APPLICATION OF THERMOSTABLE α-AMYLASE FROM *Thermomyces lanuginosus* ATCC 58157 TO NUTRITIONALLY ENHANCE STARCH BASED FOOD

By

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DECLARATION

I hereby declare that this dissertation is my own, unaided work. It is being submitted for the degree, Doctorate of Technology, to the Durban University of Technology, Durban, South Africa. It has not been submitted before for any degree or dissertation to any institution.

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Thiriloshani Padayachee

Durban

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DEDICATION

This dissertation is dedicated to my husband Kamalasen and children Kamesh, Levard and Sayuri, I thank you for your love, compassion, patience, fortitude, understanding, forbearance and unwavering loyalty and support. You surpass all expectations of husband and children. Of all my blessings you are the most valued and the greatest.

Great works are performed, not by strength, but by perseverance.

~Samuel Johnson~

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CHAPTER ONE: INTRODUCTION AND LITERATURE REVIEW

1.1 Introduction

Most people will agree with the familiar adage "You are what you Eat," but few realise it promotes a principle that is somewhat flawed. In reality, "You are what you assimilate." Even the healthiest of diets are without value, if the body cannot properly digest and assimilate its nutrients. To this end, vital biological catalysts called enzymes break down protein, carbohydrates and fats. Through their action they facilitate the digestion process and are essential to the body's absorption and complete use of food (Rachman, 1997).

In most developing countries the first food introduced in a child's diet is a porridge prepared from a cereal or cereal flour (Gopaldas *et al*., 1986). Such porridges are characterised by high viscosity and low energy content per unit volume of food, necessitating frequent feedings to meet the daily energy requirements of the child. In Africa, cereal gruels and sourdoughs are frequently used to prepare complementary foods for young children, and unfortunately have low energy and nutrient density (Lorri and Svanberg, 1993).

Among the different technological methods available to reduce the dietary bulk thereby improving the nutritional characteristics of complementary foods, α - amylase can be used to partially hydrolyse starch (Calderon *et al*., 2003). The digestibility of food is

limited in young children due to inadequate secretion of pancreatic α-amylase (MacDonald, 1988). Digestion improvement is a challenge especially in developing countries where starchy foods are also staple foods (Talamond *et al*., 2002).

Dietary starch varies greatly in digestibility and its effect on the utilisation of other nutrients. The variations appear to be due to differences in starch components and their crystallinity. Processing treatments, storage conditions, chemical modification and genetic breeding influence the digestibility of starch (Dreher *et al.,* 1984). The efficient digestion of starch is especially important to specific groups of people such as infants under six months of age and the geriatric patient. However, poor digestion of starch may have negative affects on the utilisation of protein and mineral and the availability of certain vitamins (Dreher *et al*., 1984).

Chavan and Kadam (1989) showed in their research that the nutritional quality of cereal grains is inferior due to lower protein content, deficiency of essential amino acids, lower protein and starch availabilities. However, they also showed that fermentation and treatment of cereals with enzymes for a limited period of time improves amino acid composition and vitamin content and increases protein and starch availabilities. In support of these findings, Hans (2003) demonstrated in his study the ability of *Hericium erinaceum* to degrade starch and upgrade the nutritional value of corn meal during solid state fermentation.

1.1.1 An African perspective of food and nutrition

In Sub-Saharan Africa, there is an urgent need to sustain food resources and alleviate poverty. Providing nutritionally enhanced foods to the poorest of the poor will go a long way towards achieving this objective. In Africa, feeding a starving population on the continent is a social responsibility of the citizens. Southern African food insecurity is disproportionately impacting on woman and children. Malnutrition is on the rise among children, pregnant and breastfeeding mothers.

In 2002 the United Nations Children's Fund (UNICEF) had warned that the unfolding food crisis in Southern Africa threatens to become a major humanitarian catastrophe if an immediate and adequate response is not mounted. In a region already bearing the full brunt of the HIV/AIDS pandemic, the food crisis presents new and ominous threat to the survival of the most vulnerable which are children and women. During famine, crippling and deadly diseases such as measles, cholera and polio spread more rapidly and threaten the lives of children (Chinyama *et al*., 2002).

Malnutrition is a serious problem in Africa. A national survey of the nutritional status of pre-school children in South Africa showed that one in every four children suffers from chronic malnutrition. The prevalence of malnutrition differs greatly across provinces with the Northern Province, Eastern Cape and Free State having the highest prevalence (SAVACG, 1995).

Table: 1.1 An overview of malnutrition in Sub-Saharan Africa

Country	No. of malnourished people ($x10^6$)	No. of malnourished children under 5 years ($x10^3$)
Nigeria	30	45
Eritrea	4.5	500
Zimbabwe	0.6	130
Malawai	3.2	500
Zambia	2.3	460
Lesotho	4.5	62,5
Swaziland	0.223	30
Mozambique	0.5	100

Statistics from United Nations International Childrens Fund (UNICEF) (2003) and Concern Worldwide (CWW) (2005)

Malnutrition represents the focal point of a number of Africa's social and health problems. The most prevalent diseases responsible for the high juvenile mortality include HIV-AIDS, malaria and tuberculosis. It is an established fact that the resistance to these diseases is severely compromised by malnutrition. Moreover, increasing evidence shows that adequately nourished individuals are less susceptible to the many infections associated with HIV and are less likely to have early onset of full-blown AIDS (UNICEF, 2003).

If the problem of malnutrition is not addressed, efforts to combat these diseases will be futile. A report released by UNICEF (2003) stated that Africa accounts for only 12% of the world's population; it claims 43% of the world's child deaths, 50% of maternal deaths in childbirth, 70% of people living with HIV/AIDS, and a staggering 90% of all children orphaned by AIDS. In 2001 alone, AIDS killed more than 610 000 children. An estimated 800 000 children became HIV-positive, the great majority of whom will die before age five.

Since food shortages are the root of the problem, it is imperative that available food resources be efficiently utilised. Due to a number of economic, social, geographic and cultural reasons, millions of people in Sub-Saharan Africa are affected by vitamin and mineral deficiency. Hunger and nutritional insecurity, precipitated especially by poverty, drought, conflict and other emergencies, are of major concern. These conditions reduce the ability of Africans to access foods of sufficient quantity and quality, resulting in one-third of Africans being undernourished.

Innovative approaches and technologies, such as home-fortification and small-scale village-level fortification of staple foods, can play a major role in making fortified staple foods accessible to the majority of Africans (UNICEF, 2003). Industrial food processing can, also be used to enhance various food properties i.e., increase palatability, digestibility, absorption, shelf -life and nutritional and mineral value of industrially processed foods (Rachman, 1997). Minimal progress has been made towards controlling vitamin and mineral deficiency in Sub-Saharan Africa. Decisive action in Africa to

address the vitamin and mineral deficiency problems in effective and cost-effective ways can help alleviate the associated problems (UNICEF, 2003).

1.1.2 Starch-based foods

In Africa, a wide range of products (beverages, gruels, porridges, semolina) is derived from cereal sourdoughs prepared from maize (e.g. Ogi, mawe` in Benin, Ghana and Nigeria), sorghum (e.g. Kisra in the Sudan) and millet (e.g. Binkida in Burkina-Faso, fermented semolina in Senegal). In addition, cereal sourdoughs are frequently used to prepare complementary foods for young children (Lorri and Svanberg, 1993).

Unfortunately, these sourdoughs have a low energy and nutrient density due to the high molecular mass of the polysaccharide present in these preparations (Lorri and Svanberg, 1993). In a study on the effects of natural fermentation on Ogi (sourdough) followed by the application of α-amylase, growth energetics were measured as an increase or decrease in ATP, and it was reported that sourdough displayed low ATP levels which was considerably increased after the application of the α-amylase to the Ogi (Calderon *et al*., 2003). It was also shown that dietary bulk can, be reduced to improve the nutritional characteristics of complementary foods by the addition of α- amylase, which can partially hydrolyze starch (Tre` che, 1995). Similarly, Lorri and Svanberg (1993) showed that the use of germinated cereals, combined with a *Lactobacillus* starter, allowed the energy density of cereal gruels to be improved significantly.

In the adult human intestine, the breakdown of starch occurs naturally to yield nutritionally essential carbon molecules utilised for energy and structural metabolism. The digestibility of starch-containing foods in young children is, however, limited due to an inadequate secretion of pancreatic α- amylase (MacDonald, 1988). The improvement of starch digestion in children is, therefore, a major challenge in developing countries, such as South Africa, where starch is a major ingredient of the staple diet. For this reason, the economical production of α-amylase is essential for the pre-conversion of starches into oligosaccharides (Ghosh and Chandra, 1984; McMahon *et al.*, 1999; Pederson and Nielsen, 2000) to improve juvenile starch digestion.

1.1.3 History of enzymes and food processing

In 2000 BC the Egyptians and Sumerians developed fermentation for use in brewing, bread-making and cheese-making. The history of amylases began in 1811 when the first starch degrading enzyme was discovered by Kirchhoff. This was followed by several reports of digestive amylases and malt amylases. It was much later in 1930, that Ohlsson suggested the classification of starch digestive enzymes in malt as α- and β-amylases according to the anomeric type of sugars produced by the enzyme reaction. In 1835, a Swedish chemist Jöns Jako Berzelius, carried out studies on organic catalysts. However, industrial enzymes date back to 1874 when Hansen manufactured chymosin from the stomach of calves for the manufacture of cheese.

In 1894 Jokichi Takamine, first manufactured an enzyme from a microbial source, which was a digestive enzyme taka-diatase from *Aspergillus*. The catalytic activity of enzymes was found in yeast (the word enzyme is ancient Greek for 'in yeast") and had been used for centuries. In 1926 James B. Sumner of Cornell University, made the first enzyme in pure crystalline form and was awarded a Nobel Prize in 1946 for his work on the enzyme urease, which was extracted from jack bean (Gupta *et al*., 2003 and Chemical Heritage Foundation, 2002).

The existence of enzymes was associated with the history of ancient Greece where they used enzymes from microorganisms in baking, brewing, alcohol production and cheese making (Haki and Rakshit, 2003). With better knowledge and purification of enzymes, the number of applications has increased several fold and with the availability of thermostable enzymes a number of new possibilities for industrial processes have emerged (Haki and Rakshit, 2003). These enzymes are of great significance in present day biotechnology with applications ranging from food, fermentation, textile to pulp and paper industries (Pandey *et al.*, 2000), starch hydrolysis for the production of ethanol and high fructose corn syrup, starch soil removal in laundry washing powders and dish-washing detergents, textile de-sizing, the production of modified starches, baking, and hydrolysis of oil-field drilling fluids (Richardson *et al*., 2002). Since 1980, the most widely used enzyme for these applications has been α-amylases.

Application of food enzymes is a thriving area of research that must be supported and encouraged from all perspectives. Thermostable enzymes will most definitely provide the

answers to current food related issues, in emerging economies like South Africa. The technologies we propose will help alleviate the nutritional drawbacks associated with the regular intake of starchy foods, especially in children.

1.2 Amylases

Enzymes that participate in the hydrolytic degradation of starch are collectively referred to as amylolytic enzymes or amylases. Specific enzymes classified within this group include α-amylase, β-amylase, gluco-amylase (also known as amyloglucosidase), pullulanase and inso-amylase.

Amylases are, classified into two categories, endoamylases and exoamylases (Gupta *et al*., 2002). Endoamylases catalyse hydrolysis in a random manner in the interior of the starch molecule. This action causes the formation of linear and branched oligosaccharides of various chain lengths. Exoamylases hydrolyse from the non-reducing end, successfully resulting in short end products. A large array of amylases, are involved in the complete breakdown of starch. Enzymatic degradation of starch yields glucose, maltose and other low molecular weight sugars. Also, enzymatically - mediated isomerisation of glucose yields high-fructose syrups.

Abundant supplies of starch may be obtained from seeds and tubers, such as corn, wheat, rice tapioca and potato. The widespread availability of starch from such inexpensive sources, coupled with large-scale production of amylolytic enzymes, facilitates the

production of syrups containing glucose, fructose or maltose, which are of considerable importance in the food and confectionery industry. Furthermore, they may be produced quite competitively when compared with the production of sucrose, which is obtained directly from traditional sources such as sugar-beet or sugar-cane (Gupta *et al*., 2002).

Starch may be hydrolysed by chemical or enzymatic means. Chemical hydrolysis was used formerly and involves heating in the presence of acid. However, enzymatic hydrolysis generates fewer byproducts and produces higher yields of end product compared to the chemical method. The initial step in starch hydrolysis entails disruption of the starch granule (Gupta *et al*., 2002). Solubilisation of the granules, (the process of "gelatinisation"), facilitates subsequent catalytic degradation. Gelatinisation is normally achieved by heating the starch to temperatures often in excess of 100^{o}C for several minutes. α- Amylase may be added immediately prior to the heating step, in order to render more efficient the process of granule disruption. Once the granules have been disrupted, additional α-amylase is added in order to liquefy the starch slurry. This process reduces the viscosity of the starch solution (Pandey *et al.*, 2000),

1.2.1 α- Amylase

α-Amylase activity is widely distributed in nature. α-Amylase is an endo-acting enzyme, catalyzing the random hydrolysis of internal α-1,4 glycosidic linkages present in the starch substrate (Fig 1.6). However, α - amylases which are in most demand hydrolyses the α-1,4 glycosidic bond in the interior of the molecule (Gupta *et al*., 2002). These

enzymes are incapable of hydrolyzing α-1,6 glycosidic linkages present at branch points of amylopectin chains. One exception to this is the α-amylase produced by *Thermactinomyces vulgaris*, which can hydrolyse both α-1-6 and α-1-4 glycosidic linkages.

The α-amylase family consists of a large group of starch hydrolases and related enzymes, currently known as glycosyl hydrolases family 13 (Henrissat, 1991). Thermostable α-amylases have been characterised from *Pyrococcus woesei, Pyrococcus furiosus* (Koch *et al.,* 1991) and *Thermococcus profundus* (Chung *et al*., 1995; Kwak *et al*., 1998; and Lee *et al*., 1996). The optimum activity of these enzymes is 100^{o}C and 80^{o}C respectively.

The gene encoding an extracellular α-amylase from *P. Furiosus* has been recently cloned and the recombinant enzyme expressed in *Bacillus subtilis* and in *E. coli* (Dong *et al*., 1997; Jorgenesn *et al*., 1997). The high thermostability of the pyrococcal extracellular α-amylase (thermal activity) even at 130^{o}C in the absence of metal ions, together with its unique product pattern and substrate specificity, makes this enzyme an interesting candidate for industrial application (Niehaus *et al.,* 1999).

Two of the more commonly used bacterial α-amylases are those isolated from *Bacillus amyloliquefaciens* and *Bacillus licheniformis*. *Bacillus* amylases exhibit a pH optimum close to neutrality and are stabilized by the presence of calcium ions. α-Amylase produced by *Bacillus licheniformis* is particularly suited to industrial applications because of its thermal stability. This enzyme consists of 483 amino acids and has a molecular

weight of 55.2 kDa. Its pH optimum is 6.0 and its temperature optimum is 90^{o}C. Most other α-amylases, including those produced by *B. amyloliquefaciens*, are rapidly inactivated at temperatures above 40^{o}C (Niehaus *et al.*, 1999).

Several thermostable α-amylases have already been characterised (Koch *et al*., 1991). The most thermostable α-amylase to date is from *Pyrococcus woesei*. It remained active after autoclaving for 4 h at 120^{o}C (Antranikian, 1987).

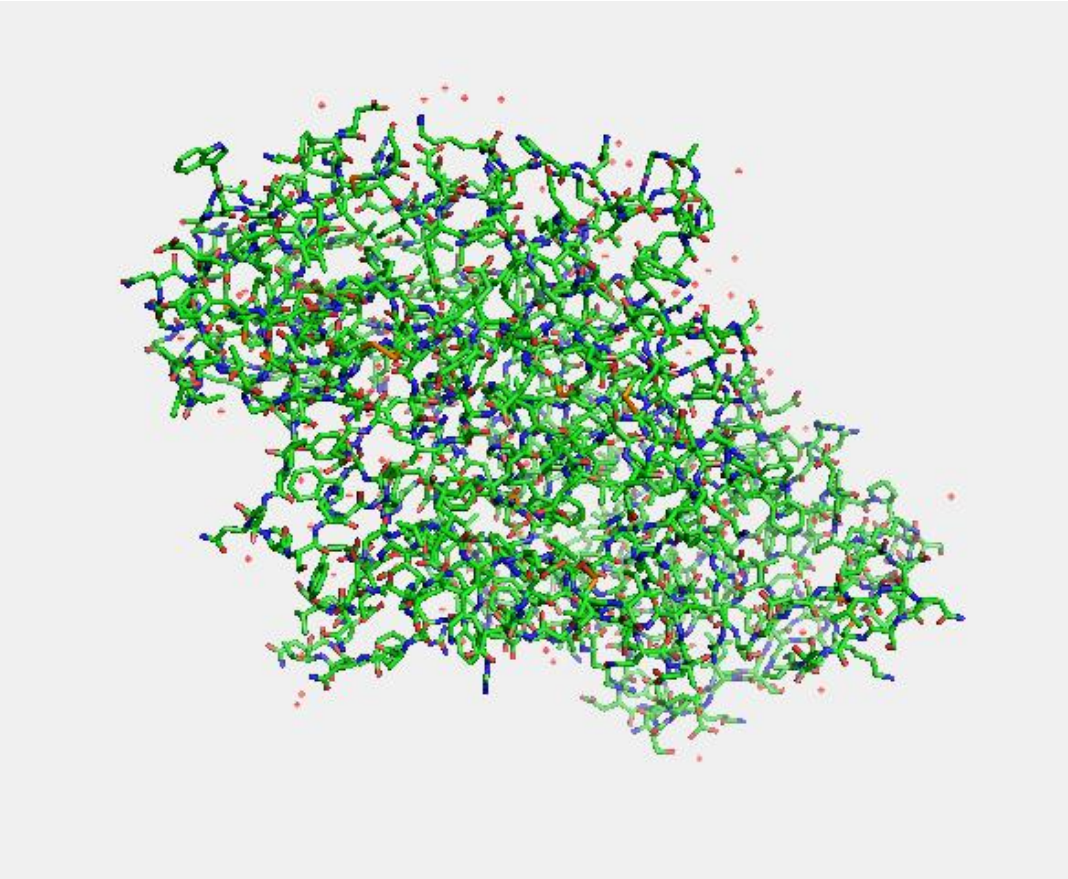

Figure 1.1 3-Dimensional representation of the molecular structure of α-amylase (Payan and Qian, 2003).

Table: 1.2 Characteristics of amylases produced by fungi

Microbial origin	Activity	Substrate	MW	T^{o} opt.	pH opt.	Application	Reference
A. oryzae	6583 U/g	spent brewing grains	ND	30	5	optimisation increase yield	Francis *et al*., 2003
T. lanuginosa	193 U/mg	corn flour, soybean meal	ND	50	5.7	hyperproduction	Rubinder *et al.*, 2002
A. wentii	6 mg/ml	soluble starch	ND	20	6.0	optimization studies	Sinha and Chakrabarty, 1978
Nocardiopsis sp.	1130 U/mg	yam	ND	70	5.0	industrial liquefaction	Stamford *et al*., 2001
H.orenii	22.32 U/mg	starch	ND	65	7.5	purification and characterization studies	Mijts and Patel, 2002
T. curata	0.39 mg/ml	starch	62	60	5.5	characterization studies	Glymph and Stutxenberger, 1977
L. kononenkoae	0,80 g/l	cross linked starch	76	70	4.5 5.0	characterization studies	Prieto *et al*., 1995
A. oryzae	11 U/ml	soluble starch	ND	60	8	control of fermentation cycle	Gigras *et al*., 2002
A. oryzae	ND	starch	52	50	4.5	production of maltohepataose	Chang *et al*., 1996
A. foetidus	ND	rice starch	42	45	50	waste water treatment	Michelena *et al*., 1984
A. flavus	50 U/ml	tapioca	53	55	6.0	starch liquefaction	Khoo *et al*., 1994
T. lanuginosus	45.19 U/mg	soluble starch	61	70	6.6	substrate homologue	Nguyen *et al*., 2002
S. alluvius	364 mg/ml	starch, pullulan	62	40	6.3	industrial ethanol	Wilson an Ingledew, 1982

ND = Not Determined
RT = Room Temperature

Table: 1.3 Characteristics of amylases produced by bacteria

Microbial origin	Activity	Substrate	MW (kDa)	T° opt	pH opt.	Application	Reference
L. fermentum	ND	amylose	106	40	5.0	inhibition of amylase	Talamond *et al.*, 2002
L. fermentum	ND	ogi - Benin maize sourdough	ND	44	4- 6	ND	Caledron *et al.*, 2003
B. licheniforms	ND	EPS - nitrophenly	45	70	4.5	substrate binding	Verhaert *et al* .,2002
P. furiosus	3.9U/mg	starch	100	100	5.5	thermostable	Dong *et al*., 1997
B. subtilis	362 U/mg	soluble starch	67	65	5.5	ND	Ohdan *et al*., 1999
B. subtilis	514 U/mg	soluble starch	47	65	5.5	ND	Ohdan *et al*., 1999
Bacillus sp.	320.5 U/mg	cyclomatodextrinase - CDase	67	50	7.5	starch hydrolysis	Lee *et al*., 2001
Thermus sp.	1596 U/mg	maltoamylase	ND	60	6.0	starch hydrolysis	Lee *et al*., 2001
Bacillus sp.	16,00 U/mg	soluble potatoe starch	55	55	8.0	liquefaction	Igarashi *et al*., 1998
Bacillus sp.	ND	various (CHO)	55	55 - 66	8.0-9.5	CHO hydrolysis	Hagihara *et al*., 2001
S. megasporus	847.33 U/mg -1	glucose, soluble starch ,raw starch (SmF)	97	85	5.5	agricultural	Dey *et al*., 1999
S. megasporus	206 U/mg-1	glucose, soluble starch , raw starch (SSF)	97	85	5.5	agricultural	Dey *et al*., 1999
A. acidocaldarius	ND	starch	160	75	3	Acid stability	Schwermann *et al*., 1994
Bacilus sp.	ND	raw starch	42	70	9	polymerisation	Lin *et al*., 1998
S. bovis	ND	potato starch	77	45	5.0	characterisation	Freer, 1993

ND = Not Determined

Table:1.4 Characteristics of amylases produced by bacteria (contd.)

Microbial origin	Activity	Substrate	MW (kDa)	T° opt.	pH opt.	Application	Reference
B. licheniformis	397 U/ml	wheat bran soya, sunflower, cotton seed meal, rice bran and husk	ND	60	7.5	commercial enzyme production	Haq *et al*., 2003
B. stearthermophilus	112 nKat/mg	starch	ND	60	7	effluent treatment	Carmelo *et al.*, 2002
Halobacillus sp.	ND	starch, dextrin, maltose, sucrose, lactose, glucose, nutrient broth.	ND	50	7.5 - 8,5	high T° commercial value	Amoozegar *et al.*, 2003
Bacillus sp.	ND	starch	ND	60	6.5	ND	Soni *et al* .,2003
H. erinaceum	179 U/g	corn meal and soybean meal	ND	25	ND	nutritional	Hans , 2003
Pyrocous sp.	ND	azo-dyed starch and starch slurry	ND	90	5.25	corn wet milling	Richardson *et al*., 2002
Pyrocous sp.	ND	azo-dyed starch and starch slurry	ND	90	5.25	corn wet milling	Richardson *et al*., 2002
Pyrocous sp.	ND	azo-dyed starch and starch slurry	ND	90	4.5	corn wet milling	Richardson *et al*., 2002
Bacillus sp.	ND	starch	55	55 - 60	8.0-9.5	resistance to chealating	Hagihara *et al*., 2001
B. stearothermophilus	14 mg/ml	bovine serum albumen	55	70 - 80	5.0-6.0	characterization studies	Vihinen and Mantsala, 1990
C. acetobutylicum	3.6 g/l	soluble starch	84	45	5.6	characterization studies	Paquet *et al* 1991
L. fermentum	5831 U/g	potato starch, glucose, fructose, sucrose	ND	45	5	cereal doughs	Caledron *et al.*, 2003
P. furiosus	ND	various starches used	52	100	4.5	non-specific	Jorgensen *et al*., 1997
Bacillus sp.	ND	soluble starch	94,5	80	9	starch, detergents liquefaction and textile industry	Burhan *et al.*, 2003
B. subtilis	3.85 mg/ml	starch	57.7	50	6.5	characterisation	Marco *et al*., 1996

ND = Not Determined

1.2. 2 Glucoamylase

Glucoamylases are ex-oacting enzymes that cleave both α-1-4 and α-1-6 bonds (Fig. 1.7). Although a number of glycoamylases are known to hydrolyse both α-1-4 and α-1-6 glycosidic linkage, their activity towards the latter is very low (Rajoka and Yasmeen, 2005). The action of this enzyme liberates one molecule of β-d-glucose at a time, causing the complete conversion of polysaccharides to glucose. This process is described as the saccharification of starch. The branching points, however, are hydrolysed at a rather slow rate (Niehaus *et al*., 1999).

These enzymes are currently used for the production of high-glucose syrup from starch-originated polysaccharides. In industrial production, the glucoamylase from *A. niger* is generally used. Because of the lack of commercially available thermostable enzymes, the process conditions after starch liquefaction must be changed, for e.g. in the production of glucose, the pH is important, therefore large amounts of salts that were added during processing, need to be removed (Bruins *et al.*, 2001). The glucose syrup produced by this method is often used by the food and related industries, in addition to the production of crystalline glucose. A further application entails conversion of some of the glucose to fructose by enzyme isomerase, thus producing high fructose-syrup. Other applications include the production of different antibiotics, amino acids, ethanol and organic edible acids (Nigam and Singh, 1995).

Similar to other enzymes, synthesis of glycoamylase can be induced by many oligomeric and dimeric sugars (Hrmova *et al.,* 1991; Magnelli and Forchiasin, 1999). Starch and maltose components in starchy subtrates are essential for formation of mRNA to support maximum formation of glycoamylase at the transcriptional level (Rajoka and Yasmeen, 2005). Numerous studies have been carried out concerning the molecular biology of glucoamylase in various organisms such as bacteria and fungi (Fowler, 1993; Devchand and Gwynne, 1991; Verdoes *et al*., 1994; Cha *et al*., 1997; Morlino *et al*., 1999 and Ma *et al*., 2000).

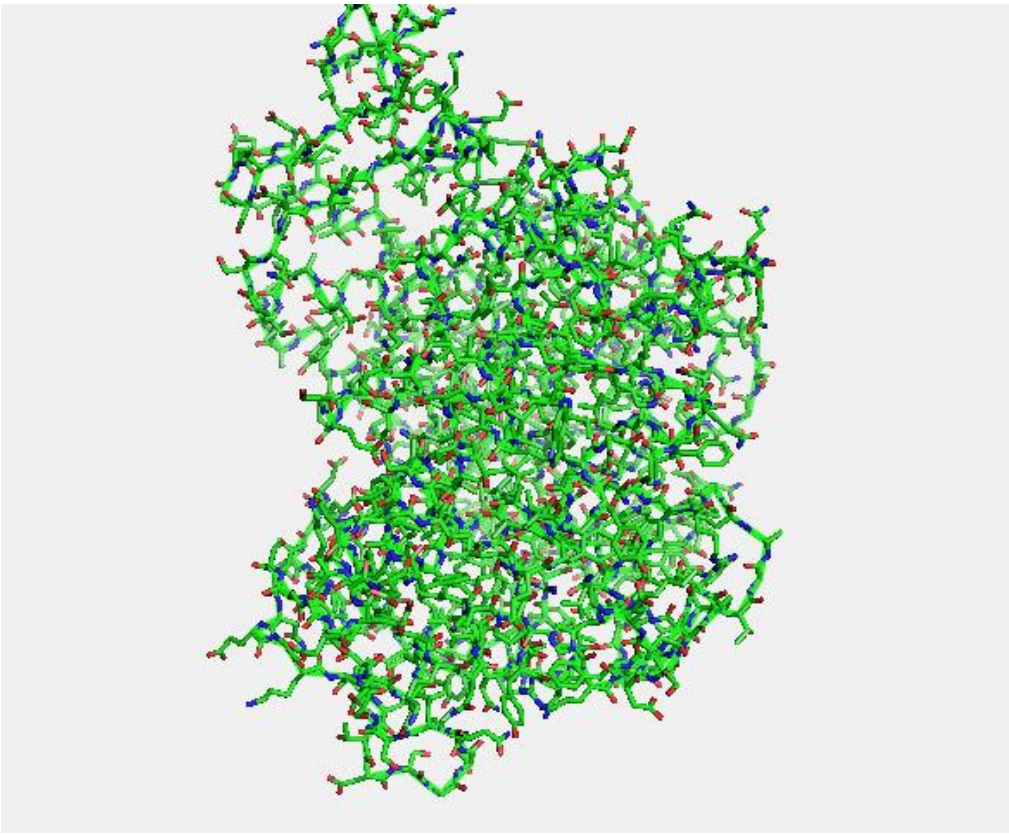

Figure 1.2 3-Dimensional representation of the molecular structure of Glucoamylase (Sevcik *et al*., 1998).

Table: 1.5 Characteristics of glucoamylases produced by fungi and bacteria

Microbial origin	Activity	Substrate	MW (kDa)	T° opt.	pH opt.	Application	Reference
T. lanuginosus	75.11 U/mg	maltose + soluble starch	75	50	4.4 -7.5	protects enzyme	Nguyen *et al.*, 2002
T. lanuginosa	99 U/mg	corn flour	ND	50	5.8	hyperproduction	Rubinder *et al.*, 2002
S. alluvius	12.67 mg/ml	starch and pullulan	155	50	5	industrial ethanol	Wilson and Ingledew, 1982
T. indicae-eudaticae	4800U/ml	soluble starch	42	60	7	saccharification	Satyanarayana *et al.*, 2004
Streptosporangum sp.	41.4 U/ml	starch	ND	70	4.5	saccharification	Stamford *et al.*, 2002
T. lanuginosus	122.2	soluble starch	71	70	5	saccharification	Nguyen *et al.*, 2000
R. marinus	2.1 nKat/ml	maltose	ND	85	5	saccharification	Gomes *et al.*, 2003
A. niger	274	maltose	ND	30	6.5	saccharification	Rajoka and Yasmeen, 2005
T. indicae-eudaticae	30 000 U/l	soluble starch	ND	40	7	saccharification	Kaur and Satyanarayana, 2004
Aspergillus sp.	3426 U/g	wheat bran	ND	50	6.5	saccharification	Soni *et al.*, 2003
A. niger	ND	waxy starch	ND	37	5	starch degradation	Yoon and Robyt, 2005
R. oligosporus	ND	soluble starch	ND	35	4.5	treatment of starch processing waste water	Jin *et al.*, 1999
A. niger	205 U/ml	dextrose	ND	25	5.5	saccharification	Papagianni and Moo-Young, 2002
S. diastaticus	33 U/ml	dextrose	ND	30	4.8	ethanol production	Verma *et al.*, 2000
E. capsularis	27 U/ml	dextrose	ND	30	4.8	ethanol production	Verma *et al.*, 2000
G. pulicaris	85 U	potato starch	ND	40	5.5	starch degradation	Marlida *et al.*, 2000

ND = Not Determined

1.2.3 Pullulanase

Type I pullulanase cleaves α-1-6 bonds in starch and is an example of a debranching enzyme. It is used in the reduction of high-glucose syrup from polysaccharides. As pullulanse is able to hydrolyze α-1-6 linkages in dextrins it is used in combination with other amylolytic enzymes (β-amylase, α-amylase, α-glucosidase and glucoamylase) in starch saccharification. Wind (1994), reported that for industrial purposes, the pullulanase from *B. acidopullulyticus* is often used, in combination with the glucoamylase from *A. niger*. Thermostable type I pullulanase from *Thermus caldophilus* and *Fervidobacterium pennavorans* (Koch *et al*., 1997), has been characterised, and the latter has been cloned into *E. coli* (Bertoldo *et al*., 1999). The purified recombinant enzyme is optimally active at 100^{o}C and extremely thermostable with a half life of 7 min at 110^{o}C (Rüdiger *et al*., 1995).

This is the only thermostable debranching enzyme known to date that attacks amylopectin, leading to the formation of longchain linear polysaccharides, which are the ideal substrate for the action of glucoamylase (Niehaus *et al*., 1999). In addition to α-1-6 bonds, Type II pullulanase cleaves α-1-4 bonds and is therefore able to saccharify starch directly into maltotetraose, maltotrise, and maltobiose. Type II pullulanase is usually referred to as amylopullulanase (Zeikus *et al.*, 1996). Thermoactive pullulanases have a temperature optima between 90^{o}C and 105^{o}C, and are remarkable thermostability even in the absence of substrate and calcium ions, which makes them valuable for the production

of glucose, crystalline dextrose syrup, dextrose syrup, maltose and maltodextrins (Caganella *et al*., 1994 and Gomes *et al*., 2003).

Table: 1.6 Characteristics of pullulanases produced by fungi and bacteria

Microbial origin	Activity	Substrate	MW (kDa)	T° opt.	pH opt.	Application	Reference
G. thermoleovorans	5200 U	starch	48	80	7	saccharification	Satyanaryana *et al*., 2004
R. marinus	33 U	maltose	ND	80	7	saccharification	Gomes *et al*., 2003
C. thermosulfurogenes	2.26 U/ml	soluble starch	ND	60	6	saccharification	Reddy *et al*., 1999
Bacillus sp.	214 U/mg	pullulan	210	50	9	saccharification	Hatada *et al*., 1996
B. acidopullulyticus	27 U.g	pullulan	ND	37	5	starch degradation	Yoon and Robyt, 2005
C. thermosulfurogenes	0.98 U	potato starch	ND	60	7.2	enzyme production	Ramesh *et al*., 2001

ND = Not Determined

Mounting social, political and environmental pressures on industry to provide alternatives to chemical-based methods, have given added impetus to the search for novel enzymes with unique and industrially-significant traits. Although there are many advantages to using enzymes as substitutes for chemical catalysts, practical applications of enzyme catalysis are few and far between. This is largely due to their relatively poor stabilities and catalytic activities under the conditions that characterize industrial processes: high temperatures, extremes of pH or non-aqueous solvents.

Enzymes that evolve from organisms may not exhibit characteristics suitable for *in vitro* application. However, the application of these enzymes in industrial processes often requires characteristics not found in enzymes isolated from their natural environments (Chen and Arnold, 1993).

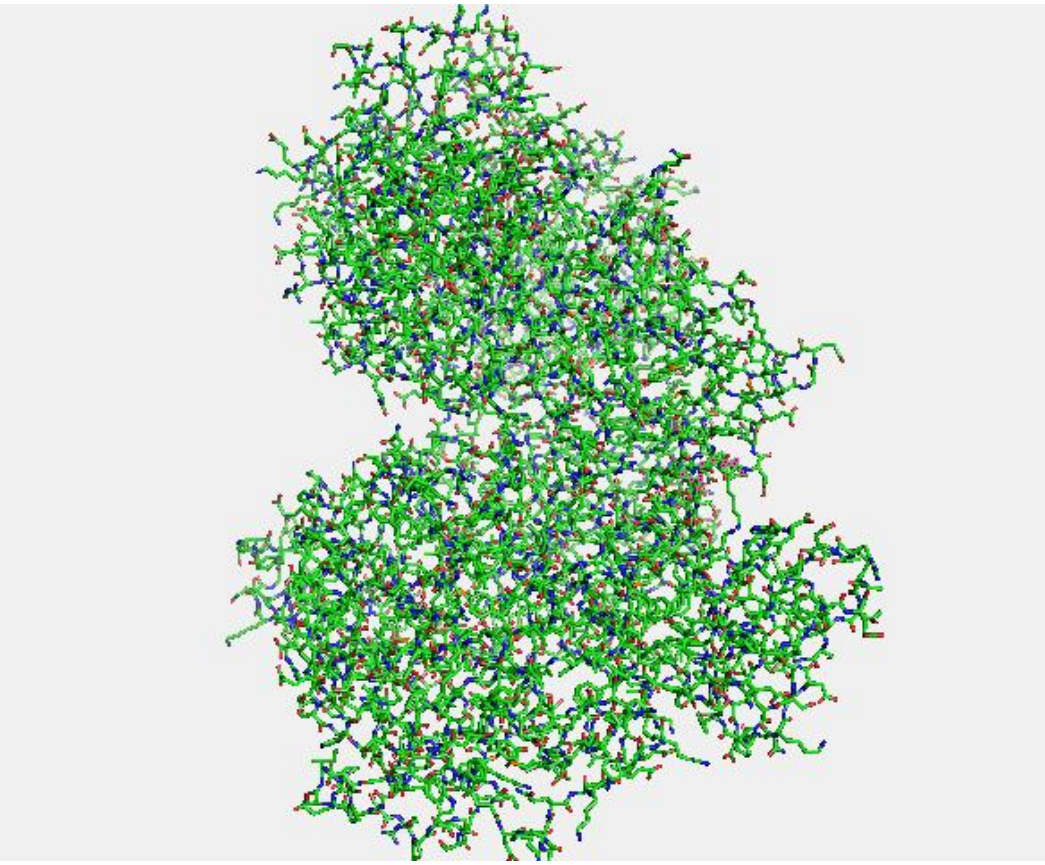

Figure 1.3 3-Dimensional representation of the molecular structure of pullulanase (Hondoh *et al*., 2003).

1.3 Production of amylase by solid state fermentation and submerged fermentation

Although the details of the specific fermentation processes adopted by different manufacturers vary, there remain two main methods for amylase production, submerged fermentation (SmF) and solid-state fermentation (SSF). Solid-state fermentation has gained renewed interest from researchers for the production of these enzymes in view of its several economic and engineering advantages and has been often employed to produce amylases (Pandey, 1992). Some of the advantages of solid-state fermentation over that of submerged fermentation are due to SSF being a simple technique, low capital investment, lower levels of catabolite repression and better product recovery (Babu and Satyanarayana, 1995; Mulimani and Ramalingam, 2000).

The SSF technique is mainly confined to process involving fungi (Aguilar and Huitron, 1986; Kadowaki *et al.*, 1997; Souza *et al*., 2001; Haq *et al*., 2002). It is believed that this technique is not suitable for bacterial cultures because of higher water activity requirements (Lonsane and Ramesh, 1990). However, successful bacterial growth using the SSF technique is known in many natural fermentations (Lonsane and Ramesh, 1990). Lonsane and Ramesh (1990) reviewed the production of bacterial thermostable α-amylase in SSF by *B. amyloliquefaciens* and *B. licheniformis.* They referred to the SSF process as the potential tool for achieving economy in enzyme production and starch hydrolysis.

1.3.1 Solid state fermentation

Commercial production of amylases are carried out in various stages, essentially because the environmental factors required for the optimum growth of the micro-organism being employed may differ from those required for the production of enzymes. These parameters include nutrient supplementation, pH of the medium, osmotic relationship, degree of aeration, temperature, inoculum size and the control of contamination during fermentation (Pandey *et al*., 2000).

The commercial success of amylases is linked to the utilization of starchy biomass as an industrial raw material for solid-state fermentation. On a dry basis, agricultural substrates like corn, wheat, sorghum and other cereal grains contain around 10 – 70% (w/w) starch, hydrolysable to glucose with a significant weight increase, offers a good resource in many fermentation processes (Soni *et al*., 2003). A number of studies have been carried out to optimize various SSF conditions with the aim of increasing α-amylase yields.

Omidiji *et al*. (1997) and Bajpai *et al*. (1992) developed a simple and cheap media based on cheesy whey, corn steep liquor and soya bean meal for α-amylase production. It was claimed that the medium could be exploited for the industrial production of α-amylase. studied the effects of different carbon sources on α-amylase production. Whereas lactose, dextran and soluble starch were found to be suitable for enzyme production, the highest enzyme yields was reported for glucose. El Helow *et al.* (2000) compared α-amylase production in three different nutritional media. In their study different patterns of enzyme

induction were obtained when beet pulp, corn cob, rice husk, wheat bran and wheat straw were used separately to partially replace the nutrient contents of the selected medium. α-amylase was maximally expressed in the presence of corn cob or wheat bran.

Hiller *et al*. (1997) demonstrated the effect of lactose and nitrogen on cell physiology and α-amylase production. Results showed cell-growth and α-amylase production patterns to be similar regardless of the limiting nutrient. Oxygen-transfer conditions and especially the dissolved oxygen tension, were reported as vital factors for α-amylase production by Milner *et al*. (1997). High aeration rates were found to be essential for good enzyme yields.

As a practice, single-stage inoculum is used for fermentation processes. Generally, it is carried out in a routine way without being given critical attention. Keeping this in mind, Milner *et al*. (1997), studied one-stage and two-stage inocula for α-amylase production and found that significantly better results were obtained using two-stage inoculum. Considerable work has also been carried out on the effect of moisture levels on bacterial α-amylase production technique (Ramesh and Lonsane, 1990). In the SSF process, the solid substrate not only supplies the nutrients to the culture, but also serves as an anchorage for the microbial cells. The moisture content of the medium changes during fermentation as a result of evaporation and metabolic activities and the moisture level of the substrate are therefore most important (Baysal *et al*., 2003).

Industrially important enzymes including amylases have traditionally been obtained from submerged cultures because of ease of handling and greater control of environmental factors. Soccol and Vandenberghe (2003) were able to increase the protein content of husks by 10.6% by SSF and Pandey (2003) reported that cultivation in SSF resulted in 4-fold-higher enzyme production than in SmF. However, solid-state fermentation constitutes an interesting alternative since the metabolites so produced are concentrated and purification procedures are less costly.

1.3.2 Submerged fermentation

SSF has developed in eastern countries over many centuries, and has enjoyed broad application in these regions. In western countries the SSF had to compete with classical submerged fermentation and because of the increasing pressure of rationalization and standardization, SSF was widely superseded by classical submerged fermentation since the 1940's. This is mainly because of problems in engineering that appear when scaling up SSF (Hölker and Lenz, 2005).

The majority of enzyme manufacturers produce enzymes using SmF techniques with enzyme titers in the range of grams per litre (Harvey and McNeil, 1993). Such levels are prerequisite if specific compounds are to be considered as commodities because product recovery costs are inversely proportional to concentration in a fermentation broth (Viniegra-González *et al*., 2003).

The application of SmF has been mainly confined to bacterial species (Pandey *et al*., 2000), the industrial exploitation of SSF for enzyme production has been confined to processes involving fungi and is generally believed that these techniques are not suitable for bacterial cultivation (Gupta *et al*., 2003). It is often stated that growth of filamentous fungi in submerged culture are quite similar to those of unicellular organisms that reproduce by binary fission. Due to practical difficulties that hinder studies of filamentous organisms in submerged culture, growth is based mainly on studies with unicellular organisms. Attachment and growth on bioreactor walls, agitators, probes and baffles lead to a degree of heterogeneity within the biomass which is more pronounced in the case of pelleted growth. Areas of growing, and non-growing biomass inside the bioreactor influences the overall growth (Gupta *et al*., 2003).

A number of processes have been reported for the production of enzymes, ethnol, and single-cell proteins using SmF (Nigam and Prabhu, 1991; Nigam *et al*., 1987a, b; Nigam *et al*., 1988; Zayed and Mostafa, 1992). Although, SSF offers numerous advantages over SmF such as simpler techniques and lower cost, there are few designs available for bioreactors operating in solid-state conditions. This is principally due to several problems encountered in the control of different parameters such as pH, temperature, aeration and oxygen transfer and moisture. SSF lacks the sophisticated control mechanisms that are usually associated with SmF (Couto and Sanromán, 2005).

The production of α-amylase has generally been carried out using SmF, however SSF systems appear to be a promising technology as was shown by Francis *et al*. (2003),

using spent brewing grains in SSF for the production of α-amylase and determining that the supplementation with Tween-80 and calcium ions enhanced α-amylase activity. Gelatinization coupled with liquefaction, which is possible by the action of thermostable amylases have also been reported in SmF (Stamford *et al*., 2001). The production of frustosyl transferase derived from microorganisms has been produced by SmF using *Aspergillus, Penicillium* and *Aureobasidium* species (Prapulla *et al*., 2000).

There are several reports dealing with extracellular lipase production by fungus such as *Rizhopus* sp., *Aspergillus* sp. and *Penicillium* sp. on different substrates under submerged conditions (Christen *et al*., 1995; Cordova *et al*., 1998; Kamini *et al*., 1998 and Miranda, *et al*., 1999). Citric acid is one of the most commonly used organic acids in food and pharmaceutical industries and can be obtained by chemical synthesis, however, the cost is much higher than using fermentation. Citric acid is mainly produced by SmF, from filamentous fungus *A. niger* (Prado *et al*., 2004).

Submerged production of α-amylase using synthetic media has been reported by many workers (Tigue *et al.,* 1995; Haq *et al*., 1997; Hamilton *et al*., 1999; Sharma and Satyanarayana, 2006; Téllez-Jurado *et al*., 2006). The contents of synthetic media such as nutrient broth, soluble starch as well as other components are very expensive (Haq *et al*., 2003).

The industrial enzyme production using SmF is often limited by the costs of substrates for the cultivation of the producer microorganisms. The use of low cost substrates, such

as agricultural wastes has been suggested as an alternative to reduce the production costs (Oliveira *et al.,* 2006). A study using sugarcane bagasse, and grass hydrolsates as carbon source for xylanase production by *B. circulans* was carried out by Bocchini *et al.* (2005) and Oliveira *et al.* (2006). High production of enzyme was achieved using bagasses hydrolysates (8.4 U/ml) and in media with grass hydrolysates (7.5 U/ml), these results were higher than when xylan was used (7.0 U/ml) and demonstrated well that agro-industrial byproducts can be used as alternatives to reduce the cost of SmF (Bocchini *et al.,* 2005).

The utilization of both brewery and meat processing wastes for amylase production by *A. niger* under submerged culture conditions produced the highest amylase (70.29 and 60.12 U/ml) and protease (6.11 and 6.03 U/ml) yields respectively. The added advantage in using these food wastes also resulted in the COD in both wastes being reduced by more than 92%. This data showed the potential use of waste from food processing as culture media for large scale production of enzymes by SmF (Hernández *et al*., 2006).

1.3.3 Purification and characterisation of amylases

Industrial enzymes produced in bulk generally require little downstream processing and hence are relatively crude preparations. The commercial use of α-amylase generally does not require purification of the enzyme, but enzyme applications in pharmaceutical and clinical sectors require high purity amylases (Gupta *et al*., 2003). The enzyme in purified

form is also a prerequisite in studies of structure-function relationships and biochemical properties.

The purification of α-amylase from microbial sources in most cases has involved classical purification methods. These methods involve separation of the culture from fermentation media, selective concentration by precipitation using ammonium sulphate or organic solvents such as chilled acetone (Gupta *et al*., 2003). The crude enzyme is then subjected to chromatography, usually affinity, ion exchange and/or gel filtration. A number of reviews are available on purification and charaterisation of α-amylase from a range of microorganisms (Windish and Mhatre, 1965; Pandey and Nigam, 2000; Vihinen and Mantsala, 1990; Fogarty and Kelly, 1990). (Table 1.7 and 1.8)

Table: 1.7 Purification strategies for α-amylases from fungi and yeast

Microorganism	Purification strategy	Fold purification/ Yield (%)	Reference
A .oryzae	DE52-Cellulose (ph 7.0), 70% $(NH_4)_2SO_4$, DE52-Cellulose (pH 7.0)	ND	Errat *et al*., 1984
A. flavus	50 -90% $(NH_4)_2SO_4$, DEAE-Sephadex A50 (pH 6.5)	13.8/70	Khoo *et al*., 1994
Cryptococcus sp.	Ultrafiltration, α-Cyclodextrin coupled with Sepharose 6B (pH 7.0)	140/78	Iefuji *et al*., 1996
L. kononenkoae	60% $(NH_4)_2SO_4$, crosslinked starch (pH 8.5), DEAE Bio-Gel A (pH 5.5)	6000/52	Prieto *et al*., 1995
S. cerevisiae	Ultrafiltration, β-Cyclodextrin linked Sepharose 6B (Epoxy activated, pH 4.5), Sephadex G-100 (pH 4.5)	5/2	De Moraes *et al*., 1999
S. alluvius	Ultrafiltration, DEAE-sephacel (pH 5.6), Sephadex G-150 (pH 5.6)	10.8/17.1	Wilson and Ingledew, 1982
T. curvata	Ultrafiltration, 75% ethanol precipitation, Sephadex G-150 (pH 8.0), DEAE Cellulose, ultrafiltration.	66/9	Glymph and Stutzenberger, 1977
T. lanuginosus	Ultrafiltration, DEAE-Trisacryl (pH 7.0), PhenylpSepharose (pH 7.0)	ND	Jenson *et al*., 1988
T. lanuginosus	Ultrafiltration, DEAE-Sephadex A50 (pH 5.0), ultrogel AcA54, DEAE-Sepgadex A50 (pH 8.0), Bio-Gel P-30	112/41	Mishra and Maheshwari, 1996

ND = Not Determined

Table: 1.8 Purification strategies for bacterial α-amylases

Microorganism	Purification strategy	Fold purification/ Yield (%)	Reference
Bacillus sp.	α-Cyclodextrin coupled Sepharose 6B (pH 6.0)	744/65	Hamilton *et al*., 1999
Bacillus sp.	Acetone precipitation, Resource Q (pH 7.0), Phenyl Sepharose CL-4B)pH 7.8)	266	Hamilton *et al*., 1999
Bacillus sp.	60% $(HH_4)_2SO_4$, DEAE Sepharose (pH 5.3), Sephadex G-75	Amy I65/13, Amy II	Mamo *et al*., 1999
B. licheniformis	65% $(NH_4)_2SO_4$, CM-Cellulose (pH 6.4)	212/42	Krishnan and Chandra, 1983
B. licheniformis	DEAE-Cellulose DE52 (pH 5.3)	33/66	Morgan and Priest, 1981
B. tearothermophlus	Adsorption on soluble starch (1%) in 10% $(NH_4)_2SO_4$, washing with Aces (pH 7.5) and 10% $(NH_4)_2SO_4$, DEAE chromatography (Zetaprep disk), ultrfiltration	ND	Vihinen and Mantsala, 1990
B. subtilis	60% $(NH_4)_2SO_4$, Sephacrly-S200 HR (pH 8.0), 60% $(NH_4)_2SO_4$, S-Sepharose	9/17	Marco *et al*., 1996
B. subtilis	Ultrafiltration	2.5	Bohdziewicz, 1996
B. subtilis	Sephacrly S-300, CM Sephadex C-50	30.85/24.8	Hayashida *et al*., 1988
L. plantarum	Untrafiltration, 50-80% $(NH_4)_2SO_4$, DEAE-Cellulose	20/35	Giraud *et al*., 1993
P. stutzeri	Concentrated by drum humidifier, 25% $(NH_4)_2SO_4$, 70% acetone	1.036	Robyt and Ackerman, 1971
S. bovis	70% $(NH_4)_2SO_4$, Sephadex G-25 (pH 7.5), MonoQ	6.9/50	Freer, 1993
T. curvata	85% $(NH_4)_2SO_4$, ultrafiltration, gel filtration (pH 6.0), DEAE-Sephacel (pH 8.0)	300	Collins *et al*., 1993
T. profundus	80% $(NH_4)_2SO_4$, DEAE-Toyopearl 650 M (pH 7.5), Superdex 200 HR (pH 7.5)	816/26	Chung *et al*., 1995

ND = Not Determined

1.4 Thermostable enzymes

1.4.1 The evolution of thermostability

Improvements in stability, particularly thermostability, are commonly sought by protein engineers. In industrial processes, high temperatures impact such benefits as increased substrate solubility, decreased viscosity of the medium, lower risk of microbial contamination or higher rates of concurrent non-enzymatic reactions (Kuchner and Arnold, 1997).

The successful implementation of enzymes as industrial biocatalysts requires the availability of suitable enzymes with high activity, specificity and stability under process conditions. However, naturally occurring enzymes are often not optimized to fulfill these requirements. Within this context, directed evolution is very effective in closing these functional discrepancies (Huimin *et al*., 2002).

Thermostable enzymes, isolated mainly from thermophilic organisms, have found a number of commercial applications because of their overall inherent stability (Dimirijan *et al*., 2001). Enzymes from thermophilic microorganisms, are referred to as thermozymes, displaying unique characteristics such as temperature, chemical and pH stability. They can be used in several industrial processes, in which they replace chemicals or mesophilic enzymes (Bruins *et al*., 2001).

Advances in this area have been possible with the isolation of a large number of beneficial thermophilic microorganisms from different exotic ecological zones of the earth and the subsequent extraction of useful enzymes from them (Groboillot, 1997; Bharat and Hoondal, 1998; Bauer *et al.,* 1999; Kohilu *et al*., 2001). The most widely used thermostable enzymes are the amylases in the starch industry (Poonam and Dalel, 1995; Crabb and Mitchinson, 1997; Emmanuel *et al*., 2000; Sarikaya *et al*., 2000). A number of other applications are in various stages of development.

The study of hypethermophilic bacteria and Archaea, which grow optimally at temperatures greater than 80^{o}C, has resulted in the discovery of many thermostable enzymes with industrial applications. These include a number of extracellular amylolytic enzymes from cultured microbes (Richardson *et al*., 2002).

Thermozymes are often used when the enzymatic process is compatible with existing (high-temperature) process conditions. The main advantages of performing processes at higher temperatures are reduced risk of microbial contamination, lower viscosity, improved transfer rates, and improved solubility of substrates (Bruins *et al*., 2001). Thermostable polymer-degrading enzymes such as amylases, pullulanases, xylanases, proteases and cellulases play an important role in food, chemical, pharmaceutical, paper, pulp and waste-treatment industries (Bruins *et al*., 2001). The application of these enzymes as biocatalysts is attractive because they are stable and active under conditions that were previously regarded as incompatible with biological materials. Furthermore, it is clear that some extremophiles, particularly those from the Archaea, have novel

metabolic pathways and so might serve as a source of enzymes with novel activities and applications (Hough and Danson, 1999).

1.4.2 Stability of thermozymes

The stability of enzymes and proteins *in vitro* remains a critical issue in Biotechnology. Both storage and operational stabilities affect the usefulness of enzyme-based products. Storage stability, or shelf life, refers to an enzyme's maintaining its catalytic properties in the period between manufacture and eventual use. Operational stability describes the persistence of enzyme activity during a process, i.e., under conditions of use (Jaenicke, 1991).

The stability of proteins is the result of a delicate balance between large stabilising and large destabilising forces. Consequently, relatively small changes in either the stabilising or destabilising forces can result in large changes in stability. A small number of extra salt bridges, hydrophobic interactions, or hydrogen bonds can confer this extra degree of stabilisation. There are, therefore, no systematic structural differences between extremely stable and normal proteins (Matthews, 1993).

The stability of enzymes is interesting from a fundamental as well as an industrial point of view. Thermozymes can function as examples for improved stability. A better understanding of the stability of thermozymes could reveal ways to stabilise other

enzymes. Engineering of mesophilic enzymes might be an option when specific functionality cannot be found among thermozymes (Bruins *et al*., 2001).

Conformational stability of proteins is the result of compromise between two opposing factors: flexibility, for the catalytic function of the enzyme, and rigidity, for conformational stability. Thermozymes are significantly more rigid than their mesophilic counterparts at room temperature. Their high rigidity protects them from unfolding and preserves their catalytically active structure. Therefore, they can be optimally active under more denaturing conditions (e.g. higher temperatures) (Bruins *et al*., 2001).

Many attempts have been made to understand the stability of extremozymes in terms of their three-dimensional structure. This approach requires high-resolution structural data for homologous enzymes from both mesophiles and extremophiles so that differences, which might result in enhanced stability of the extremozymes, can be identified by structural comparison (Hough and Danson, 1999). It is clear that there are many features that have been identified as possible contributors to increase protein thermostability. However, there are substantial differences between the conclusions reached with different proteins and there appears to be no universal rule for the structural basis of stability (Hough and Danson, 1999).

Sufficient experimental evidence i.e., sequences, mutagenesis, structure, and thermodynamics, has been accumulated on hyperthermophilic proteins in recent years to conclude that no single mechanism is responsible for the remarkable stability of

hyperthermophilic proteins. Increased thermostability must be found, instead, in a small number of highly specific mutations that often do not obey any obvious rule (Vieille and Zeikus, 2001)

1.5 Industrial enzyme applications

The effective catalytic properties of enzymes have already promoted their introduction into several industrial products and processes. Recent developments in biotechnology, particularly in areas such as protein engineering and directed evolution, have provided important tools for the efficient development of new enzymes. This has resulted in the development of enzymes with improved properties for established technical application and in the production of new enzymes tailor-made for entirely new areas of application where enzymes have not previously been used (Kirk *et al*., 2002).

The majority of currently used industrial enzymes are hydrolytic in action, being used for the degradation of various natural substances. Proteases remain the dominant enzyme type, because of their extensive use in the detergent and dairy industries. Various carbohydrases, primarily amylases and cellulases, used in industries such as the starch, textile, detergent and baking industries, represent the second largest group (Godfrey and West, 1996). The fastest growth over the past decade has been seen in the baking and animal feed industries, but growth is also being generated from applications established in a wealth of other industries spanning organic synthesis to paper and pulp and personal care (Fig. 1.2) (Kirk *et al*., 2002).

Natural microorganisms have over the years been a great source of enzyme diversity. The developments in bioinformatics and the availability of sequence data have increased immensely the efficiency of isolating an interesting gene from nature (Kirk *et al*., 2002). Rational protein engineering and the possibility of introducing small changes to proteins, on the basis of their structure and the related biochemical and biophysical properties, introduced a new valuable tool to enzyme optimisation in the 1980's. Directed evolution is the latest addition to the toolbox (Tobin *et al*., 2000). New exciting technology is predicted to out-compete the existing technologies, but we expect that time will demonstrate how the combined use of rational design, directed evolution and nature's diversity will be far superior to any technology (Kirk *et al*., 2002).

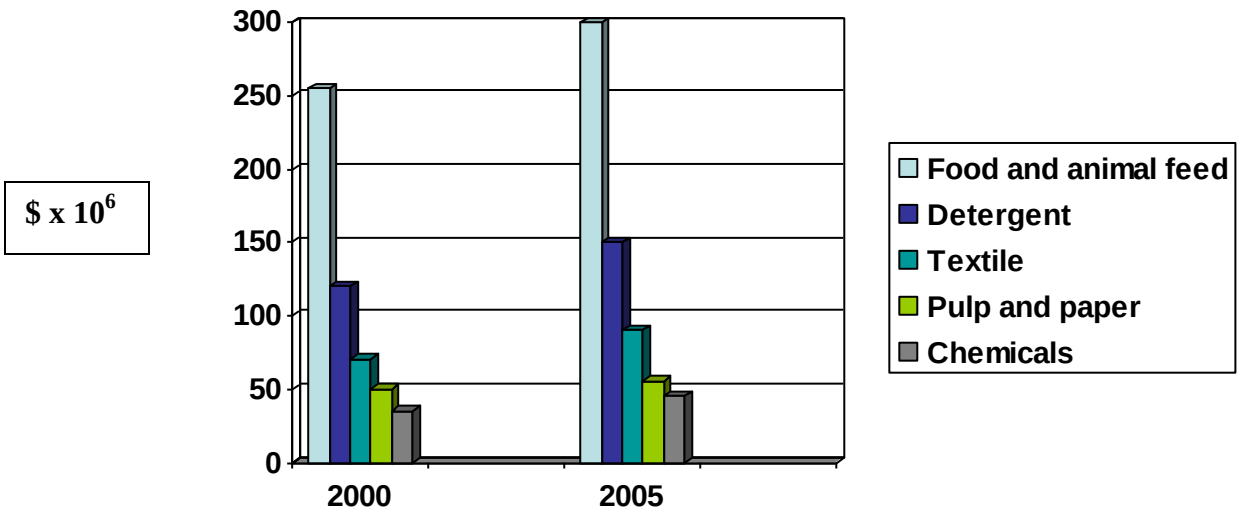

Figure 1.4 Worldwide increases in the use of enzymes in industrial applications from 2000 to 2005 (Vector Research – Global Technologies, Inc. 2005)

1.5.1 The detergent industry

Enzymes now comprise one of the ingredients of modern compact detergents. The main advantage of enzyme application in detergents is due to much milder conditions than with enzyme free detergents (Gupta *et al*., 2003). Their use as detergent additives still represents the largest application of industrial enzymes, both in terms of volume and value. The use of these enzymes in detergents affords numerous advantages, such as energy saving because they require lower temperature, and the reduction or replacement of other components that may be more harmful to the environment (Rodríguez *et al*., 2006). The major component of these enzymes, are proteases, but other and very different hydrolases are introduced to provide various benefits, such as the efficient removal of specific stains (Kirk *et al*., 2002).

Constantly, new and improved engineered versions of the "traditional" detergent enzymes, proteases and amylases, are developed. These new second and third generation enzymes are optimised to meet the requirements for performance in detergents, the composition of which is also constantly developed (Kirk *et al*., 2002). The earlier detergents were very harsh, caused injury when ingested and were not compatible with delicate china and wooden dishware. This forced the detergent industries to search for milder and more efficient solutions (Van Ee *et al*., 1992). Some of these enzymes are obtainable from renewable sources, are biodegradable and act without risking aquatic life or having a negative effect upon residual water treatments (Rodríguez *et al*., 2006).

Some of the limitations of α-amylase in detergents are that the enzyme shows sensitivity to calcium and stability is severely compromised in a low calcium environment (Gupta *et al*., 2003). One of the main problems that effects enzyme reactions is that they might undergo thermal deactivation under the conditions used for washing with the detergent (Rodríguez *et al*., 2006). In addition, most wild type α-amylases are sensitive to oxidants which are generally a component of detergent formulations. Stability against oxidants was achieved by utilising successful strategies followed with other enzymes such as protease (Tierny *et al*., 1995).

1.5.2 Starch liquefaction and saccharification

The major market for α-amylase lies in the production of starch hydrolysates such as glucose and fructose (Gupta *et al.,* 2002). The enzymatic conversion of starch to high fructose corn syrup is a well-established process and provides a beautiful example of a bioprocess in which the consecutive use of several enzymes is necessary. Because of their high sweetening property, these are used in huge quantities in the beverages industry as sweeteners for soft drinks. Enzymes used in the starch industry are also subjected to constant improvements (Van der Maarel *et al*., 2002).

The first step in the process is the conversion of starch to oligomaltodextrins by the action of α-amylase (Fig. 1.5; 1.6 and 1.7). The co-comitant injection of steam puts extreme demands on the thermostability of the enzyme. Using traditional α-amylases, the pH has to be adjusted to an undesirable high level and calcium must be added to stabilise

the enzyme. New α-amylases with optimised properties, such as, enhanced thermal stability, acid tolerance, and ability to function without the addition of calcium, have recently been developed offering obvious benefits to the industry. (Pandey *et al*., 2000; Van der Maarel *et al*., 2002; Bisgaard-Frantzen *et al*., 1999; Shaw *et al*., 1999; Declerck *et al*., 2000). Engineering efforts have also been undertaken to develop improved versions of the enzymes used later on in the process.

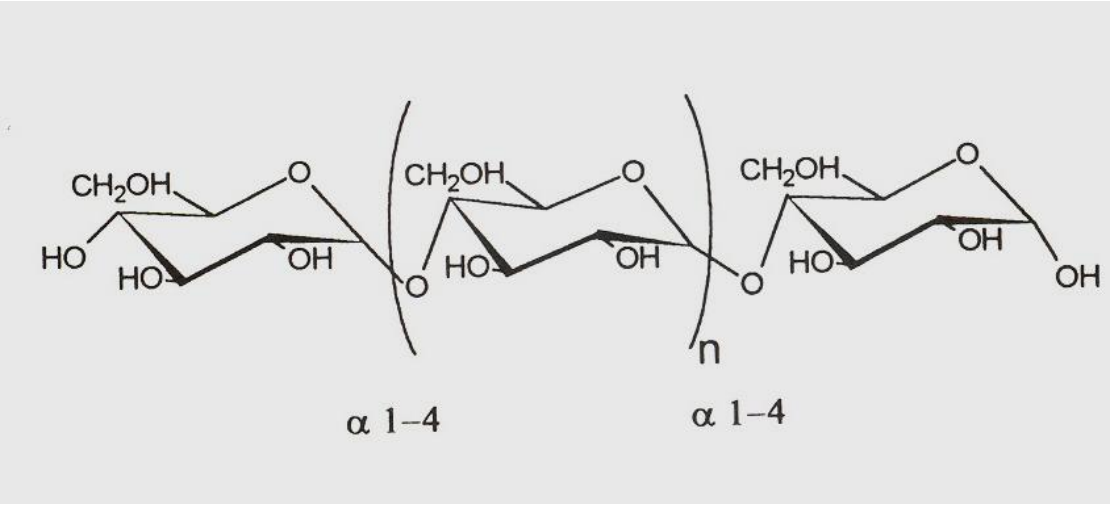

Figure 1.5 Glycosidic bonds between successive glucose molecules linked carbon atom no. 1 showing the cleveage of the α 1-4 glycosidic bonds with α-amylase resulting in liquefaction of carbohydrates (Walsh and Headon, 1996).

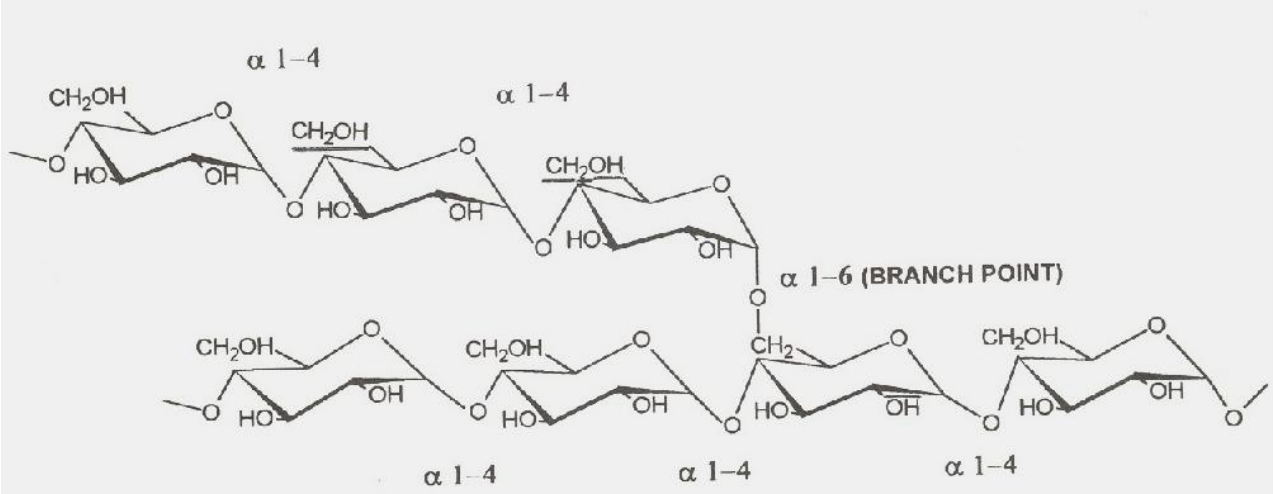

Figure 1.7 Structure of amylopectin with linear branch points consisting of α-1-6 glycosidic bonds cleaved by glucoamylase resulting in saccharification of carbohydrates (Walsh and Headon, 1996).

1.5.3 Textile applications

This industry is under considerable environmental pressure owing to its large energy and water consumption and subsequent environmental pollution. One of the most energy and water consuming steps in the processing of cotton is the scouring step, the removal of various remaining cell-wall components on the cellulose fibres performed at high temperature and under strong alkaline conditions (Kirk *et al*., 2002). As an alternative, Tzanov *et al*., (2001) developed a successful substitution of traditional chemical processes by introducing an enzyme-based process performed at much lower

temperatures and using less water. Following this, enzymes have now been introduced into most steps in the manufacturing of cotton textiles (Kirk *et al*., 2002).

In textile weaving, a starch paste is applied for warping. This gives strength to the textile at weaving. It also prevents the loss of string by friction, cutting and generation of static electricity on the string by giving softness to the surface of string due to laid down warp (Aiyer, 2005). After weaving the cloth the starch has to be removed, it is at this point of the process that α-amylase is introduced to help with the removal of the starch (Aiyer, 2005; Kirk *et al*., 2002). Kirk *et al*. (2002) reported that enzymes are used in at least seven various unit operations in textile wet processing and the manufacturing of denim.

1.5.4 Paper industry

The use of α-amylase for the production of low viscosity, high molecular weight starch for coating of paper has been reported (Bruinenberg *et al*., 1996). The use of amylases in the pulp and paper industry is in the modification of starches for coated paper. As for textiles, sizing of paper is performed to protect the paper against mechanical damage during processing. This process is said to improve the quality of the finished paper, and enhances the stiffness and the strength in paper.

Starch is subsequently used as the sizing agent, which is added to the process as a slurry. The temperature of this process is between 40^{o}C to 60^{o}C. In the process a constant viscosity of the starch is required. To maintain the viscosity of the natural starch it is

adjusted by partially degrading the polymer with α-amylase in a batch or continuous process. The conditions depend on the source of the starch and the α-amylase used (Tolan, 1996).

1.5.5 Enzymes for the food industry

The application of enzymes in the food industry, are many and diverse, ranging from texturising to flavouring. In most food applications the enzymes are applied to processed food products as processing agents upstream from the final product. Advances have been made in the optimisation of enzymes for existing applications and in the use of recombinant protein production to provide efficient mono-component enzymes that do not have potential detrimental side-effects (Kirk *et al*., 2002).

1.5.5.1 Baking industry

Enzymes such as malt and microbial α-amylases have been widely used in the baking industry (Hamer, 1995). Enzymes are used in bread and rolls to give these products a higher volume, better colour, improve flavour and as an anti-staling agent. Malt preparations have led the way and created opportunities for many enzymes to be used commercially in baking. Enzyme preparations such as proteases, lipases, xylanases, pullulanases, pentosanases, cellulases, glucose oxidase, and lipoxygenases are applied in the bread industry for various purposes, but none have been able to replace α-amylase (Monfort *et al*., 1996; Prieto *et al*., 1995). Currently, the α-amylases used in baking have

been cereal enzymes from barley malt and microbial enzymes from both fungi and bacteria (Hebeda *et al*., 1990; 1991).

Supplementation of flour with exogenous fungal α-amylase has a higher activity and enhances the rate of fermentation and reduces the viscosity of the dough. There is also an improvement in the volume and texture of the product, which also generates additional sugar in the dough, subsequently improving the taste, crust colour and toasting qualities of the bread (Van Dam and Hille, 1992).

There is an increasing focus on lipolytic enzymes (Collar *et al*., 2000). Recent findings suggest that (phospho) lipases can be used to substitute or supplement traditional emulsifiers, as the enzymes degrade polar wheat lipids to produce emulsifying lipids *in situ*. Currently the use of enzymes in the baking industry is moving towards the further understanding of bread staling and the mechanisms behind the enzymatic prevention of staling when using α-amylases and zylanases (Andreu *et al*., 1999).

Water-binding capacity and water retention in the starch and hemicellulose fractions of the bread, which are the substrates of α-amylases and zylanases, respectively, are important for maintaining softness and elasticity. The three-dimensional structure of amylases applied for anti-staling, provides an insight into the mechanism of enzyme action (Dauter *et al*., 1999). An excess of α-amylases used will result in an undesirable sticky texture to bread. Therefore, intermediate temperature stable α-amylases are used to

prevent this from occurring. They are active after starch gelatinisation and become inactive before the completion of the baking process (Gigras *et al*., 2002).

Understanding different enzyme classes provide new applications within the food industry. Enzymes have been used to enhance the quality of bread when wheat flour has been substituted with polished flour (Kim *et al*., 2006). Polished flour contains large amounts of dietary fibre, vitamins, minerals and antioxidant compounds, where as wheat flour mainly consists of the endosperm. The presence of bran has some negative effects on the texture; appearance; loaf volume and mouth feel of bread made from polished flour. The combined addition of fungal α-amylase and polished flour to bread dough increases the loaf volume by 26.3% and improves softness of bread (Kim *et al*., 2006).

1.5.6 Novel food applications

As a food component, fat contributes to some key sensory and physiological properties in products. The production of low-fat products, are normally associated with some technical problems such as poor texture, flavour and mouthfeel (Keeton, 1994). Therefore, modification of food products using fat repacers or fat mimetics is often viewed as an effective way to overcome these problems due to the reduction in fat content (Giese, 1996; Vanderveen and Glinsmann, 1992). The mouth feel for fat in a product is a phenomenon of rheology, as unlike sweetness and saltiness, there is no sensor in the mouth for fats. Instead, the sensation of fattiness is a complex interrelated phenomenon involving viscosity and flowability poperties of a food product (Ma *et al*.,

2006). Lucca and Tepper (1994) found that particles less than 3 μm in diameter were too small for human tongue to distinguish as individual particles. Instead the particles were perceived as a creamy and smooth fluid.

The hydrolysis of corn starch into fine particles with enzymes is suitable for use as a fat mimetic in low fat foods. Hydrolysed corn starch is added to produce low fat mayonnaise reducing the fat content by 60% in mayonnaise. Mayonnaise manufactured with fat mimetics, have a similar sensory quality as compared with the high fat mayonnaise (Ma *et al*., 2006).

1.5.7 Analysis in medical and clinical chemistry

With the advent of new frontiers in biotechnology, the spectrum of amylase applications has expanded into many other fields, such as clinical, medicinal and analytical chemistry (Gupta *et al*., 2003). There are several processes in the medicinal and clinical areas that involve the application of amylases. The application of a liquid stable reagent, based on α-amylase for the Ciba Corning Express clinical chemistry system has been described (Becks *et al*., 1995).

Some other processes that have been developed using amylases are for example, detection of higher oligosaccharides (Giri *et al*., 1990) and biosensors with an electrolyte ioltor semiconductor capacitor transducer for process monitoring (Menzel *et al*., 1998).

1.6 Animal feed studies

1.6.1 Use of animal models to study dietary effects on human health

Animal models are widely used in nutrition science for various reasons. The limitations in extrapolating results of animal studies, to human health are an ever ongoing debate. In general, results from animal studies are considered useful indicators, for human studies (Mawson *et al*., 1994). Animal models are advantageous in cases such as long-term studies and other investigations, which for practical or ethical reasons cannot be studied in humans (Woese *et al*., 1997).

The choice of species in animal models depends on a number of factors, including the resemblance to humans, the sensitivity to the investigated factors and cost implications. In any animal study the advantages and disadvantages of several animal species must be considered. Rat models are extensively used (Table 1.9) as a model for studies for factors affecting human health, and the cost per animal is relatively low. Another obvious choice would be minipigs, these animals are adapted to a diet more closely resembling that of humans , and some investigations are much easier to perform on pigs than on rats, however, the cost per animal is prohibitively high (Sandström *et al*., 2000).

Long-term studies using realistic diets are rather few, and in this connection the most important ones are the studies of caloric restriction, which in many rat species have shown that diet can influence the ultimate health indicator, longevity, even when no

potent chemicals are involved, nor any malnutrition (McCarter, 1995; McShane and Wise, 1996). The diet of young animals has been known for its ability to influence their health in adults (McCarter, 1995).

It is also well established that differences in nutritional status of female animals will affect the growth and development of the offspring, and that this further has consequences for their adult life, e.g. development of allergies (Leadbitter *et al*., 1999) or lung disease (Lopuhaa *et al*., 2000). It is for these reasons and others, a study that extends for at least two generations is necessary to elucidate effects that are determined during the phase of rapid growth, but manifest themselves primarily in puberty (Broadhurst *et al*., 2000).

Table: 1.9 An overview of nutritional studies using rat models

Type of Study	Number of Rats used	References
Evaluation of protein concentrations in seaweed	16	Wong *et al.* (2004)
Different sources of protein	10	James *et al.* (2002)
Protein utilization and energy digestibility	5	Eggum *et al.* (1985)
Gestation performance test	50	de Faria *et al.* (2004)
Digestibility test	48	de Faria *et al.* (2004)
Performance test – weight gain and diet consumption	200	de Faria *et al.* (2004)
Nutritional and physiological responses of rats diets of cowpea, protein isolates or starch	4	Olivera *et al.* (2003)
Resistant starches, lower plasma lipids and improved mineral retention in rats	64	Lopez *et al.* (2001)
Nutritional utilisation of chickpea by rats	4	Rubio e*t al.* (1998)
Evaluation of safety of inulin and oligofructose as dietary fibre (Toxicity)	24 M and F	Carabin and Flamm (1999)
Subacute toxicity – gavage study	18	Carabin and Flamm (1999)
Subacute toxicity – feeding study	18	Carabin and Flamm (1999)
Chronic and carcinogenicity study	50 M and F	Carabin and Flamm (1999)
Developmental and reproduction toxicity	12 to 17 F 9 rats + mother	Carabin and Flamm (1999)
Nutritional quality of masa	40	Nkama and Malleshi (2000)
Low nutritional quality of tropical crop seeds in rats	30	Proll *et al.* (1998)

M = male; F = female

1.7 Scope of this study

In Sub-Saharan Africa there is an urgent need to sustain and improve the quality of its food resources. Poverty eradication features high on the agenda of a number of world health organisations, while the number of underweight children in Africa continues to increase (Pellet, 1996). Providing nutritionally enhanced foods to the poor will help towards achieving this objective. Protein-energy malnutrition has been identified as one of the most important problems facing Africa, with maize as the staple diet (Nkama *et al*., 1995). However, a combination of several factors limits availability and the nutritional quality of maize.

During starvation, energy and protein intakes decrease by 20-30%, with most of the children in Africa having an average protein intake of only 20 g per day (Igbedioh, 1996). Energy availability also affects protein utilization because of interrelationships of protein and energy metabolism (Elwyn, 1993). The diets of inhabitants in developing regions depend mainly on cereals (maize) for both protein and dietary energy which lacks indispensable amino acids, minerals, vitamins and carbohydrates.

In light of these growing concerns an attempt was made to devise a scientific strategy to combat the nutritional shortfalls of maize meal. A multidisciplinary and concerted approach was followed within this project aimed at designing an improved thermostable amylase and applying the enzyme to nutritionally enhance maize meal.

It was envisaged that the manipulation of maize meal, by the application of enzyme technology will improve the nutritional status of this staple food. The consequences is that an alternate solution for the eradication of an ailing, poverty stricken and malnourished African population is achievable. It is possible that the boundaries defining the limits of life will extend to even greater extremes through the application of novel technologies.

1.8 Objectives of this study

1. Screening and selection of *T. lanuginosus* strains for α-amylase activity.
2. Application of solid state fermentation for the production of α-amylase using the selected strain of *T. lanuginosus.*
3. Optimisation of nutritional parameters for the enhanced production of extracellular α-amylase by *T. lanuginosus.*
4. Studies on isolation, purification and characterization of the α-amylase.
5. Application of the α-amylase in the hydrolysis of maize meal to enhance nutritional value.
6. Quantification of the nutritional advantages of the hydrolysed maize mash by conducting animal trials.

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CHAPTER TWO: SCREENING OF *T. lanuginosus* STRAINS FOR α-AMYLASE ACTIVITY

2.1 Introduction

Amylases are among the most important enzymes eg., in the food and textile industries, hence they are of great significance in present-day biotechnology (Burhan *et al*., 2003). Although amylases can be produced by several microorganisms, it remains a challenging task to obtain a strain capable of producing commercially acceptable yields. Selection of a suitable strain is the most significant factor in the amylase production process (Pandey *et al*., 2000). The major advantage of using microorganisms for the production of amylases is the economical bulk production capacity and microbes are easy to manipulate to obtain enzymes of desired characteristics (Gupta *et al*., 2003).

Commercial production of amylases is carried out in various steps, essentially because the environmental factors required for the optimum growth of the microorganism being employed for production may differ from those required for the production of enzymes (Pandey *et al*., 2000). These parameters include nutrient supplementation, pH of the medium, osmotic relationship, degree of aeration, temperature and the control of contamination during fermentation. Maintaining the purity of the medium is also a very important factor, especially when the fermentation is carried out under aerobic conditions (Pandey *et al*., 2000). The optimisation of fermentation conditions are of primary importance in the development of any fermentation process owing to their impact on the economy and practicability of the process (Francis *et al*., 2003).

The specific fermentation processes adopted vary; however, there remain two main methods for amylase production, submerged fermentation and solid-state fermentation. Solid-state fermentation has gained renewed interest from researchers for the production of these enzymes in view of its several economic and engineering advantages and has been often employed to produce amylases (Pandey, 1992). The submerged production of α-amylase, using synthetic media has been reported by many workers (Tigue *et al*., 1995; Haq *et al*., 1997; Hamilton *et al*., 1999). The contents of synthetic media such as nutrient broth, soluble starch as well as other components are very expensive and these contents might be replaced with more economically available agricultural by-products for the reduction of cost of the medium (Haq *et al*., 2003).

α-Amylases are universally distributed throughout the animal, plant and microbial kingdoms. Over few decades, considerable research has been undertaken with the extracellular α-amylase being produced by a variety of organisms (Gupta *et al*., 2003). α-Amylases have been derived from several fungi, yeast, bacteria and actinomycetes, however, enzymes from fungal and bacterial sources have dominated applications in industrial sectors (Pandey *et al*., 2000). Because of the industrial importance of amylases, there is ongoing interest in the isolation of new bacterial strains producing amylases suitable to new industrial applications, such as alkaline amylase for the detergent industry (Burhan *et al*., 2003). Amylolytic enzyme preparations are now commercially produced by *Bacillus, Aspergillus* and *Rhizopus* species for processing starch. Barnett and Fergus (1971), reported that extracellular amylolytic enzymes were produced by *T. lanuginosus* grown in starch-based medium. Since then several authors, cited by Nguyen *et al*., 2000

worked on the screening of microorganisms for enzyme production, isolation and production and characterization of the enzyme.

In this chapter the screening of known fungal strains to identify amylase degraders, using standard techniques with soluble starch as the substrate in plate assays, submerged fermentation and solid state fermentation is described.

2.2 Materials and methods

2.2.1 *Thermomyces lanuginosus* strains

Eight *Thermomyces lanuginosus* strains obtained from the American Type Culture Collection (ATCC) were screened for α-amylase production. The strains were: ATCC 16455, ATCC 22083, ATCC 26909, ATCC 34626, ATCC 38905, ATCC 58157, ATCC 58158 and ATCC 58160. The geographical origins and optimal growth conditions of the strains are shown in Table 2.1.

Table: 2.1 Fungal strains of *T. lanuginosus* and their culture conditions

STRAINS	GEOGRAPHICAL ORIGINS	GROWTH MEDIA	OPTIMUM GROWTH T ^{o}C
ATCC 16455	Rotting guayule shrubs, California	YpSs	45
ATCC 22083	Mushroom Compost, Switzerland	YpSs	45
ATCC 26909	Soil, Japan	YpSs	37
ATCC 34626	Submerged Balsa wood	PYG	45
ATCC 38905	Compost	YpSs	37
ATCC 58157	Soil, Jordan	PDA	37
ATCC 58158	Grape marc compost	PDA	37
ATCC 58160	Soil, Zambia	PDA	37

YpSs = yeast peptone soluble starch; PYG = peptone yeast glucose; PDA = potato dextrose agar

2.2.2 Maintenance of stock cultures

T. lanuginosus strains 38905, 26909, 58160, 58158 and 58157 were grown on Potato Dextrose Agar (PDA, Oxoid) plates and incubated for 5 to 7 days at 37^{o}C and ATCC strains 22083, 34626 and 16455 were incubated at 45^{o}C. Stock cultures were maintained on PDA (Oxoid) and stored at 4^{o}C.

2.2.3 Primary screening of *T. lanuginosus* strains for amylase activity

A fungal plug (0.5cm in diameter) of each of the eight ATCC strains was inoculated onto 2% starch agar plates and incubated at 50^{o}C for 3 days. α-Amylase activity was indicated by a zone of hydrolysis around the fungal growth, after staining of the plates with Lugol iodine solution (1% iodine; 2% potassium iodide w/v). Zones of hydrolysis were measured and used as a quantitative approach to select three of the highest producing strains.

2.2.4 Secondary screening of *T. lanuginosus* strains for amylase activity

The three best producing strains were further assayed to quantitatively evaluate amylase production using submerged and solid state fermentation techniques as described in sections 2.2.4.1 and 2.2.4.2.

2.2.4.1 Submerged fermentation

An Erlenmeyer flask (250 ml) containing 100 ml of starch asparagine medium at pH6 was evaluated for amylase production. Starch asparagine medium was composed of (g/l): soluble starch, 40; L-asparagine, 7.5; KH_2PO_4, 3; K_2PO_4, 2; $gSO_4.7H_2O$, 0.5; and 1 ml Vogel's trace element solution (Appendix 1) (Vogel, 1956).

All flasks were sterilized at 121^oC for 15 min. Duplicate flasks were inoculated with the respective fungal cultures and placed in a shaking incubator at 50 ^OC for 5 days with shaking at 150 rpm. Fungal broths were centrifuged and the supernarants were analysed for amylase activity as described in section 2.2.5.

2.2.4.2 Solid state fermentation

Fungal cultures were grown on PDA (Oxoid) plates for 5 days at 50 ^oC in a humidified incubator. A spore suspension (with a concentration of 4.07 x 10^7 spores/ml) for each strain (10 ml) was prepared in sterile double dionised water and added to 90 ml glucose-L-asparagine medium (pH 6) in 250 ml Erlenmeyer flasks. Cultures were incubated at 50 ^oC with shaking at 220 rpm for 2 days to obtain a homogenous mycelial pre-inoculum.

Two ml of the respective mycelial suspensions at a concentration of 4.07 x 10^7 spores/ml were used as inocula for the production of amylase in triplicate Erlenmeyer flasks (250 ml) containing a SSF medium comprising (g/100 ml): wheat bran, 10; soluble starch, 1;

soybean meal, 1 (w/w); and 1.5 ml stock salt solution (K_2HPO_4, 5.0 g; $MgSO_4.7H_2O$, 3.0 g dissolved in 7.5 ml of distilled water and with a final pH6). Distilled water was added to achieve final substrate moisture content of 80%. All flasks were sterilized at 121^oC for 15 min followed by cooling prior to inoculation. Incubation was carried out at 50^oC for 5 days.

The cooled flasks were each inoculated with 2 ml of the mycelial suspension of each of the respective strains of *T. lanuginosus* ATCC 38905, ATCC 58160 and ATCC 58157 and incubated at 50^oC for 5 days in an incubator.

After fermentation the contents of the flasks were harvested by adding 50 ml of sterile distilled water to each of the flasks followed by shaking at 250 rpm for 1 h, and refrigerated for 2 h. The contents of the flasks were filtered through muslin cloth. The residue was treated with 50 ml of sterile distilled water and filtered. Fungal filtrates for each cultures were pooled and centrifuged at 10 000 rpm for 15 min. The clear supernatant was used to determine amylase activity as described in section 2.2.5.

2.2.5 Enzyme assay

α-Amylase activity was determined according to the method described by Miller, 1959 and Bailey *et al.,*1992. α-Amylase was determined at 50^oC by mixing 0,5 ml of appropriately diluted enzyme with 0.5 ml of a 2% (w/v) soluble starch solution made up in 0.1 M phosphate buffer, pH6. After 10 min, the reaction was terminated with 1 ml of

dinitrosalicylic acid solution (DNS) (final reaction volume 200 µl). One unit of amylase activity was defined as the amount of enzyme that releases 1 mg/ml of reducing sugars (glucose equivalents) under the assay conditions.

2.3 Results

2.3.1 Primary screening for α-amylase activity

The initial screening of *T. lanuginosus* strains ATCC 16455, ATCC 22083, ATCC 26909, ATCC 34626, ATCC 38905, ATCC 58157, ATCC 58158 and ATCC 58160 revealed zones of hydrolysis on starch agar plates with a distinct halo around the growth of each of the strains. The degree of amylase activity was indicated by the width of the zone of hydrolysis around the central growth area of each culture as shown in Figs 2.1 to 2.8. From the preliminary results three strains of *T. lanuginosus* viz., ATCC 38905, ATCC 58160 and ATCC 58157 were selected for further analysis.

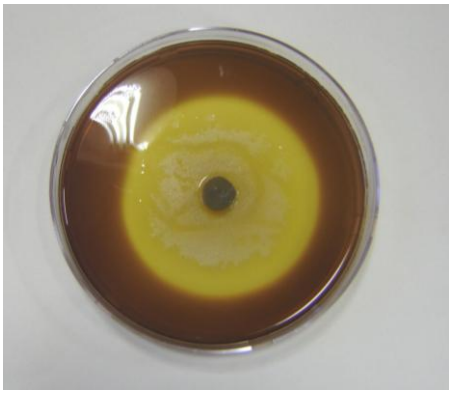

Figure 2.1 Zones of hydrolysis produced by *T. lanuginosus* ATCC 58157 after growth on starch agar plates at 50^{o}C for 3 days.

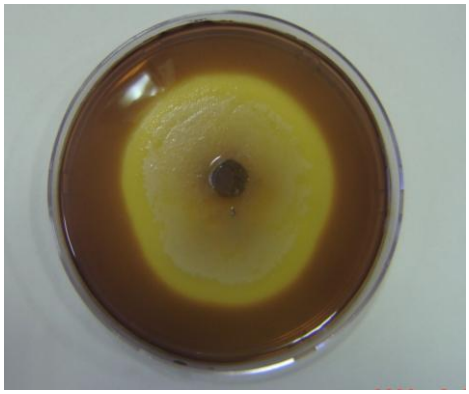

Figure 2.2 Zones of hydrolysis produced by *T. lanuginosus* ATCC 38905 after growth on starch agar plates at 50^{o}C for 3 days.

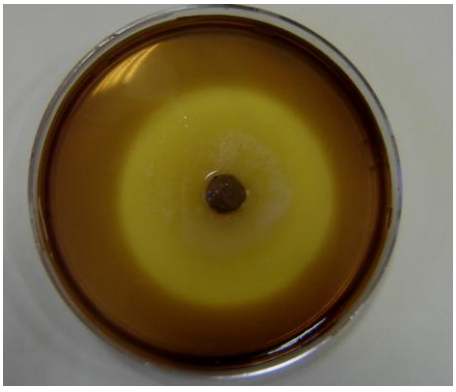

Figure 2.3 Zones of hydrolysis produced by *T. lanuginosus* ATCC 58160 after growth on starch agar plates at 50^{o}C for 3 days.

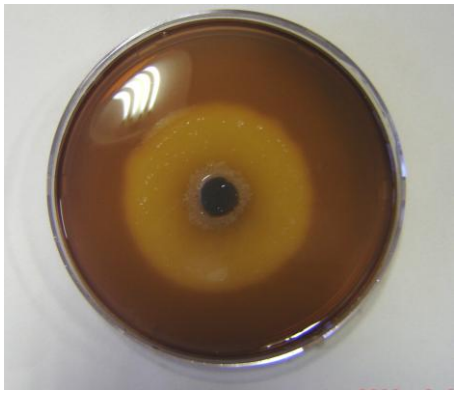

Figure 2.4 Zones of hydrolysis produced by *T. lanuginosus* ATCC 26909 after growth on starch agar plates at 50^{o}C for 3 days.

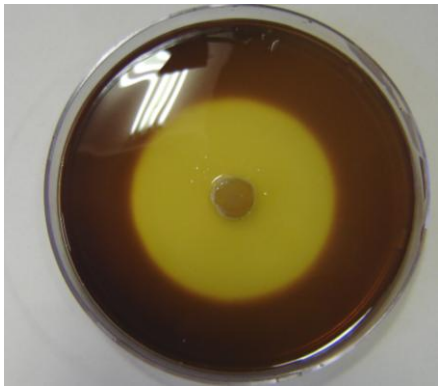

Figure 2.5 Zones of hydrolysis produced by *T. lanuginosus* ATCC 16455 after growth on starch agar plates at 50^{o}C for 3 days.

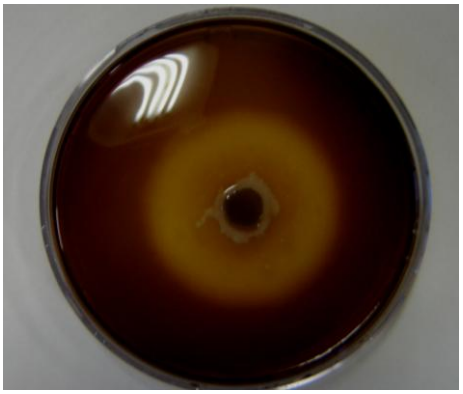

Figure 2.6 Zones of hydrolysis produced by *T. lanuginosus* ATCC 58158 after growth on starch agar plates at 50^{o}C for 3 days.

Figure 2.7 Zones of hydrolysis produced by *T. lanuginosus* ATCC 34626 after growth on starch agar plates at 50^{o}C for 3 days.

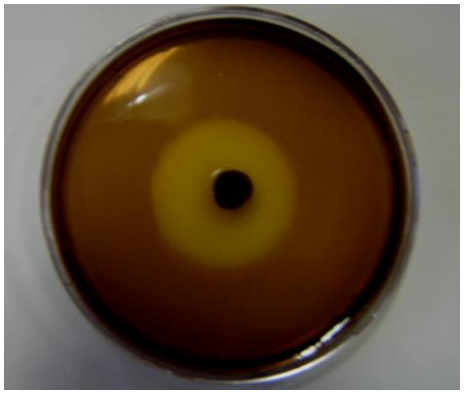

Figure 2.8 Zones of hydrolysis produced by *T. lanuginosus* ATCC 22083 after growth on starch agar plates at 50^{o}C for 3 days.

Submerged cultivations further confirmed the data obtained from the primary screen. Strains ATCC 38905, ATCC 58160 and ATCC 58157 produced the highest amounts of α-amylase (Table 2.2) and were therefore selected for further screening for a hyper - producing strain.

Table: 2.2 α-Amylase activity by strains of *T. lanuginosus*

Strains of *T. lanuginosus*	Activity (U/ml)	Zones of hydrolysis (cm)
ATCC 58157	28.7 ± 1.0	5.65
ATCC 38905	26.2 ± 1.3	5.5
ATCC 58160	25.5 ± 1.5	5.1
ATCC 26909	23.0 ± 0.6	5.0
ATCC 16455	22.3 ± 0.7	4.9
ATCC 58158	18.6 ± 0.5	4.75
ATCC 34626	13.5 ± 0.5	4.05
ATCC 22083	9.5 ± 0.3	3.65

Mean of triplicate tests ± SD

2.3.2 Secondary screening

Three hyper amylase producing strains of *T. lanuginosus* **(**ATCC 38905, ATCC 58160 and ATCC 58157) were used to determine the best fermentation technique. The results showed that the enzyme yield from SSF was higher than the enzyme yield from SmF (Table 2.3).

Table: 2.3 Amylase yields for three strains of *T. lanuginosus* by SmF and SSF

Strains of *T. lanuginosus*	SmF enzyme yields (U/ml)	SSF enzyme yields (U/ml)
ATCC 58157	28.7 ± 1.0	62.0 ± 2.4
ATCC 38905	26.2 ± 2.0	45.5 ± 3.2
ATCC 58160	25.5 ± 1.5	38.2 ± 1.8

Mean of triplicate tests ± SD

2.4 Discussion

Amylases have widely been reported to occur in microorganisms, although they are also found in plants and animals. Two major classes of amylases have been identified in microorganisms, α-amylase and glucoamylases (Pandey *et al.*, 2000). Although amylases can be produced by several microorganisms, it remains a challenging task to obtain a strain capable of producing commercially acceptable yields. Selection of suitable strains is the most significant factor in the amylase production process (Pandey *et al.*, 2000).

To ensure optimal production of α-amylase from the chosen strains the best method for fermentation had to be selected. Although the details of the specific fermentation processes adopted by different manufacturers vary, there remain two main methods for amylase production, submerged fermentation (SmF) and solid state fermentation (SSF) (Pandey *et al.*, 2000). The production of α-amylase by SmF and SSF has been thoroughly investigated and is affected by a variety of physiochemical factors (Gupta, *et al.*, 2003). Most notable among these are the composition of the growth medium, pH of the medium, phosphate concentration, inoculum age, temperature, aeration, carbon source and nitrogen source (Lonsane and Ramesh, 1990).

Increased production of the enzyme could be obtained by manipulating the growth conditions and medium composition. Sudo *et al.*, (1994) compared acid-stable α-amylase production in SmF and SSF and examined the reason why *A. kawachii* IFO 4308 produced larger amounts of acid-stable α-amylase in SSF than in SmF. Some of the SSF

characteristics were given as the major reasons for higher enzyme production in SSF. A new source of α-amylase was identified in *Pycnoporus sanguineus*. Cultivation in SSF resulted in 4-fold-higher production than in SmF (Lonsane and Ramesh, 1990). They tested more than 800 *Rhizopus* strains for α-amylase production. One of the strains showed great ability to produce a thermostable α-amylase in SSF.

The industrial production of enzymes by SSF has been confined to processes involving fungi and is generally believed that these techniques are not suitable for bacterial cultivation (Lonsane and Ramesh, 1990). Lonsane and Ramesh, 1990 also referred to the SSF process as the potential tool for achieving economy in enzyme production and starch hydrolysis. Baijpai *et al*. (1992), and Omidiji *et a.* (1997), developed simple and cheap media based on cheese whey, corn steep liquor and soya bean meal for α-amylase production. It was claimed that the medium could be exploited for the industrial production of α-amylase. The use of SSF technique in α-amylase production and its specific advantages over other methods has been discussed extensively (Lonsane and Ramesh, 1990).

The preliminary evaluation of SmF and SSF techniques for α-amylase production using the chosen strains 38905, 58157 and 58160 have indicated that SSF has the ability to produce higher titers of α-amylase then SmF. SSF increased the α-amylase production for strains 38905 and 58160 by one and a half times when compared to enzyme production by SmF and the production of α-amylase from strain 58157 was increased two fold with

SSF as apposed to SmF. Therefore, in this research project the SSF technique using wheat bran as the substrate for α-amylase production was adopted.

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CHAPTER THREE: PRODUCTION OF AMYLASE BY *Thermomyces lanuginosus* ATCC 58157 USING SOLID STATE FERMENTATION

3.1 Introduction

The term solid-state fermentation denotes cultivation of microorganisms on solid, moist substrates in the absence of a free aqueous phase; that is, at average water activity (defined as the relative humidity of the gaseous phase in equilibrium with the moist solid) significantly below one (Pandey, 2003). In a broader definition, SSF can be seen as including processes during which microorganisms are cultivated in the presence of a liquid phase at maximal substrate concentrations (Mitchell *et al.*, 2000) or on inert carriers (Ooijkaas *et al.*,2000).

Traditionally, amylase has been produced by submerged fermentation (SmF) and used in a one-way process in solution. In western countries, SSF had to compete with the technically easier SmF, which was more strongly developed from the 1940s onwards because of the necessity to produce antibiotics on a large scale. In the east, by contrast, SSF survived in many areas in the form of labour-convenient, hand-made processes; however, some of the traditional SSF processes have also been further developed to achieve higher industrial rationalisation through stronger automation (Hölker and Lenz, 2005). In recent years, however, solid state fermentation (SSF) processes have been increasingly utilized for the production of enzymes (Pandey *et al*., 1999).

A direct comparison between the SSF and SmF cultivation modes of fungi is difficult to make because the two processes differ. Studies of the production of fungal enzymes in SSF have repeatedly shown that SSF, in comparison with SmF, provides higher volumetric productivities, is less prone to problems with substrate inhibition and yields enzymes with a higher temperature or pH stability (Hölker *et al*., 2004). In the employment of microbial mixed cultures, SSF offers an option that cannot be achieved by SmF. This makes use of the fact that, during growth on natural substrates, fungal consortia secrete a broad spectrum of enzymes (Koroleva *et al.,* 2002; Stepanova *et* al., 2003; Yang *et al*., 2004). In addition, SSF offers the possibility to explore and to use interactions between fungi by properly regulating the water activity as a selection parameter depending on the different water demands of the individual co-cultivated fungi. Many SSF processes used in the food industry make use of mixed cultures that are important for the flavour of the foods produced (Fu *et al.,* 2002).

SSF has numerous advantages including productivity and may be preferred to SmF due to the simple technique, low investment, lower levels of catabolite repression and better product recovery (Babu and Satyanarayana, 1995; Mulimani and Ramalingam, 2000). The SSF technique is mainly confined to processes involving fungi (Aguilar and Huitron, 1986; Kadowaki *et al.,* 1997). It is believed that this technique is not suitable for bacterial cultures because of the higher water activity requirements (Lonsane *et al.,* 1985). However, successful bacterial growth using the SSF technique is known in a number of natural fermentations (Lonsane *et al.,* 1990 and Ramesh and Lonsane, 1991).

SSF holds tremendous potential for the production of enzymes (Pandey *et al*., 1999). It can be of particular relevance in those processes where a crude fermented product may be used as an enzyme source (Tengerdy, 1998). The selection of a particular strain, however, remains a tedious task, particularly when commercially significant enzyme yields are required. Agro-industrial residues are generally considered the best substrates for the SSF processes and enzyme production in SSF (Soni *et al.*, 2003). The commercial success of amylases is linked to the utilisation of starchy biomass as an industrial raw material. On a dry basis, agricultural substrates like corn, wheat, sorghum and other cereals contain around 60-75% (w/w) starch, hydrolysable to glucose with a significant weight increase, and offers a good resource in many fermentation processes (Soni *et al.*, 2003). The production of α-amylase by SSF is limited to the genus *Bacillus* with *B. subtilis*, *B. polymyxia*, *B. mesentericus, B. vulgarus, B. megaterium* and *B. licheniformis* being used for α-amylase production in SSF (Baysal *et al*., 2003).

Various solid substrates, such as wheat bran, soybean meal and corn cobs have been used in SSF. These substrates were employed individually without supplementing with additional carbon and nitrogen sources. Among these, wheat bran was reported to be the best (Ramesh and Lonsane, 1991). Sodhi *et al.* (2005) reported that the productivity of thermostable amylases from *Bacillus* sp. was affected by the nature of the solid substrate (wheat bran, rice bran, corn bran and combination of two brans); nature of the moistening agent; level of moisture content; incubation temperature; presence or absence of surfactant; carbon; nitrogen; mineral; amino acid and vitamin supplements. The results of their study showed that maximum α-amylase production was obtained on wheat bran supplemented with 1% (w/w) glycerol and soyabean

meal, $_{L}$-proline (0.1% w/w), vitamin B-complex (0.01%) and moistened with tap water containing 15 Tween-40. Considerable work has been carried out on the effect of moisture levels on the bacterial α-amylase production technique (Lonsane *et al.*, 1985 and Ramesh and Lonsane, 1990). In the SSF process, the solid substrate not only supplies the nutrients to the culture, but also serves as an anchorage for microbial cells. The moisture content of the medium changes during fermentation as a result of evaporation and metabolic activities and the moisture level of the substrate are therefore most important (Baysal *et al.*, 2003).

Extensive reports on the production of amylase enzymes by *T. lanuginosus* using submerged cultivation have been reported (Nguyen *et al.,* 2002; Rónaszéki *et al*., 2000; Li *et al*., 1998; Jensen *et al*., 1988). Hence, the aim of this chapter was to investigate the optimal production of amylase by *T. lanuginosus* ATCC 58157 under SSF conditions and to examine the effects of solid substrate, moisture levels, pH, incubation time and temperature, carbon, nitrogen, and metal ion supplementation on enzyme production and stability.

3.2 Materials and methods

3.2.1 Maintenance and growth of microorganism

Stock cultures of *T. lanuginosus* ATCC 58157 were maintained on PDA plates as described in section 2.2.4.2., and stored at room temperature. The inoculum was prepared by making a spore suspension of *T. lanuginosus* ATCC 58157 in sterile distilled water (10 ml) with a spore concentration of 4.07 x 10^7 spores/ml.

The experiments were conducted in 250 ml Erlenmeyer flasks with solid medium prepared as described in section 2.2.4.2. All experiments were carried out in triplicate. Distilled water and inoculum were added to achieve a final substrate moisture content of 80% as described in section 2.2.4.2. SSF was used for α-amylase production and the extraction procedure was applied as described in section 2.2.4.2.

3.2.2 Selection of solid substrate

SSF as described in section 2.2.4.2.was applied, using a spore suspension of *T. lanuginosus* ATCC 58157 as the inoculum. Commercial quality wheat bran, molasses bran, rice bran, maize meal, millet cereal, wheat flakes, barley bran, crushed maize, corncobs and crushed wheat were procured from the local market and used as solid substrates and their effect on the production of amylase was determined. All experiments were carried out in triplicate. The best solid substrate was defined as the solid substrate which resulted in the highest α-amylase yields under assay conditions.

3.2.3 Optimisation of process parameters

Various process parameters influencing enzyme production during SSF were optimised by conventional methods for maximal enzyme production and was applied to SSF as follows: incubation period (24, 48, 72, 96, 120, 144 and 168 h); incubation temperature (40^{o}C, 50°C, 60°C, 70°C and 80°C); initial total moisture content (50%, 60%, 70%, 80%, 90%, 100% and 110%); initial pH (4.0, 6.0, 6.2, 7.0, and 8.0) and inoculum level (5%, 10%, 15%, 20%, 25% and 30% [v/w]). The fermentation was carried out under standard test conditions following the SSF technique described in section 2.2.4.2, applying one variable at a time (OVAT) for each of the parameters tested.

Wheat bran (best solid substrate) was supplemented in the fermentation as follows: different carbon sources (soluble starch, sucrose, lactose, maltose, dextrose, fructose and glucose); nitrogen sources (peptone, tryptone, meat extract, ammonium sulphate, yeast extract, soybean meal, urea, ammonium sulphate and sodium nitrate) at 1% w/w; ratios of salt solution concentration to substrate weight (v/w) (1.0:10, 1.5:10, 2.0:10 and 2.5:10) and substrate weight to flask volume (w/v) (1: 50, 1:100, 1:150, 1:200, 1:250 and 1:300). Each variable was applied to the SSF procedure under standard test conditions described in section 2.2.4.2 using the OVAT approach for each parameter tested.

3.2.4 Production of amylase under optimum conditions

On the basis of the results obtained with all the optimum parameters, viz., wheat bran containing soluble starch and peptone as supplements (1% w/w); pH of 6.0; moisture content 90%; salt solution concentration of 1.5:10 (v/w); inoculum level of 10%, incubation temperature of 50^{o}C and incubation period of 120 h α-amylase was produced by SSF. The extent of improvement in the optimised medium was evaluated using basal medium as a control (Soni *et al*., 2003). The basal medium comprised of: 100 g wheat bran moistened with 125 ml distilled water, 1% (w/w) glucose, 0.5% (w/w) soybean meal and 0.01 M $MnSO_4$. The medium was thereafter inoculated with 25 ml of inoculum (4.07 x 10^7 spore/ml). The fermentation was carried out as described in section 2.2.4.2.

3.2.5 Enzyme extraction and analytical methods

After fermentation (five days), the solid biomass was treated with 50 ml of distilled water and agitated on a magnetic stirrer for 30 min. The contents were filtered through muslin cloth and the residue was again treated with another 50 ml of distilled water followed by agitation and filtration. The filtrates were pooled together and centrifuged (10,000 rpm, 15 min), and the clear supernatant was analysed for amylase activity as described in section 2.2.5.

The moisture content of the wheat bran was estimated by drying 10 g of wheat bran to a constant weight at 105°C and the dry weight was recorded. To fix the initial moisture content of the solid medium, wheat bran was soaked with the desired quantity of water. After soaking, the sample was again dried as described and the moisture content (%) was calculated as follows.

$$\text{Moisture content of initial solid medium (\%)} = \frac{\text{weight of the wheat bran - dry weight of wheat bran}}{\text{dry weight of wheat bran}} \times 100$$

3.3 Results

3.3.1 Optimisation of process parameters

In SSF, the selection of a suitable solid substrate for a fermentation process is a critical factor and thus involves the screening of a number of agro-industrial materials for microbial growth and product formation (Soni *et al.*, 2003). In this study all the substrates supported the growth of *T. lanuginosus* and the enzyme formation by the culture, while wheat bran proved superior to the other substrates. A high titer of amylase activity (211 U/g) (Table 3.1) was obtained in a medium containing wheat bran alone as the substrate. The order of substrate suitability was wheat bran, millet cereal, crushed maize, crushed wheat, corncobs, wheat flakes, maize meal, barley bran, molasses bran and rice bran (Table. 3.1). In subsequent experiments, wheat bran was used as the solid substrate for amylase production.

The incubation time for achieving the maximum enzyme level is governed by the characteristics of the culture and is based on growth rate and enzyme production. The enzyme displayed a steady increase in activity from 24 h to 120 h. *T. lanuginosus* produced high titers of enzyme (212 U/g) at 120 h of incubation (Fig. 3.1). A steady decline in enzyme activity was observed after 120 h to 168 h, with the lowest enzyme titer (120 U/g) after 168 h. The optimal temperature for maximum amylase production (213 U/g) was found to be 50°C (Fig. 3.2). The enzyme activity decreased rapidly between 50^{o}C and 80^{o}C with enzyme activity falling below 10 U/g.

Table: 3.1 Effect of different solid substrates on α-amylase production by *T. lanuginosus* ATCC 58157 in SSF at 50^{o}C for 5 days

Substrate	α-Amylase activity (U/g) [1]
Wheat bran	211.0 ± 6.0
Millet cereal	196.0 ± 5.8
Crushed wheat	170.0 ± 5.5
Crushed maize	162.5 ± 5.6
Corncobs	147.5 ± 4.6
Wheat flakes	118.5 ± 3.7
Maize meal	116.0 ± 3.2
Molasses bran	85.0 ±2.5
Barley bran	67.0 ±2.6
Rice bran	43.0 ± 2.1

[1] **Values are means of three determinations with standard deviation** (±).

A high enzyme titer (248 U/g) was attained when the initial moisture level was 90% (Fig. 3.3). An increase in moisture content of 20% resulted in a sharp decline of enzyme activity by 57% (142 U/g) (Fig. 3.3).

The maximum amylase production (231 U/g) equivalent to 100% activity (Fig. 3.4) was obtained at pH of 6.0. At pH 7.0 there was a 20% decrease in activity (183 U/g) and at pH 8.0 enzyme activity decreased by 70% (68 U/g). The inoculum level was also an important factor for the production of amylase. High inoculum levels are inhibitory in nature. The highest enzyme production (217 U/g) was obtained at an inoculum level of 10 % (v/w).

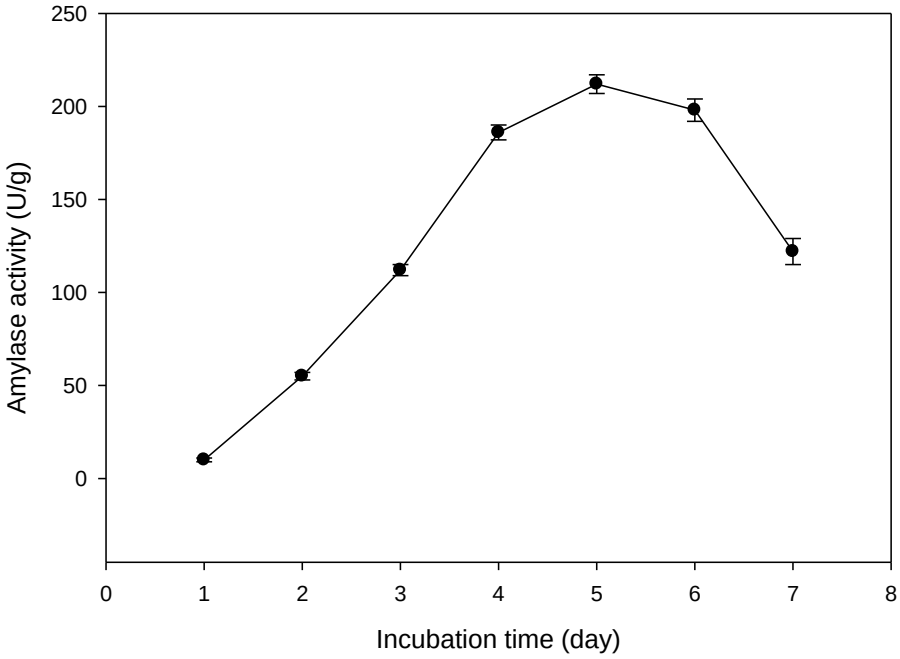

Figure 3.1 Effect of initial incubation time on α-amylase production by *T. lanuginosus* ATCC 58157 in SSF at 50°C

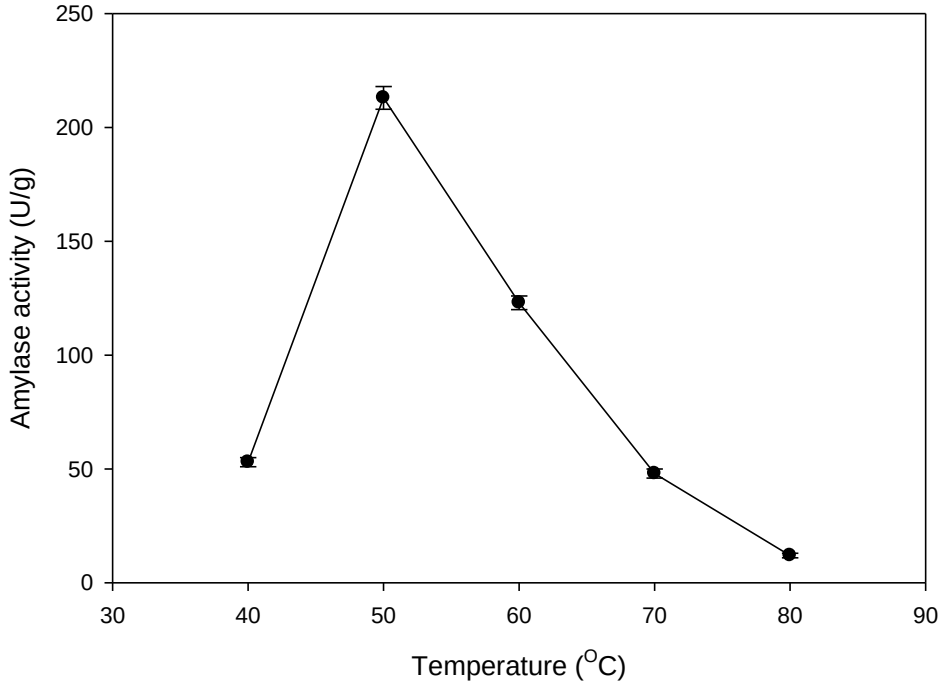

Figure 3.2 Effect of initial incubation temperature on α-amylase production by *T. lanuginosus* ATCC 58157 in SSF at pH 6.0

The influence of supplementing the solid substrate "wheat bran" with additional carbon and nitrogen sources were investigated. Of the carbon sources tested, soluble

starch increased amylase production (338 U/g) followed by sucrose (Table 3.2). Among the nitrogen sources, peptone increased amylase production (364 U/g) followed by tryptone and meat extract (Table 3.3). Of the carbon sources tested, soluble starch was the best inducer for amylase production (338 U/g) while glucose was a poor inducer at approximately 50% less. Peptone also induced the highest amylase activity (364 U/g) compared to the other complex nitrogen sources viz., meat extract, yeast extract and urea.

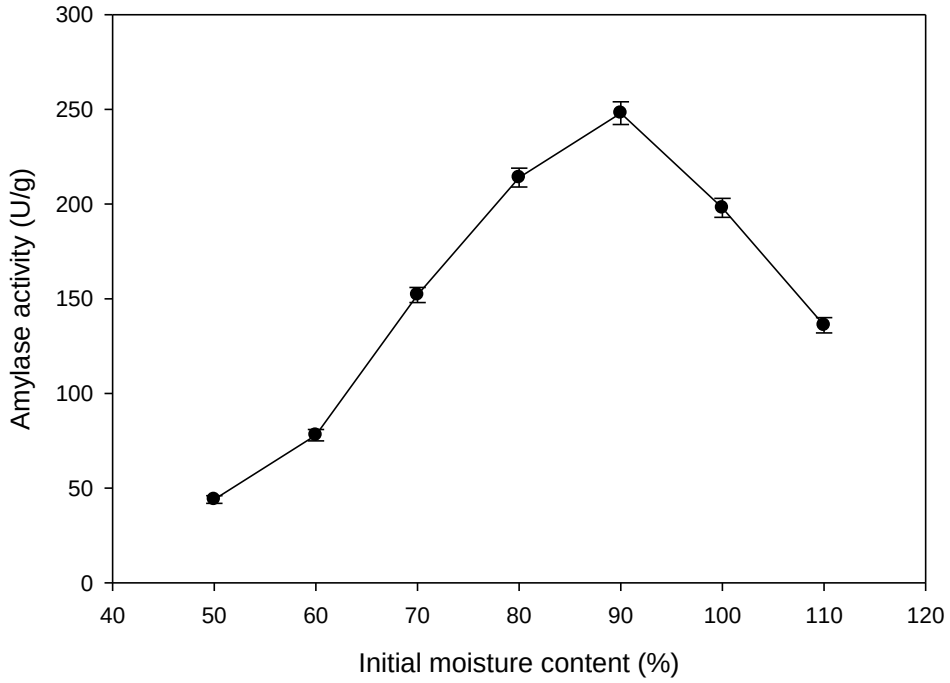

Figure 3.3 Effect of initial moisture content on α-amylase production by *T. lanuginosus* ATCC 58157 in SSF at 50°C and pH 6.0

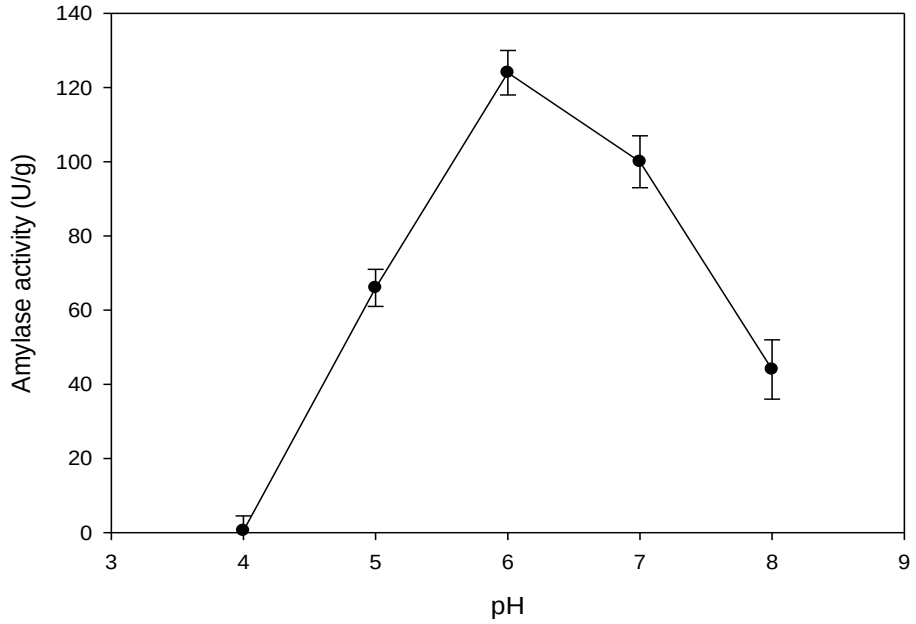

Figure 3.4 **Effect of initial culture pH on α-amylase production by *T. lanuginosus* ATCC 58157 in SSF at 50°C.**

Table: 3.2 **Effect of various supplementary carbon sources on amylase production by *T. lanuginosus* ATCC 58157 in SSF at 50°C and pH 6.0**

Carbon source (1% w/w)	Amylase activity (U/g)[1]
Soluble starch	338 ± 9.0
Sucrose	312 ± 8.5
Lactose	286 ± 7.8
Maltose	244 ± 7.2
Dextrose	228 ± 6.7
Fructose	216 ± 5.6
Glucose	142 ± 5.0
Control	211 ± 5.3

[1]Values are means of three determinations with standard deviation (±).

Table: 3.3 Effect of various supplementary nitrogen sources on amylase production by *T. lanuginosus* ATCC 58157 in SSF at 50°C and pH 6.0

Nitrogen source (1% /w)	Amylase activity (U/g)[1]
Peptone	364 ± 11.6
Tryptone	306 ± 10.4
Meat extract	278 ± 9.0
Ammonium sulphate	260 ± 8.5
Yeast extract	256 ± 8.0
Soybean meal	244 ± 7.3
Urea	230 ± 6.7
Ammonium nitrate	222 ± 6.0
Sodium nitrate	214 ± 5.3
Control	211 ± 5.4

[1]Values are means of three determinations with standard deviation (±).

Maximum enzyme production (256 U/g) was obtained at a ratio of salt solution concentration to wheat bran weight of 1.5:10 (v/w), whilst further increases in salt concentration were found to inhibit enzyme activity. The highest enzyme production (211 U/g) was observed when the ratio of substrate weight to flask volume was 1:100.

3.3.2 Production of amylase under optimized conditions

The maximum productivity of amylase (403 U/g) was achieved by utilizing wheat bran as the solid substrate under standard fermentation conditions for 120 h at 50°C, with an initial moisture content of 90%, pH of 6.0, inoculum level of 10 % (v/w), salt solution concentration of 1.5:10 (v/w) and a ratio of substrate weight to flask volume

of 1:100 with soluble starch (1% w/w) and peptone (1% w/w) as carbon and nitrogen supplements respectively.

A two-fold increase in amylase production was achieved under the optimized fermentation conditions when compared with the basal medium (control) (detailed in section 3.2.4). Although the results of these investigations are based on experiments conducted in flasks, they provide valuable information for the production of amylase by SSF.

3.4 Discussion

In industry there is a dire need for an economical and simple indigenous system for producing high titers of amylases. One alternative low cost and feasible production method is the use of SSF (Pandey *et al.,* 2000). However, there are several factors, which affect SSF processes. Among these, selection of a suitable strain, substrate and selection of process parameters (physical, chemical and biochemical) are crucial (Pandey *et al.,* 2000). In this study SSF was found to be an economical way of producing high levels of α-amylase with *T. lanuginosus* (ATCC 58157) on wheat bran as the solid substrate. The productivity levels were found to be high when compared with other published reports (Babu and Satyanarayana, 1995; Mamo and Gressesse, 1999; Ramdas *et al.,* 1996; Ramesh and Lonsane, 1987).

Since filamentous fungi are generally considered to be the most prolific producers of extracellular enzymes, attempts have been made to study α-amylase production using these fungi (Pandey *et al.,* 2000). The thermophilic fungus *T. lanuginosus* strain 1457 was reported to be an excellent producer of α-amylase (Pandey *et al.,* 2000; Jensen and Olsen, 1992; Arnesen *et al.,* 1998). It was reported that increased production of the enzyme could be obtained by manipulating the growth conditions and medium composition.

Different solid substrates were found to affect the production of enzymes (Satyanarayana, 1994). Wheat bran was previously reported to be the best substrate for glucoamylase production by an *Aspergillus* species (Ellaiah *et al.,* 2002) and was suitable for necessary manipulation to increase enzyme yields (Ellaiah *et al.,* 2002; Beckord *et al.,* 1945). In this study, all the substrates tested supported growth and

enzyme formation by *T. lanuginosus* (ATCC 58157), but wheat bran proved superior to the other substrates. Four solid substrates (tapioca, mustard oil cake, maize bran and gram bran) were tested (Babu and Satyanarayana, 1995) to investigate the possibility of replacing wheat bran as a solid substrate in SSF. Tapioca (141 U/g), mustard oil cake (105 U/g), gram bran (343 U/g) and maize bran (1267 U/g) supported poor amylase production when compared to enzyme production using wheat bran (3100 U/g) by *B. coagulans* (Babu and Satyanarayana, 1995).

The suitability of a particular substrate in a SSF process for the production of α-amylase appears to be governed by the physicochemical requirements of the microorganism used (Lonsane and Ramesh, 1990). The universal suitability of wheat bran may be due to the fact that it contains sufficient nutrients and does not aggregate even in moist conditions, thus providing a large surface area (Beckord *et al*., 1945). The results obtained in this study were consistent with those reported by Pandey *et al.* (2000). In subsequent experiments, wheat bran was used as the solid substrate for the production of α-amylase.

In SSF the incubation time is governed by characteristics of the culture and is based on growth rate and enzyme production. In most cases, the time applied for bacterial cultures was 30 h (37^{o}C) although an average of 48-50h is common and 48 h to 120 h (40^{o}C) for fungal cultures (Lonsane and Ramesh, 1990). In this study the *T. lanuginosus* ATCC 58157 produced high titers of enzyme (212 U/g) after 120 h incubation at 50^{o}C and enzyme yields compared well with other studies (Soni *et al*., 2003; Sodhi *et al*., 2005). α-Amylase production by *Bacillus* sp. in SSF showed that the organism colonized well on the solid substrate and exhibited good growth after 24

h. at 37^{o}C. Maximum enzyme production was achieved after 48 h incubation, and remained constant up to 96 h (Sodhi *et al*., 2005). In another study α-amylase production by an *Aspergillus* sp. colonized well on wheat bran, and maximum enzyme yields were achieved between 48 h and 96 h at 32^{o}C (Soni *et al*., 2003).

Previously, 30°C and 45°C were reported as optimum temperatures for amylase production for *Aspergillus flavus* and *Myceliophora thermophila*, respectively (Mukherjee and Majumdar, 1993; Sadhukhan *et al.,* 1990). In this study the optimal temperature for maximum amylase production (213 U/g) was found to be 50°C. The temperature optimum was 3^{o}C higher than that used by Nguyen *et al*. (2002), for α-amylase production by *T. lanuginosus* ATCC 34626.

The critical importance of moisture level in SSF media and its influence on the biosynthesis and secretion of enzymes can be attributed to the interference of moisture in the physical properties of the solid particles (Babu and Satyanarayana, 1995). An increase in moisture level is believed to reduce the porosity of the wheat bran, thus limiting oxygen transfer and was shown by Babu and Satyanarayana (1995). A marked improvement in enzyme production (3400 U/g) was achieved by optimising moisture content in the medium. When the moisture level was at the ratios of 1:1.5 and 1:2.0 the production peaked at 48 h; at a ratio of 1:2.5 the peak shifted further to 72 h where the highest production was recorded at this stage, 25784 U/g (Babu and Satyanarayana, 1995). Similar results were also demonstrated in this study, where a high enzyme titer (248 U/g) was attained when the initial moisture level was 90%.

A reduction in enzyme production at high initial moisture content may be due to a reduction in substrate porosity, changes in the structure of substrate particles and

reduction of gas volume. In addition, reduction in enzyme production may result from less fungal growth (Babu and Satyanarayana, 1995). Low moisture content also causes a reduction in the solubility of nutrients of the substrate and a low degree of swelling (Freniksova *et al.*, 1960).

The effects of different carbon sources on α-amylase production by *T. lanuginosus* ATCC 58157 revealed that soluble starch induced the highest enzyme titer (338 U/g) followed by sucrose and lactose. In another study the effects of various carbon sources on the production of α-amylase by *T. lanuginosus* ATCC 34626 were studied and the results did not agree with the results from the present study. Although starch is a generally accepted nutritional component for induction of amylolytic enzymes Nguyen *et al.* (2000), found that maltodextran was the best carbon source for α-amylase production. The α-amylase activity was approximately 25% higher than that of the control with starch (Nguyen *et al.*, 2000).

However, in a study reported by Pandey *et al.* (2000), higher cell density and higher specific growth rate were obtained from glucose but higher enzyme activity and higher specific enzyme activity were obtained from starch (Pandey *et al.*, 2000). Earlier workers reported soluble starch as the best carbon supplement for amylase production in *M. thermophila* D14 (Sadhukhan *et al.*, 1990) and *A. fumigatus* (Goto *et al.*, 1998). The results achieved in the present study compared well with similar studies, but the difference in the success rate of other carbon sources could be microbial species or strain dependant.

Previous findings have shown that peptone, sodium nitrate and casein hydrolysate are good nitrogen supplements for amylase production by *A. fumigatus* (Goto *et al.*,

1998), *A. niger* (Pandey *et al.*, 1994) and *A. oryzae* (Pederson and Neilson, 2000). Among the nitrogen sources used in this study, peptone increased amylase production from 211 to 364 U/g and proved to be the best nitrogen source to be used for further studies. In the investigation of the effects of different nitrogen sources on amylolytic enzyme production by Nguyen *et al.* (2000), L-asparagine was found to be the most promising and specifically for α-amylase activity yeast extract was most suitable (Nguyen *et al.*, 2000).

The level of solid substrate is vital in SSF, particularly in tray processes (Babu and Satyanarayana, 1995), and in flask experiments, as this factor together with the nature of substrates, influences porosity and aeration. The highest enzyme production (211 U/g) was observed in this study when the ratio of substrate weight to flask volume was 1:100; which is in agreement with the results reported by other investigators (Satyanarayana, 1994).

Selection of an appropriate solid substrate together with suitable carbon and nitrogen sources and other nutrients are some of the most critical stages in the development of an efficient and economic process. According to the experimental data presented here, *T. lanuginosus* ATCC 58157 could be used for the production of thermostable α-amylase, in a fermentation process using inexpensive nutrients. The production of this enzyme was increased two-fold when the commercial soluble starch of the basal medium in the fermentation was replaced with the more economically available wheat bran and further supplemented with 1% (w/w) soluble starch and 1% (w/w) peptone as the carbon and nitrogen sources respectively.

The promising ability of the strain of *T. lanuginosus* ATCC 58157 to produce high levels of α-amylase was evident. It was clear that certain organic compounds may be necessary for the biosynthesis of this enzyme at higher levels. Although the results of these investigations are based on experiments conducted in Erlenmeyer flasks, they provide valuable information for the production of amylase by SSF on a large-scale and compare well to those results reported by and quoted in this study by other researchers.

Having established the optimum parameters for α-amylase production by *Thermomyces lanuginosus* ATCC 58157 together with its biochemical characterization, the next chapter therefore focused on the purification of the α-amylase to comparatively assess biochemical data.

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CHAPTER FOUR: PURIFICATION AND CHARACTERISATION OF α-AMYLASE BY *T. lanuginosus* ATCC 58157

4.1 Introduction

Industrial enzymes produced in bulk generally require reduced downstream processing and are consequently relatively crude preparations. Commercial application of α-amylase generally does not require purification, but applications in pharmaceutical and clinical sectors require high purity amylases. The enzyme in purified form is also a prerequisite in studies of structure-function relationships and biochemical properties (Gupta *et al.*, 2003). Thus, it is significant to develop economic processes for their purification to obtain chemically pure enzymes with maximum specific activity (Pandey *et al*., 2000).

The purification of α-amylases from microbial sources in most cases has involved classical purification methods. Traditionally the purification of amylases from fermentation media has been done in several steps. These methods involve separation of the culture from the fermentation media, extraction may be required for solid media, followed by centrifugation of the culture, selective concentration by precipitation using ammonium sulphate or organic solvents such as chilled acetone. The crude enzyme is then subjected to chromatography, usually affinity, ion exchange and/or gel filtration techniques are applied (Gupta *et al*., 2003; Pandey *et al*., 2000).

Enzyme separation and purification has become increasingly important due to the evolving application of amylases in industry (Somers *et al.*, 1989). Enzyme preparations containing α-amylase (EC 3.2.1.1) and glucoamylase (EC 3.2.1.3) play an important role in the degradation of starch. They are produced commercially in bulk from micro-organisms (*Bacillus* and *Aspergillus* sp.) and represent about 25–33% of the world enzyme market, in second place after proteases. Their main application is in the production of high glucose syrup (HGS) from starch (Fogarty, 1983) and in the production of high fructose corn syrup (HFCS) (Holló and Hoschke, 1992).

Detailed studies on fungal α-amylase purification have largely been limited to a few species of mesophilic fungi (Pandey *et al.*, 2000), although the growth and amylase production from several thermophilic fungi have been described, extended data concerning their physioco-chemical properties have been published with regard to only a few species, such as *Mucor pusilus* , *Talaromyces emersonii* and *T. lanuginosus* (Jensen and Olsen, 1992; Pandey *et al.*, 2000).

Several research groups have studied *T. lanuginosus* as a potential thermostable enzyme source. The results published thus far indicate that the physico-chemical properties of amylolytic enzymes from *T. lanuginosus* vary among strains. Jensen *et al*. (1988) showed that the α-amylase from strain 1457 had a molecular a mass of 45–57 kDa by SDS-PAGE. Mishra and Maheshwari (1996) found that the α-amylase from *T. lanuginosus* IISc 91 consisted of a 24 kDa protein observed by SDS-PAGE and about 74 kDa by

gelfiltration. This means that strains of *T. lanuginosus* may secrete different forms of amylolytic enzymes.

The work in this chapter focused on the purification and characterization of α-amylase from *T. lanuginosus* ATCC 58157 in order to compare it to the crude form.

4.2. Materials and methods

4.2.1 α-Amylase production

α-Amylase was produced by *T. lanuginosus* ATCC 58157 in SSF as described in section 2.2.4.2. After fermentation the enzyme was extracted by filtration from the medium as described in section 2.2.4.2.

4.2.2 α-Amylase activity

α-Amylase activity was determined in the crude enzyme extract as described in section 2.2.5.

4.2.3 Purification of α-amylase

α-Amylase produced by *T. lanuginosus* ATCC 58157 by SSF was purified by filtration from crude enzyme extracted as described in section 4.2.2. The purification scheme is outlined in Table 4.1. Proteins in the crude enzyme extract were precipitated using 90% saturation of $(NH4)_2SO_4$. The precipitated proteins were collected by centrifugation at 55,220 *g* for 1 h and dissolved in a minimal amount of 30 mM sodium acetate buffer (pH 5.0). The undissolved portion was removed by centrifugation at 7396 *g* for 15 min.

The crude enzyme was loaded onto DEAE Sephadex A-50 columns (2.5 cm × 50 cm) equilibrated with 50 mM sodium acetate buffer pH 5.0 containing 0.2% sodium azide. The bound proteins were eluted with a linear NaCl salt gradient (0-0.5M) in the same buffer, and a flow rate of 1.75 ml/min was applied. The fractions showing α-amylase activities were collected separately and concentrated by ultrafiltration. The α-amylase fractions were applied onto a Bio-Gel P-30 gelfiltration column (2.5 cm × 80 cm) equilibrated with 50 mM sodium acetate buffer pH 5.0 and eluted with a NaCl gradient (0 – 0.1 M), in the same buffer at a flow rate of 1.25 ml/min. The α-amylase fractions were collected, concentrated and dialysed using the Amicon (USA) ultrafiltration units with PM-10 membranes against water before lyophylization. All purification steps were carried out at 4ºC.

4.2.4 SDS-PAGE

The purity of the enzyme was confirmed by SDS-PAGE according to the protocol described by Laemmli (1970). The gel system included a 12% acrylamide resolving gel and a 3% stacking gel. The protein bands were stained with 0.25% Coomassie brilliant blue G-250 (Bio-Rad, USA), in 7% (v/v) acetic acid in 50% (v/v) methanol solution. Destaining was carried out with 7% acetic acid in 50% methanol. Standard protein markers (Sigma, USA) viz., bovine serum albumin (66.2 kDa); ovalbumin (45 kDa); carbonic anhydrase (35 kDa); triose phosphate isomerase (25 kDa) and trypsin inhibitor (18.4 kDa) were used to determine the molecular weight of the purified α-amylase from *T. lanuginosus* ATCC 58157 from the gel.

4.2.5 Effect of pH and temperature on purified α-amylase activity

The optimal temperature of the purified α-amylase activity was determined by assaying the enzyme activity between 50^{o}C - 90^{o}C for 10 min at pH 6.0. The effect of pH on α-amylase activity was performed at 50^{o}C in 50 mM sodium acetate buffers (pH 4 and 5); phosphate–citrate buffers (pH 6 and 7); Tris–HCL buffer (pH 8) for 10 min under standard assay conditions as described in section 2.2.5.

4.2.6 Effect of metal ions and chemical compounds on purified α-amylase activity

The effect of metal ions on the α-amylase activity was determined by adding 5 and 10 mM of each ion ($ZnSO_4$, $CoCl_2$, $MnSO_4$, $FeSO_4$, $BaCl_2$, $CaCl_2$), and chemical compounds (disodium-EDTA and urea) was determined by pre-incubating the enzyme in the presence of the inhibitor/stimulator in equal volumes for 30 min at 50^{o}C. Thereafter, residual enzyme activity was determined as described in section 2.2.5.

4.2.7 Hydrolysis of soluble starch with purified α-amylase

Erlenmeyer flasks (100 ml), containing 10 ml of soluble starch (20%) made up in 0.1 M phosphate buffer (pH 6.0), was incubated with the purified α-amylase from *T. lanuginosus* ATCC 58157 at dose of 0.5 and 1.0 U/mg of starch. The flasks were kept in a constant temperature water bath at 50^{o}C and the extent of liquefaction was determined

at hourly intervals for 8 h, by measuring a decrease in residual starch and an increase in reducing sugar content. Residual starch content was estimated by the iodine method (Fuwa, 1954), as described in section 4.2.8. Glucose formation was assayed by the DNS method (Miller, 1959), as described in section 2.2.5.

4.2.8 Iodine method for residual starch estimation

Starch hydrolysate (1 ml) was mixed with 1 ml of iodine reagent in a total volume of 3 ml (diluted with distilled water). The iodine reagent consisted of 0.02% (w/v) iodine and 0.2% (w/v) KI in 0.5 M HCl. Distilled water (5 ml) was added and the colour that developed was measured at 590 nm in a spectrophotometer. The amount of residual starch was estimated using a standard curve prepared under the same conditions.

4.2.9 Effects of pH and temperature on purified α-amylase stability

To measure pH stability, the enzyme was pre-incubated at 50^{o}C at pH values ranging from 4 – 8 for 4 h. The various pH levels were obtained by the application of different buffer systems as described in section 4.2.5. α-Amylase activity was determined every 30 min as described in section 2.2.5.

Thermostability of the α-amylase was performed for the purified α-amylase by pre-incubating the α-amylase at optimum pH 6.0 for 4 h, at temperatures ranging between 50 and 90^{o}C. α-Amylase activity was determined every 30 min as described in section 2.2.5.

4.3 Results

4.3.1 Purification of α-amylase

The results of the purification process are summarized in Table 4.1. The α-amylase was purified 16-fold with a yield of 29% to a specific activity of 3284 U mg^{-1} of protein. The α-amylase was purified to SDS-PAGE homogeneity by fractional ammonium sulphate precipitation, ion-exchange chromatography on DEAE-Sephadex A-50 and gel filtration on Bio-Gel P-30. The enzyme showed two bands after ion exchange chromatography and one band after the gel filtration chromatography. The ion-exchange chromatography fractions and gel filtration fraction profiles are shown in Figs. 4.2 and 4.3 respectively.

4.3.2 Molecular weight of α-amylase from *T. lanuginosus* ATCC 58157

The molecular weight of the α-amylase was estimated to be 61 kDa by gel filtration on a Sephadex A-50 column (Fig.4.1). Electrophoresis of the enzyme on SDS-PAGE gave a single band of protein with a molecular weight of 61 kDa. The results showed that the α-amylase from *T. lanuginosus* ATCC 58157 consisted of a single polypeptide.

Table: 4.1 Summarised purification steps and purification results for α-amylase from *T. lanuginosus* ATCC 58157

Purification steps	Total enzyme activity (U)	Total protein (mg)	Specific enzyme activity (U/mg)	Purification fold	Recovery (%)
Fermentation supernatant	97500	468	201	1.0	100
90%$(NH_4)_2SO_4$ precipitation, dialysed	85300	332	246	1.0	87
DEAE-Sephadex A-50	41031	135	319	2.0	42
Bio-Gel P-30	28170	9	3284	16.0	29

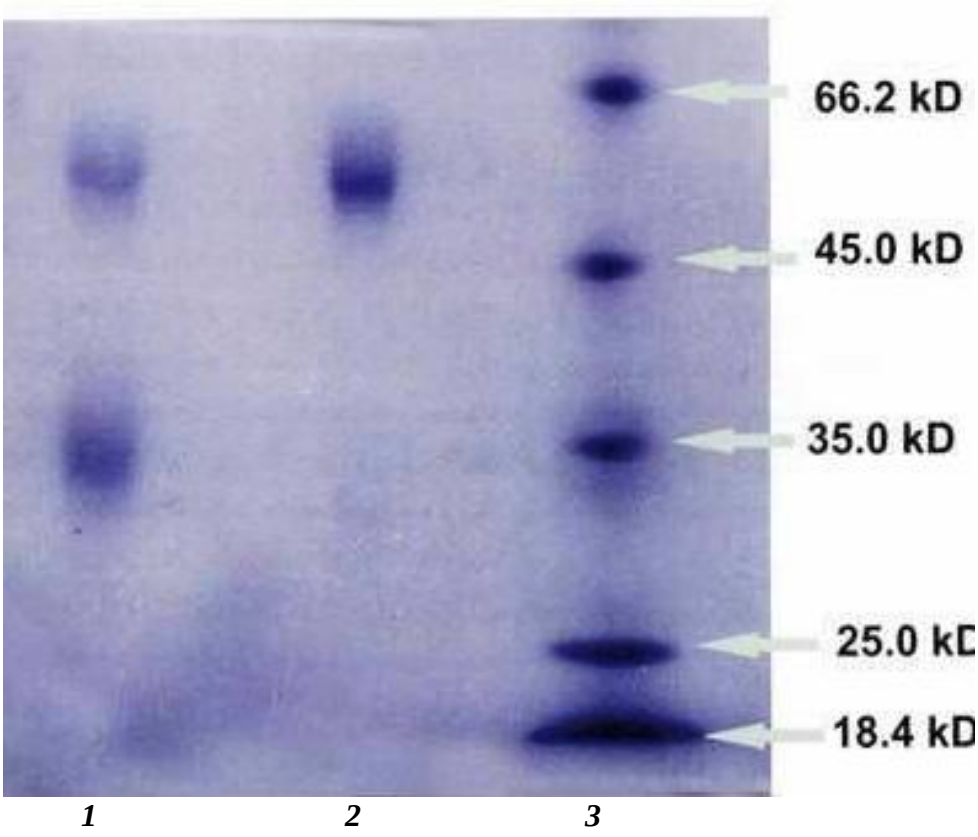

Figure 4.1 **SDS-PAGE of the purified α-amylase from *T. lanuginosus* ATCC 58157.** Lane 1: ion exchange fraction with 2 protein bands, Lane 2: gel filtration fraction with one protein band and Lane 3: molecular weight markers.

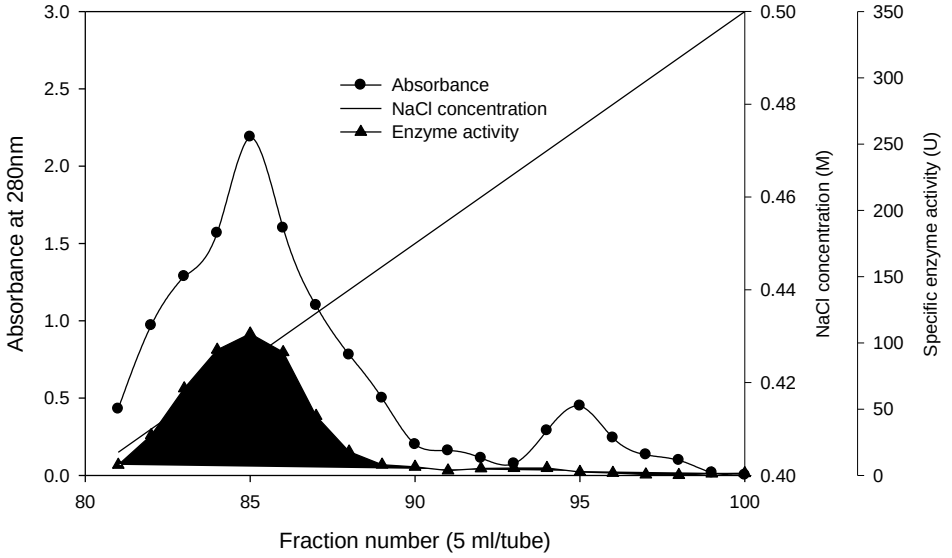

Figure 4.2 Ion-exchange elution profile of α-amylase from *T. lanuginosus* ATCC 58157

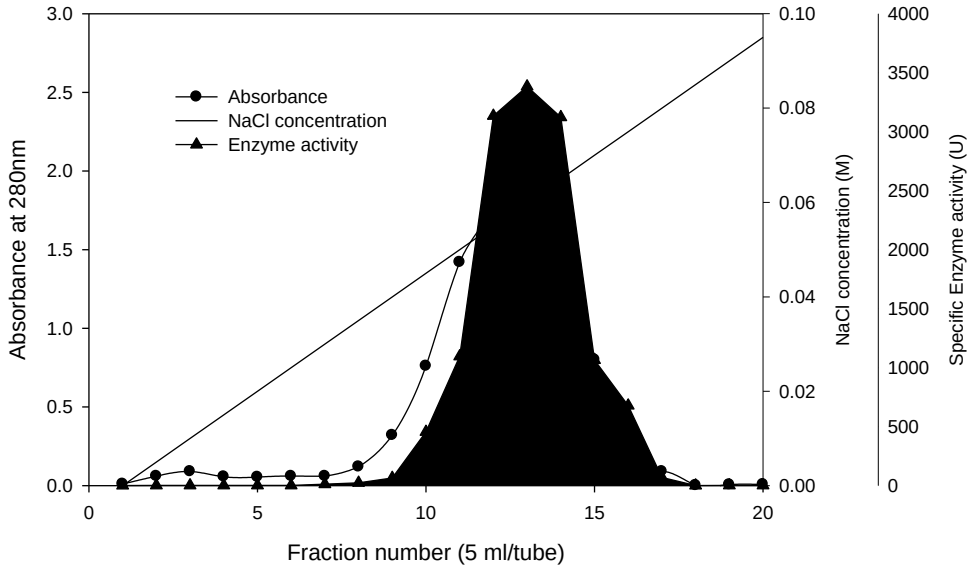

Figure 4.3 Gel filtration elution profiles of α-amylase from *T. lanuginosus* ATCC 58157

4.3.3 Effect of pH and temperature on enzyme activities

The optimum pH for α-amylase activity was established at pH 6 and the α-amylase activity decreased rapidly at pH levels higher than pH 6 (Fig. 4.4). The optimal temperature of the purified α-amylase was established at 70ºC and the α-amylase activity decreased rapidly at temperatures higher than 70°C (Fig. 4.4).

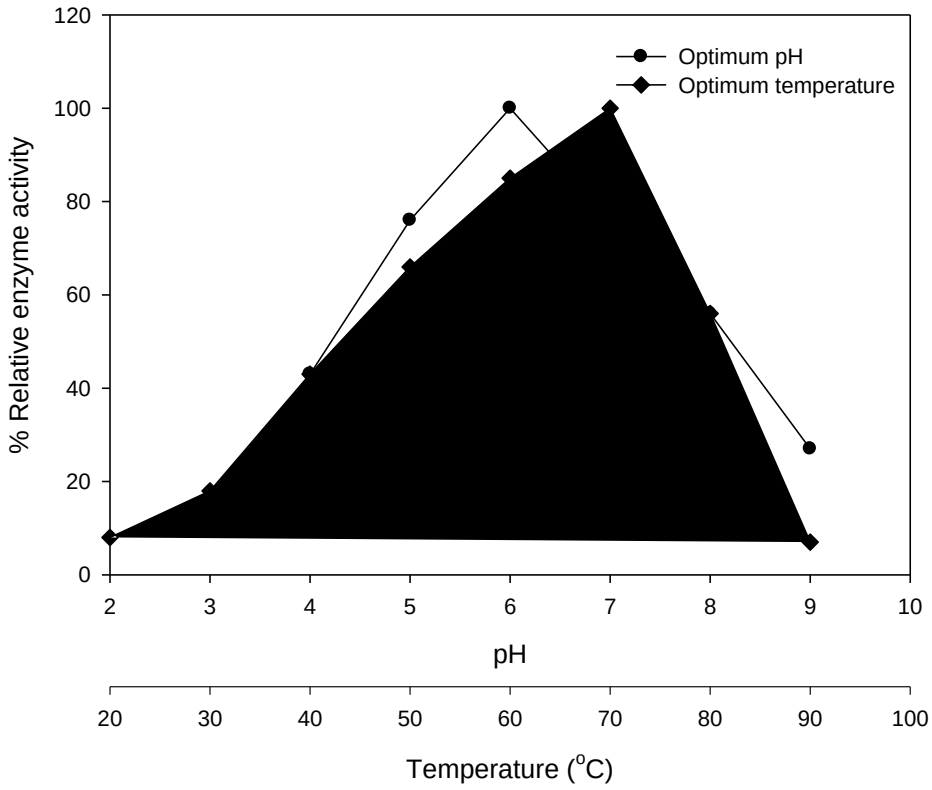

Figure 4.4 Effect of pH and temperature on activity of the purified α-amylase from *T. lanuginosus* after 10 min of exposure.

4.3.4 Effect of metal ions and chemical compounds on α-amylase activity

The effect of several metal ions and chemical compounds on α-amylase activity was measured at pH 6 and at 70^{o}C with soluble starch as the substrate in the presence of the various test substances (Table 4.2). The results showed that $MgSO_4$, $CoCl_2$ and $FeCl_3$ marginally inhibited the activity of the α-amylase, where as $ZnSO_4$ drastically inhibited the activity of the enzyme. $CaCl_2$ and $BaCl_2$ had a stimulatory effect on the α-amylase activity. EDTA and urea had minimal effect on α-amylase activity (Table. 4.2).

4.3.5 Effect of pH and temperature on purified α-amylase stability

During the thermostability assay the purified α-amylase retained 56% activity after exposure of the enzyme at 50^{o}C for 2 h and after 4 h of exposure at 50^{o}C the enzyme activity decreased to 34%. At 60^{o}C the enzyme exhibited 49% activity after an exposure time of half hour and dropped rapidly to an enzyme activity level of 1.5% after an exposure time of 3 h at 60^{o}C. At 70^{o}C, 80^{o}C and 90^{o}C the enzyme activity was denatured almost completely within the first half hour of exposure at the respective temperatures (Fig. 4.5).

The pH optima and pH stability profile were both between pH 5.0 and 6.0. The enzyme remained stable for the first 2 hours of exposure, with a 12% loss in activity after 30 min of exposure, to a final loss of 99% activity after 4 h exposure at pH 6.0. At pH 4 and pH 8 the enzyme displayed no activity after the first 30 min of exposure at the respective pH

values. At pH 5 the enzyme was 83% active after the first hour of exposure and decreased activity by 53% after 4h of exposure. At pH 7 the enzyme activity decreased by 66% after the first hour of exposure (Fig. 4.6).

Table: 4.2. The effect of metal ions and chemical compounds on purified α-amylase activity

Chemicals	Relative enzyme activity (%)
$CaCl_2$	134
$BaCl_2$	112
Urea	109
EDTA	103
$MnCl_2$	100
NaCl	100
$FeCl_3$	99
$MgSO_4$	95
$COCl_2$	92
$ZnSO_4$	24
Control	100

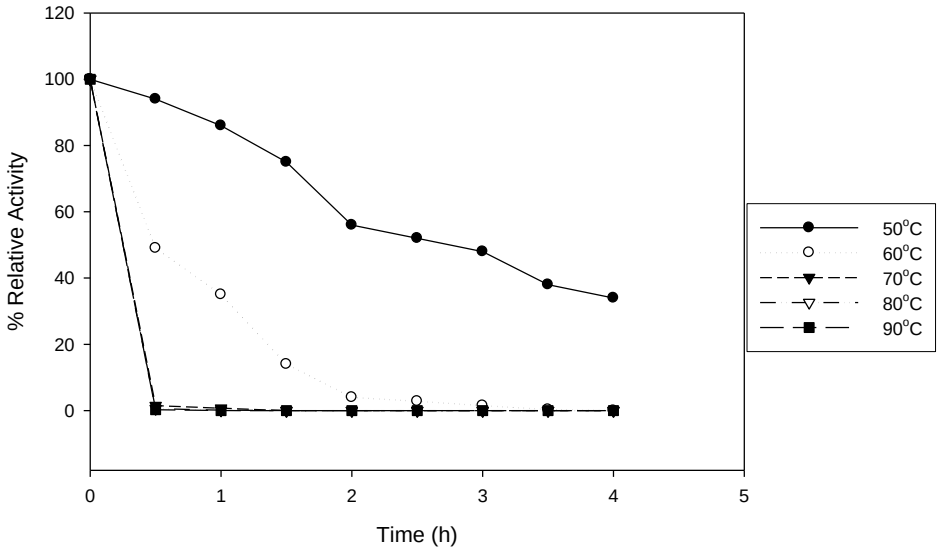

Figure 4.5 Thermostability profiles of purified α-amylase from *T. lanuginosus* ATCC 58157 at pH 6.0.

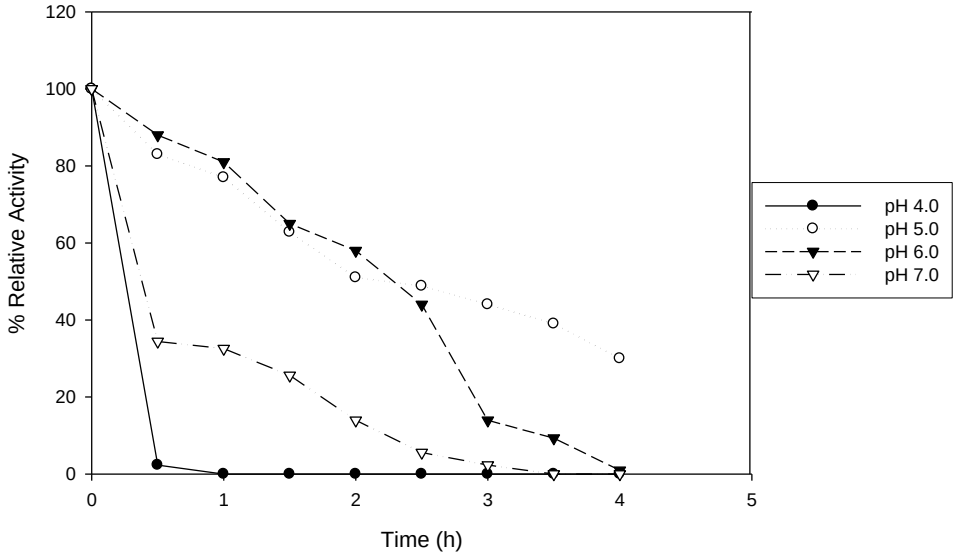

Figure 4.6 pH stability profiles of purified α-amylase from *T. lanuginosus* ATCC 58157 at 50°C.

4.3.6 Hydrolysis of soluble starch with purified α-amylase from *T. lanuginosus* ATCC 58157

The potential usefulness of the fungal α-amylase from *T. lanuginosus* in the hydrolysis of a 20% soluble starch slurry was studied, using two different concentrations of enzyme doses at 50^{o}C. The pattern of starch degradation and glucose formation along with the corresponding liquefaction with α-amylase are depicted in Fig. 4.7 and Fig. 4.8 The breakdown of starch, in the presence of fungal α-amylase, commenced immediately and continued with the hydrolysis of over 95% starch after 30 min of incubation where the level dropped from 200 to 4.4 mg/ml (at 0.5 U of enzyme/mg starch) and to 1.4 mg/ml (at 1.0 U of enzyme/mg starch). The starch remaining after 8 h incubation was 3.1 mg/ml (at 0.5 U/mg starch) and 1.0 mg/ml (at 1.0 U/mg starch) indicating overall liquefaction efficiencies of 98 and 99%, respectively, (Fig. 4.7 and Fig. 4.8). The rate of glucose formation was slow during the first 30 min of incubation and increased gradually with incubation time attaining final yields of 35 and 38 mg/ml (Fig.4.7 and Fig. 4.8) in the presence of 0.5 and 1.0 U/mg starch, respectively, after 8 h of incubation.

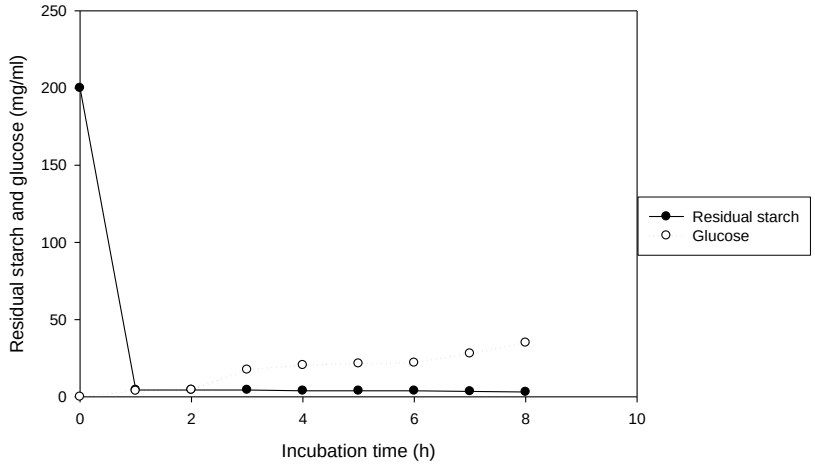

Figure 4.7 Pattern of starch disappearance and glucose formation during hydrolysis of 20% soluble starch using purified α-amylase (0.5 U/mg) from *T. lanuginosus* ATCC 58157.

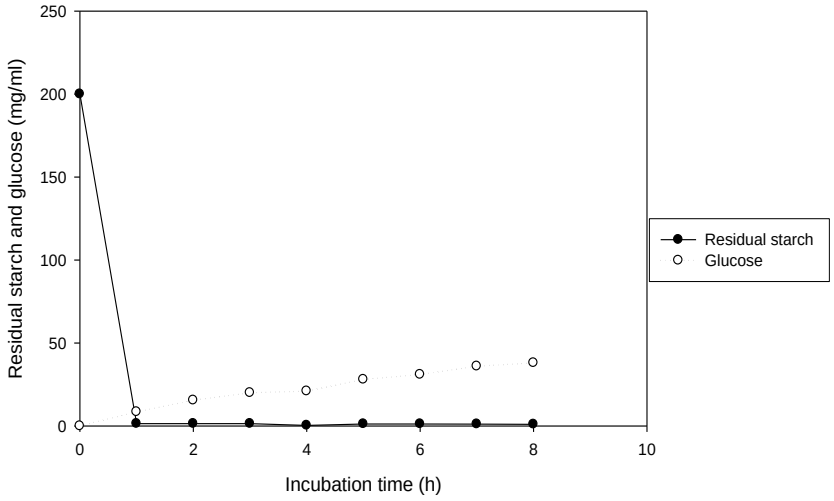

Figure 4.8 Pattern of starch disappearance and glucose formation during hydrolysis of 20% soluble starch using purified α-amylase (0.1 U/mg) from *T. lanuginosus* ATCC 58157.

4.4 Discussion

The enzymatic and physiochemical properties of α-amylase from several microorganisms have been extensively studied and described (Pandey *et al*., 2000; Fogarty and Kelly, 1979; Vihinen and Mantsala, 1989; Bohdziewicz, 1996). Although the growth and amylase production from several thermophilic fungi have been described, extended data concerning their physico-chemical properties have been published with regard to a few species viz., *M. pusilus*, *T. emersonii*, *T. lanuginosus*, *A. oryzae* and *A. awamori* (Pandey *et al.*, 2000).

The findings presented in this chapter outline the purification and characterization of α-amylase from *T. lanuginosus* ATCC 58157 by SDS-PAGE and gel filtration. In this study a single protein species was visible on SDS-PAGE stained with 0.25% Coomassie brilliant blue G-250, the enzyme was purified to apparent homogeneity. The results identified a protein band corresponding to an estimated molecular weight of 61 kDa. The results obtained in this study agree closely with those reported in other studies where α-amylase was purified from other strains of *T. lanuginosus* (Obido and Ulbrich-Hofmann, 2001; Li *et al*., 1998; Jensen *et al*., 1988).

Amylolytic enzymes from *T. lanuginosus* strains 1457 (Denmark) and ATCC 34626 have been successfully purified in previous studies. The purified glucoamylase and α-amylase were reported to have molecular weights of 70 - 76 kDa and 54 -57 kDa respectively (Ngyuen *et al*., 2002; Jensen *et al*., 1988). A purified α-amylase from *T. lanuginosus* IISc

91 (India) was reported to have a molecular weight of 42 kDa (Mishra and Maheshwari, 1996). The molecular weights of α-amylase reported in these studies confirm that the same enzyme from different species of the organism displayed different molecular weights.

The ion exchange fraction displayed two protein bands as compared to the gel filtration where only one protein band was visible. The first protein band had a corresponding estimated molecular weight of 35 kDa and it is most likely to be a different form of α-amylase. A similar result was reported previously where an α-amylase from a *T. lanuginosus* strain displayed different molecular weights by SDS-PAGE (24 kDa) and gel filtration (74 kDa). This could infer that two different forms of the same enzyme were present (Mishra and Maheshwari, 1996).

α-Amylase from *T. lanuginosus* 58157 was optimally active at 70^{o}C and displayed 69%, 84% and 60% of its peak activity at 50^{o}C, 60^{o}C and 80^{o}C respectively. In the present study the thermalstability temperature of 50^{o}C was lower than the optimum temperature of 70^{o}C. This was of concern, since starch liquefaction is generally carried out at high temperatures of between 70^{o}C - 90^{o}C, and the thermostability of α-amylase is of great significance for the efficient liquefaction of gelatinized starch. α-Amylase from a *Bacillus* sp. was reported to have a different optimum temperature 70^{o}C and thermal stability temperatures than 60^{o}C (Soni *et al*., 2003). In addition the amylase from *T. lanuginosus* 34626 displayed 70^{o}C and 55^{o}C for optimum temperature and thermal stability characteristics (Ngyuen *et al*., 2002). Therefore, it can be concluded that both

bacterial and fungal α-amylases are known to display varied optimum and thermal stability temperatures.

The relative importance of environmental factors (pH) affecting the stability of the α-amylase from *T. lanuginosus* 58157 were tested and an optimum pH of 6 and with a pH stability of 5. This was supported by other reported studies with different enzyme produced from other strains of *T. lanuginosus. T. lanuginosus* F1 and 34626 was reported to have pH stabilities ranging between 5 – 7 (Odibo and Ulbrich-Hofmann 2001; Nguyen *et al*., 2000). It was further shown that α-amylases have optimum pH and pH stability ranging from acid to alkaline, and that the enzyme has been reported to display the highest activity between pH ranges 5 and 6.

The effect of various chemical compounds and metal ions on the activity of α-amylase from *T. lanuginosus* ATCC 58157 was investigated. The results showed that $CaCl_2$ and $BaCl_2$ had stimulatory effects on the α-amylase activity and $CoCl_2$ had an inhibitory effect. To establish the enzyme characteristics with regard to stability and increased activity is vital for industrial application purpose. Stability of the enzyme to processing parameters and hence the resistance of the enzyme to be affected negatively in the presence of metal ions and other chemical compounds, suggests that the enzyme may be used as an effective additive in detergents as well as in other industrial applications (Burhan *et al*., (2003). The role of Ca^{2+} ion was investigated by Mishra and Maheshwari (1996) and Ronaszeki *et al.* (2000), and it was shown that Ca^{2+} had a positive effect on enzyme activity.

In similar studies for other strains of *T. lanuginosus* the stimulatory effect of Ca^{2+} and Ba^{2+} and the inhibitory effect of CO^{2+} were also reported (Nguyen *et al*., 2000; Lin *et al.*, 1998). An eight fold improvement of enzyme activity was reported for Ca^{2+} for an α-amylase from *T. lanuginosus* IISc 91 (Mishra and Maheshwari, 1996). It was also reported that in the presence of Ca^{2+} ions the thermostability of other amylolytic enzymes, including the amylases from *B. licheniformis, P. furiosus* and *T. litoralis* are improved (Lin *et al.*, 1998). This phenomenon was confirmed for α-amylase from other organisms (Pandey *et al.*, 2000). The reasons for these effects are not clearly understood, although metal ions often act as salt or ion bridges between two adjacent amino acid residues, resulting in the stimulatory effect that Ca^{2+} ions have on enzyme activity (Lin *et al.*, 1998).

α-Amylase from *T. lanuginosus* from ATCC 58157, exhibited a liquefying efficiency of 98% and 99% respectively for the two enzyme doses used, showing the potential use of the enzyme for the hydrolysis of starchy biomass with a starch concentration of 20%. These results compared well with a similar study by Soni *et al.* (2003) where a bacterial α-amylase exhibited a liquefying efficiency of 98% in 15% starch solution at 50^{o}C.

The potential advantage of using thermostable α-amylases in starch biotechnology industry is evident. Although many species of thermophilic fungi produce and secrete α-amylases only the α-amylase from *T. lanuginosus* have been characterized in detail (Maheshwari *et al*., 2000; Nguyen *et al*., 2002; Mishra and Maheshwari, 1996).

Having established the characteristics of the enzyme, the subsequent chapter addresses the application of the enzyme in the hydrolysis of maize mashes.

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CHAPTER FIVE: THE APPLICATION OF α-AMYLASE FROM *T. lanuginosus* ATCC 58157 FOR THE HYDROLYSIS OF MAIZE STARCH

5.1 Introduction

In Africa, cereal sourdoughs are frequently used to prepare complementary foods for young children, which unfortunately have low energy and nutrient density (Lorri and Svanberg, 1993). Amongst the different technological methods available to reduce the dietary bulk and thus to improve the nutritional characteristics of complementary foods, α-amylase can be used to partially hydrolyse starch.

Fermentation of cereals for a limited period of time improves amino acid composition and vitamin content, increases protein and starch availabilities, and lowers the level of antinutrients (Chavan and Kadam, 1989). Dietary starch varies greatly in digestibility and its effect on the utilization of other nutrients. The variation appears to be due to differences in starch components and their crystallinity. Processing treatments, storage conditions, and chemical modification influence the digestibility of starch and the poor digestibility of starch may have negative effects on the availability of certain vitamins (Dreher *et al*., 1984).

There is general agreement that cooking significantly reduces the protein digestibility of some cereals, viz., sorghum and maize (Duodu *et al*., 2002). The reasons for this are not well understood. It has been proposed by Hamaker *et al*., 1987 and Hamaker *et al*., 1986 that maize prolamins (maize proteins) may form polymeric units linked by intermolecular disulphide bonds during cooking which may be less susceptible to digestion.

The conventional method of starch hydrolysis using acid has been replaced by processes using starch liquefying and saccharifying enzymes. Usually a combination of different amylases has been used for this purpose (Soni *et al.,* 2003). α-Amylase is known to attack both insoluble starch and starch granules held in aqueous suspension (Apar and Özbek, 2003). The action pattern of various amylases on granular starches has been previously investigated, with particular attention being given to the influence of amylases. Significant degradation of native starch granules has been found with amylases from only a few microbial strains, however, most of these studies have been conducted using glucoamylases (Planchot *et al*., 1994).

In this chapter, the effects of the purified α-amylase from *T. lanuginosus* ATCC 58157 on maize hydrolysis were investigated. The digestion process was optimised using various parameters and the nutritional improvement of the maize meal was evaluated. In light of the hydrolytic effect of α-amylase on starch granules, the level of starch degradation was also observed using scanning electron microscopy (SEM).

5.2 Materials and methods

5.2.1 Application of purified α-amylase from *T. lanuginosus* ATCC 58157 in the hydrolysis of maize meal

A modified method described by Soni *et al.* (2003), was used to hydrolyse maize starch with purified α-amylase from *T. lanuginosus* ATCC 58157. Maize meal (100 g) with a particle size of 20-25 µm was dispersed in 400 ml of tap water with constant stirring in a 1 litre Erlenmeyer flask. α-Amylase (1.0 U/mg) was added to the maize suspension and incubated in a water bath at 70^{o}C for 30 min. The pre-digested maize suspension was cooked at 105^{o}C for 30 min. The gelatinized maize starch was cooled to 50^{o}C and 2 U/mg α-amylase was added. The gelatinized maize starch was held at 60^{o}C for 30 min for starch liquefaction. The hydrolysed maize starch was cooled to room temperature, centrifuged at 10 000 rpm for 15 min and the supernatant was stored at 4^{o}C for further analysis. The α-amylase assay, as described in section 2.2.5, was used to determine the reducing sugars. All experiments were done in triplicate.

5.2.2 Optimisation of hydrolysis of maize meal mash with purified α-amylase from *T. lanuginosus* ATCC 58157

Optimisation studies were carried out by studying the effect of various parameters including enzyme dose, total solid concentration, cooking time, pH of mashes,

supplementation with Ca^{2+} and Mg^{2+}, as described in section 5.2.1. The optimization variables are shown in Table 5.1.

The conversion efficiency of mashing was calculated as follows:

$$\frac{\textit{reducing sugars produced in maize meal after enzyme hydrolysis}}{\textit{reducing sugars obtained from starch in maize meal before enzyme hydrolysis}} \textbf{ X } \textbf{100}$$

Table: 5.1 Optimisation variables for enzymatic hydrolysis of maize meal with α-amylase from *T. lanuginosus* ATCC 58157

Parameters	
Pre-cooking α-amylase dose (U/mg solids)	**Post-cooking α-amylase dose (U/mg solids)**
0.5	1.0
1.0	1.0
2.0	1.0
1.0	0.5
1.0	2.0
Quantity of maize meal (g)	**Total solids in mash (%)**
50	10
75	15
100	20
Cooking period at 105^{o}C (min)	
	30
	60
	90
pH of liquefaction	
6.0	
7.0	
7.2	
Concentration of $CaCl_2.2H_2O$ in the maize mash (mM)	
Nil	
1	
5	
10	
Temperature ^{o}C of liquefaction	
37	
45	
60	
Concentration of $MgCl_2.2H_2O$ in the maize mash (mM)	
Nil	
1	
5	
10	
Mashing conditions	
Control (un-optimised)	
Optimised	

5.2.3 Hydrolysis of maize meal under optimised conditions with α-amylase from *T. lanuginosus* ATCC 58157

Mashing of maize meal was carried out by applying the optimised conditions as determined in Table 5.1. Maize meal (100 g) was homogenised in tap water (400 ml), supplemented with Ca^{2+} and Mg^{2+} (1 mM), with a magnetic stirrer. For the pre-liquefaction stage an α-amylase dose of 1.0 U/mg solids was added with cooking at 105^{o}C for 30 min. After gelatinization a second dose of α-amylase (2.0 U/mg) was added and the flasks were incubated in a water bath at 60^{o}C for a further 30 min. The method followed for the hydrolysis was as described in section 5.2.1

5.2.4 Scanning electron microscopy

The extent of starch hydrolysis at an ultrastructural level was observed by SEM. The following samples were analysed viz., undigested maize mash (control); enzyme digested maize mash and raw maize meal (negative control).

The samples were first freeze dried at -40^{o}C and then mounted onto specimen stubs with double-sided carbon-conductive adhesive tape and covered with a 10 nm layer of gold-palladium sputter coating using a Polaron E5100 sputter coater. Samples were then viewed with a Philips XL 30 ESEM, at an accelerating voltage of 15 keV. Surface roughening was enhanced by amplifying the video image signal.

5.2.5 Nutritional analysis of hydrolysed maize meal with α-amylase from *T. lanuginosus* ATCC 58157

5.2.5.1 Protein determination

Protein in hydrolysed maize mash supernatants were analysed according to the method of Lowry *et al*. (1951).

5.2.5.2 Carbohydrate (CHO) analysis using thin layer chromatography (TLC)

A preliminary assay identifying sugars present in digested and undigested maize meal, was carried out using a TLC method described by Aquino *et al.* (2003). Eleven sugar standards (glucose (D), arabinose, xylose, ribose, fructose, galactose, lactose, sucrose, mannose, maltose and β-D-glucose) (0.1% $^{w}/_{v}$) were selected as the most likely sugars found in cereals.

Silica gel aluminium plates (Merck) were spotted with 2 µl of each of the sugar standard, the supernatant of the digested and undigested samples. The plates were air dried and then placed in TLC tanks which had previously been equilibrated with the solvent system. The sugars were separated using a butanol : ethanol : water (5:3:2 $^{v}/_{v}$) solvent system for 5 h (Aquino *et al*., 2003). Thereafter the TLC plates were air dried for approximately 1 h.

A fresh stock of Anillin (Merck) spray reagent was prepared. (Appendix 2) TLC plates were evenly sprayed with the Anillin reagent, air dried and placed in a drying oven at 105^{o}C for 1 h to develop.

5.2.5.3 Quantification of carbohydrates in digested and undigested maize meal using high performance liquid chromatography (HPLC)

5.2.5.3.1 Reagents and standards

Carbohydrate content in the form of sugars, were determined according to Chávez-Serin *et al.* (2004). All chemicals used for HPLC were of analytical grade: acetonitrile (Merck), and double deionised water was purified through a Milli-Q system (Millipore, Bedford , MA, USA). Standard sugars viz., maltose, glucose, maltotriose and sucrose - (Sigma-Aldrich, Germany) were >99% pure and were stored at 4^{o}C in a vacuum desiccator, with silica gel as a desiccant.

5.2.5.3.2 Sample preparation

The supernatants collected from the optimisation study (section 5.2.2) and the digested maize meal mashes under optimised conditions (section 5.2.3) were used for analyses. Undigested maize meal served as the control, and sugar standards were used to identify liberated sugars.

Supernatants from optimization studies described in section 5.2.2 were analysed. Each sample (1 ml) was mixed with acetonitrile (1 ml) and filtered through a 0.22 µm Millipore filter before HPLC analyses. Sugar standards were prepared by dissolving 0.02g of each of the standard sugar in 5 ml double deionised water. Each of the sugar standards (1 ml) were mixed with 1 ml of acetonitrile and filtered through a 0.22 µm Millipore filter before HPLC analyses.

5.2.5.3.3 HPLC conditions

Column	:	100 NH_2 (5 µm) LiChroCart® 250-4LiChrospher® (Merck, Germany)
Mobile phase	:	acetonitrile : water (78:22 $^{v}/_{v}$)
Flow rate	:	1 ml/min
Injection volume	:	20 µl
Detection	:	RI detector
Temperature	:	30^{o}C
Run Time	:	30 min
Samples	:	maltose, glucose, maltotriose, sucrose. (0.5 – 10 mg/ml – dissolved in double de-ionised water)

5.2.5.4 Identification and quantitative analysis of water soluble vitamins using HPLC

5.2.5.4.1 Reagents and standards

Water soluble vitamins were determined according to the method of La-Chrome (Merck, 2005). Chemicals used for HPLC were of analytical grade i.e., acetonitrile (Merck), Phosphoric acid (Merck), 1-Hexanesulfonic acid, sodium salt (SHS), and double deionised water purified through a Milli-Q system (Millipore, Bedford, MA, USA). Vitamin standards viz., niacin, folic acid, thiamine, pyrodixine and riboflavin - (Sigma-Aldrich, Germany) were >99% pure and were stored at 4^{o}C in a vacuum desiccator, with silica gel as desiccant. Vitamin standards were prepared by dissolving 1 mg of each vitamin in 10 ml (100 µg/ml) double deionised water, and thereafter the standards were treated as described in section 5.2.5.3.2. Vitamin A (325 000U/g; Sigma – Aldrich, Germany) was prepared by dissolving 0.02 g in 5 ml methanol and filtered through a Millipore filter (0.22 µm) before HPLC analyses.

5.2.5.4.2 Sample preparation

Samples were collected and prepared as described in section 5.2.5.3.2. Undigested maize meal served as the control, and the vitamin standards were used to identify the vitamins.

5.2.5.4.3 HPLC conditions

Column	:	C18 column (250 mm x 4µm) LiChrosphere (Merck, Germany)
Mobile phase	:	1-Hexanesulfonis acid sodium salt + H_2O : acetonitrile ($90 : 10\ ^{v}/_{v}$)
Flow rate	:	1 ml/min
Injection volume	:	5 µl
Detection	:	210 nm - 0.16AUFS UV detector
Temperature	:	$40^{o}C$
Run time	:	25 min
Sample	:	niacin, folic, thiamine, pyridoxine, riboflavin. (0.5 – 10 mg/ml – dissolved in double de-ionised water)

5.2.5.5 Fat soluble vitamin A reagents and standard

5.2.5.5.1 Sample preparation

Vitamin A content was determined according to the method of La-Chrome (Merck, 2005). Samples were prepared as described in section 5.2.5.3.2.

5.2.5.5.2 HPLC conditions

Column	:	C18 column (250 mm x 4µm) LiChrosphere (Merck, Germany)
Mobile phase	:	methanol : water ($90 : 10\ ^{v}/_{v}$)
Flow rate	:	1.2 ml/min
Injection volume	:	22 µl
Detection	:	210 nm - 0.16AUFS UV detector
Temperature	:	ambient
Run time	:	7 min
Sample	:	vitamin A (0.02 g – dissolved in 5 ml methanol)

5.3 Results

5.3.1 Optimisation of hydrolysis of maize meal mash

The α-amylase from *T. lanuginosus* ATCC 58157 was evaluated for its ability to hydrolyze maize meal. The enzyme system produced overall sugar yields of 24, 36, 35 and 11 mg/ml for glucose, maltose, maltotriose and sucrose, respectively. Attempts were made to improve the performance of the laboratory enzyme system by optimizing the reaction parameters with a view to generate more sugars and consequently increase the overall sugar yields.

The maximum sugar yields obtained in relation to the enzyme dose was achieved when the α-amylase dose of 1.0 U/mg solids and 2.0 U/mg solids were applied at the pre- and post-cooking stages of mashing respectively (Table 5.2).

An increase in total sugars liberated as a result of hydrolysis was directly proportional to an increase in total solids. Sugar yields obtained with 10, 15 and 20% total solids were 14, 8, 12 and 18 mg/ml for glucose, maltose, maltotriose and sucrose, respectively (Table 5.2). The similarities in the conversion efficiencies suggest that the enzyme preparation from *T. lanuginosus* ATCC 58157 can be effectively used to hydrolyze maize mashes containing total solids concentrations ranging from 10 to 20%.

A cooking time of 30 min (105°C) was sufficient for gelatinization of the maize starch. Variation in cooking time had no effect on gelatinization or sugar yields of the mash (Table 5.2). Maximum sugar yields were obtained at an optimum pH of 6.0 (Table 5.2). When comparing the sugar yields achieved at optimum pH, the sugar yields at pH 7 and 7.2 were 12% and 6% lower respectively.

Supplementation of maize mash with divalent metal ions ($CaCl_2.2H_2O$ and $MgCl_2.2H_2O$ 1 mM) improved starch hydrolysis and therefore an increase in sugar yields, could be attributed to the known stimulatory effects of Ca^{+2} and Mg^{+2} compounds on amylase activity (Table 5.2). The optimum liquefaction temperature at which the highest sugar yields were obtained was 60°C (Table 5.2), 10^{o}C lower than the temperature optimum of α-amylase activity from *T. lanuginosus* ATCC 58157.

5.3.1.1 Maize mashing using optimised conditions

The optimised conditions achieved for maize mashing were, α-amylase dose pre and post cooking (1.0 and 2.0 U/mg); total solids (20%); cooking time (30 min); mashing pH (6.0); liquefaction temperature 60^{O}C and Ca^{+2} and Mg^{+2} (1 mM). Maximum sugar yields achieved for the optimised maize mash was, 23.86; 35.64; 34.47 and 10.82 (mg/ml) for glucose, maltose, maltotriose and sucrose respectively.

Table: 5.2 Optimisation of enzymatic hydrolysis of maize mashes with α-amylase from *T. lanuginosus* ATCC 58157

Parameters						
Pre-cooking α-amylase dose (U/mg solids)	**Post-cooking α-amylase dose (U/mg solids)**	**Sugars (mg/ml)**				**Protein (µg/ml)**
		Glucose	**Maltose**	**Maltotriose**	**Sucrose**	
1	5	19.24	24.57	35.04	10.90	576
5	1	19.88	25.42	25.75	7.98	600
5	10	24.73	32.54	29.59	21.75	680
10	5	27.46	37.59	29.10	21.97	882
1	10	22.74	30.70	34.67	11.14	616
0.5	1	14.25	20.84	20.72	Nil	480
1	1	16.17	19.16	23.75	3.59	528
2	1	16.89	19.00	24.13	2.73	564
1	0.5	14.68	17.48	25.80	5.23	520
1	**2**	**17.01**	**20.06**	**26.46**	**6.22**	**552**
pH						
6.0		**17.85**	**16.71**	**24.17**	**4.29**	**456**
7.0		15.86	15.04	20.77	4.27	528
7.2		16.59	16.17	22.47	4.93	552
Time (min)						
30		**17.85**	**20.6**	**26.46**	**6.22**	**552**
60		10.91	17.48	22.48	5.0	560
90		9.97	16.31	22.00	2.53	600
Concentration of $CaCl_2$ $2H_2O$ (mM)						
Nil		5.74	9.22	13.45	0.89	448
1		7.9	13.43	18.79	3.47	464
5		6.6	7.88	11.06	1.61	464
10		**9.99**	**10.54**	**14.41**	**2.54**	**484**
15		7.04	7.49	10.69	1.11	480
20		12.97	13.46	19.73	3.58	504
Concentration of $MgCl_2$ $2H_2O$ (mM)						
Nil		5.5	9.0	12.83	0.83	484
1		6.0	10.0	12.00	3.21	460
5		**6.85**	**8.67**	**10.87**	**2.18**	**488**
10		5.97	6.81	9.14	1.50	488
15		6.05	7.4	9.46	1.80	496
20		**12.97**	**13.46**	**13.02**	**2.90**	504
Temperature (^oC)						
37		6.49	11.19	15.57	3.27	484
45		8.13	14.89	20.72	2.42	480
60		9.66	17.02	23.46	3.70	488
Quantity of Maize Meal (g)						
50		6.57	3.40	6.23	9.06	512
75		10.73	6.23	9.34	15.57	518
100		13.69	8.54	12.59	18.46	562
Control (un-optimised)		0.12	0.49	Nil	1.3	320
Optimised		**23.86**	**35.64**	**34.47**	**10.82**	**558**

5.3.2 Estimation of protein content

The protein content in the hydolysed maize mash was determined for the various optimised process parameters. Protein contents of 320 µg/ml and 558 µg/ml was obtained for the undigested maize mash (control) and the optimised maize mash, respectively. A nett increase of 174% of available protein was recorded in the optimised maize mash.

When analysing the independent results for enzyme dose variables, an enzyme dose of 10 and 5 U/mg solids for pre and post digestion respectively resulted in the highest yield of available protein in the maize mash, (279%).

5.3.3 Ultrastructure of raw maize meal, undigested maize meal and maize meal digested with α-amylase from *T. lanuginosus* ATCC 58157

SEM was used to show the different states of degradation of the enzyme digested, undigested and raw maize meal. Electron micrographs of raw maize meal contained fragments of pericarp layers and germ as well as endosperm. The raw maize meal particles range in size from large groups of unbroken cells from the vitreous (v), protein-rich endosperm, and from floury (f) endosperm, as well as smaller sub-cellular fragments consisting of starch and protein bodies (Fig. 5.1).

In contrast the electron micrographs of the undigested mashed maize meal at 200x magnification, showed that the maize starch was composed of smaller, irregularly shaped

fragments with smooth surfaces and depressions. The larger granules at 1200x magnification showed irregular shapes with rough surfaces, with depressions clearly visable (Fig. 5.2).

The electron micrographs of the digested sample clearly showed the effect of the α-amylase from *T. lanuginosus* ATCC 58157 on the granular structure of maize meal. Two modes of attack were observed viz., a minor exoerosion and a major endoerosio. Individual erosion was clearly visible under higher magnification of 2000 x. External structures were detected by SEM, except, when granules broke after being weakened by complete internal hydrolysis. Enlargement of channels were visible and was indicative of the progressive exoerosion, and granules degraded by endoerosion revealed internal layered structure (Fig.5.3).

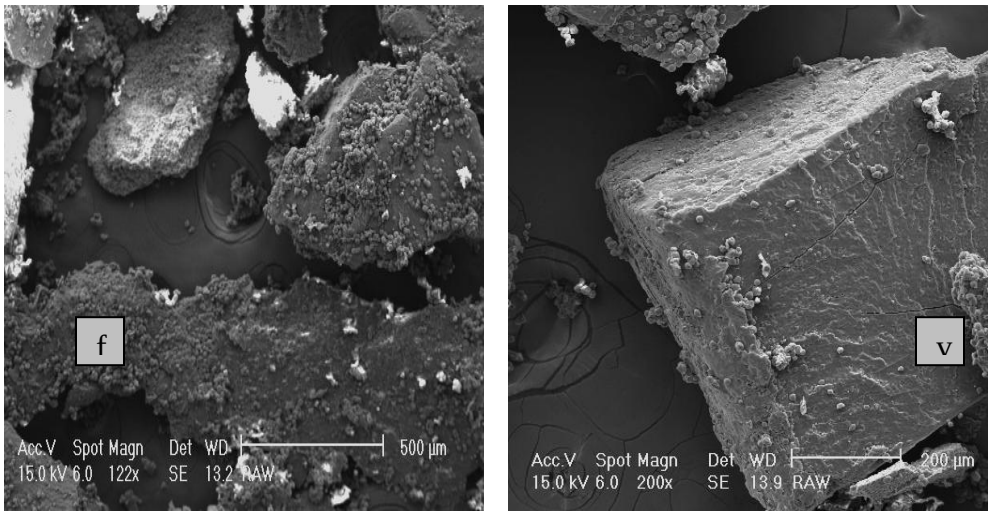

Figure 5.1 **Electron micrographs of raw maize granules showing smooth regular shaped and dense granules with floury endosperm (f) and viterous (v) protein-rich endosperm.**

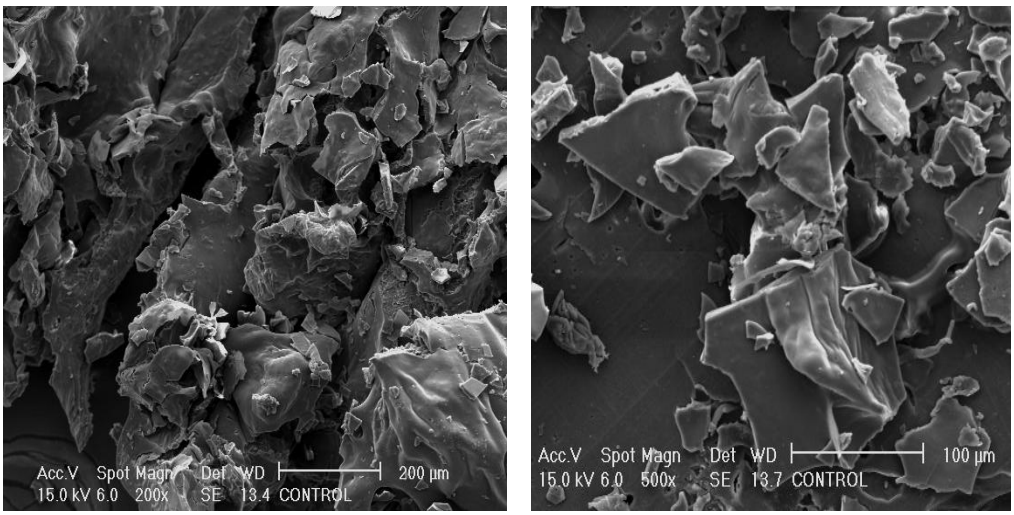

Figure 5.2 **Electron micrographs showing the granular structure of the undigested maize mash. Mashing was carried out at 105^{o}C for 30 min.**

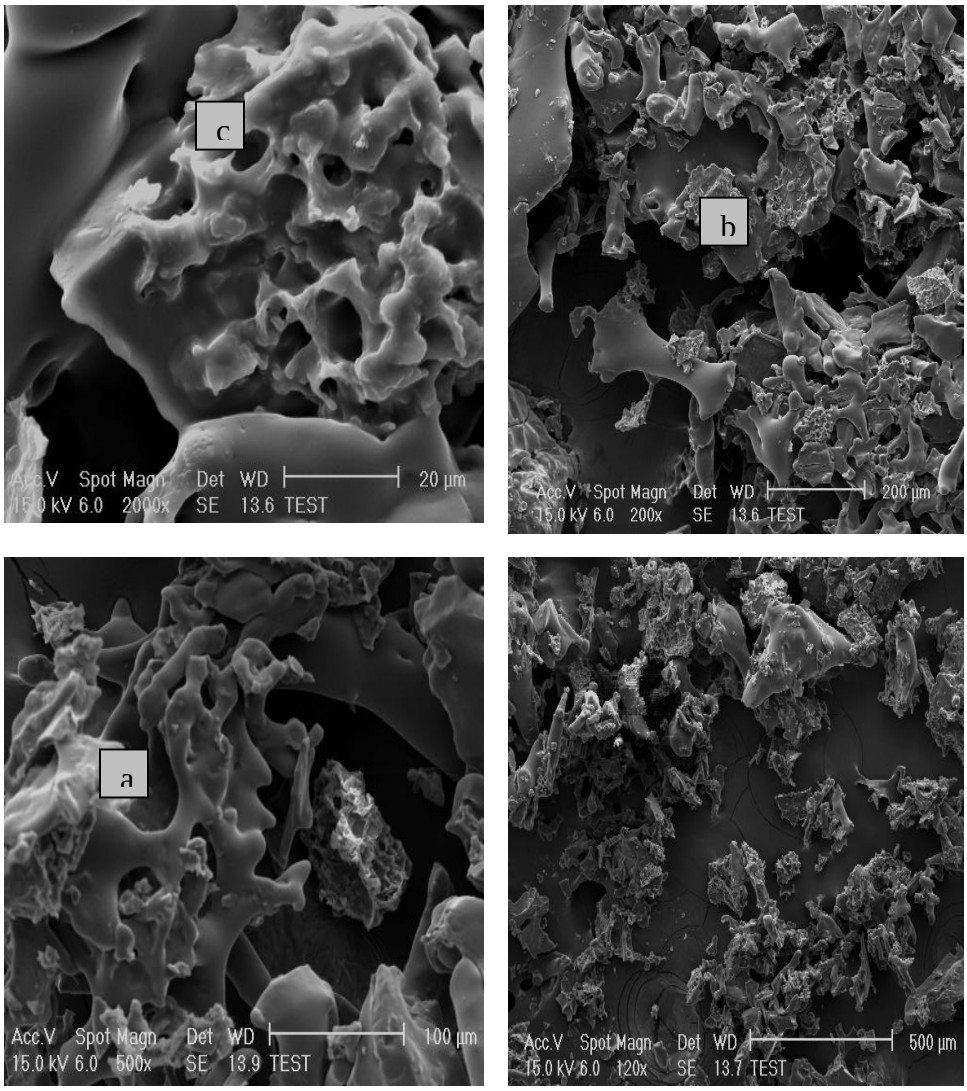

Figure 5.3 Electron micrographs showing the effect of α-amylase from *T. lanuginosus* ATCC 58157 on the granular structure of maize mash and the extent of starch hydrolysis with (a) exoerosion, (b) endoerosion and (c) enlarged channels clearly visible.

5.3.4 Carbohydrate analysis using thin layer chromatography (TLC) to identify sugars

TLC analysis was carried out as a preliminary screening technique to identify sugars present in the digested and undigested maize mash under optimised conditions. Eleven sugar standards were used to identify the sugars present in the digested and undigested maize mash. In the undigested maize sample, two sugars were separated and were identified as glucose and sucrose (data not shown). However, in the digested sample three sugars were identified i.e., maltose, glucose and maltotriose. Sucrose was not detected as a hydrolysis product using TLC because of its low concentration. However, sucrose was subsequently detected using HPLC (Fig. 5.3.5).

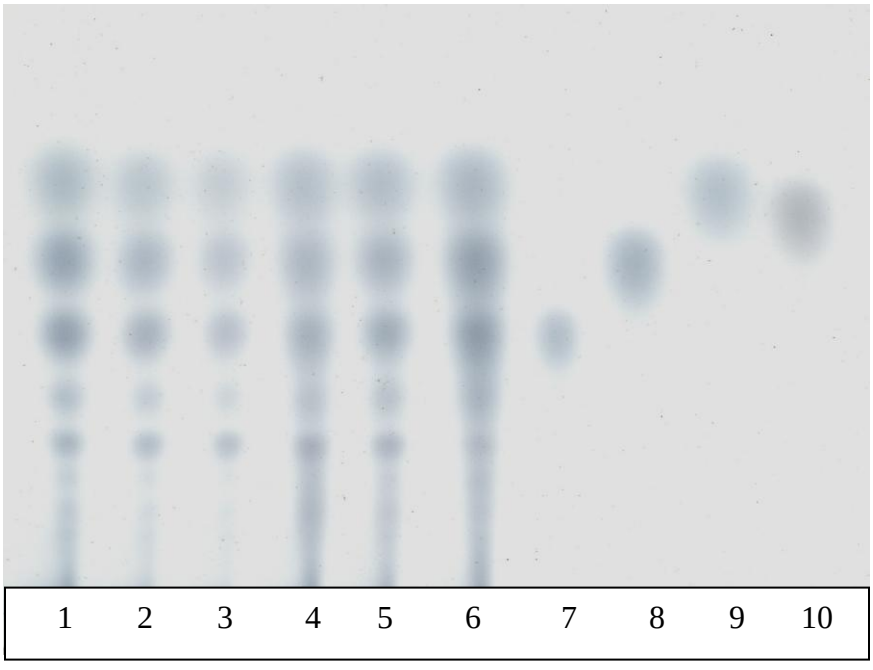

Figure 5.4 TLC plate showing the various dilutions of the optimised digested sample and the sugar standards. Lane 1-6: diluted sample; Lane 7: maltotriose; Lane 8: maltose; Lane 9: glucose.

5.3.5 Quantification of carbohydrates in digested and undigested maize meal using HPLC

The optimised and control maize mash supernatants were subjected to HPLC analysis to identify and quantify the sugars present. In the undigested sample two sugars were identified (glucose and sucrose) at concentrations of 0.12 and 1.3 mg/ml respectively (Fig.5.6). In the digested sample four sugars were identified (glucose, sucrose, maltose and maltotriose) and were present in concentrations of 23.86; 10.82; 35.64 and 34.47 (mg/ml) respectively (Fig.5.5). The retention times for each of the sugar standards correlated well with those reported for the same method and column used (Merck, 2004). Using the established retention times for maltose, glucose, sucrose and maltotriose, sugars were identified in the optimised digested (Fig. 5.5) and undigested (Fig.5.6) control.

The results also showed that with an increase in enzyme concentration there was a decrease in the concentration of the sugars. In the undigested mash only two sugars were identified being glucose and sucrose. The concentration of sucrose was higher in the digested sample than in the undigested sample (Fig. 5.6 and 5.5). The quantified HPLC results of the optimization study are shown in Table 5.2.

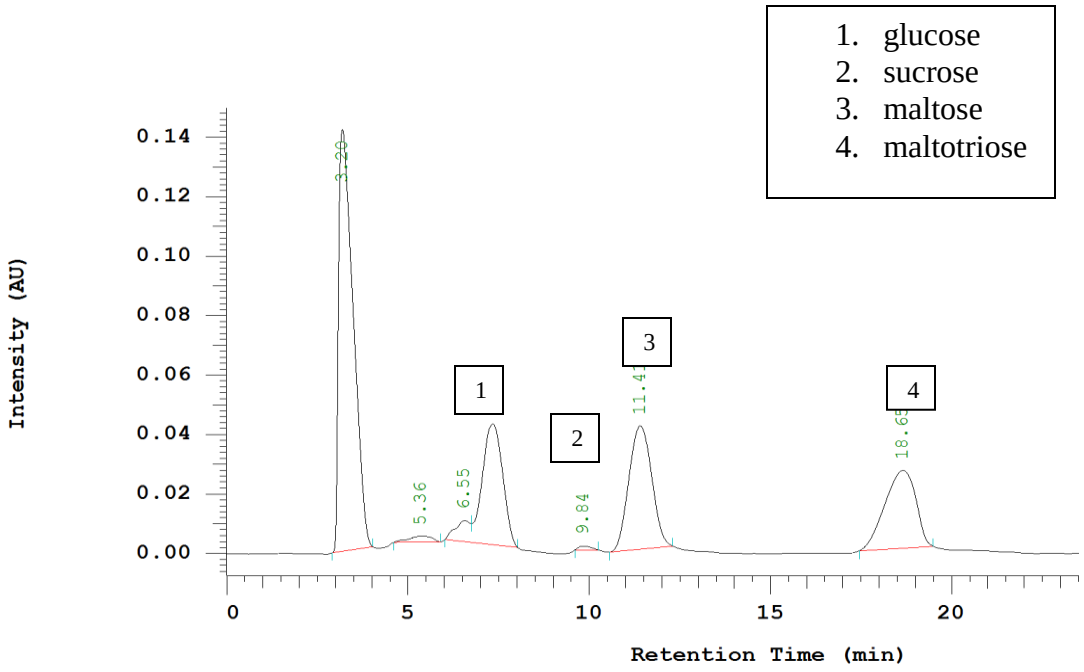

Figure 5.5 **Chromatogram of sugar analysis by HPLC- using a 100NH_2 column, acetonitrile : water mobile phase and RI detection. Sugars were separated from maize mash digested with α-amylase from *T. lanuginosus* ATCC 58157.**

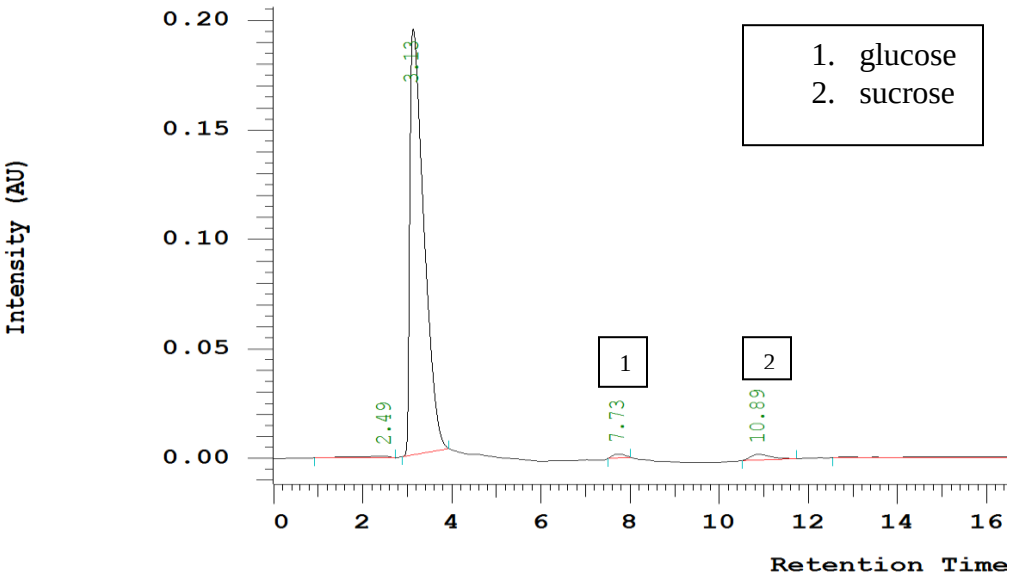

Figure 5.6 **Chromatogram of sugar analysis by HPLC- using a 100NH_2 column, acetonitrile : water mobile phase and RI detection. Sugars were separated from undigested maize mash.**

5.3.6 Identification and quantification of water soluble vitamins using HPLC

HPLC analysis was carried out on all samples derived from optimization experiments (Table 5.1), undigested control and the final optimised digested maize mashes using a C18 column and (90:10) SHS : acetonitrile mobile phase. Five water soluble vitamin standards were used viz., folic acid, niacin, thamine, riboflavin and pyridoxine. The retention times obtained for the vitamin standards correlated with those reported for the same method (Merck, 2004). Using the established retention times for niacin, folic acid, riboflavin, thiamine and pyrodoxine, the vitamins were easily identified in the optimised digested and undigested maize mash control (Fig. 5.7).

The results of the undigested maize meal showed that riboflavin, niacin, pyridoxine, thiamine and folic acid were present at very low concentrations in the control. The graph did however, show very small peaks at the retention times characteristic of these vitamins (Fig.5.8). The first two peaks on each chromatogram at retention times 1.83 and 2.01 min was consistent with all the separations and was attributed to the mobile phase. Digestion of the maize meal showed a two fold increase in niacin and pyridoxine, 55 mg increase in folic acid, and vitamins B_1 and B_2, showed the best improvement (Table. 5.3).

Table: 5.3 Effect of α-amylase from *T. lanuginosus* ATCC 58157 on available water soluble vitamins in maize mash digestion

Vitamin	Undigested maize (mg)	Digested maize (mg)
Niacin	27.21	52.82
Pyridoxine	7.17	16.61
Folic Acid	143	198
Riboflavin	0.04	2.12
Thiamine	0.2	4.53

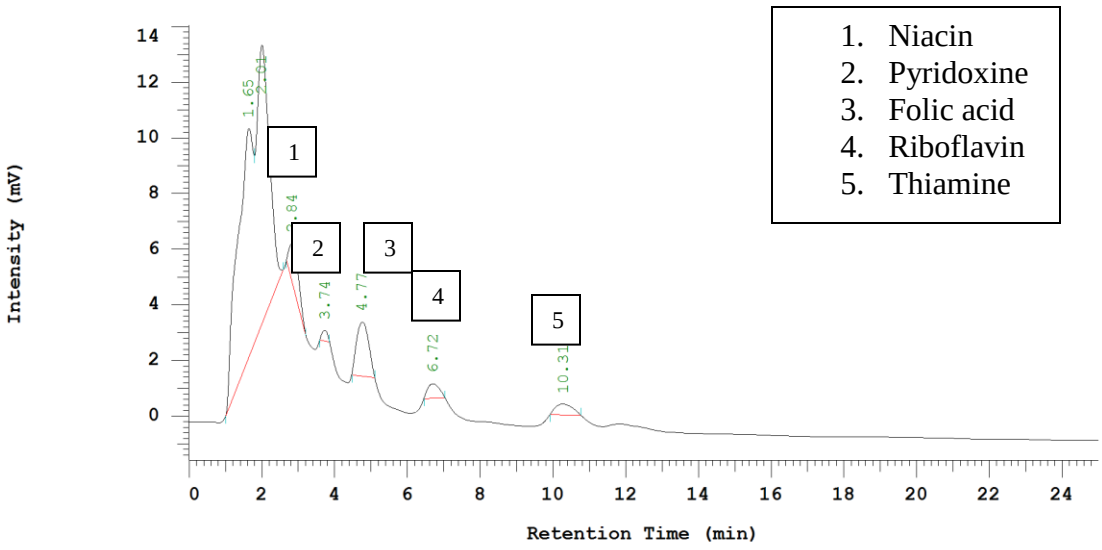

Figure 5.7 **Chromatogram of water soluble vitamin analysis from undigested maize mash by HPLC- using a C18 column, SHS : acetonitrile mobile phase UV detection.**

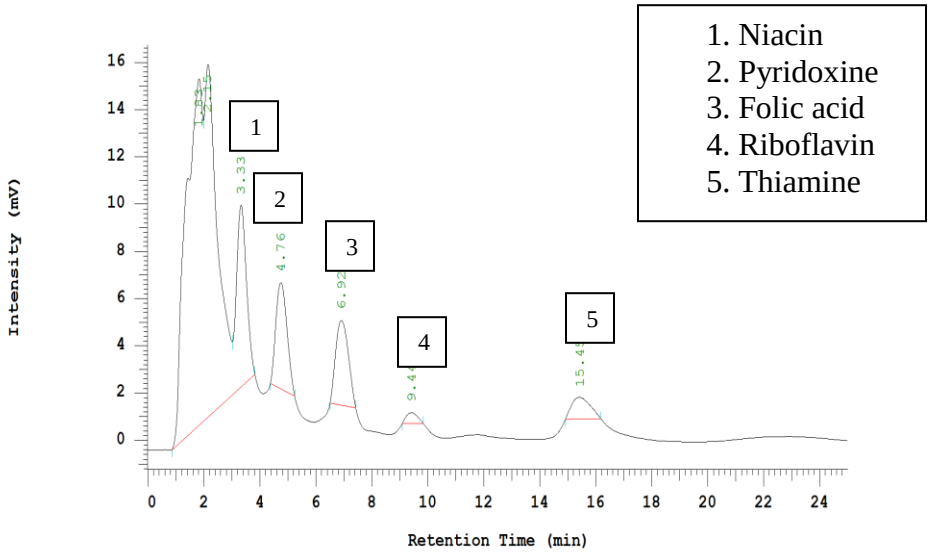

Figure 5.8 **Chromatogram of water soluble vitamin analysis by HPLC- using a C18 column, SHS : acetonitrile mobile phase UV detection. Vitamins separated from maize mash digested with α-amylase from *T. lanuginosus* ATCC 58157.**

5.3.7 Identification and quantification of fat soluble vitamin A using HPLC

The results showed good elution and peak symmetry for the vitamin A standard, undigested maize mash (control) and the digested maize mash. An increase in vitamin A content correlated with an increase in enzyme dose. A 3.5 fold increase in vitamin A content was recorded in the digested maize mash (Fig. 5.9; Fig. 5.10 and Fig. 5.11). Application of multiple pre and post amylase doses yielded a concomitant increase in vitamin A content (Table 5.4). The highest vitamin A concentration (386.84 µg) was evident at pre and post levels of 10 U/mg enzyme. However, despite a reduction of 27% in vitamin A concentration, greatest efficiency was achieved at amylase doses of 1 U/mg and 2 U/mg at pre and post applications. Of note is that the enzyme contained residual vitamin A of 53.69 µg, which confirms its negligible influence on the final vitamin A concentration after hydrolysis.

Table: 5.4 Effect of enzyme concentration on available Vitamin A in maize mash digested with α-amylase from *T. lanuginosus* ATCC 58157.

Sample No.	α-Amylase dose (U/mg)		Vitamin A (µg)[1]
	Pre	Post	
1	1	1	139.08
2	5	5	327.33
3	10	10	523.62
4	20	20	390.48
5	1	5	268.77
6	5	1	328.35
7	5	10	530.28
8	10	5	578.20
9	1	10	471.80
10	0.5	1	257.44
11	1	1	300.32
12	2	1	353.95
13	1	0.5	295.17
14	**1**	**2**	**386.84**
Raw Maize			38
Control Undigested			147.41
α-amylase			53.69

[1] values are corrected against the control undigested sample.

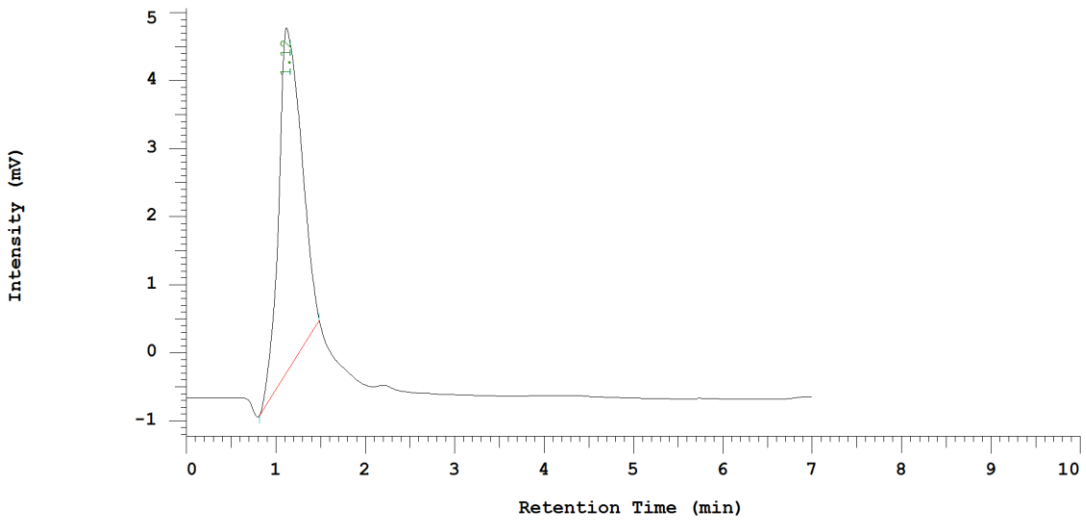

Figure 5.9 Chromatogram of fat soluble vitamin A standard by HPLC- using a C18 column, methanol : water mobile phase and UV detection.

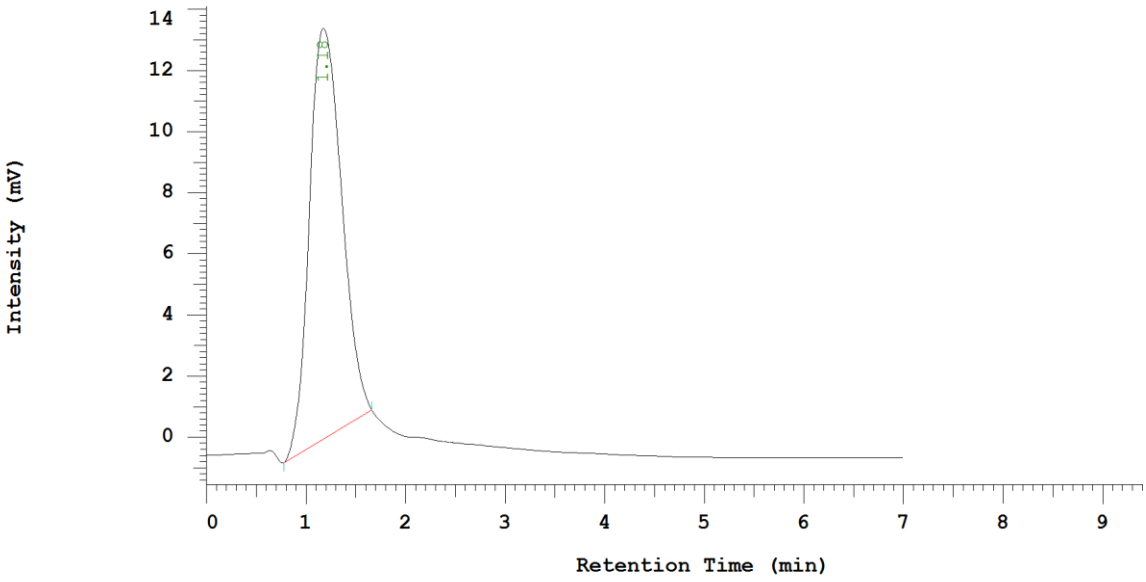

Figure 5.10 **Chromatogram of fat soluble vitamin A analysis from undigested maize mash by HPLC- using a C18 column, methanol : water mobile phase and UV detection.**

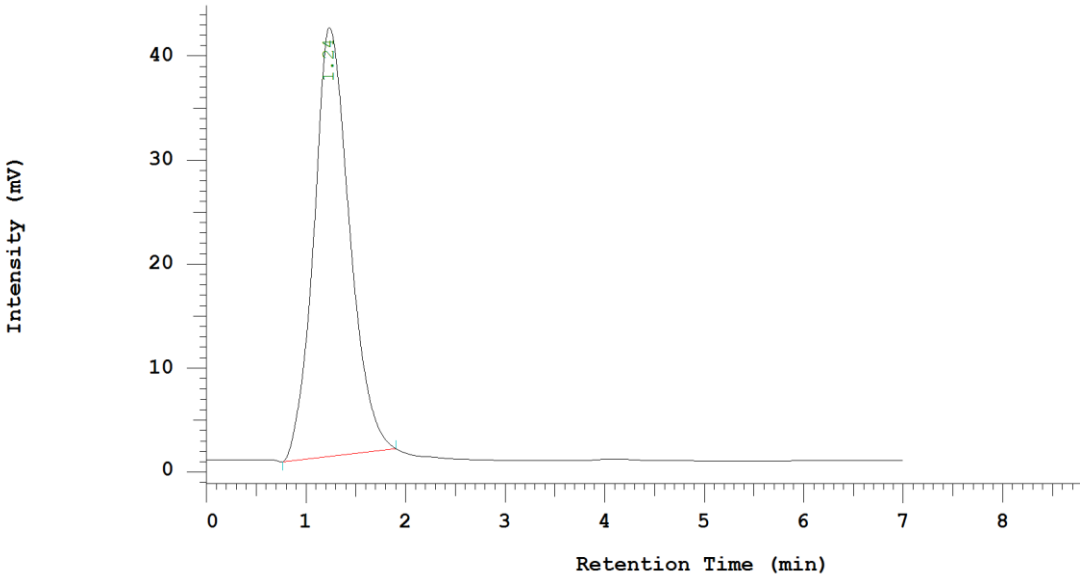

Figure 5.11 **Chromatogram of fat soluble vitamin A analysis from maize mash digested with α-amylase from *T. lanuginosus* ATCC 58157 by HPLC-a using C18 column, methanol : water mobile phase and UV detection.**

5.4 Discussion

Dietary starch varies greatly in digestibility and its effects on utilization appear to be due to differences in starch components and their crystallinity. Processing treatments, storage conditions, and chemical modification influence the digestibility of starch. Cooking often significantly improves the digestibility of poor and intermediate starches (Dreher *et al.*, 1984). Before designing a successful hydrolysis system, information describing phenomena which affect the kinetics of starch hydrolysis such as temperature, pH, enzyme concentration, is required (Apar and Özbek, 2003).

In this study, the effects of various parameters were clearly demonstrated during the enzymatic hydrolysis of maize meal. Some of the parameters that complemented the hydrolysis process viz., enzyme dose; pH; cooking time; temperature; calcium and magnesium supplementation. The optimisation of the process parameters influenced α-amylase activity. The optimum conditions were defined in this study as those variables that resulted in the highest sugar yields achieved together with an improvement in the availability of vital nutrients.

Based on the data derived from enzyme characterization studies in the previous chapter, maize hydrolysis was initiated at pH 6.0 and 60^{o}C as the optimum conditions. Additional pH variables included pH 7.0 and 7.2 and temperatures 37^{o}C and 45^{o}C based on the protocol reported by Soni *et al*. (2003).

This study showed that amylase treatment resulted in a net increase of 74% of available protein, demonstrating the value of α-amylase treatment for the enhancement of protein availability for consumption. The ramifications of such a value are of tremendous value when one considers how this could be applied to the human population. What was also evident in this study was that there was a correlation between enzyme dose and an increase in available protein. The results of this study were consistent with those reported by Duodu *et al*. (2002), in that there was an improvement in the protein availability in the hydrolysed maize meal.

Duodu *et al*. (2002) reported on the effects of cooking on maize and sorghum protein digestibility and demonstrated that cooking caused a significant reduction in protein digestibility in sorghum but had the opposite effect with maize, an effect well documented in literature (Axtell *et al*., 1981; Mertz *et al*., 1984; Hamaker *et al.*, 1987 and Hamaker *et al.*, 1986). The data was consistent with superior protein digestibility of cooked maize over sorghum (Duodu *et al*., 2002). It was also reported that the action of α-amylase in breaking down the starch structure allows the pepsin greater access to protein bodies in cooked grain. In Duodu *et al*. (2002), final analysis of the study on the treatment of maize with α-amylase, it was shown that the α-amylase did improve protein digestibility.

The mode of action of amylase on the granular starch, with particular attention being given to the influence of the amylase and its significant degradation of starch granules, was investigated in this study. Evaluation of the maize granules using SEM clearly

highlighted the degree of enzyme hydrolysis evident by the hollow granules and enlarged pores. In contrast the undigested maize granules revealed smooth, flaky surfaces. This clearly demonstrated the α-amylase penetration of the inner kernels of the maize granules, thereby enhancing nutritional availability. In support of these findings at an ultra-structural level, a similar degree of α-amylase hydrolysis on maize starch was evident from the studies of Gērard *et al*., (2001) and Planchot *et al*., (1994).

In addition, Biliaderis (1991) reported that other features of granule morphology, such as ultrastructure, crystal size and interactions between amorphous and crystalline regions, are involved in the interpretation of maize starch degradation. It can therefore be concluded that direct interpretation of maize starch granule degradation is greatly assisted by SEM.

The HPLC protocol used in this study was first tested with various mixtures of standard solutions viz., maltose, glucose, maltotriose and sucrose (4 mg/ml). The separations of the different sugars were clearly effective and were achieved in 20 min. The sugars eluted at similar retention times as compared to Chávez-Servin *et al.* (2004). The flow rate of 1 ml/min eluted the sugars, glucose (7.73 min) and sucrose (10.89 min) in the unhydrolysed maize mash, and the concentrations were calculated at 23.86 mg/ml and 10.82 mg/ml respectively. The sugars in the hydrolysed maize mash viz., glucose (7.34 min), sucrose (9.84 min), maltose (11.41 min) and maltotriose (18.63 min) at concentrations of 23.86 mg/ml, 35.64 mg/ml, 34.47 mg/ml and 10.82 mg/ml respectively. The conversion of maize starch after application of α-amylase from *T. lanuginosus* ATCC 58157 resulted in

glucose, maltose and maltotriose as the main hydrolysis products. The conversion efficiency of 20% solids was achieved at enzyme doses of 1 and 2 U/mg α-amylase.

The simultaneous determination of five water soluble vitamins viz., niacin, pyridoxine, folic acid, riboflavin and thiamine was achieved using a C18 column and HPLC protocol published by La-Chrome (Merck, 2005). The protocol was first tested using standard vitamin preparation at a concentration of 100 µg/ml. The retention times for each vitamin were confirmed by those achieved and reported for the same method by La-Chrome (Merck, 2005). The separation of the vitamins were clearly observed at 25 min with retention time for each vitamin established viz., niacin (3.33 min), pyridoxine (4.76 min), Folic acid (6.92 min), riboflavin (9.44 min) and thiamine (15.45 min) from the hydrolysed maize mash. The analysis of the unhydrolysed maize mash revealed the presents of all five vitamins at lower concentration. It was difficult to compare retention times for the vitamins using other studies since the retention times are greatly influenced by the mobile phase and detection system used.

Vitamin A detection was achieved at a retention time of 1.2 min and was consistent in the both the hydrolysed and unhydrolysed maize mash. The hydrolysed maize mash showed a 2.5 fold increase in vitamin A content. This is of great significance since the consumption of 200 g of the hydrolysed maize mash will meet 53% of the recommended daily dietary allowance (RDA) for children under 12 years of age.

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CHAPTER SIX: EVALUATION OF THE NUTRITIONAL BENEFITS OF HYDROLYSED MAIZE MEAL USING RATS IN FEED TRIALS

6.1 Introduction

Animal models are widely used in nutrition science for various reasons viz., testing of new drugs, evaluating nutritional screening interventions, screening for side effects and clinical trials for drug resistance. The limitations of extrapolating results of animal studies to human health is an ever ongoing debate. In general, results from animal studies are considered useful indicators, though, for the need of human studies (Plochberger, 1989). The choice of species in animal models depends on a number of factors, including the resemblance to humans, the sensitivity to the investigated factors and economical considerations (Plochberger, 1989).

Outbred strains of rats are used more commonly today in laboratories than in bred strains. Some common outbred strains are Wistar (albino rat), Sprague-Dawley (albino-faster growing than the Wistar) and Long-Evans (hooded rat-smaller than the Wistar or Sprague-Dawley). Rats can develop naturally occurring diseases such as diabetes and hypertension which makes them valuable in the study of those diseases as they apply to humans and they are also frequently used in behaviour and nutritional studies, toxicology studies or drug and longevity studies (Zutphen *et al*., 2001).

When breeding laboratory animals especially for nutrition trials, it is necessary to ensure their diets are appropriate, so that they can reach their maximum genetic potential. The nutrition of such animals should be made up of diets that contain ideal concentrations of several components essential for the animal's good development. Besides the diets chemical composition, the procedures related to its handling and storing, such as cleanliness, disinfection and sterilization should also be monitored and cared for, in order to avoid contamination by pathogenic microorganisms that could minimize the animal's performance (de Faria *et al*., 2004).

Nutritional screening is highly recommended for the treatment of conditions such as malnutrition. However, with the increasing emphasis on evidence-based medicine, which relies heavily on randomized controlled trials, there has been some controversy about the role of nutritional screening. The controversy surrounds the manner in which the trials are conducted and the fact that the trials do not necessarily result in improved outcomes. Such information is often inadequate or lacking from published studies, making it difficult to assess the value of specific nutritional screening tests (Elia *et al.*,2005).

In order to assess the nutritional value of the α-amylase pre-digested maize meal, the rat-model was chosen as the most appropriate cohort to mirror human consumption. Based on the appreciable increase in carbohydrates, vitamin A, water soluble vitamins, protein concentrations resulting from the enzyme hydrolysis, the work in this chapter focused on demonstrating these benefits in rats.

6.2 Materials and methods

6.2.1 Preparation of the feed

The test and unhydrolysed control feeds were prepared under optimised conditions for mashing as described in section 5.2.2. After hydrolysis the mashes were cooled, well mixed and then packed in 550 g quantities into polypropylene bags followed by freezing at -80^{o}C in a biofreezer (Snijders Scientific) for 48 hours. The test and control feed mashes were then freeze-dried at -40^{o}C in a freeze dryer (Virtis) for 48 hours, after which the freeze dried feed was ground into a flour. Both feeds were stored in airtight containers at room temperature until use.

6.2.2 The feed trial

Forty male albino Wistar rats weighing between 45 and 55 g were weaned at 21 days old and placed in individual metabolic cages for an acclimatisation period of 3 days. Lights were switched on at 06h00 and switched off at 18h00 daily for the duration of the study (21 days). Rats are nocturnal animals and it is important to simulate day and night during the study so as not to disturb eating patterns. During the acclimatisation period, 35 rats were fed a diet consisting of the control feed and fresh water on a daily basis *ad libitum* (Beynen and Coats, 2001).

The feed trial commenced after the acclimitisation period when the rats were 24 days old. This served as day zero of the feed trial wherein five rats were sacrificed by overdose of halothane inhalation (S5-Halothane, Flurothane® Zeneca) in accordance with the Standard Operating Procedure of the Biomedical Research Unit, University of Kwa-Zulu Natal, (BRU, UKZN) (2005), and the various tests evaluated as described in sections 6.2.5 and 6.2.6 to establish baseline data.

Thirty rats were subjected to the following feed trial regimen for 21 days:

i. Group 1: 15 rats were used as the positive control and were provided with 20 g of the control diet and water *ad libitum* daily.

ii. Group 2: 15 rats were used as the test rats and were provided with 20 g of the test diet and water *ad libitum* daily.

Weight determinations for both groups were recorded daily 2 h after the lights were switched on. Feed intake for both groups were also calculated daily (feed given – feed remaining). Five rats from each group were sacrificed every seven days for the duration of the trial. Blood samples were collected by cardiac puncture in accordance with the Standard Operating Procedure of the BRU, UKZN, (2005) and sent for analysis to a private pathology laboratory (Bouwer and Partners Inc.) for total cholesterol, urea, triglycerides and random blood glucose levels. The rats were then autopsied in accordance with the Standard Operating Procedure of the BRU, UKZN, (2005) and the

health status and weight of the liver, spleen, kidneys, stomach and heart were recorded. The length of the small and large intestines were also measured.

The remaining five rats served as the negative controls and were fed a normal rat diet of PVM pellets (protein, vitamins and mineral reinforced diet, Epol – compressed pellets) and fresh water daily *ad libitum* for the duration of the feed trial and sacrificed on the 21st day. Samples were collected as per test subjects and the results were used as an indication that the living environment and water provided had no adverse effect on the test subjects.

6.2.3 Statistical Methodology

SPSS version 11.5 (SPSS Inc, Chicago, Ill) and Stata version 8.0 (Stata Corporation, Texas, USA) software programmes were used for data analysis. Repeated measures of ANOVA were used to assess the body and feed weight changes for a treatment effect (time by group interaction) at various time cutoff points.

Multiple linear regressions were used to develop an equation for the relationship between time (in days) and body weight in test animals and positive controls. This was achieved using Stata's xtreg procedure with a random effects GLS regression model. For the remainder of the outcomes measured, no repeated measurements were taken (viz., measurements were made once the animal was killed), thus univariate generalized linear models were used to assess the effect of treatment group and time. Bonferroni post hoc tests were done to examine differences between multiple pair wise comparisons.

The specific objectives with regard to the statistical methodology tested were to:

i. Differentiate between the treatment groups with regard to change in body weight;
ii. Compare the feed weights of each group;
iii. Develop a regression equation to predict the body weight in the test rats and compare with the positive controls;
iv. Carry out weight measurements of the various organs to test whether the feed affected the weight of the various organs;
v. Perform length measurements of the large and small intestines to test whether the feed affected the length of the large and small intestines; and
vi. Perform measurements of blood chemicals to test whether feed affected the blood chemicals levels.

6.3 Results

6.3.1 Statistical analysis of feed trial results

6.3.1.1 Effect of hydrolysed maize mash on body weight of rats

Thirty five rats were included in this analysis (15 from the test group, 15 from the positive control group and 5 from the negative control group). There was a highly statistically significant interaction between time and treatment groups between days 0 – 21 ($p<0.001$), meaning that the effect of treatment group was dependant on the number of days. Since only 15 rats were included in the analysis up to day 21, all time points could not be examined as there were insufficient residual degrees of freedom. Therefore, the major time points of day 0, day 7, day 14 and day 21 were used to assess the trend across time (Table 6.1).

Body weights were recorded daily for all rats participating in the feed trial. Between day zero and day one, the test rats gained more weight than the positive control rats. Subsequently this resulted in the weights of the test rats to increase at a faster rate than the positive control rats over the 21 day feed trial. The body weights of the negative control rats increased exponentially, resulting in weights far above the mean body weights of the test and positive control rats. The pattern of weight gain for each group of rats remained fairly constant throughout the 21 days of the feed trial (Fig. 6.1).

Table: 6.1 Effect of body weight between test and control rats over 21 days.

Effect	Test Day	Statistic	p value
Time (Wilk's lambda)	0-7	0.006	<0.001
Time*group (Wilk'slambda)	0-7	0.003	<0.001
Group	0-7	F= 119.841	<0.001
Time (Wilk's lambda)	7-14	0.002	<0.001
Time*group (Wilk's lambda)	7-14	0.000	<0.001
Group	7-14	F= 256.9	<0.001
Time (Wilk's lambda)	14-21	0.011	<0.001
Time*group (Wilk's lambda)	14-21	0.011	<0.001
Group	14-21	F= 263.4	<0.001

*Test group

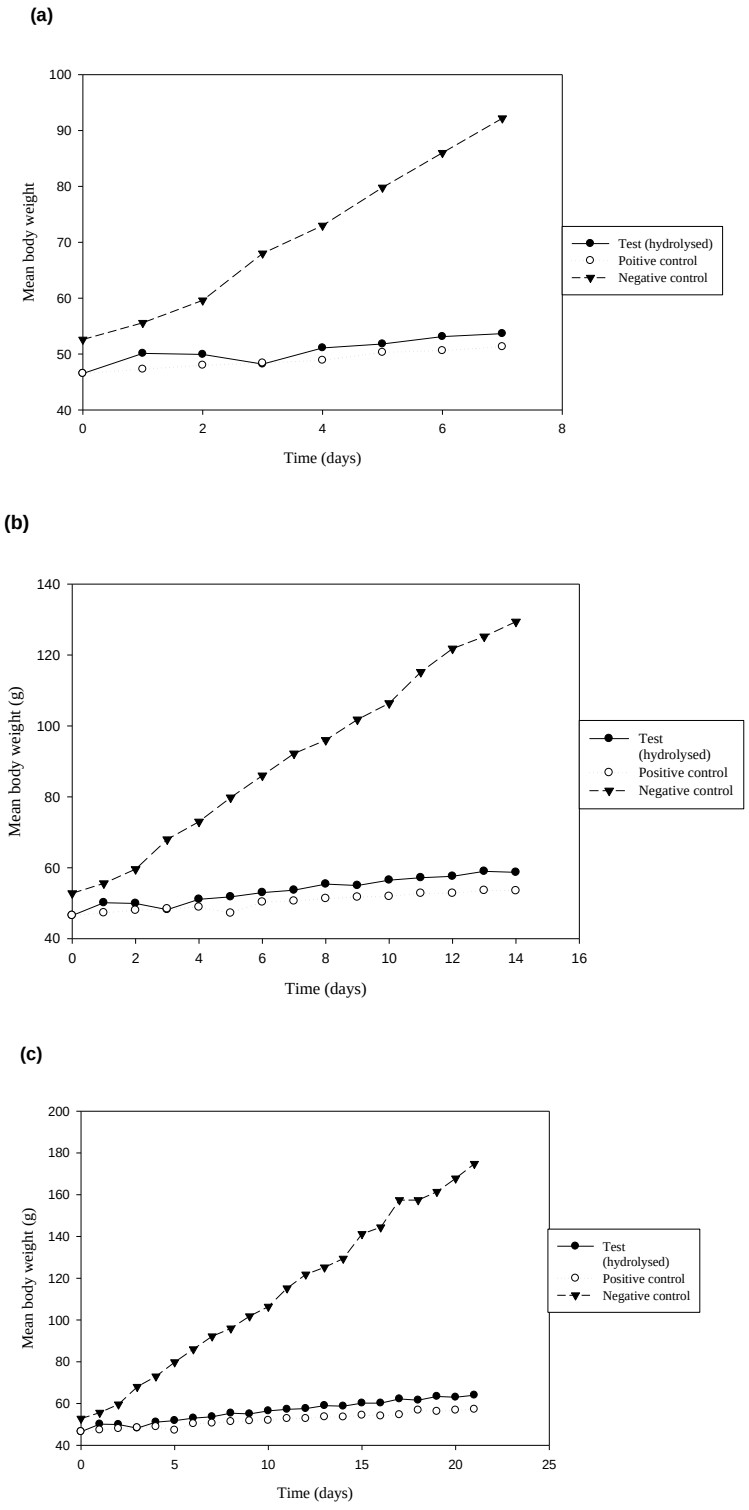

Figure 6.1 Effect of test feed (hydrolysed maize mash) and control feed (unhydrolysed maize mash) on the mean body weight of rats over durations of: (a) 7 days, (b) 14 days and (c) 21 days.

6.3.1.2 Comparison of feed consumption weights for each group of rats

The trial comprised of 15 rats in the test group and 15 rats in the positive control group that were included in the analysis (n=30). There was no difference between the feed weights of the groups over time from 0-7 days (p=0.473) although the feed weights of both groups increased significantly over time from (p.001). Twenty rats were included in this analysis from day 7-14 (n=10 from each group). There was a significant change in feed weight over time in both groups, but there was no difference between the test and positive control groups over time for 7-14 days (p=0.299). Five rats from each group were used in this analysis for 14-21 days (n=10). To increase the residual degrees of freedom, 4 time points were compared: day1, day 7, day 14 and day 21. There was a significant change over time in both the groups for 14-21 days (p=0.038). However, there was no difference between the groups over time for 14-21 days (p=0.572) (Table 6.2)

The amount of feed consumed by each test rat and positive control rat was calculated and recorded daily. Statically there was no difference in the amount of feed consumed between the test rats and control rats over 21 days. However, in comparison with the control rats there were periods where the feed consumption of the test rats was marginally higher than that of the control rats but this fluctuated daily over 21 days. Figure 6.2 shows that the groups followed roughly the same trend over time, although the profiles did intersect at various points. With a larger sample size at 21 days the interaction may have been statistically significant.

Table: 6.2 Effects of feed consumed between test and control rats over 21 days

Effect	Test Day	Statistic	p value
Time (Wilk's lambda)	0-7	0.174	<0.001
Time*group (Wilk's lambda)	0-7	0.800	0.473
Group	0-7	F=0.00	1.000
Time (Wilk's lambda)	7-14	0.055	0.009
Time*group (Wilk's lambda)	7-14	0.227	0.299
Group	7-14	F=0.891	0.358
Time (Wilk's lambda)	14-21	0.270	0.038
Time*group (Wilk's lambda)	14-21	0.733	0.572
Group	14-21	F=0.084	0.780

* Test group

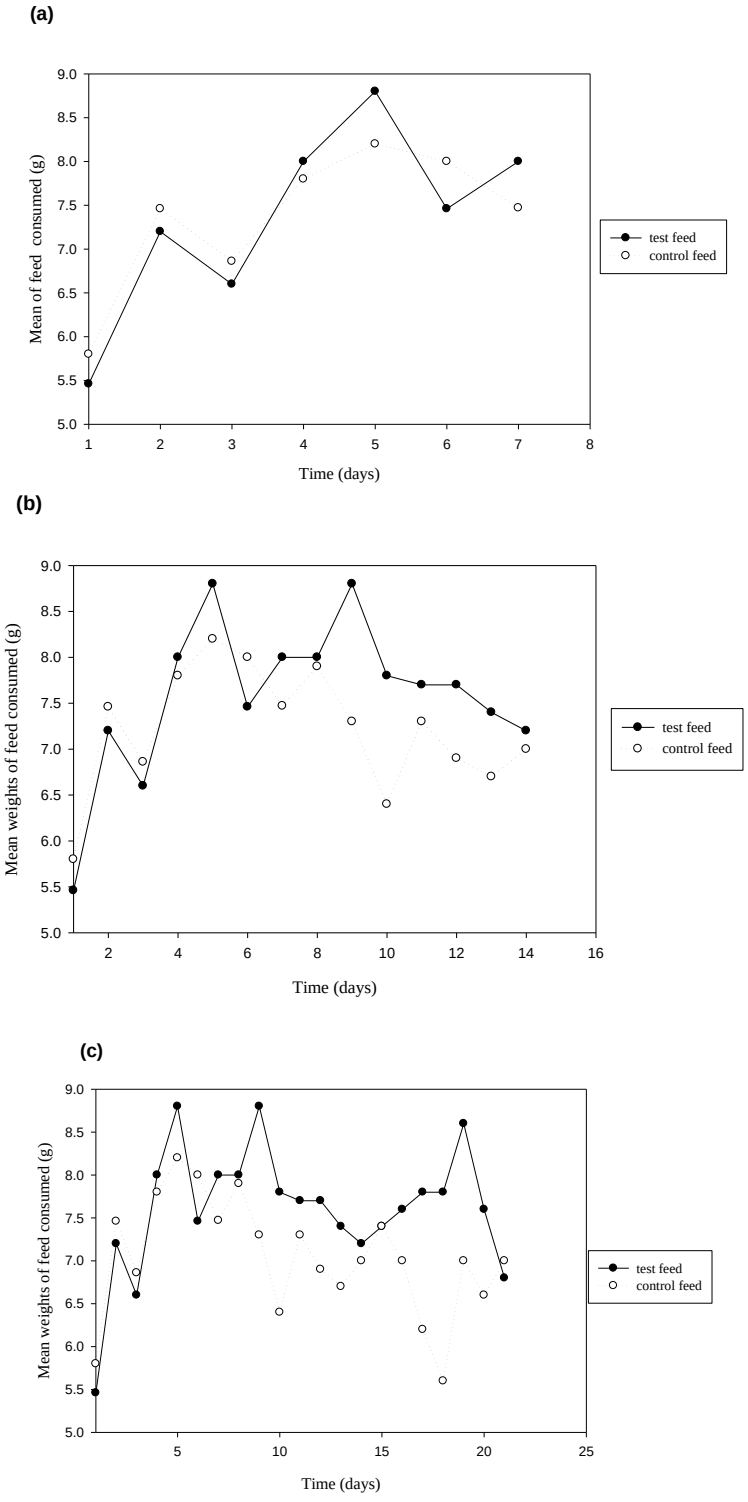

Figure 6.2 Mean weights of feed consumed daily by the test and positive control rats and shown over a durations of: (a) 7 days, (b) 14 days and (c) 21 days.

6.3.1.3 Development of a regression equation to predict body weight in the test rats compared with the positive controls.

The objective in the development of a regression equation was to predict the body weight in the test rats in comparison to that of the positive control rats at 25 days.

Table 6.3: Regression coefficients for the relationship between time and treatment group with body weight

	Coefficient	Standard error	p value
Time	0.607	0.012	<0.001
Group	3.246	1.049	0.002
constant	46.099	0.746	<0.001

According to the above table, the equation for the regression line $Y=b_0+b_1X_1+b_2X_2$ was: body weight = 46.099 + 0.607 **time** +3.246 **group**

The groups were coded 2 for the test rats and 1 for the positive control rats, the predicted weight for the test rats was calculated at 25 days (assuming the relationship between weight and time stayed the same at 25 days) would be:

Weight = 46.099 + 15.175 + 6.492

Weight = **67.77** grams

The predicted weight for positive control rats at 25 days would be:

Weight = 46.099+ 15.175 + 3.246

Weight =**64.52** grams

The effect of the regression on the group was statistically significant in the model (p=0.002) and therefore, there was a significant difference in weights between the groups

(Table 6.3). The overall r squared for the model was 0.6258. Figure 6.3 showed that the relationship between weight and time was different in the two groups, with a steeper gradient for the test subjects. The scatterplot was not generated from the regression equations controlling for the groups, therefore reading predicted values off the plot will give slightly different values to those calculated from the equation.

The relationship only holds within the range of time measured. It is highly possible that the relationship would level off after a certain time and the weights will not continue to increase in a linear fashion indefinitely. Therefore, the prediction ability of weights at times much greater than 21 days is limited as it is not known at which time point the weights begin to level out.

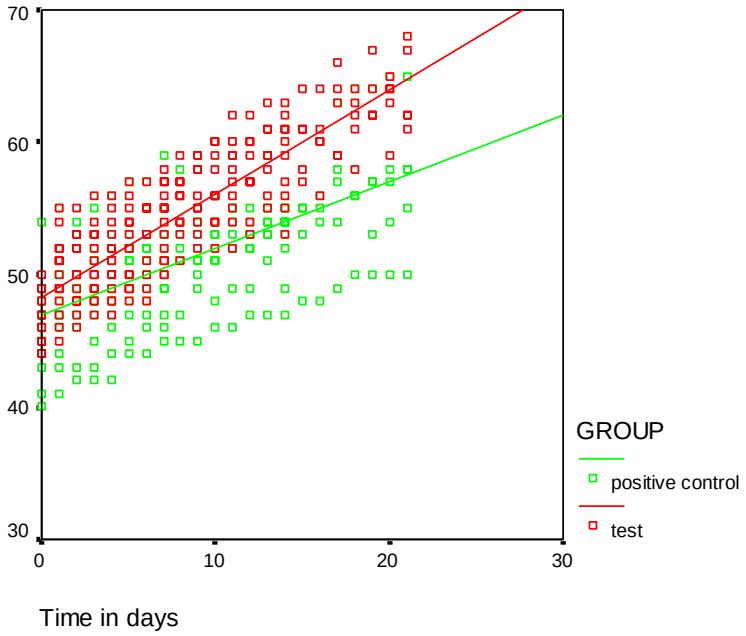

Figure 6.3 Scatter plot to demonstrate the relationship between weight and time for each test and positive control

6.3.2 Effect of hydrolysed test feed and unhydrolysed control feed on vital organs

The mean weights of vital organs of the test and control rats were calculated to determine the effect of the hydrolysed maize mash (test feed) and unhydrolysed maize mash (positive control feed) and negative control rats. The organs were also visually inspected for any gross abnormalities. There was no significant difference in the size of the spleen, kidney and heart of the test and positive control rats (Figure 6.4).

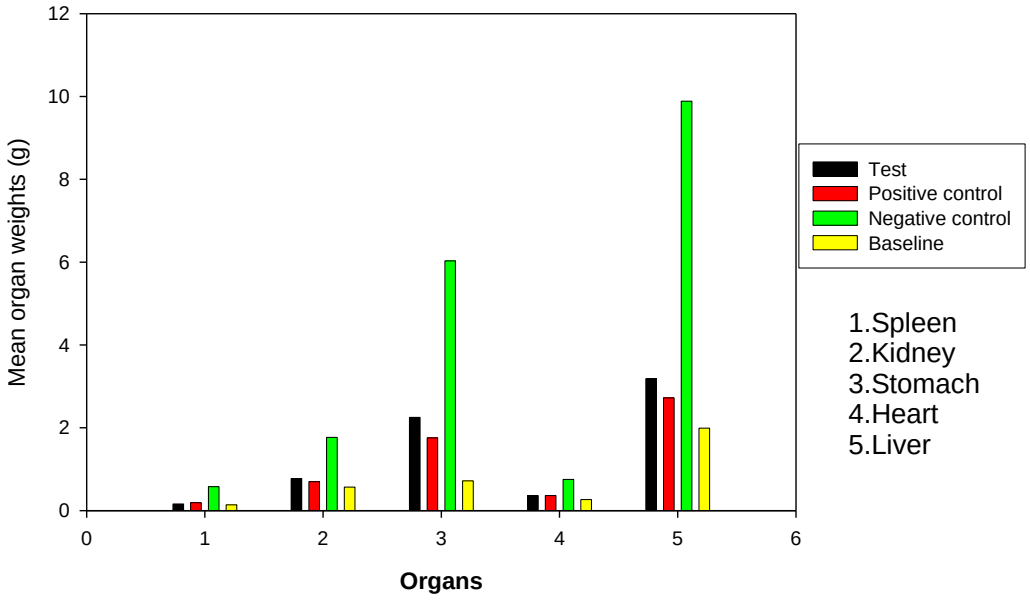

Figure 6.4 **Effect of the test feed (hydrolysed maize mash) and the control feed (unhydrolysed maize mash) on the mean weight of the organs of the rats after 21 days.**

6.3.2.1 Statistical analysis of the organ weights.

The statistical analysis for the spleen showed that the test group of rats ($p<0.001$) and the time of killing ($p=0.035$) significantly affected the spleen weight (Appendix 3, 4 and 5).

The kidney weights were significantly affected for the test group ($p<0.001$) and time of killing ($p=0.002$). There was a significant difference between the baseline group and the test group ($p=0.011$) (Appendix 6, 7 and 8).

The stomach weight was not significantly affected by time of killing ($p=0.052$). There was a significant association between the treatment group and mean weight of stomach ($p<0.001$). There was also a significant difference between the stomach weights of the baseline group and the test group ($p=0.011$), as well as between the negative control and other groups (Appendix 9, 10 and 11)

The treatment group significantly affected the weights of the hearts ($p<0.001$), but time of killing did not significantly affect heart weights overall ($p=0.231$). The baseline and the test groups were significantly different (Appendix 12, 13,14 and 15).

The treatment group was a significant factor for the weights of the livers ($p<0.001$). The day of killing was not significant overall ($p=0.286$). The liver weight were significantly higher in the test group compared to the baseline group ($p<0.001$) and the test group

compared with positive control (p=0.004), as well as in the negative control compared with all other groups (Appendix 16 and 17).

6.3.3 Effect of hydrolysed test feed and unhydrolysed control feed on the lengths of the small and large intestines of the rats

The effect of the hydrolysed and unhydrolysed maize mash on the length of the small and large intestine was evaluated by measuring the length (Fig. 6.5) of the intestines during the autopsy. The cecum was visually examined for any gross visual abnormalities. The age of the rats did not influence the length of the small intestine.

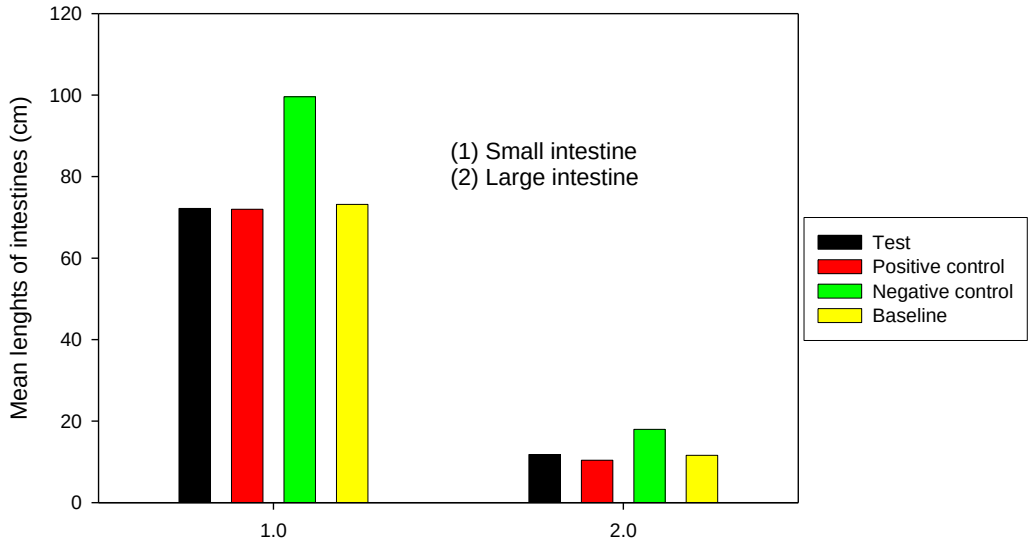

Figure 6.5 Effect of the hydrolysed maize mash and unhydrolysed maize mash on the length of the (1) small and (2) large intestines of the rats after 21 days.

6.3.3.1 Statistical analysis of the intestines

The length of the small intestine was significantly influenced by the test group ($p<0.001$) but not overall by the time of killing ($p=0.138$). The small intestine of the negative control was significantly longer than all other groups and there was no significant difference in the length of the large intestine between the test group and the positive control. The length of the small intestine was no affected by the age of the rats (Appendix 18, 19 and 20).

The test group significantly affected the length of the large intestine ($p<0.001$), but time of killing did not ($p=0.202$). The large intestine of the negative control group was significantly longer than all the other groups, and the test and positive control groups were equivalent. The length of the large intestine did not increase with the age of the rats (Appendix 21, 22 and 23).

6.3.4 The effect of hydrolysed maize mash on the biochemical status of the test and control rats

Biochemical tests were carried out on blood specimens collected from the test and control rats (Table 6.6).

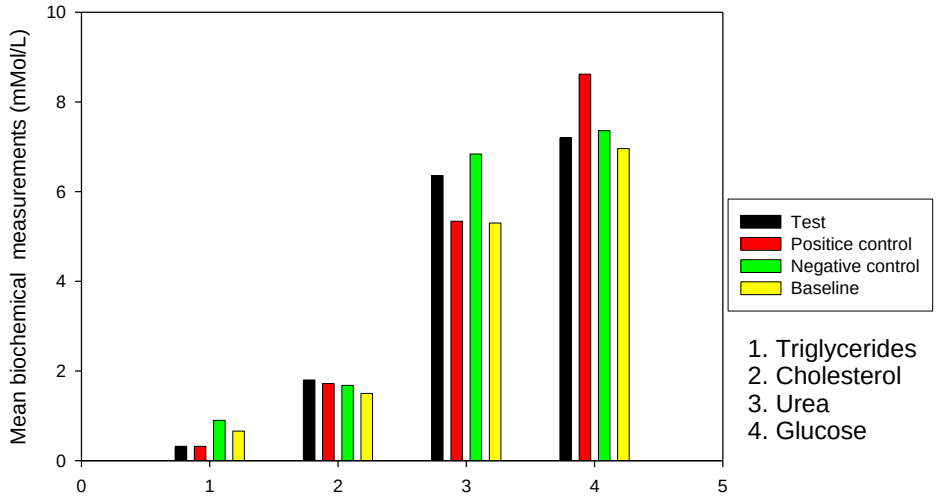

Figure 6.6 Effect of the hydrolysed maize mash (Test feed) and unhydrolysed maize mash (positive control) on the biochemical status of the test and control rats after 21 days of consumption.

6.3.4.1 Statistical analysis of biochemical test results

The test group ($p<0.001$) and time of killing ($p=0.025$) significantly influenced the triglyceride level in the rats. The baseline triglycerides were significantly higher than the positive control and test groups, but significantly lower than the negative control. The rats killed on day 0 had significantly higher triglyceride levels than all other days. Thus the triglyceride levels decreased over time, but were much higher in the negative control group than any other group (Appendix 24, 25 and 26).

The time of killing was the only significant factor for cholesterol levels ($p=0.029$). The test group did not affect the cholesterol ($p=0.709$). There was no individual group with significantly higher levels of cholesterol. At 21 days he test group had a higher mean cholesterol level than any other group. However, the difference was not statistically significant (Appendix 27, 28 and 29).

There was a statistically significant interaction between the test group and the day of killing ($p=0.003$) for urea levels. This meant that the urea level was dependant on both the treatment group and time of killing. This only applied for those groups which had measurements at different times, viz., the test group and the positive control group. The test rats showed a slight decrease between day 14 and day 21, while the positive control rats showed a very large decrease in urea between these time points. This lead to profiles which were not parallel and thus the urea level at any particular time point depended on the treatment group. In the presence of an interaction it is no longer appropriate to

examine the main effects of group and time of killing since the one was dependant on the other. The urea levels were highest for the test group on day 21 (Appendix 30, 31,32 and 35).

There was no difference in mean glucose between the treatment groups or between the age groups. The mean glucose levels in the positive controls were higher than those in the test rats at all three time points and they were higher than those of the negative control at day 21 (Appendix 33 and 34).

6.4 Discussion

Cereal grains form a major source of dietary nutrients for all people, particularly those in developing countries. However, the nutritional quality of cereal grains is inferior to animal foods, due to lower protein content, deficiency of certain essential amino acids, and lower starch and protein availabilities. Fermentation of cereals for a limited period of time has been known to improve the availability of amino acid and vitamin content, as well as increase the protein and starch availability, and lowers the level of antinutrients (Chavan and Kadam, 1989).

Animal models are widely used in nutrition science for various reasons. The limitations of extrapolating results of animal studies to human health are an ever-ongoing debate. In general, results from animal studies are considered useful indicators for the need for human studies. Animal models are advantageous in cases such as long-term studies and other investigations, which, for practical or ethical reasons cannot be studied in humans (McCarter, 1995 and McShane and Wise, 1996). Long-term studies using realistic diets are rather few, and the most important ones are the studies of caloric restriction and longevity, even when no potent chemicals are involved (McShane and Wise, 1996).

In this study the body weights and weight of consumed feed was recorded daily for 21 days to evaluate the growth performance and the influence of the feed on growth of the rats. The results of this feed trial indicate that the body weight of the rats over 21 days was proportionate to the age of the rat and quantity of feed consumed. On day one of the

feed trial the test group of rats consumed more feed than the positive controls and gained a weight advantage that was evident throughout the trial. The weight gain of the test rats was consistently higher than the positive control rats. The body weights were proportionate to the quantity of feed consumed by the test rats. Another factor influencing the increased body weights of the test rats was attributed to the high available protein content of the test feed. The test feed had a protein content that was 74% higher than that of the control feed. Since protein is directly related to normal body growth and development, the improved growth rate could also be attributed to the increased absorption of the proteins by the test rats. At the end of the 21 day feed trial the test feed rats had a 12% weight gain over the same period of time as the positive control rats. This is a significant percentage weight gain over a short period of time. This weight gain is of great significance especially in the application of sustaining malnourished individuals.

The body weights of the negative control rats increased exponentially throughout the 21 days. This was attributed to the type of feed consumed. The negative control rats were fed what was considered a normal diet - which consisted of protein, vitamins and mineral supplements in the form of PVM pellets. The negative control was not intended for the purpose of comparison in the study, but rather to monitor the influence of any possible extrinsic parameter other than those parameters entered into the trial, influencing the results of the trial either positively of negatively.

In this study, there was a steady increase in body weight of those rats that were fed the test diet as compared to those rats fed the control diet. In a similar study carried out by

Wong *et al*. (2004), where food and protein intake and weight gain was evaluated as an indication of protein uptake and absorption it was reported that the rats that were fed a protein-free diet showed a significant loss in weight as compared to those rats that were fed a high-protein diet. With reference to the study by Wong *et al*. (2004) study it can be assumed that the weight gain was indicative of the rats improved absorption of the nutrients.

The decreased consumption of the control feed by the positive control rats indicates that the increased bulk associated with the unhydrolysed maize mash extended the level of satiety in the positive control rats. This can also infer that the digestion process in the positive control diets was longer, and not necessarily more efficient, in making more nutrients available for absorption, which accounts for the lower body weights of the positive control rats. The increased consumption could also be attributed to the increased sweetness of the hydrolysed maize mash, which made it more palatable.

The livers, spleens, kidneys, hearts and stomachs were weighed to assess the growth performance of the experimental rats and to evaluate for any toxic effects on the organs. Organ weights were not significantly different between test and positive control subjects, but the organ weights of the negative control were significantly higher than the other groups. This suggested that consumption of the hyrdolysed maize had no adverse effect on the major organs of the experimental rats. The consumption of toxic substances have been known to cause gross abnormalities of the organs with regard to shape, size and colour. The enlargement of major organs is an indication that the organs are stressed due

to the consumption of the assimilated feed. Organ abnormalities are usually visually identified by the size, colour and shape of the organs (Wong *et al*., 2004). Therefore, it can be concluded that an increase in the protein and carbohydrate digestibility and absorption had no effect on the major organs of the experimental rats.

During enzyme hydrolysis starch is converted to other sugars, which could be conceived as a drawback in the application of the hydrolysed maize mash as a feed. In this study the glucose and sucrose were converted to maltose and maltotriose with small amount of glucose and sucrose residues remaining. The effect of this conversion was of concern regarding the expected effect on blood sugar levels. It was anticipated that the blood sugar levels will spike because of the ease of absorption of these sugars into the blood stream of the test rats. Without losing track of the primary objective of the study, of providing a nutritious food for the malnourished, it was of concern, that the feed may create another equally dangerous health condition viz., Type II Diabetes. Surprisingly in this study the blood sugar levels were higher in the positive controls than in the test rats.

This could be attributed to the high resistant starches as found in corn that have been known to prevent the development of non-reversible insulin resistance in rats (Lopez *et al*., 2001). This amylase-starch relationship could be the reason why the test feed with high sugar concentrations did not affect the blood sugar levels of the test rats.

It was also reported that some starches, especially those that have been through a fermentation process, have been known to lower plasma cholesterol and triglycerides

concentration (Lopez *et al*., 2001). A number of research papers have reported that oats and barley have been known to lower serum cholesterol concentrations in humans (Judd and Truswell, 1981; Anderson *et al*. 1984; Newman *et al*. 1989 a; Newman *et al*., 1986 b; Kestin *et al*. 1990; McIntosh *et al*., 1991; Ripsin *et al*., 1992; Lupton *et al*., 1994; Ikegami *et al*., 1996; Kahlon and Chow, 1997; and Delaney *et al*., 2003). This, activity was attributed to the soluble fibre fractions of these cereal grains (Delaney *et al*., 2003).

In line with Delaney's theory, it was expected that the serum cholesterol levels of the experimental rats would drop considerably because the feed was hydrolysed with enzyme and it was assumed that the maize meal would have more soluble fibre content. Therefore, it was expected to have the same effect on the plasma cholesterol of the experimental rats. It was found to have no effect on the levels of triglycerides in the test and control rats. The higher cholesterol level in the plasma of the test subjects when compared to the positive control, were insignificant.

It has also been suggested that to successfully measure the true effect of cholesterol, both cholesterol and cholic acid needs to be supplemented in the diet usually in elevated proportions. These factors are necessary to elevate serum cholesterol in rats and thus to create a more sensitive rat model for detection of cholesterol lowering activity (Delaney *et al*., 2003; Abbey *et al*., 1993; Beyen *et al*., 1986). For the results in this study to be of any significance, a study extending for at least two generations is necessary. It has been suggested that the need for extended studies is necessary to elucidate effects that are

determined during the rapid growth period, but manifests themselves primarily after puberty and adulthood (Lopuhaa *et al*., 2000).

Urea levels in this study were dependant on the treatment group and age of animal. Blood urea levels were considerably higher in the test rats than the positive control. This was an indication that degradation of protein was improved in the test rats which resulted in an increase in protein absorption.

An inverse correlation between the biological value of food and blood urea concentration in rats was previously reported by Eggum (1970). High plasma values of urea, one of the main end products of protein catabolism in mammals, are associated with disturbances in protein degradation subsequently associated with increased protein absorption (Rubio *et al*., 1995; Das and Waterlow, 1974). The increases in urea, is an indication of the increased protein absorption. If the high urea levels was an indication of adverse effects of the hydrolysed maize mash then renal or hepatic toxicity, accompanied by significant changes or other indications of damage to the kidneys or liver or any other vital organ would have been visible in this study. An increase in degradation would inadvertently result in an increase in absorption, which will be of extreme nutritional benefit.

On examining the cecum and intestines in this study, no noticeable changes were observed when compared to the control and baseline. The difference in the length of the small intestine of both the test and control was not significant. However, there was an increase in the length of the large intestine and this could be attributed to an increase in

osmotic load. Osmotic load has been known to influence the length of the large intestine (Delaney *et al*., 2003).

The normal cecum size and length of the small intestine are indicative of good absorption of nutrients in the small intestine (Newberne *et al.*, 1988). Cecal enlargement is commonly observed in rats fed high levels of fermentable carbohydrates that are either poorly digestible or slowly absorbed (Newberne *et al*. 1988). Because such substances are incompletely absorbed in the small intestines, they reach the large intestine where they are fermented by microflora. It is believed that fermentation products, including short chain fatty acids, increase osmotic load and attract water leading to distension and increased weight and length of the large intestines (Delaney *et al*. 2003).

In summary the conclusions that could be drawn from the animal trials are:

i. The increase in body weight in the test rats were significantly greater than in the positive control rats although the feed weights were similar in both groups.
ii. Increase in body weight was a linear function of time and test group and the equation derived was: body weight = 46.099+0.607 time+3.246 group.
iii. Organ weights were not significantly different between the test and positive control rats.
iv. Intestine length was not different between the test and positive control rats and did not increase with age of the animal.

v. Triglycerides levels did not differ between the test rats and the positive rats.

vi. Cholesterol was not significantly higher in the test rats than in the other groups.

vii. The urea levels were highest in the test rats and were dependant on the test group and age of the rats.

viii. Blood glucose levels were highest in the positive control group.

Of note was that this feeding trial demonstrated that the daily-consumed-controlled-amounts of hydrolysed maize meal were not associated with any obvious signs of toxicity in Wistar rats. Minimal changes were evident in the biochemical values, viz., plasma levels of urea and cholesterol. It can therefore be concluded that the consumption of hydrolysed maize meal was not a true indication of the lack of effectiveness of the test feed and that it was not likely to have any adverse effects under the conditions of its intended use. The high available protein, carbohydrates and vitamin A content of the hydrolysed maize mash are indicative characteristic improvements in the nutritional status of the maize meal and therefore satisfy the main objective of this study. Animal studies intended for human application should ideally be conducted over a long term (McCarter, 1995).

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CHAPTER SEVEN: GENERAL DISCUSSION

7.1 Research in perspective

Children are the future of every country; their situation is always of concern to policy makers, their parents and the general public. Ensuring good health of children is a universally supported goal of development. Malnutrition is the result of marginal dietary intake compounded by infection. In turn, marginal dietary intake is caused by household food insecurity, lack of clean water, lack of knowledge on good sanitation, and lack of alternative sources of income (World Bank, 2006). Having good health is an important pre-condition for escaping poverty and because improved health and sanitation contribute to growth, investment in people's health and nutrition status is fundamental to improving a country's general welfare, promoting economic growth, and reducing poverty.

Meeting primary health care needs and the nutritional requirements of children are fundamental to the achievement of sustainable development. Malnutrition in childhood is known to have long-term effects on the work capacity and intellectual performance of adults. Health consequences of inadequate nutrition are enormous. While malnutrition prevalence has decreased significantly in most other developing countries in the last decade, it has been remained unchanged for Sub-Saharan Africa.

This static trend of malnourished children, however, does not fully reflect the rapidly rising numbers of malnourished children, given Sub-Saharan Africa's high population

growth rate. Nutritional studies have found that linear growth (height-for-age) and ponderal growth (weight-for height) have different nutritional requirements. While adequate energy intake is the main reason for wasting (low-weight-for-height), stunting (low height-for-age) can be caused by numerous nutrient inadequacies (World Bank, 1997).

Just as overall dietary inadequacy (also called protein-energy malnutrition) cause stunting, so does a deficiency in many of a large number of micronutrients. Micronutrients are concentrated in specific foods and are low concentrations or absent in staple grains and legumes. Since specific foods, containing micronutrients, are often more expensive than staple foods, stunting and wasting may be affected differently by income. Nutrients such as carbohydrates, proteins, certain fats, vitamins and minerals, are essential dietary factors and malnutrition is caused by a diet that does not contain sufficient nutrients to maintain normal health, growth and development.

In Africa, serious problems associated with protein calorie malnutrition in infants and children exist. (Manna *et al.*, 1995; Gopaldas *et al*., 1988). Early weaning foods consist of starchy legumes, tubers such as cassava and sweet potato, or cereals such as corn, rice, wheat, sorghum and millet (Gopaldas *et al*., 1988). Cereal-legume based dietaries are fed to infants in the form of gruels (boiled in water). When tubers and cereals are prepared in this manner, the starchy structure binds large amounts of water, which results in gruels of high viscosity. Due to the low caloric density of cooked cereal diets, it is often difficult to

feed such diets in adequate amounts, especially to children between the ages of six months and two years (Manna *et al*., 1995).

The preparation of gruels is made with a high solid concentration to provide an adequate calorie density, which tends to be thick and viscous, thus making them difficult for a young child to eat. The addition of water to lower the concentration of solid matter makes the gruel thinner and easier to eat, but it reduces the calories per unit volume, and therefore the child has difficulty in consuming enough to satisfy their nutrition and energy needs (Manna *et al.,* 1995; Gopaldas *et al*., 1988).

Industrial manufacturing of cereal-based weaning foods reduces dietary bulk through various technologies viz., application of enzymes including the use of amylase. However, such commercial foods are not available to children in rural areas because of limited available resources (Manna *et al.,* 1995). The traditional procedure of germinating or malting reduces the dietary bulk of cereal-based weaning food considerably. This is because the amylases that are developed and activated in the germination process rapidly breaks down the starch in the cereal and reduces its water-holding capacity. Consequently, the water trapped in the gel structure is released and produces a more liquid gruel (Manna *et al.,* 1995). However, this phenomenon is only true when amylase-rich-foods (ARF) are utilized. Such ARFs are not readily available in many countries and more especially to the rural African population. There have been many reports on weaning food formulations using ARFs such as malted wheat (Gopaldas *et al*., 1988) and sorghum (Brandtzaeg *et al*., 1981). Therefore, it was envisaged that the application of α-

amylase in the pre-digestion of maize meal will offer an alternative to alleviate the problems associated with the consumption of low caloric density cereal gruels.

The most important enzymatic reactions that are carried out on an industrial scale are the enzymatic hydrolysis of starch. α-Amylase (1,4-α-D-glucanohydrolase, EC 3.2.1.1) is essential during this process, and plays a role in the liquefaction of starch and the subsequent saccharification where larger carbohydrate chains are hydrolysed and converted into smaller carbohydrates. It is, therefore, imperative to preserve the highest α-amylase activity during industrial operating conditions (Baks *et al*., 2005). Amylases are produced by numerous bacteria and fungi, but the most thoroughly studied are the amylases produced by *Bacillus* sp. (Singh and Soni 2001; Pederson *et al.*, 2000; Boes *et al.*, 1996; Agirakes *et al.*, 1992; Castro *et al*., 1992).

The commercial success of amylases is linked to utilization of starchy biomass as an industrial raw material. On a dry basis, agricultural substrates like corn, wheat, sorghum and other cereals grains contain around 60-75% (w/w) starch, hydrolysable to glucose. The conventional method of starch hydrolysis using acid has been replaced by processes using starch saccharifying enzymes, accounting for approximately 15% share in the world enzyme market (Soni *et al*., 2003).

This study involved the production of a thermostable α-amylase from *T. lanuginosus* ATCC 58157. The amylase was characterized and purified before it was used to hydrolyse maize mashes. The improvements gained were quantified and subsequently

qualified by testing their effects in an animal study using Wistar rats. It was envisaged that the overall outcome will enable such an application for human consumption with major benefits from a nutritional perspective. While this may not be immediately implementable, it will provide essential baseline data for application.

Industrially important enzymes, including amylases, have traditionally been obtained from submerged cultures, because of ease of handling and greater control of environmental factors such as temperature and pH. SSF constitutes an interesting alternative since the metabolites produced are concentrated, and the purification procedures are less expensive (Pandey *et al.,* 2000; Nigam and Singh, 1995; Sani *et al.*, 1992). Currently, enzymatic starch hydrolysis is carried out at a substrate concentration of 35-40% (wt) (Schenck, 2002). Increasing the substrate concentration has several advantages, such as increased volumetric productivity and higher α-amylase stability (Klibanov, 1998; Krishnan and Chandra, 1983).

Thermostable α-amylases have been mainly produced by SmF, although a few researchers have used SSF because it has been found to be more advantageous than SmF, and allows for more economical production of the enzyme. Therefore, SSF was used for α-amylase production in this study. Various solid substrates were evaluated for there efficiency to support the growth of *T. lanuginosus* and to ultimately produce maximum yields of the α-amylase. Of the substrates analysed, wheat bran supported good growth of the organism which resulted in the highest yields of the enzyme (211.0 U/g) and wheat bran has also shown to be a good enzyme inducer. Amylase yields, were improved two-

fold by optimizing production variables such as pH, temperature, carbon, nitrogen, moisture content, Ca^{2+} and Mg^{2+} concentrations.

Thermostability of enzymes are significant in industrial applications, because of there compatibility with existing high-temperature process conditions. The main advantage of performing processes at higher temperatures are reduced risk of microbial contamination, lower viscosity, improved transfer rates and improved solubility of substrates (Bruin *et al*., 2001). Long term thermostability of the enzyme at 50^{o}C, 60^{o}C, 70^{o}C, 80^{o}C and 90^{o}C revealed that the enzyme was stable at 50^{o}C for 2 h and the optimum temperature for the enzyme activity was established at 70^{o}C. Enzymes have been known to display different optimum and thermostability temperatures (Soni *et al*., 2003; Nguyen *et al*., 2002). However, the variation between temperature optima and thermostability of the α-amylase was of some concern, since starch liquefaction is generally carried out at 70^{o}C. The application of the α-amylase from *T. lanuginosus* ATCC 58157 in the liquefaction process indicated that the α-amylase effectively hydrolysed the maize mash. Ca^{2+} and Mg^{2+} have been known to increase temperature stability and can therefore be assumed that both these compounds improved the temperature stability of the enzyme during liquefaction (Gupta *et al*., 2003).

α-Amylase has previously been characterized as an endoenzyme, and is known to catalyse the hydrolysis in the interior of the starch molecule (Gupta *et al*., 2003). Interestingly, the effect of α-amylase *T. lanuginosus* ATCC 58157 on the maize starch granules by SEM indicated that progressive exoerosion had also occurred. α-Amylase

produced from fungi, have previously been reported to display multipliced characteristics (Mishra and Maheshwari, 1996). SEM and TEM are important tools used to characterize the insoluble residues of starches. With the help of SEM exoerosion of starch granules can be observed, while major endoerosion of thin sections of the starch can be observed using TEM. While internal structures are not always visible by SEM, TEM offers an alternate method to observing the internal ultrastructure of starch granules. Further evaluation by SEM and TEM will definitely provide a greater degree of insight into the hydrolysis of maize starch at a cellular level.

The analysis of the nutritional enhancements after starch hydrolysis in this study showed that protein content was increased by 74%. It was previously reported that low protein digestibility of cooked starches are usually attributed to the formation of disulphide-bonded oligomers during cooking (Duodu *et al*., 2002). The hydrolysis of proteins helps break them into smaller parts called peptides, which are collections of two to five amino acid chains which are theoretically absorbed faster. Therefore, it can be assumed that the proteins in the maize starch were also hydrolysed and thus being more readily available for absorption. The effect of improved absorption could also be seen by the high urea levels in the rats indicating higher protein absorption.

During maize hydrolysis, the maize was efficiently converted to high levels of glucose (monosaccharide) maltose and sucrose (disaccharides) and maltotriose (oligosaccharide) at relatively low enzyme doses. Sugars, starches and fibre are the three types of carbohydrates we eat. Not all carbohydrates are created equal. The refined and starchy

carbohydrates are digested into sugars and absorbed much faster than their higher fibre, less processed, more complex sugars.

The Glycemic Index (GI) is a method of measuring blood sugar levels with respect to carbohydrate consumption and absorption. It reflects the relative rate of blood sugar elevation and in turn affects the insulin levels in the blood. High GI foods are those foods that fill you up quickly, giving a burst of energy and then leaving you feeling lethargic in a short time. In comparison the low GI foods, fill you up, but raise the blood sugar levels slowly and gives you a feeling of fullness for longer (Coyne, 1996).

Using the blood glucose results in the feed trial it could be concluded that the unhydrolysed maize mash possibly had a High GI while the hydrolysed maize mash had a low GI and hence the constant blood sugar levels of the test rats. It would have been interesting to have measured the exact energy outputs as a result of consuming the test and positive control diets in relation to GI value.

The quantification of the various nutritional components revealed that hydrolysis of maize mash had the desired effect. All six vitamins evaluated for in the hydrloysed maize mash had improved, with the vitamin A content increasing 3.5 fold to 386.84 µg. Of great significance was the fact that consuming 200g of the hydrolysed maize mash would result in 53% of the RDA for a child under the age of 12 years being met. It is through the digestive system the body is able to make use of the vitamins, minerals and other nutrients that are consumed.

The digestive system breaks down the material until it is in a form that can be of use at a cellular level. Making sure to achieve the standard recommended daily intake levels of these nutrients will help to ensure that your body is able to make the best use of the foods consumed. The improvement in the nutritional status of maize mash offers a novel, simple way to replace the conventional fortification of maize meal with added nutrients. Extensive fortification programs have been adopted world wide to help curb the tide of malnutrition. These programs are often restricted because of the cost implications and the problems associated with reaching those most in need (UNICEF, 2003).

The increased availability of the vitamin A content of the hydrolysed maize mash in this study also offered support for the eradication of vitamin A deficiency. Vitamin A deficiency continues to be a public health problem in many developing countries. Vitamin A deficiency contributes to infant mortality from infectious diseases. The periodic distribution of vitamin A capsules, while effective for those in research is not sustainable without external assistance and rarely achieves satisfactory coverage. Under these circumstances food-based solutions due to vitamin A deficiency have been recommended, but success is limited.

An exception is the pilot programme in a district in north-east Thailand, reported by the Institute of Nutrition of Mahidol University. The programme succeeded on increasing the dietary intake of vitamin A-rich foods, raising the vitamin A status of preschool children and pregnant and lactating mothers, and developing a model for improving vitamin A status through nutrition communication (Smitasiri *et al*., 1993).

When engaging in nutritional improvement programs to eradicate the perils of malnutrition and vitamin and mineral deficiency, one must not lose sight of the safety of these foods. One of the objectives of the feed trial in this study was to determine the safety of the application of α-amylase in the hydrolysis of maize mash to human consumption. The feed trial clearly demonstrated that no adverse hepatic or toxic effects were observed, implying that the nutritionally improved hydrolysed maize mash was safe for human consumption.

Quality and safety of food are important issues, and continue to receive increasing public attention. Industrialized methods of modern food processing have the effect that unsafe food is sometimes used for human consumption prior to confirmation of its safety. Public interest on the use of enzymes and its possible allergic reactions are of growing concern. Currently there have been no reports of consumer allergies due to enzyme residues present in food. The low levels of enzyme residues present in foods unlikely to cause allergies.

Potential solutions that have been adopted by various world organisations include: (UNICEF, 2003)

i. Improving dietary intake of particularly vulnerable population groups.
ii. Fortification by the addition of essential vitamins and minerals to foods that are regularly consumed by a significant proportion of the population.
iii. Supplementation by reaching out to vulnerable groups with multi vitamin and mineral supplements in the form of tablets, capsules and syrups with serious cost implications.
iv. Control of infectious diseases.
v. Educating the communities about the kinds of foods that can increase their nutritional status.

Although, each of these available solutions contain its own difficulties; none is a complete solution in itself; and all need to be pursued simultaneously according to the particular needs and opportunities of each country (UNICEF, 2003). The focus is now on science and research to develop solutions that will work.

The consumption of the hydrolysed maize mash does not necessarily increase body weight excessively, however, it does offer to promote improved protein absorption and carbohydrate metabolism in relatively small doses which can be utilized to successfully improve the nutritional status of the malnourished population in a short time.

The implications of this study could prove to be far-reaching and most fundamentally ending malnutrition and vitamin deficiency will thus, significantly contribute to lifting successive generations of Africans from poverty.

"We have to leave behind the old thinking and act in the light of new knowledge. It is no longer a question of seeking out symptoms of sever deficiency in individuals and treating them. It is a question of reaching out to whole populations to protect them against the devastating consequences of even moderate forms of vitamin and mineral deficiency." Carol Bellamy, Executive director, UNICEF, 2003.

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APPENDICES

APPENDIX 1: Vogel's trace element solution

Citric acid$1H_2O$ 5 g

$ZnSO_4.7H_2O$ 5 g

Fe $(NH_4)_2$ $(SO_4)_2.6$ H_2O 1 g

$CuSO_4.5$ H_2O 0.25 g

Mn $SO_4.1H_2O$ 0.05 g

H_3B0_3 (anhydrous) 0.05 g

Na_2 Mo $O_4.2H_2O$ 0,05 g

Dissolve in distilled water, to a final volume of 100 ml

APPENDIX 2: Anillin reagent

Solution one :

1 g diphenylamine dissolved in 1 ml Anillin reagent and made up to a final volume of 100 ml with acetone.

Solution two:

10 ml of solution one mixed with 1 ml of 85% phosphoric acid.

Appendix 3: Effects for spleen weights between test and control rats using ANOVA

Source	Type III Sum of Squares	df	Mean Square	F	P value
Corrected Model	.846(a)	7	.121	55.394	<0.001
Intercept	1.599	1	1.599	733.094	<0.001
GROUP	.536	2	.268	122.814	<0.001
DAY KILLED	.016	2	.008	3.730	0.035
GROUP * DAY KILLED	.005	2	.002	1.094	0.347
Error	.070	32	.002		
Total	2.455	40			
Corrected Total	.915	39			

a R Squared = .924 (Adjusted R Squared = .907)

Appendix 4: Bonferroni multiple comparison post hoc tests for difference in spleen weight between test and control rats

(I) GROUP	(J) GROUP	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
baseline	test	-.004287	.0241141	1.000	-.072104	.063531
	positive control	-.002993	.0241141	1.000	-.070811	.064824
	negative control	-.437220(*)	.0295337	.000	-.520279	-.354161
test	baseline	.004287	.0241141	1.000	-.063531	.072104
	positive control	.001293	.0170513	1.000	-.046661	.049247
	negative control	-.432933(*)	.0241141	.000	-.500751	-.365116
positive control	baseline	.002993	.0241141	1.000	-.064824	.070811
	test	-.001293	.0170513	1.000	-.049247	.046661
	negative control	-.434227(*)	.0241141	.000	-.502044	-.366409
negative control	baseline	.437220(*)	.0295337	.000	.354161	.520279
	test	.432933(*)	.0241141	.000	.365116	.500751
	positive control	.434227(*)	.0241141	.000	.366409	.502044

Based on observed means.

*The mean difference is significant at the .05 level.

Appendix 5: Bonferroni multiple comparison post hoc tests for difference in spleen weight between time of killing for test and control rats

(I) day killed	(J) day killed	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
0	7	.009580	.0255769	1.000	-.062351	.081511
	14	.015870	.0255769	1.000	-.056061	.087801
	21	-.169987(*)	.0241141	.000	-.237804	-.102169
7	0	-.009580	.0255769	1.000	-.081511	.062351
	14	.006290	.0208835	1.000	-.052441	.065021
	21	-.179567(*)	.0190639	.000	-.233181	-.125952
14	0	-.015870	.0255769	1.000	-.087801	.056061
	7	-.006290	.0208835	1.000	-.065021	.052441
	21	-.185857(*)	.0190639	.000	-.239471	-.132242
21	0	.169987(*)	.0241141	.000	.102169	.237804
	7	.179567(*)	.0190639	.000	.125952	.233181
	14	.185857(*)	.0190639	.000	.132242	.239471

Based on observed means.

*The mean difference is significant at the .05 level.

Appendix 6: Effects for kidney weights between test and control rats using ANOVA

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	5.621(a)	7	.803	145.350	<0.001
Intercept	23.910	1	23.910	4328.252	<0.001
DAY KILLED	.082	2	.041	7.437	0.002
GROUP	3.600	2	1.800	325.841	<0.001
DAY KILLED * GROUP	.008	2	.004	.714	0.497
Error	.177	32	.006		
Total	30.845	40			
Corrected Total	5.797	39			

a R Squared = .970 (Adjusted R Squared = .963)

Appendix 7: Bonferroni multiple comparison post hoc tests for difference in kidney weight between test and control rats

(I) GROUP	(J) GROUP	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
baseline	test	-.131080(*)	.0383814	.011	-.239022	-.023138
	positive control	-.066253	.0383814	.564	-.174195	.041688
	negative control	-1.201440(*)	.0470075	.000	-1.333641	-1.069239
test	baseline	.131080(*)	.0383814	.011	.023138	.239022
	positive control	.064827	.0271398	.138	-.011500	.141153
	negative control	-1.070360(*)	.0383814	.000	-1.178302	-.962418
positive control	baseline	.066253	.0383814	.564	-.041688	.174195
	test	-.064827	.0271398	.138	-.141153	.011500
	negative control	-1.135187(*)	.0383814	.000	-1.243128	-1.027245
negative control	baseline	1.201440(*)	.0470075	.000	1.069239	1.333641
	test	1.070360(*)	.0383814	.000	.962418	1.178302
	positive control	1.135187(*)	.0383814	.000	1.027245	1.243128

Based on observed means.
* The mean difference is significant at the .05 level.

Appendix 8: Bonferroni multiple comparison post hoc tests for difference in kidney weight between time of killing for test and control rats

(I) day killed	(J) day killed	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
0	7	-.039500	.0407097	1.000	-.153990	.074990
	14	-.089740	.0407097	.209	-.204230	.024750
	21	-.511653(*)	.0383814	.000	-.619595	-.403712
7	0	.039500	.0407097	1.000	-.074990	.153990
	14	-.050240	.0332393	.843	-.143720	.043240
	21	-.472153(*)	.0303432	.000	-.557489	-.386818
14	0	.089740	.0407097	.209	-.024750	.204230
	7	.050240	.0332393	.843	-.043240	.143720
	21	-.421913(*)	.0303432	.000	-.507249	-.336578
21	0	.511653(*)	.0383814	.000	.403712	.619595
	7	.472153(*)	.0303432	.000	.386818	.557489
	14	.421913(*)	.0303432	.000	.336578	.507249

Based on observed means.

*The mean difference is significant at the .05 level.

Appendix 9: Effects for stomach using ANOVA

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	102.749(a)	7	14.678	30.385	<0.001
Intercept	157.287	1	157.287	325.585	<0.001
DAY KILLED	3.147	2	1.574	3.257	0.052
GROUP	57.806	2	28.903	59.829	<0.001
DAY KILLED * GROUP	1.189	2	.595	1.231	0.305
Error	15.459	32	.483		
Total	282.720	40			
Corrected Total	118.208	39			

a R Squared = .869 (Adjusted R Squared = .841)

Appendix 10: Bonferroni multiple comparison post hoc tests for difference in stomach weight between test and control rats

(I) GROUP	(J) GROUP	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
baseline	test	-1.215280(*)	.3589202	.011	-2.224687	-.205873
	positive control	-.510187	.3589202	.989	-1.519594	.499220
	negative control	-5.312560(*)	.4395857	.000	-6.548826	-4.076294
test	baseline	1.215280(*)	.3589202	.011	.205873	2.224687
	positive control	.705093	.2537949	.054	-.008665	1.418852
	negative control	-4.097280(*)	.3589202	.000	-5.106687	-3.087873
positive control	baseline	.510187	.3589202	.989	-.499220	1.519594
	test	-.705093	.2537949	.054	-1.418852	.008665
	negative control	-4.802373(*)	.3589202	.000	-5.811780	-3.792966
negative control	baseline	5.312560(*)	.4395857	.000	4.076294	6.548826
	test	4.097280(*)	.3589202	.000	3.087873	5.106687
	positive control	4.802373(*)	.3589202	.000	3.792966	5.811780

Based on observed means.

*The mean difference is significant at the .05 level.

Appendix 11: Bonferroni multiple comparison post hoc tests for difference in stomach weight between time of killing for test and control rats

(I) day killed	(J) day killed	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
0	7	-.497550	.3806924	1.000	-1.568188	.573088
	14	-.805880	.3806924	.253	-1.876518	.264758
	21	-2.627367(*)	.3589202	.000	-3.636774	-1.617960
7	0	.497550	.3806924	1.000	-.573088	1.568188
	14	-.308330	.3108340	1.000	-1.182502	.565842
	21	-2.129817(*)	.2837513	.000	-2.927823	-1.331810
14	0	.805880	.3806924	.253	-.264758	1.876518
	7	.308330	.3108340	1.000	-.565842	1.182502
	21	-1.821487(*)	.2837513	.000	-2.619493	-1.023480
21	0	2.627367(*)	.3589202	.000	1.617960	3.636774
	7	2.129817(*)	.2837513	.000	1.331810	2.927823
	14	1.821487(*)	.2837513	.000	1.023480	2.619493

Based on observed means.

*The mean difference is significant at the .05 level.

Appendix 12: Effects for heart weights between test and control rats using ANOVA

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	.824(a)	7	.118	52.323	<0.001
Intercept	5.370	1	5.370	2385.918	<0.001
DAY KILLED	.007	2	.003	1.533	0.231
GROUP	.517	2	.259	114.851	<0.001
DAY KILLED * GROUP	.006	2	.003	1.248	0.301
Error	.072	32	.002		
Total	6.750	40			
Corrected Total	.896	39			

a R Squared = .920 (Adjusted R Squared = .902)

Appendix 13: Bonferroni multiple comparison post hoc tests for difference in heart weight between treatment groups

(I) GROUP	(J) GROUP	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
baseline	test	-.094047(*)	.0244991	.003	-.162947	-.025147
	positive control	-.057707	.0244991	.149	-.126607	.011193
	negative control	-.486540(*)	.0300052	.000	-.570925	-.402155
test	baseline	.094047(*)	.0244991	.003	.025147	.162947
	positive control	.036340	.0173235	.263	-.012380	.085060
	negative control	-.392493(*)	.0244991	.000	-.461393	-.323593
positive control	baseline	.057707	.0244991	.149	-.011193	.126607
	test	-.036340	.0173235	.263	-.085060	.012380
	negative control	-.428833(*)	.0244991	.000	-.497733	-.359933
negative control	baseline	.486540(*)	.0300052	.000	.402155	.570925
	test	.392493(*)	.0244991	.000	.323593	.461393
	positive control	.428833(*)	.0244991	.000	.359933	.497733

Based on observed means.

*The mean difference is significant at the .05 level.

Appendix 14: Bonferroni multiple comparison post hoc tests for difference in heart weight between time of killing

(I) day killed	(J) day killed	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
0	7	-.060470	.0259853	.159	-.133549	.012609
	14	-.070660	.0259853	.063	-.143739	.002419
	21	-.226513(*)	.0244991	.000	-.295413	-.157613
7	0	.060470	.0259853	.159	-.012609	.133549
	14	-.010190	.0212169	1.000	-.069859	.049479
	21	-.166043(*)	.0193683	.000	-.220514	-.111573
14	0	.070660	.0259853	.063	-.002419	.143739
	7	.010190	.0212169	1.000	-.049479	.069859
	21	-.155853(*)	.0193683	.000	-.210324	-.101383
21	0	.226513(*)	.0244991	.000	.157613	.295413
	7	.166043(*)	.0193683	.000	.111573	.220514
	14	.155853(*)	.0193683	.000	.101383	.210324

Based on observed means.

*The mean difference is significant at the .05 level.

Appendix 15: Bonferroni multiple comparison post hoc tests for difference in heart weight between treatment groups

(I) GROUP	(J) GROUP	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
baseline	test	-1.144593(*)	.2387418	.000	-1.816017	-.473169
	positive control	-.503853	.2387418	.256	-1.175277	.167571
	negative control	-7.895740(*)	.2923978	.000	-8.718063	-7.073417
test	baseline	1.144593(*)	.2387418	.000	.473169	1.816017
	positive control	.640740(*)	.1688160	.004	.165972	1.115508
	negative control	-6.751147(*)	.2387418	.000	-7.422571	-6.079723
positive control	baseline	.503853	.2387418	.256	-.167571	1.175277
	test	-.640740(*)	.1688160	.004	-1.115508	-.165972
	negative control	-7.391887(*)	.2387418	.000	-8.063311	-6.720463
negative control	baseline	7.895740(*)	.2923978	.000	7.073417	8.718063
	test	6.751147(*)	.2387418	.000	6.079723	7.422571
	positive control	7.391887(*)	.2387418	.000	6.720463	8.063311

Based on observed means.

*The mean difference is significant at the .05 level.

Appendix 16: Effects of liver weights between test and control rats using ANOVA

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	232.798(a)	7	33.257	155.594	<0.001
Intercept	519.882	1	519.882	2432.298	<0.001
DAY KILLED	.557	2	.279	1.303	0.286
GROUP	163.385	2	81.692	382.203	<0.001
DAY KILLED* GROUP	.126	2	.063	.295	0.746
Error	6.840	32	.214		
Total	757.075	40			
Corrected Total	239.638	39			

a R Squared = .971 (Adjusted R Squared = .965)

Appendix 17: Bonferroni multiple comparison post hoc tests for difference in liver weight between time of killing

(I) day killed	(J) day killed	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
0	7	-.638240	.2532239	.101	-1.350393	.073913
	14	-.873510(*)	.2532239	.010	-1.585663	-.161357
	21	-3.272527(*)	.2387418	.000	-3.943951	-2.601103
7	0	.638240	.2532239	.101	-.073913	1.350393
	14	-.235270	.2067565	1.000	-.816740	.346200
	21	-2.634287(*)	.1887420	.000	-3.165094	-2.103479
14	0	.873510(*)	.2532239	.010	.161357	1.585663
	7	.235270	.2067565	1.000	-.346200	.816740
	21	-2.399017(*)	.1887420	.000	-2.929824	-1.868209
21	0	3.272527(*)	.2387418	.000	2.601103	3.943951
	7	2.634287(*)	.1887420	.000	2.103479	3.165094
	14	2.399017(*)	.1887420	.000	1.868209	2.929824

Based on observed means.

*The mean difference is significant at the .05 level.

Appendix 18: Effects of length of small intestine between test and control rats using ANOVA

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	3241.575(a)	7	463.082	35.802	<0.001
Intercept	208222.027	1	208222.027	16098.345	<0.001
GROUP	2528.333	2	1264.167	97.737	<0.001
DAY KILLED	54.600	2	27.300	2.111	0.138
GROUP * DAY KILLED	70.200	2	35.100	2.714	0.082
Error	413.900	32	12.934		
Total	236370.500	40			
Corrected Total	3655.475	39			

a R Squared = .887 (Adjusted R Squared = .862)

Appendix 19: Bonferroni multiple comparison post hoc tests for difference in small intestine length between treatment groups

(I) GROUP	(J) GROUP	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
baseline	test	.80	1.857	1.000	-4.42	6.02
	positive control	-.20	1.857	1.000	-5.42	5.02
	negative control	-26.40(*)	2.275	.000	-32.80	-20.00
test	baseline	-.80	1.857	1.000	-6.02	4.42
	positive control	-1.00	1.313	1.000	-4.69	2.69
	negative control	-27.20(*)	1.857	.000	-32.42	-21.98
positive control	baseline	.20	1.857	1.000	-5.02	5.42
	test	1.00	1.313	1.000	-2.69	4.69
	negative control	-26.20(*)	1.857	.000	-31.42	-20.98
negative control	baseline	26.40(*)	2.275	.000	20.00	32.80
	test	27.20(*)	1.857	.000	21.98	32.42
	positive control	26.20(*)	1.857	.000	20.98	31.42

Based on observed means.

*The mean difference is significant at the .05 level.

Appendix 20: Bonferroni multiple comparison post hoc tests for difference in small intestine length between time of killing

(I) day killed	(J) day killed	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
0	7	-1.60	1.970	1.000	-7.14	3.94
	14	1.40	1.970	1.000	-4.14	6.94
	21	-8.07(*)	1.857	.001	-13.29	-2.84
7	0	1.60	1.970	1.000	-3.94	7.14
	14	3.00	1.608	.428	-1.52	7.52
	21	-6.47(*)	1.468	.001	-10.60	-2.34
14	0	-1.40	1.970	1.000	-6.94	4.14
	7	-3.00	1.608	.428	-7.52	1.52
	21	-9.47(*)	1.468	.000	-13.60	-5.34
21	0	8.07(*)	1.857	.001	2.84	13.29
	7	6.47(*)	1.468	.001	2.34	10.60
	14	9.47(*)	1.468	.000	5.34	13.60

Based on observed means.

* The mean difference is significant at the .05 level.

Appendix 21: Effects of length of large intestine between test and control rats using ANOVA

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	187.100(a)	7	26.729	18.473	<0.001
Intercept	5679.245	1	5679.245	3925.180	<0.001
DAY KILLED	4.867	2	2.433	1.682	0.202
GROUP	163.500	2	81.750	56.501	<0.001
DAY KILLED * GROUP	1.400	2	.700	.484	0.621
Error	46.300	32	1.447		
Total	6433.500	40			
Corrected Total	233.400	39			

a R Squared = .802 (Adjusted R Squared = .758)

Appendix 22: Bonferroni multiple comparison post hoc tests for difference in large intestine length between test and control rats groups

(I) GROUP	(J) GROUP	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
baseline	test	-.47	.621	1.000	-2.21	1.28
	positive control	.33	.621	1.000	-1.41	2.08
	negative control	-6.40(*)	.761	.000	-8.54	-4.26
test	baseline	.47	.621	1.000	-1.28	2.21
	positive control	.80	.439	.467	-.44	2.04
	negative control	-5.93(*)	.621	.000	-7.68	-4.19
positive control	baseline	-.33	.621	1.000	-2.08	1.41
	test	-.80	.439	.467	-2.04	.44
	negative control	-6.73(*)	.621	.000	-8.48	-4.99
negative control	baseline	6.40(*)	.761	.000	4.26	8.54
	test	5.93(*)	.621	.000	4.19	7.68
	positive control	6.73(*)	.621	.000	4.99	8.48

Based on observed means.
* The mean difference is significant at the .05 level.

Appendix 23: Bonferroni multiple comparison post hoc tests for difference in large intestine length between time of killing

(I) day killed	(J) day killed	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
0	7	-.40	.659	1.000	-2.25	1.45
	14	-.30	.659	1.000	-2.15	1.55
	21	-1.80(*)	.621	.040	-3.55	-.05
7	0	.40	.659	1.000	-1.45	2.25
	14	.10	.538	1.000	-1.41	1.61
	21	-1.40(*)	.491	.045	-2.78	-.02
14	0	.30	.659	1.000	-1.55	2.15
	7	-.10	.538	1.000	-1.61	1.41
	21	-1.50(*)	.491	.027	-2.88	-.12
21	0	1.80(*)	.621	.040	.05	3.55
	7	1.40(*)	.491	.045	.02	2.78
	14	1.50(*)	.491	.027	.12	2.88

*Based on observed means.
The mean difference is significant at the .05 level.

Appendix 24: Effects for triglycerides using ANOVA

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	1.364(a)	7	.195	19.985	<0.001
Intercept	10.464	1	10.464	1073.204	<0.001
GROUP	1.121	2	.561	57.504	<0.001
DAY KILLED	.081	2	.040	4.137	0.025
GROUP * DAY KILLED	.018	2	.009	.923	0.408
Error	.312	32	.010		
Total	11.280	40			
Corrected Total	1.676	39			

a R Squared = .814 (Adjusted R Squared = .773)

Appendix 25: Bonferroni multiple comparison post hoc tests for difference in triglycerides between test and control rats

(I) GROUP	(J) GROUP	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
baseline	test	.267(*)	.0510	.000	.123	.410
	positive control	.267(*)	.0510	.000	.123	.410
	negative control	-.240(*)	.0624	.003	-.416	-.064
test	baseline	-.267(*)	.0510	.000	-.410	-.123
	positive control	.000	.0361	1.000	-.101	.101
	negative control	-.507(*)	.0510	.000	-.650	-.363
positive control	baseline	-.267(*)	.0510	.000	-.410	-.123
	test	.000	.0361	1.000	-.101	.101
	negative control	-.507(*)	.0510	.000	-.650	-.363
negative control	baseline	.240(*)	.0624	.003	.064	.416
	test	.507(*)	.0510	.000	.363	.650
	positive control	.507(*)	.0510	.000	.363	.650

Based on observed means.

*The mean difference is significant at the .05 level.

Appendix 26: Bonferroni multiple comparison post hoc tests for difference in triglycerides between time of killing.

(I) day killed	(J) day killed	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
0	7	.230(*)	.0541	.001	.078	.382
	14	.230(*)	.0541	.001	.078	.382
	21	.147(*)	.0510	.043	.003	.290
7	0	-.230(*)	.0541	.001	-.382	-.078
	14	.000	.0442	1.000	-.124	.124
	21	-.083	.0403	.281	-.197	.030
14	0	-.230(*)	.0541	.001	-.382	-.078
	7	.000	.0442	1.000	-.124	.124
	21	-.083	.0403	.281	-.197	.030
21	0	-.147(*)	.0510	.043	-.290	-.003
	7	.083	.0403	.281	-.030	.197
	14	.083	.0403	.281	-.030	.197

Based on observed means.

*The mean difference is significant at the .05 level.

Appendix 27: Effects for cholesterol using ANOVA

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	1.003(a)	7	.143	2.058	0.078
Intercept	92.137	1	92.137	1323.339	<0.001
DAY KILLED	.554	2	.277	3.978	0.029
GROUP	.048	2	.024	.347	0.709
DAY KILLED * GROUP	.266	2	.133	1.910	0.165
Error	2.228	32	.070		
Total	114.120	40			
Corrected Total	3.231	39			

a R Squared = .310 (Adjusted R Squared = .160)

Appendix 28: Bonferroni multiple comparison post hoc tests for difference in cholesterol between treatment groups

(I) GROUP	(J) GROUP	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
baseline	test	-.220	.1363	.697	-.603	.163
	positive control	-.160	.1363	1.000	-.543	.223
	negative control	-.180	.1669	1.000	-.649	.289
test	baseline	.220	.1363	.697	-.163	.603
	positive control	.060	.0964	1.000	-.211	.331
	negative control	.040	.1363	1.000	-.343	.423
positive control	baseline	.160	.1363	1.000	-.223	.543
	test	-.060	.0964	1.000	-.331	.211
	negative control	-.020	.1363	1.000	-.403	.363
negative control	baseline	.180	.1669	1.000	-.289	.649
	test	-.040	.1363	1.000	-.423	.343
	positive control	.020	.1363	1.000	-.363	.403

Based on observed means.

Appendix 29: Bonferroni multiple comparison post hoc tests for difference in cholesterol between time of killing

(I) day killed	(J) day killed	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
0	7	.000	.1445	1.000	-.406	.406
	14	-.310	.1445	.238	-.716	.096
	21	-.233	.1363	.579	-.617	.150
7	0	.000	.1445	1.000	-.406	.406
	14	-.310	.1180	.079	-.642	.022
	21	-.233	.1077	.227	-.536	.070
14	0	.310	.1445	.238	-.096	.716
	7	.310	.1180	.079	-.022	.642
	21	.077	.1077	1.000	-.226	.380
21	0	.233	.1363	.579	-.150	.617
	7	.233	.1077	.227	-.070	.536
	14	-.077	.1077	1.000	-.380	.226

Based on observed means.

Appendix 30: Effects urea levels between test and control rats using ANOVA

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	42.460(a)	7	6.066	10.018	<0.001
Intercept	1553.909	1	1553.909	2566.324	<0.001
DAY KILLED	19.973	2	9.986	16.493	<0.001
GROUP	4.864	2	2.432	4.017	0.028
DAY KILLED * GROUP	8.450	2	4.225	6.978	0.003
Error	19.376	32	.605		
Total	1889.740	40			
Corrected Total	61.836	39			

a R Squared = .687 (Adjusted R Squared = .618)

Appendix 31: Bonferroni multiple comparison post hoc tests for difference in urea between test and control groups

(I) GROUP	(J) GROUP	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
baseline	test	-1.453(*)	.4018	.006	-2.583	-.323
	positive control	-1.933(*)	.4018	.000	-3.063	-.803
	negative control	-1.520(*)	.4921	.025	-2.904	-.136
test	baseline	1.453(*)	.4018	.006	.323	2.583
	positive control	-.480	.2841	.605	-1.279	.319
	negative control	-.067	.4018	1.000	-1.197	1.063
positive control	baseline	1.933(*)	.4018	.000	.803	3.063
	test	.480	.2841	.605	-.319	1.279
	negative control	.413	.4018	1.000	-.717	1.543
negative control	baseline	1.520(*)	.4921	.025	.136	2.904
	test	.067	.4018	1.000	-1.063	1.197
	positive control	-.413	.4018	1.000	-1.543	.717

Based on observed means.
* The mean difference is significant at the .05 level.

Appendix 32: Bonferroni multiple comparison post hoc tests for difference in urea levels between time of killing for test and control rats

(I) day killed	(J) day killed	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
0	7	-2.130(*)	.4262	.000	-3.329	-.931
	14	-2.400(*)	.4262	.000	-3.599	-1.201
	21	-.873	.4018	.224	-2.003	.257
7	0	2.130(*)	.4262	.000	.931	3.329
	14	-.270	.3480	1.000	-1.249	.709
	21	1.257(*)	.3177	.002	.363	2.150
14	0	2.400(*)	.4262	.000	1.201	3.599
	7	.270	.3480	1.000	-.709	1.249
	21	1.527(*)	.3177	.000	.633	2.420
21	0	.873	.4018	.224	-.257	2.003
	7	-1.257(*)	.3177	.002	-2.150	-.363
	14	-1.527(*)	.3177	.000	-2.420	-.633

Based on observed means.
*The mean difference is significant at the .05 level.

Appendix 33: Effects of glucose between test and control rats using ANOVA

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	45.796(a)	7	6.542	1.193	0.335
Intercept	2115.726	1	2115.726	385.668	<0.001
DAY KILLED	6.093	2	3.046	.555	0.579
GROUP	23.196	2	11.598	2.114	0.137
DAY KILLED * GROUP	8.150	2	4.075	.743	0.484
Error	175.548	32	5.486		
Total	2768.560	40			
Corrected Total	221.344	39			

a R Squared = .207 (Adjusted R Squared = .033)

Appendix 34: Bonferroni multiple comparison post hoc tests for difference in glucose between test and control rats

(I) GROUP	(J) GROUP	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
baseline	test	-.433	1.2095	1.000	-3.835	2.968
	positive control	-2.153	1.2095	.507	-5.555	1.248
	negative control	-.400	1.4813	1.000	-4.566	3.766
test	baseline	.433	1.2095	1.000	-2.968	3.835
	positive control	-1.720	.8552	.317	-4.125	.685
	negative control	.033	1.2095	1.000	-3.368	3.435
positive control	baseline	2.153	1.2095	.507	-1.248	5.555
	test	1.720	.8552	.317	-.685	4.125
	negative control	1.753	1.2095	.941	-1.648	5.155
negative control	baseline	.400	1.4813	1.000	-3.766	4.566
	test	-.033	1.2095	1.000	-3.435	3.368
	positive control	-1.753	1.2095	.941	-5.155	1.648

Based on observed means.

Appendix 35: Bonferroni multiple comparison post hoc tests for difference in urea between time of killing

(I) day killed	(J) day killed	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
0	7	-1.000	1.2829	1.000	-4.608	2.608
	14	-1.930	1.2829	.854	-5.538	1.678
	21	-.767	1.2095	1.000	-4.168	2.635
7	0	1.000	1.2829	1.000	-2.608	4.608
	14	-.930	1.0475	1.000	-3.876	2.016
	21	.233	.9562	1.000	-2.456	2.922
14	0	1.930	1.2829	.854	-1.678	5.538
	7	.930	1.0475	1.000	-2.016	3.876
	21	1.163	.9562	1.000	-1.526	3.852
21	0	.767	1.2095	1.000	-2.635	4.168
	7	-.233	.9562	1.000	-2.922	2.456
	14	-1.163	.9562	1.000	-3.852	1.526

Based on observed means.