CO-PRODUCTION OF INULINASE BY *Kluyveromyces marxianus* AND *Saccharomyces cerevisiae* IN SOLID STATE FERMENTATION

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AUTHOR'S DECLARATION

This study presents original work by the author. It has not been submitted in any form to another academic institution. Where use was made of the work of others, it has been duly acknowledged in the text. The research described in this dissertation was carried out in the Department of Biotechnology, Faculty of Applied and Computer Sciences, Vaal University of Technology, South Africa, under the supervision of Prof Thiri Padayachee and Mr Prenaven Reddy

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Student's signature

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All honour and praise be to God Almighty, from whom all knowledge and wisdom is derived

This thesis is dedicated to my little boy, the love of my life, Luthando Bonga Molefe

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ABSTRACT

Solid-state fermentation (SFF) has emerged as a good method for the production of microbial enzymes such as inulinases. The use of low-cost agricultural plants and agro-industrial residues as substrates in SSF processes provides a value adding alternative to these otherwise under/or un-utilised vegetation. Production of inulinases, using various inulin-containing plant materials as carbon sources was studied using pure and mixed cultures of yeast strains. All substrates resulted in different levels of enzyme activity. A mixed culture *of Kluyveromyces marxianus* and *Saccharomyces cerevisiae* produced an extracellular exoinulinase when grown on different types of inulin-containing plant materials. Initial inulinase production was achieved as follows: 10 IU/gds (garlic cloves), 15 IU/gds (parsnips), 10 IU/gds (wheat bran) and 7 IU/gds (amadumbe) by *K. marxianus* and *S. cerevisiae* in a mixed culture. The production of inulinases by a mixed culture of *K. marxianus* and *S. cerevisiae* under SSF was further optimized by investigating initial moisture content, temperature, carbon source, nitrogen source, inoculum volume and inoculum ratio. The highest inulinase activity attained was in garlic cloves (85 IU/gds), followed by parsnips (65 IU/gds), wheat bran (37 IU/gds) and amadumbe (25 U/gds). The activities yielded 5.6 fold higher inulinase than in preliminary studies. The optimum pH and temperature of the crude enzyme were 5.0 and 50 ^{o}C, respectively. The pH and temperature stability of the enzyme was steady for 1 hour retaining about 64% activity. The average inulinase/invertase activity (I/S) ratio of 1.0 by crude inulinases was also observed after 48 hours. The crude extracellular enzyme extracts from the garlic cloves, parsnips, amadumbe and wheat bran were partially purified by ammonium sulphate precipitation and showed a specific activity of 9.03 U/mg, 0.08 U/mg, 4.12 U/mg and 0.133 U/mg respectively. The K_m and V_{max} values of the inulinase were 21.95 mM and 2.09 μM/min; 19.79 mM and 1.38 μM/min; 31.59 mM and 0.51 μM/min; and 25.74 mM and 0.23 μM/min, respectively. All extracts demonstrated potential for large-scale production of inulinase and fructose syrup.

CHAPTER 1: INTRODUCTION AND LITERATURE REVIEW

1.1 INTRODUCTION

Fructose is a six-carbon monosaccharide that is found in plant roots in a variety of forms including the free monosaccharide form; complexed with glucose to form the disaccharide sucrose; or polymerized to form fructans (Muir *et al*., 2007). Fructose is a ketose sugar used as an alternative sweetener in the food and beverage industries. It has a higher sweetening capacity than sucrose, improves calcium absorption, increases iron absorption in children and favours the removal of ethanol from the blood of alcoholics. As a result, these beneficial health effects have gained fructose an increased commercial demand (Naidoo *et al.*, 2009). The conventional production of fructose is currently based on the hydrolysis of starch by the α-amylases and the amyloglucosidases and subsequently the glucose isomerases, which catalyse the conversion of glucose to fructose. This process, however, yields only about 45% of fructose, 50% glucose and 8% oligosaccharides (Singh & Gill 2006). The use of microbial inulinases, which yield up to 95% of pure fructose in a single-step enzymatic hydrolysis of inulin, is a feasible alternative process for the production of high-fructose syrup (Nascimento *et al*., 2012, Gill *et al*., 2006).

Inulin is a linear polyfructan found in roots of tuber plants of the Liliaciae and Compositae families. It is found in high concentrations in a variety of sources such as the Jerusalem artichoke and chicory; and in low content in onion, garlic, parsnips, amadumbe (*Collocasia seculenta*), banana, asparagus and leek. Inulin is comprised of β (2→1) linked fructose residues attached to a terminal sucrose molecule. This polymer has great commercial interest as it represents a relatively inexpensive and abundant substrate for the production of high fructose syrups (Skowronek and Fiedurek 2006).

Microbial fucto-hydrolases can be divided into two categories; the endo- and exo-inulinases depending on their mode of action. Exo-inulinases (β-D-fructan Fructohydrolase EC 3.2.1.8) catalyse the hydrolysis of the terminal non-reducing 2,1-linked and 2, 6 linked β-D- fructofuranose residues of inulin, levan, raffinose and sucrose to produce fructose and glucose. Endo-inulinases (2,1-β-D-fructan fructanohydrolase; EC 3.2.1.7) are specific for inulin and catalyse the hydrolysis of the internal β-(2,1) - fructofuranosic linkages to yield inulotriose, inulotetraose, and inulopentaose (Chen *et al.*, 2009). Certain yeast and filamentous fungal species are capable of

producing exo-inulinases of high activity and great stability (Pessoni 2007). SSF offers a potential for the production of industrial enzymes such as inulinases. Although most of the commercial production and reported work on microbial inulinases involves submerged fermentation (SmF), production of inulinases in SSF results in higher, more stable enzyme yields. One great advantage of SSF is the possibility of using mixed cultures and to exploit metabolic synergisms among various fungal species (Holker *et al*., 2004).

Solid state fermentation (SSF) has been defined as a fermentation process which involves a solid matrix and is carried out in the absence of free water. However, the substrate possesses enough water to support the growth and metabolism of the microorganism (Singhania *et al*., 2009). The solid matrix could either be the source of nutrients or a support impregnated by proper nutrients that allows the development of the microorganisms (Singhania *et al*., 2009). The low moisture content also means that the fermentation can only be carried out by a limited number of microorganisms, mainly yeast and filamentous fungi, although some bacterial strains have also been identified (Pandey *et al*., 2000). Many studies in the application of SSF are focused on adding value to agro-industrial residues, which have been extensively used as physical support or source of nutrients in SSF (da Silva *et al*., 2012).

Kluyveromyces marxianus known as an inulinase hyper-producing yeast. It is also the most extensively used yeast for inulinase production. (Table 1) shows a brief indication of the production, optimization, extraction and characterisation studies of inulinases using *K. marxianus* in SSF and SmF.

Table 1:A brief indication of the production, optimization, extraction and characterisation studies of inulinases using *K. marxianus* in SSF and SmF.

Study	Inulinase activity	Reference
Production and Optimisation using submerged fermentation	17.9 U/ml 127 U/ml 176 IU/ml 208 IU/ml	Jain *et al*., 2012 Kalil *et al*., 2001 Silva-Santisteban *et al*., 2005 Silva-Santisteban *et al.*, 2009
Production and Optimisation using solid state fermentation	391.9 U/g 250 U/gds 463U/gds 189 U/gds 396.6 U/gds	Mazutti *et al*., 2006 Mazutti *et al*., 2007 Mazutti *et al*., 2010 Bender *et al*., 2006 Bender *et al*., 2008
Extraction	Only enzyme kinetics available	Treichel *et al*., 2009
Purification and characterisation	Only enzyme kinetics available Only enzyme kinetics available	Kalil *et al*., 2010 Singh[b] *et al*., 2007

Invertases (Enzyme commission number (E.C) 2.1. 26) that catalyse the hydrolysis of starch into glucose, are produced by the yeast species *S. cerevisiae,* which is also well known for its use in industrial applications for the production of sweets, and beverages such as wine and cider (Feldmann 2010). In addition to its application in the food and beverage industry, *S. cerevisiae* has been used in industrial applications for the production of various proteins and small molecule drugs. *Saccharomysces cerevisiae* serves as a model organism in research laboratories to study basic biological processes. This simple eukaryote has several advantages that make it a useful research vehicle; it grows easily and methods for cultivation under well-controlled conditions are readily available (Kolkman *et al*., 2005).

Chapter one of this study discusses the background, current knowledge and industrial application of inulinases; focusing on the origin of inulin, the benefits of inulin and microbial inulinases used for the hydrolysis of inulin. It also emphasises the two yeast strains *Kluyveromyces marxianus* and *Saccharomyces cerevisiae* used in this study for the production of the inulinase enzyme. Chapter two focuses on the evaluation of different inulin containing plant materials (garlic cloves, parsnips, wheat bran, amadumbe, onion bulbs, onion peel, and garlic peel) using a mixed culture of the *K. marxianus* and *S. cerevisiae* for maximum inulinase production. Chapter three describes the optimization of fermentation process parameters and carbon source for improved inulinase production. Chapter four investigates characterisation of crude exo-inulinases and its kinetic studies and Chapter five studies the partial purification of the crude enzyme.

1.2 LITERATURE REVIEW

1.2.1 Carbohydrates

Carbohydrates are the most abundant class of biological molecules. They occur naturally in plants, animals and microorganisms where they fulfil different structural and metabolic roles. The basic units of carbohydrates are monosaccharides e.g. D-glucose, D-ribulose and D-fructose; many of which are synthesized from simpler substances during photosynthesis in plants and photosynthesizing microorganisms. The metabolic breakdown of monosaccharides provides energy, which is used as fuel for many biological processes (Voet & Voet 2004). These simple sugars are aldehydes of straight-chain polyhydroxy alcohol or ketones containing about three carbon atoms (Prasad 2010). In nature these carbohydrates may occur as one of their chemical derivatives, usually as components of disaccharides or polysaccharides. Oligosaccharides consist of a number of covalently bonded monosaccharide units and are often associated with lipids and proteins in which they have structural and regulatory properties.

Polysaccharides, also known as glycans, are made up of covalently-linked monosaccharide units and their molecular weight may reach up to millions of daltons. Homopolysaccharides consist of one type of monosaccharide residue while heteropolysaccharides may consist of more than one. Polysaccharides are found in all types of organisms but have more prominent structural functions in plants since 80% of the plants' dry weight is made up of cellulose (Voet & Voet 2004). Non-structural carbohydrates are known as energy-rich compounds which are used for metabolism and energy storage. Inulin is one such carbohydrate.

1.2.1.1 Inulin

Inulin is a poly-dispersed carbohydrate. It is chemically composed of a mixture of oligomers and/ or polymers made up of fructose unit chains linked by a β (2,1)-D-Fructosyl fructose bonds and terminated by a single glucose unit linked by an alpha (α) D-glucopyranosoyl bond (Figure 1).

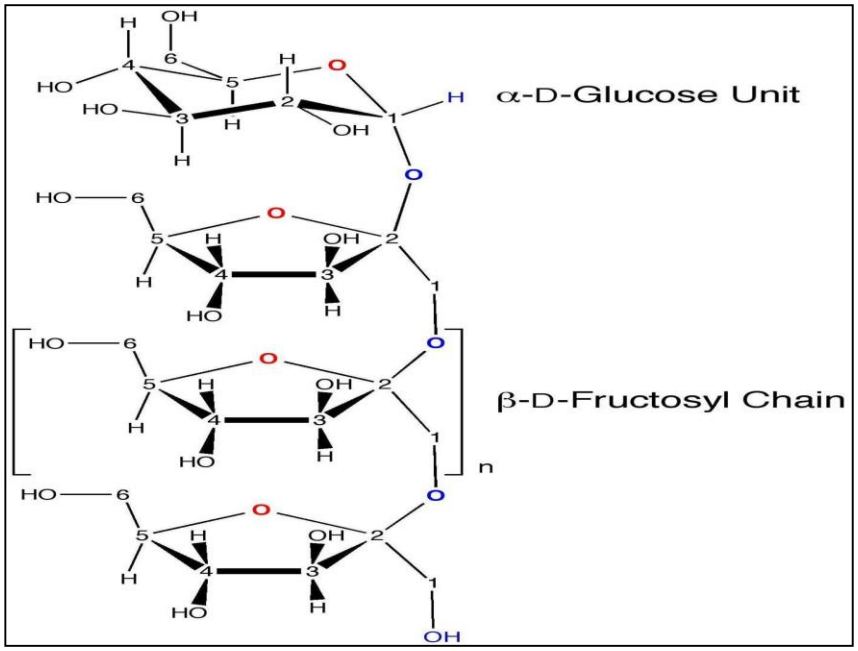

Figure 1: Chemical structure of the Inulin polymer (sourced from Barclay *et al*., 2010)

The degree of polymerization of inulin and the presence of branches play an important roles since they influence the functionality of most inulin. The degree of polymerization of plant inulin is lower (maximally 200) than that of bacterial origin and varies according to plant species, weather conditions and physiological age of the plant (Franck and De Leenheer 2005).

1.2.1.2 Natural occurrence of fructans

After starch, fructans are the most abundant non-structural polysaccharide found in nature. They are found in a variety of plants, bacteria and algae. Fructans are produced by 1200 species of grass and about 15% of flowering plants. They are widespread among the Liliaceae (3500 species) and the Compositae (25, 000 species) with β (2,1) linkage distinctively defining inulin for Compositae species. Inulin-containing plants that are commonly used for human nutrition are from the Liliaceae (onion, garlic and asparagus) and the Compositae (Jerusalem artichoke, dahlia and chicory) (Franck and De Leenheer 2005).

Table 2: Inulin content (% of fresh weight) of plants that are commonly used in human nutrition (Sourced from Franck and De Leenheer 2005)

Sources	**Edible parts**	**Dry solids content (%)**	**Inulin content (%)**
Onion	Bulb	6-12	2-6
Jerusalem artichoke	Tuber	19-25	14-19
Chicory	Root	20-25	15-20
Leek	Bulb	15-20	3-10
Garlic	Bulb	40-45	9-16
Artichoke	Leaves-heart	14-16	3-10
Banana	Fruit	24-26	03-0.7
Rye	Cereal	88-90	0.5-1*
Barley	Cereal	NA	0.5-1.5*
Dandelion	Leaves	50-55	12-15
Burdock	Root	21-25	3.5-4.0
Camas	Bulb	31-50	12-22
Murnong	Root	25-50	8-13
Yacon	Root	13-31	3-19
Salsify	Root	20-22	4-11

NA, data not available. *Estimated value.

Certain genera of bacteria can also produce fructan. Bacterial fructans are predominantly of the levan type produced by Pseudomonaceae, Enterobacteriaceae, Streptococcaceae, Actinomycetes, and Bacillaceae (Franck and De Leenheer 2005).

1.2.1.3 Benefits of inulin and Fructo-oligosaccharides

Proposed health benefits of inulin include suppressing the growth of pathogenic microorganisms in the colon; increased stool bulking capacity; prevention of constipation, increased calcium absorption; maintenance of integrity of the mucosal barrier and increased colonic mucus production; stimulation of the gastrointestinal immune system and reducing the risk of colorectal cancer (Muir *et al*., 2007).

1.2.1.4 Prebiotics

Prebiotics are a group of nutritional compounds that have the ability to promote the growth of specific beneficial bacteria in the colon. Many dietary fibres, in the form of soluble fibre compounds display some prebiotic activity; however, non-fibre compounds that exhibit prebiotic activity can also be included as prebiotics (Kelly 2008).

According to the definition by Roberfoid (2007), prebiotics are "selectively fermented ingredients that allow specific changes, both in the composition and/or activity in the gastrointestinal microflora (e.g. *Bifidobacterium* and *Lactobacillus*) that confers benefits upon the host's well-being and health". While many nutritional compounds exhibit some kind of prebiotic activity, prebiotics are currently classified into two groups; the inulin type prebiotics and the galacto-oligosaccharides (GOS) (Kelly 2008).

Prebiotics are often used in combination with probiotics that are added to the host's diet to promote health. When combined, prebiotics and probiotics have synergistic effects whereby prebiotics promote the growth of existing strains of beneficial gut bacteria and also act to improve the survival, implantation and growth of newly added probiotics (Niness 1999).

The gastrointestinal microflora ferment inulin to produce short-chain fatty acids and lactic acids, which promote other important benefits to human and animal health. The benefits include include an increase in calcium and magnesium absorption (Ohta *et al*., 1998), production of vitamin B, the reduction of serum cholesterol and the prevention of colon cancer (Kuar and Gupta 2002).

1.2.1.5 Alternative sweetener

The demand for high fructose syrup from naturally abundant fructans such as inulin as an alternative sweetener is increasing in the global food and pharmaceutical industries. The increased demand is owed to its functional and technological advantages over sucrose, which include: increased sweetness, increased water and ethanol solubility, increased hygroscopic capacity, less tendency to crystallise, lower cost based on their sweetness, lower viscosity of solutions, ease of storage, higher osmotic pressure and lower cryogenic energy storage. Its bio-availability is independent of the hormone insulin, presents the Maillard reactions at lower temperatures, and an increased sweetness with decreasing temperature (Soto *et al*., 2011).

1.2.1.6 Bio-refineries

Environmental and economic infrastructure and the depletion of fossil fuels (non-renewable energy) have led to increased research for renewable sources of energy and biochemical products (Li *et al.*, 2012). Biochemical and thermo-chemical conversion technologies can convert biomass into carbon-containing biofuels such as biodiesel and other liquids (Bonciu and Barhim 2011).

Among the biorefinery products, bioethanol has been studied extensively and thoroughly. Ethanol is the most employed biofuel, either as fuel or gasoline enhancer. It is also an excellent raw material for the production of synthetic materials (Sanchez and Cardona 2008).

Inulin rich plants have gained the attention of researchers for bioethanol production. Jerusalem artichoke (*Helianthus tuberosus*) is rich in inulin and provides a cost effective option for the production of bio-energy and biochemical products (Szambelan *et al*., 2004).

Microbial exo-inulinases are able to remove the terminal fructose residues from the non-reducing end of inulin, producing fructose and glucose which can be converted to ethanol through fermentation processes (Bonciu *et al*., 2010). Microorganisms capable of highly efficient fermentation of Jerusalem artichoke feedstocks are mostly yeast and bacteria. *Kluyveromyces marxianus* and some strains of *Saccharomyces cerevisiae* can hydrolyse and ferment inulin simultaneously to produce both inulinases and ethanol (Chi *et al*., 2011).

1.2.1.7 Food industry

Commercial inulin is derived from purified roots of chicory (*Cichorium intybus*). The *C. intybus* used for commercial inulin is of the same species that has been used for many years to produce the coffee substitute. Currently, no genetically modified organism derived from chicory roots are used (Coussement 1999).

Commercially produced inulin is of very high purity. Among the various types used, they differ with regard to their powder characteristics and carbohydrate composition. “Pure” inulin, as it is extracted from chicory roots, contains up to 10% sugars. Low sugar and high performance inulins are obtained by chromatographic methods (high performance anion exchange) or physical methods (liquid extract drying through spray drying). Commercial inulin and fructo-oligisaccharide

products are free of gluten, fat, protein and phytic acids and may contain negligible amounts of minerals and salts. These products meet today's high microbiological standards for food ingredients as they also contain no pesticides, toxins and allergens. Inulin and oligofructose are macronutrients, used as either supplements to foods or as substitutes for some nutrients. As supplements, they are added mainly for their nutritional properties and as macronutrient substitutes; they are used to replace fat and/or sugars in foods (Coussement 1999).

Inulin and oligofructose have a large number of health-promoting functions and, therefore, have a wide range of applications in foods like the confectionery, fruit, milk, desserts, yoghurts, fresh cheese, baked goods, chocolate, and ice cream and also in the preparation of fructose syrup (Kuar and Gupta 2002).

1.2.1.8 Physiological functions of inulin

The function of fructans in plants is mainly that of a long-term or short-term storage carbohydrate. Other suggested roles include cold and drought protection and also osmotic regulation (Hendry 1993; Vergauwen *et al*., 2000).

A study done by Hincha *et al*., (2000) showed that inulin-type fructan from chicory roots and dahlia tubers was able to reduce the amount of carboxyfluorescein (an indicator dye used as tracer agent) leakage from freeze-dried liposomes consisting of phosphatidylcholine. Reduced leakage of carboxyfluorescein from liposomes indicates that small carbohydrates are able to protect the membrane barrier. The fructan also reduced the amount of fusion occurring during dehydration. Contrary to this, Hincha *et al*., (2000) also showed that chicory inulin was not able to protect membranes during air-drying. The shorter-chain inulins were able to reduce carboxyfluorescein leakage from air-dried vesicles. In addition, in the dehydrated state, inulin causes an infrared frequency shift of a phosphate band, which was interpreted as an interaction with the membrane phospholipids These findings suggest that inulin-type fructan is able to protect the membrane barrier (Vereyken *et al*., 2003).

1.2.2 Microbial Enzymes

Microorganisms produce different kinds of enzymes which are used as biological catalysts for biochemical reactions in microorganisms, leading to the formation of fermentation products and

growth. In other instances, some of these enzymes become fermentation products whereby one is able to manipulate parameters to obtain high yields of the enzyme (Casida 2005).

Enzymes can either be adaptive or constitutive. Constitutive enzymes are produced in constant amounts utilizable by the cell, regardless of the presence or absence of a specific substrate within a growth medium. Adaptive enzymes are inducible enzymes, produced in usable amounts only in response to the presence of a particular enzyme substrate within a growth medium. An adaptive enzyme is produced only when it is required, in order to bring degradation or change in a particular substrate. The capacity to produce adaptive enzymes varies amongst different microorganisms (Casida 2005).

Microbial enzymes can either be endocellular (endo-enzyme) or exocellular (exo-enzyme). Endocellular enzymes are those that are produced within a cell or at the cytoplasmic membrane and retained within the cell producing it. Exocellular enzymes are released into the surrounding fermentation medium to hydrolyse and degrade polymeric substances that are too large to be transported into the cell. Most of the enzymes produced by microbial fermentations for commercial purposes are exocellular enzymes (Casida 2005).

1.2.2.1 History of Enzymes

Practical application of enzymes either in the form of vegetable-rich enzymes or in the form of microorganisms used for food preservation, baking and production of alcohol, dates back to early civilization, before the nature or the function of enzymes was fully understood by man. Cheese making can be dated as far back as 400BC (Buchholz *et al*., 2005).

The history of modern enzyme technology began in 1874 when the Danish chemist Hansen produced rennet by extracting dried calves' stomachs with saline solution. This turned out to be the first enzyme preparation produced in high quality for industrial purposes. Enzymatic processes of fermentation were the focus area of different studies in the nineteenth century which led to the development of new knowledge in this field (Shanmugan and Sathishkumar 2009). In 1833 Payen and Persoz isolated an enzyme complex 'diastase' from malt. The extract was able to convert gelatinized starch into disaccharide sugars e.g. maltose (Shanmugan and Sathishkumar 2009). In the following decade Schwann, a German physiologist isolated the first enzyme from an animal

source, pepsin, from an acid extraction of animal stomach wall (Shanmugan and Sathishkumar 2009).

Liebig, Pasteur and Kuhne contributed greatly to the history and foundation of fermentation technology. According to Liebig's theory, fermentation resulted from chemical processes and yeast was a non-viable substance continuously in the process of breaking down. Pasteur's theory on the other hand argued that fermentation did not occur unless viable substances were present. In 1897 after the death of both rivals the Buchner brothers demonstrated that cell-free yeast extracts could convert glucose into ethanol and carbon dioxide. This meant that the conversion was not as a direct result of yeast cells, but that of nonviable enzymes. In 1877 Kuhne proposed the name 'enzyme' to describe fermentation products isolated from viable organisms in which they were formed. The word itself means 'in yeast' and it is derived from the Greek 'en' meaning 'in' and 'zyme' meaning 'yeast' (Shanmugan and Sathishkumar 2009).

The developments of science during the 20th century led to novel design techniques for purification of enzymes. James Sumner and Kaj Linderstrom-Lang paved the way for the development of procedures for industrial production of enzymes. During the sixties two major breakthroughs in the enzyme industry were witnessed:

(1) Commercialization of starch-hydrolysing gluco-amylases, and

(2) The launch of the first enzyme-containing detergent.

The second breakthrough in the enzyme industry two decades later was brought about by the development in genetic engineering which saw the production and commercialization of new enzymes (Polaina and McCabe 2007).

1.2.2.2 Advantages of microbial enzymes

Microbial enzymes are increasingly dominating the commercial enzyme industry. Enzymes have numerous advantages over chemical catalysts including ability to function under relatively mild temperatures, pH range, and pressure. These advantages result in the consumption of less energy and lower operational costs since no corrosion-resistant materials are used. Enzymes are specific, stereoselective catalysts which do not produce unwanted by-products. Consequently there is less need for extensive purification of the final product. Enzymes are biodegradable and present a smaller waste-disposal problem, meaning enzyme-based processes are more environmentally

friendly compared to chemical processes. Certain enzymes are not restricted to an aqueous environment and can operate in two-phase water-organic solvent systems and in non-aqueous organic media. Operating under such conditions can also improve enzyme performance especially where substrates have limited water solubility (Waites *et al.*, 2001).

1.2.2.3 The future of microbial enzymes

Advances in the X-ray crystallography and other analytical methods in protein chemistry as well as the increasing information from genomic programs and molecular techniques are bringing powerful means to the study and modification of enzyme structure and function. Focused research in improving existing enzyme-catalysed procedures, the need to develop new technologies and the increasing demand for use and re-use of raw materials will stimulate the rational modification of enzymes to match specific requirements and also the design of enzymes with totally new properties (Polaina and McCabe 2007).

1.2.3 Microbial Inulinases

Enzymes that hydrolyse inulin (microbial inulinases) have been proposed as the most promising approach to obtain fructose syrup from inulin-rich plant materials. Inulinase activity has been reported from filamentous fungi, yeast and bacteria with the yeast *Kluyveromyces marxianus* spp. and filamentous fungi *Aspergillus* spp. Proving to have highest inulinase activity (Table 3). Other inulinase producing microorganisms that have been reported are the *Penicillium, Alternaria alternata, Rhizopus, Bacillus, Clostridium* and *Xanthomonas* species. Inulinases hydrolyses inulin by degrading the β-(2,1) linkages of inulin.

Table 3: Inulinase producing microorganisms (Sourced from Bonciu and Barhim 2011)

Microorganism	**Maximal activity**
Moulds	

Aspergillus niger	1.75g/L
	100 U[a]/ml
	52.5 IU[a]/mL
	176 U/mL
Aspergillus fumigatus	Not available
Aspergillus awamori	Not available
Aspergillus ochraceus	108 Total U
Aspergillus ficuum	193.6 U/gds[b]
Aspergillus parasiticus	2.9 U/mL
Geotrichum candidum	45.65 IU/mL
Rhizoctonia solani	1.792 U/mL
Chrysosporium pannorum	115 U/mL
Yeast	
Pichia guilliermondii	39.56 U/mL
	61.5 U/mL
	130.38 U/mL
	60.1 U/mL
Cryptococcus aureus	52.37 U/mL
	85 U/mL
	436.2 U/gds
Yarrowia lipolitica	62.85 U/mL
	22.5 U/mg
Debaryomyces hansenii	52.53 U/mL
Candida kefyr	40 U/mL
Kluyveromyces marxianus	194.1 U/mL
	127 U/mL
	176 IU/mL
	208 IU/mL
	262.9 U/mg
	1294 U/mL
	18743 U/mL
	50.2 IU/mL
	47.1 IU/mL
	250 U/gds
	47.2 U/mL
	1317 U/mL
	1139 U/mL
Bacteria	
Paenibacillus spp.	2.48 g/L
Streptomyces spp.	524 IU/L
	89 U/gds
Bacillus spp.	42.36 U/mL
Pseudomonas spp.	Not available
Arthrobacter spp.	Not available

U[a], IU[a] – inulinase activity expressed as international activity g
ds[b] – gram of dry substrate

There are primarily two types of microbial inulinases; the exo-inulinases (EC 3.2.1.7) which split off the terminal β-(2,1) fructofuranosidic bonds and the endo-inulinases (EC 3.2.1.8) which hydrolyse the internal linkages in the inulin structure to release inulo-oligosaccharides (e.g. inulotriose, inulotetraose and inulopentaose) as the main products (Figure 2) (Bonciu *et al.*, 2011). Inulinases and invertases (E.C 3.2.1.26) are both β-fructosidases which are able to hydrolyse sucrose; in addition, inulinases are also able to hydrolyse inulin (Pandey *et al.*, 1999).

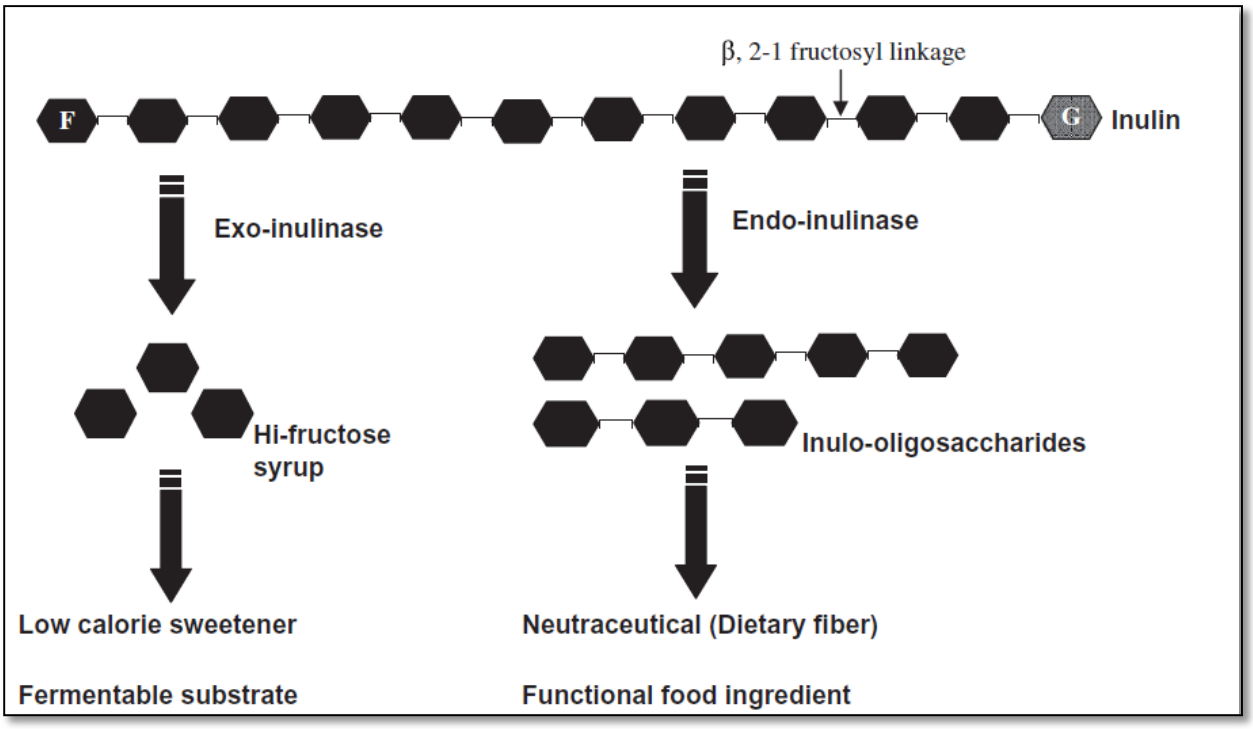

Figure 2: The Mode of action of inulinases on inulin to produce high fructose syrup and inulo – oligosaccharides (sourced from Kango and Jain 2011)

1.2.3.1 Inulinase hydrolytic activity towards sucrose

When compared to plant inulinases, microbial inulinases possess the ability to hydrolyse both sucrose and inulin. Exo-inulinases (2,1-D-fructan fructanohydrolase, E.C. 3.2.1.7) and invertases (β-Dfructofuranoside-fructohydrolase, E.C. 3.2.1.26) are both β-fructosidases which hydrolyse the β-D-2, 6- fructan linkages. However, their difference with regard to hydrolytic activity is not clear and well understood. Usually, inulinase and invertase are distinguished according to the ratio inulinase activity versus invertase (I/S) activity. If the I/S ratio is $\geq 10^2$ then the enzyme is termed

inulinases and if the ratio is $\leq 10^4$ the enzyme is an invertase (Ettalibi and Baratti 1987). The (I/S) ratio of the enzymes is dependent on the assay method used to determine their enzyme activities. The differences between S/I ratios and apparent kinetic constants are considered to be insufficient to distinguish yeast inulinase and yeast invertase (Kovaljova and Yurkevich 1978).

1.2.3.2 Inulinase production methods: solid state fermentation and submerged fermentation

The term solid state fermentation defines any fermentation process occurring in the absence or near-absence of free water, using a natural substrate, or an inert substrate as solid support (Pandey *et al*., 2000). Enzyme recovery usually requires extraction of aqueous solution, filtration and centrifugation and sometimes evaporation or precipitation of the crude enzyme solution.

Commercially, inulinase enzymes have been produced by SmF processes. However, in recent years, there have been an increasing number of reports on the production of inulinase using SSF. SSF processes have competitive advantages over SmF. These include (1) superior productivity, (2) simple technique, (3) low capital costs, (4) low energy requirement and less water output, (5) improved product recovery and (6) it is reported to be the most appropriate process for developing countries such a South Africa. SSF provides a better alternative for the production of enzymes and in recent years more enzymes have been produced employing this production method (Xiong *et al.*, 2007).

Selvakumar and Pandey (1999) reported inulinase production by *Staphylococcus* sp. RRL1 and *K. marxianus* in SSF. When wheat bran was used as a substrate, *Staphylococcus* sp. showed an inulinase activity of 107.64 IU/gds while that of *K. marxianus* was 122.88 IU/gds. When garlic powder was used as a substrate in inulinase production by SSF, Dilipkumar [a] *et al*., (2011) observed inulinase activity of 76 U/gds. Ayyachamy *et al.*, (2007) grew *Xanthomonas campestris* pv. *phaseoli* on onion peel and garlic peel in their comparative study of SSF and SmF. An inulinase activity of 117 IU/gds (garlic peel) and 101 IU/gds (onion peel) was achieved using SSF, and in SmF inulinase activity of 17.42 IU/ml was achieved using inulin as a sole carbon source (Ayyachamy *et al.*, 2007).

Another advantage of SSF becomes evident in the possibility of culturing microorganisms as mixed culture inocula. In nature, fungi (yeast and mold) grow in symbiotic relation on solid

substrates such as soils or decaying plant material. Biodegradation of these naturally occurring substrates involves contribution of vastly different enzymes produced by different microorganisms in the growth habitat (Gupte and Madamwar 1997; Koroleva *et al*., 2002; Stepanova *et al*., 2003). During mixed culture cultivation of fungal species some individual enzyme activities show a synergic increase whereas others remain unchanged. Yeasts and molds can be applied in co-culture saccharification, where fermentation can be regulated by the joint action of anaerobic and facultative anaerobic processes through the availability of oxygen (Holker and Lenz 2005). Solid state also offers the possibility to explore and to use interactions between fungi by properly regulating the water activity as a selection parameter depending on the different demands of the co-cultivated fungi. Many SSF processes used industrially make use of mixed cultures that are important for the flavour of the food produced e.g. bamboo sprouts processed by undefined mixed cultures that produce a variety of metabolites including volatile substances with aroma active properties (Holker and Lenz 2005).

1.2.3.3 Natural substrates for inulinase production

Naturally occurring inulin rich plant-materials are preferred as carbon sources for inulinase production. In recent years, however, agro-industrial residues have also gained popularity. Inulin is usually stored in bulbs, tubers, and roots in all inulin plant species except the Gramineae. Jerusalem artichoke (*Helianthus tuberosus*) and chicory of the Compositae family are the most used carbon sources for inulinase production since 50% of their dry matter is made up of inulin (Pandey *et al.,* 1999; Danilcenko *et al*., 2008; Bekers *et al*., 2008; Chi *et al.,* 2011).

Scientists have focused their attention on Jerusalem artichoke because of its availability. The increased attention can be attributed to its high tolerance to cold and drought; high tolerance to saline, wind and sand resistance; and its resistance to pests and diseases (Chi *et al*., 2011). In addition to inulin, sucrose is also a preferred pure substrate for inulinase production, while addition of pure substrate to supplement a production medium could have a positive impact on inulinase production. When Kumar *et al.,* (2005) supplemented the inulinase-production medium with sucrose, glucose, fructose, galactose, maltose and dextrose they observed maximum inulinase activity in galactose and maltose supplemented media respectively.

Agro-industrial residues and vegetable extracts are good sources for inulinase production. Carbon sources: Cassava flour, corncob, oat meal, rice straw, sugarcane bagasse, wheat bran, glucose, and sucrose were used to determine inulinase production by *Aspergillus ochraceus* and the highest inulinase activity (107 IU/gds) was obtained when sugar cane bagasse was used as a carbon source (Guimaraes *et al*., 2007).

1.2.4 Biochemical Properties of Inulinases

1.2.4.1 Molecular weight

Microbial inulinases have an average molecular weight of over 50 kDa. Sheng *et al*., (2008) purified and characterized inulinases from *Cryptococcus aureus* which had an estimated molecular weight of 60 kDa. Gong *et al*., (2008) and Chi *et al*., (2009) isolated inulinases from *Pichia guilliermondii* with a molecular weight of 50 kDa and 54 kDa respectively. Pandey *et al*., (1999) isolated an inulinase with a high molecular weight of 250 kDa.

Bacterial and yeast strains produce inulinases of similar molecular weight. Inulinases with a molecular weight of 75kDa was produced by *Arthrobacter* sp. (Kang *et al*., 1998). Inulinases produced by mould strains have a molecular weight between 50 kDa and 300 kDa. A few examples include: *Aspergillus ochraceus* (79 kDa), (Guimaraes *et al*., 2007), *Penicillium* sp. (68 kDa) (Chi *et al.*, 2009) and *Fusarium oxysporum* (300 kDa) (Pandey *et al.*, 1999).

1.2.4.2 Temperature and pH optima

Thermostability is one of the most desirable characteristics of the inulinases. Industrial processes for the production of fructose and fructo-oligsaccharides are carried out at about 60 ^{o}C (Vandamme and Derycke 1983). Most of the reported inulinases lose their activity after a few hours at this temperature (Vandamme and Derycke 1983). This lack of thermostability creates a problem in industrial applications where inulinases are used for the production of fructose since:

i. Inulin has limited solubility at room temperature, and
ii. The risk of microbial contamination is high at room temperature

The use of thermostable inulinases in industrial applications contributes towards lower production costs, since only a limited amount of enzyme is required to produce the desired product. Inulinases

from yeast, filamentous fungi and bacteria have been studied previously and research shows that some of these enzymes have optimum temperatures of 60 ºC and above (Singh *et al.*, 2006). Filamentous fungi such as *Penicillium* sp. produce three different inulinases viz. TI, II and III which have optimal temperatures between 45 ºC and 50 ºC. *Aspergillus niger* 12 had 54% residual activity when incubated at 60 ºC for 30 min. True thermostable inulinase from *A. niger* and *A. fumigatus* were reported by recent studies showing optimal activity at 60 ºC. Inulinases from most yeast species have been reported to be optimally active between 50 ºC and 55 ºC. Among the reported species *Candida kefyr, Candida salmenticensis*, *Kluyveromyces fragilis* and *Debaryomyces phaffii* showed optimal temperature of 50 ºC, 46 ºC, 55 ºC, and 50 ºC, respectively, and that of *Debaryomyces cantarellii* was 30 ºC (Kango and Jain 2011).

Most inulinases have a pH optimum between 4.0 and 5.5 (Vandamme and Derycke 1983). A pH optimum of 4.0 has been reported for inulinase from *K. marxianus* var. *bulgaricus*. Purified inulinases from *A. niger* 245 was also reported between 4.0 and 4.5. The maximum activity was observed at pH4.5 by *Kluyveromyces* sp. Y-85 inulinase. Mazutti *et al*., (2010) in their cooperative study between the crude inulinases from SmF and SSF, reported pH optimum of 4.5 and 5.0 respectively.

1.2.4.3 Kinetic properties of Inulinases

Research has shown that different inulin degrees of polimerisation and the variations during reaction processes do not allow proper estimation of the kinetic parameters and have therefore prevented inulinase kinetics from being described by Michaelis-Menten rate equation (Ricca *et al.*, 2007). A complete study on inulinase kinetics was first conducted by Focher *et al*., (1991), using inulinases from *A. ficuum*. During this study it was established that the K_2 and K_m of the kinetics were temperature dependent. Focher *et al.*, 1991 also found the Micaelis-Menten behaviour for the hydrolysis of both inulin and sucrose hydrolysis. Previous studies have shown the kinetic parameters of different inulinases from different strains and also the rate coefficient at only one temperature. It is, however, necessary to express kinetic parameters of exo- an endo-inulinases on a different basis (Azhari *et al.*, 1989).

1.2.5 Kluyveromyces marxianus

*Kluyveromyces marxianus (*previously named *Saccharomyces marxianus*), was first described by E.C. Hansen in 1888. The great majority of studies published on *K. marxianus* explore potential applications of this organism while not much effort has been aimed at its biochemical, metabolic and physiological properties. Since the 1970's an increasing number of studies have been published concerning the biological and metabolic aspects of different *K. marxianus* strains (Fonseca *et al*., 2008).

The morphology of *K. marxianus* was described by Liu (2011). The author described the yeast species as an anamorph of *Candida kyfer,* having similar characteristics of other *Candida* species. *Kluyveromyces marxianus* produces cream to brown, glossy and smooth colonies when cultured on Sabouraud dextrose agar at 25 ^{o}C. On corn meal agar at 25 ^{o}C *K. marxianus* produces highly branched pseudohyphae while microscopic evaluations show evanescent asci containing 2-4 crescent shaped spores.

In industrial bioprocesses, *K. marxianus* is produced as a nutritional yeast, a bonding agent for fodder and pet food, and as a source of ribonucleic acid. In addition it is also used to produce lactase enzyme (Liu 2011). *Kluyveromyces* sp. is used extensively for inulinase production. *Kluyveromyces marxianus* was used in many optimization studies for inulinase production in SSF by Mazutti *et al*., (2007). Mazutti *et al*., (2007) obtained a maximum inulinase activity of 250 U/gds by SSF of sugar cane bagasse and 47.2 IU/mL in submerged liquid fermentation (Mazutti *et al*., 2010). Bender *et al*., (2006) obtained inulinase activity of 444.8 IU/gds from agro-industrial residues using SSF. *K marxianus* IMB3 has been reported to possess the ability to produce ethanol at 45 ^{o}C during growth in glucose, cellobiose (Barron *et al*., 1995), sucrose (Flemming *et al*., 1993), and lactose containing media (Brady *et al*., 1995). According to Brady *et al*., (1995), *K. marxianus* IBM3 produced ethanol concentration of (8.5g/l), about 83% maximum yield when grown on medium containing 2% glucose.

1.2.6 *Saccharomyces cerevisiae*

Saccharomyces cerevisiae is one of the oldest domesticated microorganisms in history. It has extensive human use since ancient Egypt in baking of bread, beer brewing and wine making. Its

morphology was first described in 1680 by Leeuwenhoek during his microscopic studies (Feldmann 2010). Ohta *et al*., 1993 during his study for the production of hifructose using *Aspergillus niger* and *S.cerevisiae* described the vegetative cells of the *S. cerevisiae* as globose to ellipsoidal, 4.6 to 5.3 by 6.3 to 10.1um in size, and were produced by multilateral budding.

S. cerevisiae is used in industrial application for large scale ethanol production. It has previously been considered to be inulin-negative mainly because no genes encoding inulinase have been found previously in the *S. cerevisiae*. However, due to the aid of genomic tools a wide range of genotype and phenotype variations has been realised for diverse yeast populations which has led to the discovery of strains of *S. cerevisiae* possessing the ability to utilise inulin and hydrolise inulin type sugars to ethanol (Wang and Li 2012). Fermentation process using *Saccharomyces cerevisiae*, however, requires an acidic- or enzymatic hydrolysis of inulin prior to fermentation (Negro *et al*., 2011). Lim *et al*., 2011 however reported a strain of *S. cerevisiae* that efficiently utilizes inulin from Jerusalem artichoke without pretreatment for inulin hydrolysis. The strain could utilize fructo-oligosaccharides with higher degree of polymerization (DP) compared with the other fructo-oligosaccharides-fermenting *S. cerevisae* strains previously reported (Lim *et al*., 2011).

In enzyme technology, *S. cerevisiae* has been mainly used to produce invertases, both in SSF and SmF processes. The yeast *S. cerevisiae* has been used to produce recombinant proteins (Hahm and Chung 2001). *Saccharomyces cerevisiae* has also been reported in simultaneous saccharification and fermentation of raw ground corn processes by Hayashida *et al*., (1982), with a maximum production of 20.1% ethanol. Subsequently, Ohta *et al*., (1993) and Nakamura *et al*., (1996) recommended this method to produce ethanol from Jerusalem artichoke at the concentration of 21% (Ge and Zhang 2005).

Aim

The aim of this project is to produce inulinase in solid state fermentation using a mixed culture of *Kluyveromyces marxianus* and *Saccharomyces cerevisiae*

OBJECTIVES

The objectives of this project are:

- To screen natural raw substrates for inulinase activity using the DNS method for enzyme activity
- To optimize physical process parameters for maximum production of inulinases
- To partially purify the enzyme using ammonium sulphate precipitation
- To determine the Characteristics of the selected inulinase

CHAPTER 2: THE EVALUATION OF INULIN CONTAINING PLANT MATERIALS AS SUBSTRATES FOR INULINASE PRODUCTION

2.1 INTRODUCTION

Fructans are carbohydrates consisting of polymers of fructose residues. The degree of polymerization depends on the species, tissue, climate, growing conditions, storage conditions and time after harvesting of the fructan containing crop (Massadeh *et al*., 2001). Inulin is a type of fructan, found in about 36000 plants worldwide. Structurally it consists of linear chains of β (2,1)-linked fructose molecules attached to the terminal glucose residue through a sucrose-type linkage at the reducing end (Bonciu *et al*., 2011). Inulin has been extensively used by humans through consumption of edible plants and fruit. These inulin sources have gained interest as inexpensive and abundant substrates for microbial production of fructose syrup in the biopharmaceutical industry (Zhao *et al.*, 2010).

Various plants such as Jerusalem artichoke, garlic clove, garlic peel, onion bulbs, onion peel, sugarcane molasses, corn steep liquor, wheat bran, rice bran, coconut oil cake, and corn flour have been studied as substrates in inulinase production. Cost and availability of a substrate are important factors to consider when developing an economical enzyme-production process (Dilipkumar[b] *et al*., 2011). The use of mixed culture SSF with low cost inulin-containing plants provides a good alternative.

Identifying suitable substrates is one of the most important factors in designing an efficient enzymatic process with high yields of enzyme. Inulinases are enzymes that catalyse the hydrolysis of inulin to yield inulo-oligosaccharides, fructose and glucose as the main products (Bonciu *et al*., 2011). Fructose produced through enzymatic hydrolysis of inulin results in high fructose yields of about 95% compared to conventional methods such as starch hydrolysis, a three step process involving the action of α- amylase, amyloglucosydases and glucose isomerase yielding 45% fructose (Naidoo *et al*., 2009). Another method which can be used is acid hydrolysis of inulin. However, the low pH environment leads to fructose degradation and the process also gives rise to colouring of the inulin hydrolysate and formation of di-fructose anhydride, an undesirable by-product (Sharma *et al*., 2006).

Microbial preparations of inulinase are almost always accompanied by invertase activity (Mazutti *et al.*, 2006; Sharma *et al.*, 2006). Invertase splits the β-2.1fructoside linkages of sucrose. In recent years invertase has gained importance due to its various biotechnological applications in the beverage, confectionary, bakery and pharmaceutical industries (Bonciu and Barhim 2011).

Molds and yeasts have been studied extensively for inulinase production, mainly *Kluyveromyces* (Selvakumar and Pandey 1999; Bender *et al*., 2006; Mazutti *et al*., 2006; Dilipkumar *et* al., 2010; Dilipkumar[b] *et* al., 2011) and *Aspergillus* species. The bacterial species that have been studied are *Staphylococcus* (Selvakumar *and* Pandey 1999), *Streptomyces* (Dilipkumar[a] *et al*., 2011) and *Xanthomonas* (Ayyachammy *et al.*, 2007; Naidoo *et al.*, 2009*).*

Barratti and Ettalibi (1993) and Sirisansaneeyakul *et al*., (2007) reported a strong synergistic effect of exo- and endo-inulinases on inulin hydrolysis when these enzymes were used in combination. The use of mixtures of bacterial and yeast inulinases has also been reported by Cho *et al*., (2001).The present study involves the evaluation of different inulin-containing substrates (garlic cloves, garlic peel, onion bulbs, onion peel, parsnips, amadumbe (*Colocasia esculenta*) and wheat bran for optimum inulinase production in SSF using a mixed culture of *Kluyveromyces marxianus* CBS 4836 and *Saccharomyces cerevisiae* ATCC 9763.

2.2 MATERIALS AND METHODS

2.2.1 Preservation and Culturing of Strains

Kluyveromyces marxianus CBS 4836 and *Saccharomyces cerevisiae* ATCC 9763 were obtained from The Centraalbureu voor Schimmelcultures (CBS) Fungal Biodiversity Centre, The Netherlands. The strains were maintained on defined media containing (g/l): glucose 20, yeast extract 10, peptone 10, and agar 20, incubated at 37 ^{o}C for 24 hours and stored at 4 ^{o}C. The yeast cultures were sub-cultured weekly (Xiong *et al*., 2007). The master cultures were prepared in 80% glycerol stock solutions and stored at -86 °C

2.2.2 Inoculum Preparation

Erlenmeyer flasks (100 ml) containing 20 ml culture medium were inoculated with a loopful of cells and incubated at 30 ^{o}C for 12 hours on a rotary shaker operating at 120 rpm. *Kluyveromyces*

marxianus cultures were prepared in the following defined media (g/l): Yeast extract 10, peptone 20, and inulin 10. *Saccharomyces cerevisiae* cultures were prepared in the following defined media (g/l): Yeast extract 10, peptone 20, and glucose 10 (modified from Xiong *et al*., 2007). Only cultures with an optical density (OD) of 0.8 were used as inoculum in SSF.

2.2.3 Solid State Fermentation (SSF)

2.2.3.1 Substrate preparation

Garlic cloves, onion bulbs, parsnips, and amadumbe, garlic peel, onion peel and wheat bran were used to evaluate for the inulinase production. All the substrates were obtained from a local vegetable market and dried in an oven at 65 ^{o}C for 48 hours; to a moisture content of 3-4% for garlic cloves, onion bulbs, parsnips and amadumbe; and 0% moisture content for garlic peel, onion peel and wheat bran. The dried substrates were ground using a Fritch pulverisette 14 bench-top grinder to a particle size of 0.5 mm. Ten grams of solid substrates supplemented with 1 ml of an acidified mineral solution containing (mg): (0.3) $MnSO_4.H_2O$, (0.9) $FeSO_4.6H_2O$, (0.25) $ZnSO_4.7H_2O$, and (0.35) $CaCl_2$ (modified from Selvakumar and Pandey 1999) for 10 g dry substrate in 250 ml Erlenmeyer flasks, sealed with a non-absorbent cotton wool plug and autoclaved at 121 ^{o}C for 20 min..

2.2.3.2 Evaluation of inulinase activity with mixed cultures of K. marxianus and S. cerevisiae

Substrates were inoculated with 4 ml of mixed culture (2 ml *K. marxianus* and 2 ml *S. cerevisiae*) to achieve moisture content of 50% and incubated in a humidity chamber at relative humidity of 80% at 30 ^{o}C for 96 hours.

2.2.3.3 Evaluation of inulinase activity with only K. marxianus

Substrates were inoculated with 4 ml *K. marxianus* to achieve a moisture content of 50% and incubated in a humidity chamber at relative humidity of 80% at 30 ^{o}C for 96 hours.

2.2.3.4 Evaluation of inulinase activity with only S. cerevisiae

Substrates were and were inoculated with 4 ml *S. cerevisiae* to achieve a moisture content of 50% and incubated in a humidity chamber at relative humidity of 80% at 30 ^{o}C for 96 hours.

2.2.4 Extraction of Enzyme

The enzyme was extracted using 50 ml of 0.1M sodium acetate buffer at pH5.5, which was stirred on an orbital shaker at 150 rpm set at 53 ℃ for 40 min. The crude extract was filtered with a muslin cloth and centrifuged using an Eppendorf centrifuge, rotor no: 4-2-81 for 20 min at 4 ºC. The resulting clear supernatant was used for the enzyme assay (Bender *et al*., 2008).

2.2.5 Enzyme Assay

The enzyme activity was determined by measuring the reducing sugars released through the hydrolysis of chicory inulin by the supernatant. The enzyme extract (100 µl) was mixed with 900 µl of inulin solution (2% w/w) in 0.1M acetate buffer at pH4.8, incubated at 50 ℃ for 10 min and stopped by boiling in a water bath for 10 min. The enzyme reaction was assayed for reducing sugars (fructose equivalent) by the DNS method (Miller 1959). A calibration curve was prepared with fructose solutions of known strength and blanks (enzyme blank and reagent blank) were assayed simultaneously with enzyme and substrate solutions. One unit of inulinase activity was defined as the amount of enzyme which liberated 1 µmol of fructose per minute under assay conditions.

2.3 RESULTS

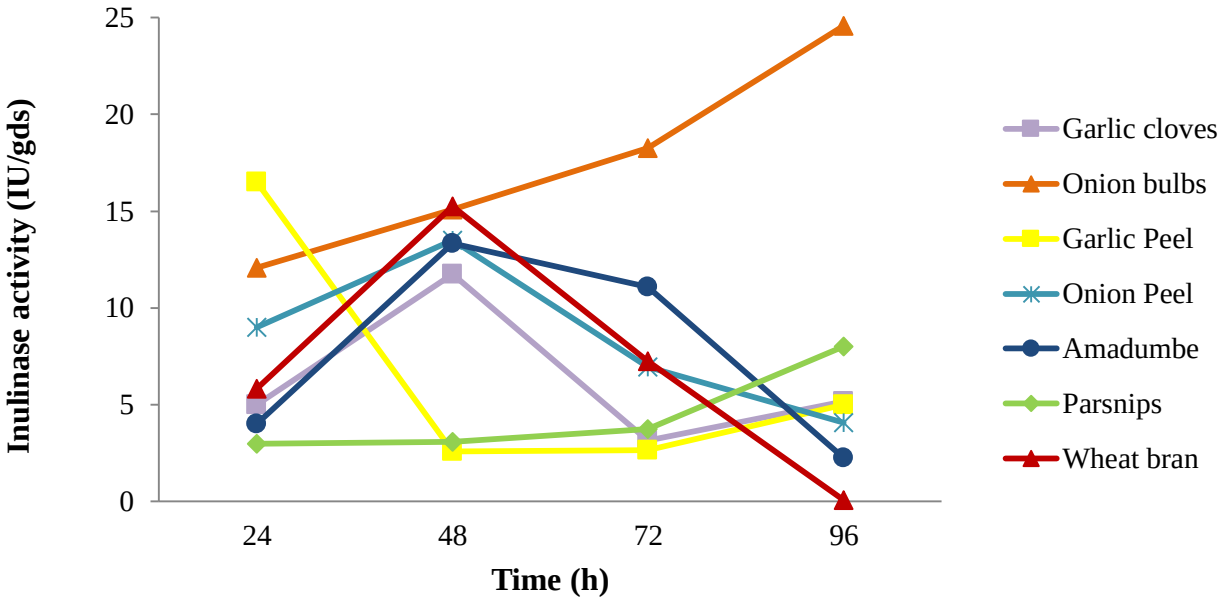

Figure 3: Evaluation of inulinase activity using a pure culture of the *K. marxianus* using inulin containing substrates

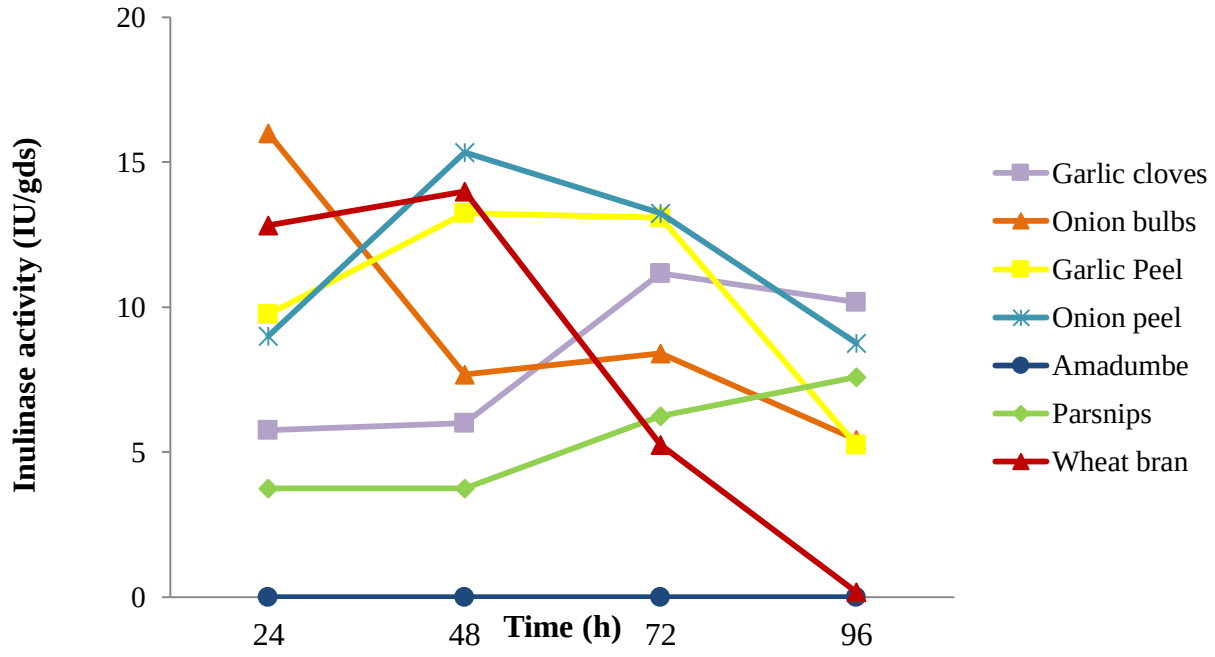

Figure 4: Evaluation of inulinase activity using a pure culture of the *S. cerevisiae* using inulin containing substrates

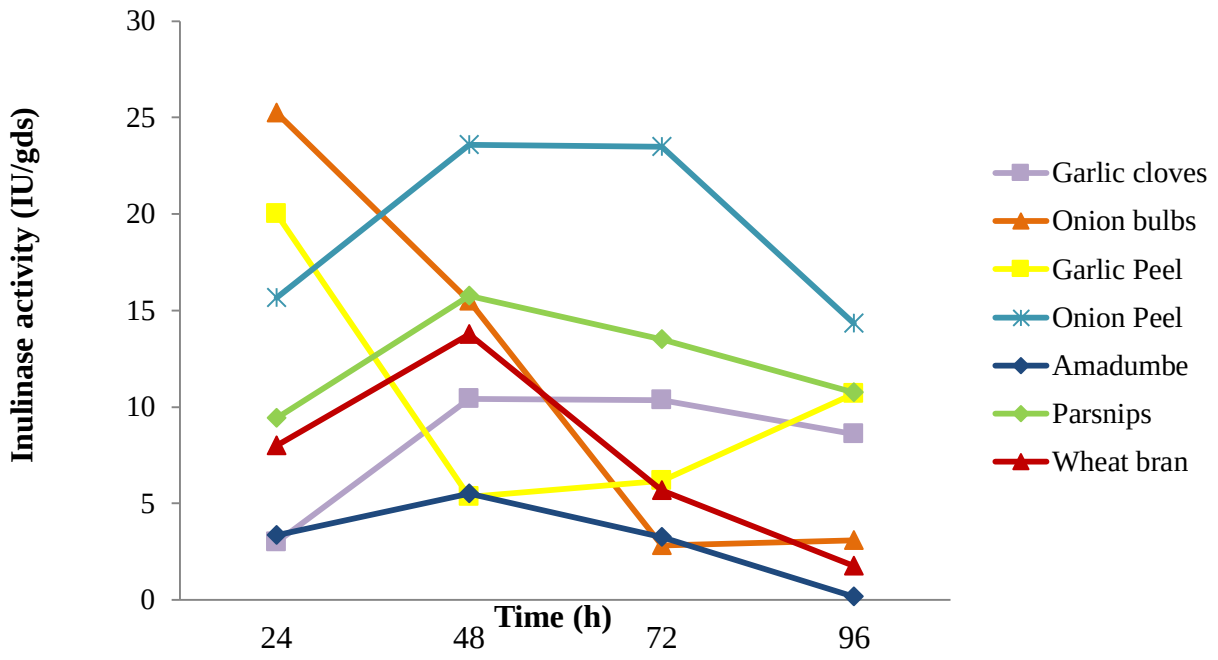

Figure 5: Evaluation of inulinase activity using a mixed culture of the *K. marxianus* and *S. cerevisiae* using using inulin containing substrates

2.4 DISCUSSION AND CONCLUSION

Selection of suitable substrates for the fermentation process is one of the most important factors in SSF and requires the evaluation of various plant materials for microbial growth and product yield (Selvakumar and Pandey 1999).

2.4.1 Evaluation of Inulinase Activity using *K. marxianus* and *S. cerevisiae* as single culture inocula

The overall enzyme activity lowered when substrates were cultured with single cultures of the yeast strains with slightly higher activities being observed in substrates inoculated with *K. marxianus.* Although high levels of inulinase activity (24 IU/gds) were produced by *K. marxianus* when onion bulbs were used as substrate, inulinase activity was determined in a shorter time on garlic peel, viz. 16 IU/gds in 24 hours (Figure 4). After 48 hours high inulinase activity had been observed in wheat bran (15 IU/gds), amadumbe (14 IU/gds) onion peel (14 IU/gds), garlic cloves (11 IU/gds) while on parsnips the lowest activity of 8 IU/gds was observed after 96 hours.

Among the different substrates used, the highest inulinase activity was observed using onion bulbs (16 IU/gds) but after 24 hours of fermentation using amadumbe as a substrate there was no significant inulinase activity (Figure 5). Growth of *S. cerevisiae* on other substrates, resulted in maximum inulinase activity after 48 hours; onion peel (15 IU/gds), wheat bran (14 IU/gds), and garlic peel (13 IU/gds). Induction of inulinase activity was determined after 96 hours on the garlic cloves (11 IU/gds) and in 72 hours on parsnips (7 IU/gds).

The maximum accumulation of inulinase was observed 48 hours after incubation in most substrates and declined thereafter. Selvakumar *et al*., (1999) and Dilipkumara[a] *et al*., (2011) reported that the maximum production of the enzyme occurs after 72 hours, while Mazutti *et al*., (2006) reported maximum inulinase activity after 96 hours when *K. marxianus* was used as an inoculum. Maxumum inulinase production was achieved in a shorter time in the present study compared to the previous studies.

2.4.2 Evaluation of Inulinase Activity using *K. marxianus* and *S. cerevisiae* in mixed culture

Inulinase production of 26 IU/gds by a mixed culture of *K. marxianus* and *S. cerevisiae* (Figure1) was observed after 24 hours with onion bulbs as a substrate followed by onion peel (23 IU/gds) after 48 hours. Garlic peel demonstrated inulinase production of 20 IU/gds in 24 hours. Inulinase activity was moderate in the parsnips (15 IU/gds), wheat bran (13 IU/gds) and garlic cloves (10 IU/gds), while the lowest activity was observed in amadumbe (3 IU/gds).

The inulinase activity was slightly higher in mixed culture using SSF as compared to pure culture in SSF. Sirisansaneeyakul *et al*., (2007) observed similar capabilities of synergism of the single and mixed inulinases on inulin hydrolysis. When onion bulbs, onion peel and garlic peel were used as substrates, the resulting inulinase activity was high in mixed culture using SSF as compared to single culture using SSF of *K. marxianus* and *S. cerevisiae*. The microbial activity on the garlic cloves, wheat bran and parsnips was similar when cultured in both mixed and single culture in SSF. Holker and Lenz (2005) stated that during co-culture of different fungal strains, some of the individual enzyme activities may show synergistic increase whereas others will remain unchanged. The amadumbe resulted in the lowest activity of all substrates, resulting in a zero activity when cultured with *S. cerevisiae*. Inulinase activity obtained on onion peel (25 IU/gds) and garlic peel

(20 IU/gds) was comparable with that of Ayyachamy *et al*., (2007) who obtained 27 IU/gds and 21 IU/ gds, respectively.

S. cerevisiae, an invertase producing yeast strain, induced high inulinase activity on the inulin containing substrates. The results indicates that the hydrolysis of inulin requires some invertase activity to release the fructose molecules from the β (2,1)-linkages. When using mixed culture in SSF, the yeast species showed increased inulinase activity, which might have resulted from a collective invertase activity from the both *K.marxianus* and *S.cerevisiae* strains.

Although a considerably high inulinase activity was observed in the onion bulbs, onion peel and garlic peel as compared to the other substrates, there was also inconsistency in enzyme activity. As a result garlic cloves, parsnips, wheat bran and amadumb were used in the optimization studies.

Dilipkumar[a] *et al*., (2011) observed the highest inulinase activity in SSF for their optimisation studies using garlic cloves (298 IU/gds) while Selvakumar and Pandey (1999) observed a high inulinase activity of 107.64 IU/gds when using wheat bran as a substrate. The present study is the first to report on the use of amadumbe and parsnips in SSF for inulinase production.

Garlic cloves and parsnips are suitable low-cost substrates for microbial production of inulinase using mixed culture SSF. The present study has shown the feasibility for scaling up inulinase production by *K. marxianus* and *S. cerevisiae* using mixed culture in SSF. It would be of added value to further explore the possibility of these yeast strains in SSF for enzyme production.

CHAPTER 3: OPTIMISATION OF THE FERMENTATION PARAMETERS FOR MAXIMUM INULINASE PRODUCTION USING MIXED CULTURE IN SSF

3.1 INTRODUCTION

Microorganisms are living organisms, and therefore, need favourable conditions to grow and multiply. Conditions may include: food source (substrate); favourable temperature, moisture content and water activity; pH and time. Inhibiting or altering any of these factors can influence growth of the microorganisms either in a positive or negative way. In natural habitats microorganisms coexist in competition for a growth limiting substrate(s), and often the outcome of such competition depends on relevant growth parameters (Fiechter 1984).

Parameters that act on growth due to mechanisms working outside of the cell's "environmental effects" such as medium components and the methods which render them available for uptake, can be divided into two categories namely physical and chemical parameters. Physical parameters include temperature, pressure, and the physical nature of the reaction mixture, including the problems of mixing and aeration in upscaled processes. The chemical parameters on the other hand include the effects of medium containing substrate, nutrients, growth factors and trace elements. Proper selection of these components and their quantities is of importance due to their potential effects on the metabolic performance of the cell (Fiechter 1984).

The establishment of the relationships between the physiology of the microorganisms and the physico-chemical factors contributes towards developing effective process models. Among several critical factors moisture and nature of solid substrate used are the most important factors affecting SSF processes. Selection of moisture depends on the microorganism used as well as the nature of the substrate. For filamentous fungi 40–60% moisture content could be sufficient, but selection of the substrate depends on several factors mainly related to the cost and availability, and thus may involve the screening of several agro-industrial residues (Singhania *et al*., 2009).
There are two reasons for optimizing process parameters:

- to ensure process stability by suppressing the influence of disturbances and
- to enhance the performance of the fermentation process

Industrial enzymes such as those used in the food and pharmaceutical industries are regulated by using composite media and regulated fermentation conditions. It is, therefore, important to study and understand the parameters involved in order to obtain optimum microbial growth and enzyme yields. Optimisation using the "one factor at a time" method involves changing the independent variables while fixing others at a particular level, to understand the impact of the particular parameter on the fermentation process (Songpim *et al*., 2011).

3.2 MATERIALS AND METHODS

3.2.1 Preservation and Culturing of Strains

The yeast cultures *Kluyveromyces marxianus* CBS 4836 and *Saccharomyces cerevisiae* ATCC 9763 were obtained from The Centraalbureu voor Schimmelcultures (CBS) fungal biodiversity centre, Netherlands. The strains were stored on defined media containing (g/l): glucose 20, yeast extract 10, peptone 10, and agar 20. To maintain the pure cultures, the cultures were streaked on the agar plate, incubated at 37 ^{o}C for 24 hours and stored at 4 ^{o}C. The yeast cultures were subcultured weekly (Xiong *et al*., 2007).

3.2.2 Inoculum Preparation

Kluyveromyces marxianus cultures were grown in the following defined media (g/l): Yeast extract 10, peptone 20, and inulin 10. *S. cerevisiae* cultures were grown in the following defined media (g/l): Yeast extract 10, peptone 20, and glucose 10. A loopful of cells was transferred from the plates to 100 ml Erlenmeyer flask containing 20 ml culture medium and incubated for 12 hours on a rotary shaker operating at 120 rpm at 30 ^{o}C (modified from Xiong *et al*., 2007). Only cultures with an optical density (OD) of 0.8 were used as inoculum in SSF.

3.2.3 Solid State Fermentation (SSF)

3.2.3.1 Substrate preparation

Garlic cloves, parsnips, amadumbe, and wheat bran were examined for their ability to produce inulinase. All the substrates were obtained from a local vegetable market and dried in an oven at 65 ^{o}C for 48 hours; to a moisture content of 3-4% for garlic cloves, onion bulbs, parsnips and amadumbe; and 0% moisture content for garlic peel, onion peel and wheat bran. The dried plant

materials were ground using a Fritch pulverisette 14 bench-top grinder to a particle size of 0.5 mm. Ten grams of the solid substrates were supplemented with 1 ml of an acidified mineral solution containing (mg): (0.3) $MnSO_4.H_2O$, (0.9) $FeSO_4.6H_2O$, (0.25) $ZnSO_4.7H_2O$, (0.35) $CaCl_2$ (modified from Selvakumar and Pandey 1999) for 10 g dry substrate in a 250 ml Erlenmeyer flasks sealed with a non-absorbent cotton wool plug and autoclaved at 121 ^oC for 20 min.

3.2.4 Extraction of Enzyme

The enzyme was extracted using 50 ml of 0.1M sodium acetate buffer (pH5.5) stirred on an orbital shaker at 150 rpm, at 53 ^oC for 40 min. The crude extract was filtered with a muslin cloth and centrifuged using an Eppendorf centrifuge, rotor no: 4-2-81 for 20 min at 4 ^oC. The resulting clear supernatant was used for the enzyme assay (Bender *et al.*, 2008).

3.2.5 Enzyme Assay

The enzyme activity was determined by measuring the reducing sugars released by the hydrolysis of chicory inulin by the supernatant. The enzyme extract (100 µl) was mixed with 900 µl of inulin solution (2% w/w) in 0.1M acetate buffer pH4.8 and incubated at 50 ^oC for 10 min. The enzyme reaction was stopped by boiling for 10 min in a water bath and assayed for reducing sugars by the DNS method (Miller 1959).

A calibration curve was prepared with fructose solution of known concentration and blanks (enzyme blank and reagent blank) were run simultaneously with enzyme and substrate solutions. One unit of inulinase activity was defined as the amount of enzyme which produced 1 µmol of fructose per munite under assay conditions.

3.2.6 Effect of Initial Moisture Content on Inulinase Production

Substrates were prepared as explained in section (3.2.3.1) by the addition of different volumes (1-3 ml) of an acidified mineral solution. Substrates were then inoculated with 4 ml of the mixed culture (2 ml *K. marxianus* and 2 ml *S. cerevisiae*). Additional deionised water was added where necessary to achieve moisture content of 40%, 50%, 60%, 65% and 70% and incubated in a humidity chamber at relative humidity of 80% at 30 °C for 48 hours.

3.2.7 Effect of Temperature on Inulinase Production

Substrates were prepared as explained in section (3.2.3.1). Thereafter, the substrates were inoculated with 4 ml of the mixed culture (2 ml *K. marxianus* and 2 ml *S. cerevisiae*) to achieve a moisture content of 50% and incubated in a humidity chamber at relative humidity of 80% at different temperatures i.e. 20 ℃, 25 ℃, 30 ℃, 40 ℃, and 45 ℃ for 48 hours.

3.2.8 Effect of Inoculum ratio on Inulinase Production

Substrates were prepared as explained in section (3.2.3.1). The various substrates were then inoculated with varying ratios of the inoculum of the *K. marxianus* and *S. cerevisiae* in mixed culture (1:3, 3:1, 1.5: 2.5, 1:1, and 2.5:1.5) of 4 ml inoculum to achieve moisture content of 50%, incubated in a humidity chamber at relative humidity of 80% at 30 ℃ for 48 hours.

3.2.9 Effect of Inoculum volume on Inulinase Production

Substrates were prepared as explained in section (3.2.3.1). Thereafter, the substrates were inoculated with varying volumes of the *K. marxianus* (1 ml, 2 ml, 3 ml and 4 ml) and *S. cerevisiae* mixed culture, and supplemented with additional deionised water to achieve moisture content of 50% and incubated in a humidity chamber at relative humidity of 80% at 30 ℃ for 48 hours.

3.2.10 Effect of Carbon concentration (Glucose) on Inulinase Production

Substrates were prepared as explained in section (3.2.3.1) and varying concentrations of glucose 0.25%, 0.5%, 0.75% and 1.0% in 250 ml Erlenmeyer flasks sealed with non-absorbent cotton wool plugs and autoclaved at 121 ^{o}C for 20 min. The substrates were then inoculated with 4 ml of mixed culture (2 ml *K. marxianus* and 2 ml *S. cerevisiae*) to achieve a moisture content of 50% and incubated in a humidity chamber at relative humidity of 80% at 30 ℃ for 48 hours.

3.2.11 Effect of Nitrogen concentration (Corn steep liquor) on Inulinase Production

Substrates were prepared as explained in section (3.2.3.1). Different concentrations of corn steep liquor at 1%, 3%, 5%, 7%, 8% and 10% were used in 250 ml Erlenmeyer flasks, which were sealed with non-absorbent cotton wool plugs and autoclaved at 121 ^{o}C for 20 min. Thereafter, the

substrates were inoculated with 4 ml of the mixed culture (2 ml *K. marxianus* and 2 ml *S. cerevisiae*) to achieve a moisture content of 50% and incubated in a humidity chamber at relative humidity of 80% at 30 °C for 48 hours.

3.2.12 Effect of dahlia inulin on inulinase activity under strict optimised conditions

The enzyme activity was determined by measuring the reducing sugars released by the hydrolysis of dahlia inulin by the supernatant. The enzyme extract (100 µl) was mixed with 900 µl of inulin solution (2% w/w) in 0.1M acetate buffer pH4.8 and incubated at 50 °C for 10 min. The enzyme reaction was stopped by boiling for 10 min in a water bath and assayed for reducing sugars by the DNS method (Miller 1959).

A calibration curve was prepared with fructose solution of known concentration and blanks (enzyme blank and reagent blank) were run simultaneously with enzyme and substrate solutions. One unit of inulinase activity was defined as the amount of enzyme which produced 1 µmol of fructose under assay conditions.

3.3 RESULTS

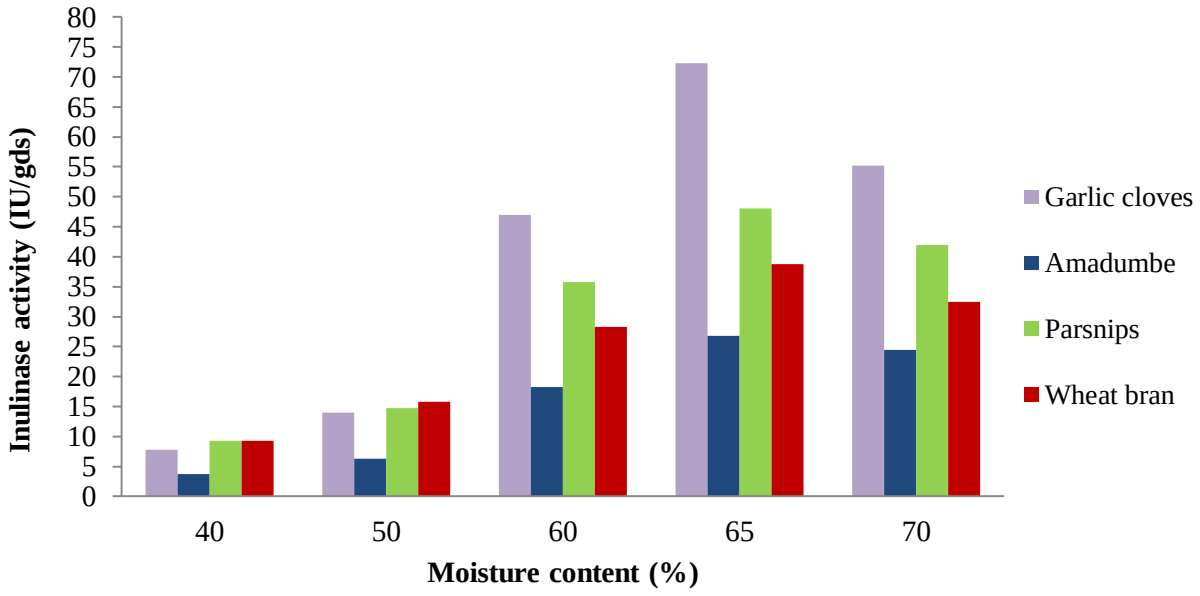

Figure 6: Effects of initial moisture content on inulinase production using different substrates

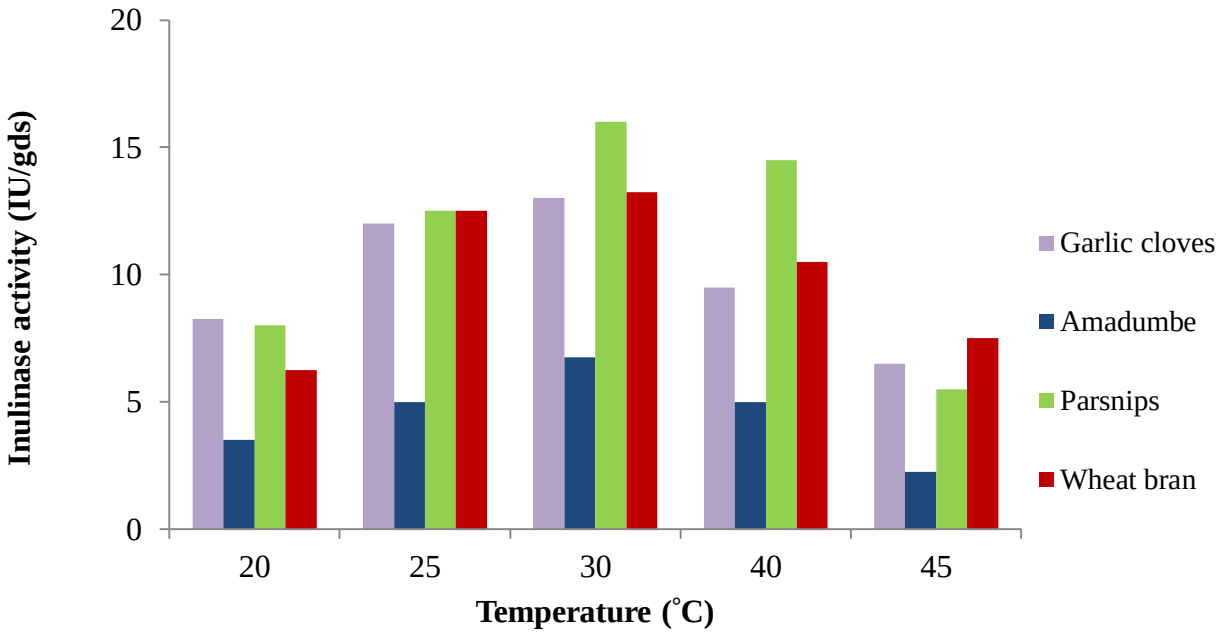

Figure 7: Effect of temperature on inulinase production using different substrates

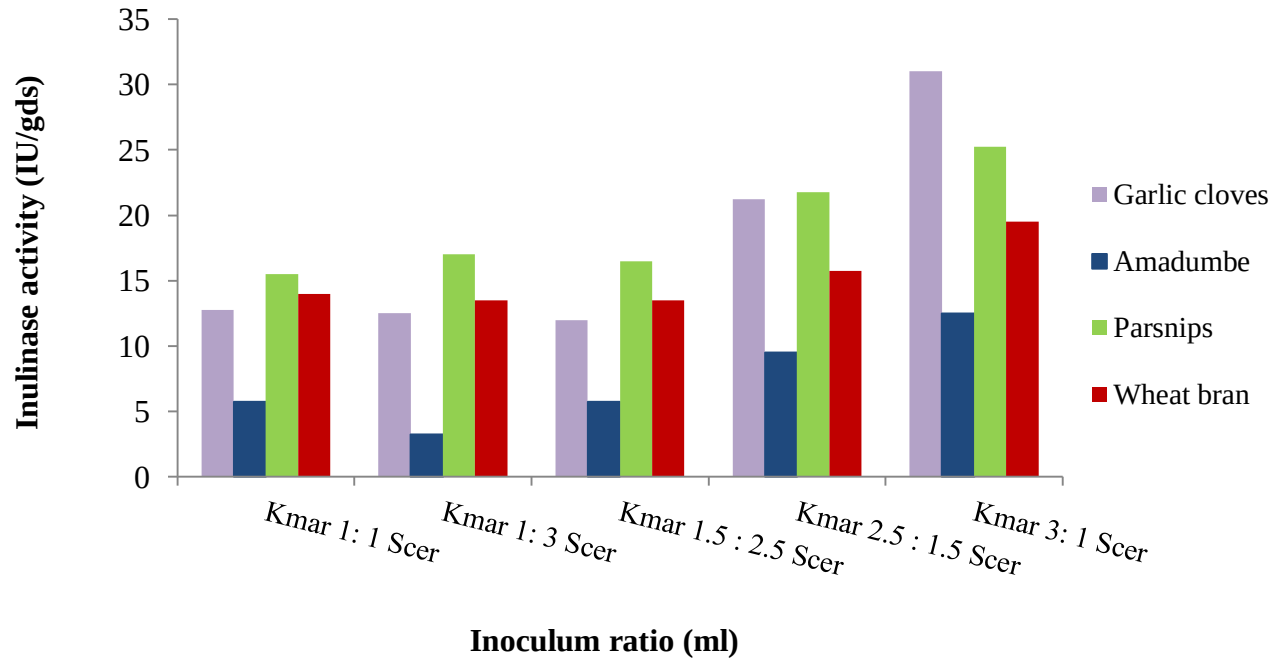

Figure 8: Effect of inoculum ratio on inulinase production using *K. marsianus* and *S. cerevisiae* cultivated in garlic cloves, amadumbe, parsnips and wheat bran

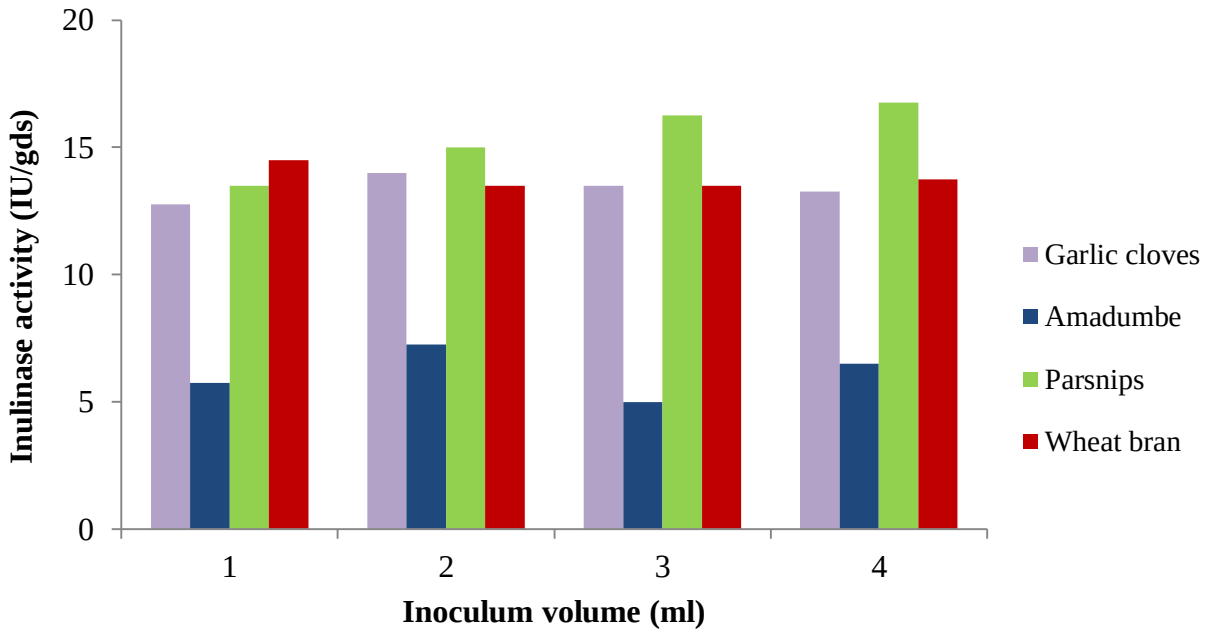

Figure 9: Effect of inoculum volume on inulinase production using *K. marsianus* and *S. cerevisiae* cultivated in garlic cloves, amadumbe, parsnips and wheat bran

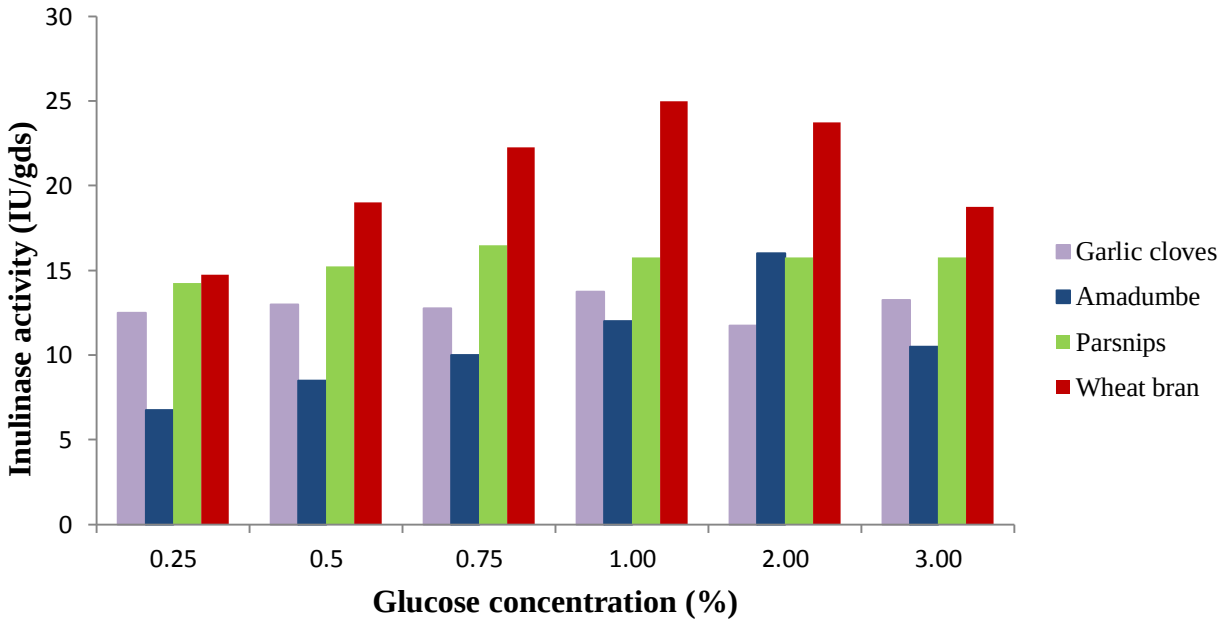

Figure 10: Effect of glucose concentration on inulinase production using *K. marsianus* and *S. cerevisiae* cultivated in garlic cloves, amadumbe, parsnips and wheat bran

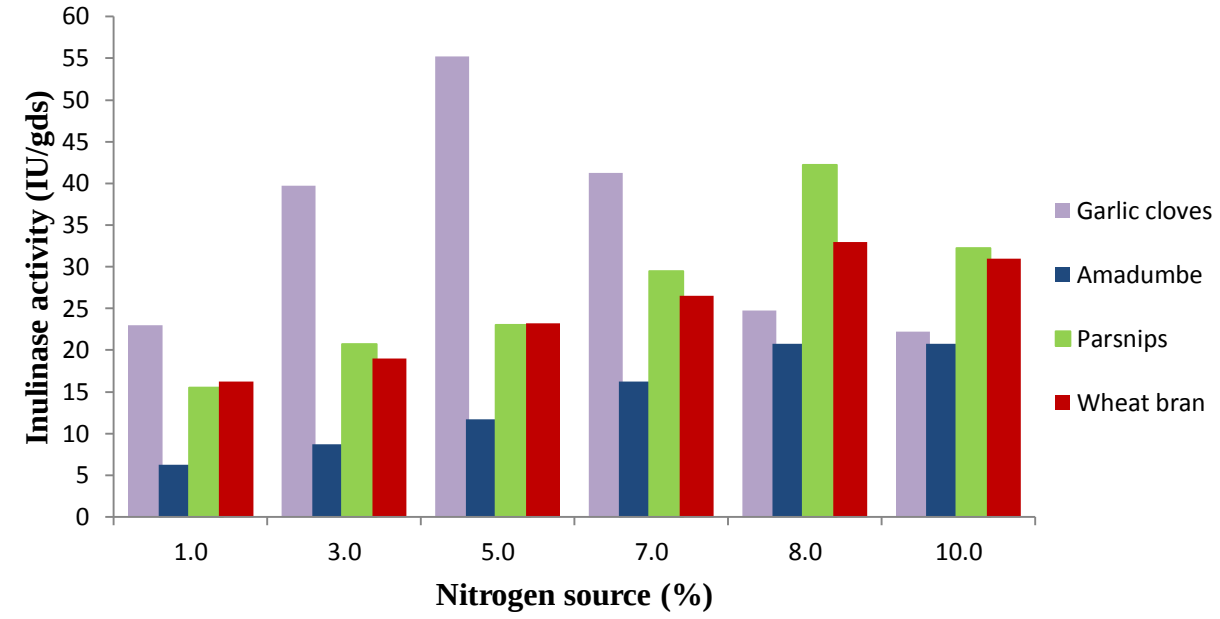

Figure 11: Effect of nitrogen source on inulinase production using *K. marxianus* and *S. cerevisiae* cultivated in garlic cloves, amadumbe, parsnips and wheat bran

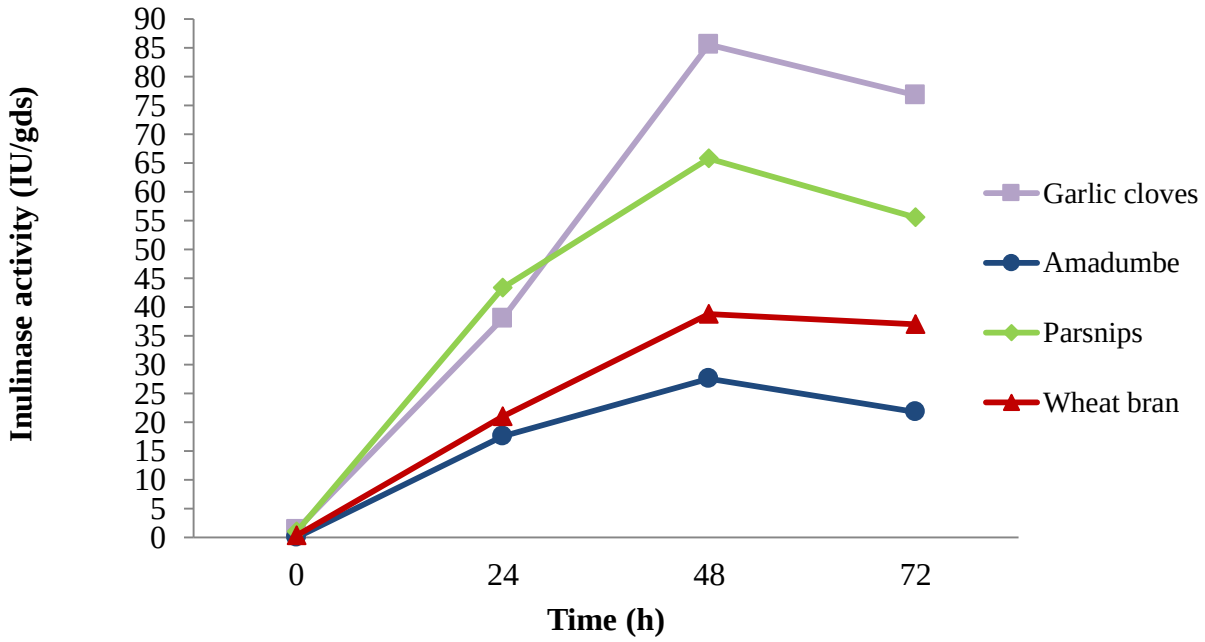

Figure 12: Inulinase production under optimised conditions using chicory inulin as a substrate for enzyme assay

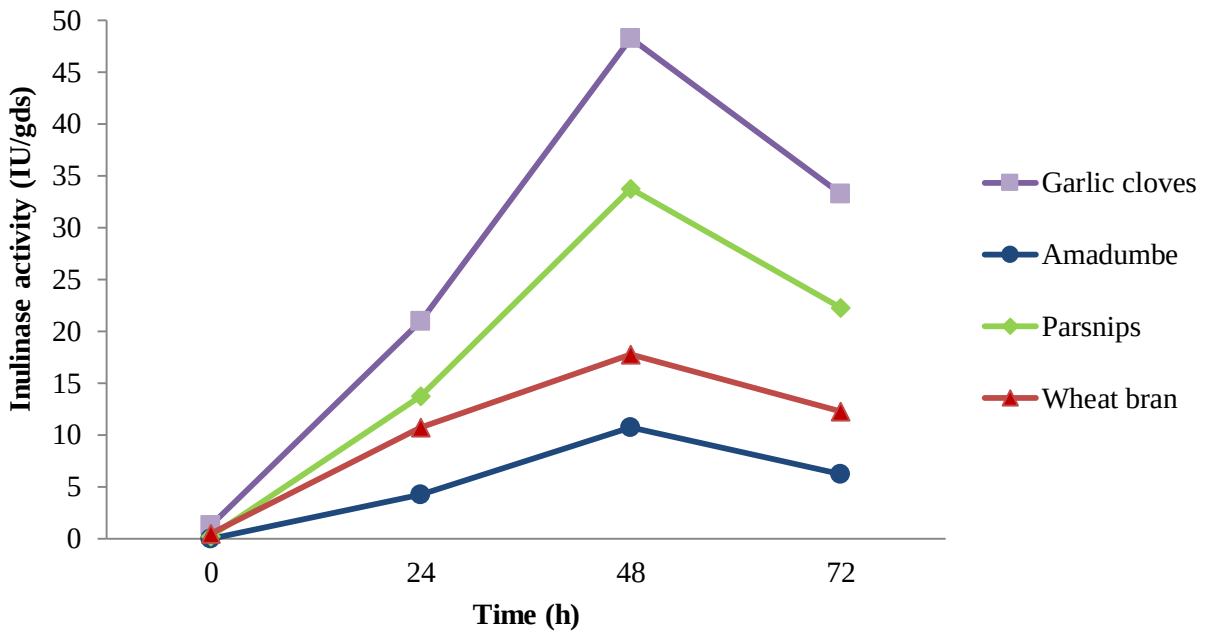

Figure 13: Inulinase production under optimized condition using Dahlia inulin as a substrate fo enzyme assay

3.4 DISCUSSION AND CONCLUSION

3.4.1 Effect of Initial Moisture Content on Inulinase Production

Inulinase production on garlic cloves, amadumbe, wheat bran and parsnips was enhanced by optimising the initial moisture content of substrates to 40%, 50%, 60%, 65% and 70%. In Figure 6 moisture content of 65% was established to be optimal for inulinase production by a mixed culture of *K. marxianus* and *S. cerevisiae*. Moisture content below 50% resulted in lower yields of inulinase. After 48 hours of incubation, the inulinase activity increased to 72 IU/gds and 48 IU/gds with garlic cloves and parsnips respectively. Inulinase activity also increased with wheat bran (38 IU/gds) and amadumbe (26 IU/gds) substrates. Selvakumar and Pandey (1999) also identified 65% as the best possible initial moisture content for maximum inulinase production.

Moisture levels below 50% resulted in a considerable decrease of inulinase activity, while levels above 50% resulted in an increased activity of the enzyme. According to Selvakumar and Pandey

(1999) low moisture content in SSF resulted in suboptimal product formation due to reduced mass transfer process.

3.4.2 Effect of Temperature on Inulinase Production

Inulinase production using the mixed culture of *K. marxianus* and was studied using five different temperatures optima i.e. (20 ºC, 25 ºC, 30 ºC, 40 ºC and 45 ºC), at initial moisture content of 50%. The initial incubation temperature of 30 ºC proved to be the best possible temperature for maximum enzyme production. Although reduced, inulinase activity was still high at 40 ºC. It is evident therefore, that the mixed culture has a wider growth temperature range and that inulinase production remained high between 25 ºC and 40 ºC.

The highest levels of inulinase activity of 16 IU/gds were obtained in parsnips followed by the garlic cloves (13 IU/gds), wheat bran (13 IU/gds) and finally amadumbe (6 IU/gds) at 30 ºC. Mazutti *et al*., (2010) obtained high inulinase activity of 391.9 IU/gds at 36 ºC when investigating inulinase activity between 30.4 ºC and 41.6 ºC using response surface methodology (RSM). Hu *et al*., (2012) produced 90% and 79.7% ethanol yield using thermo tolerant *K. marxianus* and *S. cerevisiae* grown at a temperature range of 30-40 ºC.

3.4.3 Effect of Inoculum on Inulinase Production

Garlic cloves exhibited maximum inulinase production of 29 IU/gds followed by parsnips and wheat bran, giving inulinase activity of 25 IU/gds and 19/gds respectively at an inoculum ratio of *K. marxianus* 3: 1S. *cerevisiae*. Enzyme activity attained from the amadumbe was the lowest at 12 IU/gds at inoculum ratio of *K. marxianus* 3: *1S. cerevisiae* . Inulinase activity exhibited by the mixed-culture inoculum in the garlic cloves, parsnips and wheat bran substrates, indicated that the ratio of *K. marxianus* 1: 3 *S. cerevisiae* was comparable to *K. marxianus* 1:1 *S. cerevisiae* ratio, showing no significant reduction (0.3 fold) in enzyme activity. However in the amadumbe a reduction in enzyme activity was observed showing inulinase activity of 5 IU/gds at *K. marxianus* 1:1 *S. cerevisiae* and 3 IU/gds at *K. marxianus* 1: 3 *S. cerevisiae*.

It is evident that varied inoculum volume of the mixed culture influenced inulinase production. When a high inoculum volume of *K. marxianus* was introduced into the mixed culture, inulinase activity increased; and when a low volume was introduced, the activity of inulinase reduced.

Similar observations were encountered by Massadeh *et al.*, (2001) while studying the synergism of cellulase enzymes in mixed culture SSF.

Figure 9 illustrates, growth of the mixed culture on the garlic and amadumbe yielded inulinase activity of 14 IU/gds and 7 IU/gds with an inoculum volume of 2 ml; parsnips, 16 IU/gds with a volume of 4 ml, and wheat bran, 14 IU/gds with an inoculum volume of 1 ml. Therefore, it is evident that the volume of the inoculum did not demonstrate any substantial changes on inulinase activity, as no consistent increase in inulinase yield was observed when the inoculum volume was increased, nor was there a tenable decrease in inulinase yield when inoculum volume was decreased. Narayanan *et al.*, (2013) also observed similar findings when studying the effect of inoculum volume on inulinase activity using banana peel, garlic peel, rice bran and wheat bran.

3.4.4 Effect of Carbon Source (Glucose) on Inulinase Production

Inulinase production was studied for different concentrations of carbon sources (0.25%, 0.50%, 0.75%, 1.0%, 2.0%, and 3.0%) with glucose as supplementary growth substrate. Maximal enzyme production (

Figure **10**) was achieved using wheat bran as a substrate (25 IU/gds) at 1.0% of glucose concentration followed by the amadumbe (16 IU/gds) at 2.0% glucose supplementation. Inulinase activity attained in the garlic cloves and parsnips indicated no detectable increase and remained at an average at 16 IU/gds and 13 IU/gds maximum production respectively.

The lack of increase in activity in the garlic cloves and parsnips could attributed to the high sugar (fructose and glucose) levels contained within these substrates, resulting in no increased activity when a supplementary carbon source is added. Ayyachamy *et al.*, (2007) obtained maximum inulinase activity (117 IU/gds) and (101 IU/gds) with the garlic peel and onion peel using 1% glucose as a carbon source. At 2.0% supplementation for the amadumbe and 1.0% for the wheat bran, inulinase activity dropped to 23 IU/gds and 10 IU/gds respectively. The decrease in activity could be attributed to catabolite repression due to the high concentration of glucose within the medium.

3.4.5 Effect of Nitrogen Source (Corn Steep Liquor) on Inulinase Production

Inulinase production was increased among all growth substrates with the addition of Corn steep liquor (Figure 11). At 5.0% supplementation, maximal inulinase activity was attained from the garlic cloves substrate at 55 IU/gds and parsnips (42 IU/gds) at 8.0% CSL supplementation. Using response surface methodology, Dilipkumar[a] *et al*., (2011) obtained maximum inulinase activity of 268 IU/gds by supplementing garlic cloves substrate with 5.8% CSL. Inulinase activity also increased to 33 IU/gds maximum activity when the wheat bran was supplemented with 8% CSL. Xiong *et al*., (2007) using RSM attained inulinase activity of 391.6 IU/gds by supplementing wheat bran with 10.76% CSL. Maximum inulinase activity of 20 IU/gds was also obtained from the amadumbe at 8% CSL supplementation.

The effect of the nitrogen source, in this case CSL, was significant (4.78 fold) in inulinase production as indicated by the increase in enzyme activity when increased concentrations of the nitrogen source was supplemented to the SSF medium.

3.4.6 Inulinase Production under Optimized Conditions

The results indicated that the *K. marxianus* and *S. cerevisiae* mixed culture had the ability to grow and produce high levels of inulinase under a wide range of temperature and moisture conditions, different concentrations of the nitrogen and carbon sources, inoculum volume and inoculum ratio.

Figure 12 shows inulinase production under strictly optimized conditions using chicory inulin as a substrate for enzyme assay. Under these conditions inulinase activity increased to 85 IU/gds in the garlic cloves , 65 IU/gds in the parsnips, 38 IU/gds in the wheat bran and 27 IU/gds in the amadumbe.

Inulinase activity under optimized conditions was also studied using Dahlia inulin (Figure 13). The highest inulinase activity was attained in the garlic cloves (48 IU/gds) followed by the parsnips (33IU/gds), wheat bran (17 IU/ gds) and lastly amadumbe (10 IU/gds).

The results obtained while using Dahlia inulin as a substrate demonstrated a reduction in enzyme activity compared to that of chicory inulin. The degree of polymerisation (DPn) of the Dahlia inulin is much higher than that of chicory inulin. The Dpn for chicory inulin is 8.1while that of

dahlia inulin is (29) as shown by Moerman *et al*., (2004). The present study verifies that inulinase activity was reduced under optimized environmental parameters when dahlia inulin was used as the enzyme substrate.

CHAPTER 4: CHARACTERISATION OF THE CRUDE ENZYME INULINASE

4.1 INTRODUCTION

The knowledge and understanding of the influence of temperature and pH on enzyme activity is important for determining the optimum reaction rates. High temperature and low pH may decrease the risk of contamination, improve the solubility of substrates, and reduce the colour formation of the syrup (Cazetta *et al*., 2005).

The parameters may vary according to the nature of the fermentation process (SSF or SmF); substrate; type of microbial species and purification process. In industrial enzymatic preparations it is important to know how these parameters affect the characteristics of the enzyme in order to select the best substrate, microorganism, and fermentative process that are economically feasible and that produce a product with desirable characteristics. For example, there are two major problems associated with industrial application of inulinases for production of fructo-oligosaccharides by inulin: (1) the low solubility of inulin at moderate temperatures and (2) the gret risk of microbial contamination at moderate temperatures. To avoid these risks, the industrial process for FOS production has been carried out at 60 °C (Gill *et al*., 2006).

Higher thermostability of the enzyme also reduces the cost of the production process, because a lower quantity of the enzyme is required to produce the desired product (Singh *et al*., 2006). However, inulinases are said to lose their activity after a few hours at these high temperatures, which may result in increased cost of production. Therefore, there is an increasing need for production and characterization of thermostable inulinases. Enzyme characterization is crucial to establish the operational conditions and to determine the optimum values for pH and temperature and the stability of the enzyme (Treichel *et al*., 2012).

4.2 MATERIALS AND METHODS

4.2.1 Preservation and Culturing of Strains

The yeast cultures *Kluyveromyces marxianus* CBS 4836 and *Saccharomyces cerevisiae* ATCC 9763 were obtained from The Centraalbureu voor Schimmelcultures (CBS) fungal biodiversity center, Netherlands.

The strains were stored on defined media containing (g/l): glucose 20, yeast extract 10, peptone 10, and agar 20. The cultures were streaked on the agar plates, incubated at 37 °C for 24 hours and stored at 4 °C. The yeast cultures were sub-cultured weekly (Xiong *et al*., 2007).

4.2.2 Inoculum Preparation

K. marxianus cultures were prepared in the following defined media (g/l): Yeast extract 10, peptone 20, and inulin 10. *S. cerevisiae* cultures were prepared in the following defined media (g/l): Yeast extract 10, peptone 20, and glucose 10. A loopful of cells was transferred from the plates to a 100 ml Erlenmeyer flask containing 20 ml culture medium and incubated for 12 hours on a rotary shaker operating at 120 rpm at 30 °C (modified from Xiong *et al*., 2007). Only a cultures with an optical density (OD) of 0.8 were used as inoculum in SSF.

4.2.3 Solid State Fermentation (SSF) under optimised conditions

4.2.3.1 Substrate preparation

Garlic cloves, parsnips, amadumbe, and wheat bran were used. All the substrates were obtained from a local vegetable market and dried in an oven at 65 °C for 48 hours; to a moisture content of 3-4% for garlic cloves, parsnips and amadumbe; and 0% moisture content for garlic peel and wheat bran. The dried substrates were ground using a Fritch pulverisette 14 bench-top grinder to a particle size of 0.5 mm mm.

Ten grams solid substrates were supplemented with 2.5 ml of an acidified mineral solution containing 0.3 mg $MnSO_4.H_2O$, 0.9 mg $FeSO_4.6H_2O$, 0.25 mg $ZnSO_4.7H_2O$, and 0.35 mg $CaCl_2$ (modified from Selvakumar and Pandey 1999) for 10 g dry substrate in a 250 ml Erlenmeyer flasks, sealed with a non-absorbent cotton wool plug and autoclaved at 121 ^oC for 20 min. The substrates were inoculated with 4 ml culture (3 ml *K. marxianus* and 1 ml *S. cerevisiae*) to achieve a moisture content of 65%. CSL (5% for garlic and 8% for parsnips, wheat bran and amadumber) glucose (1% wheat bran and 2% amadumbe) was also added. The substrates were incubated in a humidity chamber at relative humidity of 80% at 30 ^oC for 48 hours.

4.2.4 Extraction of Enzyme

The enzyme was extracted using 50 ml of 0.1M sodium acetate buffer pH5.5 stirred on an orbital shaker at 150 rpm, 53 °C for 40 min. The crude extract was filtered with a muslin cloth and centrifuged using an Eppendorf centrifuge, rotor no: 4-2-81 for 20 min at 4 °C. The resulting clear supernatant was used for the enzyme assay (Bender *et al.*, 2008).

4.2.5 Enzyme Assay

The enzyme activity was determined measuring the reducing sugars released by the hydrolysis of dahlia inulin by the supernatant. The enzyme extract (100 µl) was mixed with 900 µl of inulin solution (2% w/w) in 0.1M acetate buffer pH4.8 and incubated at 50 ^{o}C for 10 min. The enzyme reaction was stopped by boiling in a water bath for 10 min and assayed for reducing sugars by the DNS method (Miller 1959).

A calibration curve was prepared with a fructose solution of known strength and blanks (enzyme blank and reagent blank) were run simultaneously with enzyme and substrate solutions. One unit of inulinase activity was defined as the amount of enzyme which produced 1 µmol of fructose under the assay conditions. The ratio of inulinase (I) to invertase (S) activity was also calculated. One unit of inulinase activity was defined as the amount of enzyme which produced 1 µmol of fructose per minute under the assay conditions.

4.2.6 Determination of Optimum pH and Temperature

The effect of pH on inulinase activity was determined within the pH range of 2-9 at 50 ^{o}C for 20 min, using 0.1M acetate buffer (pH4 and pH5), 0.1 M citrate buffer (pH6), 0.1 M sodium phosphate buffer (pH7), 0.1 M Tris- HCl buffer (pH8-9). The optimum temperature for inulinase activity was determined by incubating the crude enzyme-substrate mixtures for 20 min at various temperatures (20–90 ^{o}C) in the best buffer and the liberated reducing sugars were measured.

4.2.7 Determination of pH and Thermostability of Inulinase

The pH stability of the inulinase was determined by incubating the crude enzyme in sodium acetate buffer (pH5) at 50 ^{o}C. Aliquots of 100 µl were removed at time intervals of 0, 10, 20, 30, 60, 90,

120, 150 and 180 min, respectively and assayed by incubating with 900 µl of substrate as mentioned in section 4.2.5. The thermal stability of the crude inulinase was prepared in sodium acetate buffer (pH5). The crude enzyme was incubated at different temperatures 50 ^{o}C, 60 ^{o}C, 70 ^{o}C, 80 ^{o}C and 90 ^{o}C. Aliquots of 100 µl were removed at time intervals of 0, 10, 20, 30, 60, 90, 120, 150, and 180 min and assayed for inulinase activity.

4.2.8 Determination of Enzyme Kinetics (K_m and V_{max})

The K_m value and maximum reaction velocity (V_{max}) for inulin were determined by the method of Lineweaver–Burk (1934) plots. The following calculation was used from the plots i.e.

$Y = MX + C$

Where: $C = 1/Vmax$ and $M = Km/Vmax$

$Vmax = 1/(1/Vmax)$

$Km = Vmax * Km/Vmax$

The crude inulinase was incubated with different substrate concentrations ranging from 0.2 to 4 mg/ml in 0.1M sodium acetate buffer pH5 at 45 °C and measured activity as described in section 4.2.5.

4.3 RESULTS

Table 4: Relationship between Inulinase and invertase activity of the crude extracts obtained from garlic cloves, parsnips, wheat bran and amadumbe

Carbon Source	**Inulinase Activiy (IU/gds)**	**Invertase activity (IU/gds)**	**I/S ratio**
Garlic cloves	48 .252	494.84	0.097
Parsnips	33 350	302.75	0.109
Wheat bran	17 .750	161.90	0.105
Amadumbe	10 .749	78.74	0.127

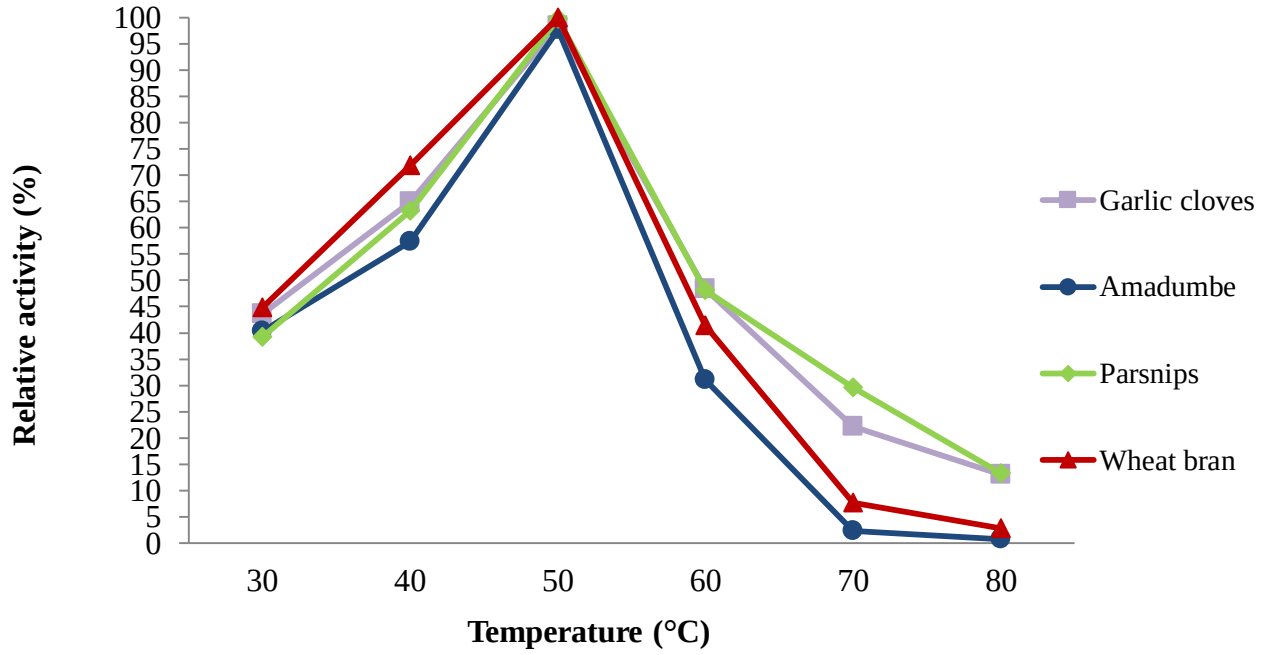

Figure 14: The effect of temperature on the activity of the crude inulinase after incubation for 20 minutes at pH5. Each value represents the average of triplicate determinations with standard deviations

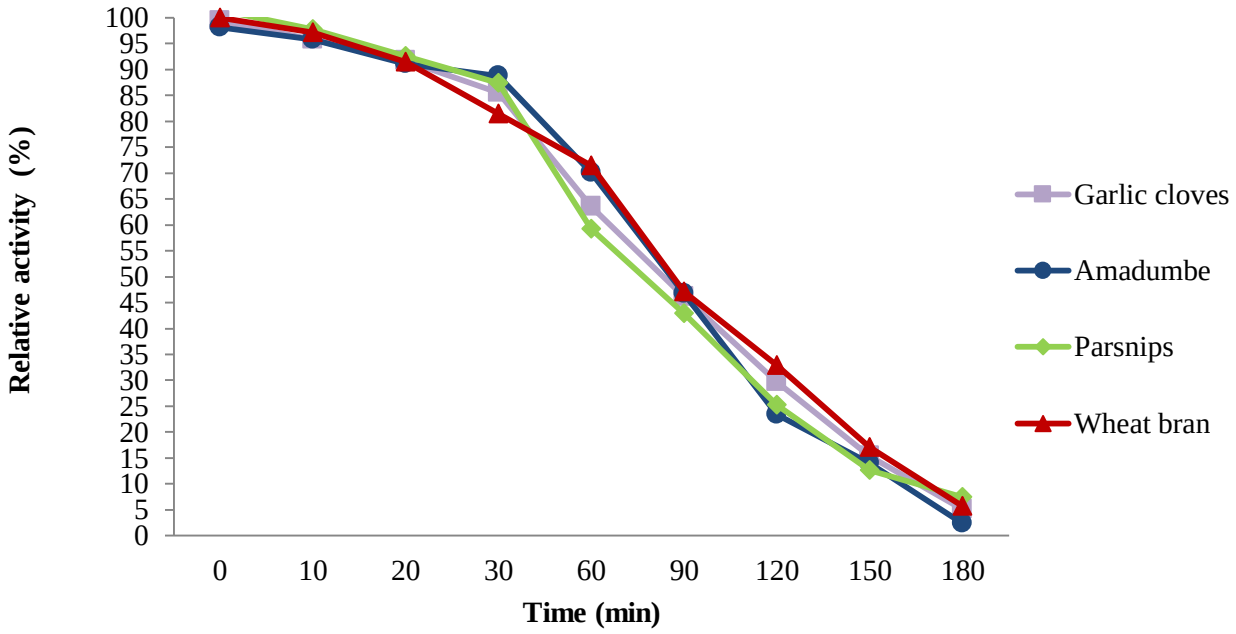

Figure 15: The effect of temperature on the activity of the crude inulinase after incubation for 3 hours at pH5. Each value represents the average of triplicate determinations with standard deviations

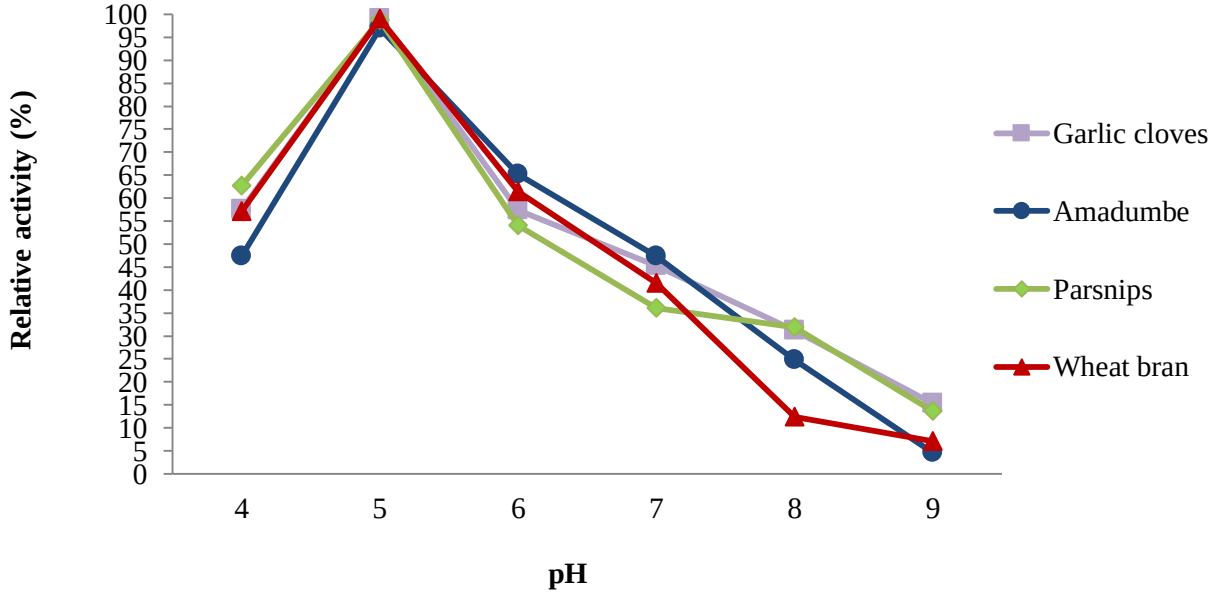

Figure 16: The effect of pH on the activity of the crude inulinase after incubation at 45 ºC for 20 minutes. Each value represents the average of triplicate determinations with standard deviations

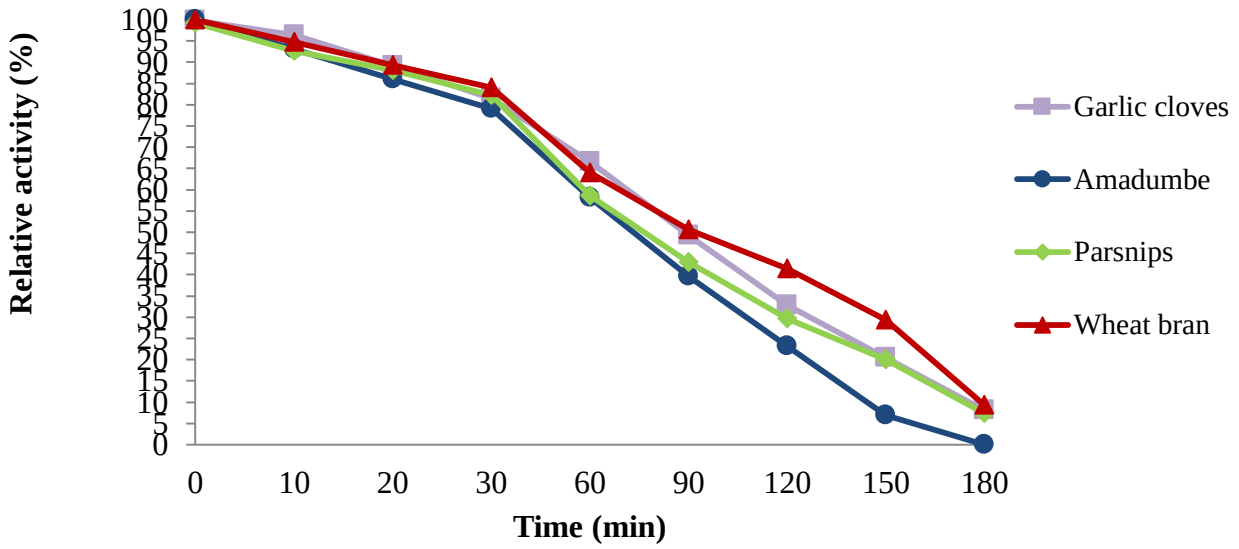

Figure 17: The effect of pH (pH5) on the activity of the crude inulinase after incubation at for 3 hours. Each value represents the average of triplicate determinations with standard deviations

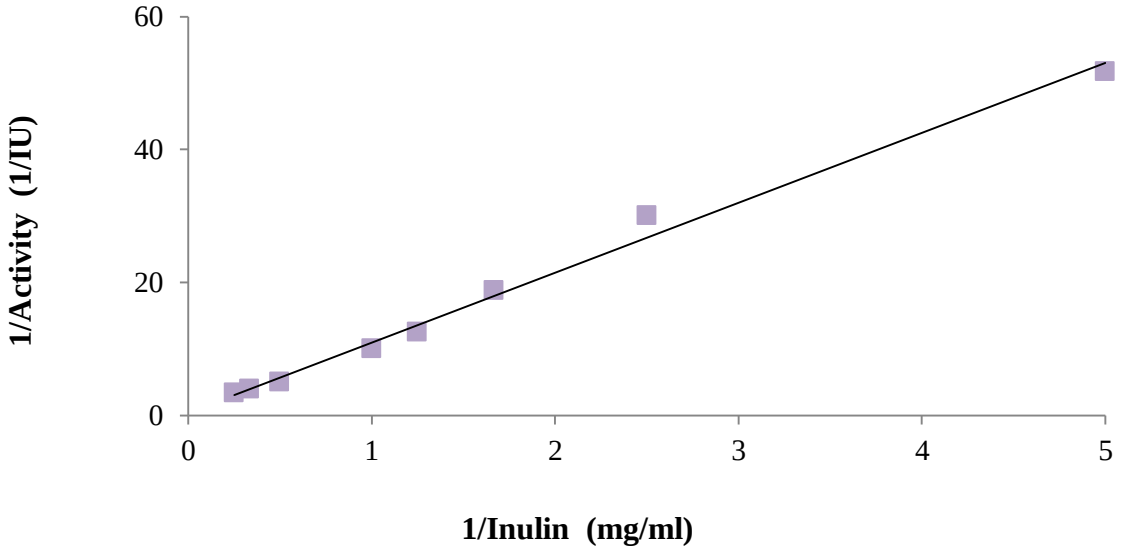

Figure 18: Determination of K_m and V_{max} of the crude inulinase from a mixed culture of *K. marxianus* and *S. cerevisiae* growing on garlic cloves at pH5 and temperature of 50 °C. Each value represents the average of triplicate determinations with standard deviation

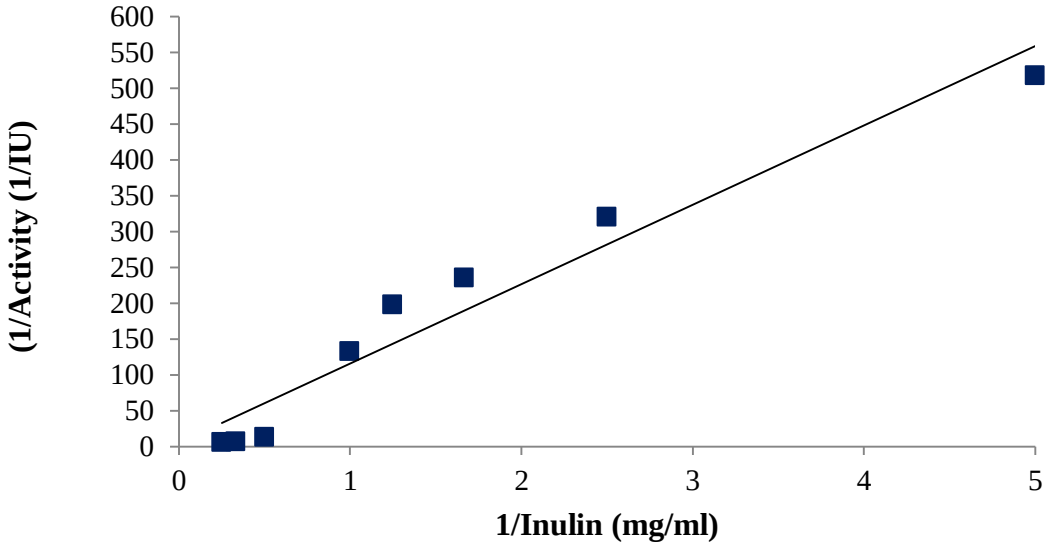

Figure 19: Determination of K_m and V_{max} of the crude inulinase from a mixed culture of *K. marxianus* and *S. cerevisiae* growing on amadumbe at pH5 and temperature of 50 ºC. Each value represents the average of triplicate determinations with standard deviations.

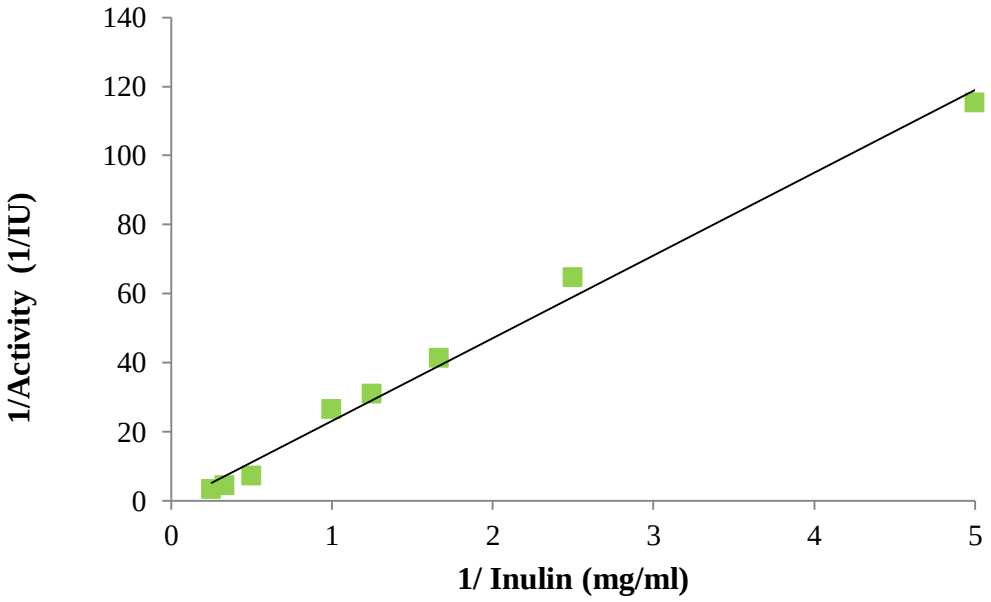

Figure 20: Determination of K_m and V_{max} of the crude inulinase from a mixed culture of *K. marxianus* and *S. cerevisiae* growing on parsnips at pH5 and temperature of 50 ºC. Each value represents the average of triplicate determinations with standard deviations

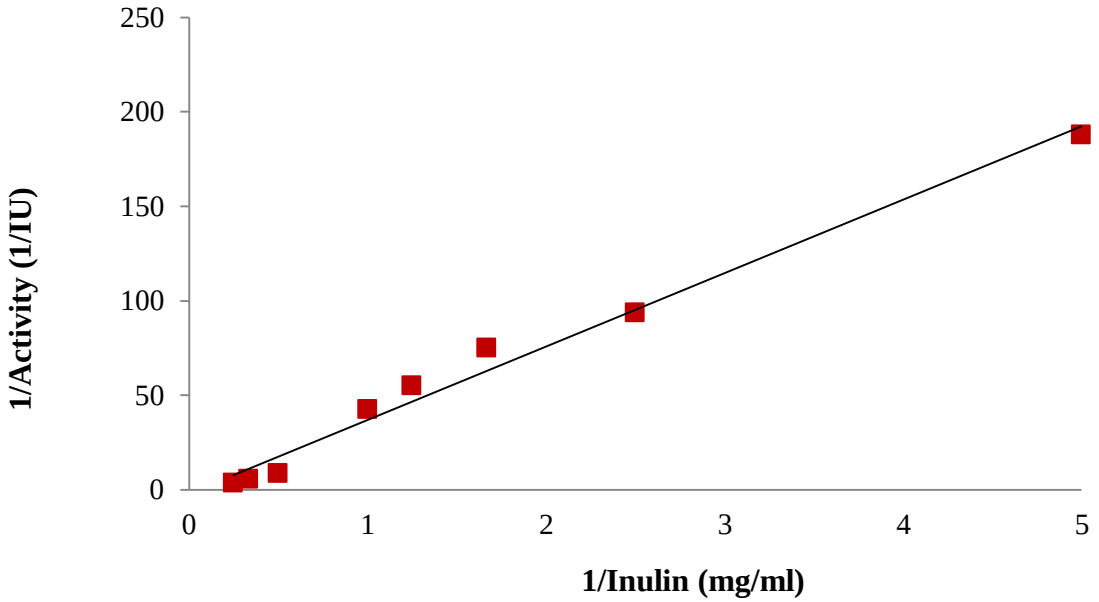

Figure 21: Determination of K_m and V_{max} of the crude inulinase from a mixed culture of *K. marxianus* and *S. cerevisiae* growing on wheat bran at pH5 and temperature of 50 ^oC. Each value represents the average of triplicate determinations with standard deviations.

4.4 DISCUSSION AND CONCLUSION

This chapter reports the characterization of the crude inulinase from the mixed culture of *K. marxianus* and *S. cerevisiae*. The inulinase exhibited high catalytic activity of inulinase, good thermal stability and low pH optimum which makes it a suitable choice for industrial applications.

4.4.1 Determination of the I/S Ratio

Microbial preparations of inulinase possess remarkable invertase activity accompanying the inulinase activity. Their catalytic activity is described in terms of I/S, ratio which describes the ratio of the activity of enzyme preparation on inulin and sucrose (Jain *et al*., 2012). The (I/S) hydrolysis ratios of the enzymes were 0.097 (garlic cloves), 0.109 (parsnips), 0.127 (amadumbe) and 0.105 (wheat bran) (Table 4). Similar I/S ratios were observed in all the crude extracts indicating a corresponding relationship between the invertase and inulinase activity. The ratios in a range of 0.02-7.9 have been reported in literature (Moriyama *et al*., 2002).

4.4.2 Optimum Temperature and Thermostability

Temperature and pH have a great influence on enzyme activity and the knowledge of this may result in the determination of optimum reaction rates for fermentation. High temperature and low pH may decrease the risk of contamination, improve the solubility of substrates, and can reduce development of undesirable colour formation in syrups (Treichel *et al*., 2012).

The inulinase activity exhibited by the crude enzyme from the inulin-containing substrates measured after incubation at temperatures of 30–80 ^{o}C (Figure 14) showed that the optimum activity was achieved at 50 ^{o}C. At 80 ^{o}C, inulinase activity could still be observed, showing 10% relativity with garlic cloves and parsnips. With the amadumbe, not much enzyme activity was initially obtained from the substrate; activity of the enzyme at 80 ^{o}C resulted in less than 1% relativity. These results suggested that high temperature levels and low concentration of the enzyme influence the catalytic activity of the enzyme. The stability of crude enzyme at 50 ^{o}C (Figure 15) was observed up to 60 minutes, where 60% of its optimum activity was retained, and decreased as time increased. After 60 minutes of incubation inulinase activity began to decrease dramatically resulting in only 40% relativity with the garlic cloves and parsnip extracts after 90 minutes incubation.

Previous research indicates that purified inulinases are optimally active at 50-60 ^{o}C. Strains of *Kluyveromyces* sp. produced inulinases within temperature optima of 50-55 ^{o}C (Mazutti *et al*., 2010; Pandey *et al*., 1999; Kushi 2000). These results indicates that the inulinase produced has comparable temperature optimum to that obtained from *K. marxianus* reported by Mazutti *et al*., (2010); Pandey *et al*., (1999) and Kushi *et al*., (2000).

4.4.3 Optimum pH and pH Stability

The pH optimum of the enzyme extracts was also studied in pH range 4-10 using acetate buffer, citrate buffer, phosphate buffer and tris-HCl buffer with suitable pH values. According to the data presented in Figure 16, optimal activity of the enzyme was obtained at pH5 and the enzyme remained stable up to 30 minute before the activity decreased. After 90 minutes of incubation at pH5 (Figure 17), stability could be observed at 50% with garlic cloves and wheat bran crude extracts and at 40% with the parsnips and amadumbe extracts. Optimum pH of inulinases from

moulds and yeasts vary in general, in the range of 4.5 – 6. Inulinases from *K. marxianus* with pH optimum of 3.5-4.75 have been reported (Mazutti *et al*., 2007; Mazutti *et al*., 2010; Kushi *et al*., 2000). Inulinase produced in the current study has similar pH ranges as those reported by Mazutti *et al*., (2007); Mazutti *et al*., (2010); Kushi *et al*., (2000).

Most industrial FOS production are carried out at 60 °C due to limited solubility of inulin at room temperature and the risk of contamination at room temperature and high pH values (Treichel *et al*., 2012). The inulinase produced has a potential to be used in industrial applications of inulinases due to its low pH optimum and stability; and also high temperature optimum and thermostability.

4.4.4 Enzyme Kinetics (K_m and V_{max})

The Lineweaver-Burk plot in Figures 18-21, shows the Michaelis-Menten constant K_m and V_{max} values for the crude inulinase from garlic cloves, parsnips, amadumbe and wheat bran. Respectively the apparent K_m values were 21.95 mM, 31.59 mM, 19.79 mM and 25.73 mM with low V_{max} values 2.08 µM/min, 1.38 µM/min, 0.51 µM/min and 0.23 µM/min. calculations we done as described in section 4.2.8.

Inulinases with both higher and lower K_m values have been reported from the *Kluyveromyces* species. The K_m for the hydrolysis of inulin by inulinases from *K. marxianus* has previously been reported as 2.28 mM (Kim *et al*., 2004) and 3.92 mM (Ku *et al*., 1994). Kushi *et al*., (2000) characterized inulinases from *Kluyveromyces* sp. with K_m value 86.9 mg/ml, Rouwenhorst *et al*., (1990) (11.9 mM) and Singh[a] *et al*., (2007) (3.92 mM). The K_m of inulinases from *M. guilliermondii* and *C. aureus* were reported at 21.10 mg/ml (Gong *et al*., 2008) and 20.06 mg/ml (Sheng *et al*., 2008). K_m values of inulinases with inulin ranges from 0.003 mM to 60 mM have also been reported (Arand *et al*., 2002; Azhari *et al*., 1989).

Lower K_m values reveal greater affinity of the enzyme towards the substrate (Singh[a] *et al*., 2007). The high K_m values of the inulinase can be attributed to higher dilution of the protein within the crude enzyme.

CHAPTER 5: PARTIAL PURIFICATION OF INULINASE USING THE AMMONIUM SULPHATE PRECIPITATION METHOD

5.1. INTRODUCTION

Microbial enzymes can be classified into three major groups according to its application: those which synthesize useful compounds; those which stereospecifically carry out important bioconversion reactions; and those which are able to hydrolyse polymers into monomers. Microbial inulinase are able to hydrolyse inulin, a plant polymer, into pure fructose and glucose (Vranesic *et al*., 2002).

Microbial inulinases can be divided into exo- and endo-acting enzymes relating to their mode of action on inulin compounds. Apart from hydrolysing sucrose and raffinose, the exo- acting exo-inulinases (β-D-fructan fructohydrolase; EC 3.2.1.80) split off the terminal fructose residues from the non-reducing end of inulin. Endo inulinases (2,1-β-D-fructan fructanohydrolase; EC 3.2.1.7) are specific for inulin and hydrolyse the terminal β-2,1-fructo furanosidic linkages to yield inulo-oligosaccharides such as inulotriose, inulotetraose and inulopentaose (Vandamme and Derycke 1983; Singh and Gill 2006). Inulin has gained great interest as a raw material for commercial production of fructose syrup and fructo-oligosaccharides. Inulin and inulinases can be used for the production of ultra-high fructose syrups where over 95% fructose yield can be produced by exo acting inulinases and oligofructoside syrup by endo-acting inulinases (Mazutti *et al*., 2006).

A number of filamentous fungi (*Aspergillus* sp., *Penicillium* sp., *Fusarium* sp., *Geotrichum* sp., *Rhizoctonia* sp., *Chrysosporium* sp.), yeasts (*Kluyveromyces* sp., *Candida* sp., *Pichia* sp., *Cryptococcus* sp., *Yarrowia* sp., *Debaryromyces* sp.), and bacteria (*Bifidobacteria* sp., *Bacillus* sp., *Pseudomonas* sp., *Clostridium* sp., *Geobacillus* sp., *Xanthomonas* sp., *Thermotoga* sp., *Streptomyces* sp., *Arthrobacter* sp.) strains have been used for the production of inulinases (Singh and Gill 2006; Bonciu *et al*., 2011).

Yeast strains, perhaps due to easy cultivation and high enzyme yields, are the most reported in literature compared to the other microorganisms. The genus of *Kluyveromyces* is the best producer of inulinases and is of great interest for the production of inulinases since it is recognized as safe (GRAS), and accepted by the Food and Drug Administration (FDA) (Mazutti *et al*., 2006; Singh

et al., 2006). The present study focuses on the partial purification and characterization of exo-inulinases from the mixed culture of *K. marxianus* and *S. cerevisiae* using ammonium sulphate precipitation. The enzyme could be used for commercial applications of inulinase.

5.2. MATERIALS AND METHODS

5.2.1. Preservation and Culturing of Strains

The yeast cultures *Kluyveromyces marxianus* CBS 4836 and *Saccharomyces cerevisiae* ATCC 9763 were obtained from The Centraalbureu voor Schimmel cultures (CBS) fungal biodiversity centre, Netherlands. The strains were stored on defined media containing (g/l): glucose 20, yeast extract 10, peptone 10, and agar 20. The cultures were streaked on the agar plates, incubated at 37 °C for 24 hours and stored at 4 °C. The yeast cultures were subcultured weekly (Xiong *et al*., 2007).

5.2.2. Inoculum Preparation

Kluyveromyces marxianus cultures were prepared in the following defined media (g/l): Yeast extract 10, peptone 20, and inulin 10. *S. cerevisiae* cultures were prepared in the following defined media (g/l): Yeast extract 10, peptone 20, and glucose 10. A loopful of cells was transferred from the plates to 100 ml Erlenmeyer flasks containing 20 ml culture medium and incubated for 12 hours on a rotary shaker operating at 120 rpm at 30 °C (modified from Xiong *et al*., 2007). Only cultures with an optical density (OD) of at least 0.8 were used as inoculum in SSF.

5.2.3. Solid state Fermentation (SSF)

5.2.3.1 Substrate preparation

Garlic cloves, parsnips, amadumbe and wheat bran were used. All the substrates were obtained from a local vegetable market and dried in an oven at 65 °C for 48 hours; to a moisture content of 3-4% for garlic cloves, parsnips and amadumbe; and 0% moisture content for garlic peel and wheat bran. The dried substrates were ground using a Fritch pulverisette 14 bench-top grinder to a particle size of 0.5 mm.

Ten grams solid substrate supplemented with 2.5 ml of an acidified mineral solution containing 0.3 mg $MnSO_4.H_2O$, 0.9 mg $FeSO_4.6H_2O$, 0.25 mg $ZnSO_4.7H_2O$, and 0.35 mg $CaCl_2$ (modified from Selvakumar and Pandey 1999) for 10 g dry substrate in 250 ml Erlenmeyer flasks, sealed with non-absorbent cotton wool plugs and autoclaved at 121 ^oC for 20 min. The substrates were inoculated with 4 ml culture (3 ml *K. marxianus* and 1 ml *S. cerevisiae*) to achieve moisture content of 65% and incubated in a humidity chamber at relative humidity of 80% at 30 ^oC for 48 hours.

5.2.4. Extraction of Enzyme

The enzyme was extracted using 50 ml of 0.1M sodium acetate buffer pH5.5, and stirred on an orbital shaker at 150 rpm, 53 °C in for 40 min. The crude extract was filtered with a muslin cloth and centrifuged using an Eppendorf centrifuge, rotor no: 4-2-81 for 20 min at 4 °C. The resulting clear supernatant was used in the enzyme assay (Bender *et al*., 2008).

5.2.5. Enzyme Assay

The enzyme activity was determined measuring the reducing sugars released by the hydrolysis of dahlia inulin by the partially purified enzyme. The enzyme extract (100 µl) was mixed with 900 µl of inulin solution (2% w/w) in 0.1M acetate buffer pH4.8 incubated at 50 °C for 10 min and the reaction stopped by boiling in a water bath for 10 min. The enzyme reaction was assayed for reducing sugars as fructose by DNS method (Miller 1959). A calibration curve was prepared with fructose solution of known strength and blanks (enzyme blank and reagent blank) were run simultaneously with enzyme and substrate solutions. For the invertase assay fructose was replaced with glucose

5.2.6. Partial Purification of the crude inulinase

A crude enzyme solution of 50 ml was first precipitated at an ammonium sulphate concentration of 0–20% of saturation at 4 °C, overnight to remove the contaminating protein by centrifugation, The supernatant was brought to 20-80% of saturation with ammonium sulphate at 4 °C for 6 h. The precipitate was dissolved in 0.1M acetate buffer (pH5.0) and the solution was then dialysed at 4 °C in 0.01 M acetate buffer (pH5.8) to desalt (Chen *et al*., 2009).

5.2.7. Sodium Dodecyl Sulphate (SDS)–Polyacrylamide Gel Electrophoresis

SDS–PAGE was performed according to a previously described method (Laemmli 1970) on vertical slab gel with 11% polyacrylamide gel containing 0.1% SDS and Tris–glycine buffer containing 0.1% SDS (pH8.3 at 18 °C) at a constant current of 10 mA. The proteins were stained with Coomassie Brilliant Blue G-250. The molecular weight of the enzyme was estimated by SDS-PAGE, using an electrophoresis Precision plus protein standard, All Blue (Biorad) 250 kDa, 150 kDa, 100 kDa, 75 kDa, 50 kDa, 37 kDa, 25 kDa, 20 kDa, 1 5kDa and 10 kDa.

5.2.8. Protein Determination

Protein content of the crude enzyme of garlic cloves, parsnip, wheat bran and amadumbe was determined as previously described by Bradford (1976), using bovine serum albumin as a standard (Sigma Chemical Co.). Then plot the standard curve by taking concentration of protein along X-axis and absorbance at 595 nm along Y-axis. From the standard curve the concentration of protein in the sample was calculated.

5.2.9. Thin Layer Chromatography

For the identification of fructose, thin layer chromatography was carried out on silica gel 60 F254 (Merck) precoated plates, using the solvent system containing (in volume ratio) ethyl ether/ acetic acid/ propanol/ formic acid/ water (25:10:5:1:15). The resulting chromatograms were visualized by spraying with phenol- sulphuric acid solution, modified from Naidoo *et al.*, 2009.

5.3. RESULTS

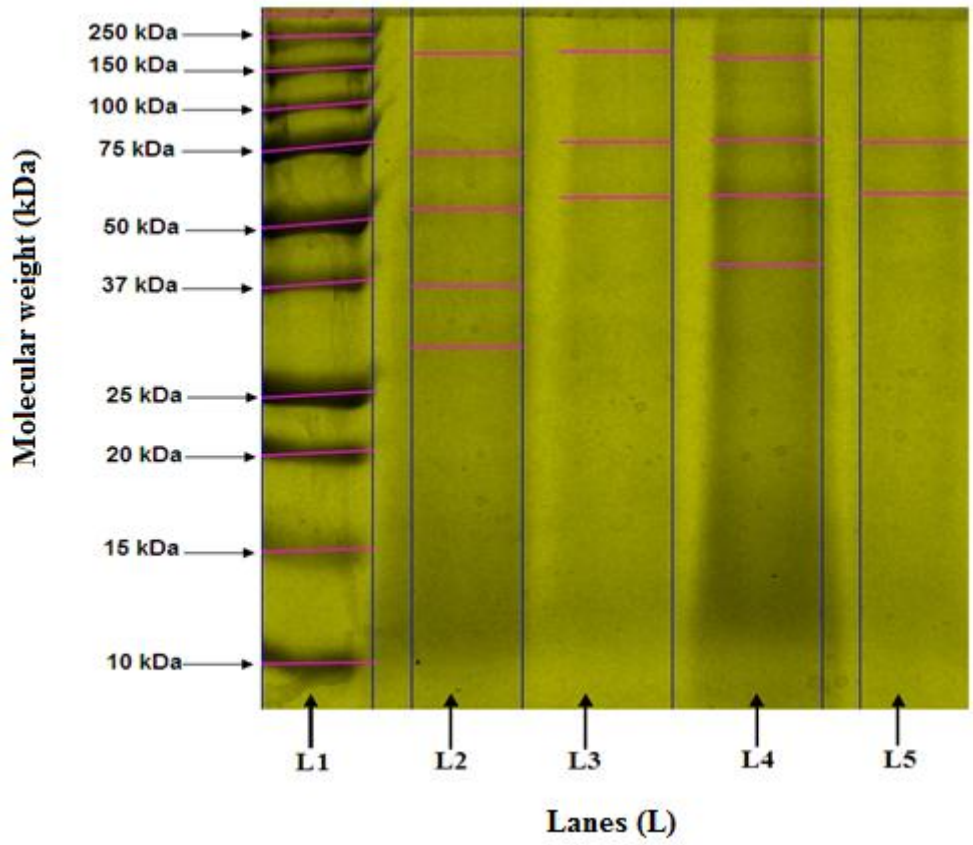

Figure 22: SDS PAGE of the partially purified *K. marxianus- S. cerevisiae* enzyme L1: Molecular weight standards, L2: Partially purified inulinase from amadumbe, L3: purified inulinase from garlic cloves, L4: purified inulinase from wheat bran, L5: purified inulinase from parsnips

Table 5: Summary of partial purification and yield of inulinase from the supernatant of the *K. marxianus* and *S. cerevisiae* mixed culture grown on garlic cloves, amadumbe, parsnips and wheat bran

Purification step	Total protein (mg)	Inulinase activity (U/gds)	Yield (%)	Specific activity (U/mg)	Purification factor
Garlic cloves					
Crude enzyme	527	88.5	100	0.168	1.0
$(NH_4)_2 SO_4$ precipitation	0.91	8.22	9.28	9.03	53.75
Amadumbe					
Crude enzyme	266	27.50	100	0.103	1.0
$(NH_4)_2 SO_4$ precipitation	195	16.30	59.27	0.08	0.78
Parsnips					
Crude enzyme	496	63.50	100	0.127	1.0
$(NH_4)_2 SO_4$ precipitation	1.18	4.87	7.68	4.12	32.44
Wheat bran					
Crude enzyme	283	33.8	100	0.19	1.0
$(NH_4)_2 SO_4$ precipitation	180	24.03	71.09	0.133	1.22

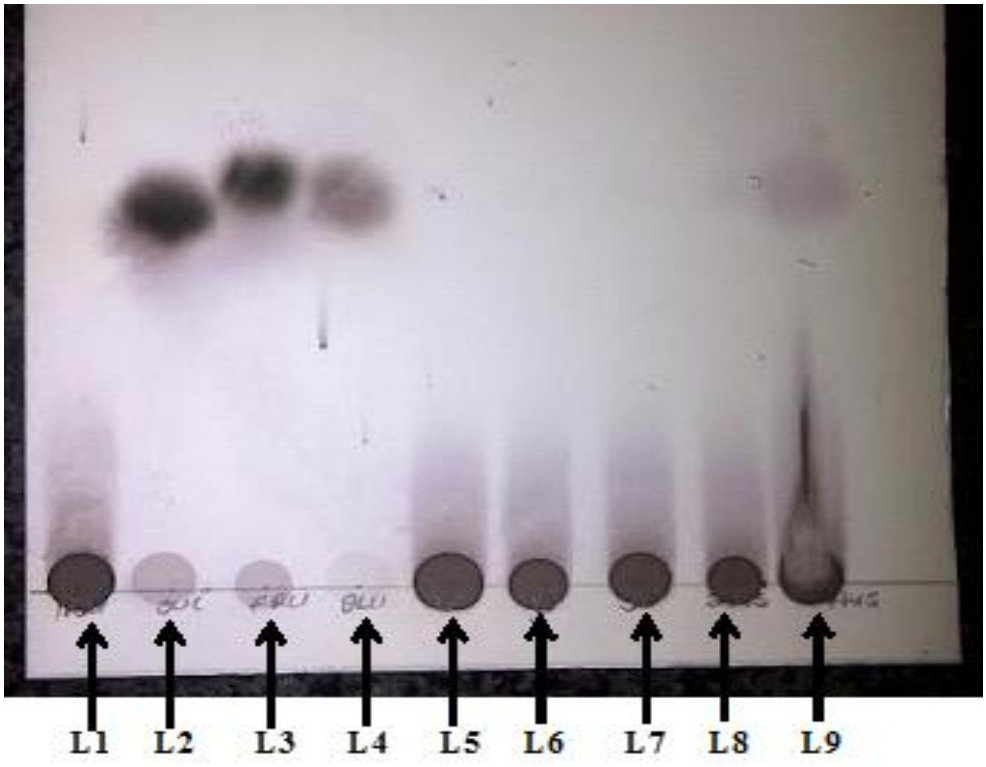

Figure 23: TLC of reaction formed during hydrolysis of inulin from a mixed culture of *K. marxianus* and *S. cerevisiae* cultivated on garlic cloves. L1- inulin, L2- sucrose, L3- fructose, L4- glucose, L5- enzyme + inulin (0 hours), L6- enzyme + inulin (10 min), L7- enzyme + inulin (30 min) , L8- enzyme + inulin (2 hours) , L9- enzyme + inulin (24 hours)

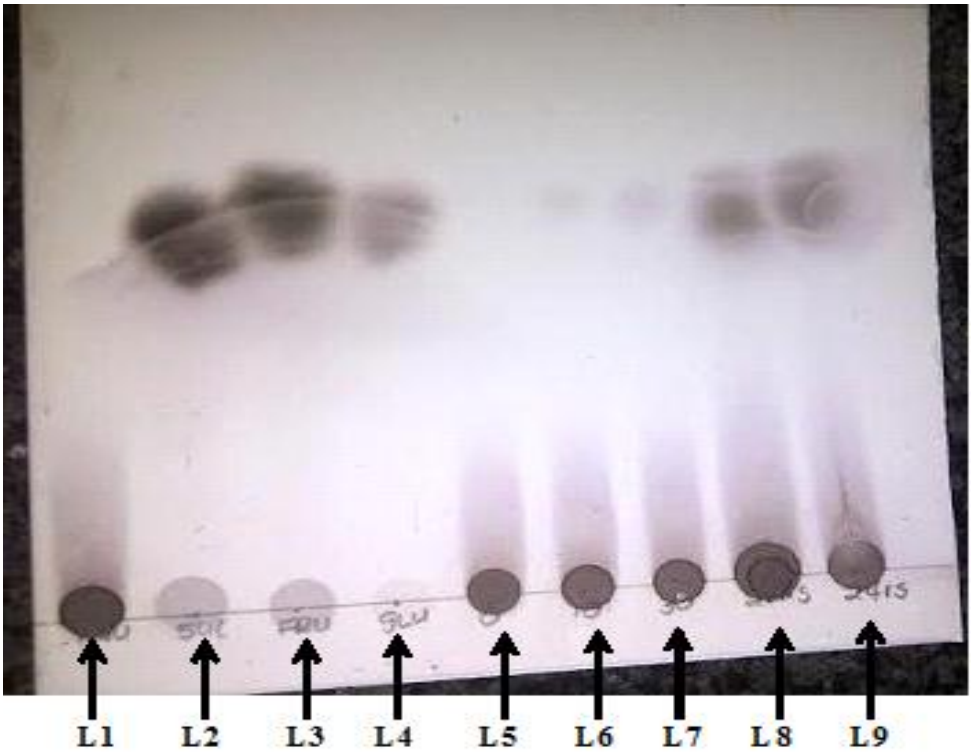

Figure 24: TLC of reaction formed during hydrolysis of inulin from a mixed culture of *K. marxianus* and *S. cerevisiae* cultivated on the amadumbe. L1- inulin, L2- sucrose, L3- fructose, L4- glucose, L5- enzyme + inulin (0 hours), L6- enzyme + inulin (10 min), L7- enzyme + inulin (30 min) , L8- enzyme + inulin (2 hours) , L9- enzyme + inulin (24 hours)

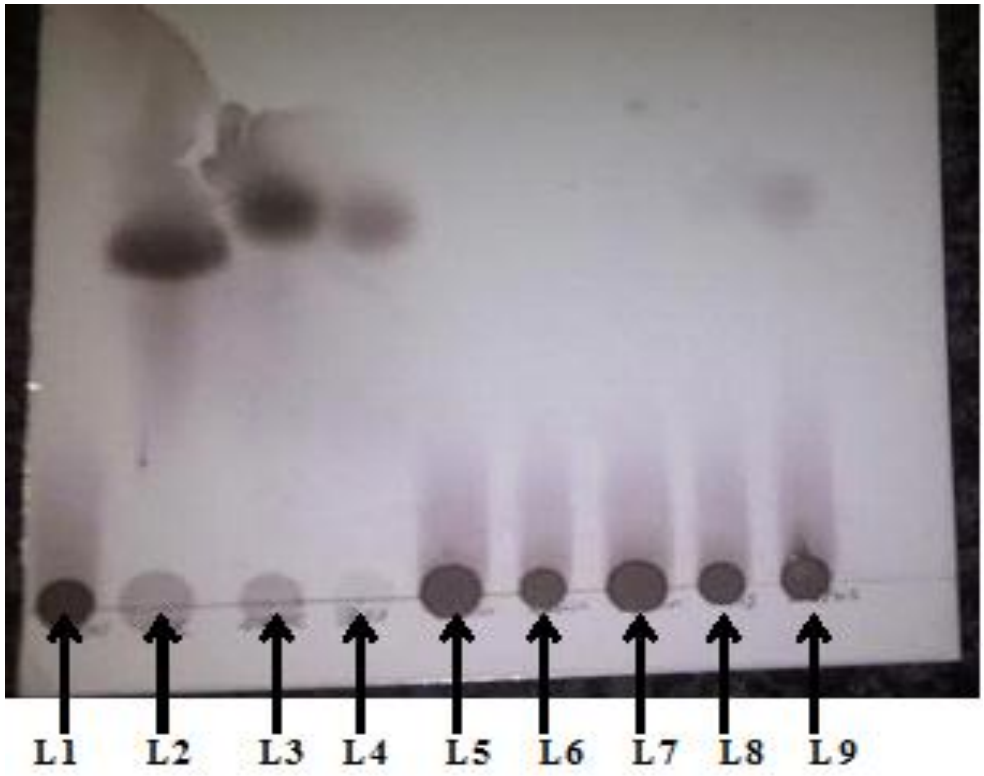

Figure 25: TLC of reaction formed during hydrolysis of inulin from a mixed culture of *K. marxianus* and *S. cerevisiae* cultivated on the parsnips. L1- inulin, L2- sucrose, L3- fructose, L4- glucose, L5- enzyme + inulin (0 hours), L6- enzyme + inulin (10 min), L7- enzyme + inulin (30 min) , L8- enzyme + inulin (2 hours) , L9- enzyme + inulin (24 hours)

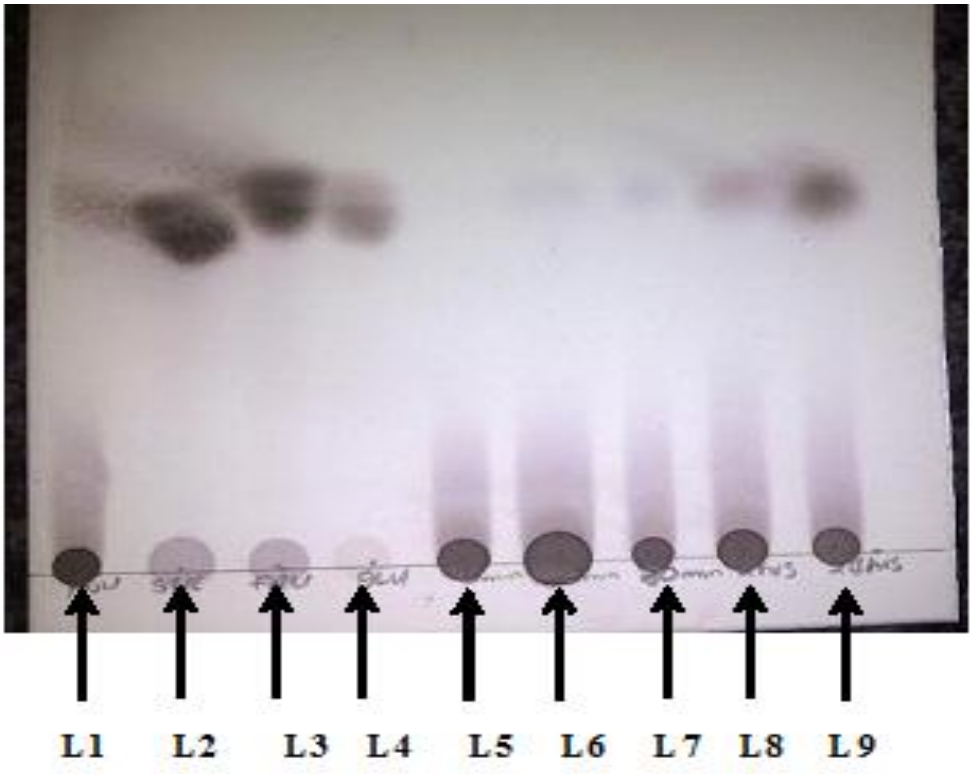

Figure 26: TLC of reaction formed during hydrolysis of inulin from a mixed culture of *K. marxianus* and *S. cerevisiae* cultivated on the wheat bran. L1- inulin, L2- sucrose, L3- fructose, L4- glucose, L5- enzyme + inulin (0 hours), L6- enzyme + inulin (10 min), L7- enzyme + inulin (30 min) , L8- enzyme + inulin (2 hours) , L9- enzyme + inulin (24 hours)

5.4 DISCUSSION AND CONCLUSION

5.4.1 Partial Purification of Inulinase

The purity of the inulinase was studied using 0.1% SDS-PAGE. After partial purification the inulinase was stained using Coomassie brilliant blue; in all of the extracts (amadumbe, garlic cloves, wheat bran and parsnips) two pattern bands were obtained (Figure 22). The molecular weight determination was carried out by comparing the values of the bands with the standard of known values obtained by SDS-PAGE. The molecular weight of the two bands formed was estimated at 55 kDa and 70 kDa respectively. Another band was also observed from the wheat bran crude extract at 40 kDa.

Most of the known inulinases from the genus *Kluyveromyces* have been reported to have a molecular mass within the region of 50 kDa (Chi *et al*., 2009). A considerable variation in molecular weight of inulinases has been reported earlier namely, *Arthrobacter* sp. (75 kDa),

Bacillus stearothermophilus KP1289 (54 kDa), *Aspergillus candidus* (54 kDa), *Penicillium* sp. TN-88 (6 8kDa), *Kluyveromyces marxianus var. bulgaricus* (57 kDa), *Streptomyces* sp. (45 kDa) (Kang *et al*., 1998; Kato *et al*., 1999; Kochhar *et al*., 1999; Kushi *et al*., 2000; Nakamura *et al*., 1997; Sharma and Gill 2007). Pandey *et al*., (1999) characterised inulinase from *K. fragilis* with a molecular weight of 250 kDa.

The yield of the purified inulinase from garlic cloves and amadumbe was 9.28% and 7.68% respectively and had specific activity of 9.03 and 4.12 U/mg, indicating a loss in protein content during salt precipitation, perhaps through inadequate precipitation of the protein. Purified inulinase from the amadumbe and wheat bran showed specific activity of 0.08 and 0.133 U/mg respectively, and product yield of 59.27 and 71.09%. Singh[b] *et al*., (2007) in their two-step partial purification study reported that inulinase from *Kluyveromyces* sp could not be precipitated successfully by ammonium sulphate saturation. However further purification will have to be conducted for a similar conclusion to be reached in this study.

5.4.2 Inulin hydrolysis by partially purified inulinase

The Retardation factor (Rf) values for the standards were as follows: inulin (0), Sucrose (0.6), Fructose (0.65), Glucose (0.63), and that of the released fructose after enzyme hydrolysis was (0.66), indicating moderate to high polarity.

TLC indicated that hydrolysis of inulin resulted in the production of fructose residues in increasing concentrations. Monosaccharide residues (fructose and glucose) were observed in increasing concentrations from 10 min to 24 hours, which suggests the presence of the exo-acting inulinase in the *K. marxianus* from the mixed culture. The results also indicate the presence of invertase from *S. cerevisiae*, since invertase possesses the ability to hydrolyse terminal inulin residues to produce release glucose and fructose.

Monosaccharides' release from the hydrolysis of inulin by partially purified inulinase from mixed culture grown on garlic cloves (Figure 23) and parsnips (Figure 25) was very low in concentration after 30 min. Increased concentrations were observed at 2 hours and after prolonged enzyme hydrolysis (24 hours).

The steps in the purification process indicated a loss in protein quantity during the purification process. Reports on inulin hydrolysis by purified inulinases from cultures grown on solid substrates of garlic cloves are yet to be reported. In addition, since parsnip is a novel substrate in this study, no previous reports on its purification exist.

TLC results of the partially purified inulinase obtained from the amadumbe and wheat bran (Figures 22 and 24, respectively) showed increased hydrolysis of inulin. Fructose and glucose residues were observed after 10 minutes and in increasing concentrations up to 24 hours.

Hydrolysis of inulin in this instance did not yield any oligosaccharides. In contrast to these findings, Kushi *et al*., (2000) obtained both monosaccharides and oligosaccharides from the hydrolysis of inulin by exo-inulinase from *Kluyveromyces marxianus var. bulgaricus*.

CHAPTER 6: GENERAL DISCUSSION

Enzymes are a part of a fast growing biocatalyst industry. In the early 21st century, the estimated value of the world enzyme market was U$ 1.3 billion (Leisola *et al*., 2011). The industrial enzyme business is rapidly growing due to improved production technologies, engineered enzyme properties, and new application fields. Industrial enzymes for food applications are produced by GRAS microorganisms in large-scale bioreactors, where sometimes the production microorgabisms and enzymes are genetically engineered for maximal productivity and optimised enzyme properties. Large-volume industrial enzymes are sold as non-purified, concentrated or granulated dry products, while those used in diagnostics and DNA technology have to be of high purity. Enzymes are used in the production of inantiopure amino acids, rare sugars, production of fructose and penicillin derivatives and other chemicals (Leisola *et al*., 2011).

Enzymes are biological catalysts synthesized by plants and living organisms. They are important synthetic and degradative catalysts used in the food, chemical, medical, detergent and textile industries. For every chemically catalysed process, there is an equivalent enzyme catalysed process (Niedleman 1984). However, enzymes present properties which make them superior to chemical catalysts. They provide high catalytic power, up to 10^{14} -10^{20} the rate of non-enzymatic reactions; they can catalyse a broad range of reactions; reactions can be conducted under mild temperature (37 ºC) conditions, pH values and pressure. They possess high specificity with regard to the reactions they catalyse; the activity of an enzyme can be regulated allosterically by intracellular concentrations of key metabolites not directly involved with the reaction; there is a vast variety of enzymes readily available in nature (Niedleman 1984).

All enzymes are proteins. An enzyme contains one or more active sites which may consist of a few amino acid residues. Enzymatic reactions with substrates take place at the active site and the rest of the protein molecule is required for maintaining the three-dimensional structure of the enzyme. Substrate specificity of an enzyme differs from molecule to molecule. Many enzymes exhibit stereochemical specificity in that they catalyse the reactions of one conformation but not the other (Niedleman 1984).

According to the International Union of Biochemistry (1979), there are six classes of enzymes determined by official taxonomic dictum. The classes include: the oxidoreductases, transferases,

hydrolases, lyases, ligases and isomerases. Hydrolytic enzymes dominate the industrial enzyme industry in numbers and are of increasing interest in industrial enzyme applications (Niedleman 1984).

Hydrolytic enzymes, e.g. inulinases, are produced by plants and microorganisms (bacteria, moulds and yeasts). The reaction of inulinases involves the action of two types of enzymes: the exo-inulinase (β-D fructan fructohydrolase) which catalyses the hydrolysis of the terminal fructose units from inulin; and the endo-inulinase (2,1-β-D fructans fructanohydrolase) that splits inulin residues into inulo-oligosaccharides (FOS). Inulinases can be used for production of high fructose syrup from the saccharification of naturally occurring inulins, and endo-acting inulinases can be used for producing inulo-oligosaccharides. Microbial preparations of inulinase possess inulinase activity accompanied by invertase activity. The resulting catalytic activity is described in terms of I/S (inulinase/invertase) ratios (Vandamme and Derycke 1983; Laloux *et al*., 1991; Pessoni *et al*., 1999).

Fructose, a six-carbon monosaccharide, is produced from the hydrolysis of inulin by exo-inulinases. Fructose is found distributed widely in plant foods in a variety of forms either as free monosaccharides, complexed with glucose to form the disaccharide sucrose, or as polymerised fructans of different degrees. Fructans comprise of oligo- and polysaccharides made up of short chains of fructose units with a single D-glucosyl unit at the nonreducing end. Fructans with DP 2-9 are referred to as fructo-oligosaccharides (FOS), and the longer chains (DP ≥10) are termed inulins.

In recent years there has been considerable research interest as fructans may have wide-ranging beneficial effects on health. Proposed health benefits of fructans include prebiotic effects such as for suppressing the growth of potential pathogens in the colon, increasing stool bulking capacity, prevention of constipation and also increasing calcium absorption, maintaining the integrity of the gut mucosal barrier and increased colonic mucus production, stimulating of the gastrointestinal immune system, and reducing the risk of colorectal cancer (Muir *et al*., 2007).

The present study was aimed at investigating the potential of a mixed culture of the yeast species *Kluyveromyces marxianus* and *Saccharomyces cerevisiae* on inulin containing plant materials for inulinase production. The first objective was to evaluate or screen the potential of various inulin-

containing plant materials for maximal inulinase activity. Garlic cloves, followed by parsnips, wheat bran and amadumbe demonstrated potential as solid substrates for synthesis of inulinases. Inulinase production using garlic has been studied extensively in SSF by Dilipkumar[a] *et al.*, (2011); Dilipkumar[b] *et al.*, (2011); maximum yield of inulinase observed was 268 U/gds and 76 U/gds respectively. The selection of suitable substrates and analysis of available material are some of the important factors in designing an effective enzymatic bioprocess. Low-cost inulin-containing plant materials are preferred compared to high-cost commercially pure substrates.

It is also important to optimize the fermentation conditions and nutrients for the production medium in order to obtain higher enzyme productivity. The optimized levels of different fermentation parameters for the garlic cloves were as follows: moisture 65%, temperature 30 ºC, inoculum ratio *K. marxianus* 3:1*S. cerevisiae*, inoculum volume 2ml, carbon source 1%, nitrogen source 5%; Wheat bran: moisture 65%, temperature 30 ºC, inoculum ratio *K. marxianus* 3:1, *S. cerevisiae*, inoculum volume 1ml, carbon source 1%, nitrogen source 10%; for the parsnips: moisture 65%, temperature 30 ºC, inoculum ratio *K. marxianus* 3:1, *S. cerevisiae*, inoculum volume 4ml, carbon source 2%, nitrogen source 8%; for the amadumbe: moisture 65%, temperature 30 ºC, inoculum ratio *K. marxianus* 3:1, *S. cerevisiae*, inoculum volume 2ml, carbon source 2%, nitrogen source 8% for the wheat bran.

Moisture plays a crucial role in solid state production of inulinase as enzyme yields increased by over 40% with substrates under moisture optimized conditions. The volume of the inoculum did not affect the yield of inulinase product irrespective of the substrate. Narayanan *et al.*, (2013) studied the effect of inoculum volume on inulinase synthesis and encountered similar observations. They concluded that, in order to obtain sufficient biomass which will inhibit growth of undesirable microbes, it is important to provide the SSF with optimal volume of inoculum. However, the volume of the inoculum should not result in excess growth of biomass thereby supressing enzyme production in the SSF. Selvakumar and Pandey (1999) and Narayanan *et al.*, (2013) also observed no increase in inulinase activity in relation to inoculum volume. The carbon source in the form of glucose, introduced to the garlic cloves and parsnips solid substrates showed no sizable effect on inulinase production during fermentation due to the high sugar concentrations contained within these plant materials.

The highest inulinase production was achieved using various fermentation parameters and media components depending on the solid substrate used. Maximal production of inulinase was attained at 82 IU/gds with the garlic cloves, 62 IU/gds with the parsnips, 38 IU/gds with the wheat bran and 25 IU/gds with the amadumbe. Maximum inulinase production was also compared using a highly polymerised Dahlia inulin (Dpn=20) as a hydrolysis substrate during inulinase assays; 37-50% reduction in activity among the substrates was observed.

The crude inulinase extracts from the mixed fungal culture was characterised in terms of pH and temperature optimum, and pH and temperature stability. The pH and thermal stability of the crude was 5% and 50% respectively with high stability in the acid to near neutral pH range. The thermal stability experiment illustrated that the crude inulinase was stable at high temperatures above 50 ºC. Inulinase exhibited high thermal stability which is common for the inulinases from *K. marxianus species*.

Crude inulinase extracts were subjected to partial purification and removal of contaminating proteins by ammonium sulphate precipitation at 20-80% saturation followed by dialysis against sodium acetate buffer pH7. After centrifugation, the protein was collected and analysed for inulinase activity. The enzyme extracts from the fermentation of amadumbe, garlic cloves, wheat bran and parsnips were purified by an average factor of 1% with activity yield from the starting crude extracts. Molecular weight of the partially purified inulinase was determined by SDS-PAGE, and two prominent bands were observed between 50 kDa and 70 kDa.

Partially purified inulinase from the mixed culture of *K. marxianus* and *S. cerevisiae* has a potential for industrial application, as it shows optimum activity in moderate temperatures and slightly acid pH. It contains hydrolytic activities which can be studied further in upscale investigations for possible HFS production of food and for the biopharmaceutical industries.

FUTURE ASPECTS

Future work of the study involves:

- Scaling up of inulinase production using a mixed culture of *K. marxianus* and *S. cerevisiae* in SSF bioreactors
- Full-scale purification and
- Immobilisation studies of the purified enzyme extract for continuous production of high fructose syrup

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