South Africa. A number of coprophilous fungal strains with unique morphological, biochemical, and physiological characteristics amenable for biotechnological utilization were isolated purified to axenicity and identified by morpho-taxonomic and molecular tools. The different dung types of mega herbivores, grazers and browsers were cultured in damp chambers. The fungal isolates demonstrated their ability to grow in moist chambers under ambient laboratory conditions. The fungal mutualistic and symbiotic competitive interactions was observed in mycelia that were in close proximity. An indication of nutrient limitation and space was evident from mycelium expansion as there was confined space for their hyphae like structures to scavenge for water and nutrients on dung substratum. Several factors were also observed in unity of coprophilous fungi such as heterotrophic nutrition, the presence of hyphae that aggregated to form fungal mycelium. Presence of chitin in their cell wall and spores formation was evident. The spores were cultured on fungal agar media MEA, PDA and chemically defined liquid basal media with inulin and sucrose as sole carbon sources respectively. The growth of fungi on dung was monitored under laboratory conditions and sporulation of fungal spores were evident under alternating light and dark from day three.

From the sixty-one (61) isolated strains of coprophilous fungi that were screened, only two species showed the greatest potential of producing fructosyltransferase (Ftase) and inulinase enzymes capable of synthesizing FOS and hydrolyzing inulin respectively. Isolate XOBP - 48 from buffalo dung displayed the highest transfructosylating ability while isolate XWRP - 2 from white rhino had showed considerable potential to produce FOS. The fungal strain XOBP - 48 was selected after the rigorous screening exercise and identified morphologically to belong to the phylum Ascomycota. It was further identified by molecular techniques of 18S rDNA to be *Aspergillus niger* and was assigned GenBank number MH445956 from NCBI database. The strain XWRP – 2 was identified as *Trichoderma asperellum* and was the second best strain showing transfructosylating ability. Additionally, the fungal isolate XOGU – 49 from goat dung in Ukulinga research farm exhibited the highest exo-inulinase ability followed by isolate XOEP – 44 from

elephant dung from Phinda game reserve. The isolate XOGU – 49 was identified to species level and showed 84 % similarity to *Mucor circinelloides*. This was an indication of a possible novel strain for inulinase production although further investigations are imperative to ascertain this claim. The coprophilous fungus *Aspergillus niger* was selected based on its ability to produce short-chain FOS after HPLC analysis resulted in 1-kestose, 1,1-kestotetraose and monomeric glucose. The predominance of FOS produced suggests that *Aspergillus niger* is a suitable candidate for FOS production.

The fructosyltransferase enzyme catalyzing the reaction was partially purified using ammonium sulphate precipitation, dialysis and HiTrap QFF column chromatography with a specific activity of 2465.5 IU/mg, 7.3 % recovery and 9.3-fold purity. The partially purified Ftase had a temperature and pH optimum at 55 °C and 6.0 respectively. The Ftase from *A. niger* showed high affinity for sucrose. The Michaelis-Menten constant exhibited was 2.08 mM and a maximal velocity of 4.72 µmole min^{-1}. The catalytic constant *k*cat was 4.7 min^{-1}, with a catalytic efficiency ($\frac{kcat}{Km}$) of 2.26 µmole min-^{1}. The enzyme was stable in the presence of metal ions Na^{+}, Ca^{2+} and K^{+} while increase in activity was evident with Mg^{2+} and Fe^{2+}. However, the presence of 10 mM of Ag^{2+} and Hg^{2+} ions in the reaction mixture inhibited enzyme activity. The FOS produced demonstrated antioxidant properties from three experimental protocols of DPPH, FRAP and Nitric oxide (NO) assays. The FOS synthesized by the coprophilous fungal Ftase demonstrated a dual purpose of FOS production and antioxidant ability. In conclusion, the present study was successful since a transferase enzyme from indigenous coprophilous fungus was obtained capable of synthesizing oligosaccharides. Even though the enzyme was purified partially a 7.3 % recovery was obtained and the enzyme showed over 95 % residual activity at optimal pH and temperature. Noteworthy, the high thermostability of temperature and pH range displayed at over 60 °C and pH range of 4.0 – 8.0 suggests the enzyme has potential for industrial exploitation at high sucrose concentration.

6.2 Future Research Direction

- It will be ideal to employ a robust sampling strategy of herbivore dung as numerous species of coprophilous fungi could be unraveled that are novel, and have biocatalytic properties. From this study, coprophilous fungi demonstrated potential for the production of enzymatic

arsenal capable of synthesizing FOS. Furthermore, exploring microbial biodiversity in nature in various terrestrial habitats in South Africa can open new vistas in fungal research.

- Few genome sequences of dung-inhabiting fungi are currently available. Among them is a classical model of *Podospora anserine* sp. The release of their entire genome sequences will facilitate comprehension of various environmental interactions including their potential for metabolomics studies. Genomic analysis will aid to investigate coprophilous fungi capable of producing bioactive secondary metabolites besides biocatalytic enzymes.
- Free radical scavenging mechanisms especially for nutritional antioxidants such as FOSs and IOSs need to be substantiated on a kinetic base both *ex vivo* and *in vivo.* This will help to account for their nutraceutical effect.
- Fermentation pathways of enzyme production for industrial applications is through submerged or solid-state culture. There is need to improve methods to study fermentation both *in vitro* and *in vivo* beyond the two methods. Through development of obscure metabolomics pathways of enzymes which may be responsive for designing optimal hybrid cultivation systems for maximal industrial application.
- There is need to further address the challenges of numerous purification steps that are costly and time consuming. One major purification obstacle is loss of enzyme recovery during different steps in purification. Application of new techniques like continuous chromatography could aid improve enzyme recovery at industrial level for more competitive approach.
- Recombinant gene technology should be considered as a predominant promising approach to boost yield of enzyme production at industrial level. This application can be used in cloning of coding systems of potential industrial enzymes and express it in an optimized strain for biotechnological exploitation. Genome shuffling is one such technology that could be used to improve the specific activity of Ftase by amplifying their genetic diversity. This can be done by incorporating mutant strain with improved phenotype followed by protoplast fusion to allow gene recombination.
- There is need to study human gut microbiome beyond *Bifidobacterium* and *Lactobacillus* by evaluating certain areas of nutrition. Nutrigenomics approach using molecular tools could be a starting point towards the future of biofunctional foods.

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Appendices

Appendix A: Chemical Composition of 3, 5-dinitrosalicylic acid (DNS) Ghose, (1987).

Component	Stock solution (0.2 g/L)
3, 5-dinitrosalicylic acid	2.0
Sodium potassium tartrate	36.4
Phenol	0.4
Sodium sulfite (Na_2SO_3)	0.1
Sodium hydroxide (NaOH)	2.0

The DNS stock reagent was prepared according to Ghose, (1987). A mixture of 2 g of 3,5 dinitrosalicylic acid, 36.4 g Rochelle salt (sodium potassium tartrate), 0.4 g Phenol, 0.1 g (Na_2SO_3) and 2.0 g of NaOH were added to 200 ml volumetric flask dissolved in 150 ml deionized water for stirring and topped up to 200 ml. The reagent was refrigerated at 4 °C in an amber bottle until used.

Citrate-phosphate buffer

The conjugate base (A-) and the weak acid (HA) was prepared by weighing separately 28.39 g of Na_2HPO_4 and dissolving in 1 L distilled water in a volumetric flask (0.2M Na_2HPO_4). Citric acid weighed was 19.21 g in 1 L volumetric flask (0.1M citric acid). 0.1 M or 100 mM of citrate-phosphate buffer was prepared by adding 631.5 ml of 0.2 M Na_2HPO_4 to 368.5 ml of 0.1 M citric acid and pH adjusted accordingly to 6.5 by HCL or 2M NaOH.

Coomassie dye preparation

The Coomassie brilliant blueG-250 reagent was prepared by dissolving 100 g of dye into 95 % ethanol, phosphoric acid 100 ml was added at a concentration of 85 % (v/v) and topped up to 1000 ml.

Preparation of DPPH

40 mg of DPPH will be dissolved in 100 ml methanol. The mixture was then diluted with 100 ml of distilled water to obtain a stock solution of methanol/water (50:50 v/v). The working DPPH solution of absorbance values 0.75-0.80 at 525 nm was be prepared by diluting 200 ml of DPPH

stock solution with approximately 800 ml methanol/water (50:50 v/v) (Serpen *et al*., 2012, Manosroi *et al*., 2014).

FRAP Solution Preparation

Dilution of 10 mM aqueous solution of TPTZ with 20 mM ferric chloride in 300 mM sodium acetate buffer (pH 3.6) in the ratio 1:1:10 (v/v/v) (Serpen *et al*., 2012, Ahmed *et al*., 2017).

Media for fructosyltransferase production.

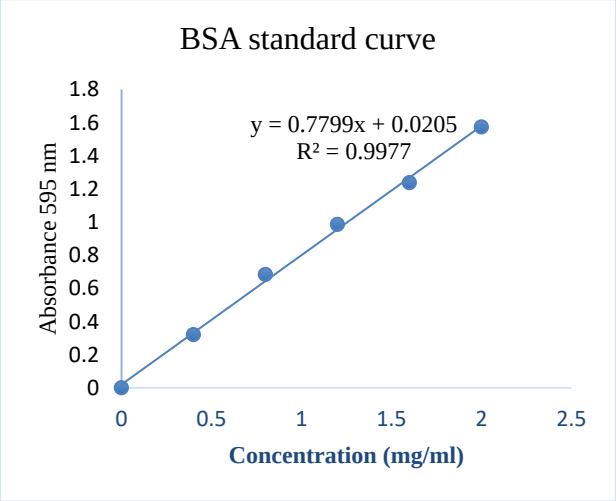
Component	Stock solution (g/L)
Sucrose	30
Sodium nitrate	5.0
Yeast extract	10
KCl	0.5
K2HPO4	1.0
MgSO4.7H2O	0.5
$FeSO_4.7H_2O$	0.01

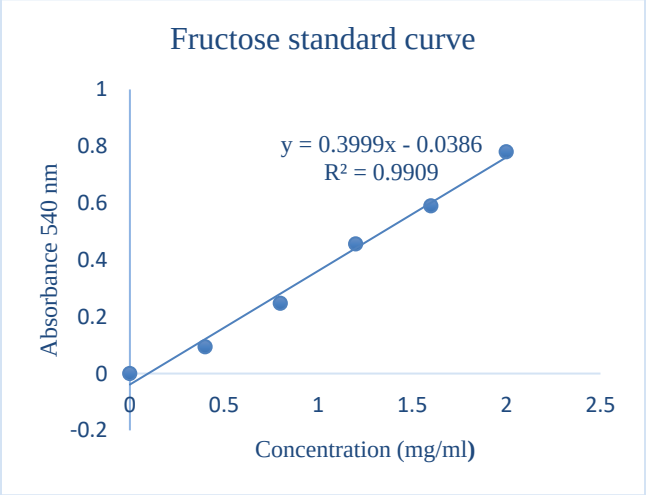
Media for inulinase production.

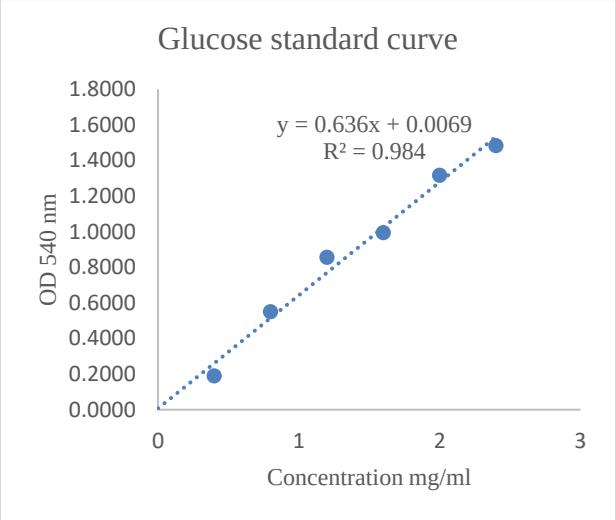
Component	Stock solution (g/L)
Inulin	10
Peptone	5.0
$NH_4H_2PO_4$	8.0
$(NH4)_2HPO_4$	4.0
KCl	0.5
MgSO4.7H2O	0.5
$FeSO_4.7H_2O$	0.01

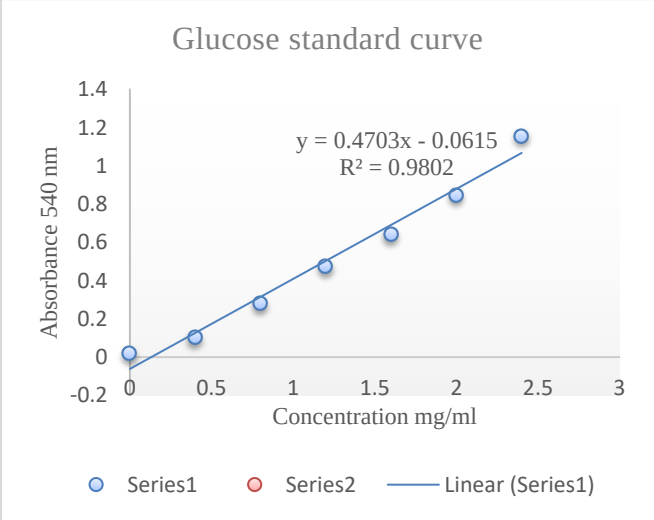
Nakamura *et al*.,(1997)

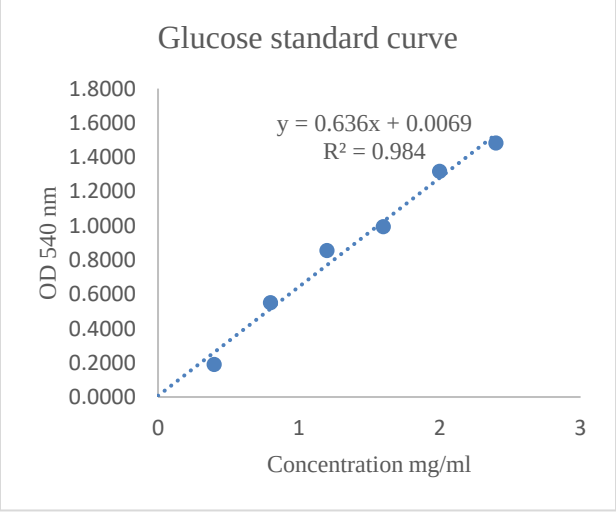
Appendix B: Standard Curves for Ftase, Inulinase and BSA assays.

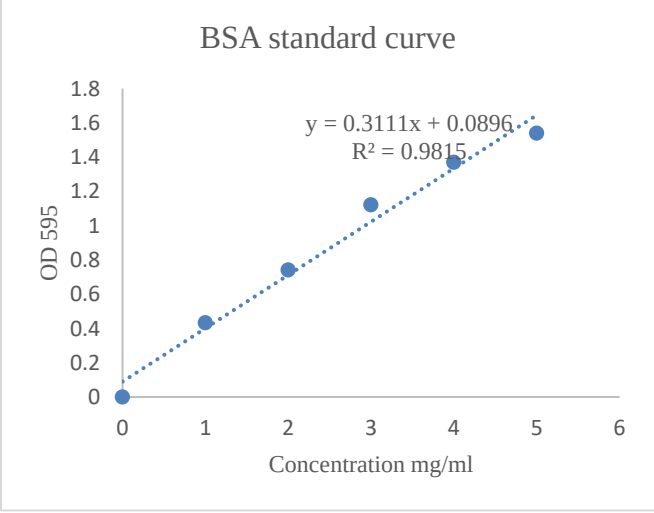


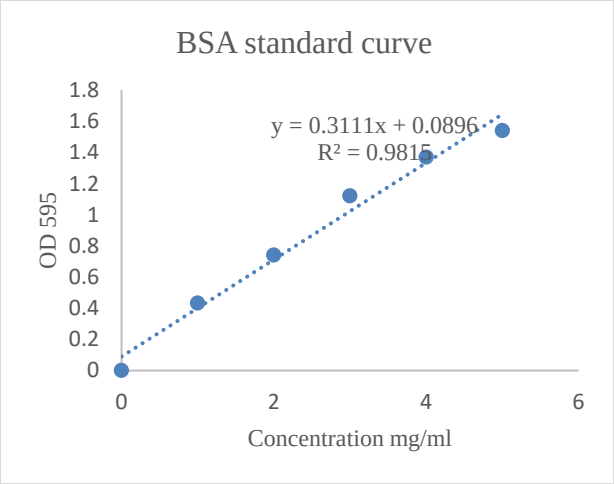











BSA standard curve
y = 0.3111x + 0.0896
R² = 0.9815
OD 595
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
0
2
4
6
Concentration mg/ml

Appendix C: Reagents for SDS and Native-PAGE.

Preparation of 12 % separating gel

Component	Stock solution
30 % Acrylamide/Bis-acrylamide solution	5 ml
Distilled water	2.2 ml
1.5M Tris Buffer (pH 8.8)	2.6 ml
10 % (w/v) SDS	0.1 ml
10 % (w/v) Ammonium persulfate (APS)	100
TEMED	10

Preparation of 5 % stacking gel

Component	Stock solution
30 % Acrylamide/Bis-acrylamide solution	0.67 ml
Distilled water	2.975 ml
0.5M Tris-HCl Buffer (pH 6.8)	1.25 ml
10 % (w/v) SDS	0.05 ml
10 % (w/v) Ammonium persulfate (APS)	0.05 ml
TEMED	0.005 ml

Appendix D: Coprophilous fungi isolates, sampling sites and herbivore species.

Isolate number	Herbivore dung species	Assigned number	Sampling site	Zone of hydrolysis (mm)
1	Rhino dung	XROT – 1	Tala game reserve	23
2	White rhino	XWRP – 2	Phinda game reserve	14
3	Rhino dung	XROT – 3	Tala game reserve	8
4	Rhino dung	XROT – 4	Tala game reserve	5
5	Rhino dung	XROT – 5	Tala game reserve	3
6	Rhino dung	XROT – 6	Tala game reserve	9
7	Rhino dung	XROT – 7	Tala game reserve	9
8	Rhino dung	XROT – 8	Tala game reserve	7
9	Goat dung	XGOU – 9	Ukulinga farm	23
10	Goat dung	XGOU – 10	Ukulinga farm	17
11	Goat dung	XGOU – 11	Ukulinga farm	20
12	Giraffe dung	XGFT – 12	Tala game reserve	23
13	Inyala dung	XIOP – 13	Phinda game reserve	15
14	Giraffe dung	XGFT – 14	Tala game reserve	9
15	Giraffe dung	XGFT – 15	Tala game reserve	13
16	Giraffe dung	XGFT – 16	Tala game reserve	14
17	Water buck	XWBT – 17	Tala game reserve	12
18	Water buck	XWBT – 18	Tala game reserve	16
19	Water buck	XWBT – 19	Tala game reserve	19
20	Bush back	XBBT – 20	Tala game reserve	8
21	Buffalo dung	XOBP – 21	Phinda game reserve	23
22	Impala dung	XIMT – 22	Tala game reserve	18
23	Impala dung	XIMT – 23	Tala game reserve	23
24	Impala dung	XIMT – 24	Tala game reserve	14
25	Impala dung	XIMT – 25	Tala game reserve	12
26	Cow dung	XOCU – 26	Ukulinga farm	11
27	Cow dung	XOCU – 27	Ukulinga farm	21

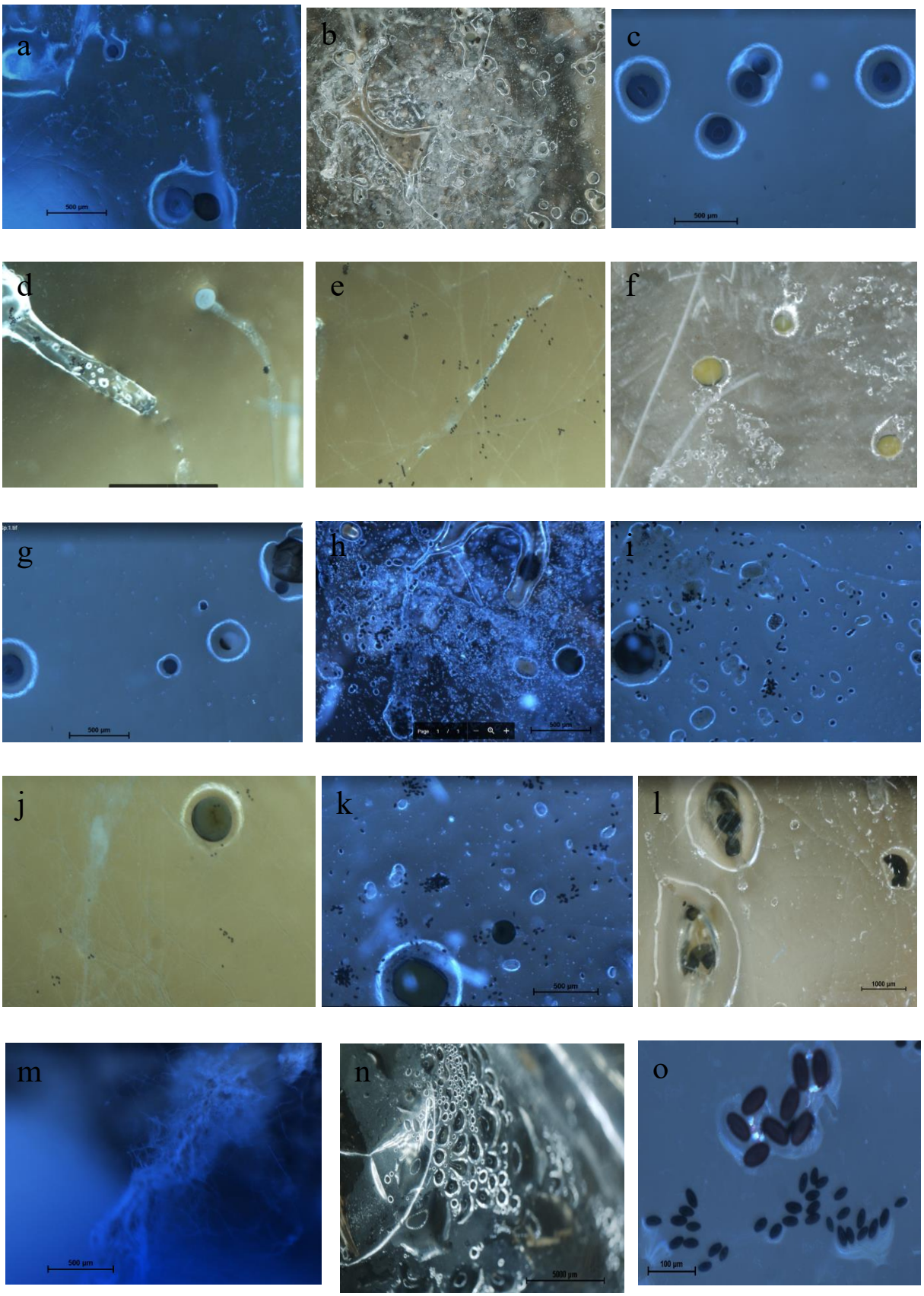
Appendix D: Continuation.

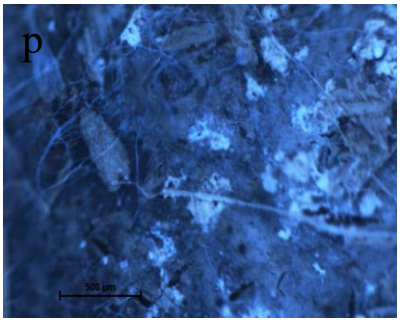
Isolate number	Herbivore dung species	Assigned number	Sampling site	Zone of hydrolysis (mm)
28	Cow dung	XOCU – 28	Ukulinga farm	9
29	Cow dung	XOCU – 29	Ukulinga farm	9
30	Zebra dung	XZBT – 30	Tala game reserve	18
31	Zebra dung	XZBT – 31	Tala game reserve	19
32	Zebra dung	XZBT – 32	Tala game reserve	18
33	Hippopotamus dung	XOHT – 33	Tala game reserve	19
34	Hippopotamus dung	XOHT – 34	Tala game reserve	14
35	Hippopotamus dung	XOHT - 35	Tala game reserve	13
36	Horse dung	XOHU – 36	Ukulinga farm	17
37	Horse dung	XOHU – 37	Ukulinga farm	11
38	Horse dung	XOHU – 38	Ukulinga farm	10
39	Horse dung	XOHU – 39	Ukulinga farm	10
40	Horse dung	XOHU – 40	Ukulinga farm	11
41	Horse dung	XOHU – 41	Ukulinga farm	7
42	Elephant dung	XOEP – 42	Phinda game reserve	24
43	Elephant dung	XOEP – 43	Phinda game reserve	25
44	Elephant dung	XOEP – 44	Phinda game reserve	21
45	Red ducker	XORP – 45	Phinda game reserve	21
46	Bush back	XOBT – 46	Tala game reserve	19
47	Bush back	XOBT – 47	Tala game reserve	
48	Buffalo dung	XOBP – 48	Phinda game reserve	25
49	Goat dung	XOGU – 49	Ukulinga farm	20
50	White rhino	XOWP – 50	Phinda game reserve	
51	Inyala dung	XOIP – 51	Phinda game reserve	11
52	Elephant dung	XOEP – 52	Phinda game reserve	
53	White rhino dung	XOWP – 53	Phinda game reserve	14
54	White rhino dung	XOWP – 54	Phinda game reserve	13

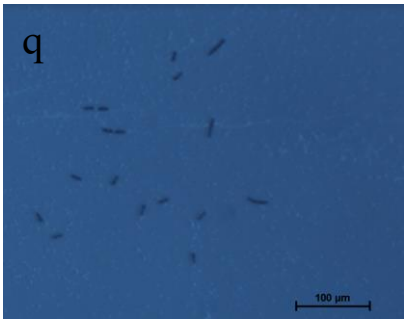
Appendix D: Continuation.

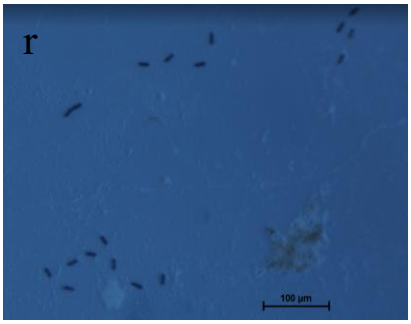
Isolate number	Herbivore dung species	Assigned number	Sampling site	Zone of hydrolysis (mm)
55	Zebra dung	XOXP - 55	Phinda game reserve	3
56	Goat dung	XOGU - 56	Ukulinga research farm	8
57	Common duiker	XOCP - 57	Phinda game reserve	7
58	White rhino	XOWP – 58	Phinda game reserve	22
59	Inyala dung	XOIP – 59	Phinda game reserve	11
60	Inyala dung	XOIP – 60	Phinda game reserve	12
61	Inyala dung	XOIP – 61	Phinda game reserve	11

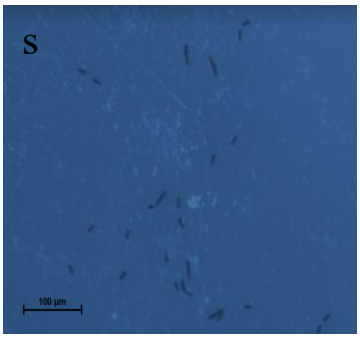
Appendix E: Morphological Identification of Coprophilous Fungal Strains.

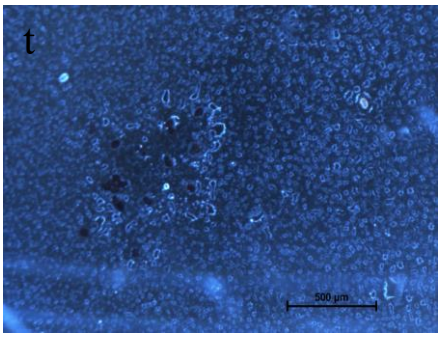


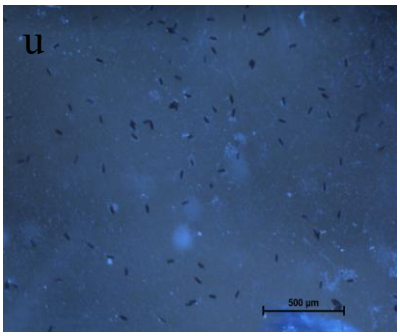






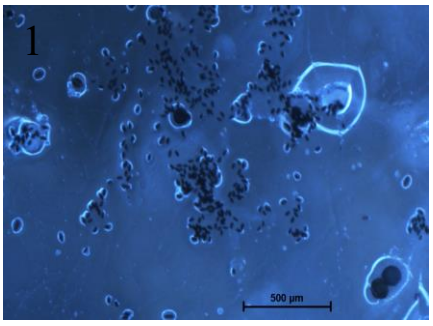


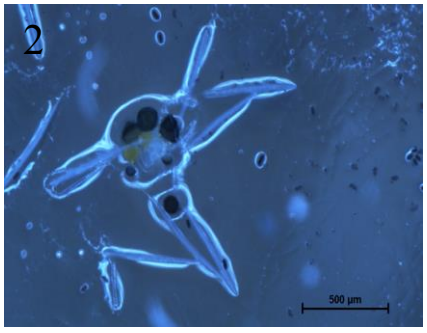


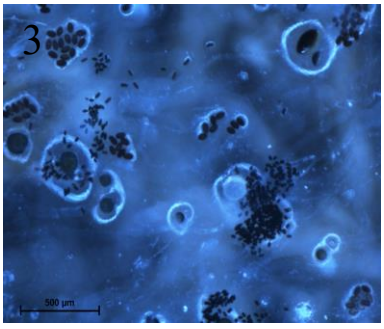


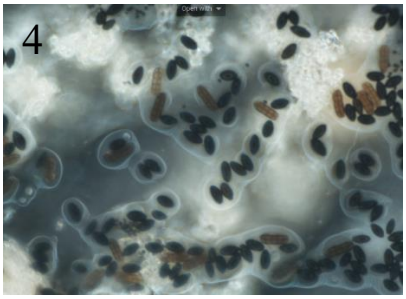
- Morphological characteristics of selected dung sources. Bushbuck, a, b c, g, cow dung d, e, f, giraffe h, zebra dung, o, n, white rhino, hippo, water buck, impala, horse, goat, u, t, s.

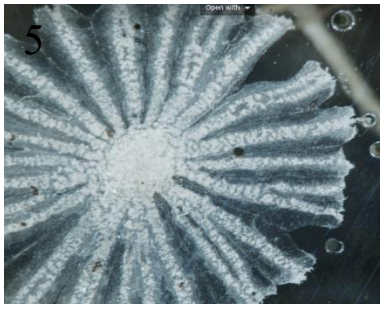
Morphological features of hind gut fermenters 1 – 5.



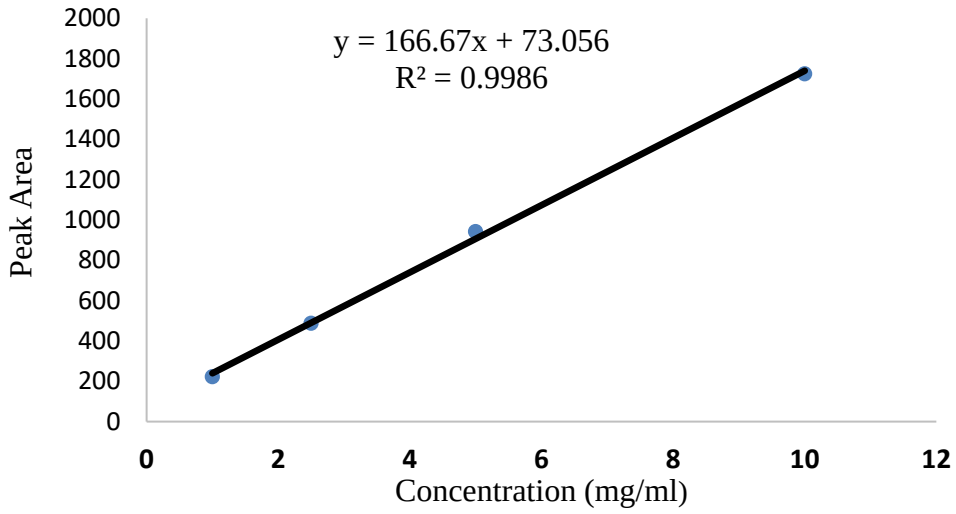




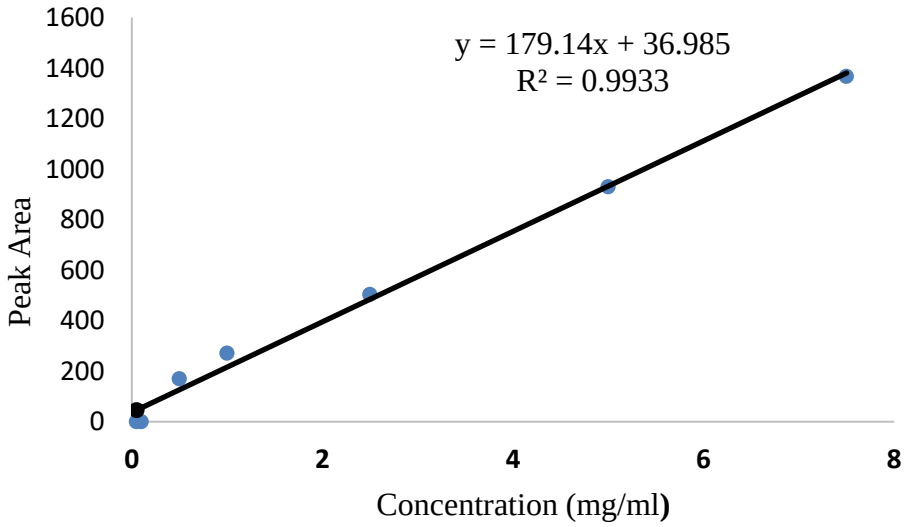




Appendix F: HPLC Standard Curves of Fructose and Glucose**.**



- Fructose calibration curve from peak areas 0-10 mg/ml



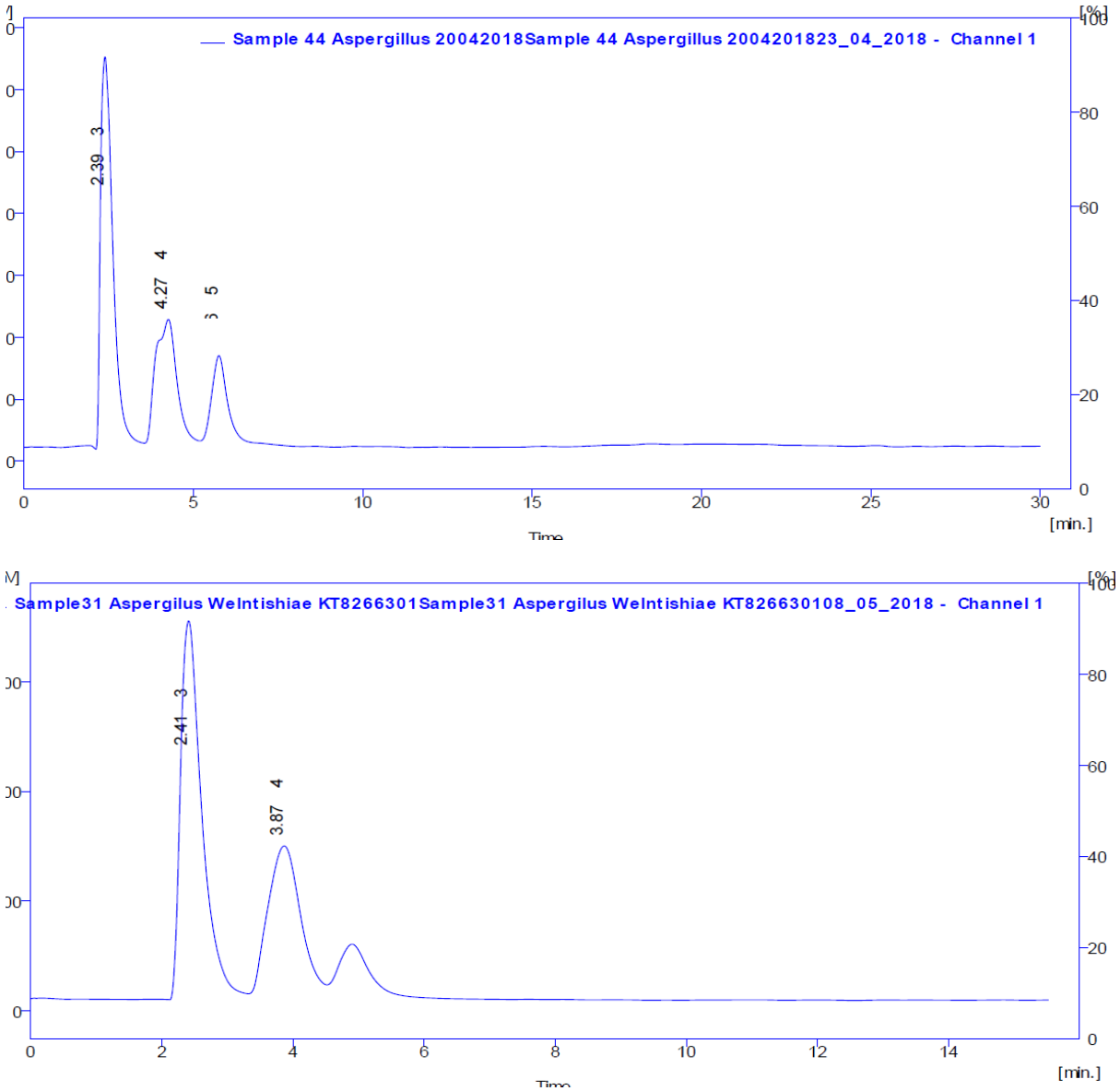
- Glucose calibration curve fom peak areas 0 – 10 mg/ml

Appendix G: Tables of Concentration of HPLC Standard Curves.

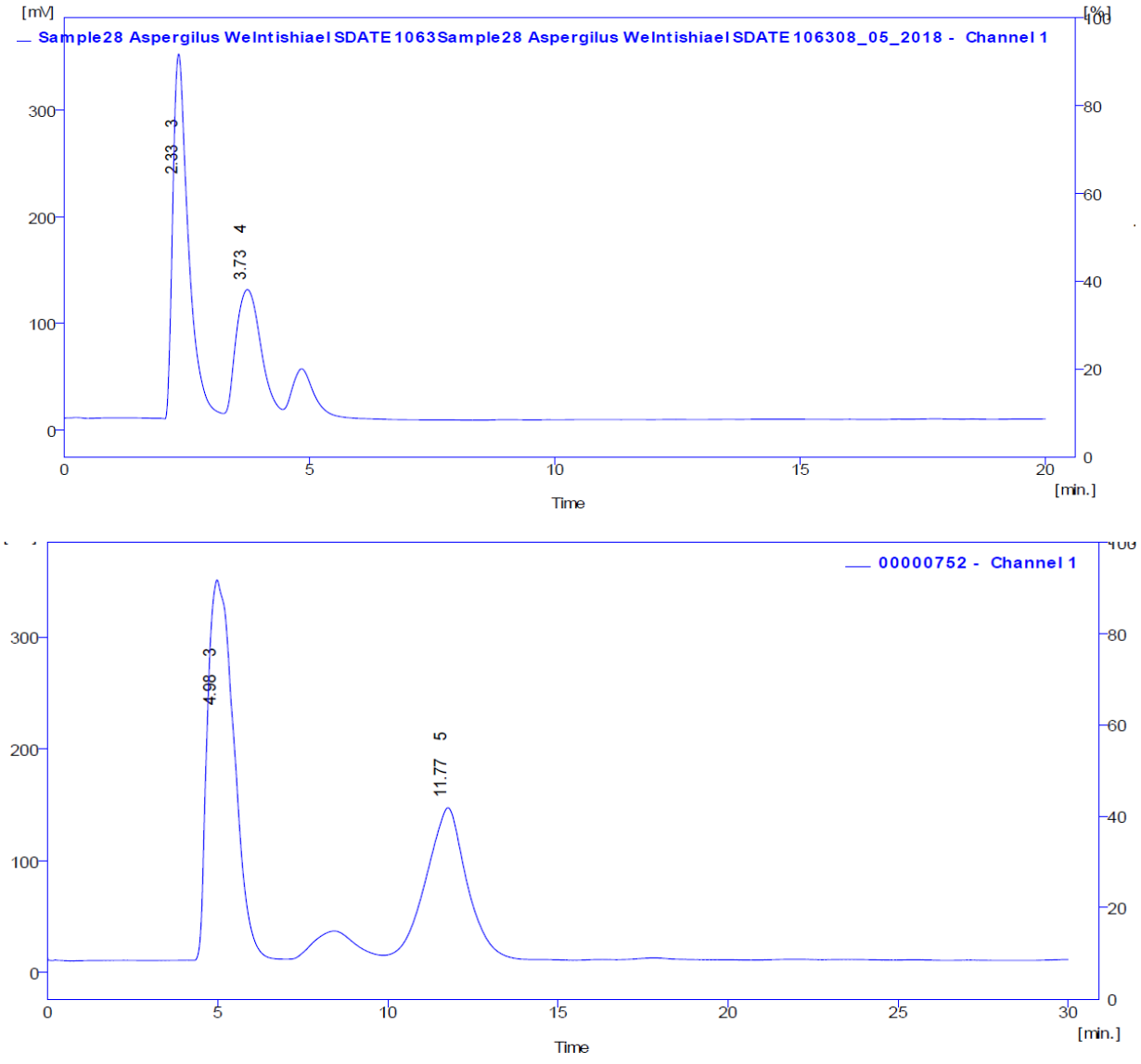
HPLC Fructose standard curve		
Retention Time (min)	Concentration (mg/ml)	Peak Area
3.85	0.05	0
3.85	0.1	0
3.85	1	222.096
3.85	2.5	487.309
3.85	5	941.733
3.85	7.5	1568.558
3.85	10	1724.41

HPLC Glucose standard curve		
Retention Time (min)	Concentration (mg/ml)	Peak Area
3.867	0.05	0
3.867	0.1	0
3.867	0.5	169.938
3.867	1	271.023
3.867	2.5	503.767
3.867	5	929.997
3.867	7.5	1366.82
3.867	9.5	1291.86

Appendix H: Chromatograms of Selected Ftase and Inulinase Producers.



- Chromatogram of sample 44 and 31 obtained from HPLC-RI showing FOS synthesis.

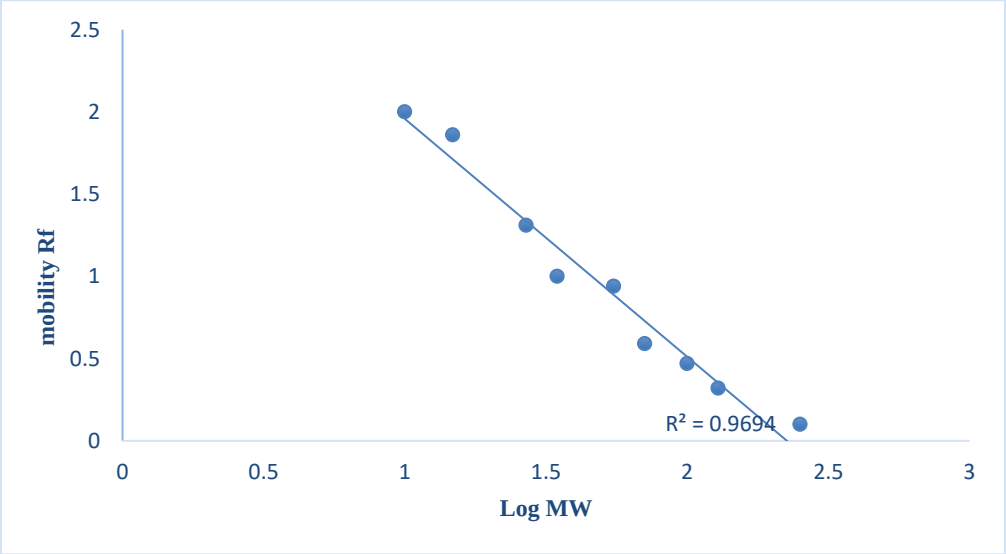


- Chromatogram of sample 28 and 45 showing inulinase potential as fructose and low level glucose was produced from inulin hydrolysis.

Appendix I: Table of log molecular weight vs Rf of standards extrapolated from the size of unknown bands.

Size kDa	log	mm	Rf	mobility (cm)
250	2.4	0.5	0.1	0.07
130	2.11	1	0.32	0.15
100	2	3	0.47	0.44
70	1.85	4	0.59	0.58
55	1.74	8	0.94	0.12
35	1.54	NB	1	NB
27	1.43		1.31	
15	1.17		1.86	
10	1		2	

[a]NB = no band seen at this MW



- Standard curve estimation of molecular weight of Ftase enzyme after anion exchange chromatography.

Appendix J: Poster Presentation.

SCREENING COPROPHILOUS FUNGI FOR FRUCTOSYLTRANSFERASE AND INULINASE BIOCATALYSTS FOR POTENTIAL BIOTECHNOLOGICAL APPLICATIONS

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Introduction

The search for novel biocatalysts from coprophilous fungi is gaining attention due to a plethora of applications in modern biotechnology. Fructosyltransferase (Ftase) and inulinase enzymes are gaining considerable attention due to their pharmaceutical and biotechnological applications for the production of nutraceuticals. It is for this reason that bioprospecting for novel sources of fructosyltransferases and inulinases is undertaken due to the low caloric value of nutraceuticals and accompanying health benefits to consumers. The aim of this research was to collect, purify and screen coprophilous fungi for the production of fructosyltransferase and inulinase enzymes for the synthesis of biofunctional prebiotics.

Materials and Methods

- Sixty-one (61) indigenous coprophilous fungal strains were isolated after repeated purification to monoculture. The coprophilous fungi were obtained from various terrestrial habitats (Ukulinga, Phinda and Tala) in KwaZulu-Natal Province, South Africa. The axenic fungal strains were partially identified using morpho-taxonomic keys and were subsequently evaluated for their ability to produce extracellular Ftase and inulinase enzymes.
- During preliminary screening, the culture filtrate was examined for transfructosylating and hydrolytic activity using 2,3,5-tiphenyl tetrazolium chloride (TTC) as a chromogenic marker and Lugol's iodine solution respectively. Zones of hydrolysis on 30 fungal isolates were observed on the TTC assay plates in diameters ranging from 15 mm to 30 mm, representing high extracellular Ftase activity. Staining and clearing zones formed after addition of iodine solution on inulin rich media indicated absence and presence of inulinolytic activity respectively. Secondary screening involved microtitre assays by DNS method of reducing sugars. Tertiary screening was the final screening step where products of biocatalysis were qualitatively detected by thin layer chromatography (TLC) and quantitatively by HPLC.

Results

Isolation, growth and screening for Ftase and inulinase production

Of the sixty-one (61) isolates, 30 isolates showed considerable zones of hydrolysis on sucrose agar indicating synthesis of FOS and ten (10) isolates secreted high concentrations of Ftase while six (6) different fungal strains showed <50 % inulinase: invertase ratio. Isolates 48, 2 ,44, 32, 21, 28, 31, 10 and 36 showed highest Ftase enzyme activity while isolate 44, 42 and 49 showed high inulinase activity. Isolate 2, 48 and 28 showed syntheses of DP3 while isolate 42 and 44 showed potential exo-inulinase activity on TLC.

Table 1 Fungal isolates of coprophilous fungi isolated from different habitats showing zones of hydrolysis.

Species Number	Sampling site	Type of herbivore	Assigned number	Zone of hydrolysis (mm)
2	Tala game reserve	White rhino	KWR-2	18
9	Ukulinga research farm	Goat dung	KGO-9	20
10	Ukulinga research farm	Goat dung	KGO-10	17
11	Ukulinga research farm	Goat dung	KGO-11	20
12	Tala game reserve	Giraffe dung	KGF-12	25
13	Phinda game reserve	Impala dung	KIP-13	15
14	Tala game reserve	Giraffe dung	KGF-14	9
16	Tala game reserve	Giraffe dung	KGF-16	14
19	Tala game reserve	Water buck	KWT-19	19
21	Phinda game reserve	Buffalo	KBP-21	10
22	Tala game reserve	Impala dung	KIMT-22	18
23	Tala game reserve	Impala dung	KIMT-23	23
26	Ukulinga research farm	Cow dung	KCU-26	9
27	Ukulinga research farm	Cow dung	KCU-27	24
28	Ukulinga research farm	Cow dung	KCU-28	9
30	Tala game reserve	Zebra	KZBT-30	9
42	Tala game reserve	Zebra	KZBT-42	19
33	Tala game reserve	Hippopotamus	KHT-33	19
36	Ukulinga research farm	Horse	KHU-36	17
37	Ukulinga research farm	Horse	KHU-37	11
42	Phinda game reserve	Elephant	KEP-42	24
43	Phinda game reserve	Elephant	KEP-43	26
44	Phinda game reserve	Elephant	KEP-44	21
45	Phinda game reserve	Red ducker	KDP-45	21
46	Tala game reserve	Rudbuck	KRT-46	19
48	Phinda game reserve	Buffalo	KBP-48	21
49	Ukulinga research farm	Goat dung	KGU-49	20
51	Phinda game reserve	Impala dung	KIP	11
58	Phinda game reserve	White rhino	KWP-58	22
60	Phinda game reserve	Impala dung	Isolate X	27

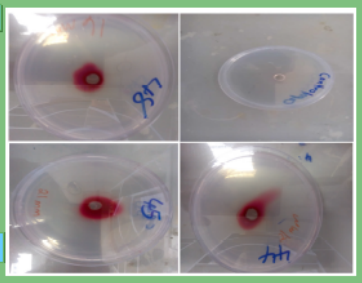

Figure 1 Triphenyltetrazolium chloride assay (TTC) a chromogenic substrate marker depicting zones of hydrolysis on sucrose agar showing presence of transferase activity.

Fig. 5 TLC showing intermediates of fructooligosaccharides synthesis. DP– Degree of polymerization.

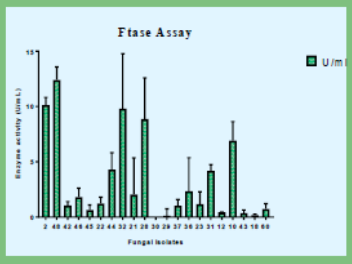

Fig. 2 Enzyme activity of selected strains with high zones of hydrolysis showing isolate 48 as the highest Ftase producer.

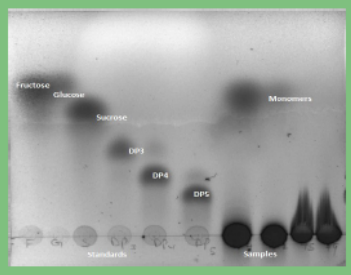

Fig. 6 TLC showing exoinulinase activity as a result of inulin hydrolysis liberating monomers (fructose).

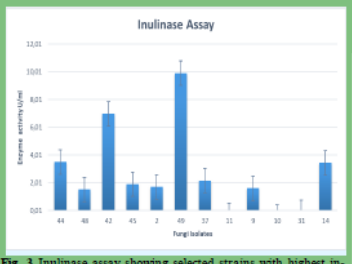

Fig. 3 Inulinase assay showing selected strains with highest inulinase activity.

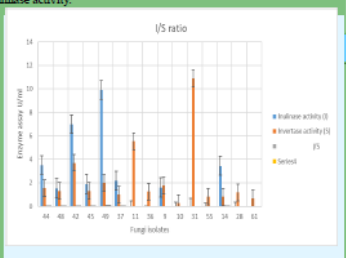

Fig. 4 Relative correlation between inulinase and invertase production of the selected strains. This shows that the selected strains have higher inulinase than invertase activity.

Discussion and Conclusion

- Larger zones of hydrolysis, elongation of sucrose to oligosaccharides and high enzyme activity are critical prerequisites for selecting suitable fungal isolates for applied biotechnological applications.
- In this study, most isolates were found suitable for biotechnological application as they possess inulinase, invertase and Ftase activity making coprophilous fungi a valuable source of fungal enzymes not yet explored.
- However, there are some distinct variation of products formation as shown on TLC making some strains more suitable for biocatalysis and further investigation in our research lab is on going.
- In conclusion, results from this study indicate that coprophilous fungi harbour a new source of transferase and hydrolytic enzymes for downstream biotechnological application.

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Prebiotics Research

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