Stabilisation and Encapsulation Studies on Xylanase for Animal Feed Improvement

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The candidate confirms that the work submitted is her own and that appropriate credit has been given where reference has been made to the work of others

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Abstract

Pig and poultry feeds contain materials that are derived from plant and animals. Most of the plant materials are indigestible because they contain non-starch polysaccharides and as a result the animal suffers from anti-nutritional effects. To reduce the anti-nutritional effects, a number of enzymes, including xylanase, are added to the feed to break down the non-starch polysaccharides. Prior to ingestion, the feed must be processed to destroy any microbial contaminants. As a consequence of this action, the enzymes become inactivated due to the high temperatures of processing. The aim of this project was to improve the quality of the feed by preventing the degradation of enzymes during processing.

In order to carry out a thorough investigation to improve the stability of xylanase, a full characterisation profile was determined first. The denaturation of xylanase when exposed to stressful conditions was monitored by circular dichroism spectroscopy. In most occasions, in the presence of low molecular weight additives, xylanase had enhanced activity and improved structural stability. Stabilisation by immobilisation on two support materials such as modified silica and chitosan improved the thermal stability of xylanase. Conditions typically reproduced within a processing cycle were used to investigate the stability of the immobilised xylanase. Microencapsulation of xylanase was also carried out by spray drying with stabilising polymers and by phase separation methods. The enzyme activities following each formulation were determined. The morphology of the microspheres produced was examined using scanning electron microscopy.

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Abbreviations

APS	3-aminopropyltriethoxysilane
BCA	Bicinchoninic acid
CD	Circular dichroism
DNSA	Dinitrosalicyclic acid
ΔG	Gibb's free energy change
ΔH	Enthalpy change
IR	Infrared
ITCPS	3-isothiocyanatopropyltriethoxysilane
NMR	Nulcear Magnetic Resonance
NSP	Non starch polysaccharide
PAGE	Polyacrylamide gel electrophoresis
PEG	Polyethylene glycol
PEI	Polyethylene imine
ΔS	Entropy change
SDS	Sodium dodecyl sulphate
SEM	Scanning electron microscopy
TEMED	Tetra methylethylenediamine
TLC	Thin layer chromatography
Tris	Tris hydroxymethylaminoethane
U	International Unit

Chapter 1

General Introduction

1.1 World-wide production of pig and poultry feed

The average human adult obtains some 16 % of their daily energy requirements and 36 % of their daily protein intake from meat, fish, dairy products (milk, cheese) and eggs (Bradford, 1999). These products are very good sources of amino acids, minerals and vitamins. In terms of global animal feed production poultry accounts for 34 % and pigs 32 % (Cowan, 1996). The growing popularity of poultry and pig meat is mainly due to the growing trend to consume white meat rather than red meat in both developing and developed countries (Holness, 1991). More poultry meat is produced than pork due to economic factors and there are very few social and religious taboos concerning the consumption of poultry products (Rose, 1997). On the contrary, pig meat is forbidden in the Islamic and Jewish faiths because pigs are considered to be unclean animals, but in some Pacific Islands pigs are associated with marriage and regarded as a source of wealth. Despite the religious issues, pork products are becoming increasingly common (Holness, 1991).

Keeping farm animals healthy and feeding them with nutritious food is an important element in manufacturing meat and meat products (Bonneau and Laarveld, 1999). Farm animals consume one third of the world-wide production of grain and they are also fed food that are inedible to humans such as crop residue, food and fibre by-products and forage (Bradford, 1999). Animal farm feeds produced in industrialised countries account for around 34 % of total world production (Cowan, 1996).

1.2 Pig and poultry nutrition

Digestion of food involves the physical, chemical and enzymatic breakdown of complex plant and animal materials. The processes taking place within the digestive tract of poultry are broadly similar to pigs and humans (Holness, 1991). The major groups of essential nutrients are energy, protein, minerals, vitamins and water.

1.2.1 Physiology of the pig digestive tract

Pigs are omnivores and will consume a wide range of feed from plant and animal sources. Pigs, like poultry are non-ruminant and unable to digest cellulose and other complex carbohydrates (Holness, 1991). A diagram of a mammal (non-ruminant) digestive system is shown in Figure 1.1.

1.2.1.2 The alimentary canal

Mouth – Food intake is ground into a pulp, moistened and mixed with saliva containing the enzyme phytalin which initiates the breakdown of starch to simple carbohydrates.

Stomach – Stomachs of adult pigs have a large microbial population, which enables them to digest large quantities of fibrous materials. The stomachs of growing pigs have little or no microbes thus their ability to digest fibre is limited. The stomach provides an acid environment and when the feed enters it buffers the stomach acid to a pH of approximately 4.5 (Cowan, 1996). The gastric juices contain the enzyme pepsin and other enzymes which begin the breakdown of proteins.

Small intestine - The digestive juices from the pancreas, liver and the small intestine complete the process of digestion. On entering the small intestine the pH rises to between 5.8 and 6.5 depending on the feed. Starch is hydrolysed to maltose. The enzymes present in the intestinal juices are maltase, lactase and sucrase. Trypsin in the pancreatic juices breaks down proteins to produce polypeptides, which are further broken down to amino acids by various peptidases. The bile, which is secreted by the liver, emulsifies fats into smaller globules that are then broken down by lipase present in both pancreatic and intestinal juices, to fatty acids and glycerol ready for absorption.

Caecum - the bacteria in the caecum can ferment small amounts of fibre to fatty acids such as acetic acid, proprionic and lactic acid (Holness, 1991).

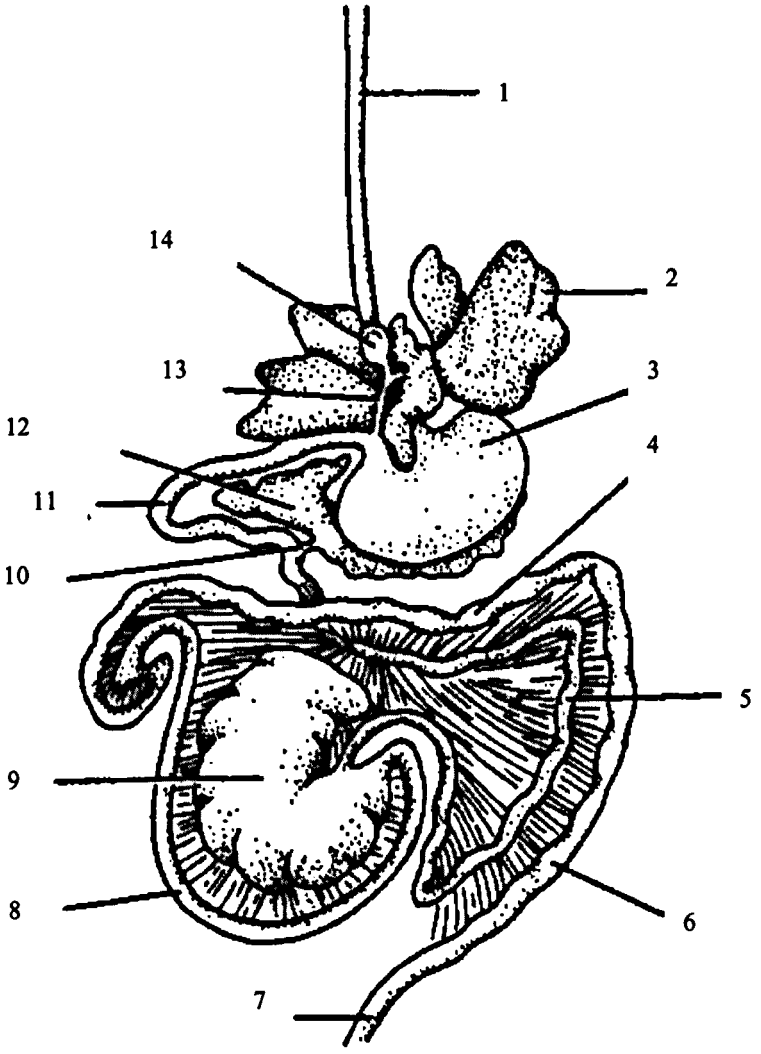


Figure 1.1 Diagram of digestive tract of a non-ruminant mammal (Withers, 1992). 1, oesophgus; 2, liver; 3, stomach; 4, transverse colon; 5, small intestine; 6, descending colon; 7, rectum; 8, ascending colon; 9 caecum; 10, pancreatic duct; 11, duodenum; 12, panceas; 13, bile duct; 14, gallbladder.

1.2.2 Physiology of the poultry digestive tract

The digestive tracts of poultry are lighter, shorter and food passes through very quickly as compared to other non-ruminants and so it is important that the poultry have an efficient digestive tract.

1.2.2.1 The alimentary canal

Mouth – the tongue and beak are important for the manipulation of the food. The salivary gland secretes mucus, which is necessary for lubrication to aid in movement of digesta. Low amounts of amylase are secreted but play an important part in the later stages.

Oesophagus and crop – Secretion of mucus and amylase for the initial stages of carbohydrate digestion occurs in the crop. The crop size ranges significantly between different species and sexes. The typical pH of crop material is 4.5.

Proventriculus and gizzard – These are the granular stomach and the muscular stomach respectively. Their function is to mechanically break up feed and secrete acid and pepsinogen, which is later converted to pepsin. The pH of pure gastric secretion is about 2, but the pH within these organs is usually higher because the secretions are diluted by digesta.

Intestine – The intestine secretes amylase, saccharidases, bile, lipase and trypsin, which are capable of digesting starch, sucrose, fat and protein. The distribution of trypsin and amylase in different segments of the chicken duodenum has been determined (Bird, 1971). The first three quarters secretes 45 % trypsin and 23 % amylase and the remaining quarter secretes 55 % and 77 % respectively (Bird, 1971). The pH in this section ranges between pH 5.7 and pH 6.4.

Ceca – The ceca is found at the junction between the small intestine and the large intestine. Its main function is the microbial fermentation of dietary fibre. The typical pH within the ceca is 5.7 (Sturkie, 1976).

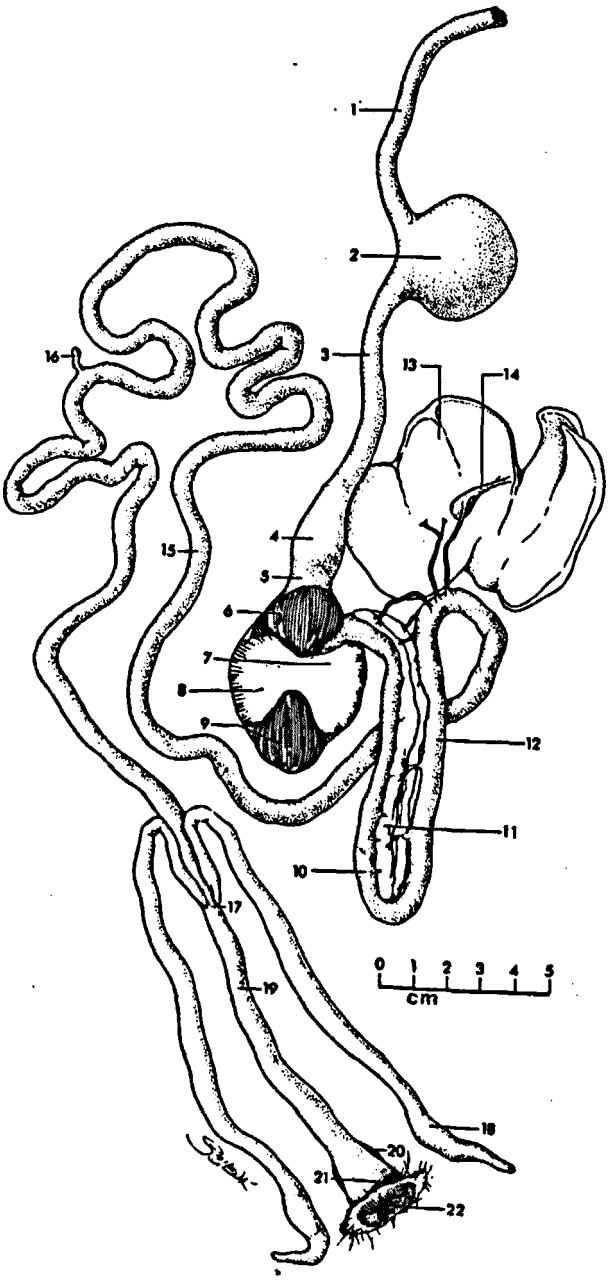


Figure 1.2 Diagram of digestive tract of a turkey.

1, pre-crop oesophgus; 2, crop; 3, post crop; 4, provenentriculus; 5, isthmus; 6, thin craniodorsal; 7, thick cranionentral; 8, thick caudodorsal muscle; 9, thin caudoventral muscle; 10, proximal duodenum; 11, pancreas; 12, distal duodenum; 13, liver; 14, gallbladder; 15, ileum; 16, Meckel's diverticulum; 17, ileocecocolic junction; 18, ceca; 19, rectum; 20, bursa of Fabricius; 21, cloaca; 22, vent (Duke, 1984).

1.2.3 Pig and poultry diets

Pigs and poultry have very similar diets. In all cases, the amount of nutrient required is dependent on their age, size, sex, practicality and the genotype of the animal (Batterham, 1994).

1.2.3.1 Energy

Energy, the most critical of all nutrients, is needed for survival, growth and reproduction. The total energy value in feed describes the gross energy, but not all the energy available in feed will be used up by the animal. Some will be lost in the excreta. The metabolised energy is the conventional system of describing the energy content available in food. For instance, wheat has a gross energy of 16.9 MJ Kg^{-1}. From wheat poultry and pigs can obtain metabolic energy of 13.1 MJ Kg^{-1} (Rose, 1997) and 14.1 MJ Kg^{-1} respectively. High levels of metabolic energy are also obtained from fats and oils.

Poultry have high levels of wheat in their diet because wheat is cheaper than maize and has a higher energy content. Maize is limited in poultry diets because it causes yellow pigmentation effects and a greater wheat content in their diet facilitates maintenance of white skin in broiler chickens (Ziggers, 1998).

1.2.3.2 Protein

Protein is needed for growth and tissue development. The balance of amino acids in proteins is very important for poultry and pig nutrition. The amino acids required for their diets can be divided into two groups, the non-essential amino acids and the essential amino acids. The non-essential amino acids are synthesised whilst the essential amino acids have to be obtained via dietary intake. In the case of poultry, some non-essential amino acids are synthesised too slowly in the body and therefore also need to be supplied in the diet (Rose, 1997). Amino acids required in the diets of both pigs and poultry are listed in Table 1.1. Pigs have a high requirement for lysine and without it they are not able to produce substantial muscle tissue. Lysine is called the limiting amino acid, which means that the amount of protein the animal can take is limited by the intake of lysine (Batterham, 1994). For poultry, only a small increase in the amount of methionine and cystine is needed to get a relatively large increase in egg output of between 50 % and 95 % Rose, (1997). Cereals and beans contribute up to 16 %

(Batterham, 1994) and 50 % (Rose, 1997) respectively of the crude proteins required in the feed. A list of classified feed components with percentage amount of protein and fibre is shown in Table 1.2.

1.2.3.3 Minerals and vitamins

The minerals required in pig and poultry feed are calcium, phosphorus, sodium, potassium, magnesium and chloride. All are required in large amounts and are essential for bone development, lactation in sows and egg laying in poultry. Phytase improves the availability of organic phosphorous found in cereal and vegetable proteins (Cowan, 1996) by breaking down phytic acid to inorganic phosphorus and inositol (Sebastian *et al*, 1998). Minerals like copper, iodine, iron, manganese, selenium and zinc are all needed in trace amounts. Vitamins are divided into two groups; fat-soluble vitamins and the water-soluble vitamins. Normally the fat-soluble vitamins like vitamin A, E, D and K are only needed in trace amounts and can be stored in the liver.

Amino- acid	**Optimum requirements**	
	chicks	**Pigs**
Lysine	1.00 (1.00)	1.00
Methionine and cystine	0.75 (0.86)	0.50
Threonine	0.63 (0.69)	0.60
Tryptophan	0.18 (0.24)	0.14
Isoleucine	0.72 (0.78)	0.54
Leucine	1.25 (1.14)	1.00
Histidine	0.40 (0.25)	0.33
Phenylalanine and tyrosine	1.21 (1.25)	0.96
Valine	0.79 (0.87)	0.70
Glycine and serine	1.31 (0.78)	-
Arginine	1.05 (1.06)	-

Table 1.1 List of essential amino acids required in domestic fowl (Rose, 1997) and growing pigs (Holness, 1991) relative to lysine. In brackets are the amino acids required for egg laying fowl.

Feedstuff classification	Examples	Crude protein (%)	Fibre (%)
Cereals	Wheat	10.8	2.8
	Maize	20.0	10.9
	Barley	-	-
	Rice	7.3	10.0
	Rye	-	-
Cereal by-products	Wheat feed	14.8	10.0
	Rice bran	13.5	13.0
	Maize gluten meal	20.0	10.9
	Maize germ meal	10.1	9.0
Protein concentrates	Soyabean meal	46.0	6.0
	Ground nut meal	48.0	6.8
	Fish meal	62.0	1.0
	Meat bone meal	45.0	2.5
Oils and fats	Soyabean oil	-	-
	Rapeseed oil	-	-
	Fish oil	-	-

Table 1.2 Basic ingredients in pig and poultry feeds with percentage amount of protein and fibre (Holness, 1991).

1.2.4 Problems caused by low quality feed

Poultry and pig feed contain large quantities of plant materials that cannot be utilised by the animals because they lack the enzymes needed to digest these materials. Without the necessary enzymes poor growth performance in chicks, and to a lesser degree in piglets is inevitable (Jeroch *et al*, 1995). The feed also affect the animals by causing excessive fermentation in the lower gut and an increase in the digesta viscosity causing less absorption of nutrients. The dominant components found in the plant materials, which causes the anti-nutritional effects are a group of compounds called non-starch

polysaccharides (NSP) or fibres. The NSP compounds that are mostly found in wheat, barley and rye are contained in the aleurone layer and the cell wall enclosing the endosperm cells. The nutrients found in the endosperm cells are the digestible starches and proteins (Figure 1.3) (Morgan *et al*, 1995). Without the enzymes needed to break down the cell walls, the animal is unable to obtain the required nutrients, and hence, anti-nutritional effects are inevitable. Cereals containing NSP prolong the digesta rate, encourage microbes to flourish (Ward, 1995), reduce the internal secretion of water, enzymes, electrolytes, lipids and decrease fat absorption (Classen *et al*, 1985). Digesta viscosity is an important constraint to digestion by interfering with the diffusion of pancreatic juices, substrate and reaction products (Morgan *et al*, 1995). The molecular size and the degree of branching of NSP are known to affect the viscosity of digesta (Morris *et al*, 1981 and 1992) and explain why feeding rye to chicks is more detrimental than feed containing barley. Morgan *et al* (1995) has reported the relation between variable molecular weight profile of soluble NSP and digesta viscosity. In contrast, the anti-nutritional effects of cereals are reduced with the age of poultry as the gut microflora adapts to utilise the NSP more efficiently (Petersen *et al*, 1999).

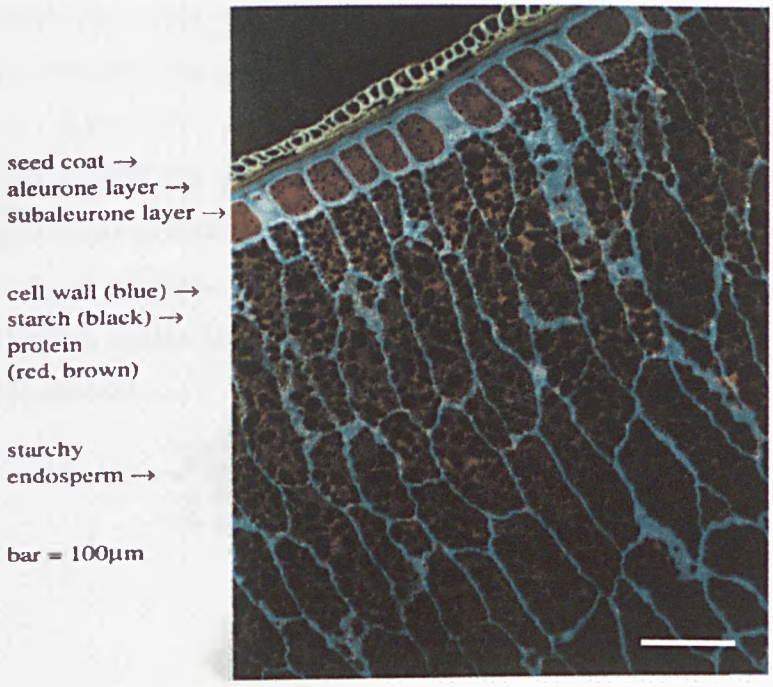


Figure 1.3 The cross section of wheat a grain revealing the aleurone layer. Walls were stained blue with Calcofluar white and protein was stained red/orange with acid Fushsin (Morgan *et al*, 1995).

1.2.4.1 Non-starch polysaccharides

Non-starch polysaccharides are indigestible or partially digestible plant carbohydrates. The cell wall of the endosperm of cereals and beans contains high levels of soluble non-starch polysaccharides. NSPs can be classified into three groups: Cellulose, non-cellulosic polymers and pectins. Cellulose, the most abundant constituent of plants, is otherwise known as insoluble fibre and has low digestibility in monogastric animals. Non-cellulosic polymers are partially soluble in water; they include polysaccharides such as arabinoxylans, mixed linked β-glucan, xyloglucan and fructans. These are also components of the cell wall of cereals. Pectins, which are mainly polygalacturonic acids, are mostly found in citrus peel of fruits and pomace of apples and are also partially soluble in water. The animal species, solubility, chemical structure and the quantity in the diet affect the digestibility of NSP. Bach-Knudsen and Hansen (1991) have reported that adult pigs are capable of digesting soluble non-cellulosic polymers to near completion but are only capable of digesting up to 60 % of cellulose. Petterson and Amon (1988) reported that broiler chickens are capable of digesting up to 32 % and 40 % of equal proportion of rye and wheat respectively in their diets.

Arabinoxylans are comprised mostly of D-xylose and arabinose but also contain ester-linked ferulic acid, acetic acid and p-coumaric acid (Figure 1.4). The biosynthesis is not well understood. Arabinoxylans consist of between 1,500 – 5,000 residues. Arabinoxylans like other NSP are synthesised by the action of glycosyltranferases in the Golgi vesicles (Stanely and Zubay, 1993). The glycosyltranferases use nucleotide sugars as donor substrates and transfer the sugar moiety to an acceptor substrate. Fibre diffraction studies indicate the molecules take up a twisted ribbon conformation with 3-fold symmetry.

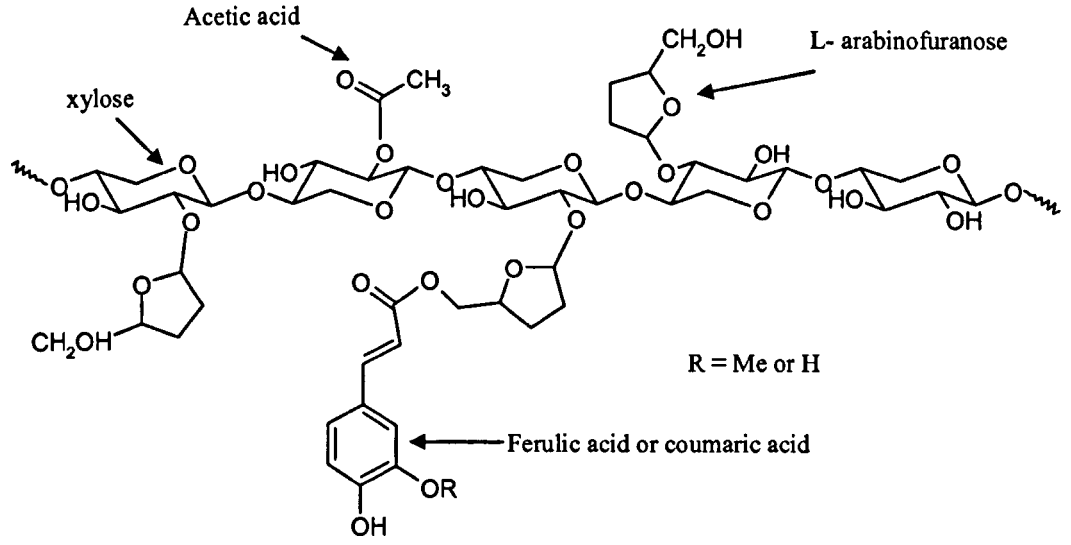


Figure 1.4 Schematic representation of the structure of cereal xylan (Ward, 1995).

β-glucans are present in barley, oats, rye and wheat in the amounts of 7 %, 5 %, 2 % and less than 1 % respectively. They contain about up to 250,000 glucose residues; 90 % of the β 1-4 links are in cellotriosyl and cellotetraosyl units joined by single β 1-3 links (Figure 1.5).

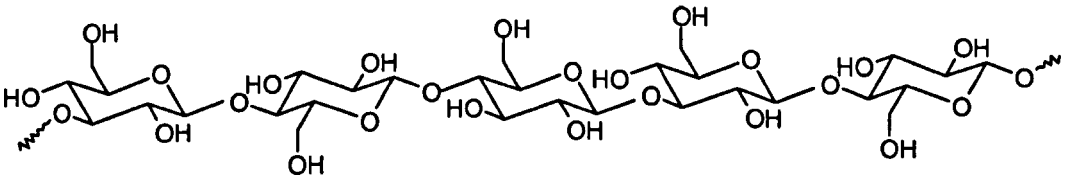


Figure 1.5 Chemical structure of β-glucan.

1.2.4.2 Other anti-nutritional factors

Galactosides are short chain carbohydrates usually found in legumes like soyabeans. They are a family of oligosaccharides, such as raffinose and stachyose (Dierick and Decuypere, 1994). The removal of galactosides from the diet of weaned pigs improved digestibility suggesting that the galactosides interfere with the absorption of nutrients. Coon *et al* (1990) had also reported that they reduce the digestibility of a soyabean meal diet and poultry cannot metabolise these oligosaccharides due to the absence of α-galactosidase enzyme activity.

Phytic acids are detected in wheat, rye, potatoes, maize, rice and other foodstuffs and are the major source of inorganic phosphorous (Dvorakova, 1998) needed for energy storage and bone calcification. A chemical structure of phytic acid is shown in Figure 1.6. Phytates strongly inhibit α-amylase activity by binding with calcium, which is necessary for the activity and stability of amylase (Knuckles and Betschart, 1987) and lead to low digestibility of starch products. To reduce the anti-nutritional effects caused by phytic acid, phytase is added to feed to hydrolyse phytates into inorganic phosphate and inositol (Sebastian *et al*, 1998). It has also been shown that calcium bound to phytic acid greatly reduces the efficiency of phytase (Dvorakova, 1998).

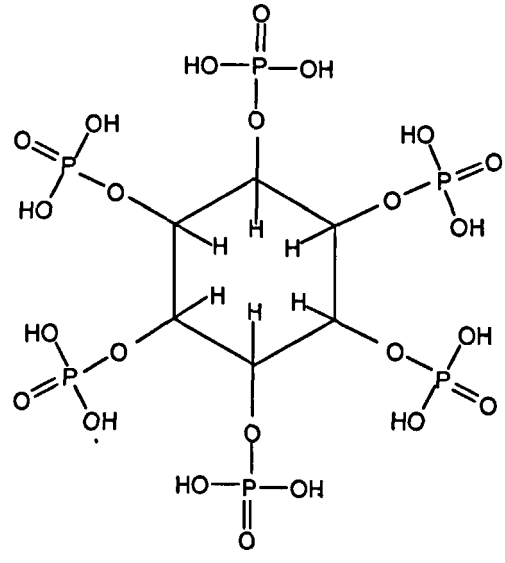


Figure 1.6 Structure of phytic acid proposed by Anderson (1914). At neutral pH phytic acid is thought to chelate Ca^{2+}, Zn^{2+}, Fe^{2+} and Mg^{2+} (Erdman, 1979).

Tannins are located in the hulls of faba beans, peas, sorghum and rapeseed and their role is to provide protection against soil organisms (Wareham *et al*, 1994). They are from plant polyphenolics, which are capable of forming stable cross-links with enzymes, carbohydrates and metal ions. Adverse affects in poultry and pigs caused by tannins include formation of complexes with dietary protein and carbohydrates, inhibition of activity of various digestive enzymes, erosion of gut epithelial cells (Holness, 1991). In

poultry small levels of tannins have adverse effects and intake of feed containing as little as 2 % (w/w) tannins can cause depression of growth and egg production.

Lectins are glycoproteins found in most plants. They are able to bind sugar moieties in the cell wall and membrane and cause agglutination, mitosis and other biological changes in cells (Pusztai *et al*, 1990; Pusztai, 1989). Most lectins are resistant to proteolysis to varying degrees and bind to glycoproteins causing damage to the gut wall (Jaffe, 1980).

Trypsin inhibitors are peptides capable of forming stable complexes with proteolytic pancreatic enzymes (Huisman, 1987) causing a reduction of protein digestibility. Soya beans and faba beans are fed to pigs and chickens in large quantities despite containing high levels of trypsin inhibitors. Trypsin inhibitors have a very rigid structure, which are maintained by extensive sulphide bonds and they are very thermally stable. Chickens are able to avoid the effects of some trypsin inhibitors at low dietary concentrations by increasing the pancreatic secretions of protease. Piglets do not respond to such a degree because there is no increase in pancreas size and so liveweight gain suffers (Wareham *et al*, 1994)

1.2.5 Solutions for feed improvement

Poor performance of cereal grain was first recognised in the 1950s. Soon after, it was known that the inability to break down the NSP was primarily due to arabinoxylans and mixed-linked β-glucans (Ward, 1995). Soaking in water was developed as the primary method to improve the nutritional feed value (Fry *et al*, 1958). Amylase and β-glutamase were originally added; xylanase was later shown to be effective and the addition of proteases and other enzymes shown also to have benefits (Ward, 1995). Morgan *et al* (1995) showed that increasing the amount of pancreatic enzymes in feed could improve the growth performance of broilers. For example, significant improvements in body weight gain could also be seen after addition of pepsin. However, the viscosity of the wheat-based diet interfered with the digestion of nutrients and the most effective treatment was the addition of enzymes to wheat based poultry diets. Feed enzymes work partly by reducing the viscosity of the intestine contents (Morgan, 1995) from 57.9 to 8.4 centipose units (cps) for barely and from 62.8 cps to 12 cps for oats

(Ward, 1995). Market research indicates that in the U.K approximately 90 % - 95 % of all broiler diets contain added enzymes (Danisco, personal communication). It is assumed that complete cleavage of the cell wall would require a number of enzymes (Chesson, 1992).

1.2.6 Enzymatic degradation of NSP

As well as reducing the amount of anti-nutritional factors, added enzymes also supplement the animals' own diet (Cowan, 1996). Their main purpose is to degrade NSP before it reaches the ileum so that available nutrients can be absorbed. The digestive tract of poultry is light, shorter and food passes through much more quickly compared to other non-ruminant species (Rose, 1997). As a consequence, the feed enzyme must function quickly in the gastric region and must be efficient in the duodenum and jejunum of the small intestine of poultry. In contrast, in pigs the digestive tract is longer and the feed experiences a longer period at milder acid pH levels. Therefore there is less need for enzymes stable at low pH. Protease and amylase secretions are low in piglets and chicks and so a surplus of these enzymes is needed. (Cowan, 1996). Because of the complex nature of NSP several enzymes with different specificities are added to the feed. A structural model of arabinoxylan with the proposed site of action of major arabinoxylan hydrolysing enzymes is shown in Figure 1.7.

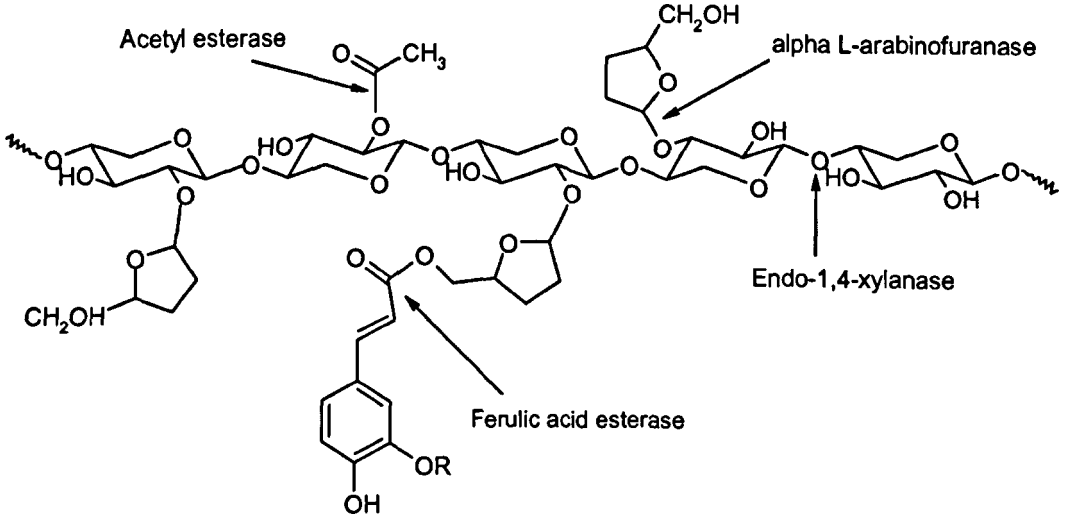


Figure 1.7 Schematic representation of cereal xylan showing the site of cleavage by xylanolytic enzymes (Ward, 1995).

Dierick (1989) concluded that the effect of adding enzymes to feed is greater in poultry than in pigs and has shown it to be more apparent in young animals than in older animals.

The major building blocks of non-cellulosic polymers are xylose and arabinose sugars, which may make up as much as 60 % (w/w) of the dry matter. Data on NSP composition of raw materials used in poultry and pig nutrition is presented in Figure 1.8.

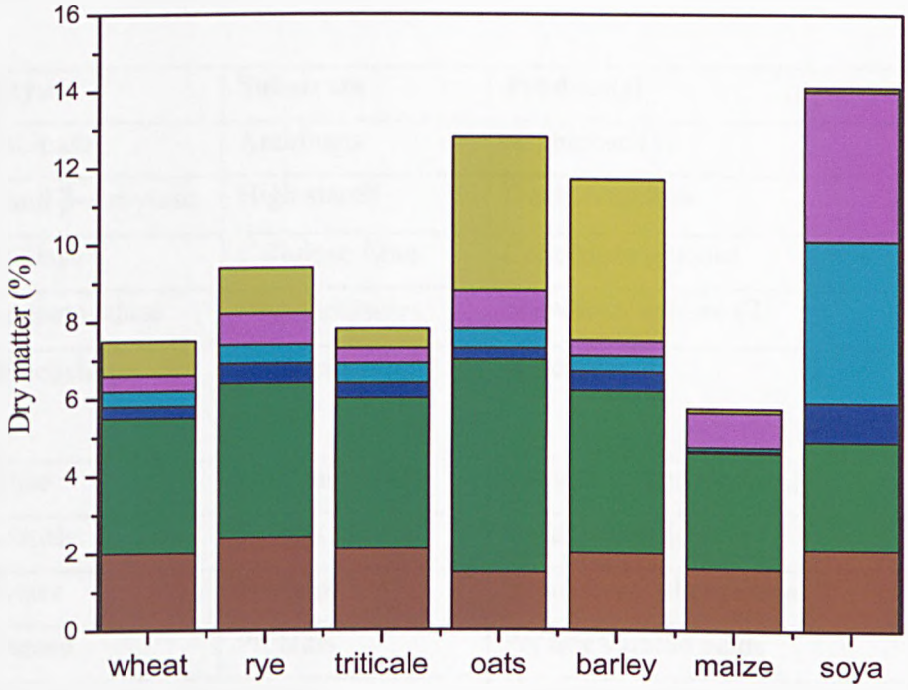


Figure 1.8 Percentage dry matter in cereals. The data shows the polysaccharide components of cereals: β-glucan; uronic acid; galactose; mannose; Xylose; arabinose (Cowan, 1996).

Cereals and vegetables used in animal feed may be divided into four main groups depending on their chemical composition. Group 1 contains cereals such as barley and oats which are the β glucan containing materials, group 2 contains wheat and rye which are the arabinoxylan containing group, group 3 contains material such as maize, which does not normally benefit from enzyme addition and group 4 contains vegetable protein sources, where the pectic material and galactosaccharides form a part of the fibre structure. Enzymes used in animal feeds are targeted at polysaccharide groups 1, 2 and 4 (Cowan, 1996). The enzymes added to both pig and poultry feeds are listed in Table 1.3.

Enzyme	Substrate	Product(s)
Arabinase	Arabinans	Arabinose (1)
α- and β-Amylase	High starch	Dextrin/maltose
Cellulase	Cellulose fibre	Cellubiose/glucose
α-galactosidase	α–galactosides	Galactose/sucrose (2)
β-glucosidase	β–glucans and NSP	Glucose
Lipase	Oils, fats, lipids	Fatty acids and glycerol
Pectinase	Pectins	D-galacturonic acid
Phytase	Phytates	Inositol and phosphates (3)
Protease	Proteins	Peptides/amino acids
Endo-1,4-xylanase	Xylan	Xylotriose/xylobiose/Xylose (4)

Table 1.3. Enzymes added to poultry and pig feeds. Data were obtained from: (1) Ward, 1995; (2) Deirick and Decuypere, 1994; (3) Sebastian *et al*, 1998; (4) Tenkanen *et al*, 1992.

1.2.7 Animal feed processing

A flow diagram illustrating the production of animal feed is shown in Figure 1.9. Added enzymes are often destroyed due to the high temperatures and steam used, and as a result an increase in intestinal viscosity is inevitable (Danicke *et al*, 1999; Nissinen *et al*,

1993; Scott *et al*, 1997). The purpose of processing is to improve the hygienic state of the feed destroying microbes such as *Salmonella* and increasing starch gelatinisation to improve pellet quality. Treatment can last from 30 seconds to 20 minutes with temperatures ranging from 75 °C to 140 °C and the moisture content ranging from 15 % to 20 % depending on the steam applied and the design of the pre-conditioner. Conditioning involves the injection of steam into the meal to raise the temperature and moisture levels and agitation (P. Steen, Danisco, personal communications). Molasses and steam are added to the meal while mixing to improve the efficiency of pelleting and the quality of the pellets (Guto, 1970). After conditioning the meal is continuously fed into the pelleting cavity. The rollers inside the cavity turn forcing the feed through the 'die' holes under extreme pressure. As the pellets are extruded, adjusted knives cut them to the desired length (de Blank *et al*, 1996; Heidenreich, 1998).

1.2.8 Current problems and possible solutions

Thermal processing decreases the activity of added enzymes which, as a consequence, increases the intestinal viscosity in broiler chickens (Nissinen *et al*, 1993; Scott *et al*, 1997). A practical way of tackling this problem was to add a surplus of enzymes to account for the loss of activity (Danicke *et al*, 1999). Addition of liquid enzyme after the pelleting stage has been recommended to avoid the deactivating effects cause by high temperatures of processing and pelleting (Danicke *et al*, 1999; Zigger, 1998). The addition of liquid enzymes could be carried out automatically which is an advantage as there is less human contact with the enzyme (Zigger, 1998). Another suggestion was to pre-digest the feed. Dierick and Deceupre (1994 and 1995) showed that there were marked improvements in the digestibility of feed in pigs fed with pre-digested feed compared with the untreated feed. Evidence of enzymes still active to some degree in the small intestine was found in both broilers and pigs. As a result, addition of liquid enzyme after pelleting was strongly recommended (Danicke *et al*, 1997). Pre-treatment of the feed improved the digestibility and the metabolic utilisation of cellulose. The consequence of such treatment is that it alters the flavour of the feed which maybe distasteful to the animal.

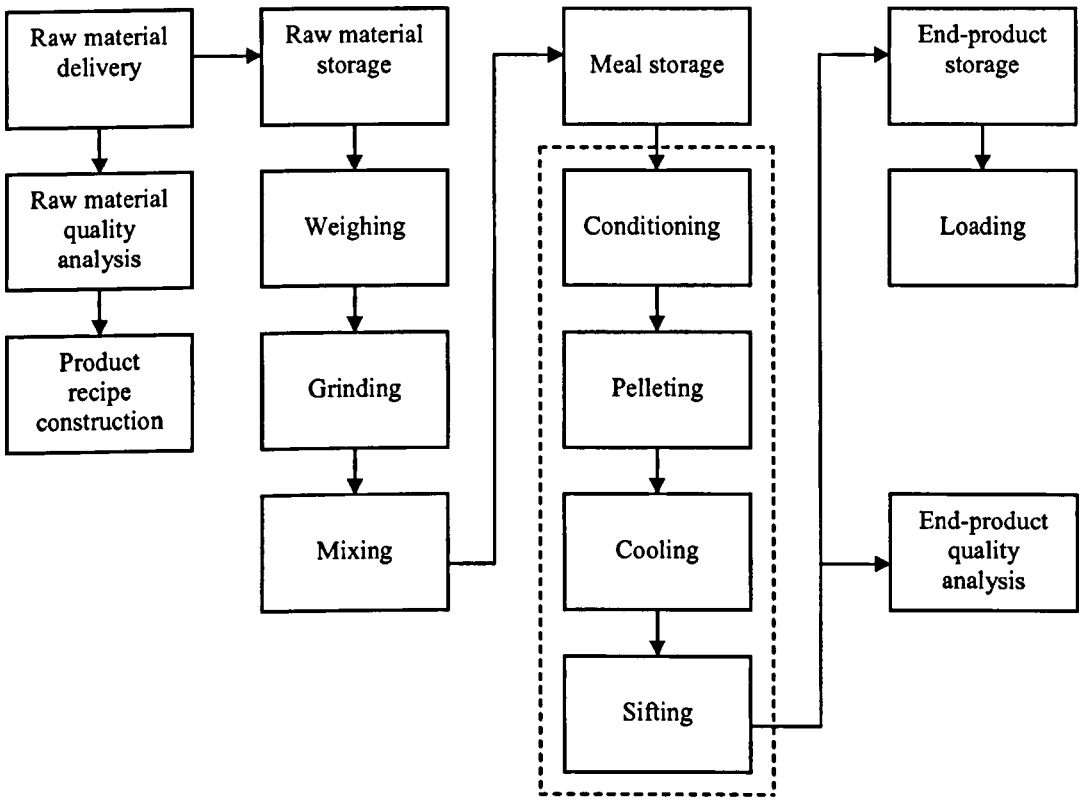


Figure 1.9. Flow diagram illustrating the production of pelleting animal feed (de Blank *et al*, 1997). The dotted box shows where added enzymes encounter the most extreme conditions.

1.3 Xylanase

Commerical enzyme products often contain xylanase as a major component (Bedford, 1994). Endo-1,4-xylanase is the enzyme of main interest within this project. Xylanase, like many other commercial enzymes is produced by fermentation using selected strains of *Trichoderma longibrachiatum* (formally known as *Trichoderma reesei*) and is responsible for the degradation of natural xylans. The enzyme prefers to hydrolyse the 1-4 linkages from the middle of the xylan-polymer chain. The main aim of this project is to stabilise the enzyme by biochemical means thereby making it more resilient to the processing temperatures used and the acid environment within livestock digestive systems.

1.3.1 Molecular details of xylanase

Endo-1,4-xylanase (EC 3.2.1.8) has a molecular mass of 21 kDa. The enzyme consists of a single 190-residue polypeptide chain folded into one domain composed of two β-sheets, A and B, which are orientated 90 ° relative to one another, and one α-helix (Havukainen *et al*, 1996 and Torronen *et al*, 1994). The dimensions of the xylanase molecule are 3.2 nm x 3.4 nm x 4.5 nm. A molecular graphic representation of xylanase is shown in Figure 1.10 and an amino acid/residue coded representation of the polypeptide chain indicating the position of the β–strand and the α-helix is shown in Figure 1.11.

Torronen (1995 and 1994) had compared the structure of xylanase with the shape of a right hand where the two β–sheets form 'fingers', and a twisted pair from one β–sheet and the α helix forms a 'palm'. The loop between β–sheet strands B7 and B8 makes a 'thumb' and part of the loop between B6a and B9 form a cord, which crosses the cleft on one side. The structure contains a total of 15 β-strands of which all have anti-parallel hydrogen bonding, except B6 and B7. Both A and B twist to form a cleft on one side of the protein (Torronen *et al*, 1994). Xylanase contains no cysteine residues and so there are no disulphide bridges. However, the molecule is held together by ionic interactions between 8 negatively charged residues (aspartate and gutamate) and 13 positively charged residues (lysine, arginine and histidine). The hydrophobic core is positioned between the β-sheets A and B, which form the 'fingers'. The hydrophilic face of the β-sheet makes the surface of the cleft. As shown in Figure 1.11 the β-strand B6 is subdivided into two shorter strands forming B6a and B6b by one residue. The two substrands are positioned at approximately 90 ° relative to one another. Other β–strands B3, B4 and B5 were also observed to have a 90 ° twist along the middle of their strands. The α-helix, which consists of only 10 residues, is situated between β–strands A6 and B4 and is packed against the hydrophobic face of β–sheet B (Torronen, and Rouvinen, 1995; Torronen *et al*, 1994).

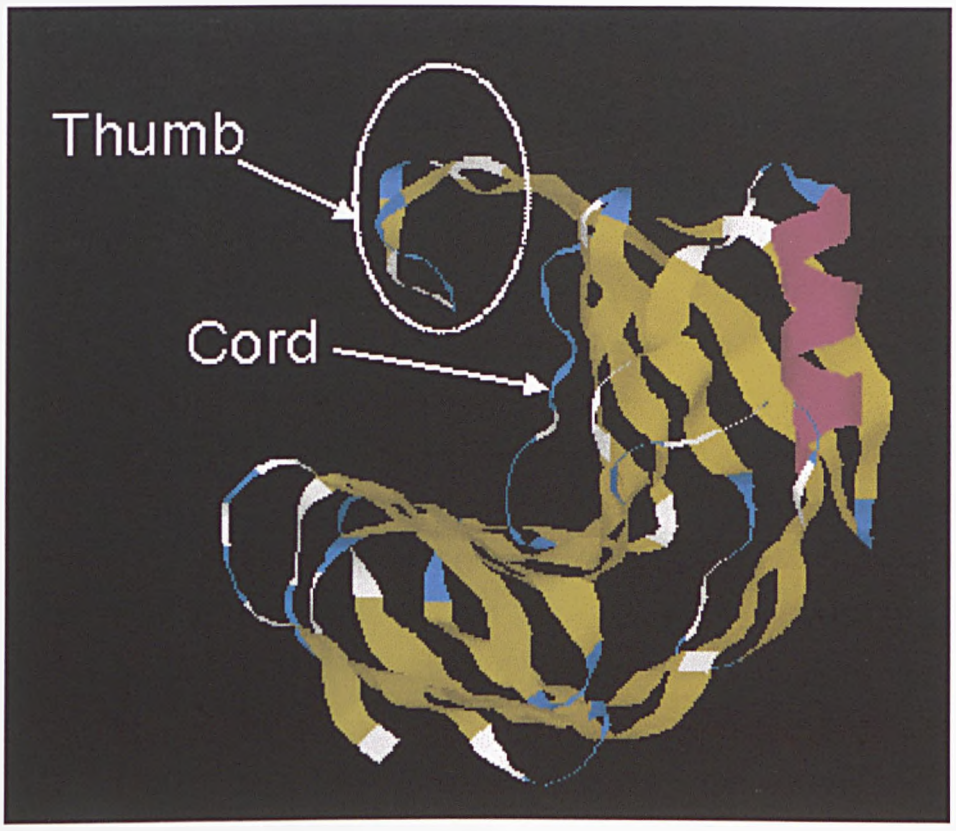


Figure 1.10 Ribbon representation of xylanase from *Trichoderma reesei* showing the position of the thumb and cord (PDB file:1XYO). The β-sheets are coloured yellow, the α-helix is coloured pink and the β-turns are blue (Torronen and Rouvinen, 1995).

```
          B1          B2          A2          A3          B3
QTIQPGTGYN NGYFYSYWND GHGGVTYTNG PGGQFSVNWS NSGNFVGGKG

          A5          B5          B6b  B6a
WQPGTKNKVI NFSGSYNPNG NSYLSVYGWS RNPLIEYYIV ENFGTYNPST

     B9          B8        thumb       B7          A6
GATKLGEVTS DGSVYDIYRT QRVNQPSIIG TATFYQYWSV RRNHRSSGSV

     Helix                     B4          A4
NTANHFNAWA QQGLTLGTMD YQIVAVEGYF SSGSASITVS
```

Figure 1.11 Amino acid sequence of xylanase from *Trichoderma reesei*. β–strand residues are coded red; α–helix residues are coded blue; the 'cord' is marked with a double line and the position of the 'thumb' is shown. The catalytic glutamate residues are highlighted and underlined (Torronen and Rouvinen, 1995).

1.3.1.1 The active site and conformational changes

Xylanase has a long groove on the surface of the protein, which acts as the binding site for the xylan chain. Torronen and Rouvinen (1995) had confirmed that Glu 86 and Glu 177 are the catalytic residues in xylanase by X-ray crystallography. Xylanase is presumed to have five sub-sites, -2, -1, +1, +2 and +3 where the positive numbers represents the reducing end and the negative number represents the non-reducing end. The cleavage takes place between sub-sites –1 and +1 and there is the possibility that the enzyme may hydrolyse xylan in different ways as shown in figure 1.12. Bieity *et al* (1993) had shown that xylanase is capable of cleaving xylopentose and xylotretose at the second and third linkages (Figure 1.12). A network of hydrogen bonds as well as hydrophobic interactions with side chains of trytophans and tyrosines holds the xylose subunits in place at sub-sites +2 and +3. There are no interactions other than the catalytic residue at the sub-sites –1 and +1.

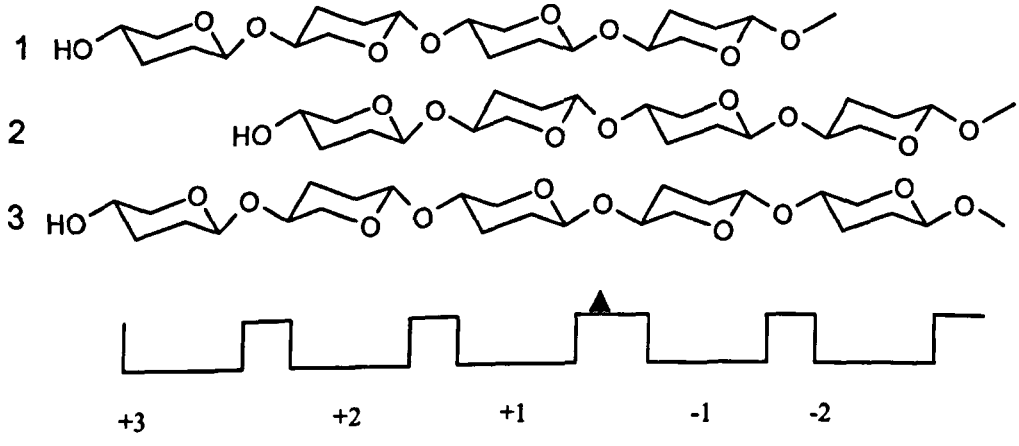


Figure 1.12 Schematic model of the active site of xylanase. The different subsites are numbered. Positive numbers are used for the reducing end and the negative numbers for the non-reducing end. The different binding positions of two (1 and 2) xylotretose and a binding position of xylopentose (3) are shown. The arrow represents the point of cleavage.

Molecular dynamic studies of xylanase have been carried out to assess the conformation changes and movements of the catalytic residues (Muhui *et al*, 1998; Havukainen *et al*, 1996; Torronen and Rouvinen, 1995; Torronen *et al*, 1994). Muhui *et al* (1998) had observed four structural changes: the upward movement of the fingers, the downward

movement of the thumb, the slight movement of the palm to the right and the backward movement of the cord. This suggested that xylanase has hinge movements between the thumb and the palm and between the palm and the fingers giving a good reason to describe the active site conformations of xylanase as opened and closed. Xylanase was found to be in its most compact state when in the presence of xylose. Torronen and Rouvinen (1995) had observed down and up movements of residue Glu 177 at pH 4.5 and pH 6.5 respectively. The conformational changes of the side-chain occur within nanoseconds, but the structural transition and domain movement may last for microseconds (Brooks *et al*, 1988).

1.3.1.2 Catalytic mechanism

The catalytic mechanism of xylanase is of an S_N1 type and is recognised to have a similar reaction mechanism to that of hen egg white lysozyme (Muliu *et al*, 1998; White and Rose, 1997). The mechanism of retaining β-glycosyl hydrolase from the evidence reviewed by White and Rose (1997) is presented in Figure 1.13 and is as follows:

1. The saccharide sub-unit at sub-site –1 induces a twisted-boat conformation. The acidic residue Glu 177 reacts by donating a proton to the glycosidic oxygen causing a transition state to develop.
2. The now positively charged carbonium ion, which resembles a half chair conformation, is stabilised by the negatively charged residue Glu 86. The β 1,4 linkage weakens.
3. The β 1,4 linkage is broken and the acidic residue Glu 177 is now a base and the pKa falls by 2-3 units. The saccharide sub-unit –1 is bound to the enzymatic residue, Glu 86, with a conformation of a covalent chair intermediate. A water molecule diffuses into the vicinity of the base catalyst, Glu 177.
4. The intrusion of the water molecule performs a nucleophilic attack on the C1 residual saccharide. The covalent species passes through a carbonium ion transition state similar to the event of stage 2. The product restores the pKa of the Glu 177 catalyst.
5. The restored Glu 177 catalyst leads to retention of anomeric configuration at C1.

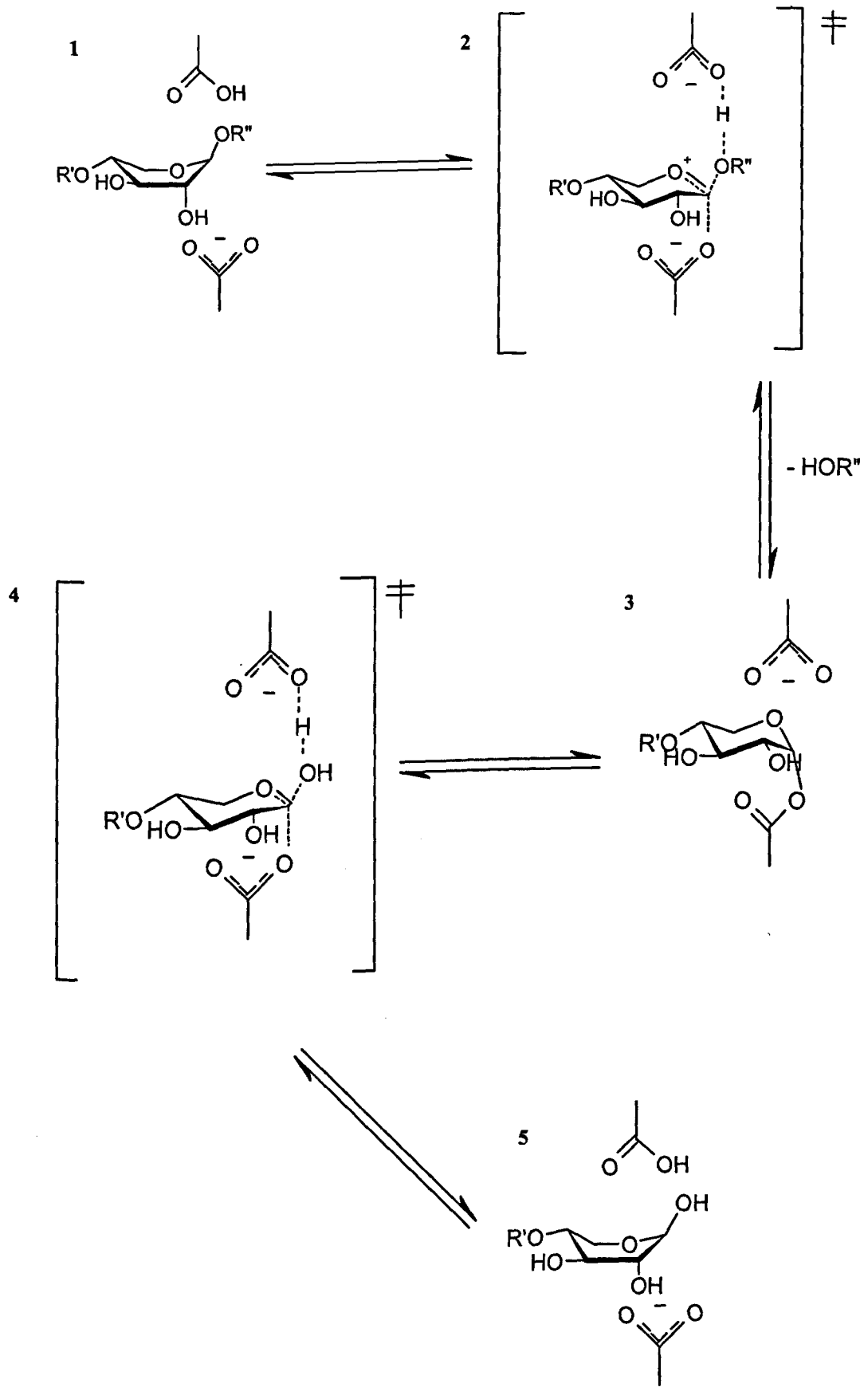


Figure 1.13 Proposed catalytic mechanism of retaining β-1,4-glycosyl hydrolases (Rose and White, 1997).

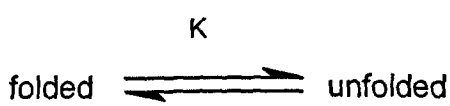
1.4 Denaturation of enzymes

Proteins play essential roles in nearly every biological process; they are very diverse in respect to their structure and function. The process of protein folding is driven by intramolecular and solvation forces and is spontaneous (Tanford, 1997; Kauzmann, 1959). Anfinsen (1973) was first to describe the folding of protein in terms of thermodynamics and had concluded that the folded state is more thermodynamically stable than the unfolded state.

Protein structures are held together by hydrogen bonding, Van der Waals forces, sulphide bridges, hydrophobic and electrostatic interactions (Kumar and Nussinov, 2001; Klibanov, 1983). Proteins are not rigid and in solution they undergo constant flexing and minor conformational changes (O'Fagain *et al*, 1988), in many cases these changes are essential for their function (Kumar and Nussinov, 2001; O'Fagain *et al*, 1988). Daniel *et al* (1996) defines denaturation as loss of tertiary and often the secondary structures in a protein and these losses can be either reversible or irreversible depending on the extent of the denaturation process. The denaturation of proteins is usually brought about by several factors such as heat, proteases, acidic or alkaline pHs, oxygen, pressure, solvent compositions and denaturing agents. Changes which occur during denaturation, can be monitored by differential scanning calorimetry, fluorescence and circular dichroism (Kumar and Nussinov, 2001), and also by measuring the residual activity over a pH or temperature range. The first step to understand protein stability is the understanding of protein denaturation itself.

1.4.1 Thermodynamics of protein unfolding

The thermodynamics of a protein folding reaction can be described as an equilibrium between the folded native protein and the unfolded denatured protein. The equilibrium between the two states is defined by the equilibrium constant:



Where

$$K = \frac{[\text{unfolded}]}{[\text{folded}]} \qquad (1)$$

The Gibbs free energy (ΔG) which is a measurement of protein stability between the two states is defined as

$$\Delta G = - RT\ln K \qquad (2)$$

and

$$\Delta G = \Delta H - T\Delta S \qquad (3)$$

where R is the universal gas constant, T is the temperature, ΔH is the change in enthalpy and ΔS is the change in entropy between the folded and unfolded states. ΔH becomes more positive at the melting temperature and is derived from the greater electrostatic interactions usually seen in thermally stable proteins (Kumar and Nussinov, 2001). ΔS of unfolding becomes higher with increasing temperature as the protein molecule shows increased motion. The enthalpy change and entropy change are both temperature dependent and the ΔG of protein folding is better expressed as

$$\Delta G_{fold}(T) = \Delta H(T) - T\,\Delta S(T) \qquad (4)$$

$$\Delta G_{fold} = \Delta H + C_p(T - T_m) - T\,[\Delta H_m/T_m + \Delta C_p \ln T/T_m] \qquad (5)$$

$$= \Delta H(1 - T/T_m) + \Delta C_p[(T_m - T) + T\ln T/T_m] \qquad (6)$$

where T_m is the melting temperature. The heat capacity change (ΔC_p) of a protein is related to the change in its accessible surface area between the folded and unfolded states and its sequence length (Myers *et al*, 1995).

1.4.2 Thermal denaturation

Under conditions of thermal stress, the weak non-covalent forces which hold the molecule together, weakens and the loss of tertiary structure begins to occur. At this stage, if heating ceases the protein molecule should reverse back to its initial

conformation. If heating continues some of the hydrogen bonds that stabilise the secondary structures begin to break and the protein unfolds. As unfolding takes place the solvent molecules interact with and form new hydrogen bonds with the amide nitrogen and the carbonyl oxygen. Aggregation then occurs, which is caused by the attraction of the hydrophobic groups of one protein molecule to the hydrophobic groups of other protein molecules. Such long-term thermal exposure results in an irreversible denaturation (Klibanov, 1983).

1.4.3 pH denaturation

Most proteins function best at a preferred pH termed the optimum pH, that can vary widely from protein to protein. The degree of inactivation can range from minor conformational changes to irreversible inactivation, depending on the incubation conditions. For example, an unfavourable pH exposure can cause ionisation of an essential amino acid group that may result in inactivation without any severe effect on the structure; simple readjustment of pH will restore activity. At either extreme pH, once far away from the protein isoelectric point, electrostatic interactions between like charges within the protein molecule results in a tendency to unfold (Volkin and Klibanov, 1989). If the pH is lowered far below the optimum the protein will lose its negative charge as it becomes protonated and hydrolysis of the peptide bonds occur; asparagine-prolyl linkages are especially vulnerable (Inglis, 1983). The resultant positively charged groups of the protein will repel from each other, areas with large charge density may force the molecule to unfold to some extent to expose the hydrophobic areas, which will the cause aggregation. At alkaline conditions partial peptide bond hydrolysis, deamidation, hydrolysis of arainine to orninthine, double bond formation, destruction of amino acid resides and formation of new amino acids are the many adverse side reactions (Whitaker and Fujimarki, 1980; Volkin and Klibanov, 1989).

1.4.4 Protease denaturation

The exposure of a protein to the low pHs of the gastric juice alters the structure of the protein which makes it easier for the proteolytic enzymes to attack at certain peptide

bonds. Also enzymes unfolded by heat are more susceptible to attack by proteases in solution (McLendon and Ranany, 1978).

1.5 Circular dichroism and the determination of protein structures

The theory behind circular dichroism (CD) is complex and the simplest way of describing the principle of how CD works is by visualising a light beam of wavelength λ passing through an optically active sample. A circular dichroism effect is said to have taken place when the sample absorbs the right-handed polarised light differently in comparison to the left- handed polarised light. The circular dichroism can be therefore defined as a measure of the difference in adsorption of the left and right circularly polarised light at wavelength λ as the light passes through an optically active or chiral sample (Wallace and Janes, 2001). It is also important to note that the direction of the polarised light does not change and the two polarised components are differently absorbed and therefore have different intensities. By means of vectors the theory can be illustrated. Figure 1.14a shows both right and left-polarised light with different intensity moving in the direction as indicated by the purple and blue arrows; the resultant vector moving in an anti-clockwise direction describes an elliptic trajectory. The ratio of the minor and major axis of the ellipse defines the tangent of angle θ and is called the ellipicity as illustrated in Figure 1.14b. The promotion of electrons to high energy levels within the optically active sample gives rise to the CD spectrum because they absorb the right and left polarised light to different extents. The CD spectrum is divided into three regions: the near UV visible region (300 nm – 700 nm), the near UV region (250 nm – 300 nm) where aromatic chains transmit energy and the tertiary structure can be examined and the far UV (180 nm – 250 nm) is the region where the changes in secondary structures can be monitored and therefore is the region of interest in this research. The groups with characteristic CD absorption are the chromophores and the amide group or peptide bonds, which form the polypeptide backbone of proteins and therefore are used to monitor secondary structures.

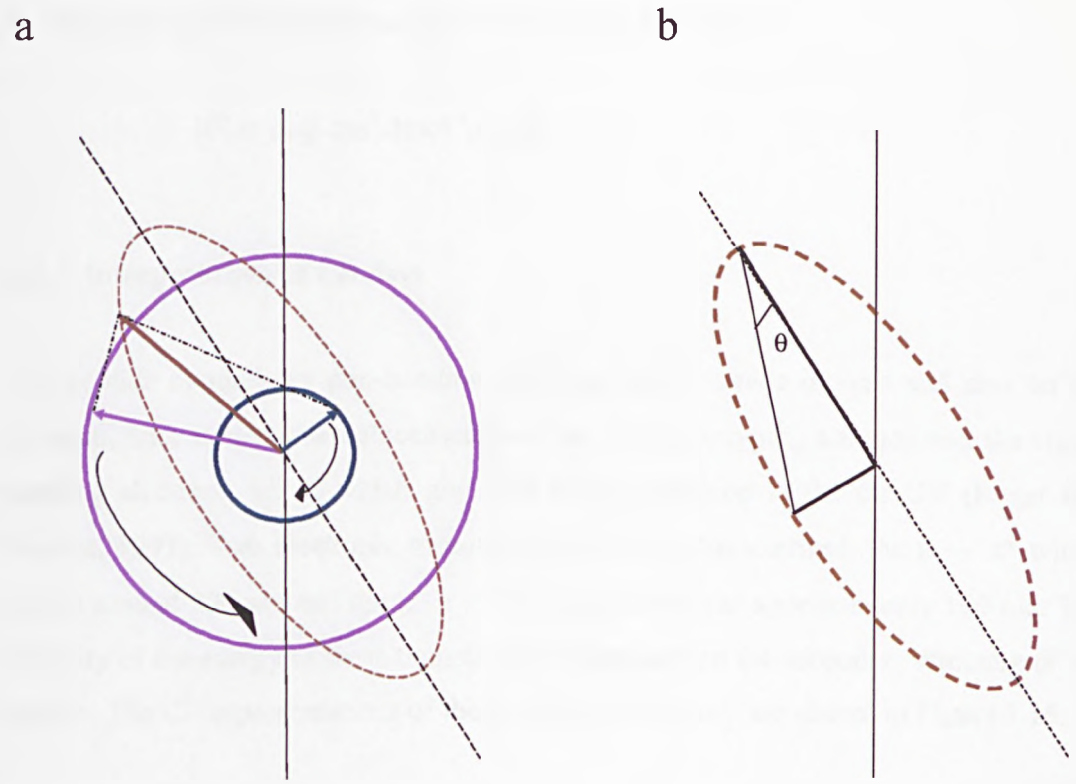


Figure 1.14 (a and b). Circular dichroism effect. (a) The purple and the blue circles represents the right handed and left handed polarised light respectively at different intensities which is represented by the size of the circles. The red ellipse represents the resultant vector moving in an anti-clock wise direction. (b) The ratio of the minor and major axis of the ellipse is the tangent of the ellipicity (θ).

The measured ellipicity (θ) is usually in millidegrees (mdeg). To standardise the ellipicity or make the data comparable, θ is normally converted to molar ellipicity [θ] and the path length of the cuvette (l), solute concentration (C), the molecular weight (M) and the number of residues must be considered as expressed in the following equation.

$$[\theta] = \theta_{mdeg} M\, C^{-1}\, l^{-1}\, residue^{-1} \qquad (7)$$

$$[\theta] = \text{mdeg. g mol}^{-1}\text{. cm}^3\text{ g}^{-1}\text{. cm}^{-1}\text{.residue}^{-1} \quad (8)$$

By simplifying the variables the molar ellipicity is defined as:

$$[\theta] = \text{deg. cm}^2\text{.dmol}^{-1}\text{.residue}^{-1} \quad (9)$$

1.5.1 Interpretation of CD data

The peptide bonds have non-bonding electrons on the amide oxygen and also on the nitrogen, with π-elections delocalised over the carbon, oxygen, nitrogen and the sigma bonding electrons, all of which give rise to the transition in the far UV (Roger and Norden, 1997). Two electronic transitions have been characterised: the n → π* which occurs around 220 nm and the π → π* transition occurs at approximately 190 nm. The intensity of the energy of these transitions is dependent on the secondary structure of the protein. The CD representations of the individual structures are shown in Figure 1.15.

1.5.1.1 α-Helix

The signals corresponding to the α-helix structure are the strongest in the far UV region. The α-helix has two negative bands; one occurs approximately 220 nm caused by n → π* transitions whilst the other is at 208 nm and is caused by α π → π *. A positive band at approximately 192 nm is also caused by the splitting on the π → π* transition. The magnitude of the bands is dependent on the length of the α helix with some discrepancies (Roger and Norden, 1997).

1.5.1.2 β–Sheet

The intensity of the bands caused by the β sheet is not as high compared with the bands produced by α helixes. The relatively low intensities are due to limited solubility of the β sheet polypeptides (Sreerama and Woody, 2000; Roger and Norden, 1997). The characteristic CD features of the β sheet are a negative band at approximately 216 nm (n → π*) and positive band at 195 nm (π→ π *). CD data of β–sheets are known to show variations with solvents and carbonyl and amide sides-chain groups.

1.5.1.3 β-Turns

Eight different type of β-turn have been characterised and types I, II and III are the most common. Typical β-turns have a weak negative red-shift band at 225 nm corresponding to the $n \rightarrow \pi^*$ transitions and a strong positive band with maxima between 200 nm and 205 nm. Unfortunately, the entire spectra of β-turns are not well documented (Johnson, 1990).

1.5.1.4 Random coils

Random coils are somewhat considered as an unordered structure and are sometimes omitted (Johnson, 1990). Roger and Norden (1997) have characterised the random coils in a protein to have a strong negative signal below 200 nm, a positive band at about 218 nm and a very weak negative band at 235 nm.

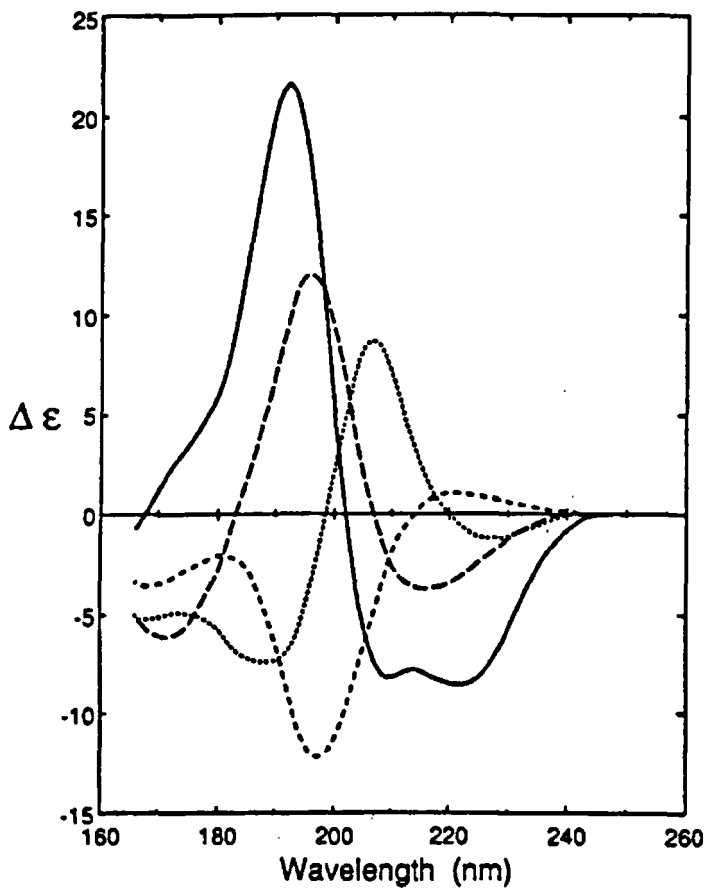


Figure 1.15. The CD for various secondary structures: α helix (—), β sheet (--); β–turn (—·—); and random coil (···) as presented in Proteins, Johnson, 1990.

1.5.1.5 Protein reference data

The CD of a protein is the sum of the characteristics from the different elements of its secondary structures. The most appropriate way of interpreting the data is by comparing and analysing the CD data with that of various known three-dimensional secondary structures that have been confirmed by NMR and X-ray crystallography (Venyaminov and Yang, 1996; Johnson, 1990). Greenfield and Fasman (1969) compared CD spectra measurements on a number of polypeptides that were presumed to have single secondary structures in solution, to give data that is presented in Figure 1.15. However, these CD spectra can only provide an estimated representation of the protein's secondary structure. There are a few important discrepancies to consider when analysing CD data. First, the reference CD spectrum of an α-helix polypeptide is assumed to be an infinitely long structure but only short α helices are found in proteins. Second, the CD data are unable to distinguish between the anti-parallel and parallel β-sheet structures in solution. Also β-sheet structures do not exist without β-turns and tend to give rise to differences in peak intensity, peak positions and crossovers. Third, proteins have many features such as chromophore side chains and distortions of secondary structure that can contribute to protein CD but are not well presented in the reference spectra.

A protein reference data is a set of normalised CD spectra of the protein of known three-dimensional structures. From the reference data one can obtain a set of Basis (Bκ) components as a spectra of pure secondary structure elements to use for structural determination.

The CD spectrum of a protein, S (λ), can be analysed as a linear combination of κ basis spectra, Bκ (λ) (Venyaminov and Yang, 1996):

$$S(\lambda) = \Sigma f_k B_k(\lambda) \qquad (10)$$

To simplify the meaning of the terms B_k represents the CD of 100 % κ structure at wavelength λ (e.g. the value of B_α is the CD of a protein which purely contains α-helix structure at wavelength λ). f represents the fraction of k secondary structure.
The CD of a protein according to equation 10 is

$$S(\lambda) = f_{\alpha}B_{\alpha}(\lambda) + f_{\beta}B_{\beta}(\lambda) + f_{R}B_{R}(\lambda) + \ldots \qquad (11)$$

Where $\Sigma f_k = 1$.

The most convenient way of analysing a protein using CD spectra is by observing the changes in secondary structure when it is exposed to conditions which cause the protein to undergo physical or chemical changes. CD spectroscopy is useful for determining secondary and tertiary structures of a protein, comparing the structure of proteins obtained from different sources, studying the conformational stability of a protein under stress and for determining whether protein-protein or ligand–protein interactions can alter the conformation of the protein. As well as using CD for structural determination one can also use it to determine the purity of the protein sample.

1.6 Ways to improve enzyme stability

1.6.1 Thermally stable enzymes

Thermally stable enzymes, otherwise known as thermozymes and hyperthermozymes, are most active and stable at temperatures of 60 °C to 80 °C or ≥80 °C respectively (Mozhaev, 1993; Veille and Zeikus, 1996). Thermozymes and hyperthermozymes are synthesised in organisms which thrive at these high temperatures and are found in deep sea vents, submarine hydrothermal areas and geothermal plants (Kumar and Nussinov, 2001). Such enzymes have been shown to have substantial biotechnological potential.

It is possible to analyse thermodynamic parameters of thermally stable enzymes, which are optimally active at ≥ 60 °C, by comparing them with their mesophilic enzyme counterparts. Similarities include their three-dimensional structures, the same catalytic mechanism and between 40 % and 80 % homogenous amino acid sequences (Veille and Zeikus, 1996). It is generally claimed that the reason why thermoyzmes are optimally activity at ≥ 60 °C is because they are more rigid than their mesozyme counterparts. Presumed dominant structural factors in protein thermal stability are better atom packing (Ishikawa *et al*, 1993), increased helical content and better salt bridge formation (Vieille and Zeikus, 1996). Increased electrostatic interaction in and around the active site and

metal binding site can help to maintain the integrity of these sites at elevated temperatures. Buried salt bridges in the protein core are found to be more stabilising than surface exposed ones due to the absence of solvent screening (Kumar *et al*, 2000). Hydrophobic interactions are thought to provide the energy required for folding in aqueous solution so extra hydrophobic interactions could increase the enthalpy at increasing temperature (Vieille and Zeikus, 1996). Surface loops are mobile and are typically the regions that are more susceptible to thermal denaturation. Deletion of the loops can reduce conformational entropy hence, the residue sequence of hyperthermozymes tends to be shorter (Thompson and Eisenberg, 1999). Proline residues located in the loops often strengthen the stabilising interactions between two adjacent core elements (Vieille and Zeikus, 1996). Thermophilic proteins tend to prefer arginine and tyrosine residues and tend to avoid asparagine, glutamine and threonine (Kumar and Nussinov, 2001).

Kumar and Nussinov (2001) have discredited some of these factors and demonstrated that stabilising factors which help to maintain function such as extra hydrophobic interactions, atomic packing and hydrogen bonds are no more common in thermally stable enzyme than in mesophilic ones. Nevertheless, hydrophobic branched resides were found to be more frequent in thermophilic proteins. Also proline substitution did not show any relevant trends.

Protein flexibility is essential for enzymes to function. Flexibility implies the movement of atoms, residues and fragments of the protein in respect to one another (Kumar *et al*, 2001). Kumar mentions that improved electrostatic interaction does not necessary reduce enzyme flexibility. Although increase in rigidity is necessary to preserve the catalytic active structure of thermozymes and hyperthermozymes, in order to function, molecular flexibility is critical and therefore thermozymes must be as flexible as mesophiles at optimum temperatures.

1.6.2 Stabilisation of protein structure

A simple method of stabilising enzymes is to add one or more stabilising agents. Stabilising agents render proteins less susceptible to oxidation, heat and pH degradation.

1.6.2.1 Sugars, polymers and polyols

Sugars, polymers and polyols have been used for many years as stabilising agents for proteins. The most common explaination as to why these carbohydrates and polymers enhance protein stability, is that they reduce the water activity by increasing the viscosity of the solution which restrict diffusion and convection (O'Fagain *et al*, 1988). The addition of sugar to aqueous solutions of enzymes strengthen the hydrophobic interactions of the amino acid non-polar residues thus increasing the rigidity and making the proteins more resistant to unfolding (Klivbanov, 1983; Fersht, 1977). A protein, which exists in four different states in aqueous solution in the absence and presence of sugars, is illustrated in Figure 1.16; the ΔG is the free energy required to convert from one state to the next as indicated by the arrows.

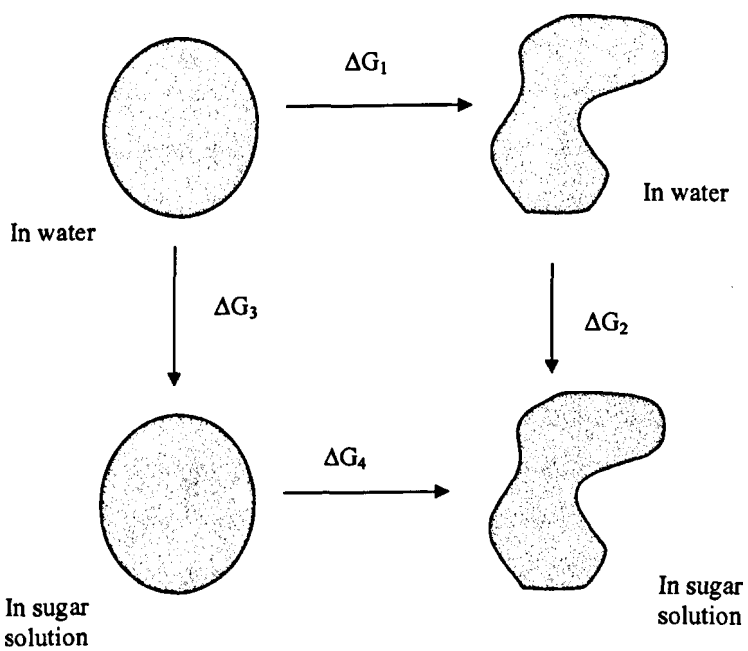


Figure 1.16 Schematic diagram of denaturation of proteins in aqueous solution in the presence and absence of sugar additives (Arakawa and Timasheff, 1982).

Arakawa and Timasheff (1982) proposed that as the native state protein unfolds, the surface area of the protein increases causing an increase in hydration. As a consequence the free energy will be greater. When the protein is in a sugar solution the preferential hydration of the sugar must also be considered assuming that identical unfolding or expansion of the protein has taken place both in the presence and absence of sugars.

Therefore:

$$\Delta G_4 > \Delta G_1 \qquad (12)$$

$$\Delta G_2 > \Delta G_3 \qquad (13)$$

and

$$\Delta G_1 + \Delta G_2 = \Delta G_3 + \Delta G_4 \qquad (14)$$

Proteins in the presence of sugars have higher transition temperatures in aqueous solution and hence, the native state is more thermodynamically stable.

1.6.2.2 Metal ions

Metal ions are electrophiles seeking to share an electron pair, which may be bound to the main chain carbonyl and amino groups of a protein. Specific binding can be achieved by amino acid carbonyl, hydroxyl and to a lesser extent, amino group side chains. Other side chains that bind to metal ions include the ring nitrogen of tryptophan, the thiol group of cysteine and the thioether group of methionine. The environment of the active site is electrostatic which tends to attract the substrate and can make the binding group stereochemically more rigid thus helping to control the action of the enzyme (Glusker *et al*, 1999). Arnald and Zhang (1994) stated that metal ions act as cross-linkers, which could offer a convenient additional stabilising interactions to disulphide bridges and that stabilisation could be observed provided that the cross-link could be formed without causing any unfavourable distortion. Handel *et al* (1993) have reported that metal ion binding significantly reduced the flexibility of four helical metalloprotein mutants. The positions of metal ions have been observed in proteins by using X-ray technology.

Klibanov (1983) describes the stabilising effect of metal ions by stating that they may bind to the protein's charged groups. Also, with increasing ionic concentration the ions

can force the hydrophobic residues from the surface into the interior therefore causing the enzyme molecule to compress, making it more resistant to thermal unfolding.

According to Hofmeister's lyotropic series the stabilising effect of cations and anions should decrease in the following order with cations and anions being additively effective:

$$(CH_3)_4N^+ > NH_4^+ > K^+ > Na^+ > Mg^{2+} > Ca^{2+} > Ba^{2+}$$
$$SO_4^{2-} > Cl^- > Br^- > NO^{3-} > ClO_4^- > SCN^-$$

Metal ions form internal crosslinking and this has a large effect on thermal stability (Arnald and Zhang, 1994). The stabilising mechanism will involve entropic destabilisation of the unfolded state and is a convenient alternative to disulphide bridges.

1.6.3 Enzyme Immobilisation

Enzyme immobilisation is a process in which the movement of the enzymes is severely restricted in space, but in such a way that catalytic activity is still preserved. Enzyme immobilisation is currently the subject of great interest since immobilised enzymes have many advantages over soluble enzymes. Enzyme immobilisation is used to produce biosensors, assist in protein synthesis, bioprocessing of biological wastes, amino acid production from biofeedstocks, hydrolysis of lactose in milk, refinements of fats and oils, production of 6-amino penicillanic acid and stereochemical resolution and purification (Katchalshi-Katzir, 1993). The main advantages of enzyme immobilisation include increased enzyme activity, reuse of the enzyme and ease of separation of the enzyme from the product (Tischer and Kasche, 1999). A variety of carriers or support materials have been used and the choice of carrier depends upon the characteristic of the enzyme, the operational conditions and support system (Bickerstaff, 1997). In general, the most desirable support material is one that is mechanically and chemically stable, has a large surface area and is resistant to microbes.

1.6.3.1 Physical entrapment

Physical entrapment is otherwise known as adsorption and occurs when the enzyme is bound to an insoluble support material by weak force such as hydrogen bonding, Van der Waals Forces and possibly by hydrophobic interactions. The procedure of physical adsorption is simple and it can be achieved quickly by mixing the enzyme with a suitable adsorbent, usually requiring no additional reagents. The process is also reversible because the enzyme is not permanently attached to the support material. Therefore, there is little damage to the enzyme. Examples of suitable absorbents are ion exchange matrices, porous carbon, clays, hydrated metal oxides, glasses and polymeric resins. In some cases to reduce the costs of materials, the adsorbent can be reused by washing off the enzyme with concentrated salt solutions after which it is re-mixed with a fresh solution of enzymes (Horwood, 1985).

The disadvantages of physical adsorption are desorption and leakage from the support material (Huckel *et al*, 1996) which may occur due to changes in conditions such as temperature, pH and ionic strength and some physical factors as variation in flow rate and agitation can also cause desorption. Overloading enzymes onto the support material and possible steric hindrance caused by the support material may cause a reduction in enzyme activity.

1.6.3.2 Covalent binding

Covent binding is a process where the enzyme or protein is covalent bonded to the insoluble support material. The process is made possible due to the functional groups on the support material and the enzyme. The most useful residue on the enzyme with an available $-NH_2$ group is lysine. These residues are common on the surfaces of protein and are highly reactive. Other residues, which have effective binding groups, include histidine, aspartate, glutamate and arginine. The functional groups, which are required for the support material to react, can vary providing they can react with the $-NH_2$ group of the enzyme under mild conditions. Examples are shown in Table 1.4. Support materials have to be modified if they do not have the required functional group (Huckel *et al*, 1996; Wirth and Hearn, 1993; Birger Anspach *et al*, 1989). Alternatively cross-linking agents can be used (Li *et al*, 2001).

Covalent binding may increase an enzyme's activity and since it increases the rigidity of enzymes it may also enhance the enzyme's stability. Another advantage is that the matrices can be re-used since there is usually minimal enzyme leakage. The turnover of the bound enzyme can be affected due to the orientation of the enzyme if the active site becomes poorly accessible. Also the enzyme can be bound in such a way that its conformation is distorted, causing inactivation (Katchalski-Katzir, 1993).

1.6.3.3 Gel-entrapment

Entrapment of enzymes within a gel is a common method for enzyme immobilisation that has been widely used for controlled release systems. The gels normally consist of a highly swollen hydrophilic polymer network that absorbs a large amount of water (Dumitriu and Chornet, 1997). A straightforward method of forming a gel is by adding a cross-linking agent to a solution containing a polymer. The strength of the gel is normally dependent on the degree of cross-linking. Some polymers are able to form gels naturally for example gelatin and gum Arabic. The entrapment of the enzyme can be via physical encagement or may involve covalent binding to the gel with the aid of a cross-linking agent (Burgess and Ponsart, 1998). A possible disadvantage of gel entrapment is the pore size distribution, which may prevent or retard the diffusion of the substrate.

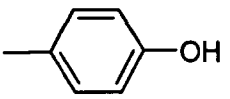
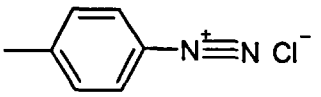
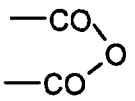
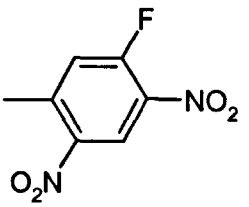
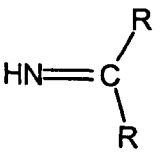
Coupling reaction	Reactive group of enzyme	Reactive group of carrier
Diazo linkage	$-NH_2$ -SH 	Diazonium salt 
Amide bond formation	$-NH_2$	Acid anhydride 
	$-NH_2$	Acyl azide $-CH_2CON_3$
	$-NH_2$	Isothiocyanate -R-NCS
	$-NH_2$	Isocyanate -R-NCO
	$-NH_2$	Acyl chloride
Arylation	$-NH_2$	3,5 nitro 2 floro benzene 
Alkylation	$-NH_2$ -SH -OH	Vinylsulphonyl $-OCH_2CH_2SO_2CH=CH_2$
Schiffs base formation	$-NH_2$	Aldehyde -CHO
Ugi reaction	$-CO_2H$ $-NH_2$	*N*-amine 
Aminidation reaction	$-NH_2$	Cyanide -CN

Table 1.4. continued...

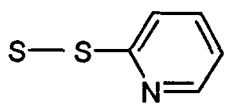
Thiol-disulphide interchange	-SH	Disulphide residue 
γ-irradiation induced coupling	Enzyme radical E•	Matrix radical M•
Amide bond formation (in presence of condensing reagents)	$-NH_2$ $-CO_2H$	Amine $-NH_2$
	$-NH_2$ $-CO_2H$	Acyl hydrazide $-CONHNH_2$

Table 1.4 Covalent binding methods for the immobilisation of enzymes (Kennedy and White, 1985).

1.6.3.4 Cross-linking agents

Cross-linking agents are chemicals with useful functional groups, which can link any two materials together by covalent binding. By using suitable crossing agents it is possible to link enzymes in two different ways. Enzymes can be linked to support materials or linked together. Both strategies can increase their stability against activation (Abdella *et al*, 1979; Noritomi *et al*, 1998; Arshady, 1990). The most suitable cross-linking agent is one that can react at room temperature or below. Glutaraldehyde and 2,3-butadione are the most commonly use cross-linking agents (Arshady, 1990) due to effectiveness and lack of expense.

1.6.4 Microencapsulation

Microencapsulation is a process in which particles are surrounded by a coating to give small capsules with useful properties (Re, 1998). Microcapsules are minute containers made from natural or synthetic materials, with a potential size ranging between 1 μm and 1 mm (Nelson, 1991; Finch, 1996 and 1985). They are normally spherical in shape if they contain gas or liquid. It can be considered as a spherical form of packing, in that

the particles are individually coated for protection against harmful environmental influences (Aggarwal *et al*, 1998).

The techniques and practises of microencapsulation cover many scientific disciplines, including colloid and physical chemistry, polymer chemistry, physics, material science, suspension, drying technology, pharmaceutical, pesticide technology and controlled release applications (Finch, 1985).

The first significant application of microencapsulation was introduced in 1954 by Green and Schleicher (1957), who were asked to develop a product that would give multiple paper copies without the need for carbon paper, rollers or ribbons. Green and Schleicher (1957) based their research on the findings of Bungenberg de Jong and Kruyt (1938) who described aspects of phase separations. They were able to apply these principles to encapsulate an intermediate colourless dye with a gelatin matrix, by a process known as coacervation. A thin layer of the microcapsules was deposited beneath the cover sheet and was placed against a paper coated with acidic clay. The rupture of the microcapsules, caused by the pressure of a pen or pencil, releases the dye intermediate, which reacts with the clay reagent forming a coloured marking (Nelson, 1991; Sparks, 1981).

1.6.4.1 Advantages of Microencapsulation

Microencapsulation can modify colour, shape, volume, density, reactivity, durability, pressure sensibility, heat sensibility and photo-sensibility of the encapsulated substance (Finch, 1996). The microcapsule is able to preserve the substance in its finely divided state, and release the substance from the enclose capsule as required. Microcapsules can also protect a core substance from the effect of UV radiation, moisture and oxygen. Zgoulli *et al* (1999) encapsulated erythromycin and clarithromycin with aminoalkyl-methacrylate and cellulose acetatephthalate, by using spray drying techniques, to mask the taste as well as preventing them from degrading in the acid environment of the stomach. Uddin *et al* (2001) have made attempts to encapsulate ascorbic acid to protect it from environmental factors such as temperature, oxygen, metal ions, UV and X-ray that affect the solubility. Microencapsulation can also be used for delayed release, for controlled release (Takada *et al*, 1994), to reduce irritations and to mask the odour of the active core component.

1.6.4.2 The microcapsule structure

Microcapsules can have a wide range of geometries and structures. They consist of mainly two parts, the core or nucleus and the wall material. Some examples are shown in Figure 1.17; the first shows a classical or mononuclear microcapsule, where a continuous coating surrounds the continuous region of the core material. If a solid material is used as a core, the resulting capsule maybe irregular in shape, however, if the core material is a liquid or a gas a simple spherical capsule is formed (Finch, 1985). The second is an example of an aggregated capsule or polynuclear structure, where the core material is subdivided into a number of parts embedded in the continuum wall material. A capsule with more the one wall material is also shown in Figure 1.17c.

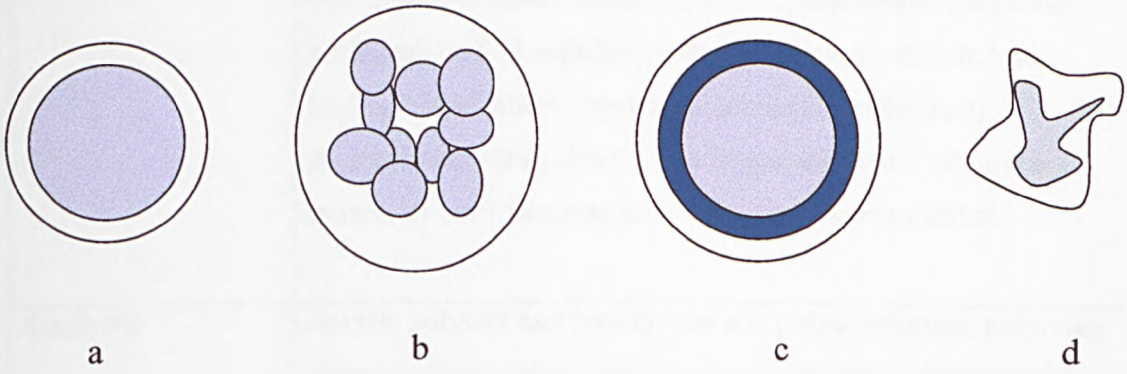


Figure 1.17 Microcapsule structures: a) mononuclear capsule; b) polynuclear capsule; c) double wall structure; d) irregular shaped mononuclear capsule (Nelson, 1991). The core material is blue and the wall materials are in white and dark blue.

Another form of microencapsulation, slightly different to that mentioned above, is the matrix particle, which is more commonly known as a microsphere. On solidification, fine particles become embedded within the hardened droplets of the polymeric wall material, forming a continuous matrix.

Wall materials (Table 1.5) can be natural, semi-synthetic or synthetic.

Material type	Examples
Natural	Gum arabic, agar, agarose, maltodextrins, sodium alginate, calcium alginate, dextran, fats and fatty acids, cetyl alcohol, milk solids, molasses, gelatin, gluten, albumin, shellac, starches, casemates, stearms, sucrose and waxes (e.g. beeswax, carnauba and spermaceti).
Semi-synthetic	Cellulose acetate, cellulose acetate butyrates, cellulose acetate phthalate, cellulose nitrate, ethylcellulose, hydroxpropylcellulose, hydroxpropyl-methylcellulose, hydroxpropylmethyl-cellulose phthalate, methyl cellulose, sodium carboxymethlcellulose, hydrogenated tallow, myristyl alcohol (1-tetradecanol), glycerylmono-ordipalmitate, hydrogenated easter oil, glyceryl mono-, di-, or tristearate and 12-hydroxystearyl alcohol.
Synthetic	Acrylic polymer and copolymers e.g. polyacrylamide, poly(alkyl cyanoacrylate) and poly(ethylene vinyl acetate), aluminum mono stearate, carboxyvinyl polymers, polyamides, poly(methyl vinyl ether maleic anhydride), poly(adipyl-L-lysine), polycarbonates, polyterephthalamide, polyvinyl acetate phthalate, poly(terephthaloyl-L-lysine), polyaryl-sulphones, poly(methylmethacrylate), poly(c-caprolactone), polyvinylpyrrolidones, polydimethylsiloxane. Polyoxyethylenes, polyesters, polyglycolic acid, polyactic acids and copolymers, polyglutatamic acid, polylysine, polystryrene,poly(styrene acrylonitrile), polyimides and poly(vinyl alcohol).

Table 1.5 Wall materials for microencapsulation (Aggarwal *et al*, 1998).

1.6.4.3 Types of micoencapsulation

Many different processes for microencapsulation have been reported with many variations. These variations depend upon the core and wall materials solubility, the size of particles, wall thickness and permeability type and rate of release of core material (Re, 1998). In general these methods can be subdivided into three main groups (Shahidi and Han, 1993): physical, physicochemical and chemical processes. The physical processes include methods such as spray drying, spray coating, prilling and extrusion. The physiochemical processes mainly involve the interaction and changes of two different phases; examples are simple and complex coacervation, organic phase separation, emulsion-solidification and liposome entrapment (Kondo, 1979). Chemical processes involve the reaction of two polymeric species at the interface between two liquids (Kondo, 1979) and examples of such processes are interfacial polymerisation and molecular inclusion.

Only spray drying and coacervation techniques will be considered here.

1.6.4.4 Spray-Drying

Spray drying is a well known technique in the food industry because it is an economical and effective method of producing many common commercial products such as powdered milk, instant coffee and instant foods. Spray drying is also particularly suitable for preparing synthetic detergents in power form. Although spray drying has been considered as a dehydration process it can also be used for encapsulation. It is also the earliest commercial form of an encapsulation technique and was used in 1927 to entrap flavours in gum arabic (Brenner, 1983).

Spray drying is defined as transformation of feed from the liquid state into dry particulate form achieved by atomising the feed through a hot drying medium (Re, 1998). The mixture of both wall and core material in the form of an emulsion or dispersion are fed into the spray dryer and undergo a process which occurs in successive stages. The first stage involves atomisation of the core/wall mixture. The atomiser is an important part of the design of the spray dryer because it effects the size distribution of the particles and the homogeneity of the spray. Secondly, the hot air flow (co-current or counter current) dries the wall material, embedding the core within (Nelson, 1991). Spray drying is a fast process and so the physical composition of the droplet is fixed.

The dried particles are carried by a stream of hot air to the bottom of the dryer and are collected (Figure 1.18). The most important characteristics of the microspheres formed are the uniformity of drop size and distribution (Re, 1998).

1.6.4.4a Spray drying of enzymes

Enzymes are normally very heat sensitive and hence mild drying temperatures are necessary. Additives such as inorganic salts are used as a protective measure to further stabilise the enzyme, minimising heat damage (Kondo, 1979).

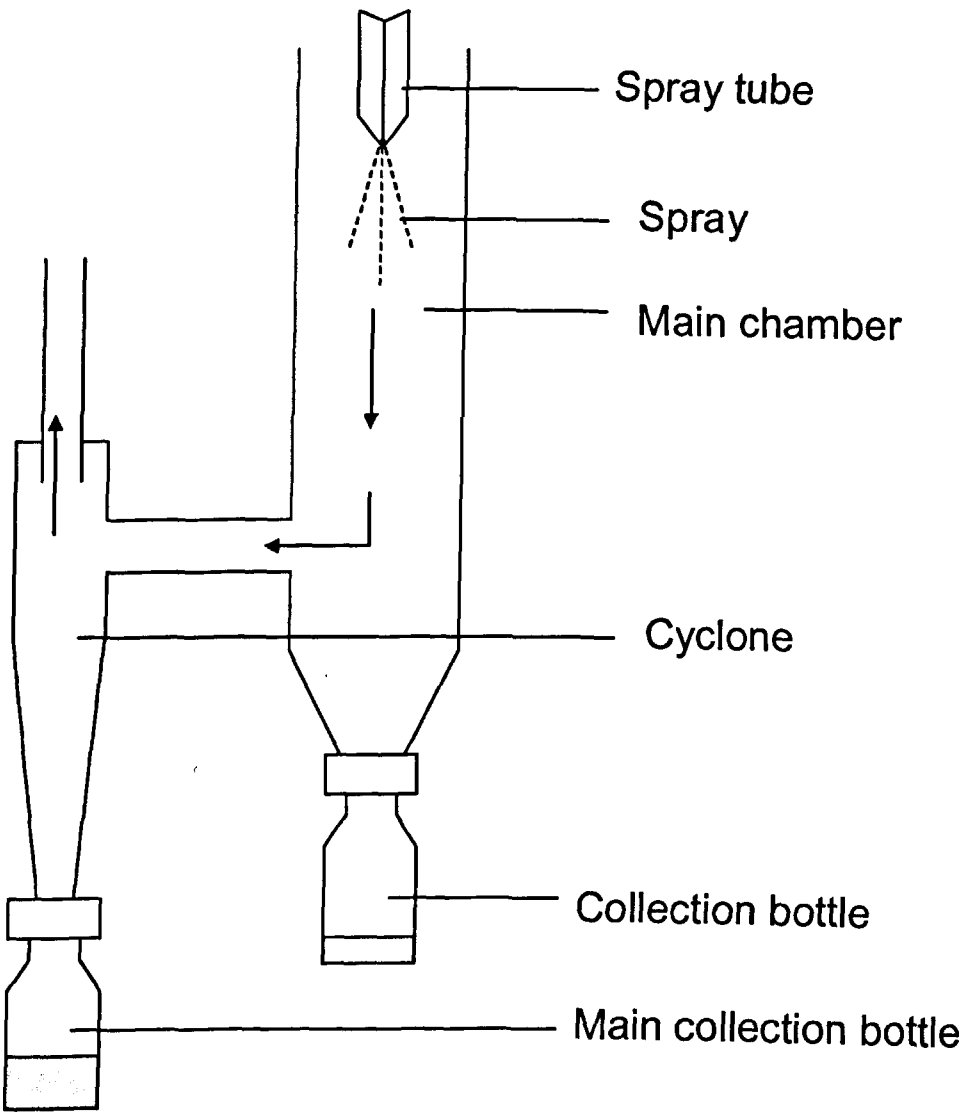


Figure 1.18 Basic schematic diagram of a flow current spray drier consisting of a drying chamber fitted with an atomiser and cyclone for product recovery (Nelson, 1991).

1.6.4.5 Phase separation

The concept of phase separation is closely related to the emulsification of a liquid forming a colloidal suspension. Emulsification is usually achieved by mechanical means such as shaking or stirring. In some cases, the addition of emulsifying agents, which are absorbed at the liquid interface to lower the interfacial tension, is necessary to stabilise the emulsion formed. The factors that affect the size of the microcapsules formed are the solvent composition, stirring speed, the type and concentration of additives used and the temperature. The loading efficiency of the core material is in most cases dependent on the solubility of the core between the two phases (Lee *et al*, 2000). An example of such an encapsulation method is presented by Kondo (1979). The starting material is the core which is dissolved in 1 part aqueous solution of appropriate concentration and is then added to 5 parts polymer dissolved in the volatile solvent benzene. The mixture is emulsified by means of a homogeniser to form a 'water-in-oil' type colloid. The water-in-oil emulsion is then further dispersed in an aqueous solution of 100 parts forming a secondary solution of water-in-oil-in-water. The temperature of the emulsion system is elevated and maintained for several hours with vigorous mixing. Finally, the benzene is evaporated and the polymer gradually precipitates resulting in the formation of microcapsules.

Coacervation phase separation can be used to encapsulate water-soluble and water insoluble liquids and solids. Coacervation can be divided into two categories: In simple coacervation a single colloidal solute is used and the capsule wall is formed by the addition of salts. Complex coaervation is formed by two colloidal solutes of opposite charge. Phase separation occurs when the pH is below their isoelectric points which cause the two molecules to interact and form an insoluble layer around the core material. Figure 1.19 shows an example of a complex type coaervation as presented by Finch (1985) where the core material (a) is dispersed into an aqueous solution of a polyelectrolyte, (b). The addition of aqueous polyelectrolyte with opposite charge initiates the deposition of the two polyelectrolytes around the core, (c) and permanent hardening of the wall material occurs when a cross-linking agent is introduced (d).

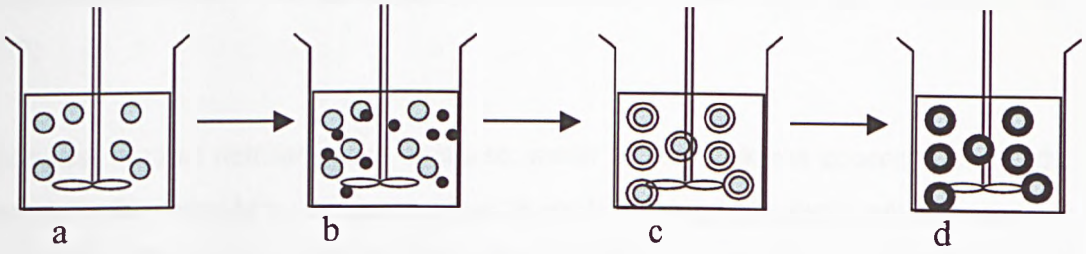


Figure 1.19 General scheme of microencapsulation in liquid phase. (a) dispersion of active phase in core medium in solvent vehicle. (b) Addition of wall material. (c) Deposition of liquid wall material. (d) Solidification of wall material (Finch, 1985).

1.7 Research aims

Prevention of the destruction of added enzymes during processing of animal feeds has been of much concern. Improvements in this area have been introduced by processing engineers by manipulating the conditions of processing or by adding the enzymes after processing. Some improvements have been brought about by mutagenesis thereby creating a genetically modified thermally stable xylanase (unpublished). In the research presented here a different approach has been investigated using immobilisation microencapsulation technology to stabilise or protect the enzyme.

It is important to take into account all of the conditions that dietary enzymes may have to survive through before reaching their point of action. For example, an enzyme may be kept in storage for up to 6 months prior to processing. During pelleting the temperature can vary between 20 °C and 120 °C. At the point of ingestion by the animal the enzyme will experience low pHs in the stomach region of pH 1.5 – pH 2.5 and pH 4.5 in poultry and pigs respectively. A pH as low as 1.5 could be detrimental to xylanase however, xylanase will not spent a considerable amount of time in the stomach. In comparison, the pH of a pig's stomach is much milder and xylanase could work better in this region. The small intestine is the most appropriate point of action in both pig and poultry since the pH ranges between pH 5.5 and pH 6.5 and body temperature remains at 39 °C and 41 °C for pig and poultry respectively (Holness, 1991; Rose, 1997). The role of the enzyme must be completed before it reaches the ileum, which is the region where most sugars are absorbed. The xylanase activity in the ileum of chicken has been estimated to

be approximately 80 % of the added enzyme activity is lost in the diet (Danicke *et al*, 1997).

Since heat is most detrimental to xylanase, much of the work has concentrated on the changes in the secondary structures as heat is applied using CD spectrometry to monitor the changes. The changes have been monitored in the presence of sugars, bivalent metal ions, polymers and polyols to determine any improvements of structural retention. The addition of stabilisers alone may not serve any practical purpose to improve enzyme stability because they are usually more effective in large quantities which as a consequence may not be acceptable in animal feeds. However, the addition of effective stabilisers can be used in various immobilisation and microencapsulation techniques to further enhance the stability and activity of xylanase. Immobilisation of xylanase should be beneficial as it could improve both thermal and pH stability. Suitable edible carrier materials to immobilise xylanase have been evaluated. Microencapsulation of xylanase could be effective by reducing or preventing the penetration of heat to the core. Controlled release of xylanase will not be essential but the complete breakdown of the wall material at the entry of the small intestine would be most ideal. Again, the chosen wall materials are edible and digestible materials. Thermal and pH stability experiments will be carried out in the laboratory prior to processing simulations.

The key aims of the research carried out have been:

1. To fully characterise endo-1,4-xylanase from *Trichoderma longibrachiatum* and to compare the results with literature values for specific activity, molecular mass, optimum temperature and pH, thermal and pH stability.

2. To investigate the unfolding of xylanase when exposed to extreme heat and at various pHs. The effects of sucrose, sorbitol, lactose, polyethylene glycol, polyethylene imine, dextran, Mg^{2+}, Ca^{2+} and Mn^{2+} on xylanase in terms of thermal stability and activity were also observed.

3. To immobilise xylanase onto inorganic support materials by physical adsorption, covalent binding and gel-entrapment and determine the thermal and pH stability of the immobilised samples were determined.

4. To investigate novel microencapsulation techniques and prepare a range of samples of xylanase have been investigated.

5. To reproduce the processing cycle in the laboratory and investigate the stability of immobilised or encapsulated xylanase during processing.

Chapter 2

Materials and Methods

2.1 General materials

Xylanase from *Trichoderma longibrachiatum* was obtained from Genencor and chicken feed sample was a gift from Danisco. The Danisco chicken feed comprised of 50 % (w/w) maize meal, 33 % (w/w) soya bean meal, 8 % (w/w) wheat meal, 4 % (w/w) vegetable oil, 1.5 % (w/w) dicalcium phoshate, 1 % (w/w) limestone flour, 1 % (w/w) pellet binder, 0.005 % (w/w) vitamins and minerals, 0.003 % (w/w) NaCl, 0.001 % (w/w) DL-methionine, 0.0003 % (w/w) choline chloride and 0.0005 % (w/w) elancoban♠. Dinitrosalicyclic acid (DNSA) and oat spelt xylan were purchased from Fluka. Sodium carboxyl methyl cellulose was obtained from Aqualon, France. Bio-gel P-60 was purchased from Bio-Rad, Hampstead, UK, and AX Xylanzyme tablets were purchased from Megazyme, Wicklow, Ireland. All other chemicals were purchased from Sigma, Aldrich or Fluka and were analytical grade or equivalent.

2.2 DNSA reagent preparation

Two differently prepared DNSA reagents were used to quantify xylose.

♠ Elancoban is a feed additive for the control of coccidiosis, a debilitating protozoal infection in poultry.

2.2.1 Danisco protocol

According to Danisco the DNSA reagent comprised of 30.0 % (w/v) sodium potassium tartrate, 1.6 % (w/v) NaOH and 1.0 % (w/v) dinitrosalicyclic acid.

2.2.2 Miller protocol

Bailey and Lab A recommended the use of Miller's reagent which consisted 21.6 % (w/v) sodium potassium tartrate, 1.4% (w/v) NaOH, 0.8 % (w/v) dinitro-salicyclic acid, 0.6 % (w/v) $HNaO_3S$ and 0.5 % (w/v) phenol (Miller 1952).

2.3 Measurement of enzyme activity

One unit (U) of xylanase activity was defined as the amount of enzyme which produced 1 μmol of reducing sugars, by hydrolysing xylan substrate, per min at 50 °C. Four different assays were used to determine the activity of the Genencor xylanase.

2.3.1 Danisco protocol

Measurements of xylanase activity according to the Danisco protocol were determined by incubating 1 ml of diluted enzyme and 1 ml of 1.2 % (w/v) oat spelt xylan dissolved in 50 mM CH_3CO_2Na buffer solution, pH 5.3, at 50 °C for 30 min. The oxidising agent used to quantify the released xylose sugar was 3 ml of dinitrosalicylic acid solution after which the absorbance (A_{540}) of the solution was measured. To determine cellulase activity within the xylanase, 1.2 % (w/v) sodium carboxymethyl cellulose was used in place of oat spelt xylan.

2.3.2 Bailey protocol

The activity of commercial xylanase was determined according to the Bailey protocol (Bailey *et al*, 1992) by incubating 1.8 ml of 1 % (w/v) birchwood xylan substrate dissolved in 50 mM CH_3CO_2Na buffer, pH 5.3 and 0.2 ml of adequately diluted enzyme at 50 °C for 5 min. The oxidising agent used to quantify xylose sugar was 3ml of dinitrosalicylic acid solution and the absorbance was measured at 540 nm. To determine

cellulase activity 1 % (w/v) sodium carboxymethyl cellulose was used in place of oat spelt xylan.

2.3.3 Lab A protocol

The quantity of reducing sugar was determined by using a protocol of an independent laboratory which will be named as Lab A in this thesis. Measurements of xylanase activity according to the Lab A protocol was determined by incubating 10 µl of diluted enzyme plus 0.99 ml of 1.2 % (w/v) oat spelt xylan dissolved in 50 mM CH_3CO_2Na buffer solution of pH 5.3 at 50 °C for 10 min. Lab A used 1.5 ml of DNSA reagent to quantify the amount of xylose released after which A_{575} was measured. To determine the cellulase activity 1.2 % (w/v) sodium carboxymethyl cellulose was used in place of oat spelt xylan.

2.3.4 Micro-titre plate format

Xylanase was assayed according to the Danisco protocol but on a smaller scale by incubating 50 µl of appropriately diluted xylanase and 50 µl of 1.2 % (w/v) oat spelt xylan in the wells of the micro-plate for 30 min at 50 °C. To quantify the oxidising sugar 150 µl of DNSA reagent (Danisco version) were used and then the absorbances were measured at 540 nm using a Titerek Multiscan titre-plate reader.

2.3.5 Xylanase detection using an insoluble dye substrate

To detect xylanase activity without quantifying the amount of reducing sugars produced, Xylanzyme AX Test Tablets were used. The active constituent in these tablets was dye cross-linked to wheat arabinoxylan. The hydrolysis of the enzyme produces soluble dyed fragments. The procedure was carried out by adding 50 µl, 75 µl and 100 µl respectively of xylanase to 1 ml of 200 mM sodium citrate-100 mM Na_2HPO_4 buffer, pH 5 and preheating to 40 °C. One Xylanzyme table was added to each test tube without stirring and then incubated for 10 min. The reaction was stopped by adding 10 ml of 2 % (w/v) Tris. The tube was shaken and the solution was filtered through Whatman N°. 1 filter paper. The filtrates were measured against a blank (one

Xylazyme tablet added to 1 ml of buffer) at 590 nm. The data were compared with a standard xylanase of activity 2,940 U ml^{-1} obtained from Danisco.

2.4 Thin layer chromatography

The products from birchwood xylan and oat spelt xylan hydrolysed by commercial xylanase were analysed by thin layer chromatography (TLC). The hydrolysis was carried out using 1 % (w/w) xylanase and 1 % (w/v) xylan substrate. Aliquots of the mixture were periodically removed and spotted onto a silica F254 TLC glass plate. The TLC plate was developed using 60 % butanol, 25 % water and 15 % acetic acid (v/v/v). Xylo-oligomers were located using concentrated sulphuric acid. Xylose was used as standard.

2.5 Purification of xylanase

2.5.1 Dialysis

Danisco xylanase, in liquid formulation was dialysed against 50 mM CH_3CO_2Na buffer, pH 5.3 using cellulose acetate dialysis tubing.

2.5.2 Gel filtration

Ten millilitres of liquid xylanase was loaded onto a 3 cm x 40 cm Bio-Gel P60 column. The xylanase was then eluted with deionised water. The protein fractions were collected and the bicinchoninic acid assay (Section 2.6.1) and dinitrosalicyclic assay (Section 2.2.1) were used to determine the protein concentration and enzyme activities respectively.

2.6 Protein assay

2.6.1 Bicinchoninic acid (BCA) assay

Protein standards were made using bovine serum albumin. The BCA working reagent and protein samples (including a blank) were mixed together at a ratio of 20:1 (v/v). The mixtures were then incubated at 60 °C for 15 min and the absorbance was measured at 562 nm.

2.6.2 Biuret assay

Protein standards were made using bovine serum albumin. Three millilitres of Biuret's reagent and 97.4 mg of spray-dried Danisco xylanase were incubated for 10 min at room temperature. The absorbances were measured at 540 nm.

2.7 Molecular mass determination

2.7.1 SDS-polyacrylamide electrophoresis (SDS-PAGE)

Discontinuous (SDS-PAGE) was used to determine the purity and the molecular mass of xylanase. Xylanase (5 µl) along with molecular markers ranging from 14.4 to 116.0 kDa were loaded on a 10 cm by 10 cm 12 % (w/v) SDS-polyacrylamide gel. The electrophoresis was carried out at 25 mA (100 V) for 1.5 h. On completion, the electrophoresis gel was placed in fixing solution comprising of 25 % (v/v) isopropanol and 10 % (v/v) acetic acid for 10 min and then placed in staining solution comprising of 0.06 % (w/v) of Coomassie Brilliant Blue R250 dissolved in distilled water, methanol, glacial acetic acid of ratio 5:5:2 (v/v/v) respectively for 1 hour. The gel was destained in 10 % (v/v) acetic acid until the background was fully clear.

2.7.2 Electrospray mass spectrometry

Samples were analysed on a Q-T (Micromass UK Ltd.) orthogonal acceleration quadruple time-of-flight mass spectrometer equipped with nano-electrospray ionisation. Samples were dissolved in 1:1 (v/v) aqueous methanol with 1 % (v/v) formic acid added. An aliquot of this solution (2 µl-3 µl) was used to fill a gold-plated, borosilicate nanospray vial, which was placed inside the ionisation source of the mass spectrometer. Position ionisation was used for the sample analyses, with a capillary voltage of 90 V and a sampling cone voltage of 40 V. Nitrogen was employed as the drying gas. The microchannel plate detector was set at 2700 V. Data were acquired over the appropriate m/z range and spectra processed using the massLynx software supplied with the mass spectrometer. The spectra were calibrated with a separate introduction of horse heart myoglobin (MW 16,951.49 Da). The experiment was performed by Dr. Alison E. Ashcroft, School of Biochemistry and Molecular Biology, University of Leeds.

2.8 Characterisation of xylanase

2.8.1 Optimum pH for xylanase

The optimum pH was determined by assaying diluted xylanase in 100 mM sodium citrate, 200 mM Na_2HPO_4 buffer solution at pHs ranging from pH 2 – pH 8.

2.8.2 Optimum temperature for xylanase

The optimum temperature was determined by assaying xylanase at temperatures ranging from 24 °C – 70 °C.

2.8.3 Thermal stability of xylanase

The thermal stability of xylanase was determined by heating a series of xylanase samples diluted in distilled water ($1:10^5$ (v/v) respectively) to 50 °C, 60 °C, 70 °C or 80 °C. Each sample was exposed for 5, 10, 15 or 20 min and then allowed to cool to

room temperature. The activities of the heat-treated diluted xylanase were measured as in Section 2.2.4.

2.8.4 pH stability of xylanase

The pH stability of xylanase was determined by diluting 1 ml of xylanase in a series of 9 ml 100 mM sodium citrate, 200 mM Na_2HPO_4 buffers at pHs ranging from pH 2 – pH 8. The samples were stored for 24 h at 4 °C and then diluted using the same buffer solution at pH 5.3 prior to the measurement of xylanase activity (Section 2.2.4).

2.9 Circular dichroism (CD) spectroscopy

2.9.1 CD spectra of xylanase

CD scanning of xylanase was performed using a Jasco spectrometer model J715 calibrated with 50 mM CH_3CO_2Na buffer, pH 5.3. For recording the secondary structure a scan of 0.4 g ml^{-1} purified xylanase was performed in the far UV range from 260 nm to 190 nm in 1 nm increments, at 50 nm min^{-1}, and a temperature of 50 °C the sample pathlength was 0.1 cm. An average of three scans was calculated and the data were subtracted from the control spectrum (without enzyme).

2.9.2 The effect of temperature

To analyse the effect of temperature on the structure of xylanase a CD spectrum was taken every 5 min, starting from 0 min to 15 min at 60 °C.

2.9.3 The effect of pH

To analyse the effect of pH on the xylanase structure CD spectra were taken on a series of samples with pHs ranging from pH 2 to pH 8. Each sample containing 0.4 g ml^{-1} of purified xylanase was incubated for 24 h at 4 °C in 100 mM sodium citrate, 200 mM Na_2PO_4 buffer solutions at the appropriate pH before scanning at 50 °C.

2.9.4 The effect of metal ions

To analyse the effect of metal ions on the structure of xylanase, 1 mM of $MgCl_2$, $CaCl_2$ or $MnCl_2$ were added to a solution of 0.4 g ml^{-1} purified xylanase in 50 mM CH_3CO_2Na buffer solution, pH 5.3. The solutions were prepared and then stored for 24 h prior to scanning in the CD spectrometer.

2.9.5 Thermal stability

To analyse the effect of metal ions on the structure of xylanase, 0.4 g ml^{-1} of purified xylanase was heated to 60 °C for 0, 5, 10 or 15 min and then allowed to cool to 50 °C prior to scanning. The experiment was also carried out in the presence of 1 mM metal ions which were added to the samples according to Table 2.1.

	Formulated buffer solution pH 5.3
1	Control 1
2	Control 1 + 1mM $MnCl_2$
3	Control 1 + 1mM $MgCl_2$
4	Control 1 + 1mM $CaCl_2$
5	Control 1 + 1mM $MnCl_2$ +1mM $MgCl_2$
6	Control 1 + 1mM $MgCl_2$ + 1mM $CaCl_2$
7	Control 1 + 1mM $MnCl_2$ + 1mM $MgCl_2$ + 1mM $CaCl_2$

Table 2.1 A list of the combination of divalent metal salts added to 50 mM CH_3CO_2Na buffer pH 5.3

2.9.6 Thermal degradation of xylanase in the presence of Mg^{2+} ions

To determine whether the presence of Mg^{2+} ions affected the thermal degradation of xylanase, the β sheet content of xylanase was monitored at 55 °C, 60 °C, 65 °C or 70 °C for 20 min. Prior to scanning, 0.4 g ml^{-1} of purified xylanase was incubated in 50 mM CH_3CO_2Na buffer solution, pH 5.3 containing 1 mM Mg^{2+} for 24 h at 4 °C.

2.9.7 Thermal degradation of xylanase in the presence of organic stabilisers

For denaturation measurements, CD scans were performed at temperatures from 40 °C to 76 °C and were also carried out in the presence of individual stabilisers or combinations stabilisers such as sucrose, sorbitol, PEG (M_w 8,000), dextran and lactose at a final concentration of 10 % (w/v). The samples were held for 1 min at the experimental temperature prior to CD scanning.

2.10 Promaxon as support material for xylanase

2.10.1 Immobilisation by physical adsorption

Promaxon is a synthetic calcium silicate mineral, $Ca_6Si_6O_{17}(OH)_2$, which is commonly known as Xonotlite. Immobilisation of xylanase onto Promaxon by physical means was carried out by mixing 3 ml of diluted xylanase at concentrations varying from 3.33 % to 33.33 % (v/v) diluted in 50 mM CH_3CO_2Na buffer solution of pH 5.3 with 0.5 g of Promaxon. The mixture was placed in a rotary evaporator (50 °C) to remove excess water and then allowed to dry overnight in a vacuum oven at room temperature. Samples were tested for xylanase activity before and after washing the xylanase loaded Promaxon.

2.10.2 Immobilisation of xylanase with glutaraldehyde to Promaxon

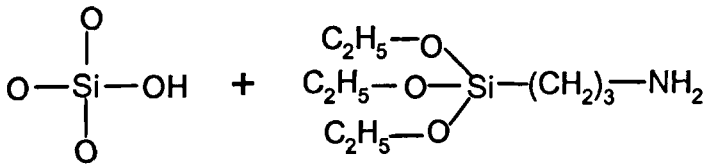
Immobilisation of xylanase to Promaxon was performed by adding 0.1 ml of Danisco xylanase to a mixture of 0.4 g Promaxon and 0.1 ml glutaraldehyde of various concentrations. The glutaraldehyde concentrations were from 0 % to 5 % (v/v). Each sample was mixed by repeated inversion for 0 min to1 h, filtered under suction and then ashed with 50 mM CH_3CO_2Na, pH 5.3. The samples were allowed to dry in a vacuum oven at room temperature overnight and then tested for xylanase activity. The washings were also kept and analysed for xylanase activity.

2.10.3 Measuring the activity of the xylanase /Promaxon matrices

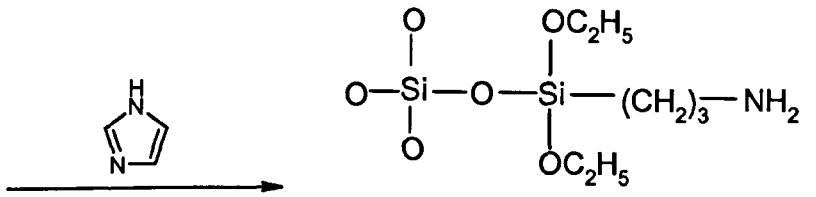
The matrices were weighed accurately to the nearest 0.100 g and suspended in 1 ml of 50 mM CH_3CO_2Na, pH 5.3, vortexed and then assayed according to Section 2.2.

2.11 Silica as a support material for xylanase

2.11.1 Synthesis of 3-aminopropyltriethoxylsilane (APS) modified silica surface



Silica surface 3-aminopropyltriethoxysilane (APS)



APS modified silica surface

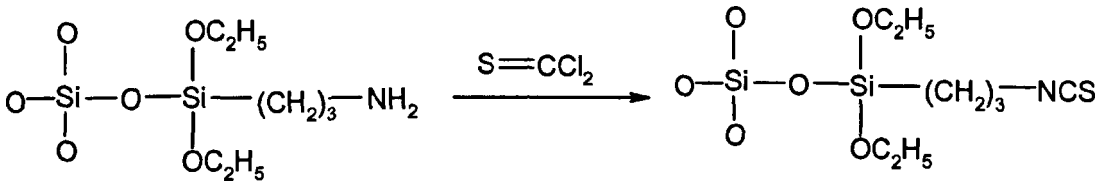
$+ C_2H_5OH$

Figure 2.1 Reaction of silica and 3-aminopropyltriethoxysilane to produce APS modified silica surface and ethanol.

Prior to synthesis, 20 g of silica (particle size 0.011 μm) was suspended in 100 ml of water and was hydro-thermically treated at 150 °C for 18 h. After treatment the silica was dried under vacuum at 108 °C for 24 h. Five grams of dried hydrated silica particles were suspended in 80 ml of toluene with 3-aminopropyltriethoxylsilane and a catalytic amount of imidiazole (0.1g). The mixture was refluxed for 24 h. After the reaction had completed, the modified silica was washed with 100 ml of toluene, 100 ml of propan-2-ol and then 100 ml of water (Figure 2.1). The compound was allowed to dry

and then was analysed using infrared spectroscopy (Huckel *et al*, 1996 and Anspach *et al*, 1989).

2.11.2 Synthesis of 3-isothiocyanatopropyltriethoxysilane (ITCPS) modified silica



APS modified silica surface

ITCPS modified silica surface

+ 2HCl

Figure 2.2 Reaction of APS modified silica and thiosphosgene to produce ITCPS modified silica and hydrochloric acid.

A solution of 1.5 g of thiosphosgene in 20 ml ethyl acetate, previously cooled in ice, was mixed with 5.0 g of 3-aminopropyltriethoxylsilane and 3 ml of triethyl amine in 80 ml of ethyl acetate. The thiosphosgene solution was then added drop wise to the mixture while stirring. The temperature of the reaction mixture was allowed to increase to room temperature and the stirring continued for 2 h (Figure 2.2). The product of the reaction was filtered under suction and then washed with 50 ml of ethyl acetate, 50 ml of water before being air dried and analysed using infrared spectroscopy (Floch *et al*, 1999).

2.11.3 Coupling of xylanase to modified silica

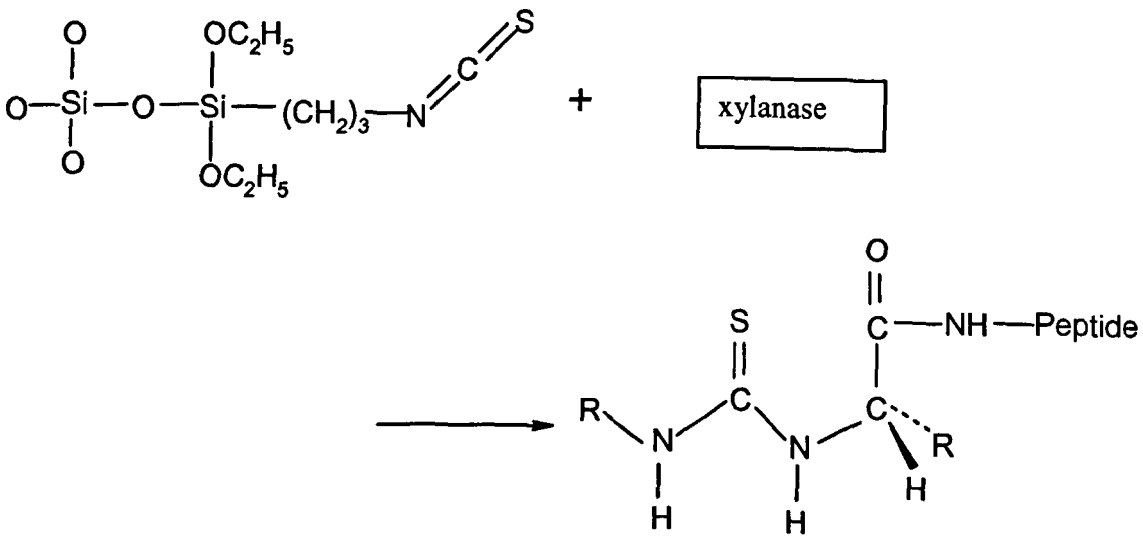


Figure 2.3 Coupling reaction of xylanase onto ITCPS modified silica.

Five grams of ITCP modified silica were suspended in 100 ml of CH_3CO_2Na buffer solution, pH 5.3 with 1 mM of $MgCl_2$, $MnCl_2$ or $CaCl_2$ or combinations of these salts. To this mixture, 0.1 ml of xylanase was added. The mixture was then shaken by inversion at room temperature for 72 h. The immobilised enzyme matrix was washed with the corresponding buffer solution and a 1 % (v/v) solution of ethanolamine dissolved in distilled water to block the exposed thiocyanato groups. Total protein and xylanase activity of the matrix and the washings were measured according to Sections 2.6.1 and 2.12.1.

2.11.4 Infrared spectroscopy

Samples were analysed on a Philips PU 9800 FTIR spectrometer. The infrared spectrum of each sample was obtained by mixing with Nujol. The samples and a blank, which consisted of only Nujol, were measured between sodium chloride plates of dimension 30 mm x 15 mm x 4 mm.

2.12 Characterisation of immobilised xylanase

2.12.1 Measuring the activity of the xylanase /silica matrices

The matrices, accurately weighed between 10 mg and 27 mg, were suspended in 1 ml of 50 mM CH_3CO_2Na buffer, pH 5.3, vortexed and then the xylanase assays were performed according to Section 2.2.1.

2.12.2 Determination of the thermal stability of silica/xylanase matrices in wet heat

The matrices, accurately weighed between 10 mg and 50 mg, were suspended in 1 ml of 50 mM CH_3CO_2Na buffer, pH 5.3. Samples were placed in a water bath at temperatures 60 °C or 70 °C for 0 min to 20 min. After heat treatment, the samples were allowed to cool to room temperature and then assayed for xylanase activity according to Section 2.12.1.

2.12.3 Determination of the thermal stability of silica/xylanase matrices in dry heat

Samples of silica/xylanase matrices were accurately weighed, between 15 mg and 37 mg, in a centrifuge tube. All sample except the ones measured for thermal stability at zero time were placed in a water bath at a temperature of 60 °C, 70 °C or 80 °C. A thermometer was place in one of the samples to measure the temperature and the timing of heat exposure was started as soon as the dry matrix had reach to the desired temperature. Samples were exposed to these temperatures between 0 min and 20 min. After heat treatment the samples were allowed to cool to room temperature and then assayed for xylanase activity according to Section 2.12.1.

2.12.4 Determination of pH stability of silica/xylanase matrices

Accurately weighed matrix samples between 15 mg and 37 mg, were suspended in 4 ml of 100 mM sodium citrate plus 200 mM Na_2HPO_4 buffer with pHs ranging from pH 2 to pH 9. The samples were stored at 4 °C for 24 hours prior to washing twice with the

same buffer solution at pH 5.3 and then assayed for xylanase activity according to section 2.12.1.

2.13 Simulated processing trials

Simulation of the processing trials was carried out in the laboratory using a retort autoclave. The immobilised and free xylanase were mixed as thoroughly as possible into the milled Danisco chicken feed at a ratio of 1 g immobilised enzyme to 1 kg of feed or 1 ml enzyme (200 U) / 1 kg of feed respectively to obtain a final activity of 5 U g^{-1} of feed. Distilled water was added to increase the moisture content to 10 %, 15 % or 20 % (w/w). For the negative control, distilled water was added instead of xylanase. The feed mixture (50 g) was sealed in cans and then heated in a retort autoclave to between 75 °C and 95 °C for 30 s to 20 min. Xylanase recovery was tested by measuring its activity as described in Section 2.2.1; 1 g of processed feed was incubated for 30 minutes with 5 ml of 1.2 % (w/v) oat-spelt xylan at 50 °C. The reaction was stopped by the addition of 5 ml 200 mM NaOH solution and reducing sugars were determined using 3, 5-dinitrosalicylic acid (Miller, 1959).

2.14 Spray drying of the xylanase/carbohydrate mixtures

2.14.1 Preparation of solutions

Solutions of maltodextrin, dextrin and chitosan were prepared by dissolving 40 g in 250 ml of 50 mM CH_3CO_2Na buffer, pH 5.3. An appropriate amount of xylanase was added to the solutions to give proportions of carbohydrate to xylanase of 4:1, 4:5, 2:1 and 1:10 (w/w) as presented in Table 2.2. Control samples, in the presence and absence of stabilisers, were also prepared.

Wall material	Additives (stabilisers and core material)	Final formulation ratio (Wall material:Additives)
Xylanase	-	-
Maltodextrin	-	-
Maltodextrin	Sucrose and *PEG	20:1:1
Maltodextrin	Sucrose	10:1
Maltodextrin	*PEG	10:1
Maltodextrin	Xylanase	4:1 and 4:5
Dextrin	-	-
Dextrin	Xylanase	2:1
Chitosan	Xylanase	1:10

Table 2.2. Formulations of samples that were spray-dried.

* PEG M_w 8,000

2.14.2 Spray-drying of solutions.

Carbohydrate/xylanase solutions were spray dried in a laboratory SD-04 Spray dryer (Lab Plant UK), with a standard 0.5 mm nozzle. The temperature and flow rate were 170 °C and 2.63 ml min^{-1} respectively while the outlet temperature was maintained between 97 °C and 104 °C. In a few cases, especially with solutions that contained stabilisers, the outlet temperature was increased to 200 °C. The solutions were fed at a rate of 2.63 ml min^{-1} and the dried powders collected. The powders were stored at room temperature in closed containers.

2.15 Microencapsulation by phase separation

2.15.1 Microencapsulation by coacervation

The preparations of complex coacervates were carried out according to Burgess and Ponsart (1998). Deionised solutions of 2 % (w/v) gelatin and 2 % (w/v) arabic gum were prepared separately and the pH values were adjusted to pH 3.9 using 100 mM HCl. The ionic strength of both solutions was maintained by the addition of 0.5 g NaCl. To each solution, 6 g of polyethylene glycol of M_w 4,000 and 0.5 ml of xylanase were added. The two polyion solutions were heated to 50 °C and then equal volumes (50 ml) were mixed together with constant stirring for 30 minutes. After mixing, 5 ml of 40 % (w/v) glutaraldehyde solution was added to the mixture and then the mixture was allowed to stir continuously for another 30 minutes. The mixture was then cooled to 5 °C in an ice bath while stirring. The coacervates were filtered under suction, washed and dried under vacuum before storing at 4 °C. For samples to be examined by microscopy, the coacervate with and without xylanase were washed with sodium acetate buffer, pH 4, and then stored in buffer solution with 5 % (w/v) NaCl.

2.15.2 Microencapsulation of xylanase with albumin

An emulsification of water-in-oil was formed with 1 ml of 50 % (v/v) xylanase diluted with distilled water in 50 ml vegetable oil using a magnetic stirrer and was allowed to mix for 15 minutes. The formation of the wall material occurred with the addition of 3 g of albumin and the mixture was allowed to mix for a further 15 minutes. The oil was then filtered off under suction and the microcapsules were washed with acetone prior to allowing to dry in air. The dried samples were then examined by SEM.

2.15.3 Melt dispersion

Three millilitres of xylanase was emulsified in 9 g of molten candellila wax at 57 °C using a motorised stirrer at 2500 rpm. Fifty millilitres of heated water of the same temperature was added rapidly while vigorously stirring. Agitation continued for about 30 sec, then the mixture was rapidly cooled using an ice bath to harden the

microcapsules. The microcapsules were collected by filtration under suction, allowed to dry in air and then stored in vials at room temperature.

2.15.4 Scanning election microscopy (SEM)

All spray-dried samples were prepared for analysis. The samples were mounted on a flat stub and coated with an alloy consisting of 60 % (w/w) gold and 40 % (w/w) palladium in order to render the sample electro-opaque. They were then examined in a Jeol JSM 5600LV scanning electron microscope. The procedure was performed by Mrs. Kath Ditchfield (Microscopy Department, BTTG).

2.16 Immobilisation of Xylanase onto Chitosan

First, a 500 ml stock solution comprising 1.3 % (w/v) chitosan, 2 % (v/v) CH_3CO_2H and 10 mM NaN_3 was prepared. The pH was 4.8. Immobilisation of xylanase onto chitosan was carried out at room temperature by adding 0 ml to 4 ml of xylanase to 20 ml of chitosan stock solution. After mixing for about 15 min, 1 ml of 40 % (w/v) glutaralderhyde was added while mixing and the solution was mixed for a further 5 min. The mixtures were then poured into 50 ml plastic containers and the resulting gels were allowed to set. The samples were dried by freeze-drying for 48 h. The xylanase activity from each sample was obtained by accurately weighing to the nearest 10 mg and suspending in 1 ml of 50 mM CH_3CO_2Na buffer, pH 5.3 and vortexing. The xylanase assays were performed according to Section 2.2.1. The binding efficiency was determined by comparing the xylanase activity of the washed and unwashed samples.

2.16.1 Determination of the thermal stability of immobilised xylanase

The chitosan/xylanase gel mixture was prepared differently from the method described in Section 2.16. The gel mixture was prepared by adding 3 µl of xylanase and 5 ml of 25 % (v/v) glutaralderhyde to 50 ml of the chitosan stock solution. Prior to allowing the gel to set, 1 ml of the mixture was injected into previously weighed 15 ml test tubes. The gels were then allowed to set and freeze-dried for 24 hours. After freeze-drying the test tubes with their contents were weighed again. The thermal stability was determined

by adding 1 ml of 50 mM CH_3CO_2Na buffer pH 5.3 to each test tube and then vortexing. The components of the test tube were exposed to temperatures between 60 °C and 80 °C for 0 min to 20 min, cooled and then assayed at 50 °C according to Section 2.2.1.

Chapter 3

Characterisation of Xylanase

3.1 Introduction

Several thousand enzymes are commercially available and many of these enzymes, including xylanase, are mass produced (Scawen, 1985). Large scale production of enzymes, in general terms, involves several steps. The first step is finding a suitable microbial source, bacterial or fungal. Those which are chosen for large scale production of enzymes are 'generally recognised as safe' (GRAS) suggesting they are non toxic, non pathogenic and do not produce antibiotics (Headon and Walsh, 1994). For production of new enzymes, rather than growing the original organism, the gene is usually transferred to a preferred GRAS organism. Once the chosen gene has been transferred the microbe is grown on large quantities using an industrial fermenter. All of the nutrients required for growth along with the maintainance of optimum pH, temperature, oxygen and a controlled rate of stirring are necessary to encourage the microorganism to grow at a fast rate. Live or dead organisms are then removed by downstream processing (Headon and Walsh, 1994). The purification process is important to ensure that live genetically modified organisms are not released from the factory and a combination of at least three chromatographic steps is usually employed. The final enzyme products are usually 95 % - 98 % (w/v) pure (Headon and Walsh, 1994) and are normally sold in powder or liquid form combined with stabilisers.

The xylanase used in this research, assumed to be produced from a selective strain of *Trichoderma longibrachiatum* (formerly known as *Trichoderma reesei)* was obtained from Genencor. The xylanase product was in the form of a liquid, and the formulation also contained 0.5 % (w/v) potassium sorbate and 0.5 % (w/v) sodium acetate. The data

presented in this chapter cover the characterisation of the Genencor xylanase and optimisation of the assay used for the determination of xylanase activity.

3.2 Xylanase activity

Three different protocols were used to determine the activity of commercial xylanase. These are detailed in Section 2.2 and are summarised in Table 3.1. The oxidising agent in all three protocols used to quantify the released xylose sugar was dinitrosalicyclic acid solution (DNSA). All of the assays were carried out in sodium acetate buffer solution, 50 mM at pH 5.3 because it has been reported that different buffer solutions can affect the activity (Royer and Nakas, 1989). Two out of the three protocols used gave similar results with a slight discrepancy of only three-percent. The activity determined by an independent laboratory, which will be named as Lab A in this thesis, was almost three times higher than the others (222,000 U ml^{-1}) possibly due to poor assay. Even though the results of Danisco and Bailey's protocols coincided with each other, there are other factors which could have a significant effect on the measurement of xylanase activity including the type of xylan used and the DNSA used.

There is evidence that some xylans are more susceptible to enzymatic attack than others (Royer and Nakas, 1989 and Bailey *et al*, 1992). According to Bailey *et al*, (1992) the susceptibility is largely due to the degree of substitution with acetic acid, 4-O-methyl glucuronic acid, and arabinose. Highly substituted xylan will produce relatively lower levels of oligo-xlyose when broken down by xylanases. Birchwood and oat-spelt xylans were used for the determination of xylanase activity in this project. Bailey *et al*, (1992) recommended birchwood xylan because of its low turbidity, extended range of linearity of the reaction using the substrate and its commercial availability. Royer and Nakas (1989) found that oat-spelt is more susceptible to enzymatic attack than aspen and larch xylan. The xylanase activity using Danisco's protocol with birchwood instead of oat-spelt xylan was 67,000 U ml^{-1} and likewise with Bailey's method using oat-spelt, the activity was 129,000 U ml^{-1}. These findings indicate that oat-spelt is a more susceptible xylan.

Xylanase activity varied between each batch of oat-spelt xylan made throughout the research which may indicate that the results were affected by the inconsistent solubility and turbidity of the substrate.

	Danisco	**Bailey *et al.***	**Lab A**
Amount of xylanase required per assay	0.001µl	0.002 µl	0.001µl
Substrate (xylans)	Oat-spelt	Birchwood	Oat-spelt
Incubation time (min)	30	5	10
Wavelength (nm)	540	540	575
Activity (U ml^{-1})	77,000(1)	80,000(2)	222,000(2)

Table 3.1 Comparison of three xylanase assays. The assays were carried out as described in Materials and Methods (section 2.2). After colour development using DNSA: DNSA[1] consisting of 30.0 % (w/v) sodium potassium tartrate, 1.6 % (w/v) sodium hydroxide and 1.0 % (w/v) dinitrosalicyclic acid; DNSA[2] according to Miller (1959)consisting of 21.6 % (w/v) sodium potassium tartrate, 1.4% (w/v) sodium hydroxide, 0.8 % (w/v) dinitrosalicyclic acid, 0.6 % (w/v) sodium bisulphite and 0.5 % (w/v) phenol. The assays were carried out in triplicate.

Oat-spelt and birchwood xylans (1 % w/v) were hydrolysed by xylanase diluted 1 in 10 and analysed by thin layer chromatography. Samples were taken at 5 min intervals for 30 min; the mobile phase used in this experiment was a mixture of n-butanol, acetic acid and water in the ratio of 60:15:25 (v/v/v). The purpose of this experiment was to determine whether xylose itself is produced during the incubation period. From the data seen in Figure 3.1 it is clear that xylose sugar is produced during the first 5 min from both xylans. It is also evident that lower mobility sugars are also produced; xylose oligomers. These are most likely indicating that the commercial xylanase has both exo and endo–acting activity.

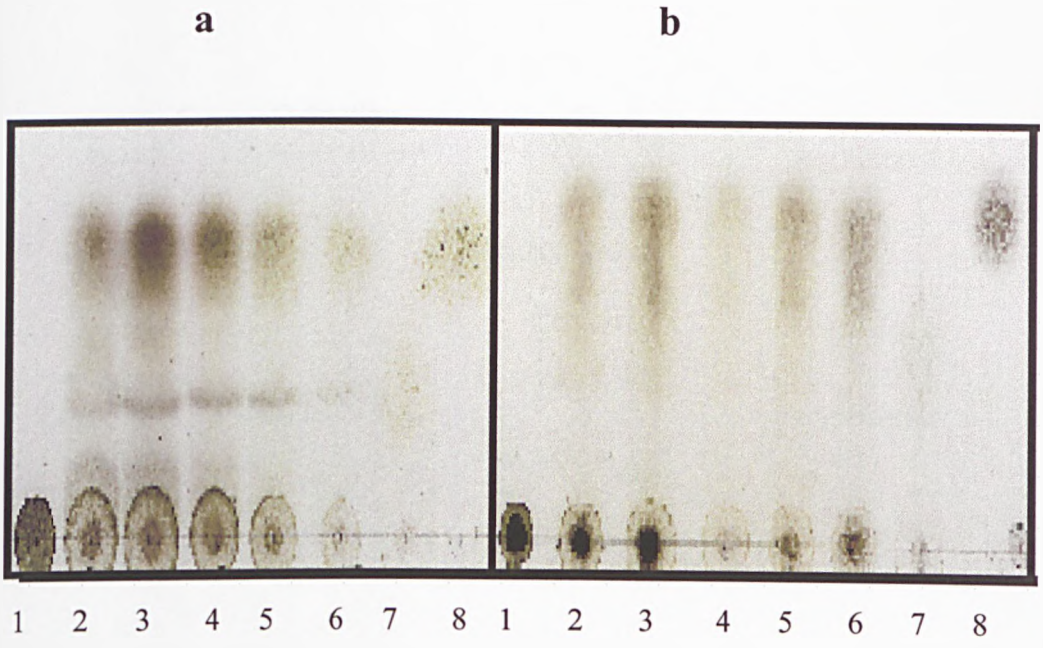


Figure 3.1 TLC analysis of the hydrolysis of (a) birchwood and (b) oat-spelt xylans. Lanes 1-7 are birchwood and oat-spelt hydrolysed for 0, 5, 10 15, 20, 25 and 30 minutes respectively; lanes 8 are xylose sugar (1% w/v).

Although the assay for reducing sugar using DNSA has been greatly criticised (Cauchon *et al*, 1983 and Breuil, *et al*, 1984) it is still the most popular method used for measuring xylanase activity (Bailey *et al*, 1992). The development of DNSA was first published in 1921 (Sumner, 1921) and was modified in 1959 (Miller, 1959). Originally, the purpose of the sulphite and phenol as to intensify the colour of the assay, the introduction of sodium potassium tartrate was to prevent the reagent from dissolving oxygen (Sumner, 1922). Miller (1959) demonstrated that sulphite successfully removed the dissolved oxygen from the solution and sodium potassium tartrate interfered with that action. Despite the interference, sodium potassium tartrate caused an enhancement of the colour. A comparison of the two DNSA reagents was made using Bailey's protocol. The results obtain by DNSA[1] (Table 3.1) was 53,000 U ml^{-1} whereas that obtained by DNSA[2] was 80,000 U ml^{-1}.

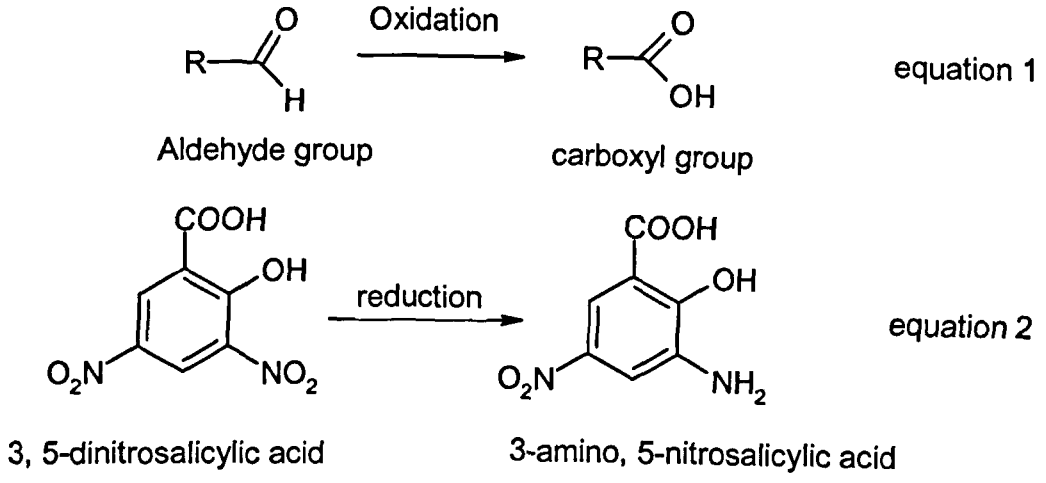


Figure 3.2 Important reactants and products in the DNSA assay responsible for the colour change indicating the presence and quantity of xylose sugar.

Oxidation of an aldehyde group to a carboxyl group by DNSA is not a stoichiometric reaction and is more complicated (Miller, 1959 and Breuil, *et al*, 1984) than that described in equations 1 and 2 (Figure 3.2).

A problem encountered while carrying out the assays (in test tube) was the nonlinear effects of dilution and therefore it was very difficult to obtain the true activity of the enzyme. Similar results have been reported (Cauchon *et al*, 1983 and Breuil, *et al*, 1984). Bailey stated that insufficient substrate in the reaction mixture is a probable cause of non-linearity in assays. All standard curves maintained linearity but there was very little or no absorbance corresponding to xylose of concentration less than 0.01 mg ml^{-1}. The standard curves have never crossed the point of origin, which has also been observed by Breuil *et al* (1984). Figure 3.3 shows the variation in the standard curves when using different methods.

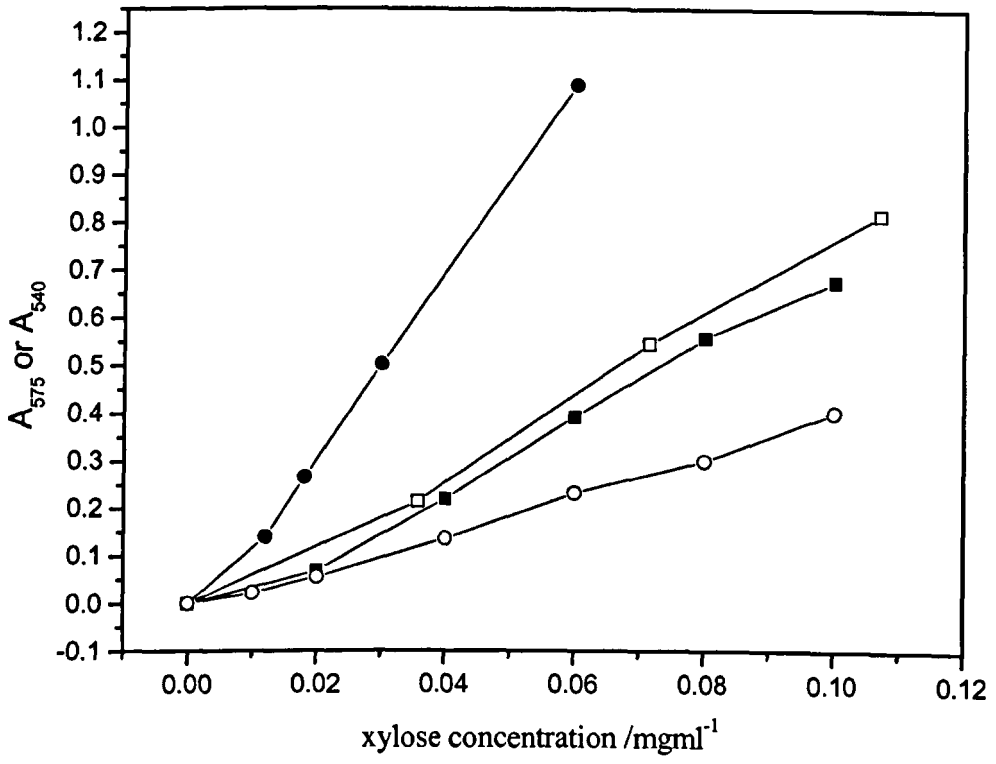


Figure 3.3 A Graph showing the variation in the standard curves. The absorption within Danisco (■), Bailey (●) and micro titre (○) assays were measured at 540 nm while the absorbance within Lab A's (□) standard assay was measured at 575 nm. Each point on the graph represents the concentration of xylose at the end of the reaction. Data are ± mean SE (n= 5).

A decision was made to perform the xylanase assay at dilutions between 1 in 10^5 and 1 in 10^6 (xylanase in buffer) according to Danisco's protocol adapted to a 96 well micro-titre plate format. The reason for the adaptation was to increase throughput. Xylanase activity appeared linear with increasing volume between 15 µl and 45 µl added as seen in Figure 3.4. These results were considered to be successful and the average activity obtained was 8,900 U ml^{-1}. The large variation in the result may have been due to the plate format since it is known to give low absorbance values.

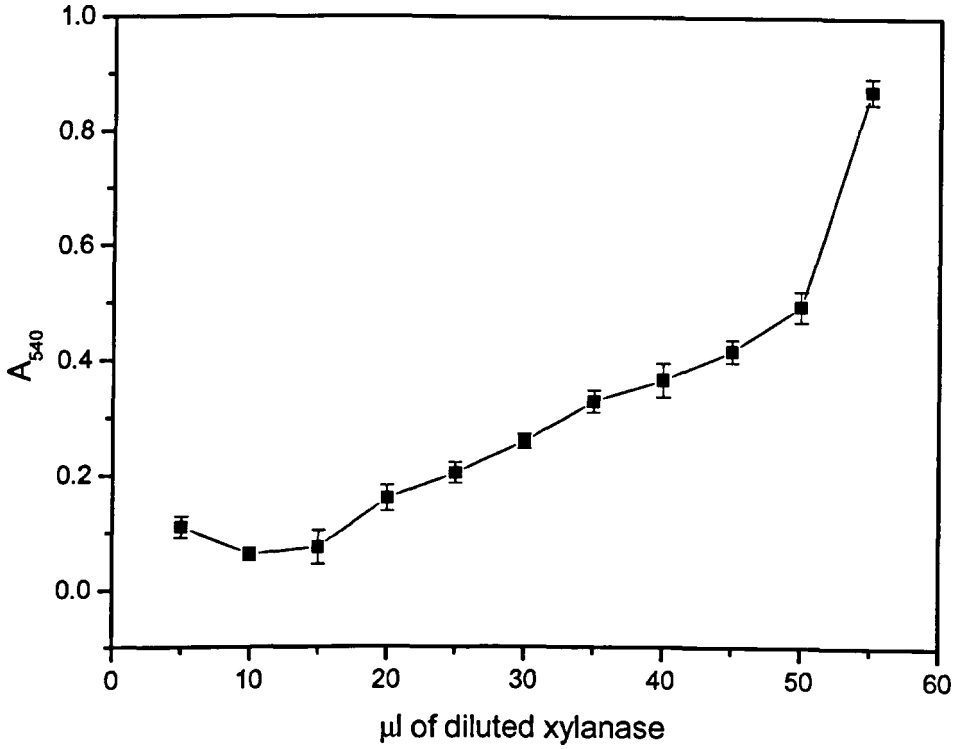


Figure 3.4 Xylanase assay carried out in the micro-titre plate format. Xylanase was assayed according to the Danisco's protocol using 5 µl to 50 µl of a 1 in 10^5 dilution of Genencor's commercial xylanase. Data are the mean ± SE (n = 4).

Several attempts were made to purify xylanase by dialysis, but it was found that the dialysis membrane was degraded due to the commercial xylanase product having some cellulolytic activity. Xylanase was assayed for cellulase activity with sodium carboxymethyl cellulose as substrate using the same methods used for the determination of xylanase activity. According to Danisco, Bailey and the Lab A method the cellulase activity of the commercial xylanase is 57 U ml^{-1}, 24 U ml^{-1} and 136 U ml^{-1} respectively. Another possibility could be due to contamination with cellulase enzyme.

3.3 Identification of xylanase

Xylanase was loaded onto a Bio-gel P60 column and purification was carried out at room temperature. The result of chromatography using sodium acetate (50 mM) of pH 5.3 for elution is shown in Figure 3.5. The bicinchoninic assay was used to determine the protein concentration of each fraction. Xylanase activity was also determined. The data clearly reveal that xylanase was eluted between fractions 24 and 34. However, there were two other peaks of protein, which showed little or no xylanase activity. The three protein peaks were further analysed using SDS-PAGE. The gel-filtration method was repeated using deionised water in preparation for subsequent mass spectroscopy analysis. Although separation took place between the second and the third peak there was no clear distinction between the first and the second. The results obtained from Figure 3.5 revealed that the specific activity of xylanase was 2230 U mg^{-1}.

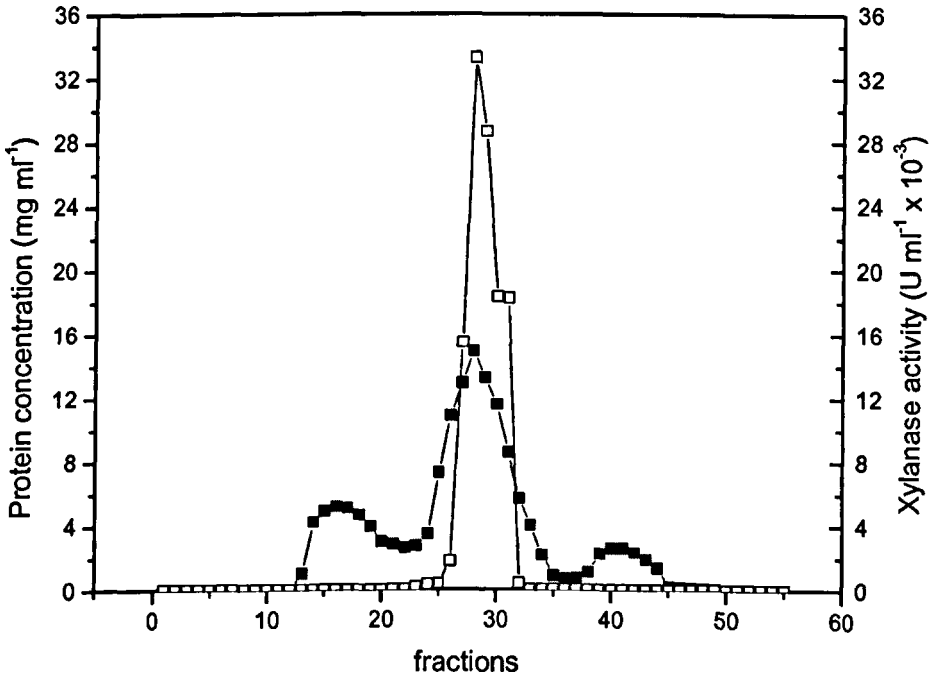


Figure 3.5 Gel filtration of xylanase revealing the protein concentration (■) and the xylanase activity (□) of each fraction. The procedures were carried out as described in Sections 2.5.2 and 2.2.4.

From the elution profile of the column chromatography as seen in Figure 3.5 and the SDS-PAGE (Figure 3.6), purified xylanase appeared to be homogeneous. The molecular mass of purified xylanase (Lane 4) was 20.3 kDa and the molecular mass of the first eluted protein (Lane 3) was approximately the same. The similarity of the results may have been due to contamination of lane 3. The molecular mass of xylanase from *Trichoderma reesei* has been reported to be 20 kDa (Tenkanen *et al*, 1992) and also 21 kDa (Torronen *et al*, 1993). No bands appeared representing the third protein, which may suggest that the concentration was too low or it may not have been a protein but a contaminant that interfered with the BCA assay.

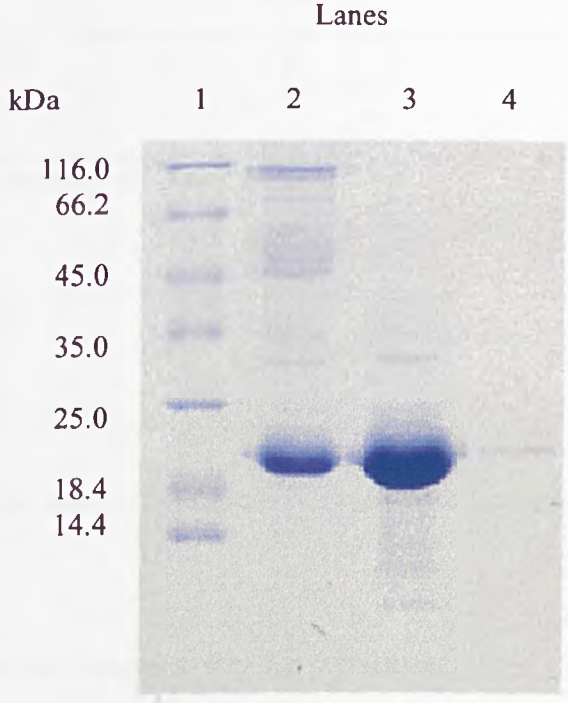


Figure 3.6. SDS polyacryamide gel analysis of xylanase fraction: Lane 1, molecular weight markers; lane 2, commercial xylanase; lane 3, first protein eluted; lane 4, purified xylanase.

The fractions shown in Figure 3.5 were treated with 5 ml sample preparation buffer and electrophoresed on a 12 % (w/v) polyacrylamide gel as described in Section 2.6. The molecular mass of xylanase was also determined by mass spectroscopy (Appendix 1), the principal peak showed a molecular mass of 20,826 +/-2 Da. According to the literature xylanase consists of 190 residues and has a molecular mass of 20,699 Da

(Torronen *et al*, 1995 and Havukainen *et al*, 1995). Discrepancies between the values may have been due to acetylation and/or water molecules, which could be bound to the enzyme by hydrogen bonding. Table 3.2 below contains a list of possible additions which could be responsible for the increase in mass. A discrepancy of 127 +/- 2 Da may also suggest that there may be an extra residue.

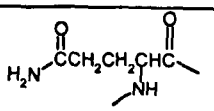
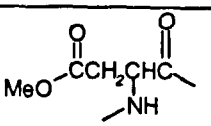
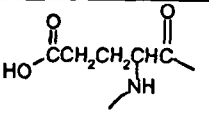
Name of group	**Structure**	**Mass**
Water	H_2O	126 (7 molecules)
acetyl		126 (3 units)
Glutamyl (Q)		128
O-Methyl Aspartamyl		129
Glutamyl (E)		129

Table 3.2 Mass values for possible chemical modifications to xylanase.

3.4 Optimum temperature for xylanase

The temperature for optimum xylanase activity was determined by assaying xylanase at temperatures from 24 °C to 70 °C over 30 min in 50 mM CH_3CO_2Na buffer solution, pH 5.3 (as outlined in section 2.8). It can be seen from Figure 3.7 that the optimum temperature under these conditions is about 50 °C. Higher activity has been observed at 55 °C but only for a short period of about 5 minutes and above 60 °C the enzyme is rapidly inactivated.

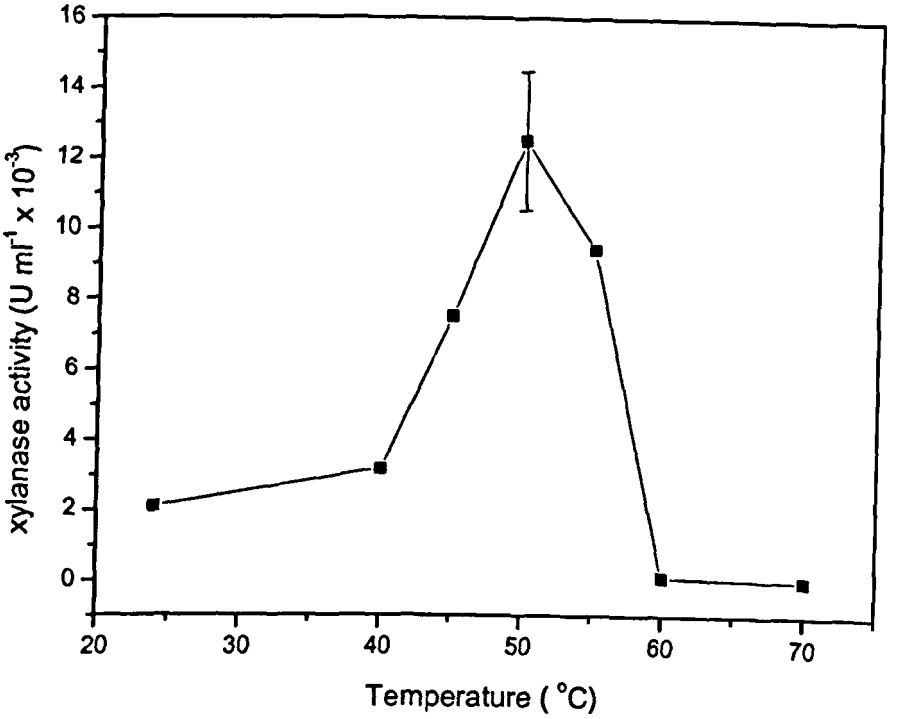


Figure 3.7 Temperature dependence of xylanase activity. Xylanase was assayed as described in section 2.8. Data are mean ± SE (n=5).

3.5 Optimum pH for xylanase

The pH for optimum xylanase activity was determined by assaying xylanase at pH 3.0 to pH 7.0. Each sample was incubated at 50 °C for 30 min in sodium citrate and sodium phosphate buffer solution of 100 mM and 200 mM respectively.

The pH optimum for xylanase was 4.5 (Figure 3.8). Tenkanen *et al* (1992) and Royer (1991) had observed pH optima at 5.0 – 5.5 and 4.8 respectively in citrate-HCl buffer.

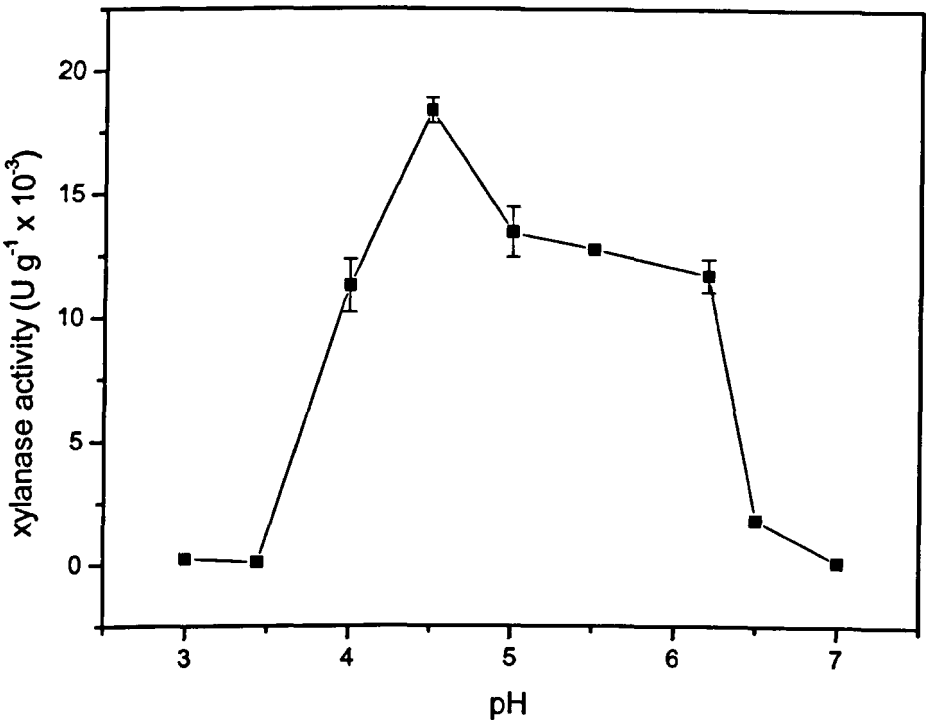


Figure 3.8 pH dependence of xylanase activity. Xylanase was assayed as described in section 2.8. Data are mean ± SE (n=5).

3.6 pH stability of xylanase

To determine the pH stability of xylanase, the enzyme was incubated at 4 °C in 100 mM sodium citrate and 200 mM sodium phosphate buffer solution with the pH ranging from pH 2 to pH 8 for 24 h. After 24 h the samples were warmed to room temperature and then diluted in 100 mM sodium citrate and 200 mM sodium phosphate buffer, pH 5.3. The result of the stability test is shown in Figure 3.9. Maximum pH stability was observed between pH 4.0 and pH 8.0 where xylanase had retained as much as 60 % of its activity.

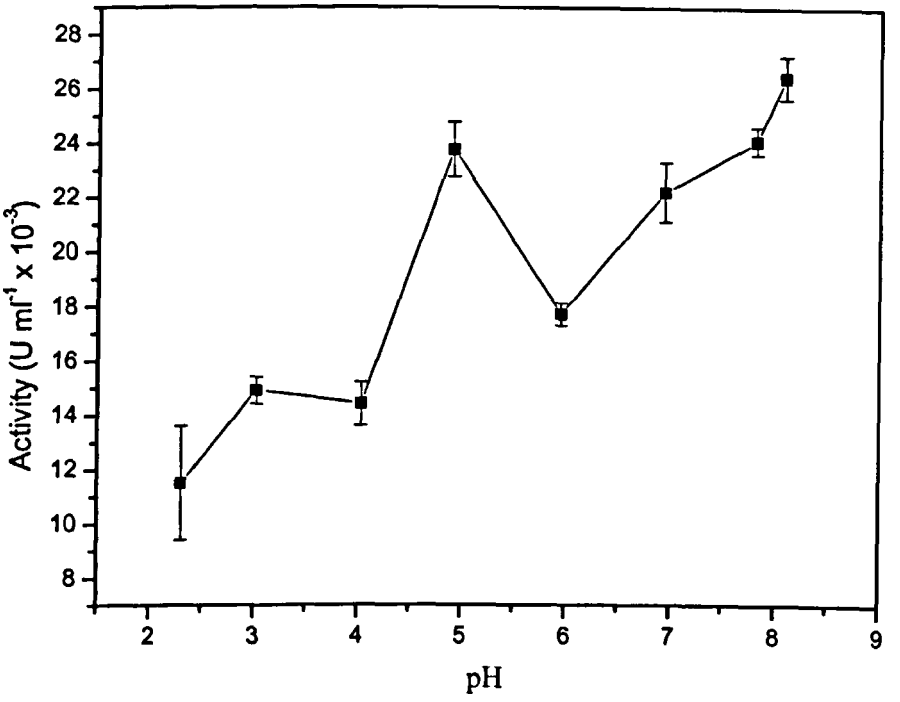


Figure 3.9 The effect of pH stability on the activity of xylanase. Commercial xylanase was assayed as described as Section 2.8.4. Data are ± mean SE (n=5).

3.7 Thermal stability of xylanase

To measure the thermal stability of xylanase, a dilution series of xylanase was exposed to temperatures of 50 °C, 55 °C and 60 °C for up to 1 h, allowed to cool to room temperature and then assayed for xylanase activity at 50 °C. The data in Figure 3.10 shows that xylanase had good thermal stability, retaining all of its activity for 1 h at pH 5.3 at 50 °C. Thermal stability of xylanase at 55 °C remained high for the first 20 min and then a steady decline to 4 % of the original activity at 60 min However, at 60 °C there was 100 % drop after 20 min.

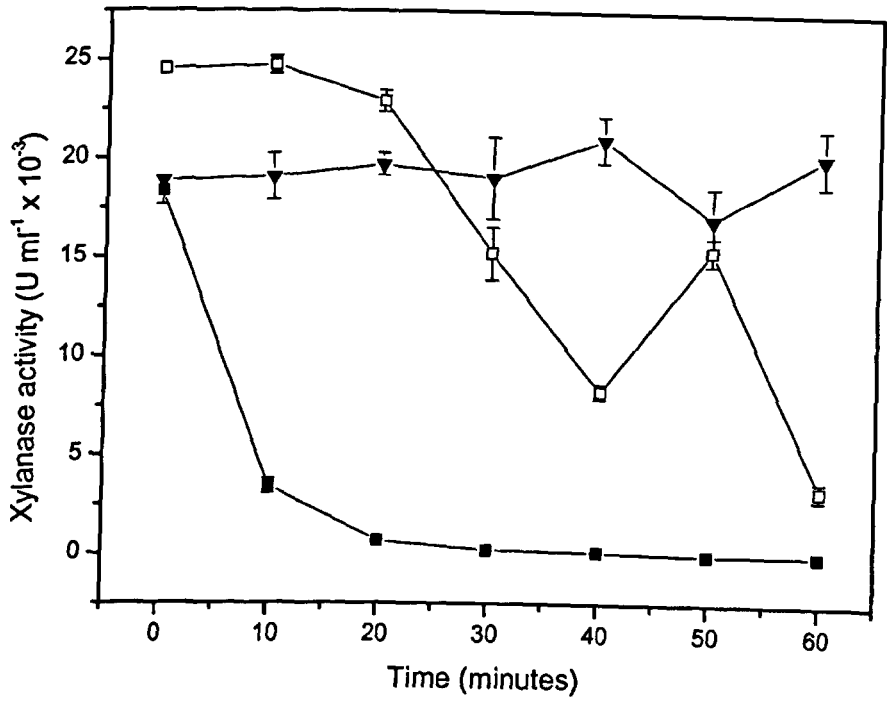


Figure 3.10 The graph reveals the effect of thermal stability at 50 °C (▼), 55 °C (□) and 60 °C (■) on the activity for 1 h prior to carrying out the activity assay. Data are ± SE (n=5).

3.8 Summary

Accurate determination of the true activity of xylanase was difficult to achieve. The three methods described in this chapter used DNSA to quantify the reducing sugar, xylose. A large variation in results was observed. The factors which contributed to these discrepancies were the type of DNSA used, the type and the preparation of the xylan substrate. A decision was made to perform the assay in a micro-titre plate format in order to increase throughput.

In order to determine the identity of xylanase the molecular mass was obtained and compared with other xylanases from the Protein Data Bank. The enzyme closely resembled that of xylanase from *Trichoderma longibrachiatum* formerly known as *Trichoderma reesei* with a slight discrepancy of 127 Da. The increase in mass may have

been due to acetylation or an additional amino acid residue. The properties of xylanase were also compared with published data of xylanase from *Trichoderma longibrachiatum*. The results reported in this chapter were in agreement with literature values. However, there was a variation of results in relation to pH optima and pH stability that has been recorded due to the different buffer conditions used.

Chapter 4

Investigation of the Structure of Xylanase under Physical and Chemical Stress

4.1 Introduction

Structural changes of proteins exposed to different chemical and physical conditions have been of great interest in the field of biochemistry. A key technique used to study the conformation of proteins is circular dichroism (CD) spectroscopy. CD is very sensitive, is able to detect different conformational changes and is a convenient method of monitoring structural changes in proteins (Sreerama and Woody, 2000; Venyaminov and Yang, 1996). CD is defined as the difference between the absorption of left and right handed circularly polarised light and is expressed as the sum of characteristics from the different elements of the secondary structure. The CD spectrum is divided into two regions, far ultraviolet (UV) and near-UV. The far-UV, from 190 nm to 250 nm, provides information on the polypeptide backbone conformation of proteins and the near-UV, from 250 nm to 300 nm, reflects the contribution of the aromatic side chain and disulphide bonds (Wallace and Janes, 2001; Sreerama and Woody, 2000; Venyaminov and Yang, 1996). The region of interest in this project is the far-UV where a strong response indicates the presence of β–sheet structures. Further representations of other secondary structures are mentioned in Section 1.5.1. CD measurements have been made in order to aid our understanding of how the structure of xylanase reacts under physical and chemical stress.

Addition of stabilisers is a very common procedure to improve the activity and stability of enzymes. In the case with metal ions, their binding provides useful routes to protein

stabilisation (Arnold and Zhang, 1994). They generally act as electrophiles seeking to share electron pairs from main and side chain groups of a protein. The number of ligands packed around the metal ion is dependant on the size of the ion (Glusker, 1991) and the effect of the metal ions on protein stability is closely related to the Hofmeister's lyotropic series as mentioned in Section 1.6.2.2. It is presumed that the role of metal ions is to stabilise the three dimensional orientation of the various domains of the protein possibly bringing to the correct relative orientation groups within the active site (Hughes, 1981). In other words, the metal ion can favourably shift the folded/unfolded equilibrium of a protein and enhance its thermodynamic stability by binding with higher affinity to the folded state than to the unfolded or the partially folded states (Arnold and Zhang, 1994).

An alternative approach is immobilisation involving physical attachment of the enzyme to a solid support material. Xylanase has been immobilised onto silica, which provided increased stability (Chapter 5). Unfortunately, CD can not be performed on materials in suspension. Accordingly, the structural behaviour of xylanase in the presence of metal ions was determined before the immobilisation process, and the aim of work presented in this chapter is to observe changes in the β–sheet structures when xylanase is exposed to 'stressors'. These include high temperatures and significant pH changes that maybe encountered during feed processing or subsequent feed use.

4.2 The effect of temperature and pH on the structure of xylanase

4.2.1 Interpretations of the spectrum

A far-UV CD spectrum for xylanase at its optimal temperature of 50 °C is shown in Figure 4.1. A moderate signal between wavelength 210 nm and 240 nm confirmed the presence of β–sheet structures. Torronen and Rouvinen (1995) had estimated that xylanase from *T. reesei* comprised of 44 % β-sheet and 5 % α-helix. The presence of the α-helix would have had a small influence on the intensity of the signal. In all cases when carrying out experiments with xylanase, random noise was seen at the lower end of the spectrum (190 nm –205 nm) and therefore this CD data was disregarded.

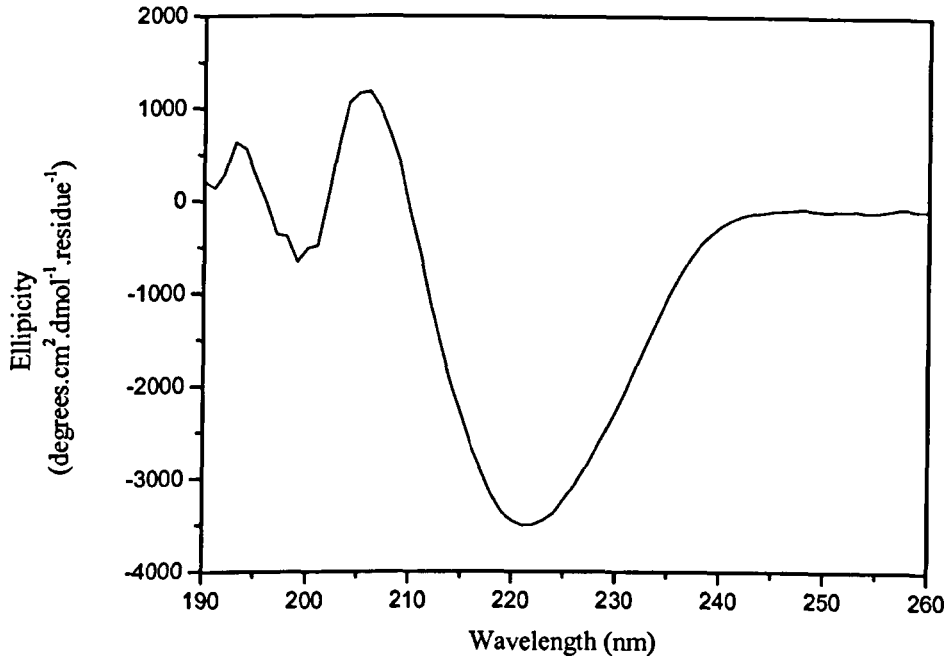


Figure 4.1 Far-UV CD spectrum of xylanase from *Trichoderma longibrachiatum*. A solution of purified xylanase (0.4 mg ml^{-1} in 50 mM CH_3CO_2Na buffer at pH 5.3) was scanned between wavelength 190 nm and 260 nm at 50 °C. The pathlength was 1mm.

4.2.2 The effect of temperature

CD scans were recorded every 5 min for xylanase dissolved in 50 mM sodium acetate buffer, pH 5.3 at 60 °C. Figure 4.2 shows that there was a decrease in the intensity of the signal between wavelengths 210 nm and 245 nm with increasing exposure at 60 °C, indicating loss of β–sheet structure with time. The data also implied that the unfolding of the protein was not proportional to time of exposure. The thermal stability of xylanase at 60 °C did show a rapid exponential decrease in activity in the first 20 min as mentioned in Section 3.7. The experiment was repeated but instead of measuring the CD every 5 min the samples were heated to 60 °C for a time then allowed to cool to 50 °C and then scanned to determine the structural stability (Figure 4.3). The data

showed that the rate of degradation was not as rapid as in the previous experiment and implies that some re-folding of xylanase is possible.

4.2.3 The effect of pH

Samples of xylanase were incubated in buffer consisting of 100 mM sodium citrate and 200 mM of Na_2HPO_4 at pHs ranging from pH 2 to pH 8 for 24 h before scanning at 50 °C. CD spectra is shown in Figure 4.4 a and b. The extremes of pH tested pH 2 and pH 8 appeared to have caused significant changes in β-sheet structure as indicated by CD spectrum between wavelengths 210 nm and 220 nm. These changes may have been due to the disruption of the random coil structure. At pH 6 there was evidence of a slight loss or unfolding of β–sheet structure. Torronen *et al* (1994) had observed conformational differences between pH 5 and pH 6. Although xylanase at pH 6 appeared to have undergone some structural changes the activity at pH 6 is still relatively high at 11,000 U ml^{-1} in comparison to an activity of 18,000 U ml^{-1} at pH 5 (Figure 3.8).

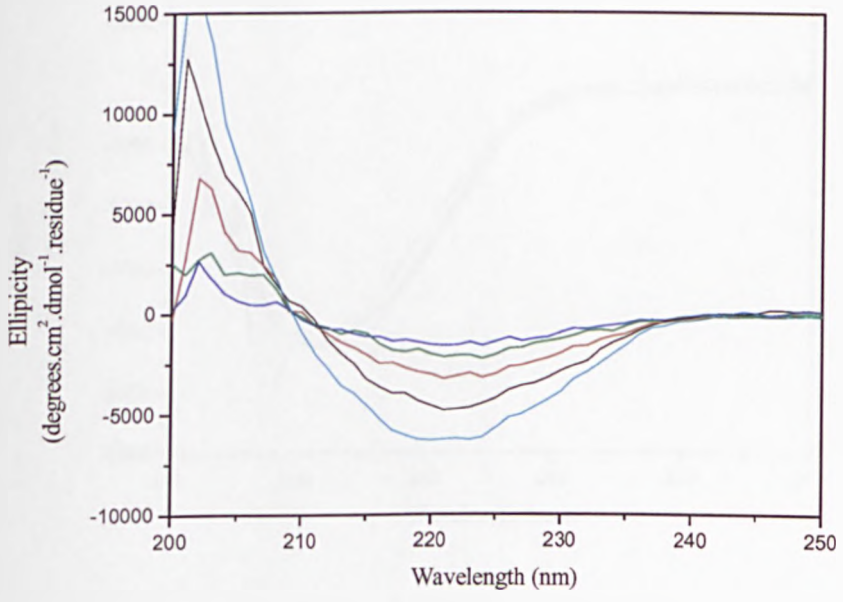


Figure 4.2 CD representation of the thermal degradation of xylanase at 60 °C. CD scans of xylanase were taken at 0 min (–), 5 min (–), 10 min (–), 15 min (–) and 20 min (–) as described in section 2.12.2.

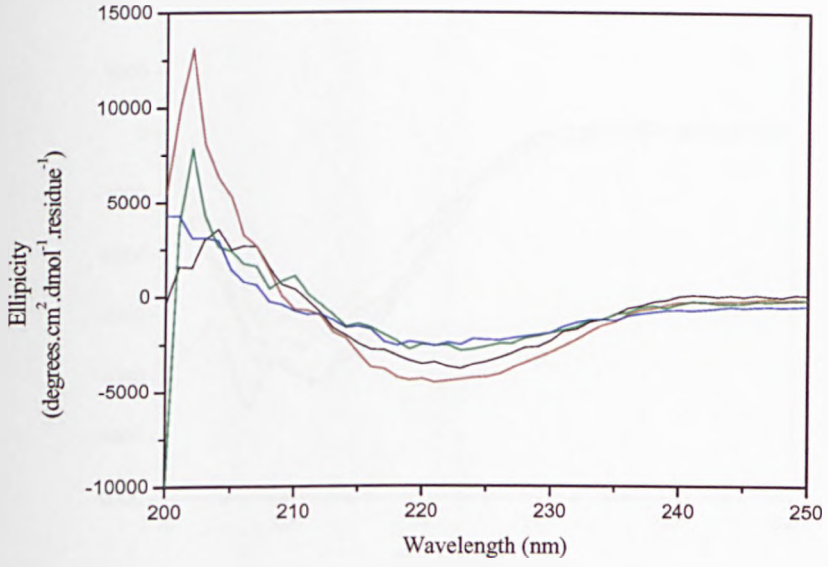


Figure 4.3 CD representation of the thermal stability of xylanase. Xylanase samples were heated to 60 °C for 0 min ( ), 5 min ( ), 10 min ( ) and 15 min ( ). Each sample was allowed to cool to 50 °C prior to scanning as described in section 2.12.4.

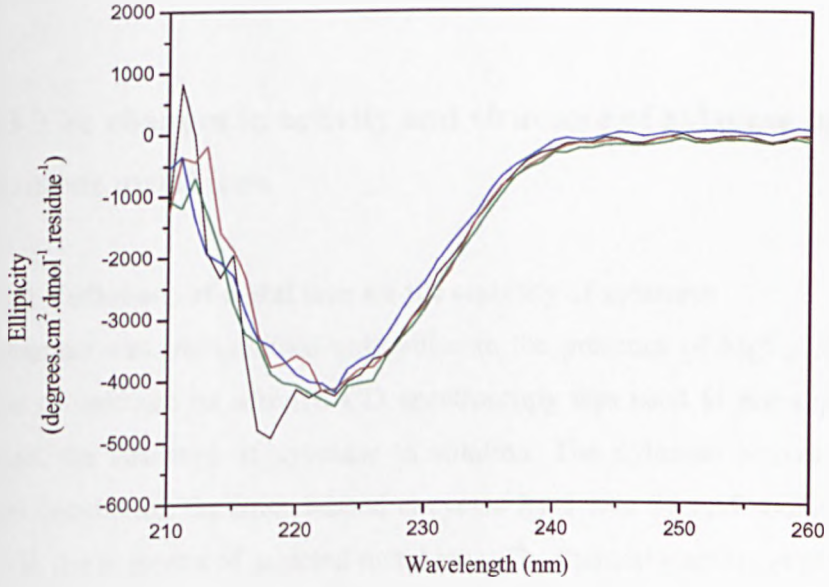


Figure 4.4a CD representation of xylanase affected by pH. CD spectrum of xylanase were taken at pH 2 (—), pH 3 (—), pH 4 (—) and pH 5 (—) at 50 °C as described in section 2.12.3.

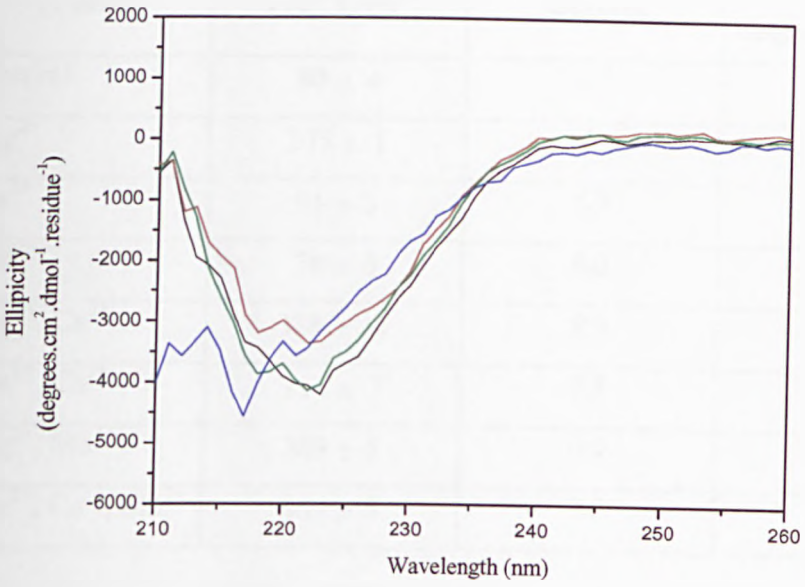


Figure 4.4b CD representation of xylanase affected by pH. CD spectrum of xylanase were taken at pH 5 (_), pH 6 (_), pH 7 (_) and pH 8 (_) at 50 °C as described in section 2.12.3.

4.3 The changes in activity and structure of xylanase in the presence of bivalent metal ions

4.3.1 Influence of metal ions on the stability of xylanase

Xylanase was immobilised onto silica in the presence of Mg^{2+}, Ca^{2+} and Mn^{2+} metal ions to enhance its activity. CD spectroscopy was used to investigate how these ions affect the structure of xylanase in solution. The xylanase activities were determined after incubating the immobilised enzymes for 1 h in 50 mM sodium acetate buffer pH 5.3 in the presence of selected metal ions. The thermal stability at 60 °C was determined relative to the control and the ellipicity at 220 nm measured. Results are presented in Table 4.1.

Metal ion (s) (1 mM)	Xylanase activity $x10^{-3}$ U ml^{-1}	$t_{½}$ at 60 °C minutes	$\theta_{220\ nm}$ $deg.cm^2.dmol^{-1}.residue^{-1}$
Control	80 ± 4	5.7	-3500
Mg^{2+}	275 ± 1	10.5	-4150
Ca^{2+}	91 ± 3	6.9	-4150
Mn^{2+}	78 ± 4	8.0	-4000
Mg^{2+}, Ca^{2+}	378 ± 11	9.5	-4400
Mn^{2+}, Ca^{2+}	114 ± 7	7.5	-4400
Mg^{2+}, Mn^{2+}	389 ± 5	6.9	-4400
Mg^{2+}, Ca^{2+}, Mn^{2+}	409 ± 8	7.5	-5100

Table 4.1 The effect of metal ions on the xylanase activity, the half time of thermal stability ($t_{½}$) at 60 °C and the ellipicity (θ) at 220 nm. Activity was determined by Danisco's method. The data presented are mean ±SE (n=3).

In general, the presence of metal ions improved the activity, thermal stability and increased the β-sheet content of xylanase. The presence of Mg^{2+} affected xylanase activity the most. Clearly the Mg^{2+} cation caused a substantial increase (3.5 fold) in activity, most probably by ligating to acidic residues on the enzyme's surface. However, the inclusion of Mn^{2+} or Ca^{2+} with the Mg^{2+}, caused a further increase in activity of around 40 % above that with Mg^{2+} alone. The reasons for this additional stimulation are difficult to explain, but must be related to some parameter other than valency, e.g. atomic radius of the hydrated ion. In most cases, the data presented in Table 4.1 suggests that the enhanced activity of xylanase was likely due to the increased β-sheet folding as indicated by the magnitude of the ellipicity at 220 nm. There seems to be no correlation between the thermal stability and the magnitude of the ellipicity but the increased metal ion concentration appeared to encourage the fold/unfold equilibrium to shift to the left, hence, enhancing xylanase activity. Overall, the data suggests that enhanced activity was not an indication of improved stability.

To further investigate how well the metal ions affect the structural stability of xylanase, samples of xylanase were held at temperatures of 60 °C for 0 min, 5 min, 10 min and 15 min, cooled and then scanned between wavelengths of 205 nm and 245 nm.

Looking back at Figures 4.2 and 4.3 the graphs show that there were marked differences between xylanase heated and scanned at 60 °C over time and xylanase exposed to 60 °C for a time and allowing the sample to cool before scanning (thermal stability). The spectra from Figure 4.2 showed consistent degradation with time unlike the spectra shown in Figure 4.3. Xylanase was also scanned in the presence of individual and combined metal ions.

In the presence of Mg^{2+} the spectra corresponding to 0 min and 5 min were very similar in appearance and more intense suggesting that Mg^{2+} encourages retention of structure and the same occurred in scans that were taken after 10 min and 15 min exposure (Figure 4.5a). However, there seems to be quite a large difference in the size of the trough of about -2,500 degrees cm^2 $dmol^{-1}$ $residues^{-1}$ at 223 nm between spectra of 5 min and 10 min which maybe an indication of a transition state during protein unfolding or perhaps, at 10 min the thermal damage had become permanent. Similar observations were made with xylanase samples in the presence of Ca^{2+} (Figure 4.5b), but in the presence of Mn^{2+}, combined Mg^{2+}, Ca^{2+} and Mg^{2+}, Mn^{2+} the effects were not as great (Figure 4.5c - e). Xylanase in the presence of combined metal ions especially with Mn^{2+} and Ca^{2+} ions (Figure 4.5f) had fairly good retention of its structural stability. The difference in ellipicity curves between the scans at 0 and 15 min was approximately 1,000 degrees cm^2 $dmol^{-1}$ $residues^{-1}$ and also it was observed that the troughs had broadened by approximately 7 nm. In addition, 0 ellipicity was observed at 203 nm rather than at 210 nm. The depths of all the troughs at 215 nm were larger than usual which may have been due to the higher protein concentrations. Xylanase in the presence of all three ions again gave similar results to samples of xylanase containing just Mg^{2+}.

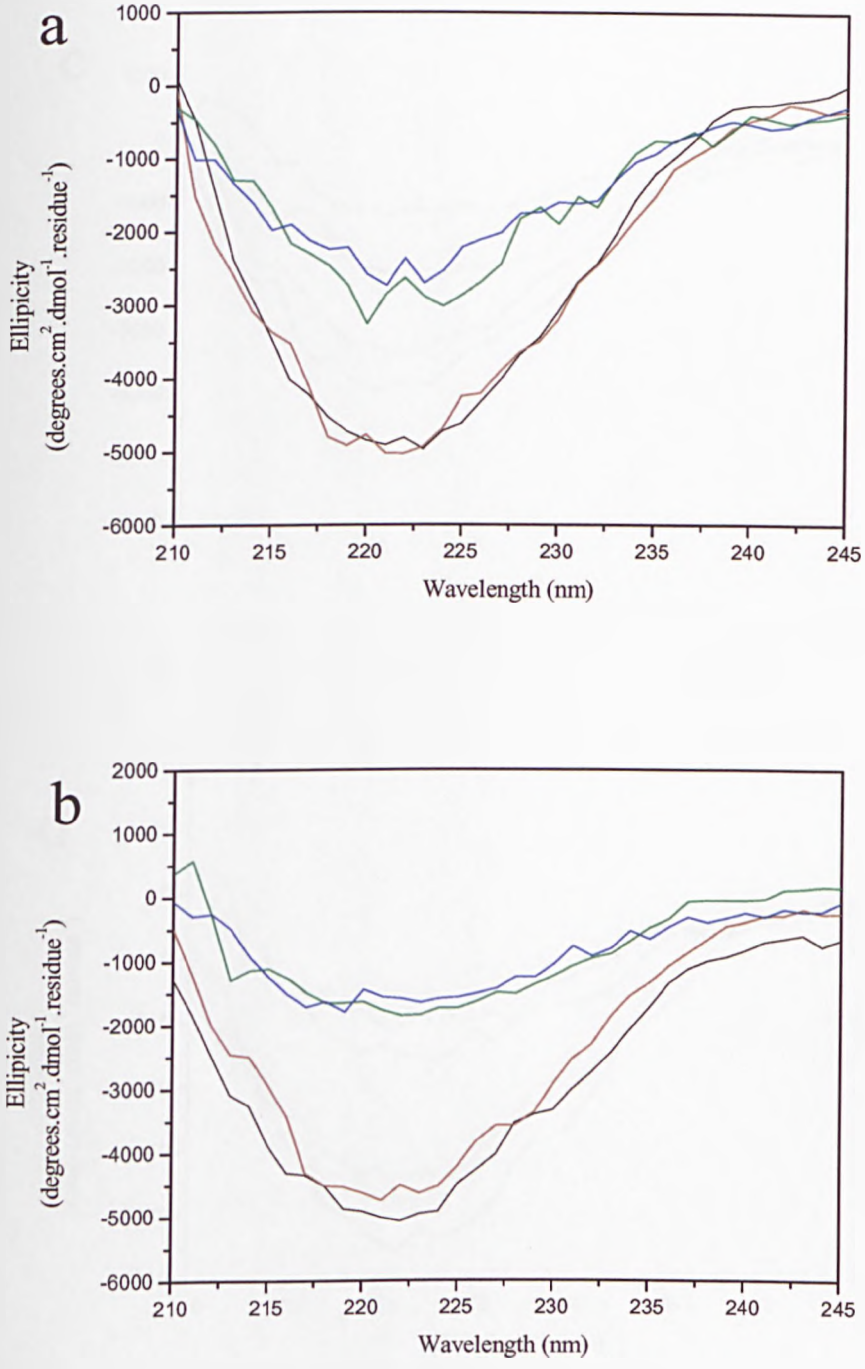


Figure 4.5 (a and b) CD representation of the thermal stability of xylanase in the presence of (a) 1 ml of Mg^{2+} and (b) 1 ml of Ca^{2+}. Xylanase samples were heated to 60 °C for 0 min ( ), 5 min ( ), 10 min ( ) and 15 min ( ) and then allowed to cooled to 50 °C prior to scanning as described in Section 2.9.5.

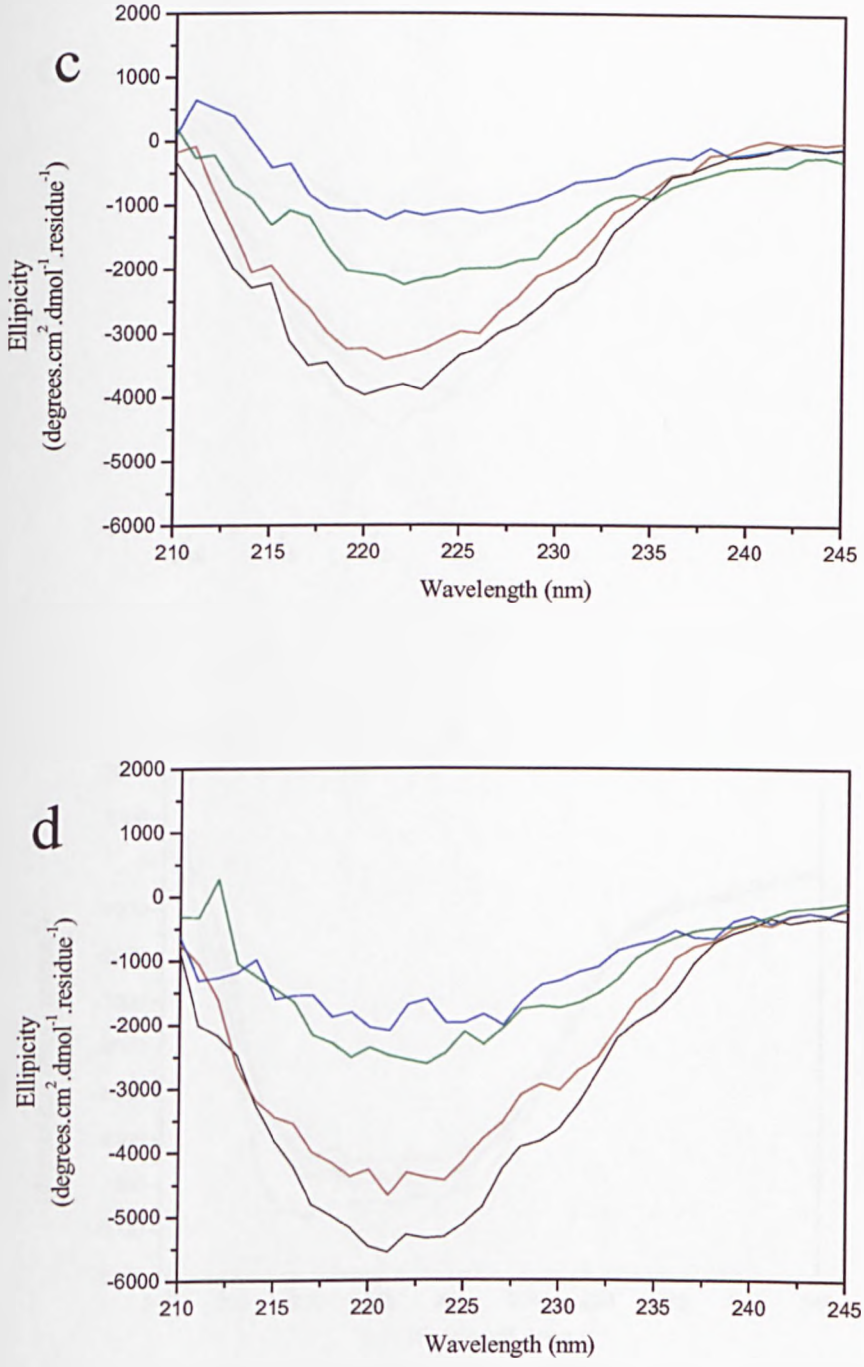


Figure 4.5 (c and d) CD representation of the thermal stability of xylanase in the presence of (c) 1ml of Mn^{2+} and (d) 1 ml of Mg^{2+} plus 1 ml of Ca^{2+}. Xylanase samples were heated to 60 °C for 0 min (—), 5 min (—), 10 min (—) and 15 min (—) and then cooled to 50 °C prior to scanning as described in Section 2.9.5.

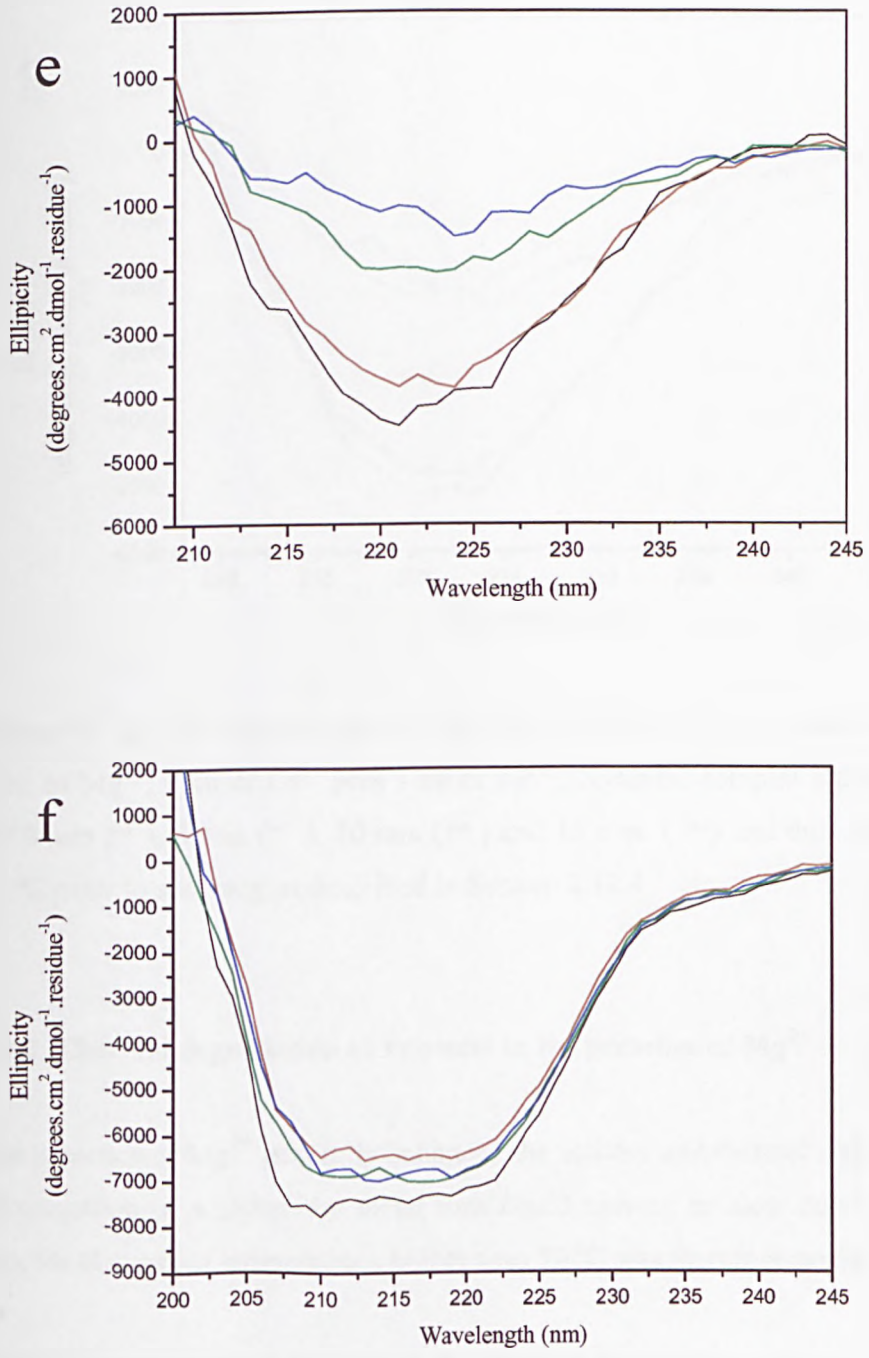


Figure 4.5 (e and f) CD representation of the thermal stability of xylanase in the presence of (e) 1 ml of Mn^{2+} plus 1 ml of Mg^{2+} and (f) 1 ml of Mn^{2+} plus 1 ml of Ca^{2+}. Xylanase samples were heated to 60 °C for 0 min (‾), 5 min (‾), 10 min (‾) and 15 min (_) and then allowed to cool to 50 °C prior to scanning as described in Section 2.9.5.

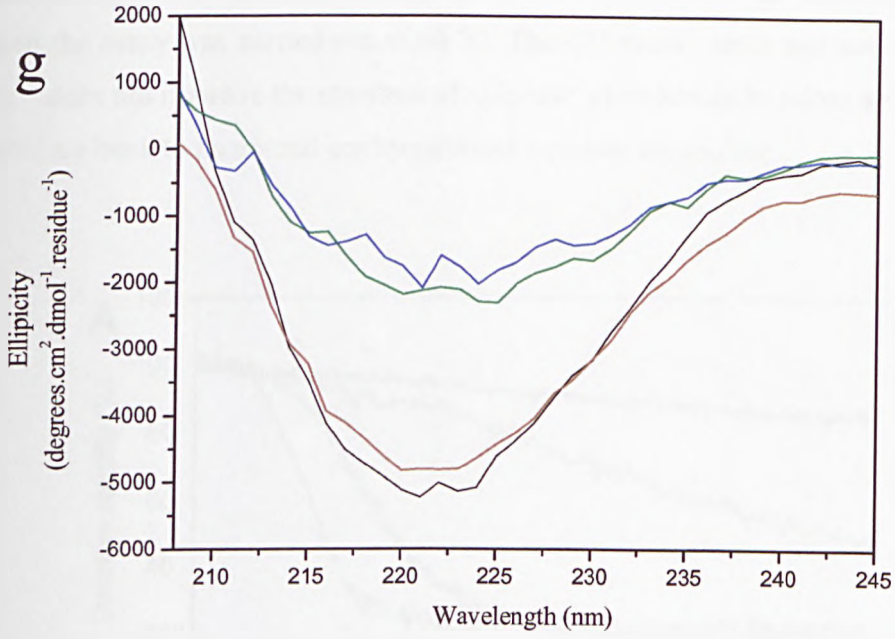


Figure 4.5 (g) CD representation of the thermal stability of xylanase in the presence of 1 ml of Mg^{2+}, 1 ml of Ca^{2+} plus 1 ml of Mn^{2+}. Xylanase samples were heated to 60 °C for 0 min (—), 5 min (—), 10 min (—) and 15 min (—) and then allowed to cool to 50 °C prior to scanning as described in Section 2.12.4.

4.3.2 Thermal degradation of xylanase in the presence of Mg^{2+}

The presence of Mg^{2+} generally enhanced the activity and thermal stability of xylanase. Investigation of whether the metal ions could prevent or slow down the degradation process at constant temperatures higher than 50 °C was therefore carried out.

Thermal degradation of xylanase in the presence and absence of Mg^{2+} was examined at 55 °C, 60 °C, 65 °C and 70 °C for 20 min with CD being measured at 223 nm. Maximum ellipicity at 223 nm represented the highest β-sheet structure. In both cases, with increasing temperature the rate of degradation increased and there was no improvement in the structural stability of xylanase in the presence of Mg^{2+} (Figure 4.6 A and B). At 55 °C the β-sheet structure had decreased to 83 % in both cases after 20 minutes. It is also important to consider that when xylanase was assayed at 55 °C,

which involved a 30-minute incubation period, the activity had decreased by only 20 % when compared to an assay carried out at 50 °C. However, no activity was observed when the assay was carried out at 60 °C. The CD experiments and assays suggest that Mg^{2+} does not improve the structure of xylanase when heated but does assist xylanase in refolding back to its normal conformational structure on cooling.

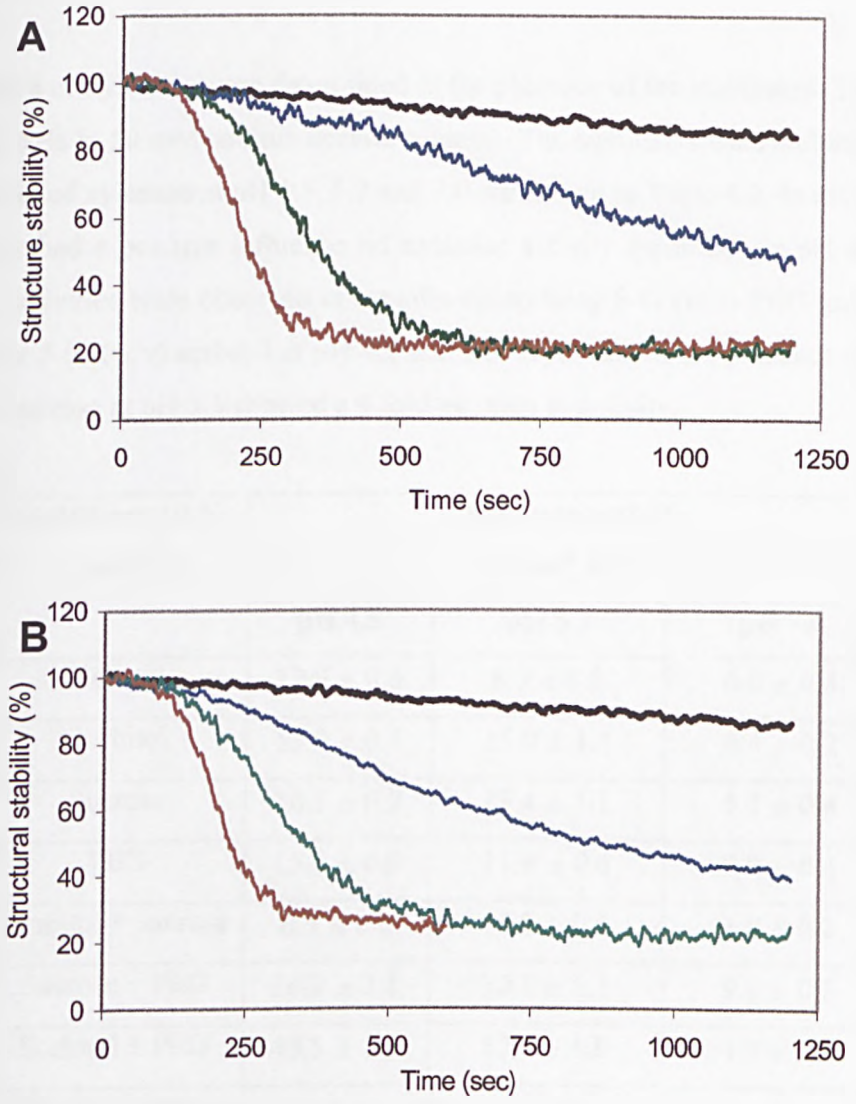


Figure 4.6 The samples in the absence of Mg^{2+} (A) and in the presence of 1 mM Mg^{2+} (B) were held at the following temperatures for 20 min: (———), 55 °C; (———), 60 °C; (———), 65 °C; (———), 70 °C prior to CD measurements at 223 nm. The starting signal was taken as 100 %.

4.4 The Effect of Organic Stabilisers

Originally, six stabilising agents were examined for prevention of thermal degradation of xylanase. These were sorbitol, sucrose, lactose, dextran, polyethylene imine and polyethylene glycol. The purpose of this investigation was to determine how well xylanase could retain its structure when exposed to heat in the presence of the stabilisers.

CD spectra of xylanase were determined in the presence of the stabilisers (10 %, w/v) at different pHs in 50 mM sodium acetate solution. The stabilisers used and their effect on the activity of xylanase at pH 4.5, 5.3 and 7.0 are shown in Table 4.2. In most cases, the stabilisers had a positive influence on xylanase activity especially at pH 4.5 and 5.3. Highest activities were observed in samples comprising 5 % (w/v) PEG and 5 % (w/v) sucrose or 5 % (w/v) sorbitol at pH 4.5 and 5.3. Xylanase in the presence of 5 % PEG and 5 % sucrose at pH 5.3 showed a 9 fold increase in activity.

Stabilisers 10 % (w/v)	**Xylanase activity U ml^{-1} $x10^{-3}$**		
	pH 4.5	**pH 5.3**	**pH 7.0**
Control	12.9 ± 0.6	8.9 ± 0.8	6.0 ± 0.5
Sorbitol	13.9 ± 0.4	15.0 ± 1.4	6.4 ± 0.2
Sucrose	10.1 ± 0.7	13.4 ± 1.1	5.1 ± 0.4
PEG	15.8 ± 0.9	11.9 ± 0.6	8.9 ± 0.4
Sorbitol + sucrose	8.5 ± 0.6	15.1 ± 0.5	1.9 ± 0.1
Sucrose + PEG	56.0 ± 3.1	70.1 ± 5.1	9.8 ± 0.5
Sorbitol + PEG	43.5 ± 1.8	17.1 ± 1.0	4.8 ± 0.2
PEI (0.1 %, w/v)	0	0	0

Table 4.2 The activity of xylanase in the presence of organic stabilisers. Xylanase activities were determined by micro-titre plate method as described in Section 2.2.4. The data presented are mean SE (n=3).

The effect of PEG on the activity may have been due to the amphiphilic nature of PEG, promoting the retention of the enzyme's optimum conformation (O'Fagain *et al*, 1988). In contract, the presence of 0.1 % PEI (w/v) had a negative affect on the xylanase activity. No activity was detected after incubating for 1 h at room temperature prior to assaying at all three pHs.

To determine how well the stabilisers retard the degradation process, CD scans of xylanase in their presence (10 % w/v) were performed after holding the samples at temperatures between 40 °C and 76 °C for 1 min. In general, without stabilisers at pH 5.3, degradation of xylanase with increasing exposure to heat caused a weakening of the far-UV CD signal at approximately 222 nm, indicating loss of β-sheet structure. At first, the protein remained stable between 40 °C and 56 °C and then a dramatic non-linear decrease in signal was observed between temperatures of 64 °C and 76 °C (Figure 4.7). Maximum β-sheet structure was observed at 40 °C, as indicated by the lowest CD value of -15 mdeg at 223 nm, but the xylanase at that temperature only had an activity of 30 % when compared with its maximum activity measured at 50 °C. Also the same was observed at 44 °C when assayed at 45 °C the xylanase activity was only 60 % of the maximum activity. Rather than stating that xylanase has a preferable conformation for maximum activity, it would be more likely that the reason for the non-proportional relation between structure and activity is that temperature affects the collision rate of molecules and, will therefore play an important role in xylanase activity. Slightly higher signals prior to heating were observed in all CD scans of xylanase containing stabilisers indicating a small increase in secondary structure.

4.4.1 Sorbitol at pH 5.3

The ellipicity at 223 nm appeared to be more consistent from temperatures of 40 °C to 56 °C. Slight reduction of the degradation process was observed and there was no total loss of structure at 76 °C (Figure 4.8).

4.4.2 PEG at pH 4.5

Major increases in retention of the β-sheet structure were observed between 60 °C and 76 °C in scans of xylanase with PEG at pH 4.5 (Figure 4.9) compared with xylanase in the absence of PEG. The significance of the data may have been due to the amphiphilic nature of PEG and the increased viscosity that may have restricted the diffusion of heat (O'Fagain *et al*, 1988). According to Nolting *et al,* (1997), PEG decreases water activity without interacting specifically with the protein and may cause an increase in absolute signal.

4.4.3 Sucrose and PEG at pH 4.5

Minimum ellipicity was significant with a value of – 24 at 223 nm but remained consistent between 40 °C and 60 °C. Again, there was a slight retention of β-sheet at higher temperatures of 68 °C, 72 °C and 76 °C and the degradation was not as sudden as the control (Figure 4.10).

4.4.4 Sucrose and PEG at pH 5.3

Highest activity 70,000 U ml^{-1} was observed for xylanase in buffer comprising of 5 % sucrose and 5 % PEG. This combination also gave the highest signal at 223 nm at 40 °C. No improvements in the retention of β–sheet structure were observed and total loss of structure was seen at 68 °C (Figure 4.11).

4.4.5 Sorbitol and PEG at pH 4.5

Despite the high activity, the minimum ellipicity was not as significant as with xylanase in the presence of 5 % sucrose and 5 % PEG, but remain consistent between 40 °C and 64 °C. Again, there was slight retention of β-sheet at higher temperatures of 68 °C, 72 °C and 76 °C (Figure 4.12).

4.4.6 Lactose at pH 5.3

Although the activity of xylanase in the presence of lactose was not determined, the CD data may support the fact that lactose would have greatly enhanced the activity in its presence. Again, the data showed consistency in minimum ellipicity values between 40 °C and 60 °C and rapidly rose between 64 °C and 72 °C. Slight retention of β-sheet was observed at 76 °C (Figure 4.13).

4.4.7 Dextran and PEG at pH 5.3

Dextran and PEG at pH 5.3 improved xylanase's β-sheet structure. Little retention of the β-sheet was observed from 68 °C to 76 °C (Figure 4.14).

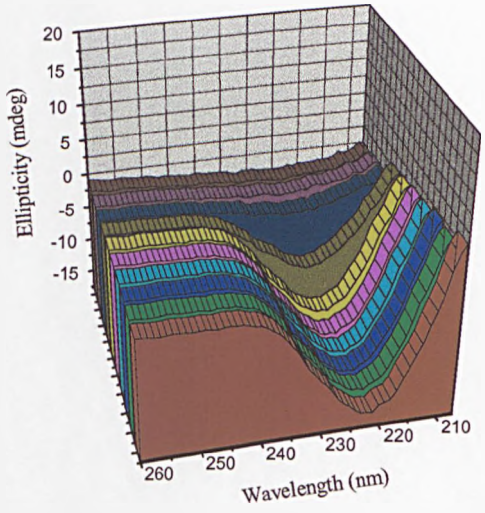


Figure 4.7 CD spectra of xylanase in absence of stabilisers in 50 mM sodium acetate buffer, pH 5.3 when heated to temperatures ranging from 40 °C to 76 °C.

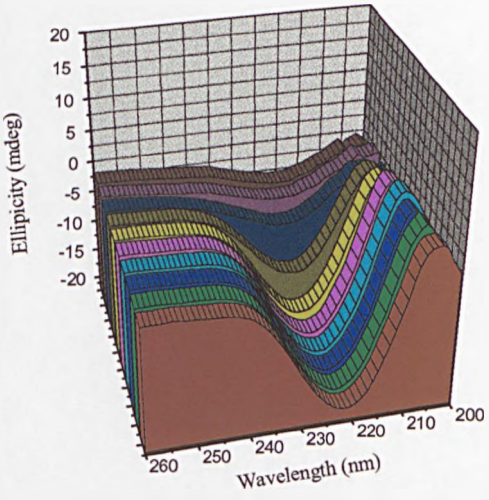


Figure 4.8 CD spectra of xylanase in the presence of 10 % (w/v) sorbitol in 50 mM sodium acetate buffer, pH 5.3 when heated to temperatures ranging from 40 °C to 76 °C.

CD spectra were recorded after the sample had been heated at the following temperature for 1 min: ( ), 40 °C; ( ), 44 °C; ( ), 48 °C; ( ), 52 °C; ( ), 56 °C; ( ), 60 °C; ( ), 64 °C; ( ), 68 °C; ( ), 72 °C; ( )76 °C.

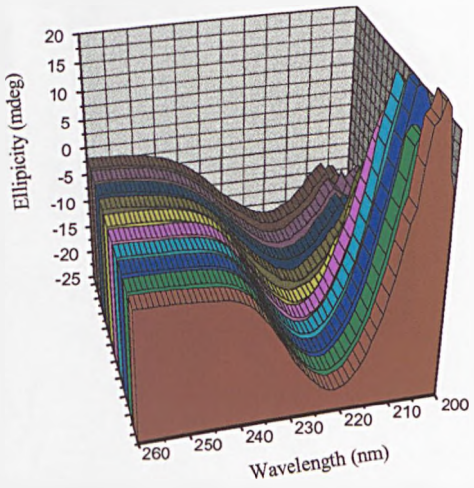


Figure 4.9 CD spectra of xylanase in the presence of 10 % (w/v) PEG in 50 mM sodium acetate buffer, pH 4.5 when heated to temperatures ranging from 40 °C to 76 °C.

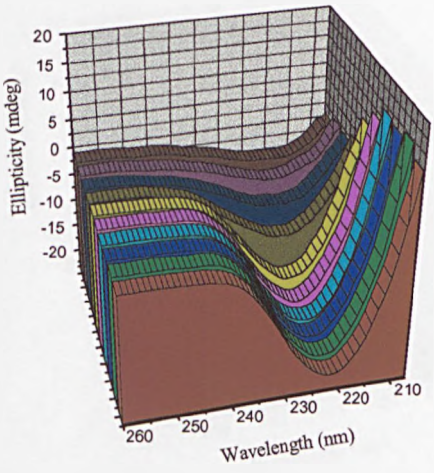


Figure 4.10 CD spectra of xylanase in the presence of 5 % (w/v) sucrose and 5 % (w/v) PEG in 50 mM sodium acetate buffer, pH 4.5 when heated to temperatures ranging from 40 °C to 76 °C.

CD spectra were recorded after the sample had been heated at the following temperature for 1 min: (, 40 °C; (, 44 °C; ( ), 48 °C; (, 52 °C; ( ), 56 °C; ( ), 60 °C; ( ), 64 °C; ( ), 68 °C; ( ), 72 °C; ( )76 °C.

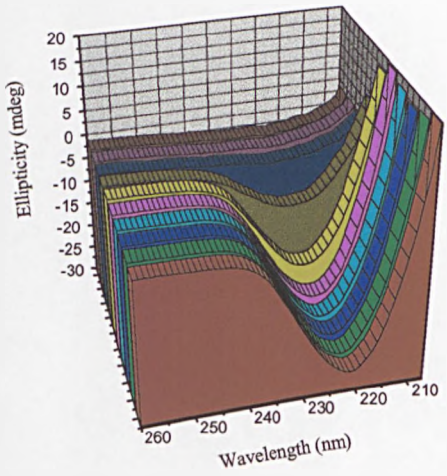


Figure 4.11 CD spectra of xylanase in the presence of 5 % (w/v) sucrose and 5 % (w/v) PEG in 50 mM sodium acetate buffer, pH 5.3 when heated to temperatures ranging from 40 °C to 76 °C.

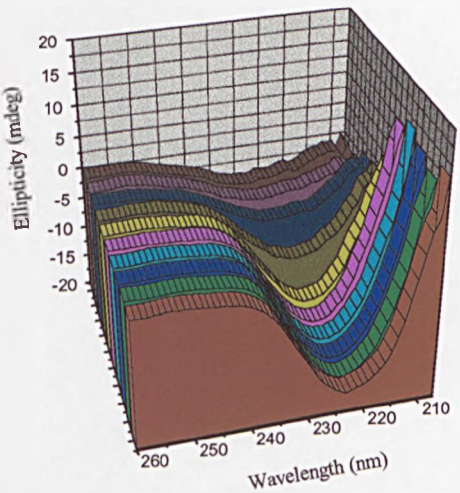


Figure 4.12 CD spectra of xylanase in the presence of 5 % (w/v) sorbitol and 5 % (w/v) PEG in 50 mM sodium acetate buffer. pH 4.5 when heated to temperatures ranging from 40 °C to 76 °C.

CD spectra were recorded after the sample had been heated at the following temperature for 1 min : ( ), 40 °C; ( ), 44 °C; ( ), 48 °C; ( ), 52 °C; ( ), 56 °C; ( ), 60 °C; ( ), 64 °C; ( ), 68 °C; ( ), 72 °C; ( )76 °C.

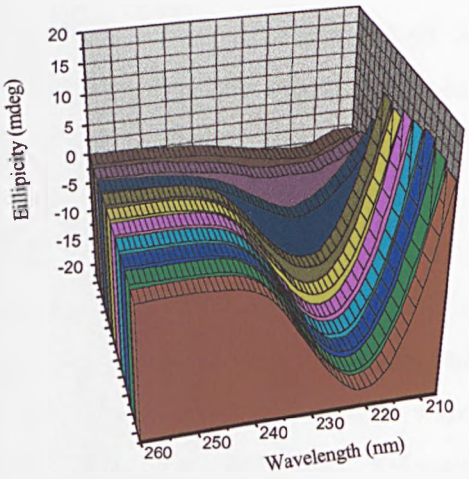


Figure 4.13 CD spectra of xylanase in the presence of 5 % (w/v) lactose in 50 mM sodium acetate buffer pH, 5.3 when heated to temperatures ranging from 40 °C to 76 °C.

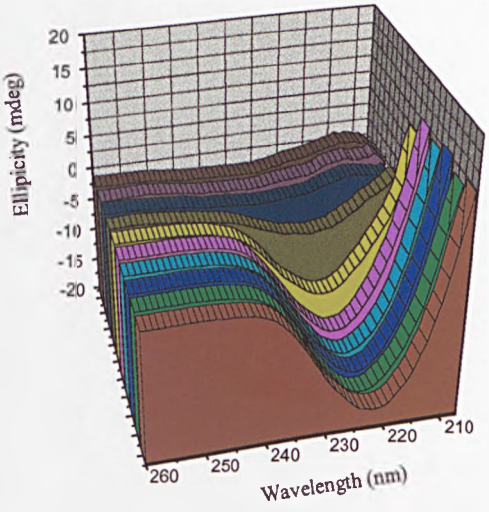


Figure 4.14 CD spectra of xylanase in the presence of 5 % dextran and 5 % (w/v) PEG in 50 mM sodium acetate buffer, pH 5.3 when heated to temperatures ranging from 40 °C to 76 °C.

CD spectra were recorded after the sample had been heated at the following temperature for 1 min : ( ), 40 °C; ( ), 44 °C; ( ), 48 °C; ( ), 52 °C; ( ), 56 °C; ( ), 60 °C; ( ), 64 °C; ( ), 68 °C; ( ), 72 °C; ( )76 °C.

4.5 Summary

Detailed investigations using CD spectroscopy confirmed that xylanase consists mainly of β-sheet and CD spectra were closely analysed see how xylanase responded to stress. The structure of xylanase was strongly affected at pH 2, pH 6 and pH 8. Progressive unfolding of the β–sheet occurred when xylanase was exposed to continuous heat at 60 °C or greater. Also when heated for a time and then cooled prior to scanning, no changes in the structure were observed after 5 minutes, but major loss occurred after 10 min suggesting that there were temporary and permanent losses in structure during the degradation process. These findings were more evident when xylanase was examined in the presence of metal ions. Xylanase retained most activity under adverse conditions in the presence of 10 % (w/w) PEG and this corresponded to a significant increase in retention of the β-sheet structure compared with xylanase in the absence of PEG.

Chapter 5

Stabilisation of Xylanase by Immobilisation

5.1 Introduction

The term 'enzyme immobilisation' can be defined as the physical confinement of an enzyme to a carrier or a support material such that the enzyme retains its catalytic activity (Katchalshi-Katzir, 1993). For a specific enzyme to be successfully immobilised, a suitable support material must be chosen carefully (Bickerstaff, 1997). The desirable properties of a support material are that it has a large surface area, is highly permeable with large pores, has a hydrophilic surface and most importantly, is chemically and mechanically stable (Huckel *et al*, 1996).

Immobilisation of an enzyme can be carried out in four ways: physical adsorption, covalent binding, entrapment and cross-linking. These methods are described in more detailed in the main Introduction (1.6.3). The advantages and disadvantages of the use of each method are summarised in Table 5.1.

The possibility of using minerals to immobilise xylanase arose from the fact that silicates are normally added to commercial chicken feeds (Feeding Stuff Regulations, 1995). The purpose of adding silicates is to aid in the grinding of hard food items in the gizzard. This action is not unusual since wild chickens normally ingest small stones or grit for the same reason (Duke, 1986). Silicates coupled with xylanase would have added benefits.

The work reported in this chapter describes attempts to improve the stability of commercial xylanase by immobilising xylanase onto mineral support materials in the presence of metal ions stabilisers.

	Advantages	Disadvantages
Physical adsorption	• Little or no damage to the enzyme. • The process is quick, simple and cheap. • No chemical changes are needed to carrier or enzyme • Easily reversible	• Desorption or leakage of the enzyme from the support. • Overloading of carrier. • Possible steric hindrance.
Covalent Binding	• May increase activity of the enzyme • The matrices can be reused time and time again. • May enhance the stability of the enzyme	• Chemical modifications may be needed to the carrier or enzyme. • Possible steric hindrance.
Entrapment	• May increase enzyme stability • Prevents loss of enzyme when washed.	• Loss of enzyme may occur during entrapment process • Diffusion of substrate and products maybe hampered by partitioning • The enzyme may have a constrained conformation in the immobilised state
Cross-linking	• May improve operational stability of the enzyme.	• Steric hindrance • The enzyme may have a constrained conformation in the immobilised state

Table 5.1 Advantages and disadvantages of different enzyme immobilisation methods (Katchalshi-Katzir, 1993).

5.2 Immobilisation of xylanase

5.2.1 Promaxon as a support material

In contrast to organic and polymeric support materials, inorganic materials in general are resistant to microbial attack. Silica and porous glass have been the predominant materials used (Anspach *et al*, 1989, Drott *et al*, 1997). The main advantages of using Promaxon are its low cost and high chemical, thermal and pH stability. It is a synthetic calcium silicate mineral, $Ca_6Si_6O_{17}(OH)_2$, which is more popularly known as Xonotlite. In regards to health and safety, Promaxon has no harmful chemical elements in its composition. Animal experiments conducted by Fruaunhofer Institute of Toxicology in Hannover (1993), showed that Promaxon is an extremely soluble material in the living body and that more than 99 % of single crystals and about 90 % of the crystal agglomerates are eliminated in two days after introduction into the lungs of a rat (Promaxon Datasheet, 1995). Promaxon is a white free flowing powder and by means of a special crystallisation process and controlled growth spherical particle are obtained. As a result it has an open inner structure surrounded by an outer shell of close knitted crystal. Promaxon particle sizes range between 25 μm and 100 μm with a pore size of 100 nm and surface area of 40 $m^2 g^{-1}$ (URL:http//www.promaxon.com) SEM images of Promaxon are shown in Figures 5.1 and 5.2.

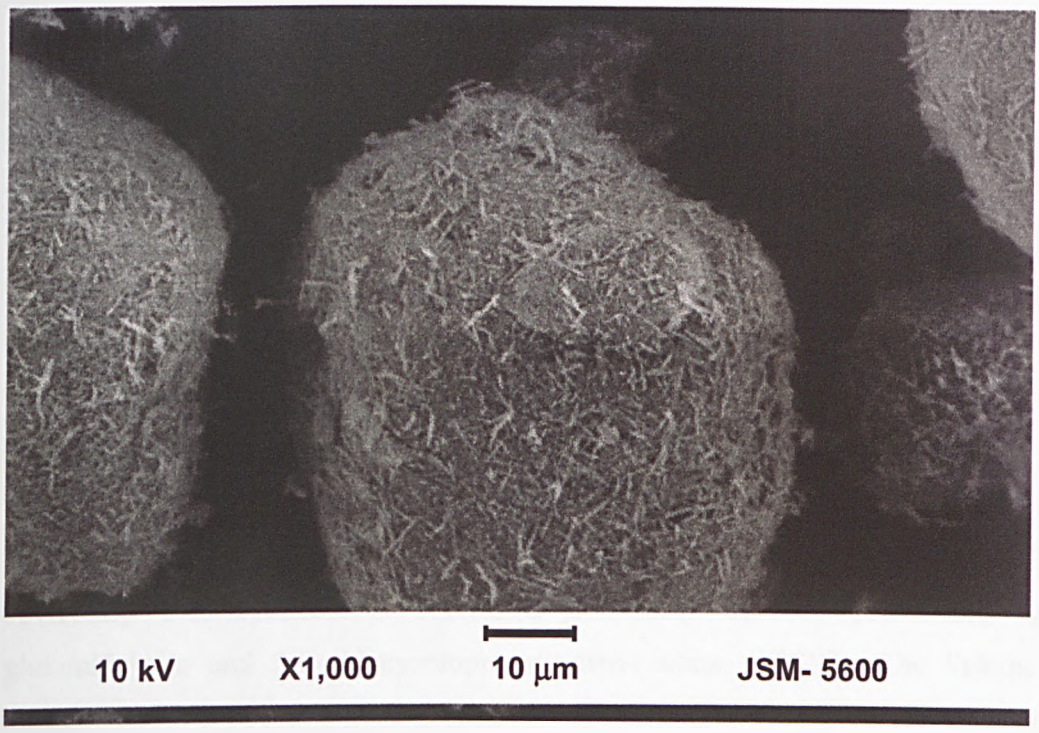


Figure 5.1. Scanning Electron Microscopy (SEM) micrograph of Promaxon particles.

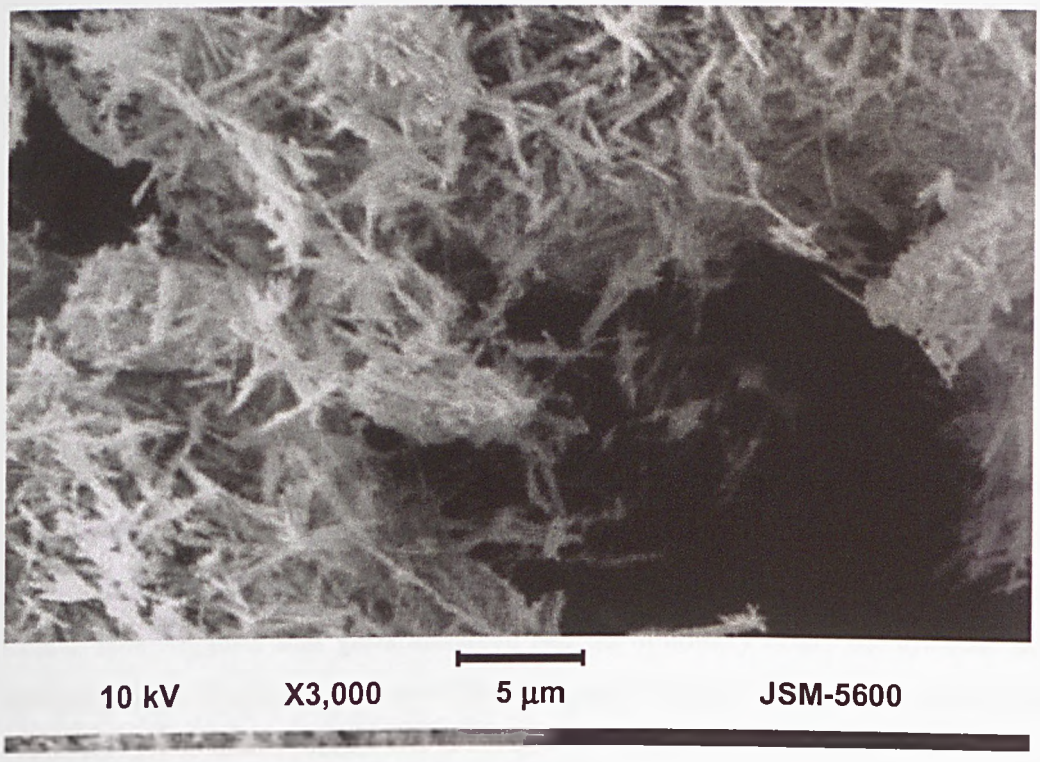


Figure 5.2. SEM of Promaxon particle at larger magnification.

5.2.2 Immobilisation by physical adsorption

Immobilisation of xylanase onto Promaxon was carried out by physical means and the detailed procedure is outlined in Section 2.10. Physical adsorption was achieved with no loss of xylanase activity. The main disadvantage experienced with this process was leakage of the enzyme which occurred during wet thermal stability tests. The leakage would have eventually led to the loss of activity with time.

5.2.3 Immobilisation onto Promaxon with the aid of a cross-linker agent

To avoid leakage and increase the stability of the enzyme, attempts were made to covalently link xylanase to Promaxon by means of the crosslinking agents, glutaraldehyde and 3-isothiocyantopropyltriethoxysilane (ITCPS). The linkage was thought feasible because of the available hydroxyl group. Among chemical cross-linking agents glutaraldehyde and 2, 3 butadione are the most frequently used (Arshady, 1990). Samples were made by adding 0.4 g of Promaxon and 0.1 ml of xylanase to a series of solutions comprising 0 % - 5 % (w/v) glutaraldehyde in 50 mM sodium acetate buffer, pH 5.3, and then mixed by inversion for 20, 30 and 60 min at room temperature. The samples were then repeatedly washed with distilled water, to eliminate unbound enzyme, and dried under vacuum overnight (as described in Section 2.11). Xylanase activity of each sample was measured as described in Section 2.10.

Data collected from the experiment revealed that high glutaraldehyde concentration had a negative effect on xylanase activity (Table 5.2). Xylanase activities were expressed in U g^{-1} of matrix. The activity of the matrices peaked sharply when 0.25 % (w/v) of glutaraldehyde solution was used at all three mixing times and an exponential decline in activity was observed at higher glutaraldehyde concentrations. The matrices with no glutaraldehyde had relativity high activities and this increased with increasing mixing times. This suggests that glutaraldehyde had an inhibitory effect on xylanase at high concentrations. Xylanase activity of the washings decreased (Table 5.3) with increasing glutaraldehyde concentration.

Percentage glutaraldehyde in solution (w/v)	Xylanase activity (U g^{-1})		
	20 min	40 min	60 min
0	1.186 ±0.025	2.281 ±0.151	3.664 ±0.687
0.025	1.142 ± 0.089	0.827 ±0.040	0.411 ±0.063
0.05	1.667 ±0.090	0.778 ±0.003	0.365 ±0.038
0.1	2.049 ±0.223	0.374 ±0.014	0.783 ± 0.097
0.25	6.629 ±0.069	3.529 ±0.004	3.972 ±0.148
0.5	4.062 ±0.054	2.519 ±0.056	3.466 ±0.162
1	3.356 ±0.155	2.125 ±0.076	3.231 ±0.012
2.5	2.913 ±0	1.659 ±0.066	2.306 ±0.015
5	1.483 ±0.048	0.941 ±0.512	1.486 ±0.116

Table 5.2 The activity of xylanase immobilised on Promaxon. Xylanase was mixed with Promaxon and 0 – 5 % diluted glutaraldehyde for 20, 40 and 60 min. The Table shows the effect of glutaraldehyde concentration and mixing times on xylanase activity. Data are mean ± S.D (n=3).

Percentage glutaraldehyde in solution (w/v)	Xylanase activity (U $ml^{-1}x10^{-3}$)		
	20 min	40 min	60 min
0	10.866 ±0	6.733 ±0	8.737 ±0
0.025	12.833 ±0.384	6.833 ±0.745	9.933 ±0.555
0.05	13.833 ±0.722	5.100 ±0.451	8.733 ±0.467
0.1	7.433 ±0.088	5.133 ±0.067	3.8 ±0.321
0.25	5.600 ±0.252	2.733 ±0.175	2.033 ±0.384
0.5	6.300 ±1.106	2.033±0.219	2.933 ±0.176
1	5.533 ±0.484	1.367 ±0.034	2.733 ±0.186
2.5	4.933 ±0.367	1.600 ±0	1.900 ±0.115
5	7.050 ±0.286	1.633 ±0.033	2.433 ±0.285

Table 5.3 The xylanase activity within the washings from the production of immobilised xylanase on Promaxon and 0 – 5 % diluted glutaraldehyde for 20, 40 and 60 min. The Table shows the effect of glutaraldehyde concentration and mixing times on xylanase activity. Data are mean ± S.D (n=3).

Efforts were then made to chemically link xylanase onto Promaxon by using ITCPS as a cross-linking agent according to Huckel *et al*, 1996, Wirth and Hearn, 1993 and Anspach *et al*, 1989. Since it has been reported that xylanase had been successfully immobilised on silica (Innocentini-Mei *et al*, 1992) attempts were made to immobilise xylanase on both silica and Promaxon in order to compare data. The same method was used on both supports as shown in the schematic in Figure 2.3.

5.3 Silica as a support material

5.3.1 Synthesis of 3-isothiocyanatopropyltriethoxysilane modified silica

Modification of silica was carried out according to Section 2.11. As with Promaxon, prior to the addition of APS, silica was hydrothermally treated to increase the number of hydroxyl groups. The success of the APS modification was confirmed by using IR analysis which revealed the presence of an amino group corresponding to a broad peak of frequency between 3100 cm^{-1} and 3700 cm^{-1} (Appendix 2). The chemical reaction and proposed mechanism for the formation of APS modified silica is shown in Figure 5.3.

Synthesis of ITCPS modified silica was carried out according to Section 2.11. APS modified silica was suspended in ethyl acetate solvent to which triethylamine was added and then 2.5 ml of thiophosgene, diluted in 50 ml of ethyl acetate, was carefully added dropwise with constant stirring for 2 h. The product was washed, dried and then analysed by using IR which showed a double peak of approximate frequencies 2125 cm^{-1} and 2200 cm^{-1} (see Appendix 2), an indication that the isothiocyanate group was present. The chemical reaction and mechanism for the formation of ITCPS modified silica are shown in Figure 5.4.

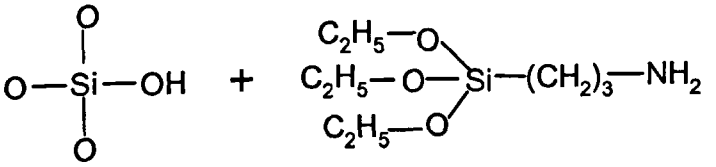
5.3.2 Immobilization of xylanase onto modififed silica

Immobilisation of xylanase was carried out using ITCPS modified silica as the support material as described in Section 2.11. The modified support material was suspended in buffer comprising sodium acetate, pH 5.3, containing 1 % (v/v) xylanase and 1 mM

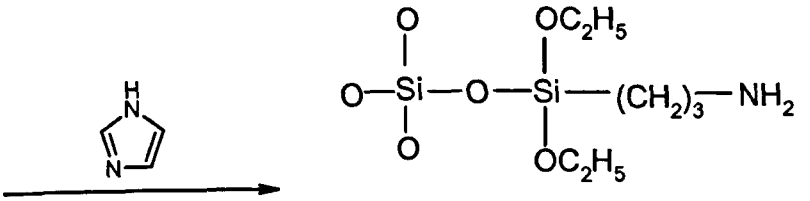
metal ions as listed in Table 5.4 to maintain the biological activity of xylanase before mixing by inversion for 72 h at room temperature. The xlanase/silica matrices were washed, dried and then stored at 4 °C. Control 1 is the immobilisation of xylanase without the presence of metal ions whilst control 2 was a blank where the active or the isothiocyanato groups of the support material were blocked using ethanolamine (Huckel *et al*, 1996). Possible chemical reactions and the mechanism for xylanase covalently binding to the modified silica surface are shown in Figure 5.5.

Attempts were made to determine the specific activity of the matrices by using the bicinchoninic acid (protein content) assay. However, the data was unreliable because of interference due to active groups of the surface of the material causing false readings.

Overall reaction



Silica surface 3-aminopropyltriethoxysilane (APS)



APS modified silica surface

Mechanism

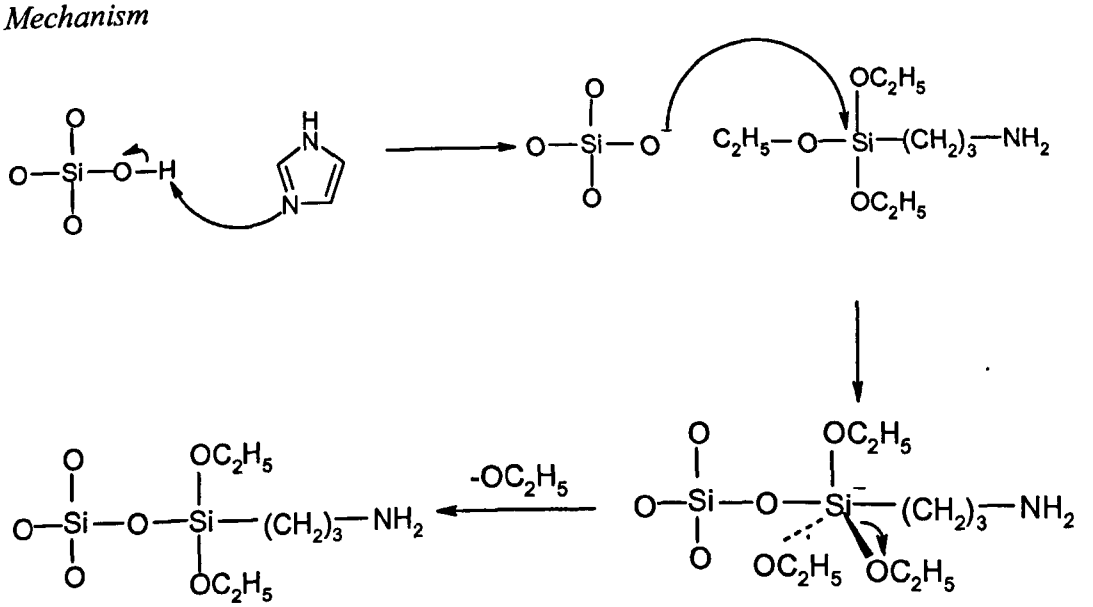
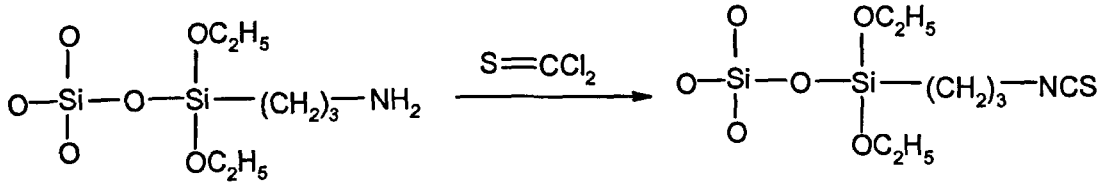


Figure 5.3 Chemical reaction and proposed mechanism for the formation of the 3-aminopropyltriethoxysilane modified silica surface (Prasad, 2002).

Overall reaction



APS modified silica surface

ITCPS modified silica surface

Mechanism

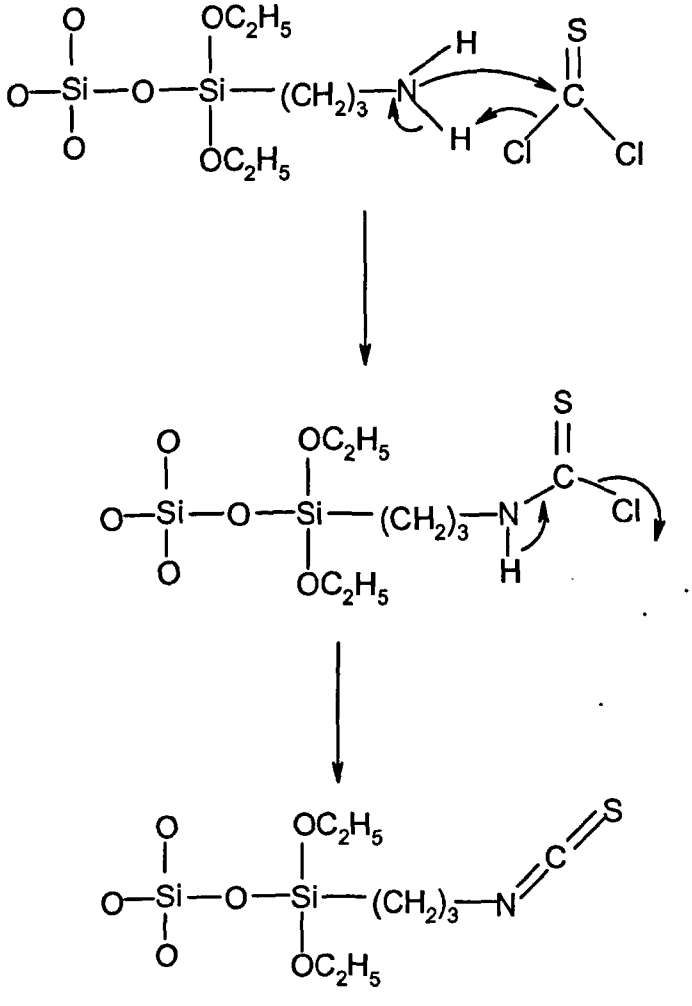
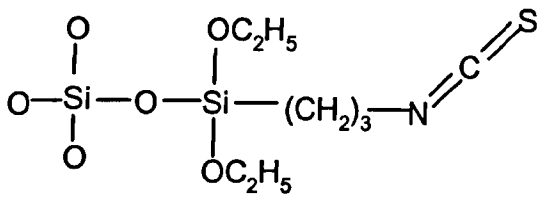


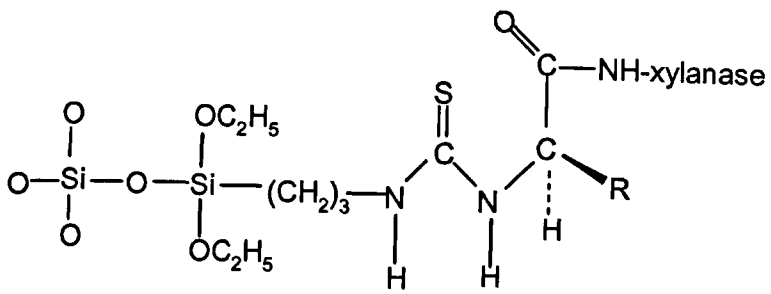
Figure 5.4 Chemical reaction and proposed mechanism for the formation of the 3-isothiocyanatopropyltriethoxysilane modified silica surface.

Overall reaction



+ XYLANASE

ITCPS modified silica surface



Xylanase immobilised on modified silica surface

Mechanism

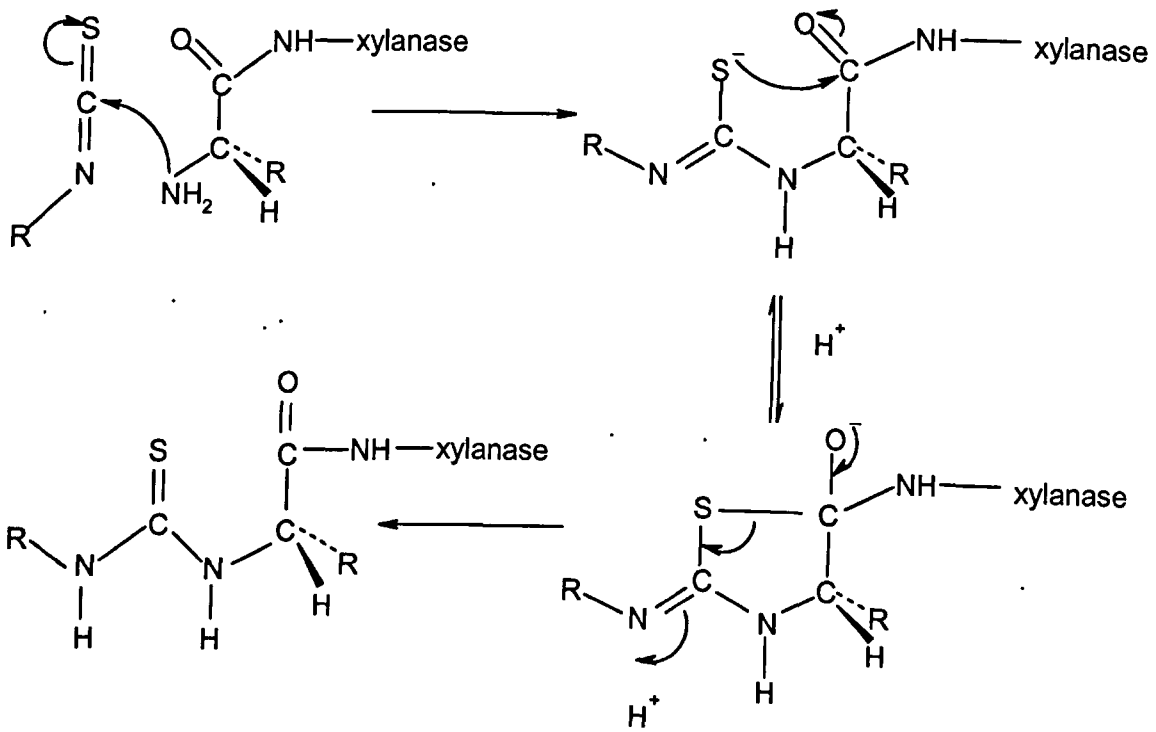


Figure 5.5 Mechanism for xylanase immobilisation onto the modified 3-isothiocyanatopropyltriethoxysilane silica surface.

The activities of the free and immobilised xylanase in the presence of bivalent metal ions at optimum conditions were determined and the data is presented in Table 5.4.

Metal ions present (1 mM)	Activity of free xylanase (U $ml^{-1}x10^{-3}$)	Activity of immobilised xylanase (U g^{-1})
Control (none)	80 ± 4	5.5 ± 0.4
Mg^{2+}	275 ± 1	6.2 ± 0.2
Ca^{2+}	91 ± 3	5.3 ± 0.3
Mn^{2+}	78 ± 4	8.3 ± 0.4
Mg^{2+},Ca^{2+}	378 ± 11	5.9 ± 0.2
Mn^{2+},Ca^{2+}	114 ± 7	5.2 ± 1.4
Mn^{2+},Mg^{2+}	389 ± 5	9.8 ± 1.0
Mg^{2+},Ca^{2+},Mn^{2+}	409 ± 8	26.0 ± 3.4

Table 5.4. The activities of free and immobilised xylanase under the influence of metal ions. Activities were measured as in Sections 2.2.1 and 2.12.1. Data are mean ±SE (n = 3).

The effects of metal ions on the activity of xylanase were determined (Table 5.4). Most individual metal ions or combinations of metal ions greatly increased the xylanase activity apart from Mn^{2+} which had little effect. The effects of Mg^{2+} and Ca^{2+} ions were to increase the activity by 244 % and 14 % respectively. Combinations of all three metal ions had the greatest effect and gave an increase of 411 % which may have been due to the higher overall ion concentration. Coolbear *et al* (1992) performed a series of proteinase activity tests with different metal ions. They deduced that these ions affect the activity according to ionic radii, ligand binding preference and co-ordination numbers of the ions.

The activities of the matrices were measured against a negative control (silica with no xylanase attached). The matrices that contained Mg^{2+} ions demonstrated the greatest xylanase activities. The presence of magnesium and manganese ions caused a 50 % and

13 % increase in activity whilst, combined Mg^{2+} and Ca^{2+} ions or Ca^{2+} ions alone both lead to a decrease of 5 % and 4 % respectively when compared with the control. Not all of the matrices followed trends similar to that of their free xylanase counterparts. This variation suggests that the activity depends on the number of modified sites produced and the success of the loading of xylanase. The matrix with Mn^{2+}, Mg^{2+} and Ca^{2+} ions in combination showed the highest activity at 26 U g^{-1} of matrix material.

5.4 Thermal stabilities of free and immobilised xylanase

A series of free and immobilised xylanase samples were subjected to temperatures of 60 °C, 70 °C and 80 °C for as long as 20 min in a solution of 50 mM CH_3CO_2Na pH 5.3, with appropriate metal ions at 1 mM. After exposure, each sample was allowed to cool to room temperature and was then analysed for xylanase activity.

5.4.1 Thermal stability of free xylanase

Free xylanase in the presence of metal ions at 60 °C showed a slight increase in thermal stability when compared to the stability of free xylanase without metal ions in solution (Figure 5.6 and Figure 5.7). The samples containing Mg^{2+} ions and combined Mg^{2+} plus Ca^{2+} ions showed the greatest stability at 60 °C over a period of 10 min with activities of 100 % and above, while the other samples lost activity at approximately the same rate as the control. The reasons why the two samples containing Mg^{2+} ions had elevated thermal stability are not fully understood, but the cause may have been due to increased stabilisation of the protein at the molecular level. Arnold and Zhang (1994) suggested that the increase in stability is due to the metal ion shifting the folding/unfolding equilibrium of the protein.

Despite the rate of denaturation, the activities, in most cases, remained relatively high during the entire 20 min. The xylanase sample with all three metal ions combination had a final activity of 33,000 U ml^{-1}. The minimum activity at the final stage was shown by xylanase in the presence of Mn^{2+} ions alone, where the activity had reduced from 78,000 U ml^{-1} to 676 U ml^{-1}. The thermal stability of xylanase in the presence of metal ions at 70 °C showed total loss of activity during the first five minutes (data not shown).

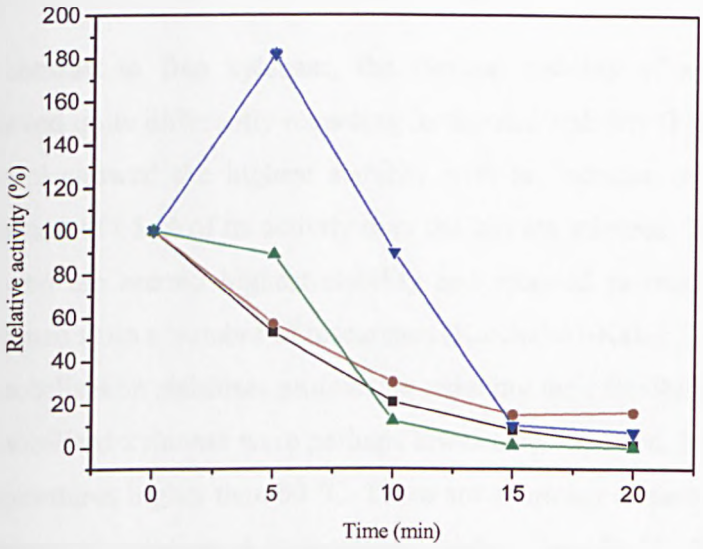


Figure 5.6 Thermal stability of xylanase at 60 °C in the presence of metal ions. The samples were subjected to a temperature of 60 °C for 0 – 20 min, then cooled before assaying at 50 °C. The following metal ions were present at 1 mM: Mg^{2+}, (▼); Ca^{2+}, (●); Mn^{2+}, (▲) and control (■ – no metal ions). The data presented are mean ± SE (n= 3).

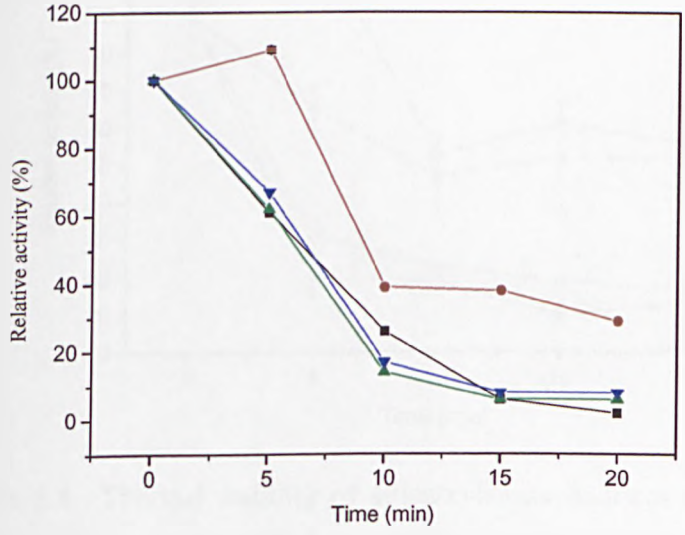


Figure 5.7 Thermal stability of xylanase at 60 °C in the presence of metal ion combinations. The samples were subjected to a temperature of 60 °C for 0 – 20 min, then cooled before assaying at 50 °C. The following metal ions were present at 1 mM: $Mg^{2+}Ca^{2+}$, (●); $Mg^{2+}Mn^{2+}$, (▲); $Mn^{2+}Ca^{2+}$, (■); and $Mg^{2+}Mn^{2+}Ca^{2+}$; (▼). The data presented are mean ± SE (n= 3).

5.4.2 Thermal stability of xylanase immobilised onto silica matrices

In contrast to free xylanase, the thermal stability of xylanase within the matrices behaved quite differently regarding its thermal stability (Figure 5.8 and Figure 5.9). The control showed the highest stability with an increase of 20 % activity initially and retention of 65 % of its activity over the last ten minutes. The matrices containing Mg^{2+} showed the second highest stability and retained as much as 60 % activity. Despite evidence from a number of researches (Katchalski-Katzir, 1993; Daniel *et al*, 1996) that immobilisation stabilises proteins by reducing their flexibility, the stability of the matrix immobilised xylanase were perhaps lower than expected. No activities were observed at temperatures higher than 60 °C. There are a number of factors that affect the stability of xylanase in solution at temperatures higher than 50 °C. First, the enzyme molecules become more flexible and therefore more prone to conformation changes. Secondly, the elevated temperature increases water activity and the protein becomes more susceptible to oxidation.

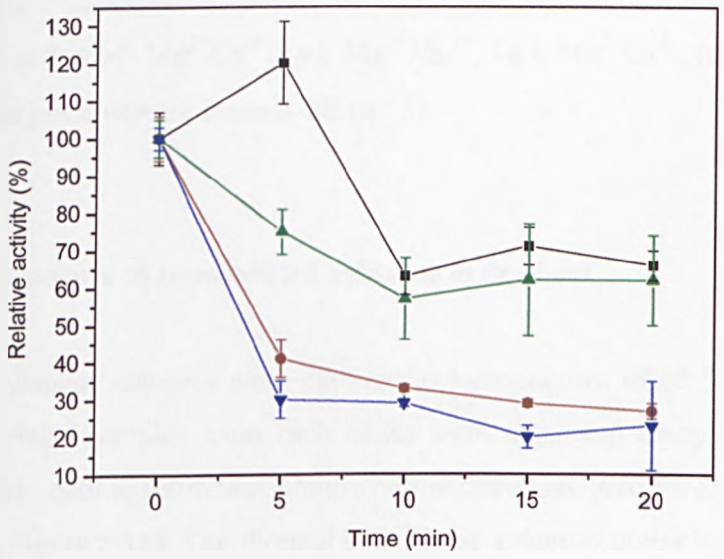


Figure 5.8 Thermal stability of silica/xylanase matrices in solution at 60 °C in the presence of metal ions. The samples were subjected to temperature of 60 °C for 0 – 20 min, then cooled before assaying at 50 °C. The following metal ions were present at 1 mM: Mg^{2+}, (▼); Ca^{2+}, (●); Mn^{2+}, (▲) and control (■– no metal ions). The data presented are mean ± SE (n= 3).

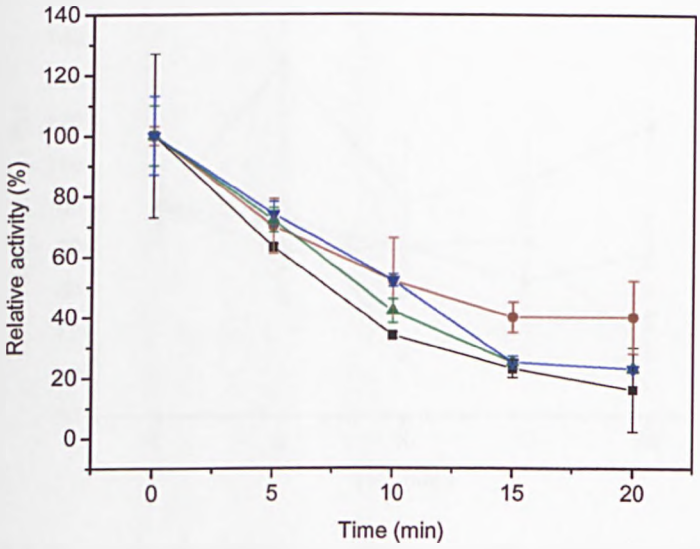


Figure 5.9 Thermal stability of xylanase on silica matrices in solution at 60 °C in the presence of combined metal ions. The samples were subjected to temperature of 60 °C for 0 – 20 min, then cooled before assaying at 50 °C. The following metal ions were present at 1 mM: $Mg^{2+}Ca^{2+}$, (●); $Mg^{2+}Mn^{2+}$, (▲); $Mn^{2+}Ca^{2+}$, (■), $Mg^{2+}Mn^{2+}Ca^{2+}$, (▼). The data presented are mean ± SE (n= 3).

5.4.3 Exposure of immobilised xylanase to dry heat

Silica/xylanase matrices were exposed to temperatures of 60 °C, 70 °C and 80 °C in sealed vials. Samples from each series were taken out every five minutes and then allowed to cool to room temperature before being analysed for xylanase activity (Figure 5.10 to Figure 5.15). The thermal stability of xylanase in dry heat at 60 °C, 70 °C and 80 °C was much greater than that of the immobilised samples exposed to wet heat. At 60 °C, the matrices were almost completely stable and up to 70 % of their activity was remained. The matrices containing calcium ions showed the greatest stability with an overall increase in activity of 20 % throughout the 20 min exposure. The lowest stability was seen in matrices in the presence of Mn^{2+} ions, where activity decreased on average by 20 %.

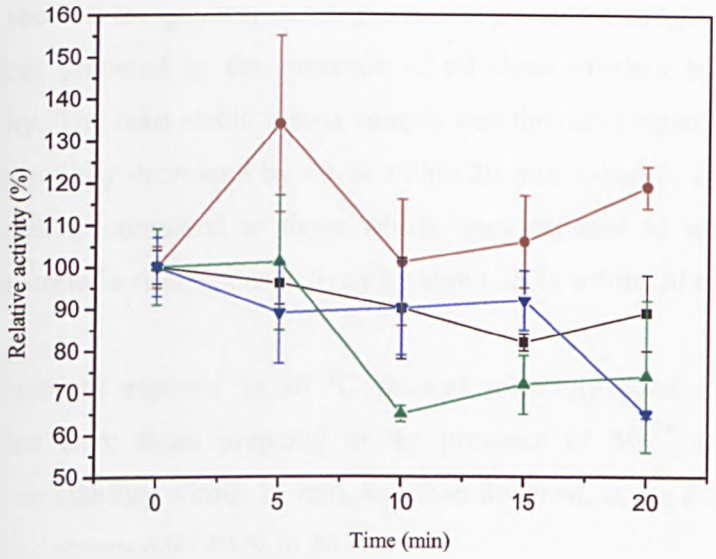


Figure 5.10 Thermal stability of silica/xylanase matrices exposed to dry heat at 60 °C in the presence of metal ions. The samples were subjected to temperature of 60 °C for 0 – 20 min, then cooled before assaying at 50 °C. The following metal ions were present at 1 mM: Mg^{2+}, (▼); Ca^{2+}, (●); Mn^{2+}, (▲) and control (■- no metal ions). The data presented are mean ± SE (n= 3).

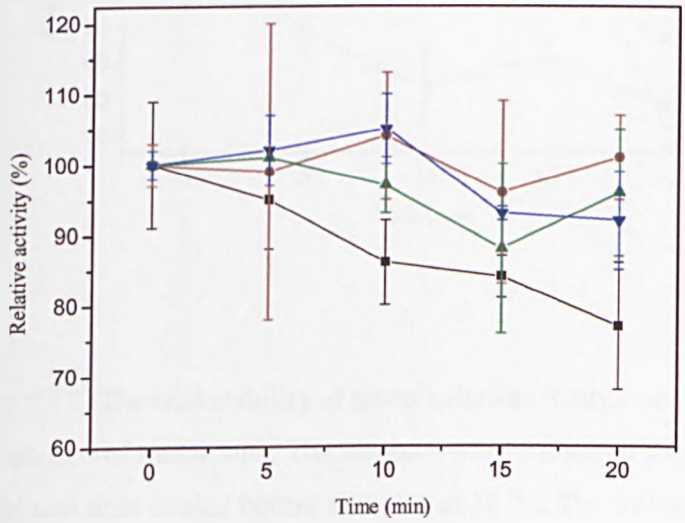


Figure 5.11 Thermal stability of silica/xylanase matrices exposed to dry heat at 60 °C in the presence of combined metal ions. The samples were subjected to temperature of 60 °C for 0 – 20 min, then cooled before assaying at 50 °C. $Mg^{2+}Ca^{2+}$; (●), $Mg^{2+}Mn^{2+}$; (▲), $Mn^{2+}Ca^{2+}$; (■) and $Mg^{2+}Mn^{2+}Ca^{2+}$; (▼). The data presented is an average ± SE (n= 3).

At 70 °C most of the dry matrices were stable. The matrices prepared in the presence of Ca^{2+} showed the greatest stability remaining stable throughout the entire 20 min. The matrices prepared in the presence of all three bivalent metal ions showed similar stability. The least stable matrix sample was the one prepared in the presence of Mg^{2+} where activity decreased by 40 % within 20 min. Overall, all matrices were relatively more stable compared to those which were exposed to wet heat. On average they demonstrated a decrease in activity by about 25 % within 20 min.

The matrices exposed to 80 °C showed relatively good stability. The most stable matrices were those prepared in the presence of Mn^{2+} and Mg^{2+}, which showed excellent stability within 15 min, and then declined, in the final 5 min. On average the activity decreased by 40 % in 20 min.

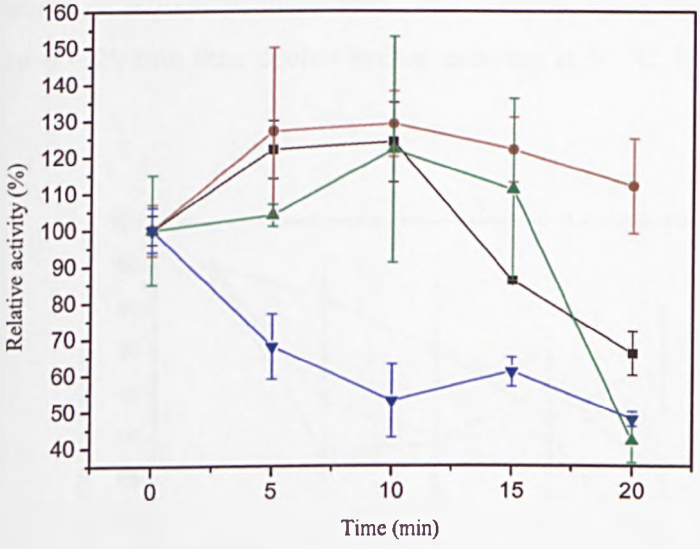


Figure 5.12 Thermal stability of silica/xylanase matrices exposed to dry heat at 70 °C in the presence of metal ions. The samples were subjected to temperature of 70 °C for 0 – 20 min and then cooled before assaying at 50 °C. The following metal ions were present at 1 mM: Mg^{2+}; (▼), Ca^{2+}; (●), Mn^{2+}; (▲) and control; (■). The data presented are mean ± SE (n= 3).

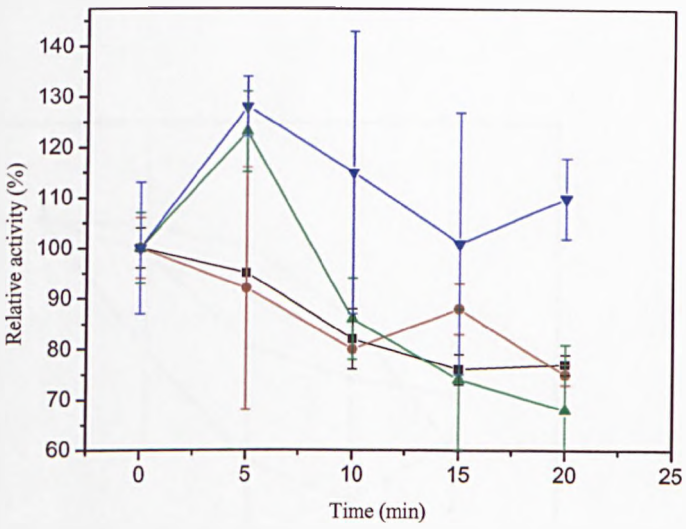
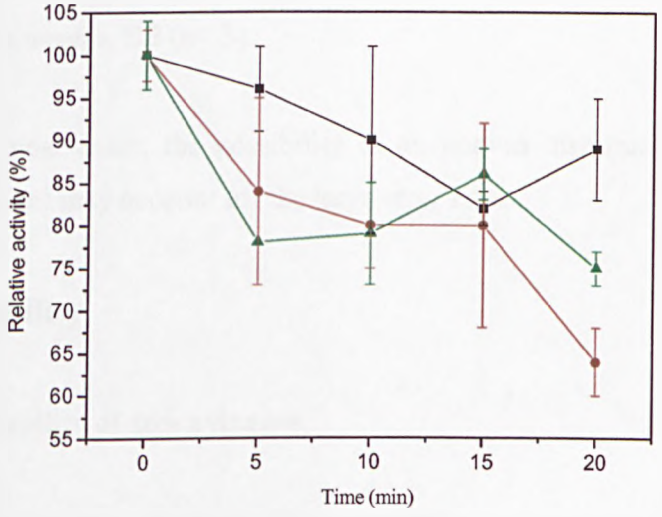


Figure 5.13 Thermal stability of silica/xylanase matrices exposed to dry heat at 70 °C in the presence of combined metal ions. The samples were subjected to temperature of 70 °C for 0 – 20 min then cooled before assaying at 50 °C. $Mg^{2+}Ca^{2+}$; (●), $Mg^{2+}Mn^{2+}$;



(▲), $Mn^{2+}Ca^{2+}$; (■) and $Mg^{2+}Mn^{2+}Ca^{2+}$; (▼). The data are mean ± SE (n= 3).

Figure 5.14 Thermal stability of silica/xylanase matrices exposed to dry heat at 80 °C in the presence of metal ions. The samples were subjected to temperature of 80 °C for 0 – 20 min, then cooled before assaying at 50 °C. The following metal ions were present at 1 mM: Mg^{2+}; (▲), Ca^{2+}; (●) and control; (■).The data presented is an average ± SE (n= 3).

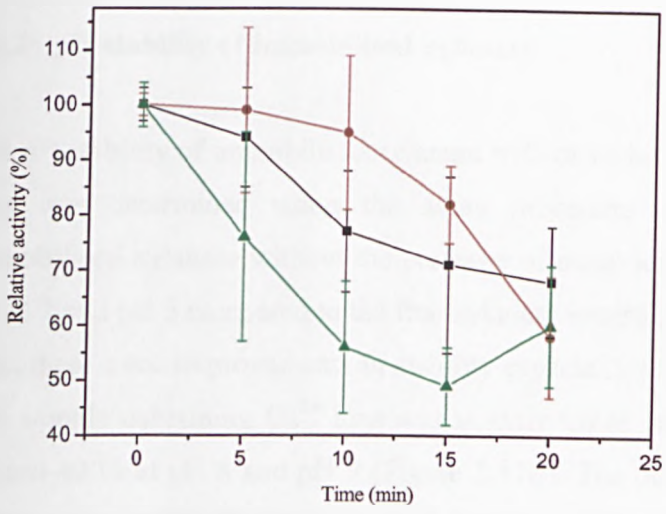


Table 5.15 Thermal stability of silica/xylanase matrices exposed to dry heat at 80 °C in the presence of combined metal ions. The samples were subjected to temperature of 80 °C for 0 – 20 min, then cooled before assaying at 50 °C. The following metal ions were present at 1 mM: $Mg^{2+}Mn^{2+}$; (●), $Mn^{2+}Ca^{2+}$; (■) and $Mg^{2+}Mn^{2+}Ca^{2+}$; (▲).The data presented are mean ± SE (n= 3).

Overall, in most cases, the possibility of an uneven distribution of xylanase on the support material may account for the large error bars.

5.5 pH stability

5.5.1 pH stability of free xylanase

The pH stability of xylanase in the presence or absence of bivalent metal ions was determined by incubating it at various pHs and then assaying at pH 5.3. The procedure is detailed in Section 2.11. Xylanase without the presence of metal ions (control) is known to be very stable when exposed to a wide pH range i.e. between pH 2.5 and 8.5 (Tenkanen *et al*, 1992). The lowest activity of the control was observed when exposed to pH 2 where it still retained about 47 % of its activity; maximum activity was observed at pH 5 and pH 8. The pH stability of xylanase showed slight improvements in the presence of metal ions, and especially in the presence of Mn^{2+} ions where the lowest

activity was 77 % at pH 2, whilst maximum activity was also at pH 5 and pH 8. Further stability was observed with xylanase and combined metal ions (Figure 5.16).

5.5.2 pH stability of immobilised xylanase

The pH stability of immobilised xylanase with or without the presence of bivalent metal ions was determined using the assay procedure as indicated in Section 2.12. Immobilised xylanase without the presence of metal ions (control) appeared less stable at pH 2 and pH 3 compared to the free xylanase control. Again, in the presence of metal ions, there were improvements in stability especially at the lower end of the pH range. The sample containing Ca^{2+} ions was slightly lower than expected with a stability of around 40 % at pH 8 and pH 9 (Figure 5.17B). The best stability was observed in the sample containing all three bivalent metal ions where maximum activity was seen between pH 6 and pH 9 and an improvement of about 20 % was also observed at pH 2 and pH 3 when compared with the control. Overall there appeared to be less fluctuation of activity along the pH range compared to that of the free xylanase samples.

5.6 Processing trials

The main purpose of immobilising xylanase was to improve its thermal and pH stability so that it is able to withstand the harsh processing conditions and is more resilient within the body of the animal when integrated in the animal feed. Plans were made to carry out processing trials in a pilot plant on chicken feed containing immobilised xylanase and to compare xylanase recovery with that of chicken feed samples containing free xylanase. Unfortunately, due to the scale of the pilot plant which only accepted a minimum amount of 10 000 kg of feed per trial, the amount of immobilised enzyme produced was insufficient for testing on that scale. An alternative method of carrying out the trials was to simulate the processing conditions by the use of a retort autoclave. The simulated processing trials were carried out at 75 °C, 85 °C and 95 °C with the moisture content of the feed ranging from 10 % to 20 %. Details of the method are given in Section 2.13.

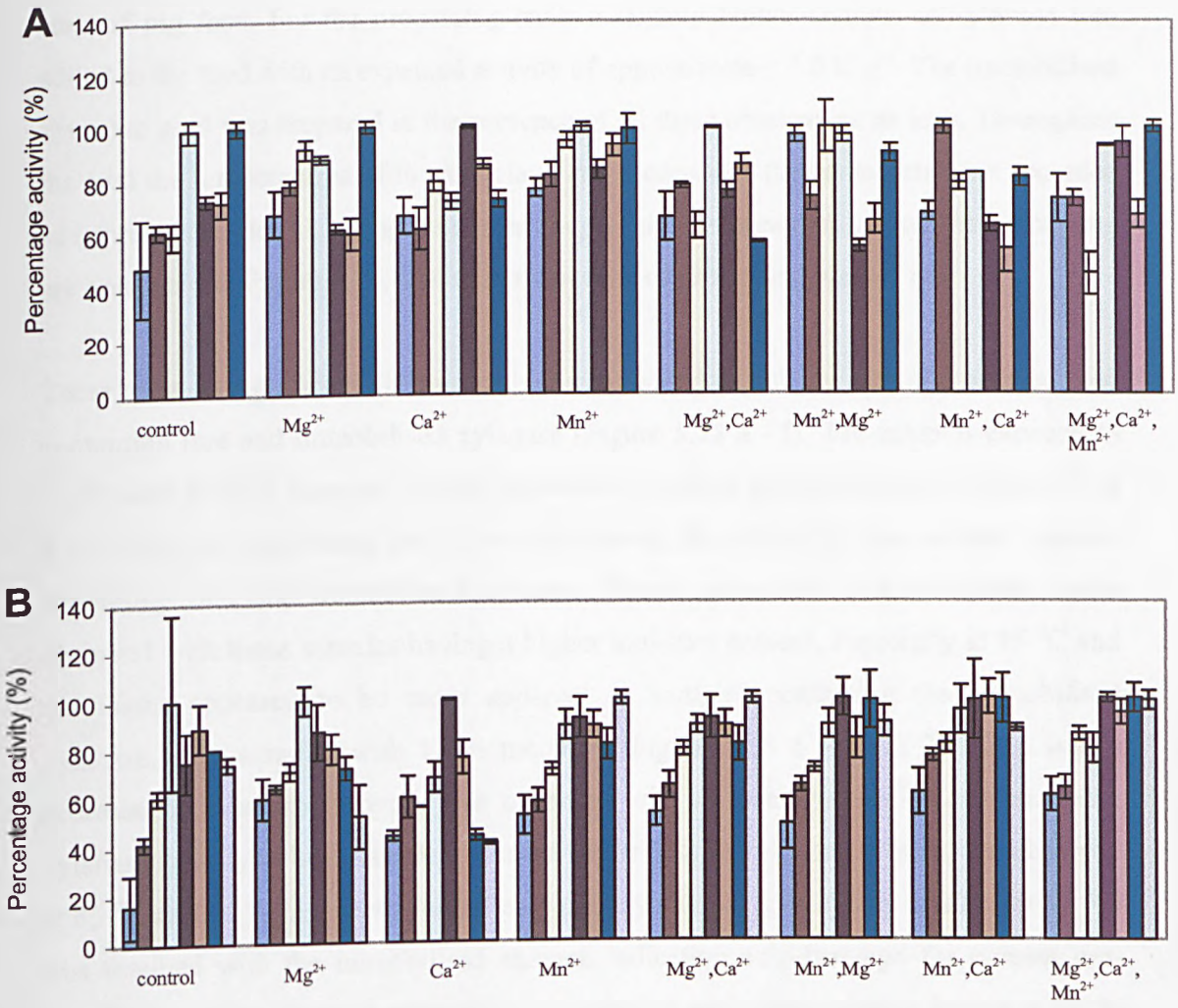


Figure 5.16 pH stability of free (A) and immobilised (B) xylanase in the presence of divalent metal ions.

Each sample was immersed in 200 mM Na_2HPO_4, 100 mM $C_6H_8O_7$, pH as indicated - ( ); pH 2, ( ); pH 3, ( ); pH 4, ( ); pH 5, ( ); pH 6, ( ); pH 7, ( ); pH 8, ( ); pH 9 plus 1 mM of Cl^- salts of divalent cations and stored for 24 hours at 4 °C and then assayed at pH 5.3 for xylanase activity. Data are mean ± SE (n = 3).

5.6.1 Simulated processing trials

The amount of xylanase added to poultry feed is usually 2.5 U g^{-1} and 4.0 U g^{-1} in the case of pig feed. For the processing trials a slightly higher amount of xylanase was added to the feed with an expected activity of approximately 5.0 U g^{-1}. The immobilised xylanase used was prepared in the presence of all three bivalent metal ions. Throughout the trial the temperatures of the autoclave and feed inside the containers were recorded and three examples showing the consistency of the temperatures during the processing are presented in Figure 5.17. The maximum time of processing was 20 min.

There were no significant differences between most data collected from processed feed containing free and immobilised xylanase (Figure 5.18 a - f). The samples exposed to 75 °C with a 10 % moisture content showed the highest enzyme recovery (Figure 5.18 a – c) with no significant loss of activity during the entire 20 min in both samples containing free and immobilised xylanase. Slight losses of xylanase activity were observed with those samples having a higher moisture content, especially at 95 °C and the losses appeared to be more apparent in samples containing the immobilised xylanase. For samples with 15 % moisture (Figure 5.18 d – f), at 75 °C, a steady decrease in xylanase recovery was observed with an average loss of a third of the xylanase activity in both samples. A more severe loss of xylanase activity was observed at 85 °C and 95 °C. However, significant preservation of activity (up to 20 % at 85 °C) was retained with the immobilised enzyme indicating an advantage for process pre-conditioning. No xylanase recovery was observed with feed samples having a 20 % moisture content. This is a strong indication that water activity is the main destructive factor. The feed containing the immobilised enzyme was expected to have lower xylanase activity because the enzyme is more restricted unlike its free form but the immobilised enzyme was expected to be more resilient to the harsh environment.

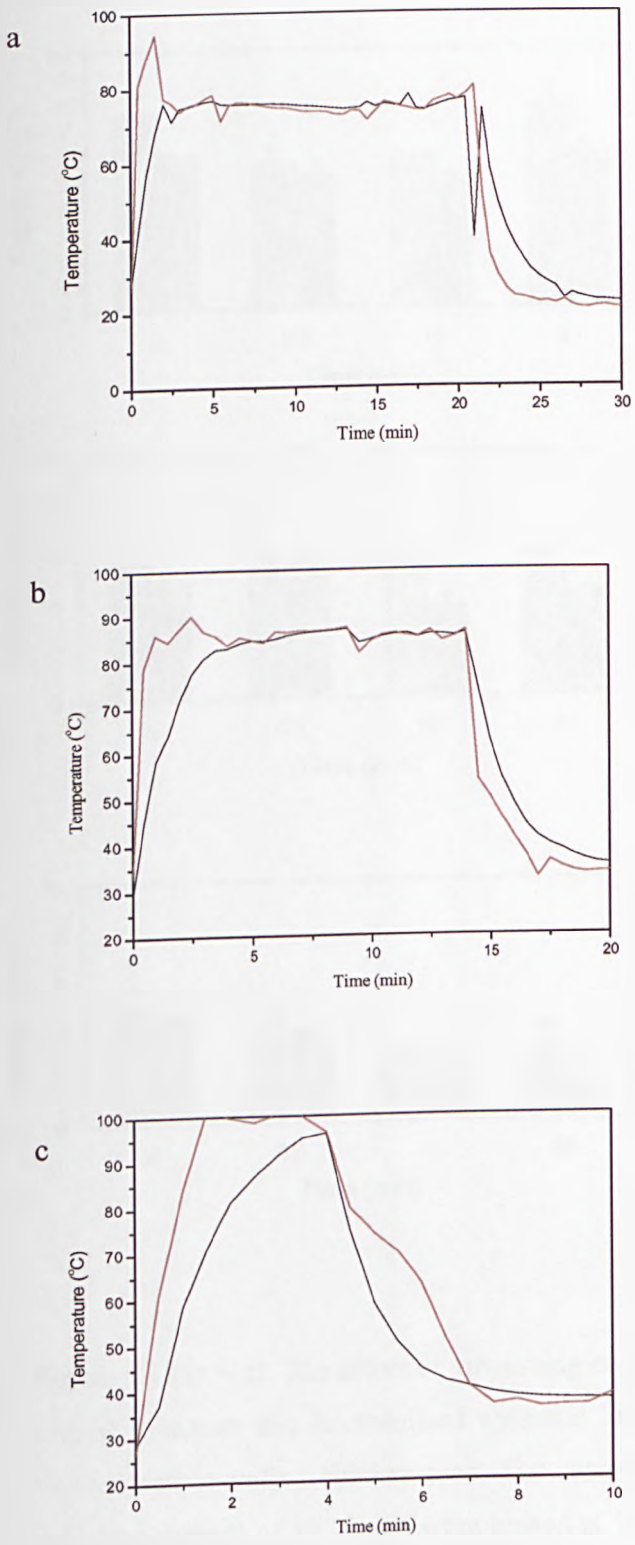


Figure 5.17 (a – c) Three examples of the temperatures during the simulated processing. The temperature of the autoclave (–) was controlled manually to sustain the optimal temperature of the samples (–). The samples were maintained at (a) 75 °C for 20 min, (b) 85 °C for 10 min and (c) 95 °C for 30 sec.

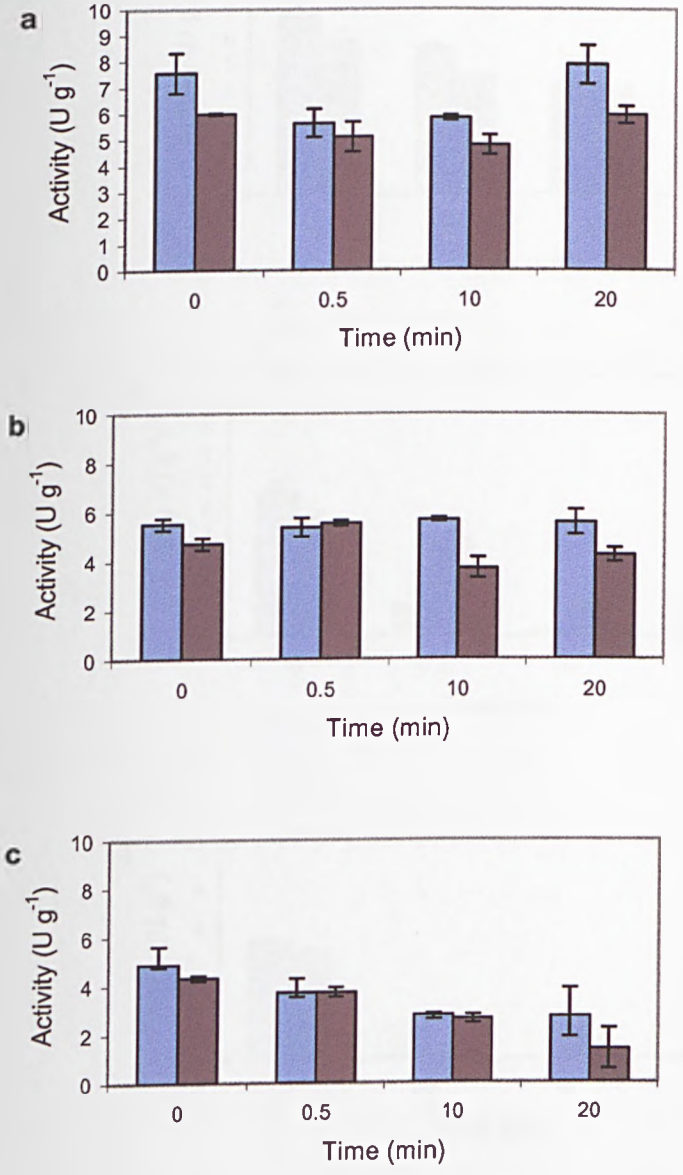


Figure 5.18 (a – c) The effect of processing conditions on xylanase activity.

Liquid xylanase and immobilised xylanase having activities of approximately 250 U were added to milled chicken feed. The samples represented in graphs a, b and c had moisture content of 10 % and were heated at 75 °C, 85 °C and 95 °C respectively prior to assaying for xylanase activity as described in Section 2.13. (▭) Free xylanase; (▬) immobilised xylanase. Data are mean ± SE (n = 2).

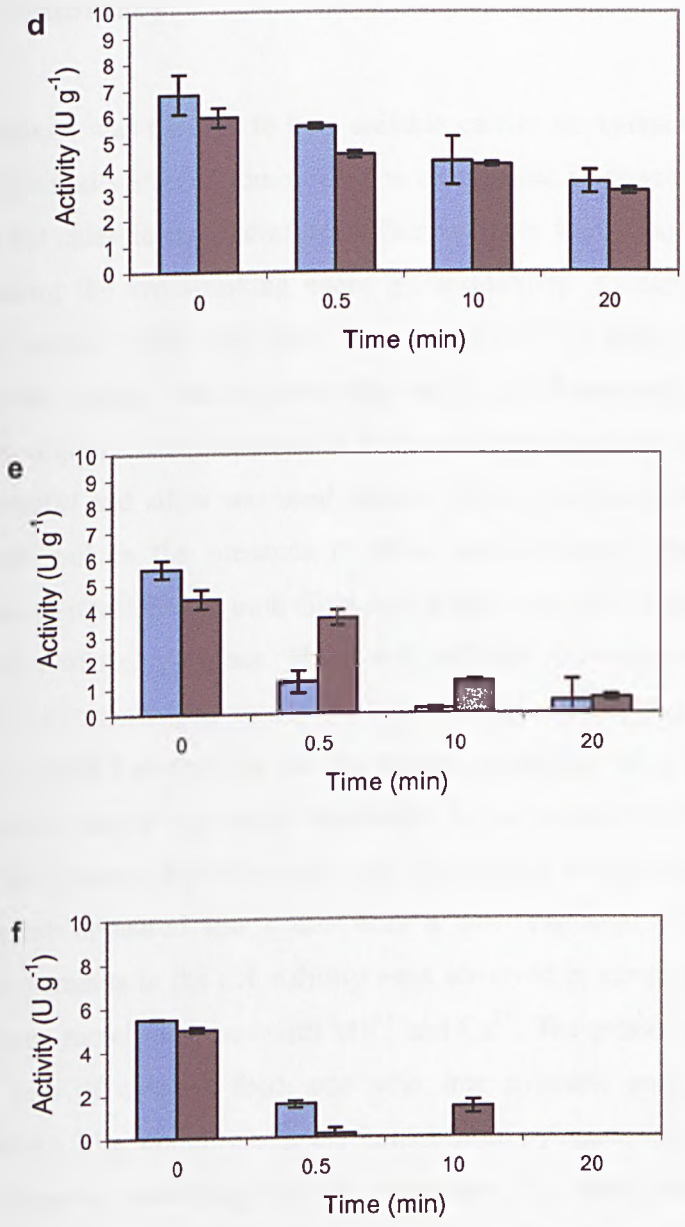


Figure 18 (d – f) The effect of processing conditions on xylanase activity.

Liquid xylanase and immobilised xylanase having activities of approximately 250 U were added to milled chicken feed. Samples represented in graphs d, e and f had a moisture content of 15 % and were heated at 75 °C, 85 °C and 95 °C respectively prior to assaying for xylanase activity as described in Section 2.13. (▭) , free xylanase; (▭) immobilised Xylanase. Data are mean ± SE (n = 2).

5.7 Summary

Promaxon was thought to be a suitable carrier for xylanase because it was chemically and thermally stable. Immobilisation of xylanase by physical adsorption onto Promaxon was not suitable and therefore further attempts were made to covalently bind xylanase by using the cross-linking agent glutaraldehyde. Xylanase recovery was low due to inactivation, which may have been caused by the high glutaraldehyde concentration. Another attempt was to chemically modify the Promaxon surface by changing the end OH group to a more reactive NCS group for binding with xylanase. The process was not successful and silica was used instead. Silica was easily modified and the process was carried out in the presence of three bivalent metal ions. The thermal stability of xylanase immobilised onto silica was determined and compared with that of the thermal stability of free xylanase. There were relatively few improvements in thermal stability with the immobilised xylanase. Good thermal stability was observed when the matrices were exposed to dry heat and the enzyme remained stable up to 80 °C. The pH stability of free xylanase was again determined in the presence of the three bivalent metal ions and the greatest improvements were observed in the presence of Mn^{2+}. The immobilised xylanase appeared less stable after a 24-h exposure at pH 2 and pH 3 but major improvements in the pH stability were observed in samples prepared in the presence of all three metal ions, and with Mn^{2+} and Ca^{2+}. The processing trials were carried out on two sets of chicken feed, one with free xylanase and the other with immobilised xylanase. The samples with the immobilised xylanase were slightly more resilient than the samples containing the free xylanase. The trials also showed that the xylanase recovery decreased with increasing moisture content.

Chapter 6

Microencapsulation of Xylanase

6.1 Introduction

Microencapsulation is a very common technique and is utilised in several scientific disciplines including colloidal chemistry, drying technology, pharmaceutics and paper-coating technology (Finch, 1985). A more general definition of microencapsulation would describe it as a process in which micro particles are totally surrounded by or embedded in a coating material to give small capsules at the microscopic level (Arshady, 1989 and Finch 1996). Microcapsules are processed in different ways which may include spray drying, coaercervation, gel-entrapment and other techniques as discussed in Chapter 1 and some of these methods have been investigated. In most cases the purpose of encapsulating a substance is to protect the active component from the external environment and to improve its shelf life by, for instance, prevention of oxidation, UV radiation and moisture (Finch, 1996). In the present work attempts were made to protect xylanase from heat, pH and moisture.

Data described in this chapter include the evaluation of potential encapsulation materials for xylanase and investigation of suitable methods for the process whilst minimising activity loss. The chemical structures of the wall materials mentioned in Chapter 6 are presented in Appendix 3.

6.2 Spray-drying

Spray drying, a method of encapsulation for the preparation of matrix type microspheres (King, 1990), has been widely used for the drying of sensitive foods and encapsulation of pharmaceutical chemicals (Shrooyen *et al*, 2001; Gibbs *et al*, 1999; Zgoulli *et al*, 1999 and He *et al*, 1999). Even though it is considered a relatively harsh method due to the high temperature of the drying gas, the evaporation of water from droplets causes a cooling effect, which compensates for the high temperature (Re, 1998). Compared with other microencapsulation techniques, spray drying offers a relatively simple continuous process. The active material in solution is mixed with the stabilising polymer matrix material and then spray-dried (Re, 1998). Millqvist-Fureby (1999) spray-dried trypsin with an inlet temperature of 180 °C and outlet temperature between 65 °C and 70 °C and recorded a recovery of trypsin activity of 87 %.

In all cases, spray–drying of the samples gave lower yields than expected. The maximum yield was impossible to determine because a large quantity of the product adhered to the inner surface of the glass chambers of the spray dryer and was difficult to retrieve. The physical states of the spray-dried samples are indicated in Table 6.1. Most were dry powders apart from the spray-dried xylanase and the chitosan/enzyme microcapsules, which appeared as clusters.

6.2.1 Spray drying of xylanase

Spray drying of commercial xylanase was carried out without polymers to obtain dry xylanase samples for examination using SEM. At first, the spray-dried xylanase appeared as yellow/brown clusters but with closer observation the texture of the sample was more of a thick paste. Unfortunately, the xylanase sample was unsuitable for SEM analysis. The protein content of the spray-dried samples was determined using Biuret's reagent and the amount of protein in 97.4 mg of spray-dried sample was 43.2 mg. The activity of the spray-dried sample was 52 U ml^{-1} and was determined by re-dissolving 1 g of spray-dried xylanase in 1 ml of 50 mM CH_3CO_2Na of pH 5.3.

Sample	Percentage yield (%)	State of the product	Size of the particles (μm)
Xylanase enzyme	46.7	Moist clusters	-
Maltodextrin	58.3	Dry powder	1 – 45
Maltodextrin/xylanase (ratio 4:5 w/w)	47.0	Dry Powder	2 – 15
Maltodextrin/xylanase (ratio 4:1 w/w)	42.3	Dry powder	2 – 15
Dextrin/xylanase (ratio 2:1 w/w)	36.1	Dry powder	10 – 30
Chitosan/xylanase (ratio 1:10 w/w)	6.1	Moist clusters	5 -10

Table 6.1 Characteristics of xylanase loaded microspheres prepared by spray-drying.

6.2.2 Xylanase spray-dried with maltodextrin

Maltodextrin was spray-dried using the conditions as described in Section 2.14. The formulations with maltodextrin are listed in Table 2.2 and the formulations containing of maltodextrin and xylanase of ratio 4:5 and 4:1 (w/v) gave the highest yield in grams of 47.0 % and 42.3 % respectively. The products were white powders in appearance, dry and homogenous. The activities of the spray-dried products were determined initially by re-dissolving in CH_3CO_2Na buffer, pH 5.3, and then assayed using Danisco's protocol. All products, including the negative control, gave positive results, which implied that the spray-dried maltodextrin had broken down to reducing sugars. The next approach was to find a method of detecting xylanase activity without the detection of sugars. Megazyme tablets (Section 2.3.5), which contained an insoluble dye substrate that releases the blue dye when attacked by an enzyme (Lee and Lee, 1997) were used. This test finally revealed that there was no xylanase activity in any of the samples. The presence of stabilisers such as sucrose and PEG was thought to improve the durability of the enzyme but the formulations containing 10 % (w/v)

stabilisers failed to form dry products. Instead the products were highly viscous liquids and the presence of stabilisers had prevented the product from drying.

Spray-dried samples were examined, where possible, under the SEM to observe the conditions of the microcapsules or matrices formed. Maltodextrin was also examined under SEM prior to spray-drying to observe any physical changes that had been caused by the spray-drier (Figure 6.1). Spray drying of maltodextrin caused extensive changes producing spherical microspheres (Figure 6.2) from large irregular shaped particles. The variation in particle size was thought to be caused by the inlet temperature affecting the expansion of droplets. As the temperature increased greater expansion of the droplet occurred. This is a characteristic of the matrix material and the design of the spray dryer according to Re (1998).

SEM analysis showed that the maltodextrin/xylanase spray-dried particles were spherical and individualised i.e. were not in clusters. For the spray dried maltodextrin samples without xylanase, most of the particles had sizes ranging from 0.5 μm to 25 μm and appeared wrinkled or indented. The indentation may have been generated by the change in pressure due to the cooling, suggesting that the core was hollow. Larger particles, with sizes of 25 μm and above, were completely spherical with very smooth surfaces. A large microsphere shown in Figure 6.2 has an open surface revealing that it was also hollow. This may indicate that the shell surfaces of larger microspheres have thick walls and are therefore more able to withstand the reducing pressure caused by the effect of cooling. There were no obvious differences in the appearances of microspheres with 1:4 and 4:5 (w/w) maltodextrin/xylanase ratios, although the size of particles within both samples were more uniform than the maltodextrin only microspheres, ranging from 1 μm to 20 μm . The surfaces appeared less shrivelled and craters could be observed on some particles (Figure 6.3), but there was no evidence of porosity as suggested by Re (1998).

6.2.3 Xylanase spray-dried with dextrin

Dextrin, produced from corn, was chosen as a suitable wall material. The conditions in which the dextrin containing samples were spray-dried are described in Section 2.14. Powdered dextrin was difficult to dissolve in CH_3CO_2Na buffer pH 5.3 at room

temperature and so heating at 50 °C was utilised to improve its solubility. Dextrin containing mixtures were fed into the spray dryer, despite being incompletely dissolved, the products were collected as dry white powders. The percentage yield from a 2:1 (w/w) ratio of dextrin and xylanase was 36 %. Again, no xylanase activity was detected with the spray-dried samples. A similar experiment of spray-drying trypsin with dextrin was performed by Millqvist-Fureby *et al* (1999) who had experienced minimal loss of activity but did not present microscopy of the encapsulated enzyme.

SEM analysis showed that the spray drying of commercial xylanase with dextrin did not yield proper spheres (Figures 6.4 and 6.5). Dextrin was unable to dissolve therefore there was no difference in size between the dextrin and the dextrin/xylanase microspheres. The only difference observed between the two samples was that spray-dried dextrin had individual particles and the spray-dried dextrin/xylanase particles were clustered. The clustered appearance of the dextrin/xylanase sample may have been an indication of imperfect mixing of the constituents and therefore can not be regarded as representing proper matrix particles. The dextrin particles appeared to be covered by xylanase enzyme and the sample had not completely dried.

6.2.4 Xylanase spray-dried with chitosan

Chitosan is a hydrophilic biopolymer produced by hydrolysing the aminoacetyl groups of chitin, a main component of shellfish (Shahidi *et al*, 1999; Kas, 1997). Chitosan usage is growing, particularly within the pharmaceutical industry where its applications ranged from wound healing to reducing blood cholesterol (Kas, 1997). Most importantly, chitosan has anti-bacterial properties and is biodegradable (Shahidi *et al*, 1999; He *et al*, 1999).

Spray drying of xylanase with chitosan was carried out according to Section 2.14. At first, while preparing the liquid feed mixture of 2 % (w/v) chitosan dissolved in 2 % (v/v) acetic acid, the solution was too viscous to be fed into the spray-dryer. The solution was then diluted by adding more acetic acid reducing the chitosan concentration to 0.2 % (w/v). After the mixing was complete, xylanase was added to the solution and the mixture was spray-dried. Not surprisingly, the yield was very low at 6 %. The product appeared light brown in colour and clustered but was dry enough for examination using SEM.

The morphology of the spray-dried chitosan/xylanase sample analysed using SEM had revealed that the microspheres were strongly clustered (Figure 6.5) indicating there was too much moisture in the sample. He *et al* (1999) produced spherical and individualised chitosan microspheres, which had an average diameter of 3.75 μm. Comparing the two micrographs, it is obvious that the abnormality shown in Figure 6.6 was mainly due to the formulation ratio of 1:10 (w/w) (chitosan/xylanase).

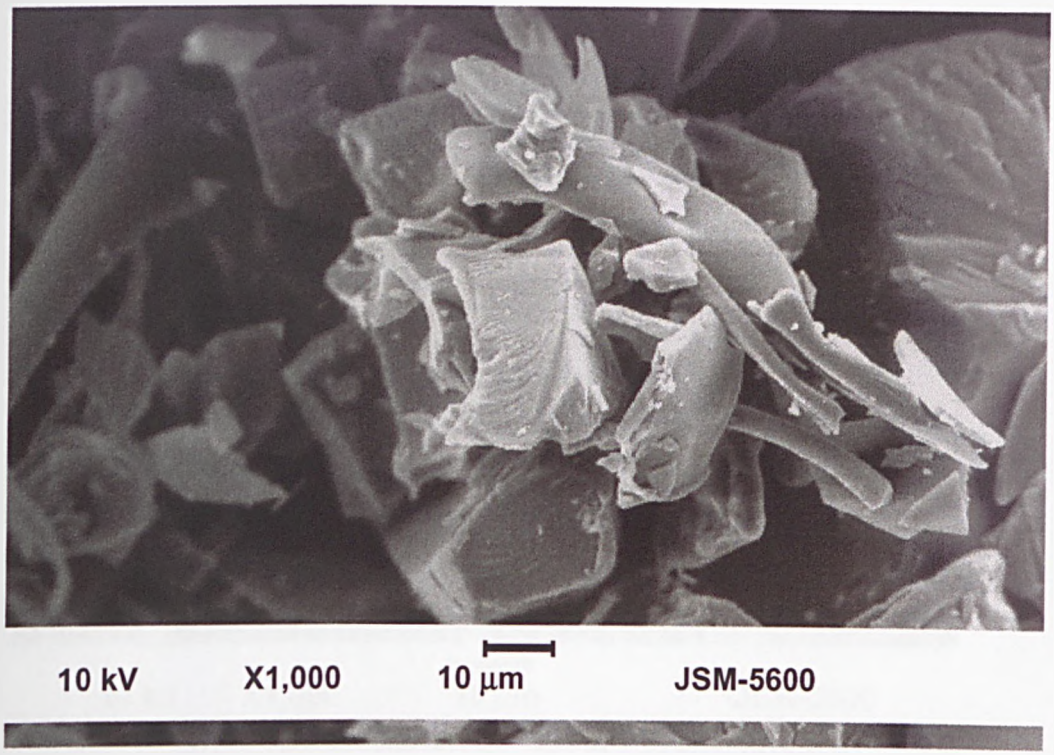


Figure 6.1 SEM micrograph of maltodextrin prior to spray-drying.

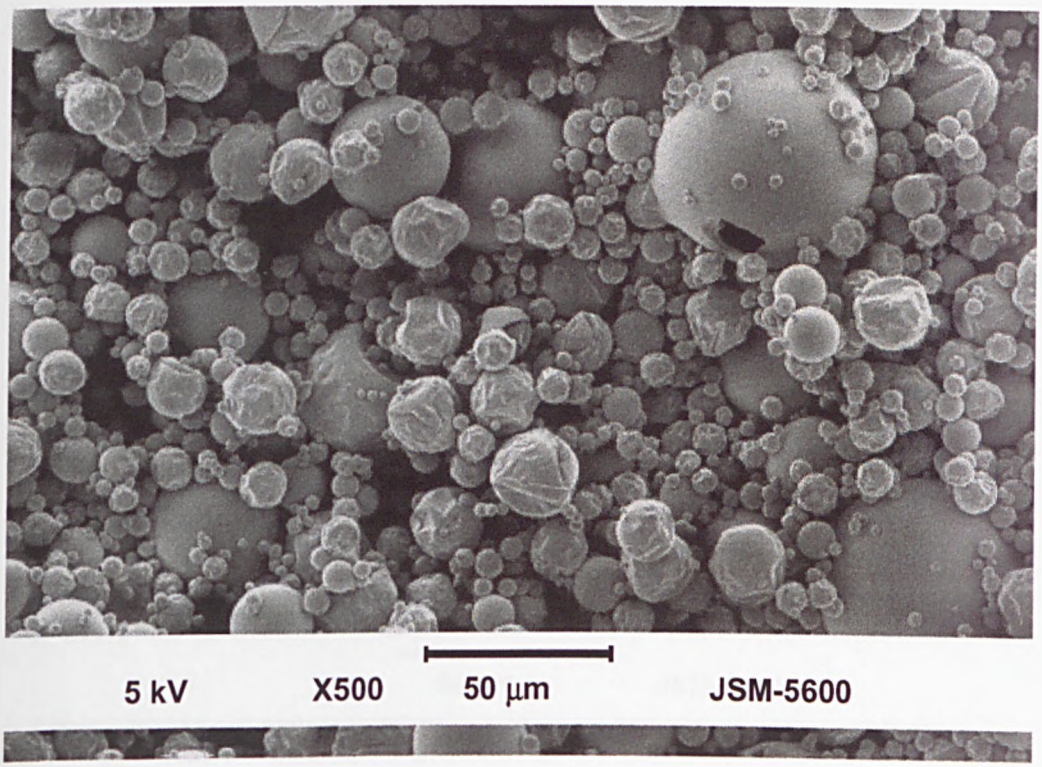


Figure 6.2 SEM micrograph of spray-dried maltodextrin from solution. The drying outlet temperature was 97 °C.

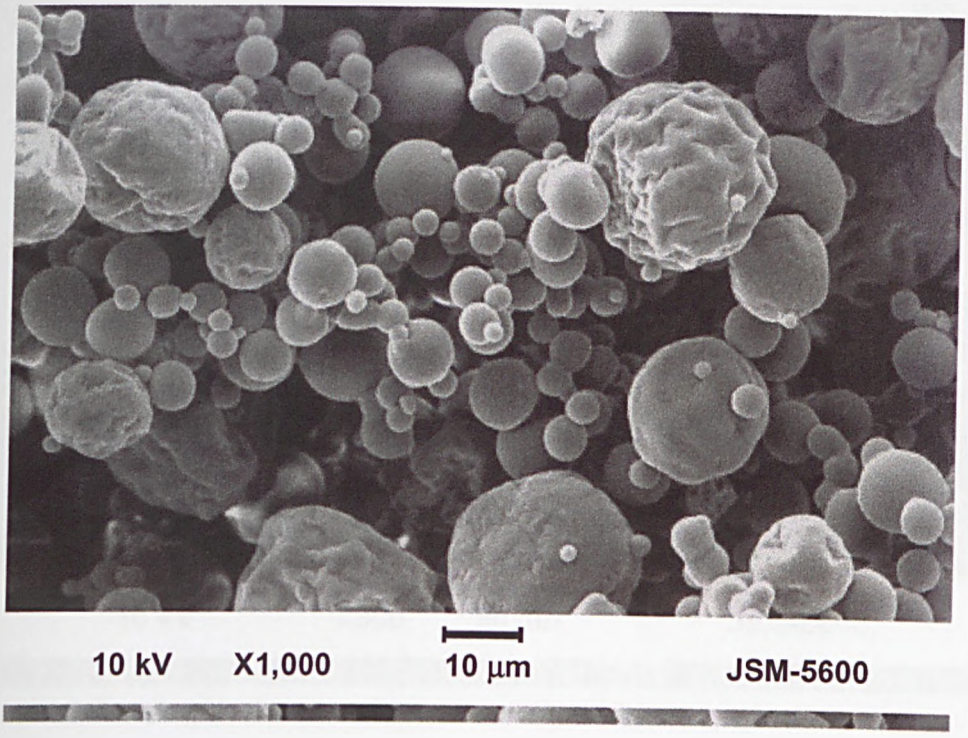


Figure 6.3 SEM micrograph of spray-dried maltodextrin and xylanase at a 1:4 (w/w) ratio from solution. The spray drier outlet temperature was 97 °C.

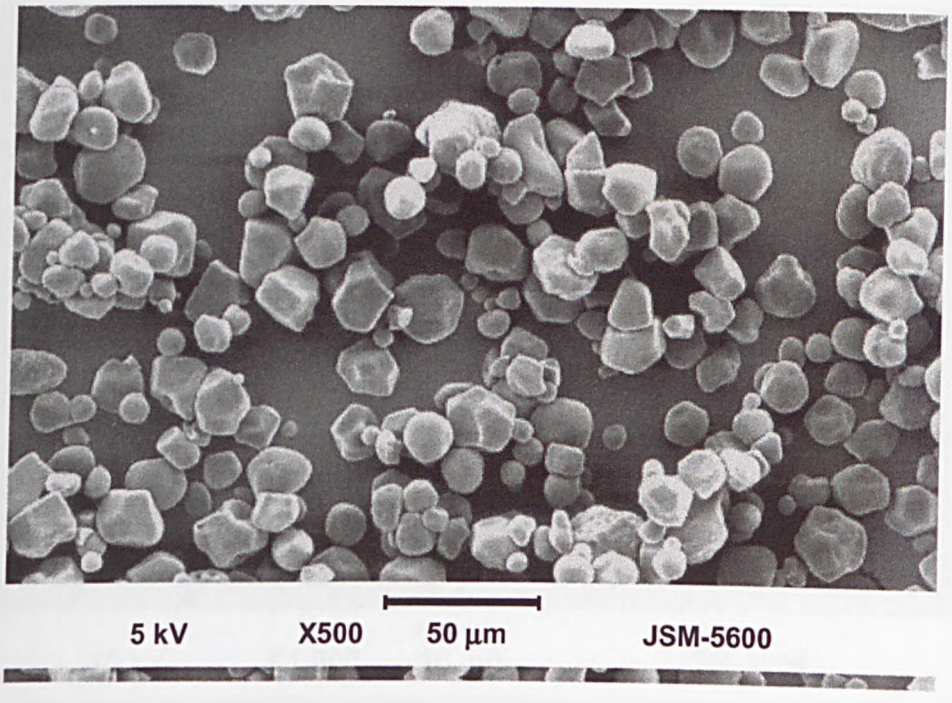


Figure 6.4 SEM micrograph of spray-dried dextrin form solution. The spray drier outlet temperature was 97 °C.

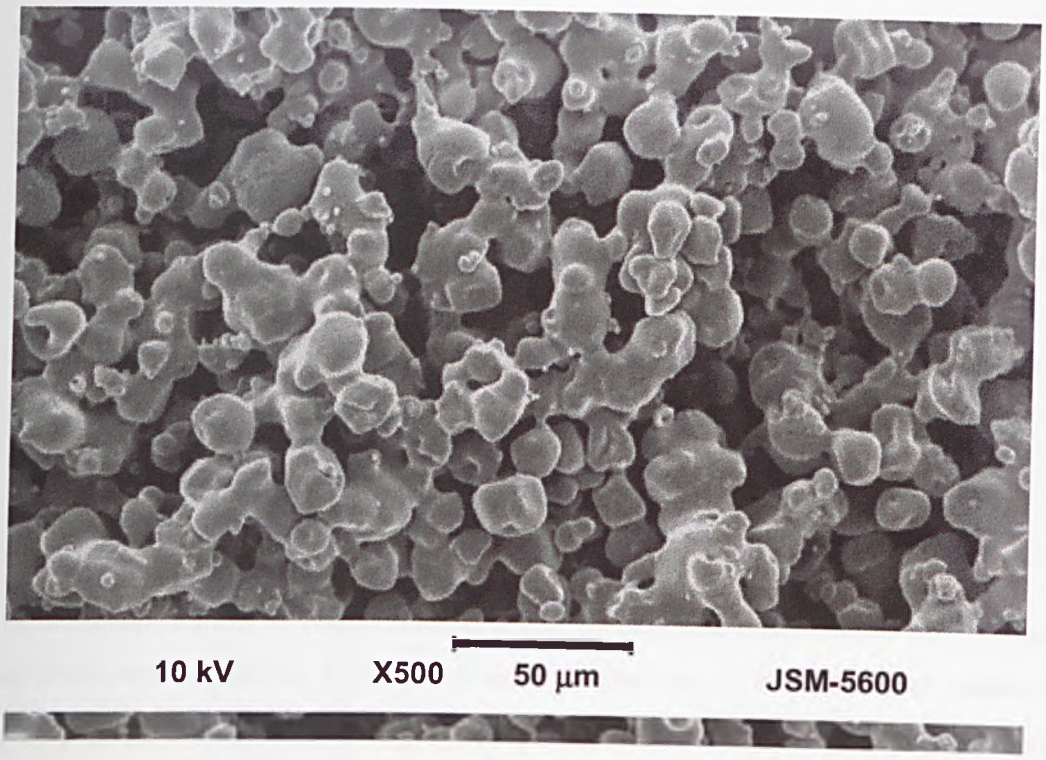


Figure 6.5 SEM micrograph of spray-dried dextrin and xylanase at a 2:1 (w/w) ratio from solution. The spray drier outtlet temperature was 97 °C.

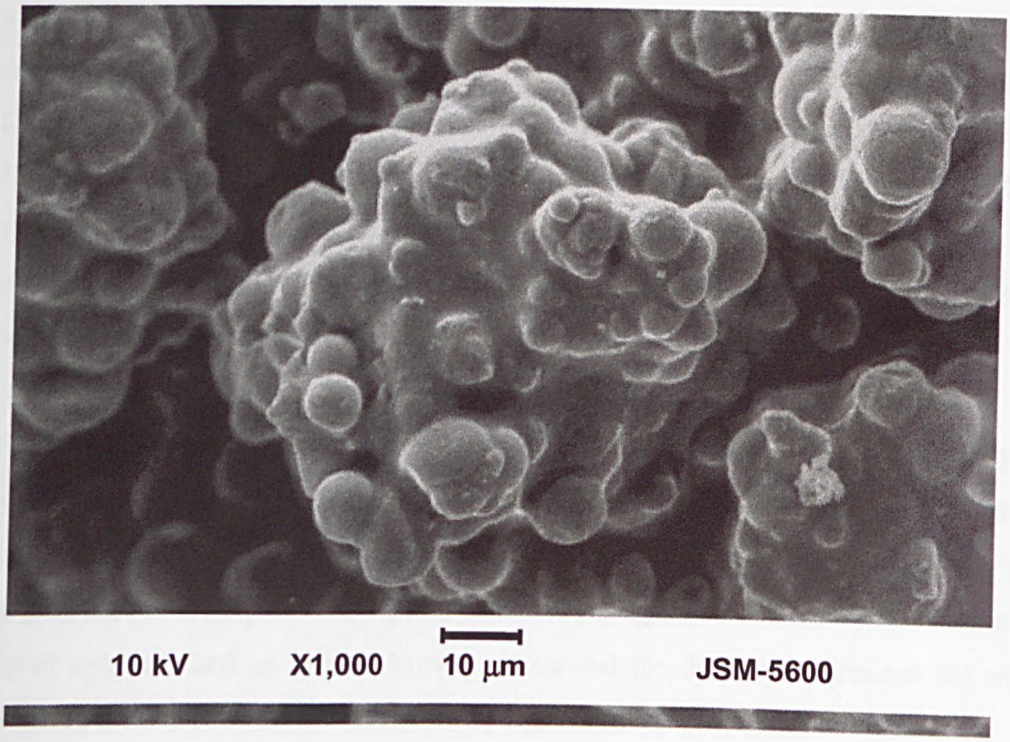


Figure 6.6. SEM micrograph of spray-dried chitosan and xylanase at a 1:10 (w/w) ratio from solution. The spray drier outlet temperature was 97 °C.

6.3 Microencapsulation by phase separation

Microencapsules can be formed in a process in which the phase separation occurs between two different systems, for instance oil in water, and with vigorous mixing an emulsion is formed. Hardening of the tiny globules or microspheres formed can be initiated by change in temperature or by adding a second substance such as concentrated aqueous salt solution (Kondo, 1979). This process produces mononuclear and multinuclear type microcapsules.

6.3.1 Complex coacervation

Microencapsulation of commercial xylanase by complex coacervation was carried out as mentioned in Section 2.15.1. The main components of the coacervate system were 2 % (w/v) gelatin, 2 % (w/v) arabic gum, 6 % (w/w) polyethylene glycol (PEG) and the phase separation was initiated by a rapid temperature change from 50 °C to 4 °C. Glutaraldehyde was then added as a cross-linking agent to harden the microcapsules so they were unable to reverse back to a single-phase. After being filtered and washed, the end product appeared as a homogeneous gel and the microcapsules were difficult to separate. Drying under vacuum did not improve the state of the product as the microcapsules were still stuck together and coacervates were best stored in solution. The coacervation process was repeated several times in the presence and absence of PEG. The development of the microcapsules did not occur in the absence of PEG but instead, large clumps of gel clusters were formed. Low pH (from pH 3 to pH 4) was also found necessary to form the coacervates, no phase change or microcapsules were observed when the system was above pH 4.

The morphology of the microcapsules in two samples, one in the presence of xylanase and the other in the absence of xylanase, was investigated by examining the samples under a light microscope. The diameter of the microcapsules ranged from 3 μm to 15 μm. The microcapsules had good spherocity (Figures 6.7 and 6.8). The commercial liquid xylanase had an intense brown colour and the difference between the samples could be clearly seen showing that the xylanase had become encapsulated.

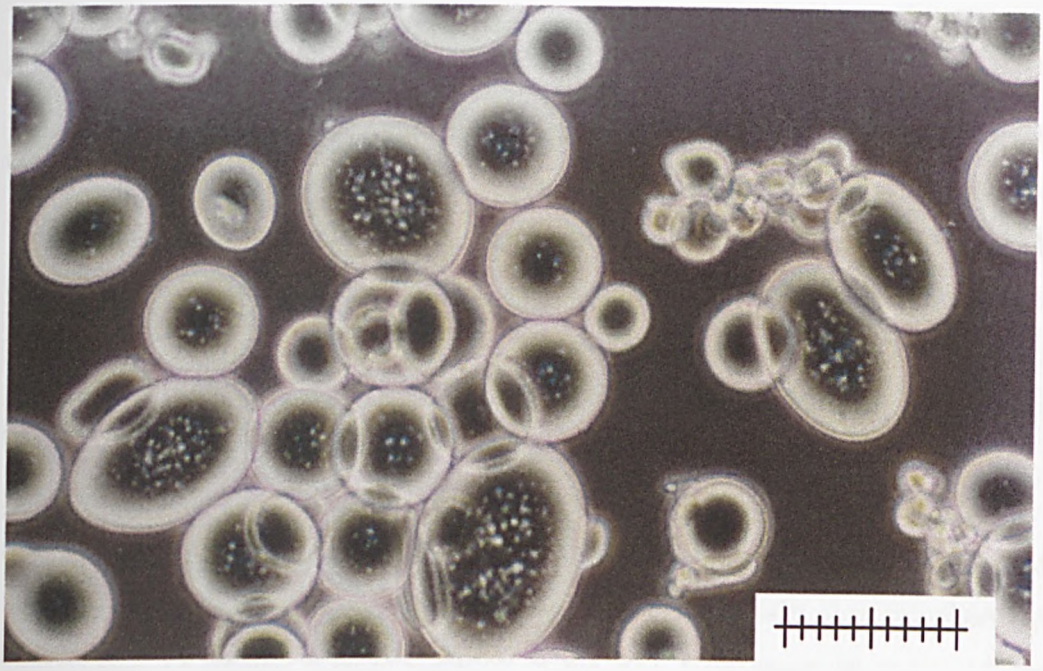


Figure 6.7 Microscopy of xylanase encapsulated coacervates. The main components of the coacervates were 2 % (w/v) gelatin, 2 % (w/v) arabic gum and 6 % (w/v) PEG crosslinked with 2 % (v/v) glutaraldehyde. The scale bar is 10 μm.

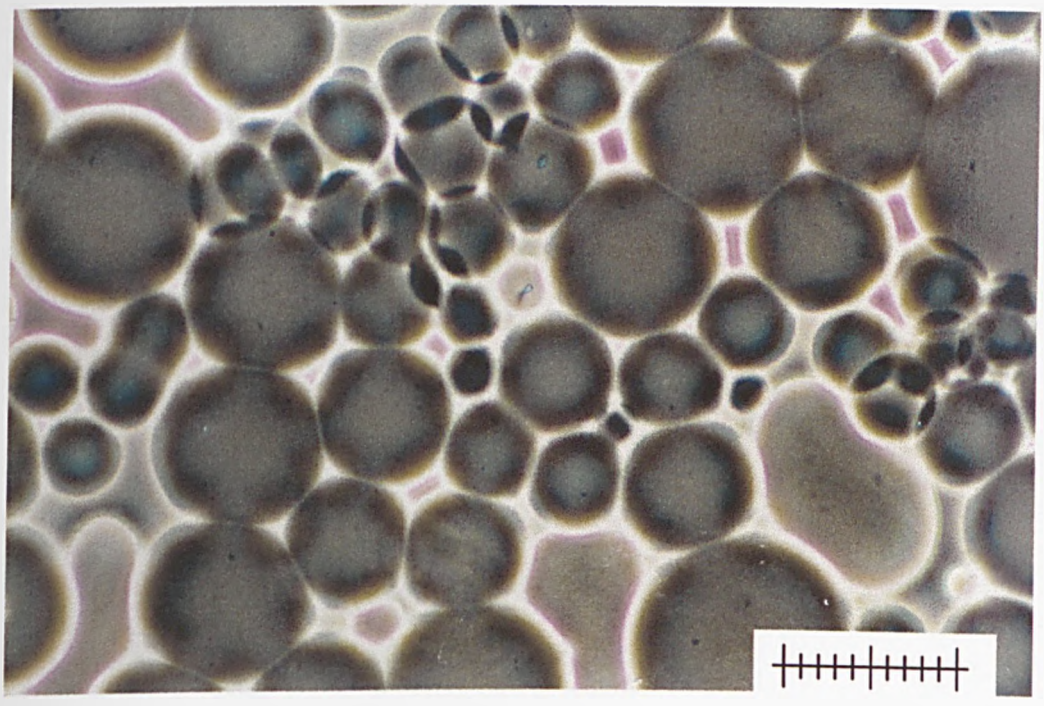


Figure 6.8 Microscopy of coacervates without xylanase. The main components of the coacervates were 2 % (w/v) gelatin, 2 % (w/v) arabic gum and 6 % (w/v) PEG crosslinked with 2 % (v/v) glutaraldehyde. The scale bar is 10 μm.

6.3.2 Aqueous or organic phase separations

Microencapsulation of xylanase using albumin as the wall material was accomplished from a water-in-oil type system. The organic phase separations experiment was carried out as mentioned in Section 2.15.2. The process of encapsulation was very simple and quick, and was achieved by forming an emulsion of diluted commercial xylanase in vegetable oil after which the addition of albumin caused an immediate wall to form between the two phases. After drying, the yellow microcapsules appeared slightly clustered but were easily separated by applying pressure.

The morphology of the microcapsules showed good spherocity with moderate indentations (Figure 6.9) and slightly resembled the spray-dried microspheres shown previously. There was a large variation in the size, from about 10 μm to 60 μm and some of the microcapsules appeared individualised while others formed clusters. Figure 6.10 shows a closer view of an individual microcapsule, which was a perfectly formed sphere that does not appear to be totally smooth on the surface. It was important to note that each sphere contained aqueous solution inside. Albumin, which was obtained from chicken eggs, contains many hydrophobic residues, which is reason why albumin can adapt to such a change. The polypeptide chain changes conformation at the interface such that hydrophobic residues surround the droplet thus stabilising the suspension (Arshady 1990 and Arshady 1988).

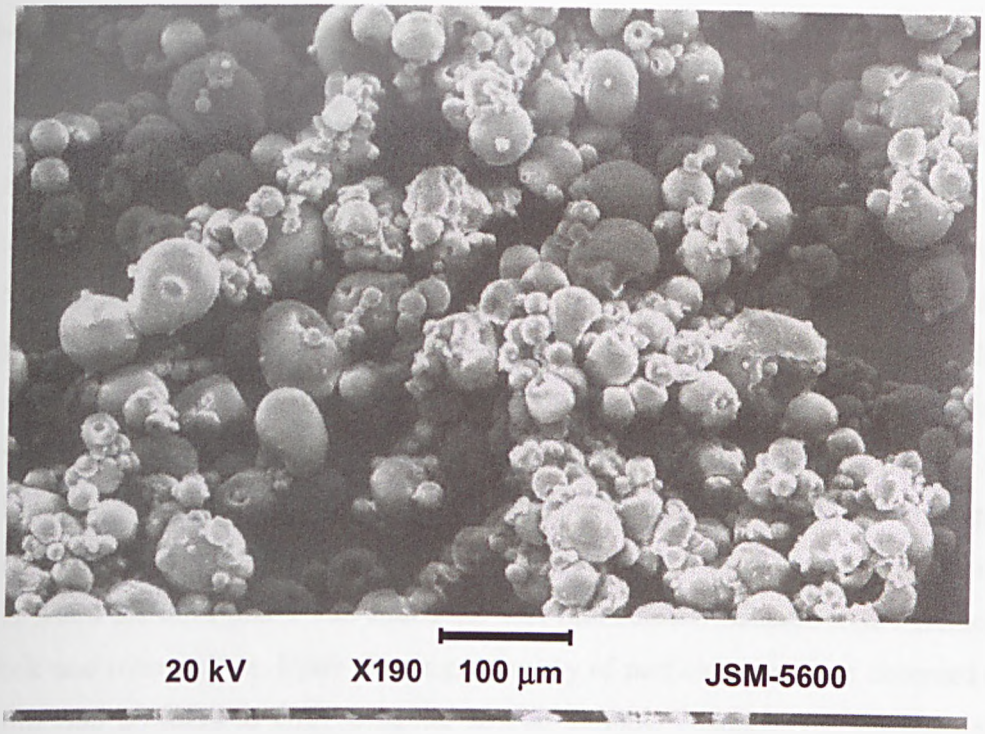


Figure 6.9. SEM micrograph of albumin microspheres.

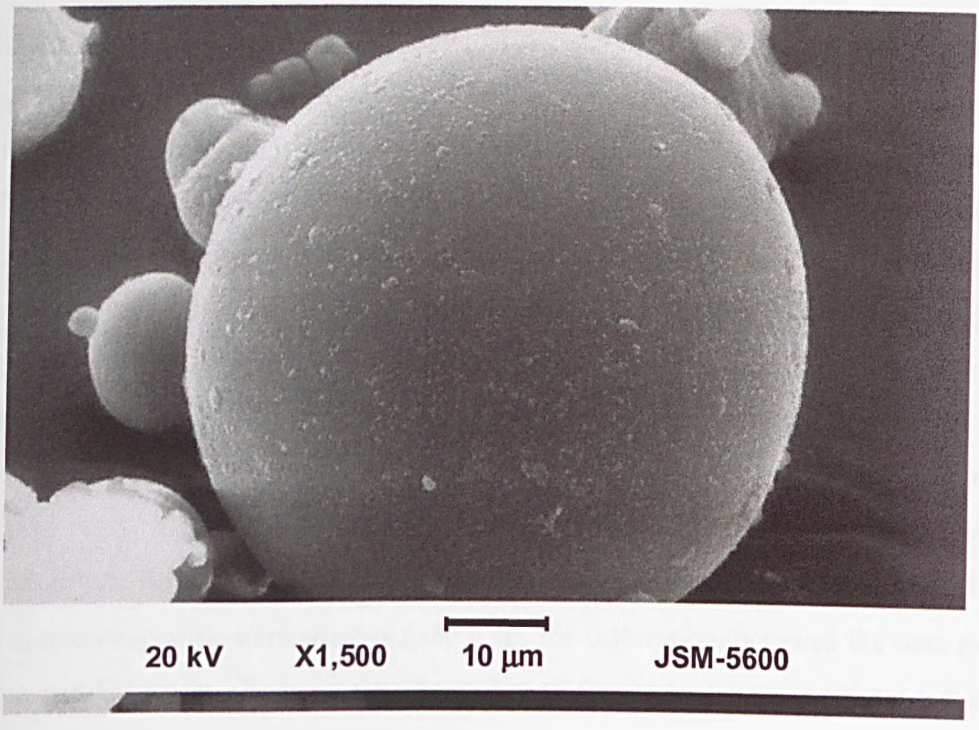


Figure 6.10. SEM micrograph of albumin microspheres.

6.3.4 Microencapsulation prepared by melt dispersion.

Microencapsulation by melt dispersion is another phase separation technique, which is obtained from a water-in-oil-in-water (three-phase) system. Uddin *et al* (2001) used the same technique to encapsulate ascorbic acid with carnauba wax with successful results, resulting in the controlled release of ascorbic acid over 2 h. Melt dispersion was carried out to encapsulate xylanase according to Uddin *et al* (2001) and the method is described in more detail in Section 2.15.3. Instead of using carnauba wax, candelilla wax was used as the oil phase. The experiment was difficult to manage as the melting point of candelilla wax is 57 °C. In order to avoid thermal damage to the enzyme the experiment had to be carried out quickly. An emulsion was formed after the addition of xylanase but when the third phase was added the wax immediately adhered to the surface of the flask and rotary blade. Upon filtering, a variety of particle sizes were observed ranging from fine powders to large irregular shaped clumps. Disregarding the large particles formed, the yield was quite small at 24 %. Only the fine particles were collected and a small sample was examined using SEM.

Figure 6.11 shows a surface of the candelilla microcapsule. The surface was rough with many craters but no pores could be seen. From a wider prospective, the number of irregular candelilla particles observed was in the same proportion as microcapsules (Figure 6.12). The microcapsule shown in Figure 6.10 resembled that of Uddin *et al* (2001) who mentioned that the aggregation was insignificant. Despite the evidence provided by Uddin *et al* of good release rate for the entrapped ascorbate, there was no release of xylanase after intermittently shaking in 50 mM sodium acetate buffer, pH 5.3 for 1 h. It was presumed that there was some loss of xylanase in the preparation process, due to its solubility in water. The main reason for complete loss in activity was due to exposure to high temperatures during the molten stages of the preparation. The candelilla xylanase microcapsules ranged in size between 75 μm and 150 μm. Uddin *et al*'s, microcapsules were smaller (~50 μm), the differences between the data presented here and Uddin *et al*'s may have been due to the type of wax used and the speed of mixing during the encapsulation process.

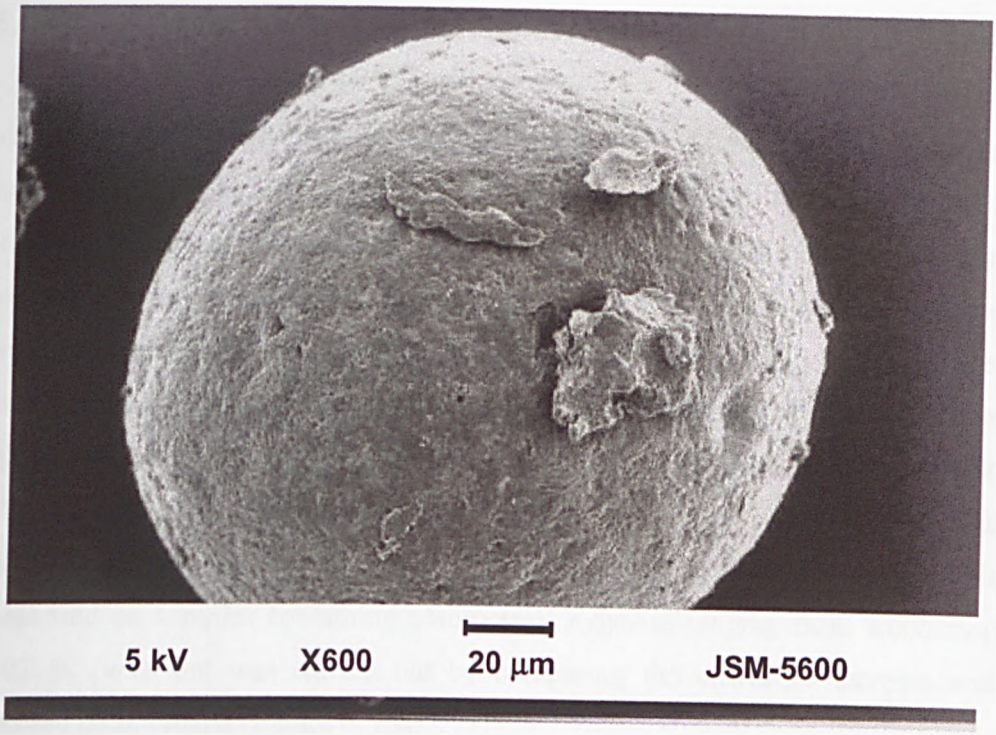


Figure 6.11. SEM micrograph of candelilla wax encapsulated xylanase.

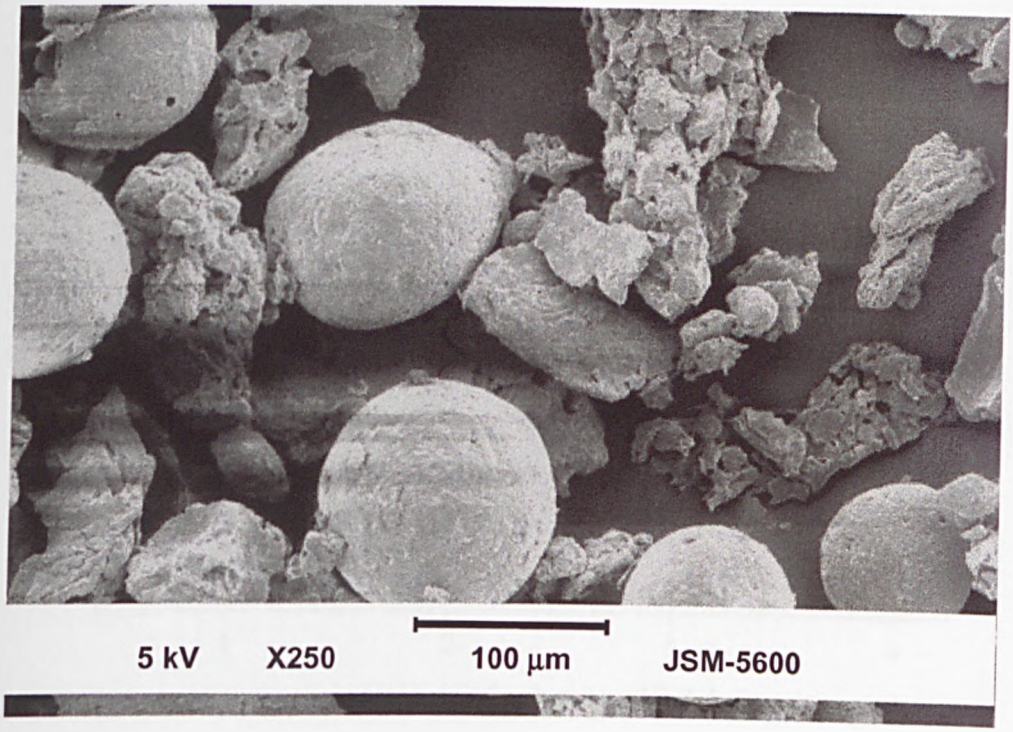


Figure 6.12. SEM micrograph of candelilla wax encapsulated xylanase.

6.4 Gel entrapment and immobilisation of xylanase onto chitosan

Immobilisation of xylanase onto chitosan was made possible by the use of a cross-linking agent, glutaraldehyde. As previously mentioned, chitosan, even at low concentrations of 2 % (w/v), forms a very viscous solution. Dumitri and Chornet (1997) successfully immobilised xylanase onto chitosan-xanthan hydrogels without the use of a cross-linking agent, and reported that the immobilised xylanase showed elevated thermal stability. Modifications were made to the Dumitri and Chornet (1997) protocol by adding 0.3 % (v/v) glutaraldehyde in 1.3 % (w/v) chitosan solution to form a gel. The gel, also containing xylanase, was freeze-dried before experimental tests were carried out. The binding efficiency of xylanase onto chitosan after freeze-drying was measured on samples containing commercial xylanase ranging from 0.005 % (v/v) to 0.02 % (v/v) and was carried out by comparing the unwashed samples with their washed counterparts (Figure 6. 13).

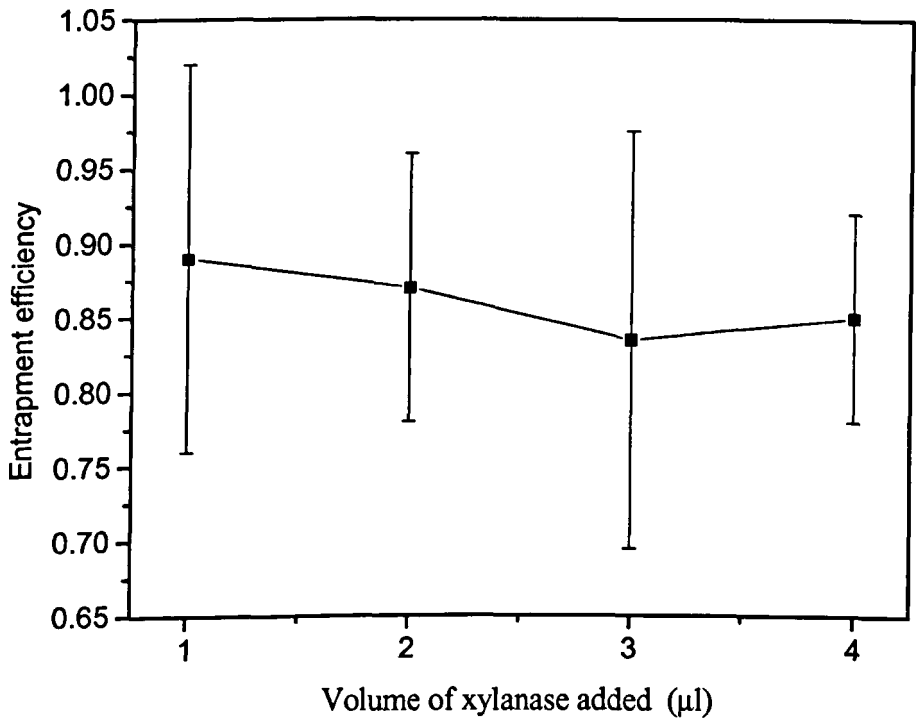


Figure 6.13 Entrapment efficiency of xylanase in freeze-dried chitosan. Immobilisation was carried out by adding xylanase (1 µl to 4 µl) and 1 ml of 40 % (v/v) glutaraldehyde to 20 ml of 1.3 % (v/v) chitosan. The data presented are mean ±SD (n = 3).

When carrying out the DNSA assay, it was apparent that chitosan had reducing sugar properties, which may account for the large error bars. There was no significant decrease in entrapment efficiency with increasing xylanase concentration. Overall the average entrapment efficiency was 0.86.

6.4.1 Thermal stability of xylanase immobilised onto chitosan

Thermal stability was tested to determine how well the glutaraldehyde immobilised enzyme could sustain temperatures above the optimum temperature of the free enzyme (50 °C). Samples were heated to 60 °C and 70 °C for between 5 min and 20 min then cooled and assayed at 50 °C. The heat treatments were carried out in 1 ml 50 mM CH_3CO_2Na buffer, pH 5.3. The data is presented in Figure 6.14. Immobilised xylanase remained completely stable at 60 °C for the period of 20 min. Increase in activity was observed after exposure at 60 °C. Good thermal stability was also observed at

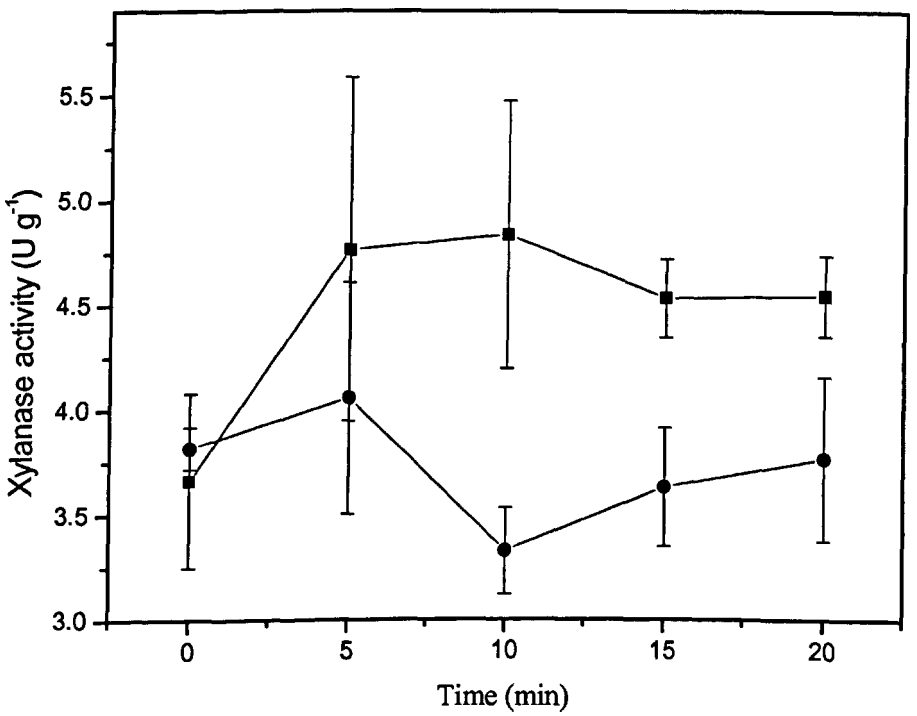


Figure 6.14 Thermal stability of freeze-dried xylanase immobilised onto chitosan. The samples were subjected to temperatures of 60 °C, (■) and 70 °C, (●) for 0 – 20 min, then cooled and assayed at 50 °C. The data presented are mean ±SD (n = 3).

70 °C, most of the xylanase activity was retained for the entire 20 min. Meanwhile, the negative control maintained a constant value of 2.0 U g^{-1} (not shown). Thermal stability tests were carried out at 80 °C also but no xylanase activity was observed after 5 min.

6.5 Summary

Maltodextrin was the most suitable polymer material for spray drying producing spherical and individualised microspheres. Dextrin did not dissolve well in water and therefore was unable to form proper microspheres, whilst chitosan was too viscous. Overall, the spray-drying method was found to be unsuitable for microencapsulation of xylanase since the high temperature of the spray dryer deactivated the enzyme and also the product yield was always low. Phase separations methods did not involve heating to temperatures above 50 °C except in one case, melt dispersion which was considered unsuitable. In all cases, under close examination good spherical microspheres were observed and mononuclear or multinuclear type microcapsules were produced. Although chitosan was not suitable for spray-drying, chitosan was able to produced excellent gels onto which xylanase could be immobilised with the aid of a cross-linking agent. The immobilised xylanase had increased thermal stability and was able to withstand temperatures up to 70 °C for up to 20 min, the typical maximum processing time, with no loss of activity.

Chapter 7

Discussion and Future Work

The aims of this project were to provide different ways to improve the thermal stability of xylanase in order to make the enzyme more resilient to the high temperatures encountered in processing which is an inevitable part animal feed production. The work has focused on immobilisation, microencapsulation and formulation techniques.

7.1 Identification of xylanase

Xylanase used in this project was commercially produced and could have been obtained from one of many different sources (Headon and Walsh, 1994; Wong *et al*, 1988). Although the reaction catalysed by all xylanases is the same, enzymes from different sources possess different characteristics (Lin *et al*, 1999; Tenkanen *et al*, 1992). Therefore, it was necessary to confirm the identity of xylanase before any investigations took place. The molecular profile of xylanase indicated the source to be *Trichoderma longibrachiatum* (formally *T. reesei*) and the data obtained agreed with most of the literature values. However there was a variation in the optimum pH of xylanase that ranged between pH 4.5 and pH 5.5 which is likely to have been due to the buffer used. For example, in this study the optimum pH of xylanase in 50 mM sodium acetate was pH 4.5 (Chapter 4) whilst in 100 mM sodium citrate and 200 mM sodium phosphate buffer the optimum pH was 5.0 (Chapter 3). In addition, the measured activity of xylanase was dependent on the purity and the type of xylan substrate and the formulation of the DNSA regent used. Such noticeable variations in assay conditions are not unusual but make comparisons of xylanase activities in different laboratories difficult (Royer and Nakas, 1989; Bailey *et al*, 1992).

7.2 Denaturation and activation of xylanase

Stabilisation of enzymes has been of great interest industrially and has been extensively examined over several decades. The events of protein folding can be best described thermodynamically where an equilibrium is present between the folded and the partially unfolded states. At ambient temperatures, the native or folded state is thermodynamically more stable and so the equilibrium tends to shift towards the native state (Anfinsen, 1973; Pace, 1990). Permanent or irreversible denaturation occurs when there is loss of secondary and primary structures (Volkin and Klivibanov, 1989; Daniel *et al*, 1996). The events of protein folding and protein inactivation are illustrated in Figure 7.1. Protein inactivation is not only dependent on the external environment such as unfavourable pHs and extreme heat but also on the nature of the protein (Volkin and Klibanov, 1989). Therefore, stability can be defined as the ability of a protein to retain its conformation and function after exposure to unfavourable conditions. CD analysis showed that reversible and irreversible unfolding of xylanase had taken place (Figures 4.3 and 4.5); CD spectra of xylanase taken after exposure to 60 °C for 5 min and 0 min were similar. The same was also seen after 10 min and 15 min but as a lower signal at 223 nm (see Figure 4.5 (a, b and g)). These observations may indicate that some degradation took place but when exposed for 15 min the enzyme was able to partially refold back to the same conformational state as the enzyme exposed for 10 min. This suggests that irreversible unfolding of xylanase occurred in stages, which is the most likely situation. The refolding of xylanase after exposure to 60 °C was more apparent in the presence of bivalent metal ions (Figure 4.5). Apparently the activity of xylanase was not at its optimum after the 5 min exposure at 60 °C although there was evidence of xylanase refolding back to the native state. An explanation for this observation could be that minor irreversible changes in secondary structures essential for activity had occurred, but these were not detected via CD. This seems to contradict the thermodynamics of protein folding (Klibanov and Mozhaev, 1978). However, the thermal stability of xylanase had improved in the presence of bivalent metal ions, especially Mg^{2+} (Figure 5.6), where after being exposed for 10 min to the same conditions no activity loss was observed although CD analysis indicated the xylanase structure had altered. This may suggest that the role of the metal ions was mainly to assist xylanase in refolding back to its native state after exposure, rather than to prevent it from unfolding.

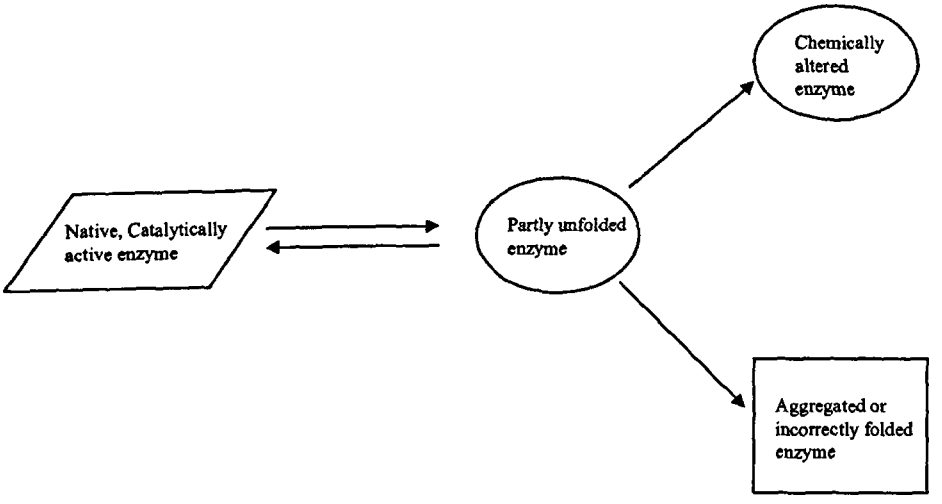


Figure 7.1 Diagram of the events occurring during thermal inactivation of enzymes (Volkin and Klibanov, 1989).

Additives are well recognised to improve the stability of proteins by shifting the equilibrium of the "native protein ↔ thermally-unfolded protein" to the left, in other words, they stabilise proteins against thermal unfolding (Arakawa and Timasheff, 1982; Klibanov, 1983; Volkin and Klibanov, 1989). CD spectra of xylanase in the presence of organic stabilisers showed lower ellipicity at ~223 nm, increased β–sheet retention and higher transition temperatures (T_m) during exposure to varying temperatures from 40 °C to 76 °C compared with the CD spectrum of xylanase without stabilisers. The degree of unfolding of xylanase in the presence of the additives varied, depending on the formulation used indicating that some additive formulations are more effective at stabilising xylanase than others.

Another observation was the enhancement of xylanase activity in the presence of additives. In one case there was an apparent nine-fold increase in xylanase activity which was observed in the presence of 5 % (w/w) PEG and 5 % (w/w) sucrose in 50 mM $NaCH_3CO_2$, pH 5.3. Although the reason for enhanced activity can not be definitely ascertained, it is likely to be due to conformational change (Park *et al*, 2000).

In most cases, the measured ellipicities at 223 nm of xylanase in the presence of additives were significantly lower which may be an indication of increased β-sheet content. Also, there appeared to be a correlation between magnitude of ellipicity at 223 nm and xylanase activity. Since enzyme activity is dependent on conformational stability, again, thermodynamics can be used to explain why some additives enhance the activity of enzymes (Pace, 1990). As explained in Section 1.6.2.1, additives such as sugars are able to increase the Gibb's free energy between the folded and unfolded states by altering the degree of hydration causing the equilibrium to shift toward the folded state. Therefore, one can assume that the increase in activity was due the increase in folded protein molecules. It is important to remember that some additives can have a destabilising effect, which could eventually lead to deactivation (Volkin and Klibanov, 1989) and usually occurs when the inhibitor binds competitively to the active site to form an irreversible conformationally tight complex with the enzyme. Of the additives used in this study, PEI was the only additive that caused the deactivation of xylanase even at the low concentration of 0.1 % (w/w).

7.3 Immobilisation of xylanase

A structural advantage possessed by thermozymes compared with mesozymes is that they have a more rigid structure and therefore are more resistant to thermal unfolding (Vielle and Zeikus, 1996). To attempt to increase the rigidity of xylanase, xylanase was covalently immobilised onto modified silica. This procedure was made possible via surface primary amine groups on the enzyme becoming attached to the ITCPS modified silica.

The activity of immobilised xylanase was 5.5 U g^{-1} and when immobilised in the presence of bivalent metal ions the activity was enhanced by up to four-fold. To determine whether immobilisation had affected the activity of xylanase a comparison between the specific activity of the immobilised and the equivalent free xylanase was attempted. Unfortunately, due to the interference of the active groups present such as -NCS, -NH_2 and -OH the protein content data were unreliable. The activity of the immobilised xylanase was not expected to be greater than the equivalent free xylanase because of factors such as possible distortion of the active site or the active site may

have been inaccessible due to the orientation of xylanase while attached to the support material. Another factor that should be considered is the limitation in the diffusion rate of the substrate and product (Tischer and Kasche, 1999; Volkin and Klibanov, 1989). Increasing the activity by loading more enzymes onto to a support material with a greater surface area, e.g. porous silica or smaller silica particles (Tischer and Kasche, 1999) would be an effective way of increasing overall activity.

Although there were slight improvements compared with that of free xylanase, the thermal stability of xylanase immobilised onto modified silica when immersed in solution was expected to be somewhat higher. Significant improvements were observed when the immobilised xylanase samples were exposed to dry heat, which suggests that increased water activity, not heat, was the main destructive factor. The thermal stability of xylanase when immobilised onto chitosan was significantly better and not only did it show consistent stability at 60 °C reasonable stability at 70 °C while immersed in solution for 20 minutes was also observed. Considering the differences in behaviour between xylanase immobilised onto silica and chitosan support materials, there are almost certainly other factors that contribute to improving the stability of xylanase such as the nature of the support material, cross-linking agent used, the number of binding sites available on the support material and choice of immobilisation method. Gel-entrapment techniques are well known to produce highly improved enzyme stability (Dumitriu and Chornet, 1997; Avnir *et al*, 1994). Klibanov (1983) had suggested that increased stability is due to the type of support material used the ideal materials will have a surface complementary to that of the enzyme molecule. Within the gel, the enzymes are chemically attached to a three-dimensional polymeric lattice (Klibanov, 1983) and not just attached on one side. Therefore, the gel-entrapped enzymes are more rigid, and hence more thermally stable. Chitosan was a more complementary carrier than modified silica for xylanase and further development of this matrix would be worthwhile.

Both free and immobilised xylanase had good pH stability after exposure to pHs ranging from pH 3 to pH 8 with the immobilised xylanase having the lowest stability at pH 2. It would be interesting to determine stability after exposure for extended periods at the body temperature of the chickens and pigs (typically 41 °C and 39 °C respectively).

Based on the data presented from the simulated processing trials, the immobilised xylanase was slightly more resilient than the free xylanase. It was apparent that the higher the temperature, longer processing time and higher moisture content, the lower the xylanase recovery. Despite efforts to produce the most realistic simulation, the conditions used not entirely representative of industrial feed processing. Other important factors such as pressure and the particle size of the feed play essential roles in processing. During the processing, high-velocity (15 m sec^{-1}) steam is injected into the meal to raise the temperature and moisture content while the meal is agitated, suggesting that the feed reaches its target temperature very rapidly, unlike in the simulated trials. The pressure and temperature of the steam can vary, which is mainly influenced by the configuration of the steam pipework. Superheated steam is most likely to be used in feed processing because it is less likely to reduce its pressure when in contact with the feed, therefore does not give off as much latent heat. As a result, the feed can be exposed to high temperature for a longer period (P. Steen, Danisco, personal communications). Table 7.1 lists the different types of processes used. The effect of the superheated steam on the immobilised and free xylanase could be less detrimental as long as the moisture content and temperature is kept below 16 % and 95 °C respectively.

7.4 Microencapsulation of xylanase

Technically, microencapsulation could offer several advantages over immobilisation provided that the microcapsules or microspheres are in a solid state and dry. An additional advantage is that it can provide a protective barrier between proteases and other enzymes (Gibbs *et al*, 1999).

Unlike immobilised enzymes, if the wall material is water impermeable, the core of microcapsule can remain dry thus reducing the sudden impact of steam and heat during processing.

Feed conditioning method	Time of exposure at conditioning temperature (sec)	Meal temperature (°C)	Steam temperature (°C) and pressure (bar)	Comments
Barrel	20 – 120	70 – 95	125 – 155 (1.3 – 4.5)	Most common type of conditioning, used in all feed mills
Long term	120 – 900	70 – 95	125 – 155 (1.3 – 4.5)	Long term conditioner (LTC) used in feed manufacturing for increased retention time. Steam is not applied at the LTC; a barrel type conditioner is used directly above the LTC
Friction	1 – 20	70 – 95	125 – 155 (1.3 – 4.5)	Elevated temperatures are achieved by a compression ring assembly.
Expander	1 - 10	100 - 150	125 – 185 (1.3 – 10)	Elevated temperatures are due to the pressure (10 – 40 bar) induced by an annular gap configuration. With the expander it is also possible to inject steam into the barrel of the conditioner at up to 10 bar, (185 °C). Typical processing temperature for pig/poultry diets is 105 – 110 °C.

Table 7.1 Variations in conditioning process methods highlighting the differences in times and temperatures required by animal feed manufacturers (P. Steen, Danisco personal communication).

Spray drying was initially considered to be the most convenient method to encapsulate xylanase and the process has economical advantages. There are many examples of spray drying of heat sensitive enzymes and even bacteria with good recovery and survival rates (Burgass and Ponsart, 1998; Millqvist-Fureby *et al*, 1999; Amiet-Charpentier *et al*, 1998). Encapsulating enzymes with a suitable carbohydrate wall material could have a stabilising effect as well as offering protection from the processing environment.

The drying quality of the product during spray drying was greatly influenced by the inlet temperature of the dryer. By increasing the inlet temperature, the rate of the film formation on the surface of the droplet increased. Consequently, the build up of steam in the interior of the drying droplet caused the microspheres to expand (Re, 1998). This 'balloon-type' expansion, resulted in the formation of cracks, pores and craters on the surface of the matrix as revealed by the SEMs in Chapter 6, and upon cooling the pressure inside the microspheres reduced, resulting in a shrivelled appearance. The conditions of the spray dryer were controlled manually but little or no improvements could be made to preserve the activity of xylanase. The drying quality of the product improved with increasing inlet temperature, which as a consequence, caused the destruction of xylanase. Similar observations have been published showing less drastic effects on enzyme stability (Amiet-Charpentier *et al*, 1998). Despite the failures of preventing the loss of xylanase activity during spray drying, the idea of using this method to encapsulate other enzymes should not be discouraged. As mentioned previously, other enzymes have been spray dried with minimal loss of activity. These contrasting data may be attributed to variations of the design of the spray dryer (Re, 1998). The process of spray drying is dependent on the geometry of the dryer and the design of the inlet and outlet channels and can be adapted to the specifications needed (Straatsma *et al*, 1999). Most of the specifications and properties of spray dryers are fixed by the manufacturer (Kondo, 1979).

Alternative methods, which could be use instead of spray drying, are spray cooling and spray chilling. Both of these methods are primarily used to encapsulate water-soluble solid food additives such as vitamins, minerals and flavours. In contrast to spray drying, spray cooling and spray chilling uses air cooled to ambient or refrigerated temperatures below the solidification point of the molten fat or wax coating. The two methods differ in the melting point of the carrier used; spray chilling uses hydrogenated vegetable oil

with a melting point range between 32 °C and 42 °C, whilst in spray cooling, the normal melting point range is between 45 °C and 122 °C (Taylor, 1983). Encapsulating xylanase using these two methods could be feasible provided that xylanase was in a dry state, for example using xylanase adsorbed on a carrier material like Promaxon. In this case, if vegetable oil or wax was used then the high temperature of processing would cause melting and destruction of the microcapsule wall. Alternative wall materials that could be used are gum and gel mixtures along with a cross-linking agent to cause solidification on cooling.

Microencapsulation of xylanase by phase separation methods was found to be feasible as confirmed by light microscopy. The disadvantage of this type of microencapsuation is that the xylanase within the capsules was still in solution, making it more susceptible to microbial attack and if the heat of processing penetrates though the outer wall the enzyme would quickly degrade due to the increased water activity. An obvious solution would therefore be to quickly dehydrate the microcapsules by spray drying (Burgess and Ponsart, 1998) or by freeze-drying.

Overall, microecapsulation and immobilisation of enzymes are beneficial to the animal feed industry offering advantages in improvement of the quality and assimilation of low-grade feed. Both techniques offer the advantage of avoiding direct contact of xylanase with denaturing agents.

7.5 Future work

In addition to the points discussed previously, recommendations for further work are discussed in this section. Results presented in this thesis showed that the presence of organic additives improved the structural retention of xylanase when exposed to heat. The additives may have affected xylanase in other ways such as causing shifts in optimal temperature and pH as well as improving the thermal and pH stability. Changes in these characteristics could be determined by measuring the residual activity of xylanase during and after stressful conditions (e.g. heat, pH).

Further investigations on the microcapsules prepared by phase separations may prove fruitful. In particular dehydration by freeze-drying will be necessary to reduce the water content and to improve the thermal and storage stability prior to laboratory testing and processing trials. In addition, attempts should be made to optimise immobilisation and encapsulation of xylanase using appropriate additives and other support materials. Another characteristic and desired benefit, which is found in both immobilised and encapsulated enzymes, is improvement in storage stability, and further tests should be carried out to determine how long the immobilised xylanase could remain active.

Attempts to investigate and develop other immobilisation and microencapsulation techniques to optimise the stability of xylanase should be continued. New approaches such as chemical modification of xylanase and preparation of cross-linked crystals could also be attempted. Considerable benefits could be gained if the newly developed modified xylanase is cheap and easy to produce. As expected, the newly developed xylanase will have to undergo various standard 'food safety' testing before *in vivo* trials. It may be possible the immobilised or encapsulated xylanase could react differently when ingested by the animal. Further investigations should be carried out to determine the behaviour of the immobilised or encapsulated xylanase *in vivo*.

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Appendix 1

Electro-spray mass spectrometry of xylanase after purification by gel-filtration.

Figure 1A-1 Electro-spray mass spectra of xylanase. The numbers above the peaks (A10, A9...B10, B9...) represents the molecular charge associated with the peaks.

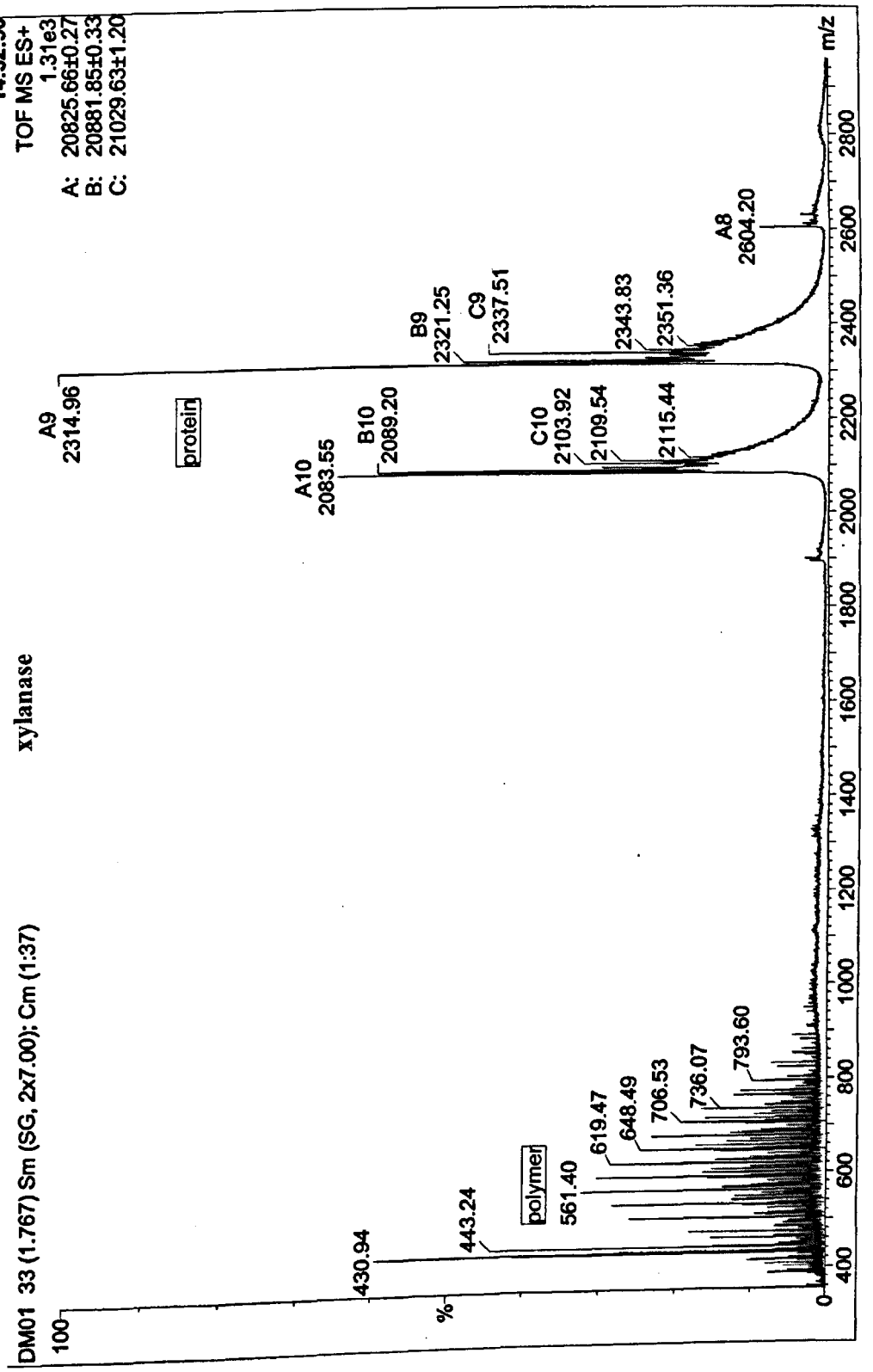
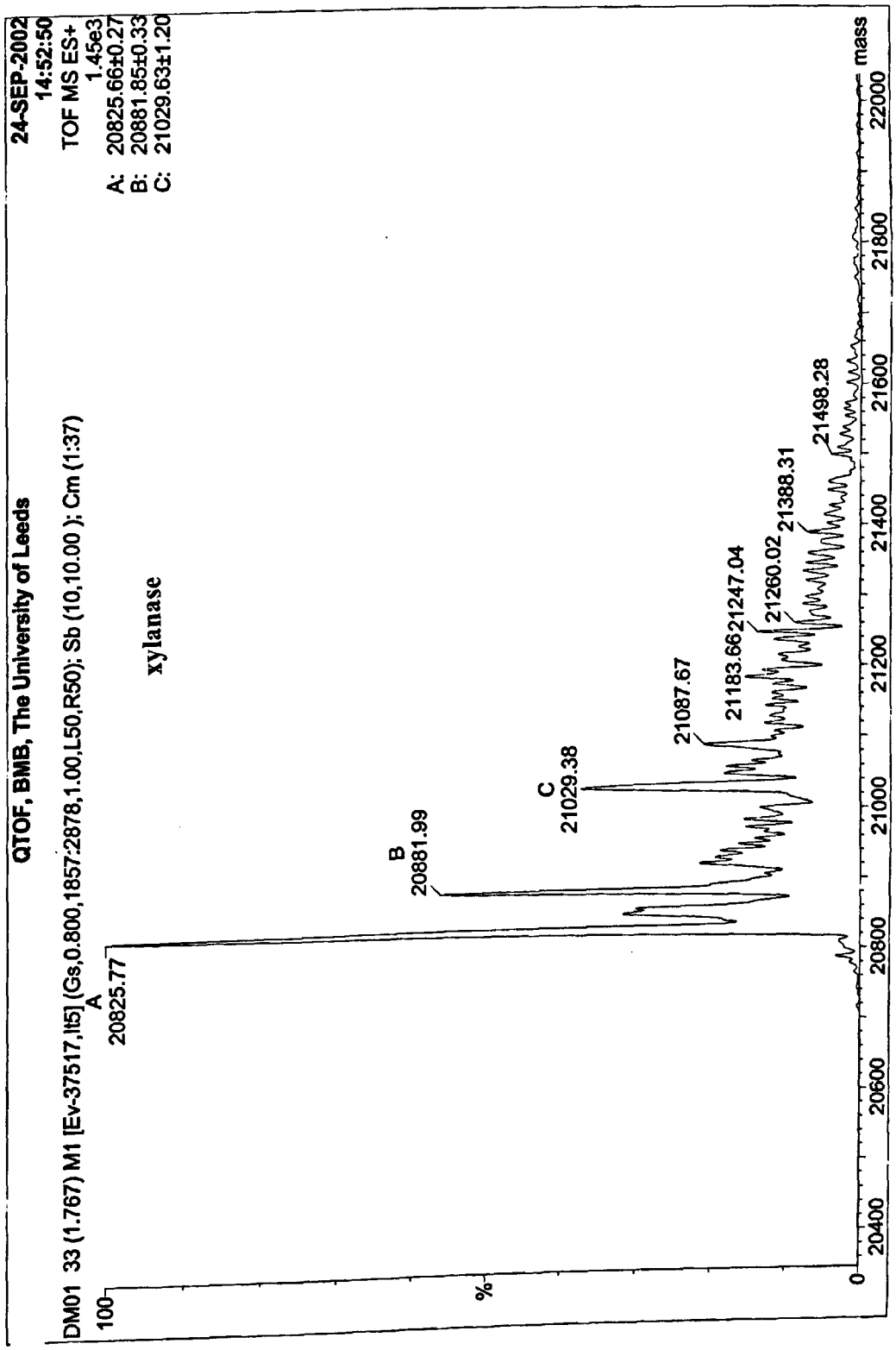


Figure 1A-1 Mass spectral data of xylanase



Appendix 2

Infra red spectra of silica before and after modifications to 3-aminopropyltriethoxy-silane and 3-isothiocyantopropyltriethoxysilane silica.

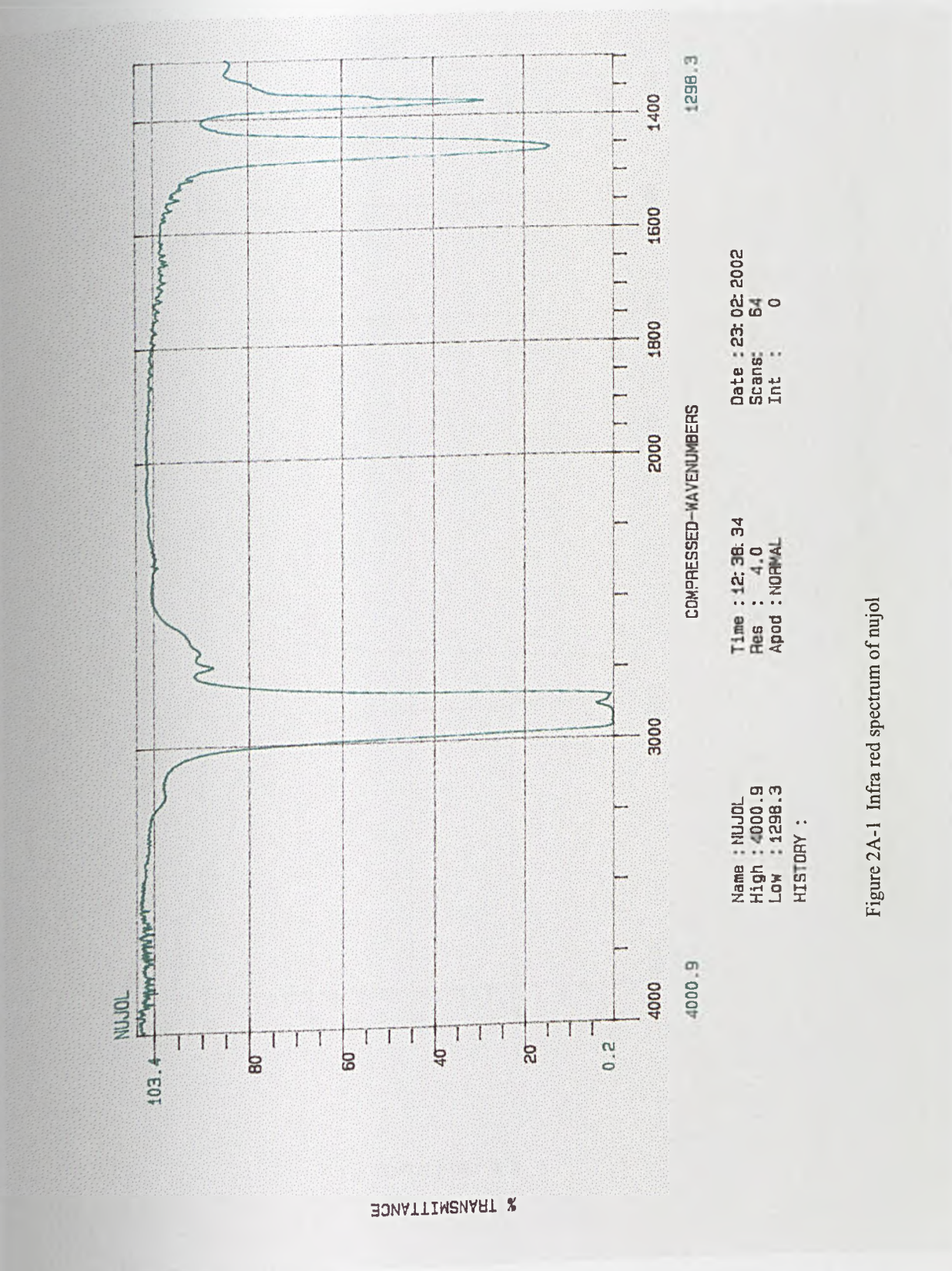


Figure 2A-1 Infra red spectrum of nujol

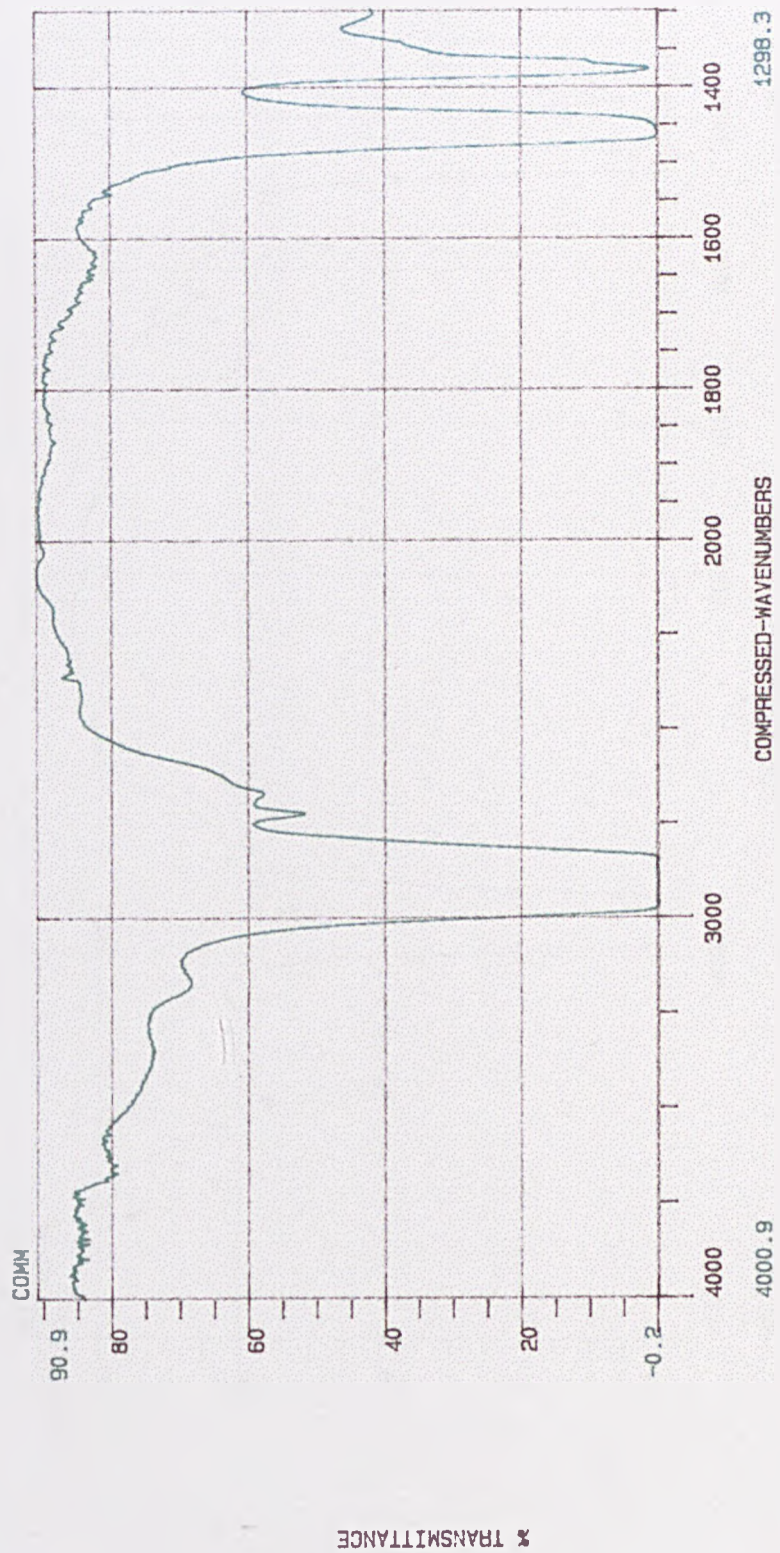


Figure 2A-2 Infra red spectrum of silica, particle size 0.011 microns, obtainced from aldich (catalogue number 38,127-6).

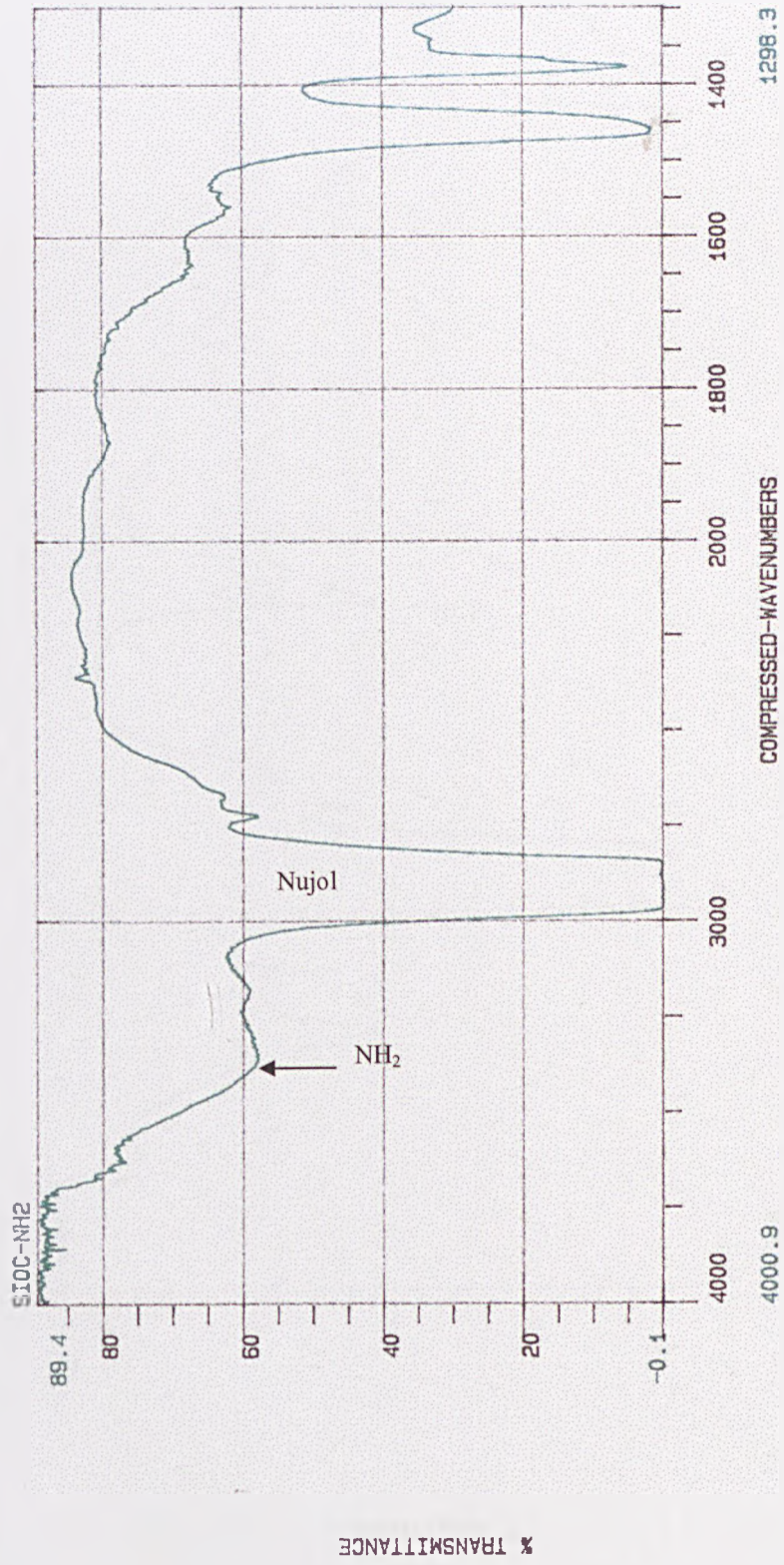


Figure 2A-3 Infra red spectrum of 3-aminopropyltriethoxysilane

Figure 2A-4

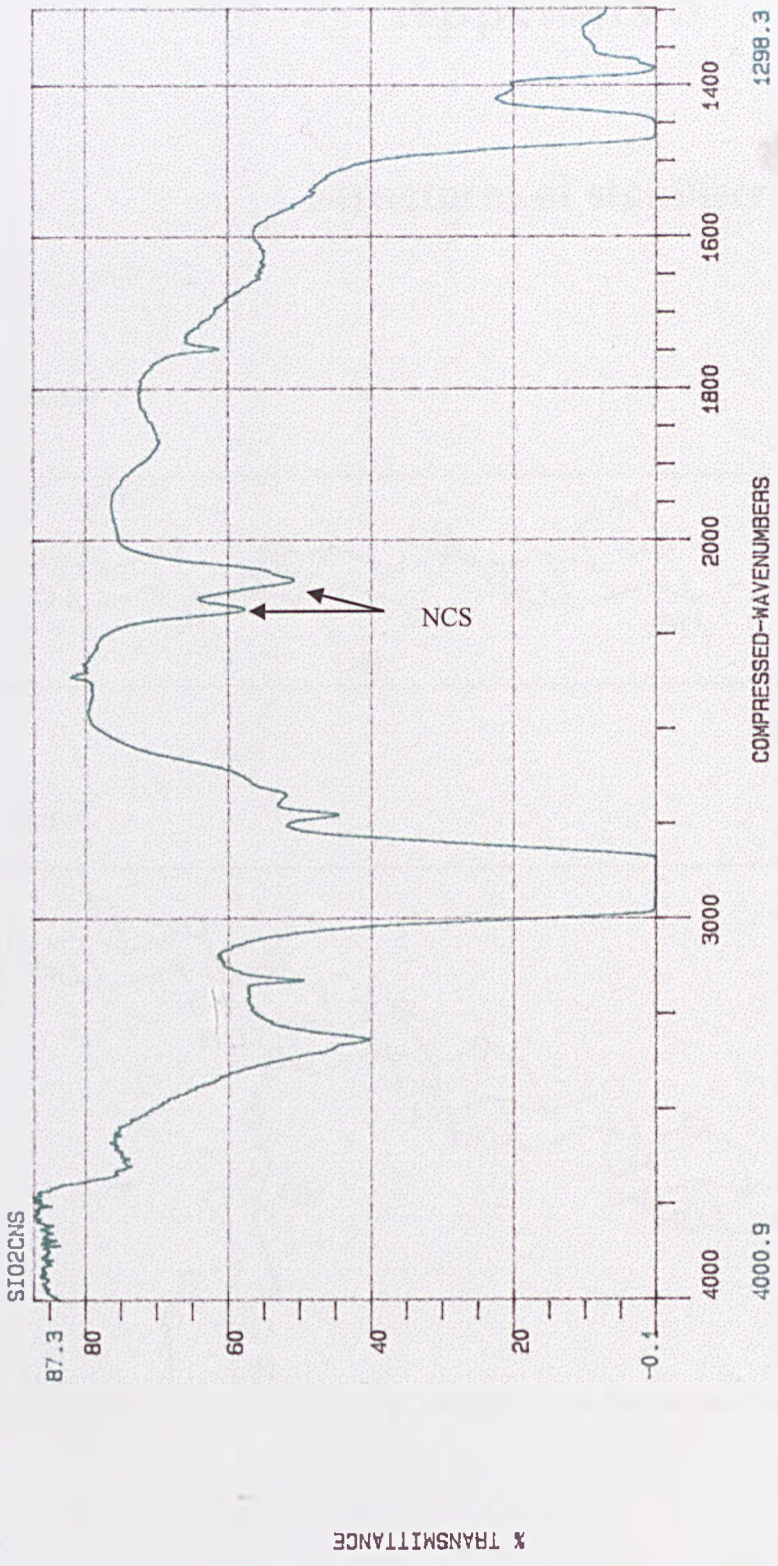
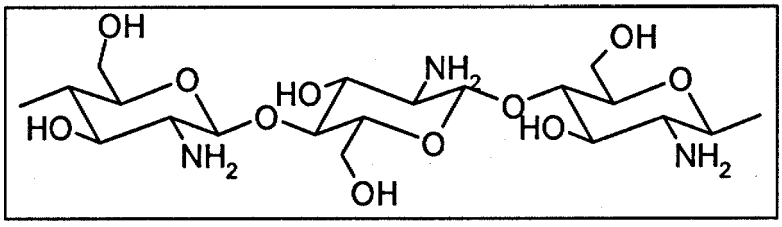


Figure 2A-4 Infra red spectrum of 3-isothiocyantopropylthrichoxysilane

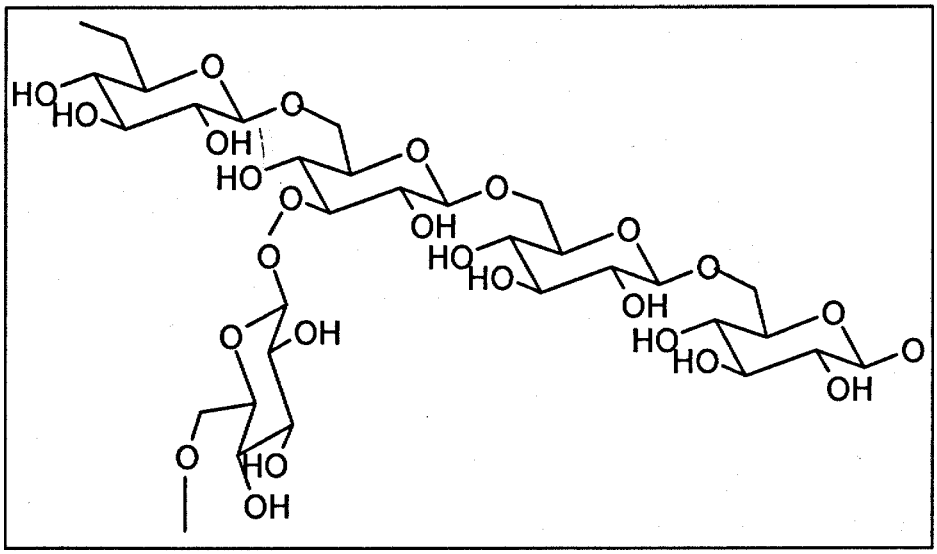
Appendix 3

Structures of stabilisers

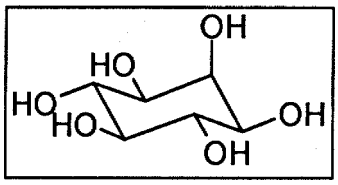
Chitosan



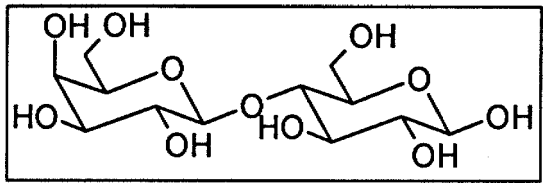
Dextran



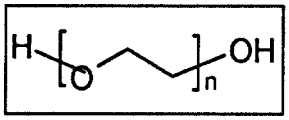
inositol



Lactose



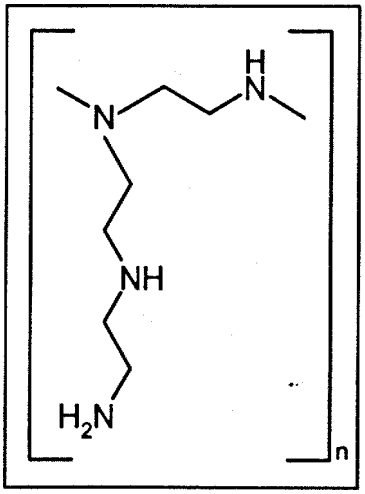
Polyethylene glycol



M_w = 4,000, an average value of n is between 68 – 84.

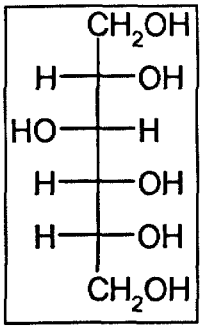
M_w = 8,000, an average value of n is between 158 – 204 (Merk index, 1996).

Polyethylene imine



M_w = 750,000, average value of n is 5200.

D-sorbitol



Sucrose