ENHANCED PRODUCTION OF INULINASE FROM

Xanthomonas campestris pv. *phaseoli*

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Submitted in complete fulfillment for the Degree of Master of Technology (Biotechnology) in the Department of Biotechnology and Food Technology, Durban University of Technology, Durban, South Africa

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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 and Food Technology, Faculty of Applied Sciences, Durban University of Technology, South Africa, under the supervision of **Prof Suren Singh** and **Prof Kugen Permaul**

_____

Student's signature

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ABSTRACT

Xanthomonas campestris pv *phaseoli* produced an extracellular endoinulinase on various carbon sources. The highest inulinase production of 9.24 ± 0.03 IU ml^{-1} by *X. campestris* pv. *phaseoli* was attained using an optimized medium comprising of 3% sucrose and 2.5% tryptone. Inulinase production in *X. campestris* pv. *phaseoli* was further enhanced through ethylmethanesulfonate mutagenesis. The resulting mutant, *X. campestris* pv. *phaseoli* KM 24 demonstrated enhanced inulinase production of 22.09 ± 0.03 IU ml^{-1} after 24 h, which was 2.4 - fold higher than that of the wild type. Inulinase production by this mutant was scaled up in a 5 L fermenter yielding a final activity of 21.87 ± 0.03 IU ml^{-1} with an inulinase/invertase (I/S) ratio of 2.6 after 18 h. Maximum volumetric (21 865 IU l^{-1} h^{-1}) and specific (119 025 IU g^{-1} h^{-1}) productivities of inulinase were attained in a fermenter after 18 h growth. Inulin hydrolysis by the crude inulinase and subsequent detection of mono- and oligosaccharides indicated the presence of an endoinulinase. The extracellular endoinulinase from the mutant KM 24 was purified to homogeneity by gel filtration chromatography and had a specific activity of 174.74 U/mg. The optimum pH and temperature of the purified enzyme were found to be 6.0 and 50^{o}C, respectively. The enzyme was stable up to 60^{o}C, retaining over 60% activity for 30 min, but activity rapidly declined at temperatures above 60^{o}C. The pure inulinase enzyme was also found to be stable between pH 6-9. The Lineweaver–Burk plots showed that the apparent K_m and V_{max} values of the inulinase for inulin were 1.15 mg/ml and 0.15µM/min, respectively. The K_{cat} value was found to be 0.145 min^{-1} with an enzyme catalytic efficiency of 0.126 mg^{-1}.ml.min^{-1}. This mutant demonstrated good potential for large scale production of inulinase and fructooligosaccharides.

CHAPTER 1

1.1. INTRODUCTION

D-fructose is a safe alternate sweetener used mostly in the food and beverage industries. This ketose is two times sweeter than sucrose, improves calcium absorption and favours the removal of ethanol from the blood of alcoholics (Kaur and Gupta, 2002). Therefore these favourable functionalities have increased the commercial demand of this sugar. Currently, D-fructose is obtained through acid hydrolysis of inulin and multi-enzymatic hydrolysis of starch (45% yield). An efficient alternative process is the hydrolysis of inulin, a β-2,1-linked fructan (terminated by a glucose residue), by microbial inulinases, which yields 95% of fructose as compared to conventional methods (Mazutti *et al*., 2005).

Inulin is usually found as a reserve fructopolymer in the roots and tubers of plants belonging to *Liliaceae* or *Compositae*. Asparagus, garlic, leek, onion, Jerusalem artichoke and chicory are fructan-containing plant species (Van Loo *et al*., 1995). Inulin has received huge interest as a renewable raw material for fructose syrup and inulooligosaccharides (IOS)/ fructooligosaccharides (FOS) production (Yun, 1996). Inulinases (fructofuranosyl hydrolases) are produced by bacteria, fungi, yeasts and plants and three types of inulinases have been described *viz.,* endoinulinase, exoinulinase and fructotransferase. However, inulin hydrolysis is generally carried out by endo- and exo-inulinases.

Endoinulinase is more specific for inulin and hydrolyses the internal β-2,1 fructofuranosidic linkages to yield FOS whereas exoinulinase splits off the terminal fructose units from inulin (Vandamme and Deryeke, 1983; Singh and Gill, 2006). Production of FOS by microbial inulinases is a cost effective method (Cho *et al*., 2001), which can be used in biorefineries and biofuel sectors to produce commodity chemicals in addition to food industries. Researchers are still striving to isolate potential inulinolytic microbial strains or improve the efficacy of available strains to meet

the commercial demands. Strain improvement has been conventionally achieved through mutation and selection (Bai *et al*., 2004). Mutagenesis with physical and/or chemical agents has been used successfully to improve the production of microbial metabolites and enzymes (Nakamura *et al*., 1994; Sharma *et al*., 2005). Among the bacteria, *Xanthomonas oryzae* is one of the highest endoinulinase producers releasing FOS from a wide range of substrates.

Xanthomonas campestris, on the other hand, is used for the production of xanthan gum (biopolymer), which can be used in food and other industrial sectors. Several studies have been reported on the optimization of *X. campestris* culture conditions for biopolymer production. The xanthan gum production ability of *X. campestris* PTCC 1473 was successfully enhanced through chemical mutagenesis (EMS) and the mutant showed a 30% improvement in xanthan yield (Kamal *et al*., 2003). Inulinase production by *X. campestris* pv. *phaseoli* has been reported from our research group (Ayyachamy *et al*., 2007). The present study was focused on improving the inulinase productivity level of *Xanthomonas campestris* pathovar *phaseoli* (*Xcp*) through chemical mutagenesis.

In this study, chapter one reviews the current knowledge, origin and industrial application of inulinases. Much emphasis is placed on inulin hydrolysis, the benefits of inulin and microbial inulinases used for FOS production. It also highlights *Xanthomonas campestris* pathovar *phaseoli*, used in this study, for the production of inulinase and FOS. Chapter two reveals the approach taken to optimize the nutritional and growth conditions of *X. campestris* pv. *phaseoli* for maximum inulinase production. Chapter three deals with strain improvement for enhanced inulinase production through ethylmethanesulfonate (EMS) mutagenesis and endoinulinase production by *X. campestris* pv. *phaseoli* KM 24 mutant at a laboratory scale fermenter level. Chapter four describes purification and characterization of endoinulinase and its kinetics studies.

1.2. LITERATURE REVIEW

1.2.1. MICROBIAL ENZYMES

1.2.1.1. Enzymes in antiquity

The use of enzymes dates back many thousands of years ago. The oldest known reference to the commercial use of enzymes comes from a description of wine making in the Codex of Hammurabi (ancient Babylon, circa 2100 B. C.). The use of microorganisms as enzyme sources for fermentation was widespread among ancient people. References to these processes can be found in writings not only from Babylon but also from the early civilizations of Rome, Greece, Egypt, China and India. Ancient texts also contain a number of references to the related products that were processed based on the enzymatic conversion. All microorganisms produce a large number of enzymes for their unique metabolic activities. But the absolute and relative amounts of the various individual enzymes produced vary markedly between species and even between strains of the same species. Hence it is customary to select strains for the commercial production of specific enzymes which have the capability of producing high amounts of the desired products.

Commercial enzymes are produced from moulds, bacteria and yeasts. Biologically active enzymes may therefore be extracted from any living organism. A wide range of sources are used for commercial enzyme production from *Actinoplanes* to *Zymomonas,* from spinach to snake venom.

1.2.1.2. Advantages of microbial enzymes

Of the hundred or so enzymes being used industrially, over one half is from fungi and yeasts, and over a third is from bacteria and the remainder divided between animal (8%) and plant (4%) sources. A very much larger number of enzymes find use in chemical analysis and clinical diagnosis. Non-microbial sources provide a larger proportion of these, at the present time. Microbes are preferred over plants and animals as sources of enzymes because they are generally

cheaper to produce and a microbial enzyme contents are more predictable and controllable. Reliable supplies of raw materials of constant composition are more easily arranged for enzyme production of microbial origin and their enzymes are more easily retrievable.

It is also a fact that plant and animal tissues contain more potentially harmful material than microbes; including phenolic compounds (from plants) and endogenous enzyme inhibitors and proteases. Microbial enzymes have several distinct advantages for industrial applications:

i. They are of natural origin and are usually non-toxic,
ii. they have great specificity of action; hence can bring about reactions not otherwise easily carried out,
iii. they work under mild conditions and moderate temperature and near neutral pH, thus not requiring drastic conditions, which necessitate specialized, expensive equipment,
iv. they are able to act even at low concentrations, and the rate of reaction can be easily controlled by adjusting temperature, pH and the amount of enzyme employed, and
v. they are easily inactivated when the reaction has gone as far as required.

1.2.1.3. The future of microbial enzymes

Industrial uses of enzymes have increased greatly during the past few decades. Prospects are excellent for continuing increased usage of currently available enzymes in many industrial applications, and in new uses, and of new enzymes for many purposes. Currently much enzyme research is underway by various institutes and industries including manufacturers. Such research is devoted to come across novel enzymes and improved methods for specific industrial needs. Continuous usage of old and improved/modified enzymes acting on various substrates would result from such research.

1.2.2. CARBOHYDRATES

Carbohydrates are vastly distributed on earth and because they are present in a wide variety of substances and materials, it is possible that they may occur in many different forms. Carbohydrates play an imperative role in photosynthesis, whereby the sun's energy is converted into chemical energy. The production and conversion of carbohydrates are essential parts of the carbon cycle of our planet which leads to the estimation that approximately $3.4x10^{14}$ kg of carbohydrates are synthesized by plants and photosynthesizing microorganisms per annum. Therefore it is not surprising that carbohydrates make up an important part of our diet and form more than half of the calories consumed daily.

Carbohydrates fall into two categories which are structural and non-structural components of organisms. Structural carbohydrates include the components of the cell wall and cytoskeleton and non-structural carbohydrates are known as energy rich compounds which are used for metabolism and energy storage compounds. One type of non-structural bacterial carbohydrate in the form of common and special fructose polymers is called inulin.

1.2.2.1. Inulin

Inulin is one of the polysaccharides of plant origin used as a substrate for the production of fructose syrups in food industries. Inulin is a linear chain of fructose residues linked by a β-2,1 glycosidic bond with a terminal sucrose residue.

1.2.2.2. Origin of fructans

Dicot plants (family *Asteraceae*) synthesize linear inulin consisting of one terminal glucose residue and a variable number of fructose residues exclusively linked by β-2,1 bonds. The chain length of the inulin (Fig. 1) deposited in storage organs and inilin content (Table 1) varies between species. The inulin stored in Jerusalem artichoke (*Helianthus tuberosus*) tubers and chicory

(*Cichorium intybus*) taproots has a rather low mean in the degree of polymerization (DP), of about 10-30. Inulin of the highest DP in *Asteraceae* has been found in globe artichoke (*Cynara scolymus*) roots reaching up to 200 fructose residues (Praznik and Beck, 1985 and Hellwege *et al*., 2000).

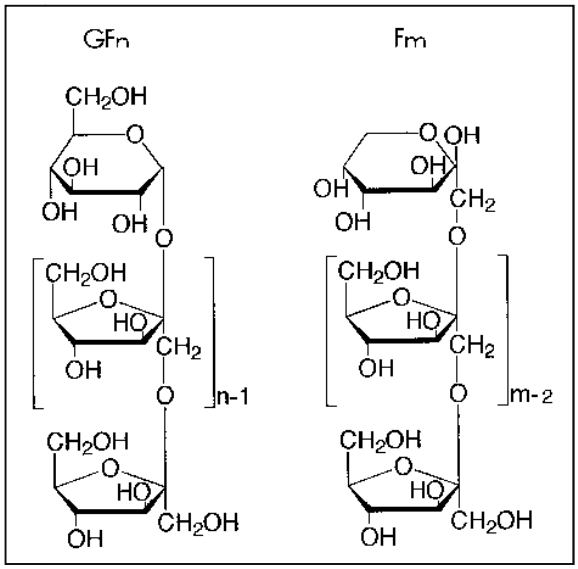

Figure 1: Structure of two inulin molecules, one with a glucopyranosyl residue, and the other with fructopyranosyl residue at one end (Rusu *et al*., 2006)

Monocot plants belonging to families *Poaceae, Alliaceae, Asparagaceae, Agavaceae, Amaryllidaceae, Heamodoraceae* and *Iridaceae* produce more complex fructans. Temperate grasses (*Poaceae*) form structures that vary from linear levan, referred to as phlein, for instance in big bluegrass (*Poa secunda*) (Chatterton and Harrison 1997 and Wei *et al*., 2002), to highly branched levan having both β-2,6 and β-2,1 fructosyl- fructose linkages, referred to as graminan, for instance in wheat (*Triticum aestivum*) and barley (*Hordeum vulgare*) (Bancal *et al*., 1992). An inulooligosaccharide chain attached to the C6 glucosyl residue of sucrose may also be present in graminans, for instance in oat (*Avena sativa*), tall fescue (*Festuca arundinacea*) and *Lolium* species (Livingston *et al*., 1993; Luscher and Nelson, 1995; Pavis *et al*., 2001). The members of *Lilliaceae* and *Asparagaceae*, for instance onion (*Allium cepa*) and asparagus (*Asparagus officinalis*) produce inulin neoseries.

These are also referred to as neoinulins and consist of two linear β-2,1 linked fructosyl chains, one attached to the fructose residue of the sucrose starter, the other attached to the glucose residue at the C6 position (Shiomi, 1989 & Vijn *et al*., 1997). Diverse fructan structures have been reported in *Agavaceae* with inulin identified as the principal reserve carbohydrate in *Agave americana*. It is also noted that a complex mixture of fructooligosaccharides, inulins, neoinulins and branched fructans, containing both β- (2-1) and β- (2-6) fructosyl fructan linkages (Lopez *et al*., 2003) are stored in the stems of *Agave vera cruz* and *Agave tequilana.*

Table 1: Inulin content (% of fresh weight) of some plants

Source	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	Leaf	14-16	3-10
Banana	Fruit	24-26	0.3-0.7
Rye	Cereal	88-90	0.5-1*
Barley	Cereal	NA	0.5-1.5*
Dandelion	Leaf	50-55*	12-15
Burdock	Root	21-25	3.5-4.0
Camas	Bulb	31-50	12-22
Murnong	Root	25-28	8-13
Yacon	Root	13-31	3-19
Salsify	Root	20-22	4-11

NA; Not available *- Estimated Value; Source: Franck and De Leenheer (2004)

Fructans comprise of both oligosaccharides and polysaccharides that mainly have fructose residues. Fructans synthesized in nature, are water soluble and non-reducing sugars in which from one up to more than a hundred thousand fructose units are attached to the precursor sucrose molecule. A broad range of microorganisms synthesize fructans. Many Gram-positive and Gram-negative bacteria produce levan, while inulin synthesis has been reported so far only in the Gram-positive species *Streptococcus mutans*, *Lactobacillus reuteri* and *Leuconostoc citreum*. The largest fructans in nature are bacterial levan and inulin with a DP ranging from 10^4 to 10^6.

Fungal species that produce fructans are basically introduced in the genera *Aspergillus*, *Aureobasidium*, *Penicillium*, *Fusarium*, *Pestalotiopsis*, *Myrothecium*, *Trichoderma* and *Phytophora*. Fungal fructans consist of a linear β-2,1-linked chain, with the predominant occurrence FOS (Hang and Woodams, 1995; Kurakake *et al*., 1996).

1.2.2.3. Benefits of inulin and FOS

Inulin has many applications in the food, neutraceuticals and non food industries. Fructans of different size potentially have different uses. In actual fact, only FOS and inulin are currently produced on a commercial level. These fructans are considered functional foods and are marketed in developed countries due to an increasing demand. By concept, functional foods have an added health value above their nutritional properties (Roberfroid, 2000).

Fructose, being sweeter than sucrose is therefore used in many foods, pharmaceuticals, and beverages. However, the chemical approach of fructose production is currently associated with some drawbacks (Gill *et al*., 2006; Pandey *et al*., 1999). Fructose can also be produced from starch by enzymatic methods by the use of α-amylase, amyloglucosidase and glucose isomerase (Gong *et al*., 2007). The best procedure involves the use of microbial inulinase, which, after one-step enzymatic hydrolysis of inulin, yields 95% pure fructose. Therefore the exoinulinase from different microorganisms are used for the production of ultra-high fructose syrup from inulin and inulin-containing materials. It is interesting to note that only monosaccharides (glucose and fructose) were released from inulin by the action of the purified inulinases from both the marine yeast *C. aureus* G7a and *P. guilliermondii* strain 1 (Sheng *et al*., 2009; Gong *et al*., 2008).

Therefore, it would be appropriate to state that the inulinases from the two marine yeasts may have great potential use in the direct digestion of inulin in food and fermentation industries. The monosaccharides and oligosaccharides were also detected after inulin hydrolysis for more than 2 h

by the purified exoinulinase produced by *K. marxianus* var. *bulgaricus* (Kushi et *al*., 2000). The purified inulinase produced by *B. polymyxa* MGL21 acted on inulin via an exo attack to successfully produce fructose (Kwon *et al*, 2003).

1.2.2.3.1. Prebiotics

As a typical representative of prebiotics, fructans have a proven bifidogenic effect in animals and humans. Due to the fact that the β- fructosyl linkages cannot be hydrolyzed by the digestive enzymes in the upper part of the human gastrointestinal tract, these sugars have low caloric value and dietary fibre-like properties. Once in the colon, fructans are selectively metabolized by resident bifidobacteria and lactobacilli that produce β-fructofuranosidases, which are the main representatives of the beneficial colonic microflora. Due to proliferation of healthy bacteria in the gut, competitive exclusive of pathogens, such as *Escherichia coli, Clostridium sp.* and *Salmonella sp*. occurs. Short-chain fructans serve as more convenient substrates for rapid growth of bifidobacteria (Van der Meulen *et al*., 2004) and (Kilian *et al*., 2002), whereas branched fructans are claimed to provide for a long-lasting source of energy (Weyens *et al*., 2004). Fructans with a low and medium degree of polymerization are important primarily because of their functional properties, but in addition they have applications in the food industries.

1.2.2.3.2. Medical benefits

The bifidobacteria and lactobacilli release short chain fatty acids and lactic acids from inulin, which in turn provoke other important associated benefits to human and animal health and these include an increase in calcium and magnesium absorption (Ohta *et al*., 1998), the production of B-vitamins, the reduction of serum cholesterol, the prevention of colon cancer (Kaur and Gupta, 1999).

1.2.2.3.3. Alternate sweetener

For instance, the trisaccharide 1-kestose has a natural sweet taste, and in a blend with other low caloric sweeteners it can replace sucrose in certain specific uses. Inulin also, when extracted from chicory is extensively used as a food ingredient mainly because of its excellent characteristics as a dietary fibre, and fat replacer and neutral taste.

1.2.2.3.4. Biorefineries

Bacterial levan and inulin have other potential food and non-food applications. Levan is more soluble than inulin and produces viscous solutions in water and this property makes levan especially attractive in some cases; as an emulsifier or encapsulating agent in a wide range of products, including textiles coatings and detergents, cosmetics, biodegradable plastics, glues and biorefineries and biofuels (Calazans, 2000).

Ethanol is the most employed liquid biofuel either as a fuel or as a gasoline enhancer (Sanchez and Cardona, 2008). It is also an excellent raw material for synthetic chemicals. Jerusalem artichoke tuber is one of the best raw materials for fuel ethanol production. The flocculating yeast lacking the pathway for fructose utilization by integration of the FLO1 flocculation gene in the ribosomal DNA of a hexokinase–deficient (hxk1, hkx2) *Saccharomyces cerevisiae* strain was used for simultaneous production of ethanol and fructose from glucose /fructose mixtures or from Jerusalem artichoke hydrolysates containing 6% of sugars (Remize *et al*., 1998).

Ethanol was also produced from Jerusalem artichoke mashed tubers using *K. fragilis*, a yeast with an active inulinase, together with either a commercial distillery yeast, *S.cerevisiae*, or a bacterium *Zymomonas mobilis*. After batch fermentation, the best ethanol concentration of 0.48 g/g for the mixed population and 0.46 g/g for the single population were obtained.

The results show that industrial ethanol producing strains of *S. cerevisiae* as well as the bacterium *Z. mobilis* in mixed fermentation with a yeast with inulinase activity, namely, *K. fragilis*, yields up to 12% more than a single strain process (Szambelan *et al*., 2004).

1.2.2.3.5. Food industry

FOS are non-cariogenic as they are not used by *Streptococcus mutans* to form acids and insoluble glucans that are the main culprits in dental caries. Due to the large number of health promoting functions of inulin and FOS, these have wide applications in various types of foods like confectionery, fruit preparations, milk desserts, yoghurts and fresh cheese, baked goods, chocolate, ice-cream and sauces. Inulin can also be used in the preparation of fructose syrups (Kaur and Gupta, 2002).

The body responds to fructose in a different way than to glucose and sucrose. Fructose is more satiating and due to the fact that it is sweeter than sucrose, this makes it useful in foods and beverages for the health conscious.

Fructose is also ideal for use in diabetic foods as it has very little effect on blood glucose and only a negligible effect on the secretion of insulin. The principle use for glucose isomerase is in the production of high fructose syrups from glucose which is usually derived from maize or corn starch. This is also possible with pure glucose or molasses using both invertase and isomerase (Kaur and Gupta, 2002).

Enzymatic treatments are now a major way of producing sweeteners, including syrups derived from sucrose or starch that contain mixtures of glucose, maltose, fructose and other sugars. High fructose syrups (HFS) from maize starch have now eclipsed sucrose as a major sweetener used in the US food industry.

Currently for production of HFS, three production processes exist with similar technology and pathways but has three different start materials:

i. starch (the industrial sources are maize and other cereals),
ii. molassses (from sugar cane or sugar beet), and
iii. cellulose (it is in pilot scale but has successful development)

Glucose has 70-75% the sweetening strength of beet sugar (sucrose), but fructose is twice as sweet as sucrose. Thus, processes for the manufacture of fructose are of considerable value.

1.2.2.4. Physiological function of inulin

Despite major advances having been made in the elucidation of the metabolism of fructans, their precise physiological function remains a subject of debate. The most documented role is that of a long-term reserve carbohydrate stored in underground, over-wintering organs. Two other functions are often quoted: viz as a cryoprotectant, and an osmotic regulator. Together these roles allow not only survival but also growth under conditions of water shortage, whether induced by drought or low temperatures (Hendry and Wallace, 1993).

De Roover *et al*., (2000) have reported on the effects of drought on inulin metabolism in chicory. Glucose, fructose and sucrose contents were increased in the roots and leaves of stressed plants, whereas the inulin concentration was found to be ten-fold higher than in control plants, with inulin content being normal in the roots but absent in the leaves. In the cold environment (3 weeks at 4°C), chicory inulin is clearly degraded.

The role of fructans as true cryptoprotectors is under discussion, as the increase in hexoses and sucrose upon depolymerization of the fructan would only account for a freezing point decrease of 0.2-0.5°C (Van Den Ende, 1996).

In contrast, inulin was seen to interact directly with membrane lipids upon freeze-drying, thereby preserving the membranes in a liquid crystalline phase at room temperature and preventing phase transition and solute leakage during rehydration (Hinchu *et al*., 2000).

1.2.2.5. Microbial inulinases

Inulinases can be produced by a host of microorganisms, including fungi, yeast and bacteria (Table 2). Among them, however, *Aspergillus* sp. (filamemtous fungi) and *Kluyveromyces* sp. (diploid yeast) are apparently the most common choices for commercial application.

There are two types of inulinases, which are endo and exo-inulinase. Endo-inulinase is specific for inulin and hydrolyzes the internal β-2,1- fructofuranosidic linkages to yield inulotriose, inulotetraose and inulopentaose as the main products. In contrast, exo-inulinase split the terminal fructose units in sucrose, raffinose and inulin (Vandamme and Deryeke, 1983). Inulinases which are β-2,1-D-fructan fructanohydrolase (EC 3.2.1.7) are different from invertases (EC 3.2.1.26). Both types of enzymes are β- fructosidases which are active on sucrose but, in addition, inulinases are also able to hydrolyze inulin (Vandamme and Deryeke, 1983; Pandey *et al*., 1999). Inulinases are usually thermo stable and commercially available for industrial applications (Ettalibi and Baratti, 2001). Microbial inulinases play an imperative role in the hydrolysis of inulin for its commercial exploitation (Table 3). Endoinulinases and endolevanases have absolute substrate specificity for inulin and levan, respectively. These enzymes split at random the internal β-linkages of the polymer yielding a mixture of oligofructans of different sizes (Murakami *et al*., 1992).

Table 2: Inulinase yield from different microorganisms, expressed in U ml^{-1} if not otherwise specified; Data marked by "*" are expressed in U g^{-1} (Adapted from Ricca *et al*., 2007)

Microorganisms	Inulinase activity (IU ml^{-1})	References
FUNGI		
Aspergillus sp.	75	Pandey *et al*., 1999
A. aureus MTCC 151	160	Pandey *et al*., 1999
A. ficuum	3000*	Pandey *et al*., 1999
A. fischeri MTCC 150	1-1.2	Pandey *et al*., 1999
A. flavus MTCC 277	1-1.2	Pandey *et al*., 1999
A. nidulans MTCC 344	1-1.2	Pandey *et al*., 1999
A. niger	60	Poorna and Kulkarni.1995
A. niger 817	0.0685	Pandey *et al*., 1999
A. niger A42	4600*	Pandey *et al*., 1999
A. niger MTCC 281	1-1.2	Pandey *et al*., 1999
A. niger mutant 817	160	Pandey *et al*., 1999
A. niger mutant selection	35.18	Skowronek and Fiedurek, 2003
A. niger mutant UV1	120	Pandey *et al*., 1999
A. parasiticus	2.9	Ertan *et al*., 2003
Cladosporium sp.	10.9	Pandey *et al*., 1999
Fusarium sp.	0.080	Pandey *et al*., 1999
Penicillium sp.	50	Pandey *et al*., 1999
Penicillium sp. 91-4	3.74	Pandey *et al*., 1999
P. rugolosum	54	Pandey *et al*., 1999
P. spinulosum	1.67	Ertan *et al*., 2003
P. trzebinskii	11	Pandey *et al*., 1999
Streptomyces sp.	32	Pandey *et al*., 1999
S. rochei E 87	1	Pandey *et al*., 1999
Trichoderma viride	1.18	Ertan *et al*., 2003
BACTERIA		
Bacillus sp.	5.14	Pandey *et al*., 1999
B. subtilis 430 A	50-70	Pandey *et al*., 1999
Cladosporium acetobutylicum IFP 912	43.7	Pandey *et al*., 1999
C. acetobutylicum ABKn8	6.06	Pandey *et al*., 1999
C. thermosuccinogenes	0.011	Pandey *et al*., 1999
Flavobacterium mulivorum	0.456	Pandey *et al*., 1999
Pseudomonas sp. 65	15*	Pandey *et al*., 1999
Staphylococcus sp.	0.634	Gill *et al*., 2003
Streptomyces sp. GNDU 1	0.552	Gill *et al*., 2006
Streptomyces sp. ALKC4	0.524	Sharma *et al*., 2007
Streptomyces sp. AlkC4	9400*	Pandey *et al*., 1999
YEASTS		
C. pseudotropicalis IP513	25000*	Pandey *et al*., 1999
Kluyveromyces fragilis	7	Pandey *et al*., 1999
K. fragilis ATCC 12424	355	Pandey *et al*., 1999
K. lactis	43.7	Pandey *et al*., 1999
K. marxianus	56000*	Pandey *et al*., 1999
K. marxianus	176	Santisteban and Filho (2005)
K. marxianus	127	Kalil *et al*., 2001
K. marxianus ATCC 36907	260	Pandey *et al*., 1999
K. marxianus ATCC 52466	0.418	Pandey *et al*., 1999
K. marxianus CDBB-L-278	82	Pandey *et al*., 1999
K. marxianus var. *bulgaricus*	107	Kushi *et al*., 2000
K. marxianus var. *marxianus* CBS 6556	3000	Pandey *et al*., 1999
K. marxianus CBS 6556	58000*	Pandey *et al*., 1999
K. marxianus UCD (FST) 55-82	212	Pandey *et al*., 1999
Pichia guilliermondii	60.1	Gong *et al*., 2006

Table 3: Mode of action of inulinase of different microbial sources (Adapted from Ricca *et al*., 2007)

Source	Action	References
Awamori var 2250	Exo	Arand *et al*., 2002
A.candidus	Exo	Kochhar *et al*., 1999
A. ficuum	Endo-Exo	Zittan, 1981
A. fumigatus	Exo	Gill *et al*., 2006
A. niger	Exo	Derycke and Vandamme, 1984
A. parasiticus	Exo	Ertan *et al*., 2003
C. pannorum	Endo	Pandey *et al*., 1999
Debaromyces cantarellii	Exo	Beluche *et al.,* 1980
Geobacillus stearothermophilus	Exo	Tsujimoto *et al*., 2003
Kluyveromyces fragilis	Exo	Pandey *et al*., 1999
Paenibacillus sp.	Endo	Gern *et al*., 1999
Penicillium spinulosum	Exo	Ertan *et al*., 2003
Pichia guilliermondii	Exo	Gong *et al*., 2007
Streptomyces sp.	Exo	Sharma and Gill, 2007
Trichoderma viride	Endo	Ertan *et al*., 2003

In industry, enzymes have always competed and will still compete with conventional chemical catalysts. Potential advantages of enzymes are their high specificity, high activity under mild environmental conditions and high turnover number; their biodegradable nature and their label as a natural product have now also become very important assets (Polastro, 1989). Drawbacks are inherent to their complex molecular structure that increases their monetary value in production and their intrinsically unstable nature (Illanes, 1999). Fructans are metabolized by a wide range of bacteria and fungi. These organisms utilize the fructans as an alternative carbon source when more energetic sugars (for instance glucose and sucrose) are limited or exhausted. Levan and inulin of microbial origin get hydrolyzed by the action of extracellular endo and exofructanases. Most levanases characterized so far function as fructose or levanbiose-producing exohydrolases.

Fructose-releasing levanases have been identified in the species *Bacillus subtilis* (Martin *et al*., 1987)*, Bacteriodes fragilis* (Blatch and Woods, 1993)*, Paenibacillus polymyxa* (Bazzate *et al*., 1994)*, Bacillus stearothermophilus* (Li *et al*., 1997) and *Gluconacetobacter diazotrophicus* (Menendez *et al*., 2002). In addition to levan, these enzymes commonly hydrolyze inulin, raffinose and sucrose, although with different substrate preferences. In all cases the enzyme attacks the substrate molecule from the fructose end and releases fructose as the sole reaction product.

By contrast, levanbiose-producing levanases split levan mainly into levanbiose and hardly hydrolyze the β-2,1- linkages of inulin, raffinose or sucrose (Kang *et al*., 1999 and Saito *et al*., 2003). In plants, the breakdown of fructans is accomplished by a complex of fructan exohydrolases (FEHs). Two isoforms of 1-FEH have been identified to hydrolyse inulin in chicory roots (Van den Ende *et al*., 2001). More recently, three cDNAs from wheat encoding two 1-FEHs and one 6 and1-FEH were associated with the breakdown of branched graminan-type fructans containing both β-2,1 or β-2,6 fructosyl linkages (Kawakami *et al*., 2005). The 6 and 1-FEH-type of enzyme in wheat crowns were suggested to fulfill a crucial role in the modulation of fructan content and DP under cold stress. The expression of FEH genes is mainly induced after defoliation or by freezing temperature (Asego and de Carvalho, 2004 & Michiels *et al*., 2004). Hormones like gibberellin and ABA have been found to be important for FEH regulation (Yang *et al*., 2004). Plant FEHs, in contrast to microbial exofructanases, are unable to hydrolyze sucrose. This sugar is, otherwise, a strong competitive inhibitor of FEH enzymes and may regulate their activity in vivo avoiding fructan hydrolysis when more energetic substrates are available.

1.2.2.6. Inulinase hydrolytic activity towards sucrose

Enzymes capable of hydrolyzing β-1,2-fructan links and known as inulinases often show a certain activity towards β-2,6-fructan links, which means that they can hydrolyze sucrose into fructose and glucose. Sucrose hydrolytic enzymes are called invertases (E.C. 3.2.1.26, β-D-fructo-furanoside-fructo-hydrolase) and present specific characteristics. It is difficult and controversial to make a distinction between inulinases and invertases, which constitute the group of β-fructosidases. It is a common practice to distinguish inulinases from invertases according to the ratio α inulinase activity/invertase activity; if $\alpha > 10^{-2}$ the enzyme is referred to as an inulinase; if $\alpha < 10^{-4}$ it is considered as invertase (Baratti and Ettalibi, 1987). Invertase activity is observed in enzymes coming from all kinds of microorganisms and also in β-fructosidases from vegetable origin, as reported by Rutherford and Deacon (1972).

The values of α measured during experimental works on inulinase from different sources confirm the results reported by Pandey *et al*. (1999), i.e. invertase activity is greater in enzymes produced by yeasts than in those obtained from fungi. Derycke and Vandamme (1984) studied inulinase from *A. niger* and found α=1.2, while Rhee *et al*. (1989) studied inulinase from *A. ficuum* and found α=9.4. some strains, such as *A. niger* 12. *Penicillium* sp. and *Debaryomyces cantarellii*, due to their substrate specificity, are reported to possess two or more types of inulinase (Vandamme and Derycke, 1983).

It is important to note that there are a few cases of true inulinases or true invertases; generally an enzyme shows both the activities, although to a different extent. Clearly an invertase activity of inulinase is desirable, since it is necessary to hydrolyze the ultimate link between glucose and fructose residues remaining after the completion of inulinase action on an inulin molecule. Ettalibi and Baratti (1990; 1987), separated inulinases and invertases in a preparation from *A. ficuum*. Due to the similarities of the responses to inhibitors, pH and temperature, in terms of inulinase activity, the authors postulated that inulinases possess common catalytic sites, but different binding sites for inulin and sucrose hydrolysis.

1.2.2.7. Inulinase production methods

Some of the methods employed for inulinase production include submerged cultivation (SMC) and solid state cultivation (SSC). In the case of submerged cultivation compact closed fermenters are used and require minimum labour but the use of high air pressure. This method also requires considerable power for air compressors and agitators. It demands careful control as contamination is frequently a serious problem. SSC may be defined as a fermentation process where the microorganisms grow in solid substrates with low water concentration (Pandey, 1999). Finally the enzyme recovery would include filtration or centrifugation and perhaps evaporation and/or precipitation.

SSC requires more space for trays and involves more hand labour. Only a little power is sufficient to run the system and minimum control is necessary as contamination is not a major problem. Enzyme recovery will require extraction with aqueous solution, filtration or centrifugation and perhaps evaporation and/or precipitation. *Staphylococcus* sp. RRL1 and *Kluyveromyces marxianus* ATCC 52466 were able to produce extracellular inulinase using SMC with inulin being the carbon source. *Staphylococcus* sp. showed an inulinase activity (618 U L^{-1}) and *Kluyveromyces marxianus* showed an activity of 470.4 U L^{-1} (Selvakumar and Pandey, 1999).

The production of enzymes by SSC has gained much attention in biotechnology studies for production of lipases (Di Luccio *et al*., 2004); inulinases (Pandey 2009), proteases (Couri *et al*., 2000) and more. The use of low cost residues, higher productivities, low energy requirements, lower wastewater production, extended stability of products and low production costs are some of the main advantages of SSC (Gessesse and Mamo, 1999). The selection of a suitable microbe is an important aspect of SSC for the production of enzymes (Pandey, 2003).

Due to the fact that sugarcane bagasse is an abundant by-product of the sugar industries, it was used as a carbon source for inulinase production by *Kluyveromyces marxianus* NRRL Y-7571, using SSC. It was concluded that the highest enzyme production (391.9 U g^{-1}) was observed at 36°C with corn steep liquor as the nitrogen source (Mazutti *et al*., 2006). A comparative study on inulinase production by *X. campestris* pv. *phaseoli* using both SSC and SMC was conducted. It was found that the highest inulinase production of 17.42 IU ml-1 was achieved during SMC with inulin as the sole carbon source. However, the inulinase production using SSC was 6.7 fold higher and 5.8 fold higher using garlic and onion, respectively as a solid substarte (Ayyachamy *et al*., 2007).

1.2.3. BIOCHEMICAL PROPERTIES OF INULINASE

1.2.3.1. Molecular weight

Inulinase molecular weight (MW) is strictly related to the producing-microorganism. Kaur *et al*. (1994) reported a MW of 250 kDa for an inulinase from *K. fragilis*. MW data for inulinase from four different strains of *Aspergillus* have been determined as follows: *A.niger* was found to have an inulinase of 300 kDa; *A.ficuum* (53 kDa), *A candidus* (54 kDa) (Kochlar *et al*., 1999) and *A. awamori* var. 2250 (69 kDa) (Anand *et al*., 2002).

Takahashi *et al*. (1985) purified exoinulinase (83 kDa) to homogeneity from *Streptococcus salivarius*. The pure enzyme was able to hydrolyze levan, inulin and several β-2,1-linkage-containing oligosaccharides such as sucrose and raffinose. A thermophilc *Bacillus stearothermophilus* KP1289 produced an induced extracellular inulinase with a molecular mass of 54 kDa and a half-life of 10 min (Kato *et al*., 1999). An extracellular endoinulinase of 139 kDa was purified from *X. oryzae*, which converted inulin into FOS at 50°C (Cho and Yun, 2002).

1.2.3.2. Temperature and pH optima

It is certain that effects of temperature and pH on inulinase activity and stability, depends mainly on the strain used as a source of enzyme production. Pandey *et al*., (1999) noted that fungal inulinases exhibited an optimum pH between 4.5 and 7.0, yeast inulinases between 4.4 and 6.5 and bacterial inulinases between 4.8 and 7.0. Optimum temperature values are generally higher for bacteria and yeast than for fungi. Information about the shapes of the curves of activity vs. temperature and pH, rather than only optimum values, is also very important from an industrial point of view, since large ranges mean great flexibility in operating conditions. However optimal temperature and pH values are not necessarily those that would be used in an industrial process, because many other factors, such as enzyme stability, the risk of contamination, inulin solubility and colour formation, contribute to define the optimal values of operating conditions.

pH values as low as the optimal ones seem to be suggested because they avoid colouring formation, while temperature values that maximize enzyme activity may endanger its stability, due to thermal deactivation. In this regard the need for a high temperature stable enzyme is obvious because it would permit thermal conditions favouring inulin solubility, which may be a limiting factor and contrasting contamination, without massive deactivation of the catalyst.

Unfortunately, there is insufficient literature reporting information on both activity and stability versus temperature. Data about denaturation of inulinase from *A. ficuum* were reported from Focher *et al*. (1991). They conducted a study on thermal deactivation in which they determined the parameters for the Arrhenius equation describing the kinetics of inulinase deactivation, and these were described by 1st order rate equation. An inulinase from *A. fumigatus* was obtained and compared to a commercial inulinase produced by *A. niger*. The former was proven to be more stable and have a temperature optimum of 60°C with an appreciable stability even at higher temperatures (Gill *et al*., 2006). Remarkably, an inulinase from *Streptomyces* sp. has been produced with a temperature optimum of 70°C and no activity loss after 6 h incubation at that temperature (Sharma *et al*., 2007).

1.2.3.3. Kinetics properties of inulinase

According to Derycke and Vandamme (1984) many researchers concluded that the different inulin DP and its variation during the reaction progress do not allow a precise estimation of the kinetic parameters and, in general, prevent inulinase kinetics from being described by a Michaelis-Menten type rate equation (Ricca *et al*., 2007). A complete kinetic study was conducted by Focher *et al*. (1991) on inulinase from *A. ficuum*. They found the dependence of k_2 and K_m with respect to temperature. They found a Michaelis-Menten behaviour for both inulin and sucrose hydrolysis activity. Several authors have evaluated kinetic parameters of inulinase from different strains, but only at one temperature; observed values in terms of Michaelis constant (K_m) and rate coefficient

(k_2). Azhari *et al*. (1989) pointed out the necessity to express kinetic parameters of exo- and endo-inulinase on a different basis.

1.2.3.4 Effect of substrate concentration on inulin hydrolysis

The substrate concentration influences the rate of hydrolysis by inulinase for many reasons. From a kinetic point of view, a high concentration of substrate determines a high rate of reaction. Several studies have reported that the rate of reaction increases with inulin concentration and no inhibition effect was observed at any concentration (Zittan, 1981; Focher *et al*., 1991 and Uhm *et al*., 1982), even though Zittan (1981) noticed that inulin concentration values higher than 12.5% no longer affected the rate of hydrolysis. A significant inulin concentration is also desirable because the presence of substrate has been proven to stabilize the enzyme. Zittan (1981) and Uhm *et al*. (1982) have reported that storage of an inulinase from *K. fragilis*, in the presence of inulin at 55ºC for 4 h, resulted in no loss of activity, while deactivation occurred in the same interval of time at T>10ºC in the absence of substrate and, in particular, the enzyme retained only 20% of its maximum activity when stored for 4 h at 55ºC without inulin.

In any case, high inulin concentrations are limited by its low solubility. The solubility of pure inulin at 50ºC is about 1% (w/w), which is very low, fortunately in an industrial process, plant inulin characterized by a lower average DP, but high solubility, will most likely be used. Zittan (1981) suggested an inulin concentration value of 13% (w/w) and Uhm *et al*., (1982) reported inulin solubilisation up to 16%, but any concentration above 10% rapidly led to precipitation. Another important property of inulin influencing the rate of hydrolysis is the DP (Ricca, 2007). Derycke and Vandamme (1984) demonstrated that the hydrolysis was much faster when a less refined inulin substrate extracted from chicory (DP=8) was used than a pure inulin (DP=24) was processed. The reason for this evidence could be that, since exo-inulinases act on one end of the inulin molecule, the higher the DP is, the less available ends are within the reacting mixture.

1.2.4. *Xanthomonas campestris*

The genus *Xanthomonas* Dowson 1939 consists of bacteria described as being Gram-negative, obligately aerobic, non-fermentative rods. Being a popular plant pathogen, this bacterium is capable of moving through the infected plant by means of a single polar flagellum, which renders it motile. Based on microbiological classification, this genus can be separated into at least five separate species and over 125 variants, or pathovars, of *X. campestris*, are essentially indistinguishable from each other, except for their plant host range. To maintain order in differentiating among strains of *X. campestris* by host range, the rank of pathovar has been assigned in addition to the species name. The primary means by which strains can be differentiated as pathovars is by inoculation of the plants which serve as susceptible hosts to a given pathovar. The convention for naming pathovars of *X. campestris* has generally been to name it for the host from which it was first isolated. A particular pathovar designation suggests a limited host range for a given strain.

1.2.4.1 Xanthan production

Xanthan gum is a complex microbial exo-polysaccharide industrially produced from glucose via fermentation by the plant pathogenic bacterium, *Xanthomonas campestris* pv. *campestris*. It was first discovered in the late 1950s by US scientists and is the first biopolymer to be produced industrially and it was not until 1969 that the FDA issued the final approval for the use of xanthan gum in food products. The demand for xanthan gum produced by *Xanthomanas campestris* has increased steadily every year and is estimated to grow continuously at an annual rate of 5-10%. Xanthan gum is widely used in a broad range of industries, such as in food, toiletries, cosmetics, oil recovery and as water-based paints; due to its rheological properties and is used as a rheological control agent in aqueous systems and as a stabilizer for emulsions and suspensions. The amazing properties of xanthan gum is the ability to form high viscosity solutions at low shear

forces, highly pseudoplastic, and may also display a viscosity yield value (Yoshida and Tanner, 1993).

Inulinase has been isolated from *X. campestris* p.v. *phaseoli* which is a popular plant pathogen. *X. campestris* pv. *phaseoli* is a motile, aerobic, gram-negative rod, 0.4-0.9x0.6-2.6 μm, with a single polar flagellum. Agar colonies are convex, yellow and wet shining. In culture on complex medium or medium containing tyrosine, a brown, diffusible pigment is produced by so-called fuscans strains. A novel *Xanthomonas* sp was proven capable of producing an endoinulinase, to be used for FOS production from inulin. An activity of 11 U ml^{-1} was achieved through batch cultivation (Park *et al*., 1999).

Comparative studies on endoinulinase production using chicory roots were investigated using *Xanthomonas* sp and *Pseudomonas* sp. Inulinase production was higher in *Xanthomonas* sp. (15 U ml^{-1}) than *Pseudomonas* sp. (3 U ml^{-1}) (Park and Yun, 2001). *Xanthomonas oryzae* No.5 was also found to produce an endoinulinase and FOS from chicory extract (Cho *et al*., 2000). This proved the ability of the *Xanthomonas* crude endoinulinase to stoichiometrically convert inulin to IOS/FOS without the formation of by-products.

Therefore the results reveal an important observation that, with the crude endoinulinase, it is possible to achieve almost 100% substrate conversion. *X. campestris* pv. *phaseoli* was capable of producing 17.42 U ml^{-1} of inulinase in submerged cultivation using production medium with inulin as the carbon source (Ayyachamy *et al*., 2007).

1.2.5. STRAIN IMPROVEMENT

It is imperative to enhance the production of enzymes that exhibit industrial potential. One of /the methods implemented to achieve this is mutation. Mutations are permanent, transmissible changes

to the genetic material (usually DNA or RNA) of a cell. Mutations can be caused by copying errors in the genetic material during cell division and by exposure to radiation, chemicals, or viruses, or can occur deliberately under cellular control during the processes such as meiosis or hyper mutation.

Mutations are considered the driving force of evolution, where less favourable (or deleterious) mutations are removed from the gene pool by natural selection, while more favourable (or beneficial) ones tend to accumulate. Neutral mutations do not affect the organism's chances of survival in its natural environment and can accumulate over time, which might result in what is known as punctuated equilibrium; the modern interpretation of classic evolutionary theory. It should be noted that, contrary to science fiction, the overwhelming majority of mutations have no real effect.

1.2.5.1. Mutagenic techniques

Mutagenesis is the source of all genetic variations, but no single mutagenic treatment will give all possible types of mutation. There are two major types of mutagens, which are the physical methods that include UV, X-ray and gamma radiation. Chemical methods include ethyl methyl sulfonate (EMS), nitrosoguanidine (NTG) and mustards. The type of mutations induced in any cell type depends on: The type of DNA damage caused by the mutagen. Most mutagens will cause more than one type of DNA damage. Some mutagens will produce more than one type of DNA damage than others. DNA repair systems may be non-mutagenic (error free) or mutagenic (error prone). Some mechanisms include photo reactivation, excision repair, recombination repair and SOS repair. EMS (ethylmethane sulphonate) is the mutagen of choice due to the fact that its effects have been well studied and it is known to generate almost exclusively G/C to A/T point mutations. These mutations may lead to a complete or partial loss of gene function or, less frequently, to some other alteration of normal gene function.

Mutations are randomly distributed in the genome and a high degree of mutation saturation can be achieved with a mutagen like EMS that does not cause a lot of collateral DNA damage. *Pichi guilliermondii* strain 1, isolated from the surface of marine alga, was found to secrete a large amount of inulinase into the production medium which was seawater containing 4% inulin and 0.5% yeast extract (Gong *et al*., 2007). Under the optimal conditions, over 61.5 ± 0.4 U ml^{-1} of inulinase activity was observed after 48 h. In order to isolate the overproducers of the marine yeast, its haploid cells were treated by using UV light and LiCl and the resulted mutant (M 30) showed an increased inulinase production of 115 ± 1.1 U ml^{-1}(Guo *et al*., 2009). The conidia of *A. niger* 13/36, an active producer of inulinase, were subjected to mutagenesis with U.V and N-methyl-N'-nitrosoguanidine (NTG) and the products were analysed for inulinase activity by a diffusion plate method and the mutants grown under stress conditions showed significantly higher inulinase activity (about 1.22-4.5 fold) as compared with the parent strain (Skowronek and Fiedurek, 2003).

The project was designed to initially optimize inulinase production in the parent strain of *Xanthomonas campestris* pv *phaseoli*, which was accomplished by adjusting the growth parameters of the organism as well as the nutrients supplied to the organism for maximum yield of inulinase, as demonstrated in chapter two. The development of an even higher inulinase producer was established in the third chapter by use of a chemical mutagenic technique that involved the application of ethylmethyl sulphonate to the bacterium. A suitable screening method was crucial in determining whether or not the chemical mutagenesis proved to be a successful process. The mutant displaying the highest inulinase activity was then used in the up-scale process by fermentation of inulinase in a 5 L fermenter. During this part of the study, various parameters were observed. Finally chapter four uncovers the entire purification process, which was carried out by column chromatography and thereafter the pH and temperature optima and stabilities as well as the kinetics of the successful mutant were then determined under normal assay conditions.

1.3. AIM

To enhance and up-scale inulinase production from *Xanthomonas campestris* pv. *phaseoli*

1.4. OBJECTIVES

- To produce inulinase from *Xanthomonas campestris* pv. *phaseoli*
- To optimize production of inulinase
- To enhance inulinase production by chemical mutagenesis
- To up-scale inulinase production in a fermenter
- To determine whether the enzyme is an exo- or endoinulinase
- To purifiy the inulinase enzyme

CHAPTER 2: OPTIMIZATION OF GROWTH PARAMETERS OF *Xanthomonas campestris* pv *phaseoli* FOR INULINASE PRODUCTION

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2.1. INTRODUCTION

Of all living organisms, microbes are the most versatile and diversified in their nutritional requirements. Humans and other animals require complex carbon containing compounds as nutrients, whereas in microorganisms, some may grow with a few inorganic substances, while others are more like higher organisms in their need for complex organic compounds. But, all living organism share some common nutritional needs like, carbon, nitrogen and water. Water is principally very important, because microbes can absorb nutrients only when the chemicals are dissolved in water. In addition to macro elements, bacteria also require micronutrients for their metabolic activities.

In order to grow and characterize the physiological and biochemical properties of all organisms, laboratory cultivation of microbial cells is essential. They need a variety of chemical elements as nutrients for both the synthesis and normal functions of cellular compounds, which exist in nature as either organic or inorganic form. The requirement of nutrients for growth and synthesis of specific compounds varies among bacteria. Hence, the optimization of nutrients required is mandatory not only for the growth of inulinase producers but also to attain high level of inulinase synthesis. Inulinase is usually produced when the organism is grown in inulin containing medium. However, the addition of other organic compounds at lower concentration is also needed for an organism to synthesize other cellular constituents.

In lieu of the above the work in this chapter focused on assessing a combination of macro and micronutrients to attain optimum growth and inulinase synthesis in the selected inulinolytic bacterium as well as optimizing associated physical parameters.

2.2. MATERIALS AND METHODS

2.2.1. Chemicals

All chemicals and medium constituents used were of analytical grade. Pure inulin (prepared from chicory roots) was obtained from Sigma Chemical Co, USA.

2.2.2. Bacterium used

Xanthomonas campestris pv *phaseoli* (Xcp) was obtained from the culture collection of the Department of Microbiology, University of Kwa-Zulu Natal, Durban, South Africa. The strain was periodically subcultured in nutrient agar and maintained in 15% glycerol at -70^{o}C.

2.2.3. Medium used for inulinase and FOS production

Inulinase production medium (IPM) consisting of (g L^{-1}) inulin 20, yeast extract 20, $(NH_4)_2$ HPO_4 5, $NH_4H_2PO_4$ 2, $MnCl_2$ 0.5, KCl 0.5, $MgSO_4$ 0.5 and $FeSO_4$ 0.01 (pH 7.0) was used throughout the study (Cho *et al*., 2001). For inulinase production, 50 mL of IPM was dispensed into 250 mL Erlenmeyer flasks and inoculated with 10% (v/v) of a 16 h Xcp culture. Flasks were incubated at 37°C with shaking at 150 rpm for 120 h. Samples were withdrawn every 12 h, centrifuged at 10 000 x g and the supernatant was used for determining inulinase, invertase activities and FOS. All experiments were carried out in triplicate throughout the study.

2.2.4. Determination of enzyme activity and FOS

Inulinase and invertase activities were determined by quantifying the amount of reducing sugars released from inulin and sucrose, respectively, using the dinitrosalicylic acid reagent method (Miller, 1959). The assay mixture comprised 0.05 mL of appropriately diluted enzyme and 0.95 mL of substrate (0.5% inulin for determining inulinase activity and 0.5% sucrose for invertase activity) prepared in sodium acetate buffer (0.05 M, pH 5). After incubation at 45ºC for 10 min, the liberated reducing sugars were determined as fructose equivalents. One enzyme unit (U) is defined as the amount of enzyme needed to release one µmol of fructose per min under standard assay conditions. The ratio of inulinase (I) to invertase (S) activity was also calculated. FOS was calculated by quantifying the reducing sugars (Miller, 1959) released from inulin during the bacterial growth.

2.2.5. Effect of carbon and nitrogen sources on inulinase and FOS production

Fifty mL of inulinase production medium (IPM) was prepared in 250 mL Erlenmeyer flasks and inulin, glucose, fructose, sucrose or lactose were added as sole carbon sources at 2% final concentrations. Different nitrogen sources *viz*., peptone, beef extract, tryptone, yeast extract or urea was added at a 2% concentration in IPM containing 3% sucrose as the sole carbon source. Further optimization of sucrose (0.5% - 6%) and tryptone (0.5% - 6%) concentration was carried out. 10% (v/v) of 16 h old Xcp cultures (with an absorbance value of 0.8 at 600 nm) grown on nutrient broth at 37°C under shaking condition (150 rpm) was used as an inoculum and incubated at 37 °C with shaking at 150 rpm for 120 h.

2.2.6. Effect of temperature and pH on inulinase and FOS production

Fifty mL of IPM containing optimized concentrations of carbon source (3% sucrose for inulinase and 3% inulin for FOS production) and nitrogen source (2.5% tryptone) was prepared in 250 mL

Erlenmeyer flasks. For determination of the effect of pH on inulinase and FOS production, Xcp was grown at pH values from 4.0 to 9.0. The optimum growth temperature was determined by incubating the inoculated flasks (pH 7) at temperatures from 25 to 40^{o}C with shaking at 150 rpm for 120 h.

2.2.7. Inulinase production under optimized conditions

One hundred ml of IPM with sucrose (3%) was prepared in 500 ml Erlenmeyer flasks. Tryptone (2.5%) was added as a nitrogen source. The other ingredients were the same as in IPM. Inoculum from Xcp was added at 10 % (v/v) and incubated under shaking condition (150 rpm) for 120 h. Culture filtrate drawn at every 24 h was checked for extracellular inulinase activity as described in 2.2.4.

2.3. RESULTS AND DISCUSSION

2.3.1. Effect of various growth parameters on inulinase production by *Xcp*

Inulinase activity produced by Xcp on various sugars was determined after 24 h and then declined. Similar trends in inulinase production were observed in *Xanthomonas* sp. and *Pseudomonas* sp (Park and Yun, 2001). Therefore, a 24 h incubation period was chosen to evaluate the maximum inulinase production level. Among the different carbon sources tested, the highest inulinase activity (5.19 ± 0.03 U mL^{-1}) was observed on sucrose followed by fructose (4.22 ± 0.04 U mL^{-1}). Growth on lactose and glucose yielded inulinase activities of 1.3 ± 0.04 and 1.1 ± 0.04 U mL^{-1}, respectively. Inulin was found to be a poor inducer of inulinase (1.47 ± 0.05 U mL^{-1}) (Table 4) and FOS production of 5.6 mg mL^{-1} was observed in medium containing inulin. Inulin has also been observed as a poor inulinase inducer in *Streptomyces* sp. GNDU (Gill *et al*., 2003) and *Aspergillus niger* 245 (Cruz *et al*., 1998). Of the various concentrations of sucrose tested, the maximum inulinase activity of 6.07 ± 0.05 U mL^{-1} was observed at 3% (Table. 5).

Table 4: Effect of various carbon sources on inulinase production

Carbon source (2%)	Inulinase activity ($IUml^{-1}$)*
Sucrose	5.19± 0.03
Inulin	1.47± 0.05
Fructose	4.22± 0.04
Lactose	1.30± 0.04
Glucose	1.10± 0.04

***Each value represents the mean of triplicate determinations with ± SD**

Table 5: Effect of various concentrations of sucrose on inulinase production

Sucrose concentration (%)	Inulinase activity ($IU\ ml^{-1}$)*
0.5	2.82 ± 0.04
1.0	3.96 ± 0.03
1.5	5.19 ± 0.07
2.0	5.28 ± 0.04
2.5	5.90 ± 0.03
3.0	6.07 ± 0.05
3.5	5.10 ± 0.03
4.0	3.17 ± 0.03
4.5	3.17 ± 0.02
5.0	1.58 ± 0.02
5.5	1.14 ± 0.04
6.0	0.44 ± 0.03

***Each value represents the mean of triplicate determinations with ± SD**

Table 6: Effect of various nitrogen sources on inulinase production

Nitrogen source (2%)	Inulinase activity ($IUml^{-1}$)*
Beet extract	5.37± 0.06
Peptone	4.40± 0.05
Tryptone	6.25± 0.06
Yeast extract	5.46± 0.06
Urea	5.10± 0.05

***Each value represents the mean of triplicate determinations with ± SD**

Aspergillus japonicus FCL 119T also showed the maximum inulinase production in a medium containing 3% sucrose (Dorta *et al*., 2006). At higher sugar concentrations, inulinase synthesis was suppressed in Xcp possibly due to catabolite repression (Vandamme and Derycke, 1983 and Singh and Gill, 2006). Reduction in inulinase and inulooligosaccharides yield was also observed in *Xanthomonas oryzae* No. 5, when the cells were grown at higher concentration of the sugar (Cho *et al*., 2001).

Among the different nitrogen sources used at 2% concentration for inulinase production by Xcp with a 3% sucrose medium, the highest inulinase activity, 6.25 ± 0.06 U mL^{-1}, was obtained in tryptone followed by yeast extract, 5.46 ± 0.06 U mL^{-1} (Table 6)

Table 7: Effect of various concentrations of tryptone on inulinase production

Tryptone concentration %	Inulinase activity (U mL^{-1})*
0.5	1.07 ± 0.04
1.0	5.37 ± 0.03
1.5	5.72 ± 0.03
2.0	6.60 ± 0.04
2.5	6.69 ± 0.09
3.0	4.75 ± 0.05
3.5	4.93 ± 0.03
4.0	4.65 ± 0.04
4.5	4.14 ± 0.06
5.0	3.96 ± 0.06
5.5	3.34 ± 0.06
6.0	2.82 ± 0.04

***Each value represents the mean of triplicate determinations with ± SD**

In the presence of other nitrogen sources such as peptone, beef extract and urea, inulinase activities reached 4.40 ± 0.05, 5.37 ± 0.06 and 5.10 ± 0.05 U mL^{-1}, respectively. As tryptone resulted in the highest activity, inulinase production at various tryptone concentrations was evaluated. The highest inulinase activity of 6.69 ± 0.09 U mL^{-1} was observed at 2.5% tryptone and a further increase in tryptone concentration did not show any enhancement in inulinase production (Table 7). In contrast, yeast extract was found to be more effective than tryptone in inulinase production by *Streptomyces* sp. (Gill *et al*., 2003) and *Pichia guilliermondii* (Gong *et al*., 2007). No

considerable difference in FOS production was observed when tryptone concentration was varied. FOS production decreased by 20% at higher concentrations (3.5 -4%) of inulin.

Xcp grown on IPM at varying pH showed that the optimum inulinase production occurred at pH 7. Inulinase production (9.2 ± 0.03 U mL^{-1}) was highest at pH 7 and a reduction in inulinase synthesis was observed under acidic and alkaline conditions (Fig. 2). By contrast, the maximal inulinase production by *Arthrobacter* was observed at acidic pH (Elyachioui *et al*., 1992).

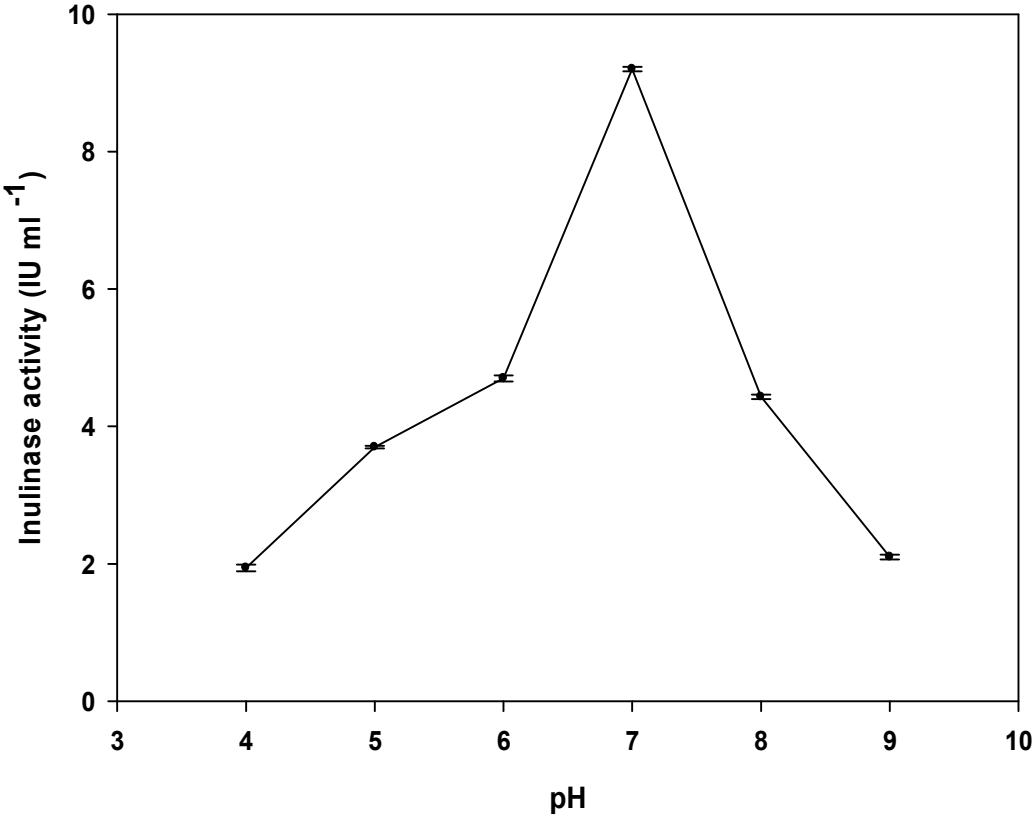

Figure 2: Effect of initial pH of the production medium on inulinase production by *Xcp* after incubation at 37°C with shaking at 150 rpm for 24 h. Each value represents the mean of triplicate determinations with ± SD

The highest level of inulinase synthesis (9.24 ± 0.03 U mL^{-1}) was observed at 35 - 40^{o}C (Fig. 3). Similar trends in inulinase production by *Xanthomonas* sp. were reported by Park *et al*. 1999. In view of the fact that, the inulinase production is growth associated, these results indicate that the medium pH and growth temperature play a vital role in the synthesis of inulinase by Xcp.

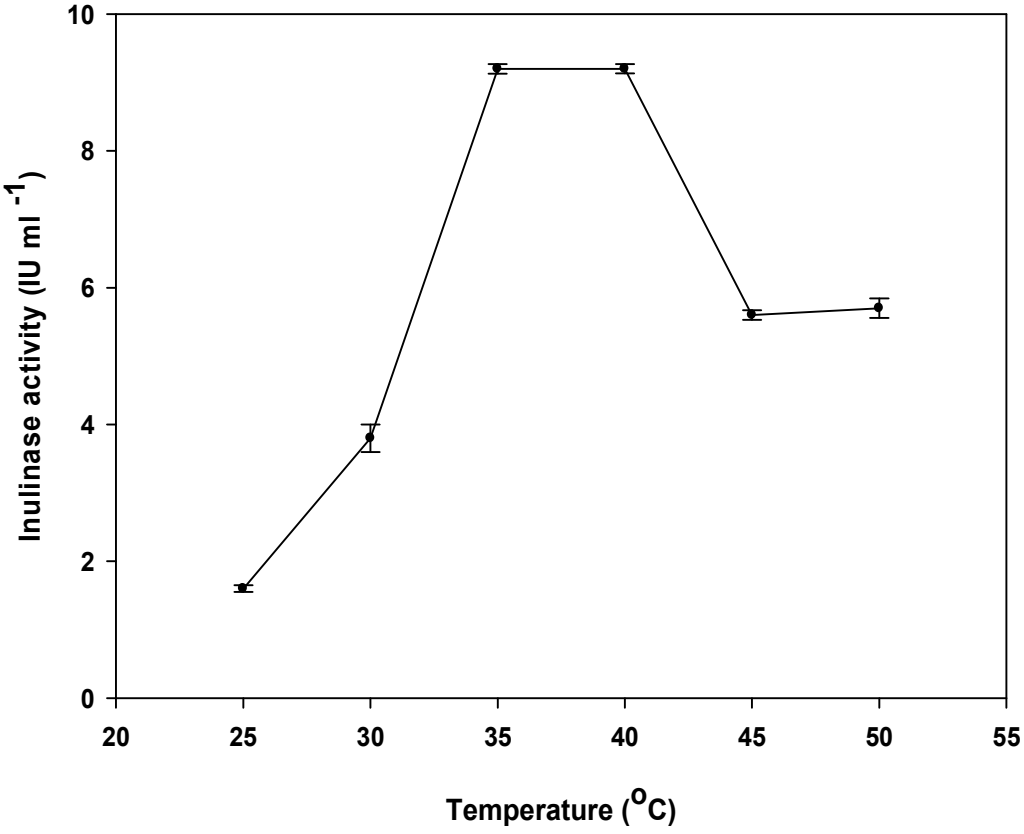

Figure 3: Effect of the growth temperature of *Xanthomonas campestris* pv. *phaseoli* on inulinase production at pH 7 with shaking at 150 rpm for 24 h. Each value represents the mean of triplicate determinations with ± SD

All the above experimental results show that the organism *Xcp* has the ability to grow in different carbon and nitrogen sources, wide range of pH and temperature conditions and can be cultivated easily. High levels of inulinase production can be obtained under optimized growth conditions.

CHAPTER 3: STRAIN IMPROVEMENT THROUGH MUTAGENESIS AND LAB SCALE PRODUCTION OF INULINASE

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3.1. INTRODUCTION

With microbial mutation for enhanced enzyme or metabolite production, it is not possible to predict what type of mutation is needed to give an improved yield in a particular strain. Therefore, it is common to use several types of mutagenic treatment during screening to yield as wide a range of mutants as possible. Other considerations in choosing a mutagen include ease of use and safety. If mutagen dose is too high, fewer mutants per survivor will be produced and may also give rise to other unwanted mutations. It is best to construct a plot for a particular mutagen and strain and then select the optimal dose starting mutagenesis experiment.

Strain improvement through mutagenesis can be divided into three classes: spontaneous, induced and target-selected. In spontaneous mutagenesis, mutations are often unintentionally employed, without the deliberate introduction of a mutagenic agent. The nature and frequency of the most common type of spontaneous mutations can vary between strains. Spontaneous mutations occur without exposure to any obvious mutagenic agent. Sometimes DNA nucleotides shift without warning to a different chemical form (known as an isomer) which in turn will form a different series of hydrogen bonds with its partner. This leads to mistakes at the time of DNA replication. Chemical mutagens change the sequence of bases in a DNA gene as they mimic the correct nucleotide bases in a DNA molecule, but fail to base pair correctly during DNA replication. These mutagens also remove parts of the nucleotide (such as the amino group on adenine), again causing

improper base pairing during DNA replication and they also add hydrocarbon groups to various nucleotides.

Enzymatic microbial hydrolysis of insoluble polymers can be detected as a clear zone surrounding a bacterial colony or by staining of soluble polymers after growth to reveal a zone of hydrolysis (Castro *et al*., 1995). Alternate methods for microbial screening are based on dye coupling to suitable polymeric substrates. When the dye-polymer conjugate is hydrolyzed, the oligosaccharide-dye molecules diffuse from the colony zone producing pale or colourless halos (Dhawale *et al*., 1982). The most useful plate technique for screening inulinase producing microorganisms is based on the capability of carbohydrate assimilation (Yokota *et al*., 1991).

3.2. MATERIALS AND METHODS

3.2.1. Microorganism used

A loopful of *Xanthomonas campestris* pv. *phaseoli* (Xcp) stock was streaked onto nutrient agar plates and incubated at 37°C overnight. A single colony was then inoculated into nutrient broth until it reached an absorbance value of 0.8 (approximately 10^8 cells per ml) at 600 nm.

3.2.2. Strain improvement through chemical mutagenesis

Chemical mutagenesis was carried out using EMS as described by Kamal *et al*. (2003) with slight modifications. Xcp was grown in nutrient broth until it reached an absorbance value of 0.8 (approximately 108 cells mL-1) at 600 nm. Five mL of the culture was centrifuged at 10 000xg for 15 min and the bacterial pellet was washed twice with buffer (1.05% K2HPO4, 0.45% KH2PO4, 0.1% (NH4)2SO4 and 0.05% sodium citrate, pH 7), then resuspended again in 2.5 mL of the same buffer. Forty micro litres of EMS (0.05% - Sigma Chemical, Co, USA) was added and incubated at 37°C (150 rpm) for 1 h and subsequently 4 mL of sodium thiosulphate (10%) was added to

quench the mutagen. Finally, 0.5 mL of bacterial cell suspension was transferred into 10 mL nutrient broth and incubated at 37°C overnight to recover the mutants. Bacterial cultures were then serially diluted in 0.85% NaCl solution and 100 µl from each dilution was spread on nutrient agar plates and incubated at 37°C for 24 h. Optimized inulinase/FOS production medium was used to test the mutants for their ability to produce inulinase and FOS. Mutants were grown in test tubes containing 10 mL of optimized IPM (pH 7) at 37 oC (150 rpm). A sample was withdrawn after 24 h and centrifuged at 10 000 g and the supernatant was used for inulinase activity, invertase activity and FOS determination.

3.2.3. Inulinase, invertase and fos production by xcp KM 24 mutant in a fermenter

The study was carried out in a vertical 5 L glass fermenter (Minifors, Switzerland) with a working volume of 3 L medium containing carbon (3% sucrose for inulinase and 3% inulin for FOS production) and 2.5% tryptone as a nitrogen source. A seed culture of *Xcp* KM 24 (16 h old) was prepared in nutrient broth, inoculated at 10% (v/v) concentration and grown for 120h at 150 rpm, 1.5 vvm (using the cascade mode) and 37^{o}C respectively. pH changes and cell growth were monitored at 6 h intervals. Twenty millilitres of the culture was withdrawn and centrifuged at 10 000 x g for 15 min. The supernatant was used to determine inulinase and invertase activities, reducing sugars (Miller, 1959), total sugars (Dubois *et al*., 1956) and FOS (Miller, 1959 and Dubois *et al*., 1956). Bacterial cell biomass was quantified using a biomass probe (Aber Inst, UK).

3.2.4. Thin layer chromatography (TLC)

The products of inulin hydrolysis with crude inulinase of Xcp KM 24 were studied by performing the enzyme reaction (0.5 mL of crude inulinase and 0.5 mL of 0.5% (w/v) inulin) at 45^{o}C for 24 h. Pre-coated TLC plates (Silica gel 60, Merck, Germany) spotted with samples were developed with the solvent system, ethyl acetate: acetic acid: 2-propanol: formic acid: water (25: 10: 5: 1: 15 v/v). Sucrose (Sigma, USA), glucose (Sigma, USA), fructose (Merck, Germany), 1-kestose, 1, 1-

kestotetraose and 1, 1, 1-kestopentaose (Megazyme, Ireland) were used as standards. After pouring the detection reagent containing 1% (v/w) orcinol and 10% (v/v) sulphuric acid in absolute ethanol, the TLC plate was heated at 100^{o}C for 5 min and sugars were detected.

3.3. RESULTS AND DISCUSSION

3.3.1. Strain improvement through chemical mutagenesis

One hundred and fifty mutants obtained after subjecting Xcp to the chemical mutagen (EMS) were tested for inulinase and FOS production using IPM. Among the 150 mutants, 62 strains showed an improved inulinase (41%) and FOS (50%) production and 88 strains showed decreased (59%) inulinase production than the parent strain. The mutant, Xcp KM 24 showed the highest inulinase production of 22.09 ± 0.03 U mL^{-1} (2.4 fold higher than the wild type) and FOS production of 11.9 mg mL^{-1} (2.1 fold higher than the wild type). Similar results on improved inulinase production through mutagenesis have been reported for *Aspergillus niger* (Nakamura *et al*., 1994 and Vishwanathan, 1995) and *Penicillium purpurogenum* (Sharma *et al*., 2005). This mutant Xcp KM 24 was tested thrice for its stability in inulinase and FOS production and it was very stable. Inulinase production by a mutant was also confirmed further by subcuturing the strain after four months, which showed similar inulinase activity and FOS yield.

3.3.2. Inulinase, invertase and fos production in a 5 l fermenter

Xcp KM 24 was grown in a fermenter under controlled growth conditions and studied for inulinase; invertase and FOS production over a period of 120 h. Inulinase and invertase production were increased up to 18 h and gradually decreased afterwards (Fig. 4b). The highest inulinase and invertase activities of 21.9 ± 0.03 and 8.4 ± 0.8 U mL^{-1}, respectively, with an inulinase/invertase (I/S) ratio of 2.6 were observed in the late exponential stage (Fig. 4d). In contrast, the maximum inulinase activity of 17.42 U.mL^{-1} was observed only after 48 h in our previous study, where the

Xcp wild type strain was grown on inulin as a sole carbon source (Ayyachamy *et al*., 2007). Similarly, the highest inulinase production (77 $U.mL^{-1}$) by *X. oryzae* from inulin during late exponential growth phase has been observed (Cho and Yun 2002).

Maximum volumetric (21 865 $U\ l^{-1}\ h^{-1}$) and specific (119 025 $U\ g^{-1}\ h^{-1}$) productivities of inulinase were attained after 18 h in a fermenter (Fig. 5d). In this study, the maximum biomass (0.26 $g\ L^{-1}$) were reached after 24 h, whilst the pH of the culture medium remained between 6.7-7.0 over 18 h with a concomitant higher inulinase production (Fig. 4a).

After 24 h, the pH decreased to 5.15, which may have affected the inulinase production suggesting that the maximum inulinase synthesis occurred at pH 7. Although, inulinase production declined, small amount of sucrose utilization was observed during the late log phase of Xcp KM 24. However, this did not contribute to cell biomass improvement.

Most of the microbial preparations of inulinase are accompanied with significant invertase activity. In general, the catalytic activity of inulinase (I) and invertase (S) is described in terms of I/S ratio (Vandamme and Derycke, 1983 and Ettalibi and Baratti, 1987). The I/S ratio was found to be maximum (5.1) when an inulinase activity of 12.1 $U\ mL^{-1}$ and invertase activity of 2.4 $U\ mL^{-1}$ observed during exponential phase of the mutant and declined afterwards (Fig 4c). The crude inulinase of Xcp KM 24 had a high I/S ratio (5.1) which indicated that the enzyme has more affinity to inulin than sucrose. For fructooligosaccharides production, the enzyme preparations should contain a high I/S ratio (Vandamme and Derycke, 1983 and Ettalibi and Baratti, 1987). Xcp KM 24 inulinase also demonstrated the highest catalytic activity of inulinase which can be used for FOS production.

The optimal inulinase production was found at 150 rpm and 1.5 vvm, respectively. At lower agitation rates, the inulinase production was decreased by 31% (75 rpm) and 21% (100 rpm). Similar trends in inulinase production by *X. oryzae* were observed (Cho and Yun, 2002). In contrast, the maximum inulinase production by *Kluyveromyces marxianus* was observed in a fermenter operated at higher agitation (450 rpm) rate (Silva-santisteban and Filho, 2005). Total sugar analysis after 66 h indicated that 97% of the sugar was utilized by Xcp KM 24 during inulinase production (Fig. 4d). Inulinase production levels in the fermenter were comparable with shake flask experiments.

A maximum FOS production of 11.9 g L^{-1} h^{-1} (Fig. 5a) and specific productivity of 72 g g^{-1} h^{-1} FOS from inulin was observed in a fermenter (Fig. 5c). The maximum FOS production by Xcp KM 24 was observed after 24 h (Fig. 5a). In contrast, the maximum concentration of FOS reached only after 72 h in *A. niger* IMI 303386 (Nguyen, 1999) The highest yield of FOS (60%) was observed in Xcp KM 24, whereas in *Pseudomonas* sp. 80% of FOS yield was achieved from chicory juice at a higher substrate concentration (Park *et al*., 1998). The possible reason could be the non-availability of sugars other than fructan, which could have arrested bacterial growth and FOS production. The maximum inulinase activity of 12.4 ± 0.02 U mL^{-1} was observed in inulin medium (Fig. 5a) after 30 h, which was lower than our previous study (Ayyachamy *et al*., 2007). The reduction in inulinase production by Xcp KM 24 could be due to a higher concentrations of inulin (3%) used in this study. In addition, the bacterial biomass (0.204 g L^{-1}) obtained was also lower (Fig. 5b) than sucrose grown cultures.

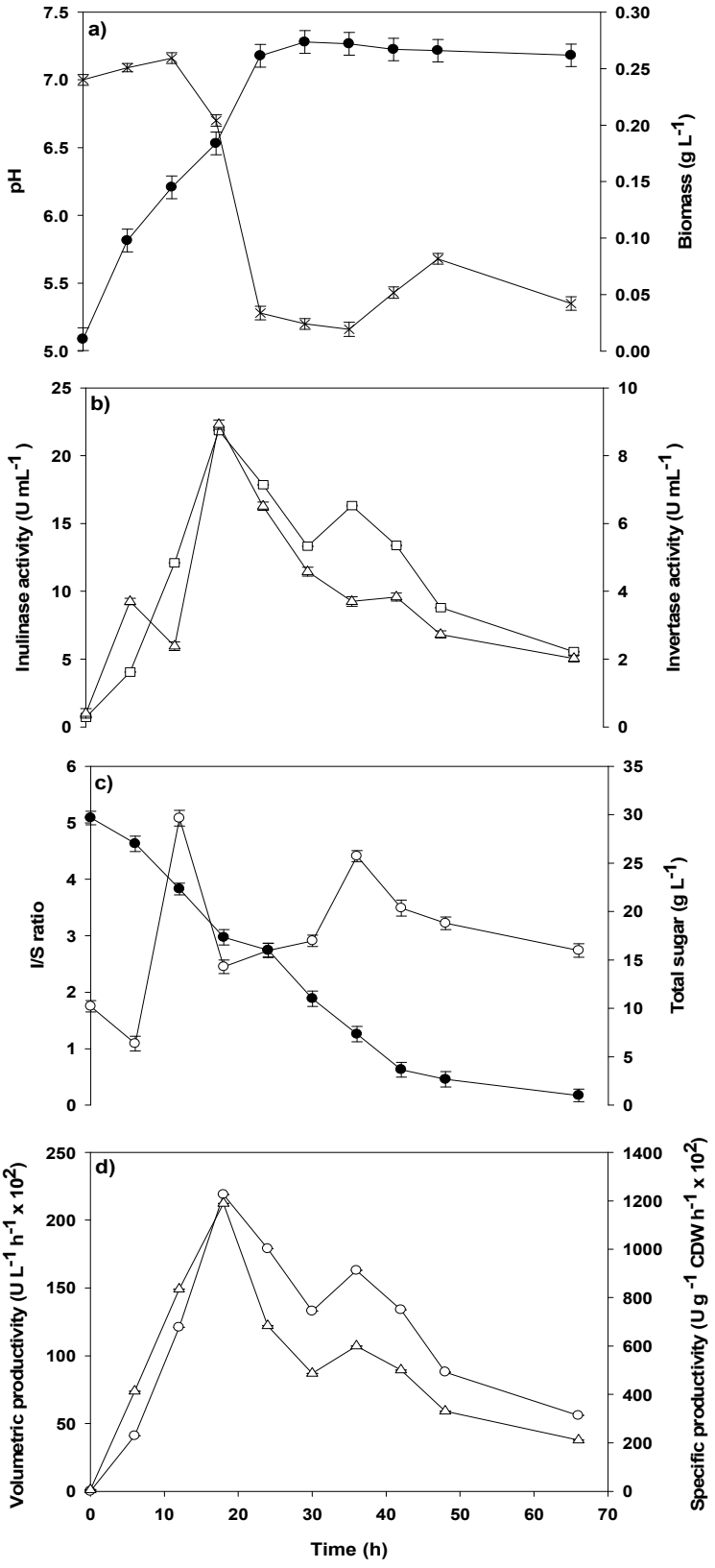

Figure 4: Laboratory scale production of inulinase by Xcp KM 24 in a 5 L fermenter.

The cultures were grown on IPM (containing 3% sucrose and 2.5% tryptone, w/v) at 37 ^{o}C for 120 h. Agitation and aeration rates were maintained at 150 rpm and 1.5 vvm, respectively with the evaluation of the following parameters: a) pH (X) and biomass (●), b) Inulinase (□) and invertase (△), c) I/S ratio (○) and total sugar (●), d) Volumetric productivity of inulinase (○) and specific productivity of inulinase (△). Each value represents the mean of triplicate determinations.

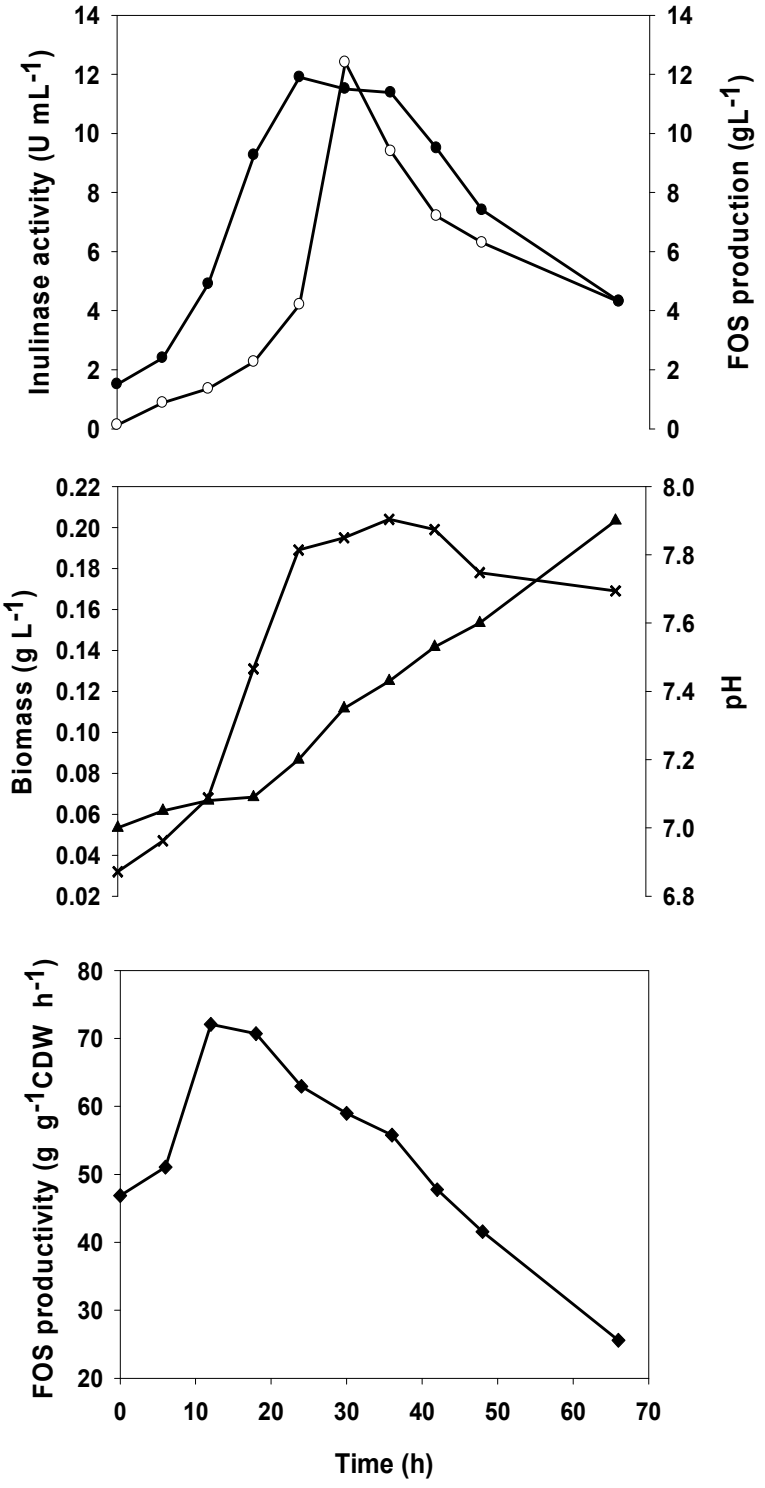

Figure 5: Laboratory scale production of fructooligosaccharides by Xcp KM 24 in a 5 L fermenter. The cultures were grown on IPM (containing 3% inulin and 2.5% tryptone, w/v) at 37°C for 120 h. Agitation and aeration rates were maintained at 150 rpm and 1.5 vvm, respectively with the evaluation of the following parameters: a) Inulinase (○) and FOS production (●) b) Biomass (●) and pH (X), c) Specific productivity of FOS (□). Each value represents the mean of triplicate determinations.

3.3.3. Inulin hydrolysis by crude inulinase

TLC analysis of inulin hydrolyzed products showed that oligosaccharides were the predominant end product over a hydrolysis time period of 10 min to 2 h and rapid conversion of these oligosaccharides into monosaccharides occurred after 24 h (Fig. 6).

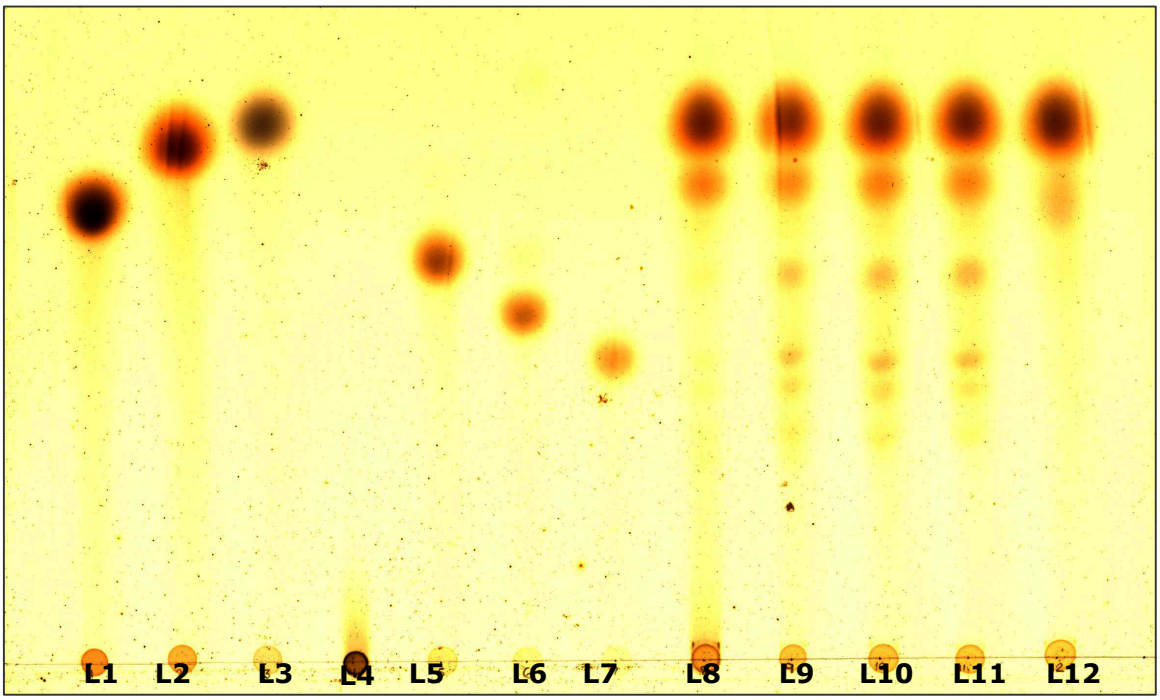

Figure 6: TLC of reaction products formed during hydrolysis of inulin by crude inulinase from *X. campestris* KM 24. L1 - Sucrose, L2 - Glucose, L3 - Fructose, L4 – Inulin, L5 – 1-Kestose, L6- 1,1-Kestotetraose, L7- 1,1,1-Kestopentaose, L8 - enzyme + inulin (0h), L9- enzyme + inulin (10 min), L10 - enzyme + inulin (30 min), L11- enzyme + inulin (2 h), L12 - enzyme + inulin (24 h).

Oligosaccharides with varying degrees of polymerization were observed in 10 min, 30 min and 2 h hydrolysate samples and this hydrolysis pattern suggests that the presence of an endoinulinase in Xcp KM 24. The prolonged enzyme hydrolysis (24 h) revealed only mono (glucose and fructose) and disaccharides (sucrose) in the hydrolysates, which point to the presence of an exoinulinase also. This will require confirmation by purification and characterization of the enzymes. In contrast, inulin hydrolysis by an extracellular inulinase of *Rhizopus* sp. resulted in the occurrence of fructose and oligosaccharides after 24 h incubation (Ohta *et al*., 2002). Fructose formation was completely absent when inulin was hydrolyzed with crude endoinulinase of *Xanthomonas oryzae*

No 5 (Cho *et al*., 2001). Since the crude inulinase (culture filtrate) obtained from an 18 h old mutant (grown on 3% sucrose) was used as enzyme source in this study, the presence of glucose, fructose and sucrose was observed at 0 h.

CHAPTER FOUR: PURIFICATION AND CHARACTERIZATION OF ENDOINULINASE FROM *Xanthomonas campestris* pathovar *phaseoli* MUTANT KM 24

4.1. INTRODUCTION

Inulin has received great interest as a renewable raw material for fructose syrup and fructooligosaccharides (FOS) production (Yun, 1996). Inulinases are widely used in food and pharmaceutical industries. Microbial inulinases can be divided into exo- and endo-acting enzymes according to their modes of action on inulin. Endoinulinases (2,1- β -D-fructan fructanohydrolase; EC 3.2.1.7) are specific for inulin and hydrolyse the internal β-2,1-fructofuranosidic linkages to yield inulooligosaccharides as the main products, e.g. inulotriose, inulotetraose, and inulopentaose. Exoinulinases (β-D-fructan fructohydrolase; EC 3.2.1.80) successively split off terminal fructose units from the non-reducing end of inulin, and also hydrolyse sucrose and raffinose (Vandamme and Derycke, 1983 and Singh and Gill, 2006). Therefore, inulin and inulinase can be used for production of either ultra-high fructose syrups, with D-fructose content over 95% by exo-enzymatic hydrolysis, or for production of oligofructoside syrups by endo-enzymatic hydrolysis (Mazutti *et al*., 2006). Inulinases are produced by few bacteria (*Xanthomonas* sp., *Bacillus* sp., *Pseudomonas* sp., *Thermotoga* sp., *Bifidobacterium* sp., *Geobacillus* sp., *Clostridium* sp.), fungi (*Aspergillus* sp., *Penicillium* sp., *Fusarium* sp.) and yeast (*Kluyveromyces* sp.) (Singh and Gill, 2006). In our previous studies, we have reported the optimization of the nutritional and growth parameters of *X. campestris* pathovar *phaseoli* for endoinulinase production using the submerged and solid state cultivations (Ayyachamy *et al*., 2007). Endoinulinase and FOS production level was further enhanced through ethylmethanesulfonate (EMS) mutagenesis of *X. campestris* pathovar *phaseoli* and the mutant was named *X. campestris* pathovar *phaseoli*, mutant KM 24 (Xcp KM 24) (Naidoo *et al*., 2009). The present study therefore focused on the purification and

characterization of endoinulinase from Xcp KM 24 using gel filtration chromatography. Since the importance and potential applications of FOS is growing globally, this endoinulinase could be used for commercial applications like for large scale FOS (alternate sweetener) production and would help in reducing the overall production cost.

Enzymes are only active over a limited pH range and in most cases at a definite optimum pH. This optimum may be due to a true reversible effect of the enzyme velocity (V), an effect of pH on the affinity and, an effect of pH on the stability of the enzyme. Most pH effects are due to changes in the ionization of the components of the system as the pH changes. The optimum pHs of the purified inulinases from fungi and yeast are in the range of 4.5-6.0 (Sheng *et al*. 2009; Gong *et al*. 2008; Pandey *et al*. 1999; Zhang *et al*. 2005; Singh *et al*., 2007).

Several different effects of temperature on the enzyme reaction velocity have been reported (Dixon and Webb, 1979). These include effects on the thermostability, affinity of the enzyme for a substrate and on the actual velocity of the breakdown of the enzyme-substrate complex. Temperature optima can be determined by the balance between the effect of temperature on the rate of reaction and its effect on the rate of inactivation of the enzyme (Dixon and Webb, 1979).

The optimal temperature of the purified enzyme from the marine yeast *C. aureus* G7a is 50ºC and the enzyme is very stable up to 65ºC (Sheng *et al*., 2009). From these results, the inulinase seemed to have considerable thermostability. However, the inulinase activity produced by *P. guilliermondii* strain 1 is the highest at 60ºC and the enzyme is very stable up to 60ºC (Gong *et al*., 2008). Inulinase from terrestrial microorganisms, in general shows the highest activity below 50ºC whereas optimum temperature is mostly between 30ºC and 55ºC (Pandey *et al*. 1999; Kushi *et al*. 2000; Zhang *et al*. 2005; Singh *et al*. 2007).

It has been reported that most of the inulinases from fungi have more than 50 kDa of molecular weight (Pandey *et al*., 1999). For instance the molecular mass of the purified inulinase from the supernatant of the cell of the cell culture of the marine yeast *C. aureus* G7a was estimated to be 60.0 kDa (Sheng *et al*., 2009), while the molecular mass of the purified inulinase from *P. guilliermondii* strain 1 was estimated to be 50.0 kDa (Gong *et al*., 2008). However it has been reported that the extracellular inulinase from the terrestrial yeast *K. fragilis* has 250 kDa of molecular weight. The molecular mass of the purified exoinulinase from bacteria was estimated to be approximately 54.0 kDa (Tsujimoto *et al*., 2003; Kwon *et al*., 2000). This suggests that molecular weight of the exoinulinases from bacteria is almost the same as the exoinulinases from yeasts. The endoinulinases from fungi and bacteria were also purified and characterized. For example, the endoinulinase produced by *Penicillium* sp. TN-88 has an apparent molecular weight of 68.0 kDa (Nakamura *et al*., 1997) while the molecular mass of the extracellular endoinulinase from *Arthrobacter* sp. S37 is 75 kDa (Kang *et al*., 1998).

Measurements of enzymatic reactions are used to characterize enzymes with regard to their substrate affinities and maximal velocity rates. By measuring the rate of substrate utilization (V) at different substrate concentrations (s), K_m and V_{max} can be calculated by the method of Lineweaver-Burk, Eadie/Hofstee or Wilkinson (Counotte and Prins, 1979; Lineweaver and Burk, 1934).

4.2. MATERIALS AND METHODS

4.2.1. Bacterium

Xcp KM 24 was obtained from the culture collection at the Department of Microbiology at the University of Kwa- Zulu Natal, Durban, South Africa (Naidoo *et al*., 2009). The strain was maintained in 70% glycerol at -70^{o}C. The medium used for exoinulinase production (IPM) contained in gL^{-1}: inulin 20, yeast extract 20, $(NH_4)_2$ HPO_4 5, $NH_4H_2PO_4$ 2, $MnCl_2$ 0.5, KCl 0.5,

$MgSO_4$ 0.5 and $FeSO_4$ 0.01 and pH was adjusted to 7.0 (Cho *et al*., 2001). Pure inulin prepared from chicory roots was obtained from Sigma Chemical Co, USA. For extracellular endoinulinase production, 50 ml of IPM was dispensed into 250 ml Erlenmeyer flasks and inoculated with 10% (v/v) of a 16 h old Xcp KM 24 culture. Flasks were incubated in a shaker at 37^{o}C at 150 rpm for 120 h. Samples were withdrawn every 12 h, centrifuged at 10,000 g and the supernatant was used for determining inulinase activity (Ayyachamy *et al*., 2007 and Naidoo *et al*., 2009).

4.2.2. Inulinase activity

Inulinase activity was determined by quantifying the amount of reducing sugars released from inulin using the DNS reagent (Miller, 1959). The reaction mixture containing 0.1 mL of crude enzyme extract and 0.9 mL of sodium acetate buffer (0.1 M, pH 5.5) containing 2% inulin was incubated at 50^{o}C for 20 min. One inulinase unit (IU) was defined as the amount of enzyme that produces one micromole of fructose equivalent per minute under standard assay conditions.

4.2.3. Purification of inulinase

To begin the purification process, 1000 ml of inulinase production medium was subjected to ammonium sulphate precipitation at 4ºC overnight. The protein precipitated at 40-80% saturation was collected by centrifugation and the pellet was dissolved in 5 ml of sodium phosphate buffer (pH 7) and dialyzed against the same buffer. After concentrating the sample by ultrafiltration (Centricon Plus-20 Centrifugal filter cut-off of 30 kDa, Amicon- Bioseparation), the sample was chromatographed on a Sephadex G-100 column eluted with 50mM phosphate buffer at flow rate of 1ml/min. One millilitre of enzyme sample (1mg/ml) was loaded onto the column and 4 ml fractions were collected and assayed for protein and inulinase activity. Active fractions were pooled and mixed with 4 volumes of acetone. The precipitate was dissolved in the sodium phosphate buffer (pH 7), assayed for inulinase activity and used for SDS-PAGE.

4.2.4. Sodium dodecyl sulphate –polyacrylamide gel electrophoresis (SDS- PAGE)

SDS–PAGE was performed according to Laemmli (Laemmli, 1970) on vertical slab 12% polyacrylamide gel containing 0.1% SDS and Tris–glycine buffer (1M) containing 0.1% SDS. The inulinase proteins were stained with Coomassie Brilliant Blue G-250. The molecular weight of each inulinase was estimated by SDS–PAGE, using an electrophoresis calibration kit (Sigma M3913), with bovine serum albumin (66 kDa), chicken egg ovalbumin (45 kDa), rabbit muscle glyceraldehyde-3-phosphate dehydrogenase (36 kDa), bovine erythrocytes carbonic anhydrase (29 kDa), bovine pancreas trypsinogen (24 kDa), trypsin inhibitor (20 kDa), bovine milk α-Lactalbumin (14.2) and bovine lung aprotinin, (6.5 kDa) as standards.

4.2.5. Protein determination

Protein content was determined as previously described by Bradford (Bradford, 1976) using bovine serum albumin as standard (Sigma Chemical Co., St. Louis, MO, USA).

4.2.6. Determination of optimum pH and temperature

In order to determine the optimum pH of the purified inulinase (1µM), 0.5% (w/v) substrate inulin solutions were prepared in the following buffers (100 mM): citrate buffer (pH 4-6); sodium phosphate buffer (pH 7); Tris-HCl (pH 8 and 9) and glycine NaOH (pH 10). The enzyme was incubated with the substrate at 50°C for 20 min. To determine optimum temperature, the enzyme (1µM) was incubated with the substrate prepared in the most appropriate pH buffer. The assay mixture containing 50 µl of the enzyme solution and 950 µl of the substrate solution in sodium phosphate buffer (100 mM, pH 7) was incubated at temperatures ranging from 25 to 90ºC.

4.2.7. Determination of pH and temperature stability

The pH stability of the enzyme was determined by incubating 1ml (10 µM) of the enzyme at pH 4, 5, 6, 7, 8 and 9 at 50ºC. Aliquots of 100µl were removed at time intervals of 0, 10, 20, 30, 60, 90, 120, 150 and 180 min, respectively and assayed by incubating with 900µl of substrate as mentioned above. For temperature stability of the purified enzyme, the substrate was prepared in the most suitable pH buffer. Inulinase (10 µM) was then incubated at different temperatures 50ºC, 60ºC, 60ºC, 80ºC, and 90ºC. Aliquots of 100µl were then removed at the time intervals of 0, 10, 20, 30, 60, 90, 120, 150 and 180 min and assayed for inulinase activity.

4.2.8. Determination of K_m, V_{max} and K_{cat} values

Inulinase (1µM) was incubated with different substrate concentrations ranging from 0.2-15 µg/ml in sodium phosphate buffer, pH 7 at 45ºC and the activity was measured as described previously. The K_m and the V_{max} values were determined from the Lineweaver-Burk plot by applying the Michaelis-Menten equation (Equation 1) using the software, Origin 7 (Origin Lab. U.S.A.). The k_{cat} (catalytic efficiency or turnover number of the enzyme) value was also determined by applying equation 2. Enzyme catalytic efficiency was calculated by applying the equation k_{cat}/K_m.

$$1/V_0=(K_m/V_{max})(1/[S])+1/V_{max} \qquad \text{Equation 1}$$

$$k_{cat}=V_{max}/[E]_T \qquad \text{Equation 2}$$

Where $[E]_T$ is the total enzyme concentration (1µM), [S] is the substrate concentration, V_0 is the initial velocity, V_{max} is the maximum velocity and K_M is the Michaelis-Menten constant.

4.2.9. Thin layer chromatography (TLC)

The products of inulin hydrolysis with purified endoinulinase of Xcp KM 24 were studied by performing the enzyme reaction [0.5 ml of purified inulinase and 0.5 ml of inulin (0.5% w/v)] at 45ºC for 24 h. Pre-coated TLC plates (Silica gel 60, Merck, Germany) spotted with samples were developed with the solvent system, ethyl acetate:acetic acid:2-propanol:formic acid:water (25:10:5:1:15 v/v). Sucrose (Sigma, USA), glucose (Sigma, USA), fructose (Merck, Germany), 1-kestose, 1, 1-kestotetraose and 1, 1, 1-kestopentaose (Megazyme, Ireland) were used as standards. After pouring the detection reagent containing 1% (v/w) orcinol and 10% (v/v) sulphuric acid in absolute ethanol, the TLC plate was heated at 100ºC for 5 min and sugars were detected (Naidoo *et al*., 2009).

4.3. RESULTS AND DISCUSSION

4.3.1. Purification of inulinase

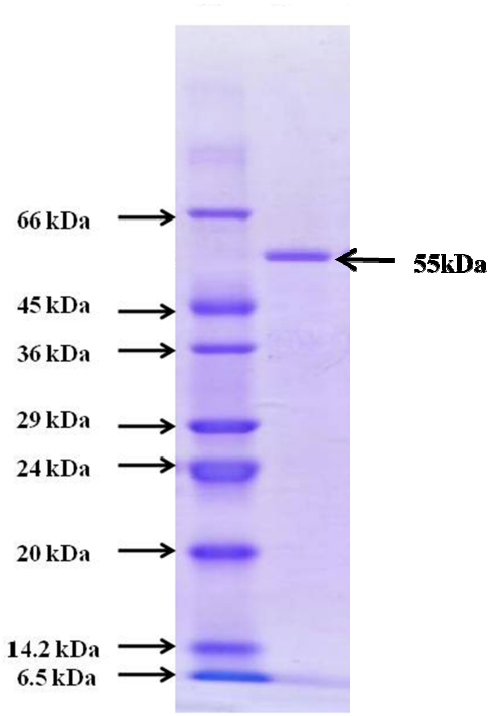

Figure 7: SDS PAGE of *Xanthomonas campestris* pv. *phaseoli* Lane 1: Molecular weight standards, Lane 2: Purified endoinulinase

Table 8: Summary of purification and yield of endo-inulinase from the supernatant of *Xanthomonas campestris* pv. *phaseoli*

Purification step	Total protein (mg)	Endoinulinase activity (U/ml)	Yield (%)	Specific activity (U/mg)	Purification factor
Crude culture filtrate	0.217	22.12	100	102	1
$(NH_4)_2SO_4$ precipitation	0.179	18.76	85.2	104.8	1.02
Sephadex G-100	0.097	16.95	77	174.74	1.74

The present study reports the purification and characterization of an endoinulinase from Xcp KM 24. Xcp KM 24 endoinulinase also demonstrated the highest catalytic activity of inulinase which can be used for FOS production. The molecular weight of inulinase reported in the present study is 55kDa.

A considerable variation in molecular weight has been earlier reported namely, *Arthrobacter* sp. (75 kDa), *Bacillus stearothermophilus* KP1289 (54 kDa), *Aspergillus candidus* (54 kDa), *Penicillium* sp. TN-88 (68 kDa), *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 and Sharma and Gill, 2007). Ettalibi and Baratti (1987) reported that five exoinulinases from *Aspergillus ficuum* showed the same molecular weight of 74 kDa and three endoinulinases had a molecular weight of 64 kDa (Ettalibi and Baratti, 1987). Chen *et al* (2009) have reported five enzymes from *Aspergillus ficuum* JNSP5-06, with molecular weights of 70 kDa, 40 kDa, 46 kDa, 34 kDa and 31 kDa, respectively (Han-*Qing et al*., 2009). Park *et al.* (1999) reported a novel inulinolytic strain of *Xanthomonas* sp., which produced an endoinulinase optimally active at 45°C and pH 6.0 (Park *et al*., 1999). An endoinulinase produced by Xcp KM 24 was purified to homogeneity in two steps, ammonium sulphate precipitation and Sephadex G-100 column chromatography with 77% yield and had a specific activity of 174.74 U/mg. The

summary of the enzyme purification and the yield is shown in Table 8. The final enzyme preparation was homogeneous on SDS-PAGE, which showed molecular mass of 55 kDa (Fig. 7).

4.3.2. Optimum temperature and thermostability

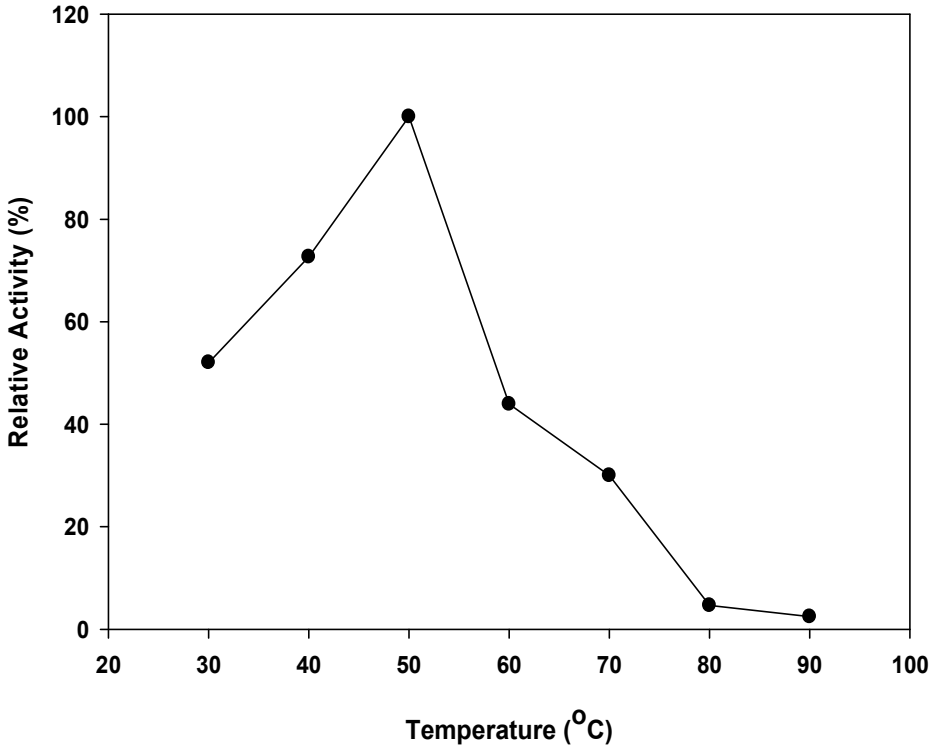

Figure 8: Effect of temperature on the activity of endoinulinase in the culture supernatant of *Xcp* mutant KM 24 after incubation for 20 min. Each value represents the mean of triplicate determinations with ± SD

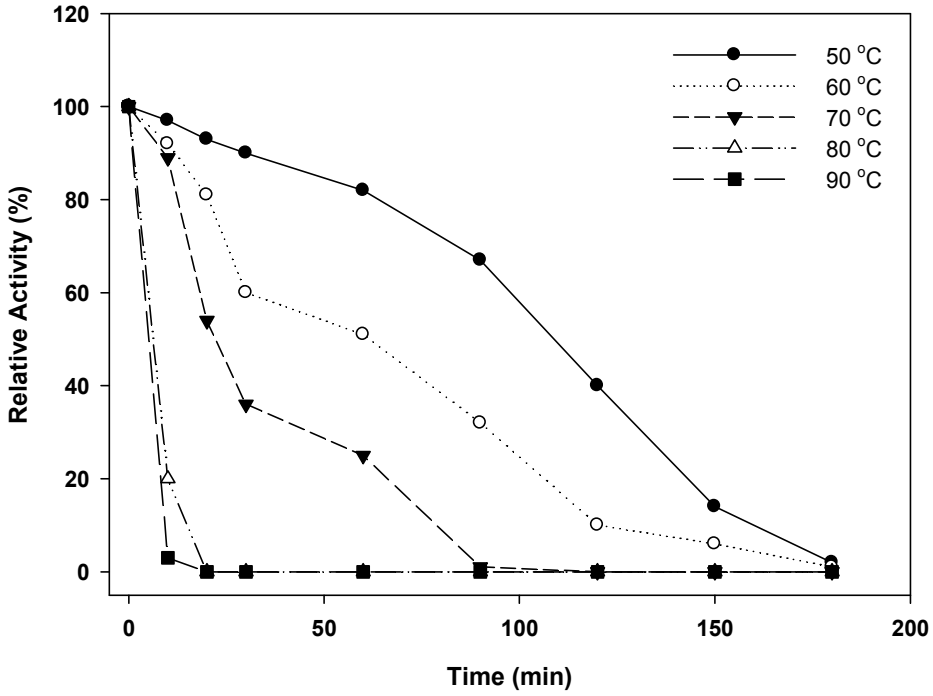

Figure 9: Effect of temperature on the stability of endoinulinase in the culture supernatant of *Xcp* mutant KM 24 after incubation for 3h. Each value represents the mean of triplicate determinations with ± SD

The inulinase activity measured as a function of temperature from 40°C to 90°C shows the activity was the highest at 50°C (Fig. 8). As shown in Fig. 9, the enzyme was stable up to 60°C, keeping over 60% activity for 30 min, but inactivated rapidly at temperatures above this. At 90°C, the enzyme lost its total activity within 10 min. The optimum temperature for activity of the enzyme was 90–95°C. The gene encoding for a 432-residue polypeptide of about 50 kDa protein was expressed in *Escherichia coli* and the recombinant enzyme hydrolyzed inulin quantitatively in an exo-type fashion. The optimal temperature of the purified enzyme from the marine yeast *C. aureus* G7a is 50ºC and the enzyme is very stable up to 65ºC (Sheng *et al*., 2009). However, the inulinase activity produced by *P. guilliermondii* strain 1 is the highest at 60ºC and the enzyme is very stable up to 60ºC (Gong *et al*., 2008). The optimum pH of the purified inulinases from fungi and yeast are reported to be in the range of 4.5-6.0 (Sheng *et al*., 2009; Gong *et al*., 2008; Singh *et al*., 2007; Zhang *et al*., 2005 and Pandey *et al*., 1999).

4.3.3. pH optimum and pH stability

The inulinase activity was measured in the pH range of 4-10 in buffers with the same ionic concentrations. The results in Fig. 10 show that maximum activity was observed at pH 6.0. The activity of the purified enzyme was stable between pH 6.0 and 9.0 (Fig. 11). After 2h, more than 40% and 45% of the residual activity remained at pH 6.0 and 9.0 at 50^{o}C, respectively.

An extracellular endoinulinase of 139 kDa, which converted inulin into inulooligosaccharides, was later purified from *Xanthomonas oryzae* (Cho and Yun, 2002) and was optimally active at pH 7.5 and 50 °C, stable over a pH range of 6.0–9.0. Takahashi *et al*. (1985) purified an exoinulinase of 83 kDa from *Streptococcus salivarius* with pH optimum of 7.0 (Takahashi *et al*., 1985). The inulin-inducible inulinase of *Clostridium acetobutylicum* was reported to produce both extra- and intracellularly with the pH and temperature optima of 5.5 and 47 °C, respectively (Looten *et al*., 1987). The endoinulinase from *C. thermoautotrophicum* was maximally active at 60 °C and neutral pH (Drent and Gottschal, 1991). *Bifidobacterium longum*, *Bacillus infantis* and *Bacillus angulatum* were also reported to produce chicory fructooligosaccharides metabolizing activity (Durieux *et al*., 2001). The D-fructofuranosidase of *B. infantis* was a monomeric protein of 70 kDa and possessed both inulinase and invertase activities (Warchol *et al*., 2002) and the purified endoinulinase showed the optimum pH and temperature, 6.0 and 37°C, respectively. The gene encoding for one of the most thermostable bacterial inulinases, which retained 85 % of its initial activity after 5 h at 80 °C and pH 7.0, was cloned from *Thermotoga maritima* (Liebl *et al*., 1998).

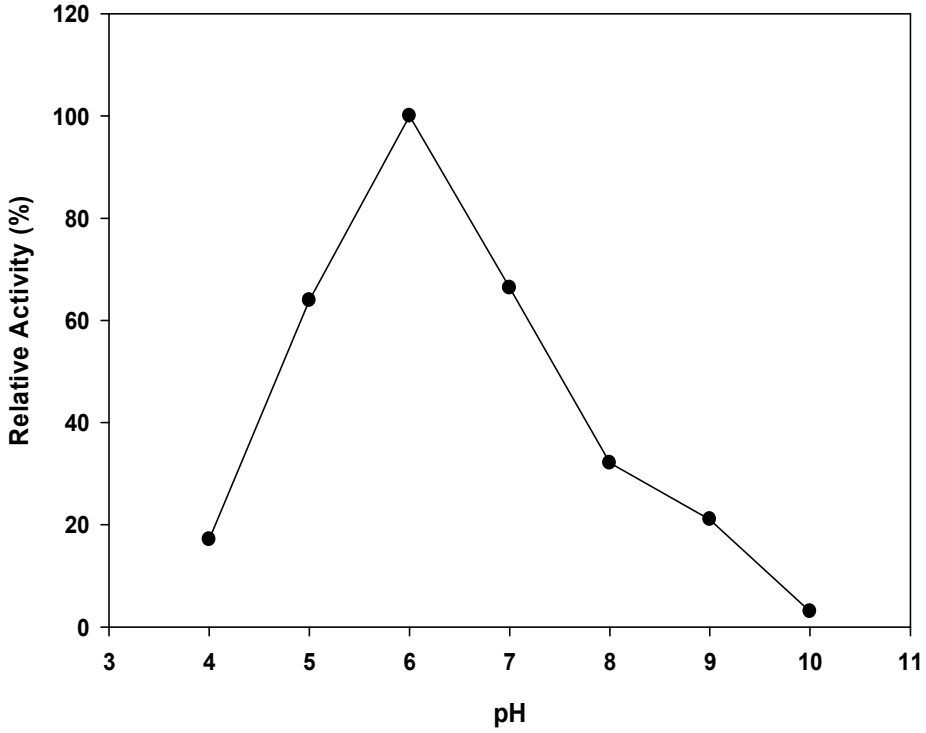

Figure 10: Effect of pH on the activity of endoinulinase in the culture supernatant of *Xcp* mutant KM 24 after incubation at 45ºC for 20 min. Each value represents the mean of triplicate determinations with ± SD

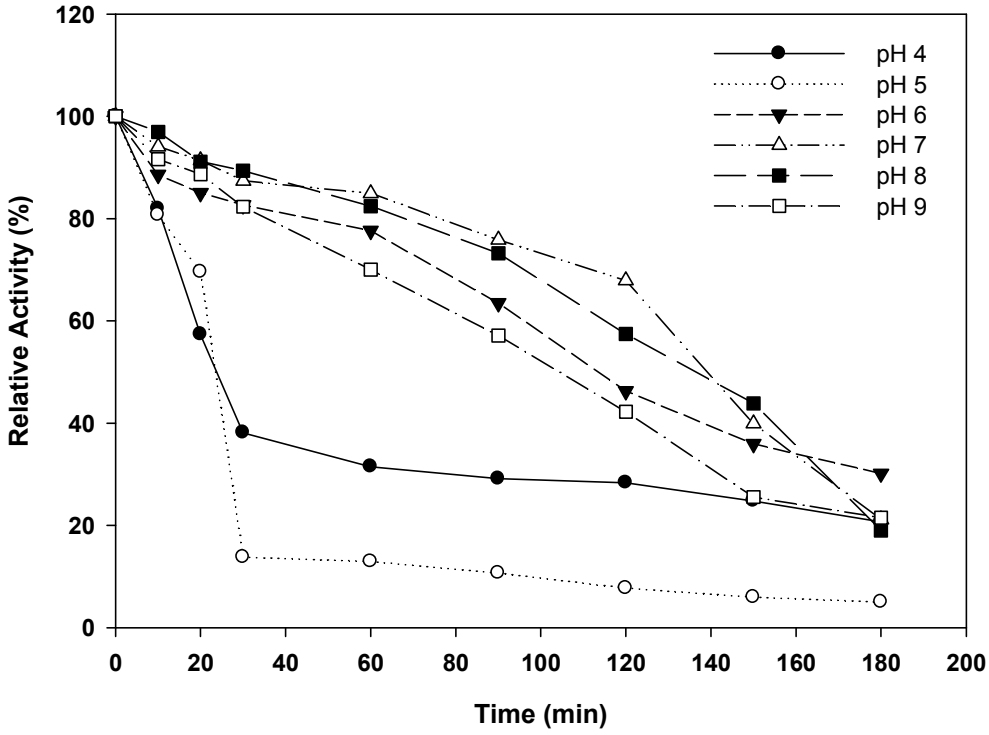

Figure 11: Effect of pH on the stability of endoinulinase in the culture supernatant of *Xcp* mutant KM 24 at a temperature of 45°C for 3h. Each value represents the mean of triplicate determinations with ± SD

4.3.4. Kinetic properties

The Lineweaver–Burk plots showed that the apparent K_m and V_{max} values of the inulinase for inulin were 1.15 mg/ml and 0.15 μM/min, respectively (Fig. 12). The k_{cat} value was found to be 0.145 min^{-1}. Enzyme catalytic efficiency calculated was found to be 0.126 $mg^{-1}.ml.min^{-1}$. The K_m, 1.15mg/ml for this enzyme is lower than that of other reported inulinases. For example apparent K_m values of the two endoinulinase, Endo-I and Endo-II, produced by *Aspergillus ficuum* JNSP5-06 were 14.8 mg/ml and 25.6 mg/ml, respectively (Han-qing *et al*., 2009). The K_m value of inulinase from *P. guilliermondii* strain1 for inulin was 21.1 mg/ml (Zhang *et al*., 2009). *Streptomyces* sp. ALKC4 endoinulinase showed K_m (1.63 mM) and V_{max} (450 mM) for inulin which is almost the same as the enzyme reported in this study (Sharma and Gill, 2007).

Debaromyces cantarelli (15 mM) (Beluche *et al*., 1980), *Candida salmenticensis* (17 mM) (Guiraud *et al*., 1980) and *A. ficuum* (10–15 mM) (Ettalibi and Baratti, 1987) showed higher K_m than the present reported inulinase which makes it a better candidate for inulin hydrolysis. Because of lower K_m value and high thermal stability Xcp KM 24 endoinulinase has the ability to use for commercial applications like for large scale FOS (alternate sweetener) production and would help in reducing the overall production cost.

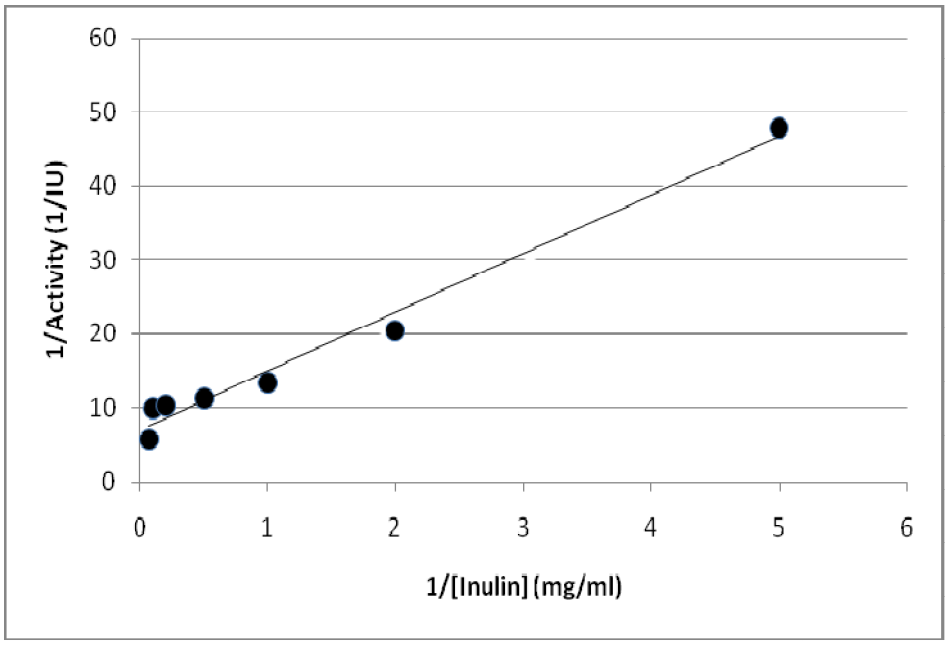

Figure 12: Determination of K_m, V_{max} and K_{cat} of the purified inulinase from *Xcp* mutant KM 24 at pH 7 and temperature of 50°C. Each value represents the mean of triplicate determinations with ± SD

4.3.5. Product of inulin hydrolysis by purified inulinase

Thin layer chromatography analysis of inulin-hydrolyzed products showed that oligosaccharides were the predominant end product over a hydrolysis time period of 10 min to 2h (Fig. 6). Oligosaccharides with varying degrees of polymerization were observed in hydrolysates at 10 min, 30 min and 2 h and this confirmed the mode of action of endoinulinase.

CHAPTER FIVE: GENERAL DISCUSSION

In the face of growing health awareness at governmental and regulatory levels various industries are increasingly evaluating biotechnology as a technically and commercially sound tool to improve overall sustainability. However, few natural biocatalysts are capable of meeting the requirements of modern biotechnological processes. So, in order to increase their utility, it is necessary to increase their production by techniques such as mutation of their hosts.

Biocatalysts, like other catalysts, are able to increase the rate at which a reaction takes place, but it does not affect the thermodynamics of the reaction. They do, however, offer some unique characteristics over conventional catalysts. For instance, they are generally more efficient, whereby a lower concentration of enzyme is needed; they can also be modified to increase selectivity, stability and activity; they are more selective; milder reaction conditions can be used and they are environmentally friendly because they can be completely degraded in the environment.

The most important advantage of a biocatalyst is the high selectivity. This selectivity is often chiral, positional or functional group specific. This property of high selectivity is very desirable in chemical synthesis as it may offer benefits such as reduced or no use of protecting groups, minimized side reactions, easier separation and fewer environmental problems. The other advantages, like high catalytic efficiency and mild operational conditions are also just as attractive in commercial applications. The characteristics of limited operating regions, substrate or product inhibition and reactions in aqueous solutions have often been considered as the most serious drawbacks of biocatalysts. Many of these drawbacks, however, turn out to be misconceptions and prejudices (Faber, 1997 and Rozzell, 1999).

Both isolated enzymes and whole cells can be used as biocatalysts. When comparing isolated enzymes to whole cells, it is evident that the isolated enzymes offer several benefits, including simpler reaction apparatus, higher productivity owing to higher catalyst concentration, and a simpler process for product purification (Rozzell, 1999). For the longest time, it was thought that only enzymes that were produced in abundance by cells could be used for industrial applications.

Functional foods and their ingredients have a beneficial effect on host health as they can reduce the risk of chronic disease beyond their nutritive value (Ziemer and Gibson, 1998; Saarela *et al*., 2002). Any food can be rendered functional by addition of a potential health-promoting entity, reducing or removing concentrations of harmful components and/or improving the source of the availability of one or more components. The first generation of functional foods was based on enrichment or fortification with minerals or vitamins. However, the concept has eventually moved towards food ingredients exerting a positive effect on the gut microbiota, including probiotics and prebiotics (Ziemer and Gibson, 1998).

This study involved the production of inulinase which is capable of hydrolysing the substrate inulin. The degradation of inulin yields fructose and in some cases fructose and fructooligosacchrides, depending on the nature of the inulinase enzyme. Inulinases are fructofuranosyl hydrolases produced by an array of microorganisms including plants, bacteria, yeasts and moulds (Vandamme and Derycke, 1983). Inulin can also be hydrolysed by acid (pH 1.0-2.0) at a temperature of 80-100°C. However the low pH results in degradation of fructose and the process also allows for the formation of di- fructose anhydrides. The di- fructose anhydrides are coloured and have no sweetening capacity (Barthomeuf *et al*., 1991). Therefore acid hydrolysis is not recommended because of this undesirable final product. The conventional introduction of fructose is based on amylolysis of starch with α-amylase and amyloglucosidase followed by glucose isomerase, which catalyses the conversion of glucose to fructose. However, this process

only yields about 45% of fructose at best, the rest being approximately 50% glucose and finally about 8% oligosaccharides. Though ion exchange chromatography techniques have been developed for enrichment of fructose, these techniques only increase the cost of production (Gill *et al*., 2006).

Thus the use of microbial inulinases was proposed as the most promising approach to obtain pure fructose syrups from inulin. Inulin is degraded by the inulinase, which cleaves glycoside bonds to form largely (95%) D-fructose by a single step process and is attractive for the industrial production of high fructose syrups as well as the production of fructooligosaccharides. Fructooligosaccharides actually constitute one of the most popular functional food components because of their bifidogenic and health promoting properties.

The high- fructose syrup obtained from enzymatic hydrolysis of inulin can, very well be used for the production of ethanol in addition (Guiraud *et al*., 1981 and Ohta *et al*., 1993). Several researchers have reported the use of microbial inulinase for hydrolysis of plant inulin for the production of high-fructose syrup and the utilization of the fructose rich hydrolysate for fermentation (Nakamura *et al*., 1995 and Vandamme and Derycke, 1983).

The study focused on the production of inulinase from the bacterium, *Xanthomonas campestris* pathovar *phaseoli*. The first objective was to increase inulinase production by optimizing the growth parameters and nutrients of the production medium. Temperature and pH play a vital role in the synthesis of biocatalysts. It was found that inulinase production maximum when the microorganism was grown at 35-40°C and at a pH of 7. It is also imperative to choose the best components for production medium when testing for enzyme production, as this impacts largely on whether or not the highest enzyme production is attained. Five different carbon and nitrogen sources were tested in the production medium. Once the most suitable carbon and nitrogen source

was determined, the concentration of both these was optimized as well. It was confirmed that the most suitable carbon and nitrogen sources were sucrose and tryptone, respectively. Inulinasse production, using various naturally occurring inulin-containing agricultural plants as carbon sources was studied with an indigenously isolated actinomycete strain. It was evident that all the substrates depicted some activity but levels of production varied among them. The maximum inulinase activity was observed with garlic (*Allium sativum*) (524 IU ml^{-1}) the enzyme activity was about 1.6-fold higher than the activity obtained by using pure inulin, which was commercially obtained, as the carbon source Sharma *et al*. (2006). Singh *et al*., 2006 carried out a study on the production of inulinase from *Kluyveromyces marxianus* YS-1 using the root extract of *Asparagus racemosus* as the source of inulin. It was concluded that an increase of 6-fold in shake flask experiments and an increase of 7-fold in a bioreactor, was achieved, using the Asparagus root extract. In a study conducted by Kango (2008), it was found that an infusion prepared from tap roots of dandelion was able to support maximum inulinase production (52.5 IU ml^{-1}) as compared to pure chicory inulin and other complex substrates such as sucrose, onion and garlic. Dandelion root extract has been reported to contain inulin and oligofructans (Trojanova *et al*., 2004). This is a very interesting alternative, since, these carbon sources are economical, easily available, which are an attractive feedstock for large-scale fermentation, whereas pure inulin is only available in limited quantities and at a very high cost.

The highest inulinase production was achieved using a production medium containing 3% sucrose as the carbon source and 2.5% tryptone as the nitrogen source. The bacterium was then subjected to chemical mutagenesis using EMS (ethylmethyl sulphonate), in an attempt to enhance inulinase production. Following first round mutagenesis, 300 mutants were selected and assayed for inulinase production. The highest inulinase producer was then subjected to second round mutagenesis and assayed once again. It was noted from screening that only 41% showed an increased inulinase activity. The objective behind this aspect of the study was to determine

whether or not the EMS has the ability to mutate the microorganism in such a manner as to enhance the production of the enzyme. Determining the highest inulinase producer was difficult as this was a tedious process and required the assays to be carried out in triplicate. However, both rounds of mutagenesis proved to be successful. According to Skowronek and Fiedurek, 2003, *Aspergillus niger* was biochemically mutated, using both UV and NTG. The most active mutants grown under stress conditions showed significantly higher inulinase activity (approximately 1.2-4.5 fold) when compared with the parent strain. These results do not differ much to the present study, therefore it would be safe to say that this is an indication that chemical mutagenesis led to the successful enhancement of inulinase production.

Xanthomonas mutant KM24 was applied to a 5 L fermenter with all optimized conditions scaled up. The aim of conducting enzyme production in a fermenter is to provide optimal growth conditions for the host microorganism and, in turn, to mass produce the biocatalysts that show good potential for industrial applications. The fermentation run was carried out over a period of 120 h and samples were extracted every 6 hours and analysed for inulinase and invertase production. Various other parameters were monitored over this entire period of time, which included observations on pH; inulinase activity; invertase activity; I/S ratio; Biomass; Total sugars, Volumetric productivity, Specific productivity and FOS productivity. The optimization of culture conditions for higher inulinase production by *Kluyveromyces* sp. Y-85 and scaling-up was undertaken by Wei *et al*., 1998. In this study, optimum conditions were achieved by response surface method, followed by fermentation in a 15 L fermenter, which was later up-scaled to a 1000 L fermenter.

The conditions in both fermenters were uniform and the highest inulinase activity, after 5 cycles, in the 1000 L fermenter was found to be 68.9 U ml^{-1}. In contrast, for the present study, the fermenter used was only of a 5 L capacity and only 2 cycles were carried out. This could have

impacted significantly on the production of inulinase and a higher inulinase activity could be achieved if more experiments are run in a larger fermenter. Therefore, much thought would go into planning future work regarding this endo-inulinase in order to achieve a higher yield.

In the hope of determining whether the inulinase enzyme is exo or endo- acting, the hydrolysis of inulin was carried out by incubating the enzyme with the inulin substrate and then analyzing the products of hydrolysis by means of thin layer chromatography. The reaction products formed during this process showed evidence of inulooligosaccharides production. This confirmed that the enzyme was an endoinulinase. It was also noticed that after 2h, inulooligosaccharides production did not occur. This is probably due to the fact that the enzyme is only active on the inulin substrate for up to 2 hours.

After fermentation, the desired enzyme would generally undergo a purification process. The desired enzyme is purified from the cell extracts based on their chemical and physical properties. The most frequently used purification techniques include electrophoresis, centrifugation, and chromatography. The centrifugation process separates the enzymes based on their differences in mass whereas electrophoresis separates enzymes in relation their differences in charge.

The final phase of this study entailed the purification of the endoinulinase by column chromatography. The purification process involved a number of steps for the elimination of unwanted proteins. These included ammonium sulphate precipitation at 40-80% saturation. This was followed by dialysis against a sodium phosphate buffer at pH 7. After concentration of the protein by ultrafiltration the sample was applied to a Sephadex chromatography column and fractions were then collected and analysed for inulinase activity.

In another study, an extracellular exo-inulinase from *Streptomyces* sp. was purified from the culture broth by ammonium sulphate precipitation, followed by successive chromatographies on DEAE- Sephacel and ConA-CL Agarose columns. The enzyme was purified 18-fold with 4.8% activity yield from the starting culture broth. The purified enzyme gave a single band on gel electrophoresis and its molecular weight was estimated to be 45 kDa (Sharma and Gill, 2006).

Cho and Yun (2002) discovered an extracellular endoinulinase from *Xanthomonas oryzae* No. 5, which was able to convert inulin to inulooligosaccharides. This enzyme was purified from the culture broth by ammonium sulphate precipitation, followed by column chromatography on Phenyl-Sepharose and DEAE- Sephacel. The enzyme was purified 29-fold with a yield of 5.5% from the starting culture broth. This purifired enzyme also gave a single band on gel electrophoresis and its molecular weight was estimated to be 139 kDa.

Another endoinulinase was isolated from the filamentous fungus *Rhizopus* sp. Strain TN-88. This extracellular inulinase was purified from the culture filtrate and grown media containing inulin. Purification was carried out by use of DEAE-Cellulofine A-500 and Sephacryl S-200 HP chromatographies. By means of SDS-PAGE, the molecular weight of the enzyme was estimated to be 83 kDa (Ohta *et al*., 2002).

According to Kang *et al*. (1998), *Arthrobacter* sp. S37 was also able to produce an extracellular endo-inulinase. They succeeded in purifying the enzyme 63-fold and a single band was evident on SDS-PAGE after activity staining. The molecular weight was estimated to be 75 kDa.

From the above, it is evident that there is no correlation between the type of the inulinase and molecular weight, irrespective of the source of the enzyme.

The number of enzyme properties that need to be altered to suit industrial process conditions and perhaps the reluctance of chemists to use enzymes may be hampering enzyme commercialization (Schoeman *et al*., 2003). Continued growth of the industrial enzyme market is dependent on technological innovation – the identification and characterization of new enzymes from natural sources, the modification of these enzymes for optimal performance in selected applications, and high level expression of the enzyme. Biocatalytic technologies will ultimately gain universal acceptance when enzyme are perceived to be robust, specific and inexpensive. As high throughput screening technologies, ever more intelligent assay design and process-conscious research are brought to bear, the utility and economics of enzymes should mandate the use of these mild, selective catalysts.

Xcp KM 24 has the ability to produce inulinase from sucrose and fructan rich plant biomass and it does not require inulin (specific substrate) for inulinase induction. This constitutive type of inulinase expression is an added advantage for commercial applications. *X. campestris* has been used for biopolymer production. In addition, this can also be used for inulinase and FOS production. Xcp KM 24 can be effectively used for dual applications by selectively growing the organism on sucrose to produce high titre of inulinase and inulin-rich substrates for FOS and as well as inulinase production. Inulinase production by Xcp KM 24 could be scaled up for commercial production. In addition to inulinase, this mutant could be a potential candidate for large scale fructooligosaccharides (alternate sweetener) production, which can also be used as a feed stock in biorefineries and biofuels industrial sectors. Utilization of inulin rich alternate cheap substrates for inulinase and FOS production would help in reducing the overall production cost.

When natural enzyme are harnessed for industrial application, a common observation is that they are often not well-suited to their tasks based on a variety of reasons, which include poor substrate solubility and competing chemical reactions. Reflecting their participation in complex biochemical

networks inside living cells, enzymes are often limited by their own substrates or products, either of which may severely limit the productivity of a biocatalytic process. Evolution is usually the reason, as enzymes are optimized and often highly specialized for specific functions within the context of a living organism. Consequently, several strategies have been envisaged to modify the properties of natural enzymes (Bergquist *et al*., 2005).

The advent of modern biotechnology, particularly large-scale fermentation and recombinant DNA technology, has not only made enzymes economically feasible, but in many cases it has been used to create enzymes that perform under decidedly unnatural conditions, such as in organic solvents. Commercial enzyme products were originally developed as single fermentation broths of naturally-occurring organisms. Often the resulting products were complex mixtures of secreted enzymes produced at relatively low yields (Arnold, 1996). Today, over 90% of industrial enzymes are produced recombinantly to maximize product purity and economy of production. Expression is carried out in fungal or bacterial hosts that have been modified to remove unwanted side activities and to maximize expression of heterologous genes (Cherry and Fidantsef, 2003). In addition, a growing number of these heterologous genes have been modified to optimize the catalytic performance of enzymes they encode under application-relevant conditions. Protein engineering technologies involves creating new proteins by modifying existing ones and its importance in industry continues to grow as the number of applications or proteins expands, and the technology to efficiently discover proteins with useful properties is better able to address industrially-relevant problems (Rubingh, 1997).

FUTURE PERSPECTIVES

Inulin and oligofructose have many interesting functional attributes that are useful in formulating the food of today and tomorrow. Nutritional concepts have been revolutionized in recent years with today's consumer being extremely health conscious. There is therefore a demand for food that is both pleasantly tasty as well as low in fat and calories, together with the reduction of the risk of diseases. Inulin has been well investigated with respect to its sources as well as its characteristics as a process substrate. On the other hand, as far as the properties of inulinases are concerned, even though exhaustive research has been published, further work is still necessary due to complexity of the enzyme.

This investigation clearly reveals that *Xanthomonas campestris* pv. *phaseoli* is a promising organism for inulinase production and industrial production of inulooligosaccharide from inulin. The inulinase production ability of this organism was successfully enhanced several fold by chemical mutagenesis. Scale-up studies for inulinase production will be a viable option especially with reference to the limited data available in literature. Also, considering that the enzyme was easily purified, future work on the inulinase enzyme will include isolation of the inulinase gene, modifying it and cloning it into a more effective producer. These factors will provide a wealth of information in the dynamic field of enzyme technology.

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